Structure–Activity Relationship Study and NMR Analysis of Fluorobenzoyl Pentapeptide GPR54 Agonists

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ABSTRACT:

GPR54 is a Gq-protein coupled receptor involved in cancer metastasis and regulation of the endocrine system. GPR54 activation by endogenous ligands attenuates the mobility of carcinomas and stimulates the secretion of gonadotropin-releasing hormone. GPR54 agonists are, therefore, potential therapeutic candidates for cancer metastasis and hormonal diseases. Pentapeptide derivatives of kisspeptin C-terminus were identified as potent GPR54 agonists in our previous studies. In the present study, we investigated the structure-activity relationship of a variety of pentapeptides having various fluorine-substituted benzoyl groups at the N-terminus. Among these, a 4-fluorobenzoyl derivative was the most potent agonist. On the other hand, the derivatives having multiple fluoro-substituting groups showed less binding affinity. NMR analysis of these peptides and their N-terminal partial structures suggested that fluorine substituents affect the benzoyl conformation. o-Monofluorobenzoyl is likely to be in a coplanar conformation due to the intramolecular CF—HN hydrogen bonding between o-fluorine and amide hydrogen; the o,o-difluorobenzoyl moiety exists in a distorted conformation probably due to the steric hindrance and/or electrostatic repulsion between two o-fluorine atoms and carbonyl oxygen. © *2008 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 90: 503–511, 2008.*

Keywords: GPR54; kisspeptin; structure-activity relationship; CF—HN hydrogen bonding

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INTRODUCTION

GPR54[1] (hOT7T175,[2] AXOR12[3]) was originally identified as an orphan receptor with sequence homologies (>40%) to galanin receptors. Although galanin does not bind to GPR54, endogenous peptides, designated metastins[2] or kisspeptins,[4] were isolated as natural ligands from human placenta. Kisspeptins, including N-terminally truncated analogues (kisspeptin-14, -13), share a common C-terminal decapeptide sequence and an Arg-Phe-NH_2 motif of the RF-amide peptide family.[5] The GPR54/kisspeptin system initially attracted interest in cancer biology because kisspeptins are derived from a single precursor protein encoded by a metastasis-suppressor gene *KiSS-1*.[6,7] Several groups independently demonstrated a correlation between increased *KiSS-1* expression and disease progression in thyroid,[8] breast[9] and hepatocellular[10] cancers. Other studies indicated that kisspeptins attenuated the mobility and metastatic behavior of several cancer cells that express GPR54, including melanoma,[2] thyroid,[11] and pancreatic cancer cell lines.[12] Although GPR54's anti-metastatic mechanism has not been determined, recent research suggests that GPR54 signaling suppresses cancer metastasis by induction of apoptosis in malignant cells[13] and by blocking CXCR4-mediated signaling and chemotaxis.[14] In addition,

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kisspeptins suppressed the expression and proteolytic activity of matrix-metalloproteinases,[15,16] which are known to be relevant to cancer metastasis. Thus, these indicate a therapeutic potential of GPR54 agonists as anti-metastatic agents.

On the other hand, the GPR54/kisspeptin system in puberty and reproduction has been intensively investigated, starting from the report that GPR54 loss-of-function mutations cause idiopathic hypogonadotropic hypogonadism (IHH) and deficient pubertal onset.[17–20] After this breakthrough, a number of studies revealed roles of kisspeptins as a gatekeeper of hormonal secretion. Peripheral and central administration of kisspeptins induces the production of gonadotropin-releasing hormone (GnRH)[21,22] and elicits GnRH-dependent luteinizing hormone (LH) secretion.[23–26] Recent evidence indicates that GPR54-mediated stimulation of the hypothalamic-pituitary-gonadal axis is required for the onset of puberty,[27] which is triggered by the direct action of kisspeptin on GnRH neurons.[28] Therefore, GPR54 agonists could be potential agents for puberty initiation in the case of pubertal delay and for induction of ovulation in those with anovulation.[29]

In spite of the potential utility of GPR54 agonists as anti-metastasis agents and for hormonal therapy, there are only a few reports on the development of the GPR54 ligands.[30] In recent years, we have engaged in the development of novel GPR54 agonists with low molecular-weight. Through the structure-activity relationship study on kisspeptin-10, the most potent GPR54 agonistic kisspeptin, we identified several pentapeptide analogues as novel GPR54 agonists.[31–34] These possess an N-terminal aromatic acyl group as a pharmacophore for GPR54 activation, and the substituents on the aromatic ring influence the potency of pentapeptide analogues for GPR54 activation.[34] Previous quantitative structure-activity relationship (QSAR) analysis indicated that electron-withdrawing substituents were favored at the *p*-position on N-terminal benzoyl group for potent GPR54 agonistic activities.[34] For example, 4-fluorobenzoyl analogue **1** was the most potent agonist (EC_{50} = 0.69 n*M*) among pentapeptide analogues (see Figure 1). On the other hand, pentafluorobenzoyl analogue **2**, which was designed on the expectation that an electron-deficient aromatic group may contribute to high bioactivity, did not exhibit significant agonistic activity (EC_{50} > 100 n*M*). These fluorobenzoyl moieties are observed in several bioactive compounds such as carbonic anhydrase inhibitors[35] and CXCR4 antagonists.[36] In the present study, we evaluated the GPR54 binding affinities and functional activity of fluorinated benzoyl pentapeptides to elucidate the effects of fluorine-substituent(s) on benzoyl moieties and to obtain the information for the development of novel GPR54 ligands. In addition, in order to investigate the conformational preference and limitation of the benzoyl moieties within pentapeptide derivatives, NMR analysis of peptides and their N-terminal partial structures was carried out.

