SAR and QSAR Studies on the N-Terminally Acylated Pentapeptide Agonists for GPR54

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Kisspeptins (KPs) play important roles in the regulation of physiological and pathological states through activation of the cognate receptor GPR54. Our previous studies to downsize KP agonists to the essential GPR54 pharmacophore identified peptides **1**−**3** as low molecular weight GPR54 agonists. In this study, the effect of N-terminal acyl groups on the activity of a series of analogues (R-Phe-Gly-Leu-Arg-Trp-NH_2) was investigated in order to develop novel potent GPR54 agonists. Among the compounds developed, the most potent agonistic activity for GPR54 was observed for N-terminal 4-fluorobenzoyl analogue **29**. Using quantitative structure−activity relationship studies, it was demonstrated that the inductively negative and small substituents were preferred at the 4-position of N-terminal benzoyl groups.

Introduction

GPR54 (OT7T175, AXOR12) is a member of the G-protein coupled receptor (GPCR) superfamily that was originally identified as an orphan receptor sharing a modest sequence homology with galanin receptors.[1] Its endogenous ligands, called kisspeptin (KP) or metastin (KP-54), and its N-terminally truncated peptides, such as KP-10, were identified by three independent groups.[2−4] Because the amino acid sequences of the agonists correspond to those of peptide fragments encoded by the *KiSS-1* gene, a known metastasis suppressor gene,[5] these naturally occurring peptides were considered to be potential candidates for antimetastatic agents for several carcinomas. There have been multiple reports indicating that KPs attenuated or inhibited the mobility and metastic behavior of several cancer cells that express GPR54, including melanoma,[2] thyroid,[6] and pancreatic cancer cell lines,[7] in vitro and/or in vivo. It was recently demonstrated that GPR54 activation by KP-10 suppressed CXCR4 signaling, which has been implicated in the homing mechanisms involved in cancer metastasis and directed migration induced by a chemotactic gradient of the exclusive CXCR4 ligand, stromal cell derived factor 1 (SDF-1, CXCL12).[8]

Recently, the role of KPs in modulating hormone secretion involved in the onset of puberty has been discovered. KPs prompts the pulsatile release of gonadotropin-releasing hormone (GnRH) from hypothalamus by direct activation of GnRH neurons via signaling mediated by GPR54.[9−12] Investigations to date suggest that this increase in the pulsatile release of GnRH is involved in the initiation of mammalian puberty.[13−16] This is supported by evidence that the inherited loss of GPR54 function leads to the deficient sexual development, called isolated hypogonadotropic hypogonadism (IHH), in humans and in (knockout) mice.[17−19] Thus, the GPR54/KPs axis is important for the neuroendocrine regulation of puberty and reproduction in mammals.

Although critical roles for GPR54 and KPs in normal and pathologic physiology have been identified, there are few reports on the development of novel ligands (agonists/antagonists) for GPR54 using medicinal chemistry or genetic engineering approaches. Such ligands could contribute to elucidating mechanism for the induction of GPR54 signaling by KPs as well as development of therapeutic agents for preventing cancer metastasis and IHH. Previously, Clements et al. reported that invertebrate-derived FRMF-related neuropeptides, NRNGLRW-NH_2 and NRNFLRW-NH_2, showed higher affinity for GPR54 compared with pEGLRW-NH_2 (pE = pyroglutamic acid) and NRNFLRF-NH_2, respectively.[20] On the basis of the observation that an N-terminal basic group and a C-terminal RW-amide motif are critical for GPR54 agonist activity, we examined a series of truncated KP-10 analogs and identified compounds **1**−**3**, resulting in the identification of pentapeptide agonists of GPR54 that are equipotent to KP-54 (Figure 1).[21,22]

Peptides **1**−**3** are composed of N-terminal basic 4-(aminomethyl)benzoyl derivatives and common C-terminal KP-like pentapeptide. It is worth noting that the N-terminal acyl moieties may play an important role in the binding and activation of GPR54, because peptides **1**−**3** showed significantly higher bioactivities compared to KP-9. Herein, we report the structure−activity relationship (SAR) for N-terminal acyl groups of pentapeptide GPR54 agonists. In addition, the data were rationalized by quantitative structure−activity relationship (QSAR) studies using classical QSAR and 3D-CoMFA analyses.

Results and Discussion

Chemistry. Synthesis of pentapeptide analogues was conducted using standard methods that were reported for the production of compounds **1**−**3**.[22] The protected pentapeptide [H-Phe-Gly-Leu-Arg(Pbf)-Trp-resin; Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl] chain was constructed on Rink-amide resin by standard Fmoc-based solid-phase peptide synthesis. Several carboxylic acids were condensed on the N-terminal amino group of the protected resin. For the preparation of peptides **4** or **18**, reductive amination using benzaldehyde and $NaBH_3CN$ or *N*-acetylation with Ac_2O was performed, respectively, after removal of the Fmoc group of Fmoc-Amb [Amb = 4-(aminomethyl)benzoic acid]. Pentapeptide analogues were released from the resin by treatment with 1 M TMSBr-thioanisole in TFA in the presence of *m*-cresol and 1,2-ethanedithiol as scavengers. Purification of the resulting crude peptides by RP-HPLC yielded the desired products. Peptides

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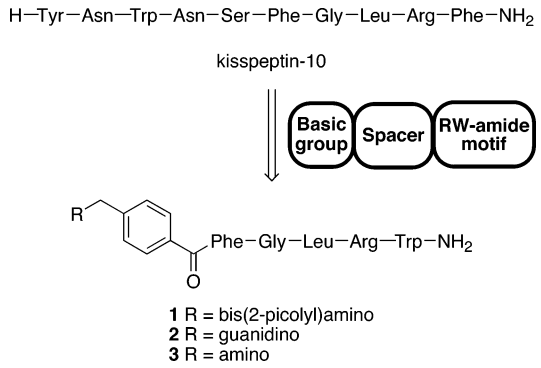


Figure 1. Structures of KP-10 and downsized derivatives **1**−**3**.

5, **6**, and **23** were obtained as byproducts for the preparation of **1**, **4**, and **22**, respectively.

Assessment of GPR54 Agonistic Activity of Synthetic Pentapeptide Analogues Using Flipr Technology. To evaluate the GPR54 agonistic activities of synthetic pentapeptide analogues, the mobilization of intracellular Ca^{2+} ion levels following stimulation of CHO transfectants stably expressing GPR54 on the cell surface by peptides was monitored by spectrofluorometry.[21] The agonistic activity of each compound was calculated as the following three values: percent activity [relative maximum agonistic activity induced by 10 nM of each compound ($n = 3$) compared with the maximum signal induced by the addition of 1 μM KP-10 ($n = 3$)], EC_{50} value (the concentration needed for 50% of the full agonistic activity induced by 1 μM KP-10), and Q value [EC_{50} ratios (Q) were calculated as EC_{50}(compound)/EC_{50}(KP-10)]. Q values are derived in order to indicate the relative agonistic activity, since the EC_{50} values of control peptides may vary for each plate due to the assay conditions (N.B., the EC_{50} of KP-10 varied among the assay plates in the range of 0.56−1.1 nM).

SAR Studies. Table 1 summarizes the GPR54 agonistic activities of a series of N-terminal acylated pentapeptide analogues (R-Phe-Gly-Leu-Arg-Trp-NH_2) to evaluate the structure−activity relationships. N-Terminal acyl substructures (R) are shown in Chart 1. In our previous studies, peptides **1**−**3** were identified as potent GPR54 agonists, which were designed on the basis of the presumption that a basic group(s) at the N-terminus of pentapeptides was needed for GPR54 agonistic activity.[21,22] For further optimization of N-terminal basic groups, several pentapeptide analogues (**4**−**10**) possessing a basic group(s) at the N-termini were prepared and evaluated for biological activity. Peptides **5**−**10** exhibited agonistic activities ($Q = 2.8$−5.6) similar to compounds **1**−**3**, while bis-benzyl modification of the amino group on Amb in peptide **4** led to the loss of bioactivity. The monopicolylamino derivative **5** was less potent than bis-picolylamino derivative **1** and the primary amino derivative **3** in terms of Q values. *N*-Picolyl derivatives **1** and **5** showed higher agonistic activities than *N*-benzyl derivatives **4** and **6**, respectively. Peptide **3** containing an aliphatic primary amino group showed lower agonistic activity than the guanidino (**2**) and aromatic amino (**8**) group containing derivatives, suggesting that the alkalinity constant of the functional group does not correlate with the degree of agonistic activity. The position of the amino constituent seems to be permissive, because peptides **8**−**10** kept good agonistic activities ($Q = 2.8$−4.2). As such, any apparent correlation between bioactivity and the characters of basic groups was not detected.