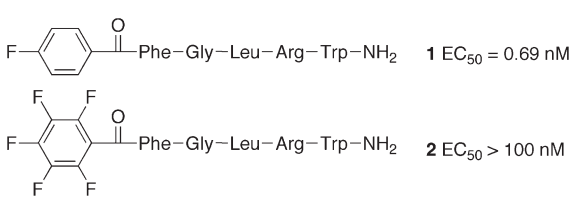

FIGURE 1 Structures of pentapeptide GPR54 ligands developed in the preceding research; 4-fluorobenzoyl **1** and pentafluorobenzoyl analogues **2**.

RESULTS AND DISCUSSION

Synthesis and Biological Evaluation of Pentapeptide Derivatives

The method of synthesis of pentapeptide derivatives was essentially the same as that reported previously.[34] After construction of pentapeptides on Rink-amide resin by Fmoc-based solid-phase peptide synthesis, the resulting N-terminal amino group was condensed with fluorinated benzoic acids by the same method as amino acid coupling. Peptide release from the solid-support and final deprotection were conducted by treatment with 1*M* TMSBr-thioanisole/TFA cocktail in the presence of 1,2-ethanedithiol and *m*-cresol as scavengers. RP-HPLC purification followed by freeze-drying afforded the desired pentapeptide derivatives as a powder of TFA salts. GPR54 binding affinity of the peptides was evaluated by competitive binding assay using [^{125}I]kisspeptin-15.[2,37] *Q* values were calculated as IC_{50}(compound)/IC_{50} (kisspeptin-10) to compare the binding affinity among GPR54 ligands. To identify whether the ligands function as agonists or antagonists, the agonistic activity was evaluated by monitoring the intracellular Ca^{2+} ion flux induced by GPR54 activation.[32] Relative maximum agonistic activity (% activity) induced by 1 μM of each peptide was calculated against the maximum signal induced by the addition of 10 n*M* kisspeptin-10. All biological values are summarized in Table I.

Among a series of pentapeptide derivatives, the most potent receptor binding affinity was observed with 4-fluorobenzoyl analogue **1** ($Q = 3.5$). Although the receptor binding affinity of pentapeptides differs with the number of fluorine substitutions on the benzoyl group, Ca^{2+} ion flux levels induced by high concentration (1 μM) of each compound were similar, indicating that these peptides are full agonists. These results suggest that GPR54 agonistic activity mainly correlated with receptor binding affinity such that a similar

Table I Receptor Binding Affinities and Agonistic Activities of N-Terminal Fluorobenzoyl Pentapeptide GPR54 Agonists (R-Phe-Gly-Leu-Arg-Trp-NH_2) and Kisspeptin-10

Peptide [R][a]	IC_{50} (n*M*)[b]	*Q*[c]	% Activity[d]
3 [Bz]	0.59	5.6	108.7 ± 4.4
4 [Bz(2-F)]	0.80	12.2	112.6 ± 1.3
5 [Bz(3-F)]	0.96	14.8	109.6 ± 1.0
1 [Bz(4-F)]	0.26	3.5	125.0 ± 7.7
6 [Bz(2,3-F)]	1.1	17.3	112.7 ± 4.1
7 [Bz(2,4-F)]	0.65	10.1	118.1 ± 4.9
8 [Bz(2,5-F)]	6.7	81.6	119.0 ± 6.3
9 [Bz(3,5-F)]	14	176.6	114.8 ± 11.4
10 [Bz(2,3,4-F)]	1.7	20.3	123.1 ± 12.1
11 [Bz(2,4,5-F)]	8.7	106.4	118.3 ± 6.2
12 [Bz(2,4,6-F)]	4.4	53.2	120.4 ± 5.1
2 [Bz(2,3,4,5,6-F)]	80	1087.5	84.0 ± 5.7
Kisspeptin-10	0.065–0.11	1	—

[a] The numbers in parenthesis indicate the substituted positions with fluorine atom(s).

[b] IC_{50} values mean the concentration needed for 50% inhibition of receptor binding of [^{125}I]kisspeptin-15.

[c] *Q* values are calculated as $Q = IC_{50}$(compound)/IC_{50}(kisspeptin-10).

[d] % Activities are based on the relative maximum agonistic activity induced by 1 μM of the compounds (%). Maximum agonistic activity signal at 10 n*M* kisspeptin-10 was used as reference (100%).

correlation was reported on alanine-scanning of kisspeptin-10 by Orsini et al.[30]

We investigated the relationship between the fluorine-substituted position and the binding affinity (Table II). Modification at *o*-position with fluorine led to a decrease in GPR54 binding affinity by 0.19–0.86 fold. In particular, transformation of *o*-monofluorinated analogue **7** into *o,o*-difluorinated analogue **12** led the decrease (0.19-fold) of binding affinity compared with other *o*-fluorination (0.34–0.86 fold). Substitution with fluorine at the *m*-position was also unfavorable for receptor binding. Modification of this position led to decreased bioactivity, indicating that *m*-substituent including electronegative functional groups is disadvantageous for potent bioactivity. On the other hand, *p*-fluorination was preferred in the absence of substituents at the *m*-position (1.2–1.6 fold), while *m,p*-difluoro derivatives exhibited lower binding affinity (0.46–0.85 fold). Although a previous QSAR study demonstrated that an inductively electronegative functional group is favorable at the *p*-position,[34] an additional *o*- or *m*-substituted fluorine is disadvantageous for bioactivity.

NMR Study on the N-Terminal Partial Structure and Its Parent Compound

It has been reported that the *o*-fluorine atom on the benzamide structure could form a hydrogen bonding with an intramolecular amide hydrogen,[38–40] which would limit C—C bond rotation of the benzoyl moiety. To clarify the structure-activity relationship, conformations of fluorobenzoyl moieties were investigated by NMR and IR.

N-benzoylphenylalanine methyl esters **13–24**, which are N-terminal partial structures of pentapeptide GPR54 agonists, were analyzed to identify whether N-terminal benzamide moieties could form an intramolecular CF—HN interaction. The esters **13–24** were prepared by condensation of substituted benzoic acids and phenylalanine methyl ester. NMR spectra of the phenylalanine derivatives **13–24** were measured in $CDCl_3$ and dimethylsulfoxide (DMSO) (Table III). These solvents correspond to the hydrophobic and hydrophilic environments,[41,42] respectively, in which the ligands may exist for binding to the receptor. According to the results from NMR study in $CDCl_3$, the molecules **13–24** may be classified into three groups: *o*-unsubstituted (**13**, **15**, **16**, **20**), *o*-monofluorinated (**14**, **17–19**, **21**, **22**), and *o,o*-difluorinated derivatives (**23**, **24**). An additional long-range spin–spin coupling between the aromatic fluorine and phenylalanine α-proton was observed only in *o*-monofluorinated derivatives, which supports the presence of interaction between the fluorine and the amide hydrogen (the long-range *J* coupling in 2-fluoro-*N*-methylbenzamide was reported[38]). In addition, a significant downfield shift of NH signals was observed in *o*-monofluorinated derivatives compared to *o*-unsubstituted and *o,o*-difluorinated derivatives

Table II Comparative Binding Affinity of Pentapeptide Analogues (R-Phe-Gly-Leu-Arg-Trp-NH_2) to GPR54 by an Additional Fluorination

Peptide [R]	*Q*[a]	Peptide [R]	*Q*[a]
o-substitution			
3 [Bz]	5.6	**4** [Bz(2-F)]	12.2
5 [Bz(3-F)]	14.8	**6** [Bz(2,3-F)]	17.3
1 [Bz(4-F)]	3.5	**7** [Bz(2,4-F)]	10.1
7 [Bz(2,4-F)]	10.1	**12** [Bz(2,4,6-F)]	53.2
m-substitution			
3 [Bz]	5.6	**5** [Bz(3-F)]	14.8
4 [Bz(2-F)]	12.2	**6** [Bz(2,3-F)]	17.3
4 [Bz(2-F)]	12.2	**8** [Bz(2,5-F)]	81.6
5 [Bz(3-F)]	14.8	**9** [Bz(3,5-F)]	106.4
7 [Bz(2,4-F)]	10.1	**10** [Bz(2,3,4-F)]	20.3
7 [Bz(2,4-F)]	10.1	**11** [Bz(2,4,5-F)]	176.6
p-substitution			
3 [Bz]	5.6	**1** [Bz(4-F)]	3.5
4 [Bz(2-F)]	12.2	**7** [Bz(2,4-F)]	10.1
6 [Bz(2,3-F)]	17.3	**10** [Bz(2,3,4-F)]	20.3
8 [Bz(2,5-F)]	81.6	**11** [Bz(2,4,5-F)]	176.6

[a] *Q* values are calculated as $Q = IC_{50}$(compound)/IC_{50}(kisspeptin-10).

Table III ^{1}H NMR Data of *N*-Benzoyl Phenylalanine Derivatives 13–24[a]

	$CDCl_3$			DMSO		
Compound [R]	δ_{NH}	$\Delta(\delta_{NH})^b$	αH^c	δ_{NH}	$\Delta(\delta_{NH})^b$	αH^c
13 [Bz]	6.53	0.00	ddd	8.76	0.00	ddd
14 [Bz(2-F)]	7.12	+0.59	dddd	8.59	−0.18	ddd
15 [Bz(3-F)]	6.49	−0.04	ddd	8.88	+0.12	ddd
16 [Bz(4-F)]	6.45	−0.08	ddd	8.81	+0.05	ddd
17 [Bz(2,3-F)]	7.00	+0.47	dddd	8.83	+0.07	ddd
18 [Bz(2,4-F)]	7.05	+0.54	dddd	8.62	−0.15	ddd
19 [Bz(2,5-F)]	7.16	+0.63	dddd	8.72	−0.04	ddd
20 [Bz(3,5-F)]	6.44	−0.09	ddd	8.97	+0.21	ddd
21 [Bz(2,3,4-F)]	6.94	+0.41	dddd	8.83	+0.07	ddd
22 [Bz(2,4,5-F)]	7.07	+0.54	dddd	8.72	−0.05	ddd
23 [Bz(2,3,6-F)]	6.41	−0.12	ddd	9.16	+0.40	ddd
24 [Bz(2,3,4,5,6-F)]	6.41	−0.12	ddd	9.37	+0.61	ddd

[a] Spectra were measured in 1 m*M* solution at 37°C.
[b] The downfield change of the chemical shift relative to that of **13**.
[c] Peak shape of α-proton in the phenylalanine moiety (d = doublet).

(see Figure 2). This result also supports the presence of intramolecular hydrogen bonding in the *o*-monofluorinated derivatives (CF—HN hydrogen bonding), resulting in the coplanar phenyl ring and planar amide groups (see Figure 3). CF—HN hydrogen bonding of *o*-monofluorinated derivatives was also suggested by the IR spectra (ATR), in which NH stretching frequency was shifted to higher wavenumbers (3400–3500 cm^{-1}).[43] In addition, the expected NH stretching frequency around 3300–3400 cm^{-1} was also observed (see experimental section). Detection of two NH stretching frequencies implies the concomitant presence of two conformations with and without intramolecular hydrogen bonding in *o*-monofluorinated derivatives.[44] On the other hand, no evidence indicating the presence of the intramolecular interaction within *o,o*-difluorinated derivatives was obtained in NMR and IR spectra. This seems to be attributed to the steric hindrance and/or electrostatic repulsion between two *o*-fluorine atoms and carbonyl oxygen of the amide.[45] This distorted conformation may contribute to the low receptor binding affinity of pentafluorobenzoyl derivative **2**.