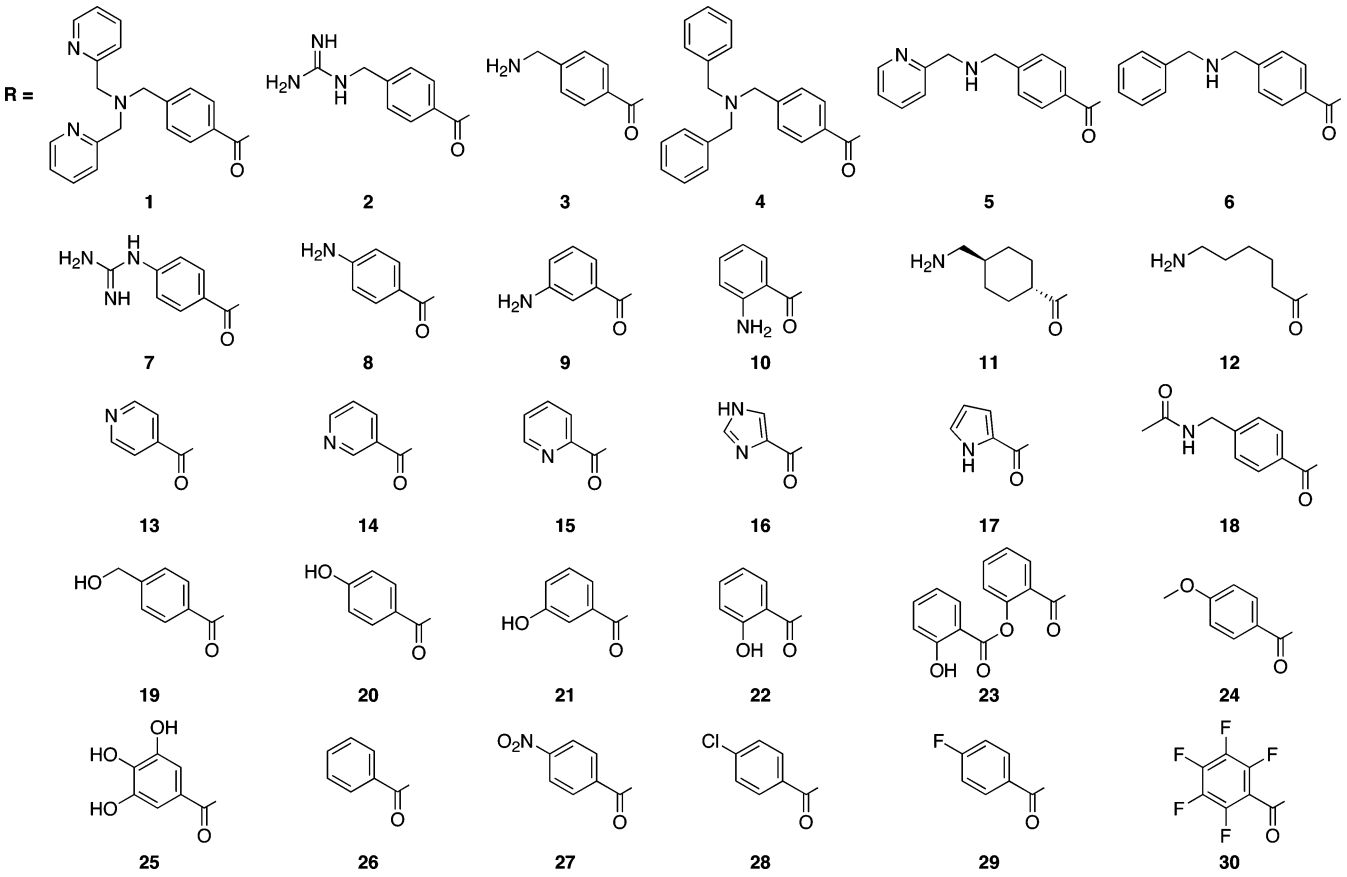
Next, we focused on the aromatic ring of Amb in **1**−**3** that potentially serves as a linkage between N-terminal basic groups and the five-residue core peptide sequence. We synthesized two

Table 1. Structure and Bioactivities of KP-10 and Synthetic Pentapeptide Analogues (R-Phe-Gly-Leu-Arg-Trp-NH_2)

compound	R	% activity[a]	EC_{50} (nM)	Q[b]
kisspeptin-10	(H-YNWNSFGLRF-NH_2)	−	0.56−1.1	1
1	4-(*N*,*N*-bis(2-picolyl)aminomethyl)benzoyl	88.9 ± 2.6	3.3	3.1
2	4-(guanidinomethyl)benzoyl	93.7 ± 1.8	1.4	1.6
3	4-(aminomethyl)benzoyl	96.5 ± 0.3	3.1	3.4
4	4-(*N*,*N*-bis(benzyl)aminomethyl)benzoyl	9.3 ± 0.4	NT	−
5	4-(*N*-(2-picolyl)aminomethyl)benzoyl	89.3 ± 0.3	2.7	4.5
6	4-(*N*-benzylaminomethyl)benzoyl	86.5 ± 1.4	3.3	5.6
7	4-guanidinobenzoyl	91.2 ± 0.9	1.7	2.9
8	4-aminobenzoyl	90.4 ± 0.5	1.6	2.9
9	3-aminobenzoyl	88.5 ± 2.1	4.7	4.2
10	2-aminobenzoyl	94.0 ± 1.7	3.1	2.8
11	*trans*-4-(aminomethyl)cyclohexylcarbonyl	9.9 ± 1.2	NT	−
12	6-aminohexanoyl	1.6 ± 0.1	NT	−
13	4-pyridinecarbonyl	101.9 ± 0.6	1.7	2.4
14	3-pyridinecarbonyl	93.5 ± 1.6	3.0	4.1
15	2-pyridinecarbonyl	104.5 ± 1.2	0.8	1.3
16	1*H*-4-imidazolecarbonyl	88.4 ± 0.2	1.7	3.0
17	2-pyrrolecarbonyl	95.8 ± 0.7	2.0	1.7
18	4-(*N*-acetylaminomethyl)benzoyl	101.3 ± 1.3	0.99	1.6
19	4-(hydroxymethyl)benzoyl	95.4 ± 1.1	3.0	4.7
20	4-hydroxybenzoyl	94.6 ± 0.7	2.1	3.0
21	3-hydroxybenzoyl	99.7 ± 0.6	1.2	1.6
22	2-hydroxybenzoyl	90.1 ± 0.6	5.0	6.6
23	2-(*O*-(2-hydroxybenzoyl)hydroxy)benzoyl	92.1 ± 1.4	3.0	4.0
24	4-methoxybenzoyl	104.0 ± 0.9	1.2	1.6
25	3,4,5-trihydroxybenzoyl	1.0 ± 0.3	>100	−
26	benzoyl	99.9 ± 0.5	0.99	1.6
27	4-nitrobenzoyl	94.3 ± 1.3	1.0	1.5
28	4-chlorobenzoyl	91.8 ± 0.7	1.3	1.3
29	4-fluorobenzoyl	95.6 ± 1.2	0.69	0.63
30	pentafluorobenzoyl	0.6 ± 0.0	>100	−