Next, NMR analysis of the phenylalanine derivatives **13–24** was conducted in DMSO, a representative hydrophilic environment. In all spectra including *o*-monofluorinated derivatives, no long-range spin-spin couplings were observed, which indicated the absence of intramolecular hydrogen bonding between *o*-fluorine and amide hydrogen in DMSO.[38] Weak H-F(sp^2) hydrogen bonding[46] may be disturbed in DMSO because of the hydrophilic character.[38] No significant differences of chemical shifts of amide protons were observed between *o*-monofluorinated and *o*-unsubstituted derivatives, while chemical shifts of the amide protons in *o,o*-difluorinated derivatives were significantly shifted to lower fields (see Figure 2), which was in sharp contrast to the experiments in $CDCl_3$.

NMR analysis of whole structures of pentapeptide GPR54 agonists was also conducted in DMSO (Table IV). Chemical shifts of the phenylalanine amide proton were in the range of 8.30–9.14 ppm among these peptides, while amide protons of the other amino acids were similar. Similar characteristic chemical shifts were also observed among the phenylalanine α-protons. These findings suggest that fluorine substituents influence solely the environment around the N-terminal. No couplings indicating the presence of a CF—HN interaction were observed in the NMR spectra of *o*-unsubstituted (**1**, **3**, **5**, and **9**) and *o,o*-difluorinated derivatives (**2** and **12**), which is consistent with the data from model experiments using **13–24**. On the other hand, additional *J* coupling of phenylalanine amide hydrogen was observed in four *o*-monofluori-

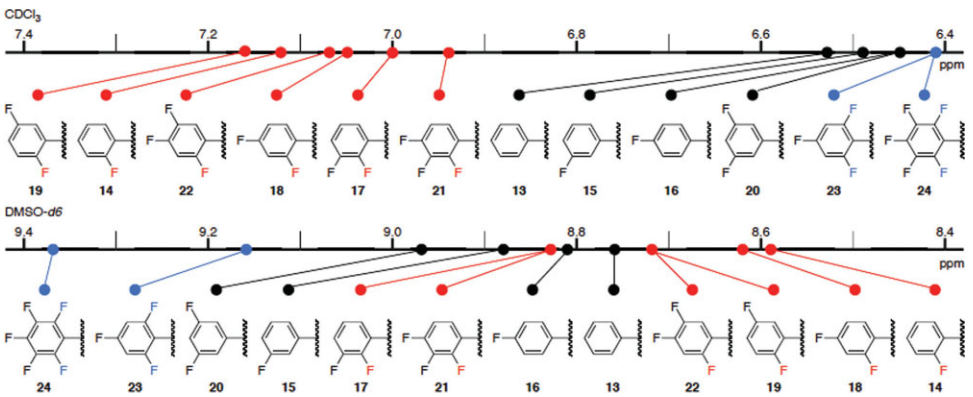

FIGURE 2 Chemical shifts (ppm) of amide proton in *N*-benzoyl phenylalanine derivatives **13–24** in $CDCl_3$ or DMSO-d_6. *o*-Unsubstituted, *o*-monofluorinated and *o,o*-difluorinated analogues are represented by black, red, and blue dots, respectively.

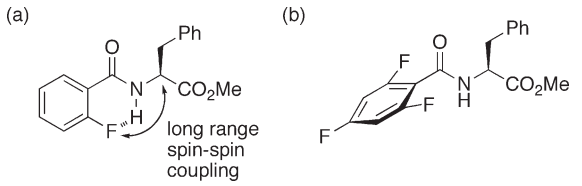

FIGURE 3 Plausible conformations of *N*-benzoyl phenylalanine derivatives in chloroform. (a) Coplanar structure of *o*-monofluorinated derivative **14**. (b) Distorted conformation of *o*,*o*-difluorinated derivative **23**.

nated analogues (**4**, **7**, **8**, and **11**). This coupling indicates the presence of an intramolecular interaction between the amide hydrogen and *o*-fluorine, which could be assisted by the C-terminal tetrapeptide region. No similar intramolecular CF—HN interaction was observed in two other *o*-monofluorinated analogues **6** and **10** having additional fluorine adjacent to the *o*-fluorine. The additional fluorine may disturb the formation of intramolecular CF—HN hydrogen bonding by reducing the electron density of the *o*-fluorine atom. These conformational restrictions of the N-terminal benzoyl group by fluorination could affect molecular recognition by GPR54.

CONCLUSIONS

A structure-activity relationship study on *N*-fluorobenzoyl pentapeptides with GPR54 agonistic activities was carried out. Multiple fluoro-substitutions on the benzoyl group led to a decrease in binding affinity for GPR54. In particular, fluorine substitutions at the *o*- or *m*-position were unfavorable to the bioactivity. On the other hand, maximum agonistic activity is independent of the fluorine substituents on the benzoyl group. NMR-based analyses of the N-terminal partial structures and their parent pentapeptides demonstrated that the *o*-monofluorobenzoyl moiety predominantly exhibits a planar conformation by forming an intramolecular interaction between fluorine and amide hydrogen, while the *o*,*o*-difluorobenzoyl moiety exists in a distorted conformation. These results indicate that both electron-withdrawing and steric effects by fluorine substituents on the benzamide moiety could control the peptide conformation and bioactivity. Information from this research is useful for development of novel GPR54 ligands.

EXPERIMENTAL SECTION

General Synthetic Approach

IR spectra were determined on a JASCO FT/IR-4100 spectrometer. ^{1}H NMR spectra were recorded using a JEOL AL-400 spectrometer. Chemical shifts are reported in δ(ppm) relative to Me_4Si (in $CDCl_3$, DMSO-d_6) as internal standard. ^{13}C NMR spectra were recorded using a JEOL AL-400 and referenced to the residual $CHCl_3$ signal. ^{19}F NMR spectra were recorded using a JEOL AL-400 and referenced to the internal $CFCl_3$ (δ_F 0.00 ppm). Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer. Optical rotations were measured with a JASCO P-1020 polarimeter. Melting points (uncorrected) were measured by a hot stage melting point apparatus. For column chromatography, Wakosil C-300 was employed. For HPLC separations, a Cosmosil 5C18-ARII analytical (4.6 × 250 mm, flow rate 1 mL/min) column or a Cosmosil 5C18-ARII preparative (20 × 250 mm, flow rate 10 mL/min) column was

Table IV ^{1}**H NMR Data of Pentapeptide Analogues (R-Phe-Gly-Leu-Arg-Trp-NH$_2$)**[a]

	Phe				Gly		Leu		Arg		Trp	
Compound [R]	δ_{NH}	$\Delta(\delta_{NH})$[b]	H_N[c]	$\delta_{\alpha H}$	δ_{NH}	$\delta_{\alpha H}$	δ_{NH}	$\delta_{\alpha H}$	δ_{NH}	$\delta_{\alpha H}$	δ_{NH}	$\delta_{\alpha H}$
3 [Bz]	8.59	0.00	d	4.66	8.35	3.71, 3.79	7.81	4.32	8.01	4.25	7.73	4.46
4 [Bz(2-F)]	8.30	−0.29	dd	4.71	8.35	3.74, 3.80	7.86	4.33	8.03	4.25	7.73	4.46
5 [Bz(3-F)]	8.71	+0.12	d	4.68	8.37	3.71, 3.79	7.79	4.33	8.02	4.25	7.73	4.46
1 [Bz(4-F)]	8.64	+0.05	d	4.66	8.36	3.71, 3.79	7.80	4.32	8.02	4.25	7.73	4.46
6 [Bz(2,3-F)]	8.56	−0.03	d	4.71	8.36	3.75, 3.80	7.86	4.34	8.04	4.25	7.73	4.46
7 [Bz(2,4-F)]	8.32	−0.27	dd	4.70	8.36	3.74, 3.80	7.85	4.33	8.03	4.25	7.73	4.46
8 [Bz(2,5-F)]	8.45	−0.14	dd	4.70	8.36	3.75, 3.80	7.85	4.34	8.03	4.25	7.73	4.46
9 [Bz(3,5-F)]	8.81	+0.22	d	4.68	8.39	3.71, 3.79	7.79	4.33	8.03	4.25	7.73	4.46
10 [Bz(2,3,4-F)]	8.56	−0.03	d	4.71	8.38	3.75, 3.81	7.87	4.34	8.05	4.25	7.74	4.46
11 [Bz(2,4,5-F)]	8.45	−0.14	dd	4.70	8.37	3.74, 3.80	7.85	4.34	8.04	4.25	7.74	4.46
12 [Bz(2,3,6-F)]	8.94	+0.35	d	4.73	8.32	3.78, 3.78	7.88	4.34	8.05	4.25	7.74	4.46
2 [Bz(2,3,4,5,6-F)]	9.14	+0.55	d	4.80	8.43	3.79, 3.79	7.92	4.35	8.07	4.26	7.74	4.46

[a] Spectra were measured in 1 m*M* solution at 37°C in DMSO.
[b] The downfield change of the chemical shift relative to that of **3**.
[c] Peak shape of amido proton of the phenylalanine (d = doublet).

employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA solution (v/v) and 0.1% TFA in CH_3CN (v/v) was used for HPLC elution.

General Synthetic Procedure of Benzoyl Pentapeptide Analogues 1-12

The protected peptide-resin was manually constructed using Fmoc-based solid-phase synthesis on a Rink amide-resin (0.60 mmol/g, 83 mg, 0.05 mmol). Fmoc-protected amino acid and benzoic acid derivatives (0.15 mmol, 3.0 equiv.) were successively condensed using 1,3-diisopropylcarbodiimide (DIPCDI; 23 μL, 0.15 mmol, 3.0 equiv.) in the presence of *N*-hydroxybenzotriazole monohydrate ($HOBt \cdot H_2O$; 46 mg, 0.3 mmol, 6.0 equiv.). The resulting protected resin was treated with 1*M* TMSBr-thioanisole/TFA (3.6 mL) in the presence of *m*-cresol (200 μL, 38 equiv.) and 1,2-ethanedithiol (200 μL, 48 equiv.) at 4°C for 1 h. After removal of the resin by filtration, the filtrate was poured into ice-cold dry diethyl ether (45 mL). The resulting powder was collected by centrifugation and then washed three times with ice-cold dry diethyl ether (45 mL x 3). The crude product was purified by preparative HPLC to afford the expected peptide as a white powder.

Bz(2-F)-Phe-Gly-Leu-Arg-Trp-NH_2 (4). 2-Fluorobenzoic acid (21 mg, 0.15 mmol, 3.0 equiv.) was used for N-terminal acylation. Title peptide **4** was yielded as a TFA salt (14.0 mg, 31% yield from Rink-amide resin): $[\alpha]_D^{21}$ −10.4 (c 0.10, CH_3OH); HRMS (FAB), *m/z* calcd for $C_{41}H_{52}FN_{10}O_6$ $(M+H^+)$ 799.4050, found: 799.4048.

Bz(3-F)-Phe-Gly-Leu-Arg-Trp-NH_2 (5). 3-Fluorobenzoic acid (21 mg, 0.15 mmol, 3.0 equiv.) was used for N-terminal acylation. Title peptide **5** was yielded as a TFA salt (14.4 mg, 32% yield from Rink-amide resin): $[\alpha]_D^{21}$ −13.5 (c 0.11, CH_3OH); HRMS (FAB), *m/z* calcd for $C_{41}H_{52}FN_{10}O_6$ $(M+H^+)$ 799.4050, found: 799.4044.