[a] Percent activity values are based on the relative maximum agonistic activity induced by 10 nM of the compounds (%). The maximum agonistic activity signal at 1 μM KP-10 was used as reference (100%). [b] Q values are calculated as EC_{50}(compound)/EC_{50}(KP-10).

Chart 1. N-terminal Acyl Substructures (R) of Synthetic Pentapeptide Analogues (R-Phe-Gly-Leu-Arg-Trp-NH_2)



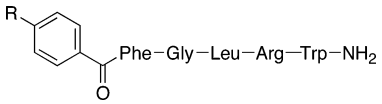
analogues containing an aliphatic acyl group instead of Amb in **3**, a cyclic *trans*-4-(aminomethyl)cyclohexyl carbonyl (**11**) or an acyclic 6-aminohexanoyl (**12**) group. These two analogues lacked GPR54 agonistic activity, indicating that the N-terminal aromatic ring plays an important role in activation of GPR54. Pentapeptide analogues **13**−**17** that contain several aromatic heterocyclic groups at the N-termini were also designed. All of them induced significant GPR54 activation. In particular, 2-pyridinecarbonyl derivative **15** was a highly potent GPR54 agonist (Q = 1.3). Moreover, it is notable that the 2-pyrrolecarbonyl derivative **17** also exhibited high agonistic activity (Q = 1.7), although the pyrrole ring does not possess basic functionality. This fact prompted us to re-evaluate the importance of N-terminal basic group(s) for GPR54 agonists. The *N*-acetylated Amb-containing analogue **18** was found to be a more potent agonist than peptide **3**, indicating that the N-terminal basic group is not required for the bioactivity. This result also suggests that several functional groups may be feasible for substitution on the N-terminal aromatic ring.

We prepared a series of pentapeptide analogues **19**−**25**, which possess oxygen-containing moieties on the N-terminal benzoyl group. Replacement of the aminomethyl group in peptide **3** by a hydroxymethyl group (**19**) maintained the agonistic activity, proving that hydroxy group was tolerable in the N-terminal functional group. Monohydroxy-substituted benzoyl analogues **20**−**22** and a doubly acylated analogue **23** showed potent agonistic activities (Q = 1.6−6.6). The finding that the 4-methoxybenzoyl analogue **24** had agonistic activity comparable to that of the hydroxy congener **20** suggested that this constituent does not interact with the receptor as a hydrogen-bonding donor. On the other hand, the 3,4,5-trihydroxybenzoyl analogue **25** did not stimulate receptor signaling, although the mechanism for this lack of activity is not clear. Interestingly, even the unsubstituted benzoyl analogue **26** exhibited GPR54 agonistic activity (Q = 1.6) nearly equipotent to KP-10. This implies that substituents on the benzoyl group may not be necessarily required for GPR54 stimulation.

We also introduced three different electron-withdrawing structures (a nitro group for **27**, a chlorine atom for **28**, and a fluorine atom for **29**, respectively) at the 4-position of benzoyl groups. These three peptides **27**−**29** exhibited highly potent GPR54 agonistic activities (Q = 0.63−1.5). Out of them, the 4-fluorobenzoyl analogue **29** was a more potent GPR54 agonist than any other compounds reported thus far, including KP-10. Whereas we presumed that the electron-deficient aromatic ring of the pentafluorophenyl group could enhance the bioactivity, peptide **30** lacked GPR54 agonistic activity. These results suggest that the suitable introduction of electron-withdrawing substitution onto the N-terminal aromatic ring improves agonistic activity. On the other hand, multisubstituted benzoyl analogues **25** and **30** did not exhibit agonistic activity, indicating that irrelevant multisubstitution on the benzoyl group resulted in loss of bioactivity.