Bz(2,3-F)-Phe-Gly-Leu-Arg-Trp-NH_2 (6). 2,3-Difluorobenzoic acid (24 mg, 0.15 mmol, 3.0 equiv.) was used for N-terminal acylation. Title peptide **6** was yielded as a TFA salt (16.1 mg, 35% yield from Rink-amide resin): $[\alpha]_D^{21}$ −12.4 (c 0.10, CH_3OH); HRMS (FAB), *m/z* calcd for $C_{41}H_{51}F_2N_{10}O_6$ $(M+H^+)$ 817.3956, found: 817.3960.

Bz(2,4-F)-Phe-Gly-Leu-Arg-Trp-NH_2 (7). 2,4-Difluorobenzoic acid (24 mg, 0.15 mmol, 3.0 equiv.) was used for N-terminal acylation. Title peptide **7** was yielded as a TFA salt (14.7 mg, 32% yield from Rink-amide resin): $[\alpha]_D^{21}$ −16.4 (c 0.11, CH_3OH); HRMS (FAB), *m/z* calcd for $C_{41}H_{51}F_2N_{10}O_6$ $(M+H^+)$ 817.3956, found: 817.3954.

Bz(2,5-F)-Phe-Gly-Leu-Arg-Trp-NH_2 (8). 2,5-Difluorobenzoic acid (24 mg, 0.15 mmol, 3.0 equiv.) was used for N-terminal acylation. Title peptide **8** was yielded as a TFA salt (15.7 mg, 34% yield from Rink-amide resin): $[\alpha]_D^{21}$ −7.7 (c 0.10, CH_3OH); HRMS (FAB), *m/z* calcd for $C_{41}H_{51}F_2N_{10}O_6$ $(M+H^+)$ 817.3956, found: 817.3955.

Bz(3,5-F)-Phe-Gly-Leu-Arg-Trp-NH_2 (9). 3,5-Difluorobenzoic acid (24 mg, 0.15 mmol, 3.0 equiv.) was used for N-terminal acylation. Title peptide **9** was yielded as a TFA salt (12.2 mg, 26% yield from Rink-amide resin): $[\alpha]_D^{21}$ −13.1 (c 0.11, CH_3OH); HRMS (FAB), *m/z* calcd for $C_{41}H_{51}F_2N_{10}O_6$ $(M+H^+)$ 817.3956, found: 817.3964.

Bz(2,3,4-F)-Phe-Gly-Leu-Arg-Trp-NH_2 (10). 2,3,4-Trifluorobenzoic acid (26 mg, 0.15 mmol, 3.0 equiv.) was used for N-terminal acylation. Title peptide **10** was yielded as a TFA salt (14.0 mg, 30% yield from Rink-amide resin): $[\alpha]_D^{21}$ −11.9 (c 0.11, CH_3OH); HRMS (FAB), *m/z* calcd for $C_{41}H_{50}F_3N_{10}O_6$ $(M+H^+)$ 835.3861, found: 835.3868.

Bz(2,4,5-F)-Phe-Gly-Leu-Arg-Trp-NH_2 (11). 2,4,5-Trifluorobenzoic acid (26 mg, 0.15 mmol, 3.0 equiv.) was used for N-terminal acylation. Title peptide **11** was yielded as a TFA salt (13.2 mg, 28% yield from Rink-amide resin): $[\alpha]_D^{21}$ −7.0 (c 0.12, CH_3OH); HRMS (FAB), *m/z* calcd for $C_{41}H_{50}F_3N_{10}O_6$ $(M+H^+)$ 835.3861, found: 835.3856.

Bz(2,4,6-F)-Phe-Gly-Leu-Arg-Trp-NH_2 (12). 2,4,6-Trifluorobenzoic acid (26 mg, 0.15 mmol, 3.0 equiv.) was used for N-terminal acylation. Title peptide **12** was yielded as a TFA salt (18.1 mg, 38% yield from Rink-amide resin): $[\alpha]_D^{21}$ −3.0 (c 0.12, CH_3OH); HRMS (FAB), *m/z* calcd for $C_{41}H_{50}F_3N_{10}O_6$ $(M+H^+)$ 835.3861, found: 835.3867.

General Synthetic Procedure of Methyl (*S*)-2-(*N*-benzoylamino)-3-Phenylpropionate Derivatives 13-24

$HOBt \cdot H_2O$ (1.0 equiv.), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride ($EDC \cdot HCl$; 1.0 equiv.), *N,N*-diisopropylethylamine (*i*-Pr_2NEt; 4.0 equiv.), and methyl (*S*)-2-amino-3-phenylpropionate hydrochloride salt ($HCl \cdot$H-Phe-OMe; 1.0 equiv.) were successively added to the solution of a substituted benzoic acid (1.0 equiv.) in DMF (0.1*M*) at 4°C, and the mixture was stirred for 12 h at room temperature. After removal of DMF under the reduced pressure, the crude residue was extracted with EtOAc. The extract was washed with saturated citric acid, brine, 5% $NaHCO_3$, brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by chromatography over silica gel with *n*-hexane-EtOAc (2:1) gave the desired compound.

Methyl (S)-2-(N-benzoylamino)-3-phenylpropionate (13) [Bz-Phe-OMe]. The title compound **13** was obtained from benzoic acid (916 mg, 7.50 mmol) as colorless crystals (1.99 g, 94% yield): mp 83–85°C; $[\alpha]_D^{19}$ +70.5 (c 0.57, CH_2Cl_2); FTIR (ATR) n 3322 (CONH), 1741 (C=O), 1640 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.23 (dd, J = 13.9, 5.6 Hz, 1H), 3.30 (dd, J = 13.9, 5.6 Hz, 1H), 3.76 (s, 3H), 5.09 (ddd, J = 7.6, 5.6, 5.6 Hz, 1H), 6.59 (d, J = 7.6 Hz, 1H), 7.10–7.77 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.9, 52.4, 53.5, 127.0, 127.2, 128.6, 128.6, 129.3, 131.7, 133.9, 135.8, 166.8, 172.0. Anal. Calcd for $C_{17}H_{17}NO_3$: H, 6.05; C, 72.07; N, 4.94. Found: H, 6.05; C, 71.83; N, 4.94.

Methyl (S)-2-[N-(2-fluorobenzoyl)amino]-3-phenylpropionate (14) [Bz(2-F)-Phe-OMe]. The title compound **14** was obtained from 2-fluorobenzoic acid (1.05 g, 7.50 mmol) as colorless crystals (2.07 g, 92% yield): mp 57–59°C; $[\alpha]_D^{20}$ +40.4 (c 0.60, CH_2Cl_2); FTIR (ATR) n 3445 (CONH), 3313 (CONH), 1742

(C=O), 1660 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.21 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.28 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.75 (s, 3H), 5.05–5.12 (m, 1H), 7.06–8.10 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.9, 52.4, 53.9, 116.1, 120.5, 124.7, 127.2, 128.6, 129.2, 132.0, 133.5, 135.8, 160.8, 162.8, 171.7; ^{19}F NMR (375 MHz, $CDCl_3$) δ −113.5. Anal. Calcd for $C_{17}H_{16}FNO_3$: H, 5.35; C, 67.76; N, 4.65. Found: H, 5.34; C, 67.71; N, 4.61.

Methyl (S)-2-[N-(3-fluorobenzoyl)amino]-3-phenylpropionate (15) [Bz(3-F)-Phe-OMe]. The title compound **15** was obtained from 3-fluorobenzoic acid (1.05 g, 7.50 mmol) as colorless crystals (2.24 g, 99% yield): mp 70–72°C; $[\alpha]_D^{20}$ +74.2 (c 0.60, CH_2Cl_2); FTIR (ATR) n 3325 (CONH), 1742 (C=O), 1644 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.22 (dd, $J = 13.9$, 5.6 Hz, 1H), 3.29 (dd, $J = 13.9$, 5.6 Hz, 1H), 3.77 (s, 3H), 5.07 (ddd, $J = 7.6$, 5.6, 5.6 Hz, 1H), 6.58 (d, $J = 7.6$ Hz, 1H) 7.15–7.73 (m, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.8, 52.5, 53.6, 114.4, 118.8, 122.4, 127.3, 128.7, 129.3, 130.3, 135.7, 136.2, 162.7, 165.5, 171.9; ^{19}F NMR (375 MHz, $CDCl_3$) δ −112.1. Anal. Calcd for $C_{17}H_{16}FNO_3$: H, 5.35; C, 67.76; N, 4.65. Found: H, 5.37; C, 67.88; N, 4.57.

Methyl (S)-2-[N-(4-fluorobenzoyl)amino]-3-phenylpropionate (16) [Bz(4-F)-Phe-OMe]. The title compound **16** was obtained from 4-fluorobenzoic acid (1.05 g, 7.50 mmol) as colorless crystals (2.18 g, 97% yield): mp 81–83°C; $[\alpha]_D^{20}$ +56.0 (c 0.60, CH_2Cl_2); FTIR (ATR) n 3310 (CONH), 1742 (C=O), 1639 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.21 (dd, $J = 13.9$, 5.6 Hz, 1H), 3.29 (dd, $J = 13.9$, 5.6 Hz, 1H), 3.77 (s, 3H), 5.07 (ddd, $J = 7.6$, 5.6, 5.6 Hz, 1H), 6.55 (d, $J = 7.6$ Hz, 1H) 7.05–7.77 (m, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.8, 52.4, 53.5, 115.6, 127.2, 128.6, 129.3, 129.3, 130.0, 135.7, 164.9, 165.7, 172.0; ^{19}F NMR (375 MHz, $CDCl_3$) δ −108.1. Anal. Calcd for $C_{17}H_{16}FNO_3$: H, 5.35; C, 67.76; N, 4.65. Found: H, 5.36; C, 67.51; N, 4.53.

Methyl (S)-2-[N-(2,3-difluorobenzoyl)amino]-3-phenylpropionate (17) [Bz(2,3-F)-Phe-OMe]. The title compound **17** was obtained from 2,3-difluorobenzoic acid (316 mg, 2.00 mmol) as colorless crystals (589 mg, 92% yield): mp 79–81°C; $[\alpha]_D^{20}$ +47.4 (c 0.64, CH_2Cl_2); FTIR (ATR) n 3438 (CONH), 3311 (CONH), 1742 (C=O), 1660 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.21 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.29 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.76 (s, 3H), 5.08 (dddd, $J = 7.6$, 5.9, 5.9, 1.7 Hz, 1H), 6.97–7.09 (m, 1H) 7.12–7.80 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.8, 52.4, 54.0, 120.5, 122.8, 124.3, 126.3, 127.3, 128.7, 129.2, 135.6, 149.1, 150.5, 161.8, 171.5; ^{19}F NMR (375 MHz, $CDCl_3$) δ −139.7, −138.0. Anal. Calcd for $C_{17}H_{15}F_2NO_3$: H, 4.74; C, 63.95; N, 4.39. Found: H, 4.82; C, 63.65; N, 4.38.