Classical QSAR Study. To investigate the quantitative relationships between GPR54 agonistic activity and the electronic and steric effects of constituents on the N-terminal benzoyl group, the log Q values were analyzed by a classical QSAR strategy (Table 2). In all equations, n is the number of compounds used for the regression analyses, s is the standard deviation, r is the correlation coefficient, q is the leave-one-out cross-validated correlation coefficient, and the values in parentheses are 95% confidence intervals. The following eq 1, in which the field-inductive effect ($F^{23,24}$) and the bulkiness

Table 2. Bioactivities and Chemical Parameters of 4-Substituted-benzoyl-containing Derivatives



				observed		calculated −log *Q* ($\Delta_{-\log Q}$)[b]	
compound	R	*F*	E_S	*Q*[a]	−log *Q*	eq 1	eq 2
3	H_2NCH_2	0.04	−1.21[c]	3.4	−0.531	−0.624 (+0.093)	−0.636 (+0.105)
8	H_2N	0.08	−0.61	2.9	−0.462	−0.405 (−0.057)	−0.368 (−0.094)
19	$HOCH_2$	0.03	−1.21	4.7	−0.672	−0.634 (−0.038)	−0.647 (+0.013)
20	HO	0.33	−0.55	3.0	−0.477	−0.139 (−0.338)	−
24	MeO	0.29	−0.55	1.6	−0.204	−0.179 (−0.025)	−0.109 (−0.095)
26	H	0.00	0.00	1.6	−0.204	−0.302 (+0.098)	−0.232 (+0.028)
27	O_2N	0.65	−1.77[d]	1.5	−0.176	−0.187 (+0.011)	−0.155 (−0.021)
28	Cl	0.42	−0.97	1.3	−0.114	−0.176 (+0.062)	−0.118 (+0.004)
29	F	0.45	−0.46	0.63	0.201	0.006 (+0.195)	0.105 (+0.096)

[a] *Q* values were calculated as $Q = EC_{50}(\text{compound})/EC_{50}(\text{KP-10})$. [b] $\Delta_{-\log Q}$ values were calculated as (observed −log *Q*) − (calculated −log *Q*). [c] E_S value of $HOCH_2$ was used for QSAR analysis. [d] The average E_S value of O_2N (−1.01 and −2.52).

(E_S[25,26]) were significant, was formulated for the activity of 4-monosubstituted and unsubstituted benzoyl derivatives.

$$-\log Q = 0.99(\pm 0.71)F + 0.30(\pm 0.31)E_S - 0.30(\pm 0.28) \quad (1)$$

$$n = 9,\ s = 0.17,\ r^2 = 0.68,\ q^2 = 0.41$$

The residual value ($\Delta_{-\log Q}$) from (observed −log *Q*) − (calculated −log *Q*) of each compound is shown in Table 2. Among this spectrum of compounds, the residual value of 4-hydroxybenzoyl analogue **20** was remarkably higher than any others. The acidity of the hydroxy group of **20** is relatively high because of the presence of a carbonyl group on the *p*-position. Therefore, the hydroxy group of **20** was considered to be partially in the dissociated state (phenoxy ion) under the biological assay conditions (pH = 7.4). However, the values of chemical parameters shown in Table 2 are only for nonionic states and not for the dissociated state. Thus, the *F* and calculated −log *Q* of **20** should be smaller. For the above reason, the other eight compounds, except **20**, were analyzed again.

$$-\log Q = 1.13(\pm 0.39)F + 0.37(\pm 0.17)E_S - 0.23(\pm 0.16) \quad (2)$$

$$n = 8,\ s = 0.089,\ r^2 = 0.93,\ q^2 = 0.82$$

Equation 2 indicates the excellent correlation ($r^2 = 0.93$) of −log *Q* with *F* and E_S values, although the number of compounds used for analysis was small ($n = 8$). The positive slope of *F* in eq 2 showed that the more inductively electronegative the substituent at the 4-position of the N-terminal benzoyl group, the higher the GPR54 agonistic activity of the compound. Equation 2 represents the bulky substituent at the 4-position of the benzoyl group as being unfavorable for this activity, since the bulkier substituting group has the smaller E_S value. According to this correlation, substitution of fluorine at the 4-position of the benzoyl group conferred the highest GPR54 agonistic activity.

In eqs 1 and 2, addition of the resonance effect (*R*)[23] of the 4-substituents was insignificant, probably because of the insufficient number of compounds analyzed. Besides, the use of Hammett σ_{para}[27] in place of *F* did not give significant equations. These facts suggest that the effect of the 4-substituents may not be that on the 1-substituent because the resonance effect of the 4-substituents on reactivity of the 1-substituent is usually large. Since the field effect works for neighboring groups, the 4-substituents may affect adjacent substituents electronically. More details are discussed in the next section.

Table 3. CoMFA-Predicted −log *Q* Values for Pentapeptide GPR54 Agonists Using Eq 3

	−log *Q*		
compound	observed	calculated[a]	residual value[b]
2	−0.204	−0.233	0.028
3	−0.531	−0.463	−0.068
6	−0.748	−0.777	0.029
7	−0.462	−0.463	0.001
8	−0.462	−0.340	−0.123
9	−0.623	−0.587	−0.036
10	−0.447	−0.398	−0.049
13	−0.380	−0.336	−0.044
14	−0.613	−0.547	−0.066
16	−0.477	−0.536	0.059
17	−0.230	−0.197	−0.034
18	−0.204	−0.233	0.028
19	−0.672	−0.748	0.076
20	−0.477	−0.418	−0.060
21	−0.204	−0.218	0.014
22	−0.820	−0.890	0.070
24	−0.204	−0.223	0.018
26	−0.204	−0.146	−0.058
27	−0.176	−0.143	−0.034
28	−0.114	−0.225	0.111
29	0.201	0.064	0.137

[a] Caluculated −log *Q* values using eq 3. [b] Residual values were calculated as (observed −log *Q*) − (calculated −log *Q*).

3D-QSAR with CoMFA. Although the above QSAR study using chemical parameters of substituents is quite useful, 3D-QSAR analyses of synthetic pentapeptide analogues were carried out to analyze several scaffold compounds inclusively using the comparative molecular field analysis (CoMFA) method. The CoMFA model was constructed using 21 compounds of all 25 *Q* value calculable compounds for the training set with four compounds set aside for testing models (Tables 3, 4). These four compounds were selected at random.

As a result, the following equation was yielded.

$$-\log Q = [\text{CoMFA field term}] - 0.450 \quad (3)$$

$$n = 21,\ s = 0.076,\ r^2 = 0.93,\ m = 5,\ q^2 = 0.45,\ s_{CV} = 0.21$$

(steric, 49.4%; electrostatic, 50.6%)

In eq 3, the CoMFA results were represented by the leave-one-out cross-validated correlation coefficient (*q*), the number

Table 4. Comparison of CoMFA-Predicted vs Experimentally Observed Values of −log *Q* for Test Set Compounds

	−log *Q*		
compound	observed	predicted[a]	residual value[b]
1	−0.491	−0.670	0.179
5	−0.653	−0.780	0.127
15	−0.114	−0.203	0.089
23	−0.602	−0.559	−0.043

[a] Predicted −log *Q* values using eq 3. [b] Residual values were calculated as (observed −log *Q*) − (predicted −log *Q*).

of components (*m*), the conventional correlation coefficient (*r*), and the standard deviation (*s*), in addition to the relative contribution (%) of descriptors to the correlation equation. Observed −log *Q* values, calculated −log *Q* values using eq 3, and residual values of each compound are summarized in Table 3. The satisfied predictive ability of this equation can be seen from the testing model (Table 4). The result was visualized by contour maps using connected lattice points having an equivalent coefficient level for each molecular field.

CoMFA analysis was in agreement with classical QSAR analysis, showing that electronegative (red region in Figure 2) and small (yellow region) substituents were preferable at the 4-position of the benzoyl group. On the other hand, the blue regions (favoring electropositive substituents) and green regions (favoring bulky substituents) were observed around protons of the *o*- and *m*-positions of the amide group. This implies that the hydrogen atoms of the benzoyl group may be involved in the interaction with an aromatic system of the receptor such as edge-to-face CH/π interaction.[28] It is supported by the fact that multisubstituted benzoyl analogues **25** and **30**, in which hydrogen atoms on the benzene ring were substituted, were not active for GPR54.

Among aminobenzoyl analogues **8**−**10** and pyridinecarbonyl analogues **13**−**15**, 3-substituted analogues **9** and **14** showed lower agonistic activities than their regioisomers, suggesting that *m*-substituents play a deleterious role and that *m*-hydrogen atoms of the amide group are especially important for receptor activation. On the other hand, in the case of hydroxybenzoyl analogues **20**−**22**, 3-hydroxybenzoyl analogue **21** was a more potent agonist compared to its regioisomers. This enhanced activity may be caused by the differences between the acidity of aromatic hydroxy groups or by interaction of the hydrogen of hydroxy group at the 3-position with the receptor. If aromatic residues of the receptor interact with hydrogen atoms on the N-terminal aromatic ring, it is likely that substitution of the 4-position influences the electron density of adjacent hydrogens, especially *m*-hydrogens of the amide group, and their accessibility to the receptor. That is, the inductively electronegative 4-substituents could enhance the interaction of adjacent hydrogen atoms with the receptor by increasing the acidity, and bulky groups might attenuate GPR54 agonistic activities by prevention of the interaction between the ligand hydrogen atoms and the receptor.

Matsushima et al. reported the structure−activity relationship of heptapeptides, SFLLRNP derivatives that activate the thrombin receptor.[28] (Multi)fluorinated Phe was substituted for Phe, and the ability of the peptides to induce the platelet aggregation of human platelets was evaluated. The 4-fluorophenylalanine isomer was most potent among the tested peptides, whereas the pentafluorophenylalanine isomer was completely inactive, similar to the result obtained in this study (compound **29** and **30**). On the basis of the variation in the activity of variously fluorinated peptides, it was concluded that the edge-to-face CH/π interaction of 2,3- or 5,6-hydrogens of Phe2 with aromatic residues in the receptor plays an important role in activation of the thrombin receptor. This report strongly suggests involvement of CH/π in the interaction of GPR54 agonists with the receptor.

Furthermore, the CoMFA green and blue regions were mainly observed on the peptide bond plane, suggesting that the higher agonistic activity of **15** than the isomers **13** and **14** may require a conformation in which the N-terminal aromatic ring is in the plane of the peptide bond. This conformation may be able to form hydrogen bonding between the pyridine nitrogen atom and the amide hydrogen. Although more investigations are required for clarification of the role of N-terminal acyl groups for GPR54 agonistic activity, the results in structure−activity relationships provide important insights for the development of more potent agonists.

Conclusion

In this study, we carried out SAR and QSAR studies on N-terminal acyl groups of pentapeptide analogues as GPR54 agonists to investigate the mechanism for GPR54 stimulation. As a result, it was demonstrated that the aromatic acyl group and the substituents contributed to the agonistic activity. QSAR studies indicate that the inductive electronegative and small substituents at the 4-position of the N-terminal benzoyl group were preferred for higher agonistic activity. 4-Fluorobenzoyl analogue **29** was the most potent agonist reported so far. On the basis of the results from this study, we are now trying to identify novel antagonists and inverse agonists for GPR54 by more extensive investigation of the substituents on the N-terminal aromatic ring.

Experimental

General Synthetic. Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer. Optical rotations were measured with a Horiba high-sensitivity 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-10ADvp (Shimadzu Corp., 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. The purity of each peptide compound was more than 95%, as determined by HPLC analysis. 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).

Synthesis of Pentapeptide Analogues. Peptides **1**−**3** were synthesized using Fmoc-based solid-phase synthesis followed by N-terminal modifications as previously described.[22] Procedures for N-terminal modifications in the synthesis of other compounds are given below.

Peptide 4. After deprotection of the Fmoc group of Amb, the protected resin (0.05 mmol) was reacted with benzaldehyde (15 μL, 0.15 mmol) in DMF/MeOH (2 mL, 1:1) for 1 h followed by reduction with $NaBH_3CN$ (16 mg, 0.25 mmol) in DMF/MeOH/AcOH (2 mL, 9:9:2) for 30 min. This operation was repeated twice.

Peptides 7−**17, 19**−**30.** After deprotection of the Fmoc group of Phe, the protected resin (0.1 mmol) was reacted with carboxylic acids (0.5 mmol) and 1,3-diisopropylcarbodiimide (DIPCDI) (78 μL, 0.5 mmol) in the presence of *N*-hydroxybenzotriazole (HOBt) (153 mg, 1.0 mmol) in DMF. Then, the Fmoc group was removed by treatment of 20% (v/v) piperidine−DMF for 30 min for the

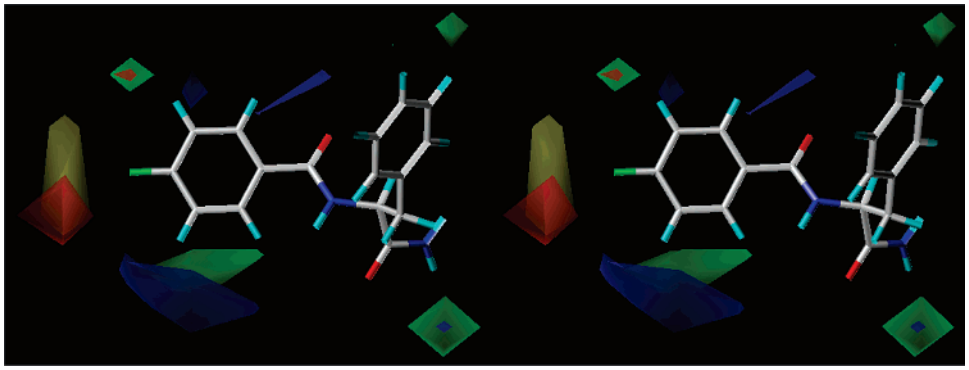


Figure 2. Stereoviews of CoMFA analysis, drawn according to eq 3, shown with 4-fluorobenzoyl-Phe-NH_2 (derived from **29**). The contours are shown to surround regions where a higher steric bulk increases (green) or decreases (yellow) or where a positive (blue) or a negative (red) electrostatic potential increases the activity.

synthesis of **8**, **11**, and **12**. Removal of other protecting groups (Boc group for **9** and **10**, TBS group for **19**) was conducted simultaneously with cleavage of peptides from the resin.

Peptide 18. After deprotection of the Fmoc group of Amb, the protected resin (0.1 mmol) was reacted with Ac_2O (80 μL, 1.0 mmol) in the presence *i*-Pr_2NEt (82 μL, 0.5 mmol).

Measurement of $[Ca^{2+}]_i$ Using Flipr Technology. A CHO/dhfr transfectant cell line stably expressing GPR54 was prepared as previously described.[22] The fluorochrome mixture was prepared by addition of two vials of Fluo3-AM (50 mg/vial) in 21 mL of DMSO and 21 mL of 20% Pluronic acid to 10 mL of HANKS/HBSS (prepared from 9.8 g of HANKS, 0.35 g of sodium hydrogen carbonate, and 20 mL of 1 M HEPES, pH 7.4) containing 2.5 mM Probenecid and 1% FBS.

GPR54/CHO cells (3.0×10^4 cells/200 μL/well) were inoculated in 10% dFBS/DMEM onto a 96-well plate for FLIPR analysis (black plate clear bottom, Coster, Inc.), followed by incubation at 37 °C overnight in 5% CO_2. After the medium was removed, 100 μL of the pigment mixture was dispensed into each/well of the plate, followed by incubation at 37 °C for an hour in 5% CO_2. 1 mM peptide in DMSO 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 analysis (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 four 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 the FLIPR device (Molecular Devices, Inc.) and 0.05 mL of a sample from the sample plate was automatically transferred to the cell plate. Mobilization of intracellular calcium ions was measured over a 40 s interval.

Classical QSAR. Analyses were performed using QREG2.05.[29] Swain−Lupton *F* and Taft E_S were used as electronic and steric parameters, respectively, which were cited from literature values.[30] The E_S value of $HOCH_2$ in **19** was used for analysis of **3** because the E_S value of H_2NCH_2 was not cited. Although two E_S values (−1.01 and −2.52) are reported for O_2N, the average value (−1.77) was used for analysis.

Molecular Modeling. All computations were performed using the molecular modeling software package SYBYL, version 6.9 (Tripos Co., St. Louis, MO). All compounds were analyzed as protected phenylalanine amide (R-Phe-NH_2) derivatives truncated from their common C-terminal tetrapeptide sequence to simplify computations (Figure 3). Guanidino groups of **2** and **7** and aliphatic amino groups of **1**, **3**, **5**, and **6** were positively charged, while each state (ionic and nonionic) model was made for phenol analogues **20**−**23**. In the case of the compounds possessing asymmetric aromatic rings (**9**, **10**, **14**−**17**, and **21**−**23**), two models were made: the functional group on the aromatic ring was directed for the carbonyl oxygen atom or for the amide hydrogen atom. The SYBYL systematic search for all rotatable bonds identified several low-energy conformers of peptides **1**−**3**, **5**, **6**, and **19** having a

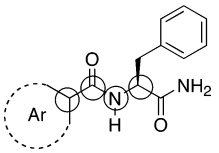


Figure 3. Structure of models for QSAR analysis and the atoms used for superposition (open circles).

4-substituent. The conformers were then fully reoptimized by the MOPAC/PM3 method to give the stable conformations. We selected one model for each compound, which provided the better correlation for CoMFA.

Superposition. The optimized conformation of the unsubstituted benzoyl derivative (derived from **26**) was selected as a reference standard on which the other compounds were superposed for calculating the molecular-field descriptors. The key atoms for superposition were the α-amide nitrogen atom and the α-carbon atom of Phe, and the carbonyl carbon atom and the adjacent carbon atom of the aromatic ring (Figure 3, circled atoms).

CoMFA Analysis. The analyses were conducted with the 'Advanced CoMFA' module of SYBYL. The lattice spacing was 2 Å, and an sp^3 carbon with a +1 charge was used as probe to estimate the electrostatic and steric molecular fields, respectively. The models were superposed in the lattice space of 22.9 Å × 19.8 Å × 23.1 Å ($X = -20.9$ to 2.0, $Y = -4.8$ to 15.0, $Z = -12.1$ to 11.0). The electrostatic and steric potential energies at each lattice point were calculated using Coulombic and Lennard-Jones potential functions, respectively. We initially selected the number of components in the set from the cross-validation (the leave-one-out method) setting the column filtering at 2 kcal/mol, and then performed the analysis using the optimum number of latent variables which was deduced from the cross-validation tests without actual cross-validation.

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Supporting Information Available: Characterization data of novel synthetic compounds and HPLC charts of **4**−**30**. This material is available free of charge via the Internet at http://pubs.acs.org.

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