Methyl (S)-2-[N-(2,4-difluorobenzoyl)amino]-3-phenylpropionate (18) [Bz(2,4-F)-Phe-OMe]. The title compound **18** was obtained from 2,4-difluorobenzoic acid (1.19 g, 7.50 mmol) as colorless crystals (2.24 g, 93% yield): mp 43–45°C; $[\alpha]_D^{20}$ +37.1 (c 0.64, CH_2Cl_2); FTIR (ATR) n 3444 (CONH), 3317 (CONH) 1739 (C=O), 1661 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.20 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.27 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.76 (s, 3H), 5.07 (dddd, $J = 7.6$, 5.9, 5.9, 2.0 Hz, 1H), 6.85 (ddd, $J = 12.0$, 8.5, 2.4 Hz, 1H), 6.94–7.02 (m, 1H), 7.03–7.12 (m, 1H) 7.13–7.33 (m, 5H), 8.05–8.13 (dt, $J = 9.0$, 6.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.9, 52.4, 53.9, 104.3, 112.3, 117.0, 127.2, 128.7, 129.2, 133.8, 135.7, 161.1, 161.9, 165.0, 171.7; ^{19}F NMR (375 MHz, $CDCl_3$) δ −109.1, −104.0. Anal. Calcd for $C_{17}H_{15}F_2NO_3$: H, 4.74; C, 63.95; N, 4.39. Found: H, 4.87; C, 63.80; N, 4.46.

Methyl (S)-2-[N-(2,5-difluorobenzoyl)amino]-3-phenylpropionate (19) [Bz(2,5-F)-Phe-OMe]. The title compound **19** was obtained from 2,5-difluorobenzoic acid (316 mg, 2.00 mmol) as colorless crystals (556 mg, 87% yield): mp 44–46°C; $[\alpha]_D^{21}$ +47.0 (c 0.64, CH_2Cl_2); FTIR (ATR) n 3439 (CONH), 3317 (CONH), 1739 (C=O), 1661 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.20 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.27 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.76 (s, 3H), 5.06 (dddd, $J = 7.6$, 5.9, 5.9, 2.0 Hz, 1H), 7.02–7.33 (m, 8H), 7.74 (ddd, $J = 8.8$, 5.9, 3.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.8, 52.4, 54.0, 117.5, 118.1, 120.0, 121.9, 127.2, 128.6, 129.2, 135.6, 156.6, 158.8, 161.6, 171.6; ^{19}F NMR (375 MHz, $CDCl_3$) δ −119.3, −117.5. Anal. Calcd for $C_{17}H_{15}F_2NO_3$: H, 4.74; C, 63.95; N, 4.39. Found: H, 4.83; C, 63.95; N, 4.46.

Methyl (S)-2-[N-(3,5-difluorobenzoyl)amino]-3-phenylpropionate (20) [Bz(3,5-F)-Phe-OMe]. The title compound **20** was obtained from 3,5-difluorobenzoic acid (1.19 g, 7.50 mmol) as colorless crystals (2.12 g, 88% yield): mp 84–86°C; $[\alpha]_D^{21}$ +58.3 (c 0.64, CH_2Cl_2); FTIR (ATR) n 3323 (CONH), 1743 (C=O), 1647 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.21 (dd, $J = 13.9$, 5.6 Hz, 1H), 3.29 (dd, $J = 13.9$, 5.6 Hz, 1H), 3.78 (s, 3H), 5.04 (ddd, $J = 7.6$, 5.6, 5.6 Hz, 1H), 6.58 (d, $J = 7.6$ Hz, 1H), 6.91–7.34 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.7, 52.5, 53.6, 107.1, 110.3, 127.3, 128.7, 129.2, 135.5, 137.2, 162.9, 164.4, 171.8; ^{19}F NMR (375 MHz, $CDCl_3$) δ −108.3. Anal. Calcd for $C_{17}H_{15}F_2NO_3$: H, 4.74; C, 63.95; N, 4.39. Found: H, 4.90; C, 63.83; N, 4.22.

Methyl (S)-2-[N-(2,3,4-trifluorobenzoyl)amino]-3-phenylpropionate (21) [Bz(2,3,4-F)-Phe-OMe]. The title compound **21** was obtained from 2,3,4-trifluorobenzoic acid (352 mg, 2.00 mmol) as colorless crystals (574 mg, 85% yield): mp 82–84°C; $[\alpha]_D^{21}$ +40.4 (c 0.67, CH_2Cl_2); FTIR (ATR) n 3438 (CONH), 3311 (CONH), 1742 (C=O), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.20 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.28 (dd, $J = 13.9$, 5.9 Hz, 1H), 3.76 (s, 3H), 5.06 (dddd, $J = 7.3$, 5.9, 5.9, 1.7 Hz, 1H), 6.91–7.01 (m, 1H), 7.07 (ddt, $J = 6.8$, 2.0, 8.8 Hz, 1H), 7.11–7.35 (m, 5H), 7.75–7.85 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.8, 52.5, 54.0, 112.8, 118.2, 125.8, 127.4, 128.8, 129.2, 135.5, 139.7, 150.2, 153.4, 161.1, 171.5; ^{19}F NMR (375 MHz, $CDCl_3$) δ −159.6, −135.0, −128.1. Anal. Calcd for $C_{17}H_{14}F_3NO_3$: H, 4.18; C, 60.54; N, 4.15. Found: H, 4.28; C, 60.31; N, 4.16.

Methyl (S)-2-[N-(2,4,5-trifluorobenzoyl)amino]-3-phenylpropionate (22) [Bz(2,4,5-F)-Phe-OMe]. The title compound **22** was obtained from 2,4,5-trifluorobenzoic acid (352 mg, 2.00 mmol) as colorless crystals (589 mg, 87% yield): mp 59–61°C; $[\alpha]_D^{21}$ +48.2 (c 0.67, CH_2Cl_2); FTIR (ATR) n 3450 (CONH), 3317 (CONH), 1743 (C=O), 1665 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.19 (dd, $J = 13.9$, 5.6 Hz, 1H), 3.27 (dd, $J = 13.9$, 5.6 Hz, 1H), 3.76 (s, 3H), 5.05 (dddd, $J = 7.6$, 5.6, 5.6, 2.0 Hz, 1H), 6.98 (dt, $J = 6.1$, 10.1 Hz, 1H), 7.05–7.34 (m, 6H), 7.86–7.96 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.8, 52.5, 54.0, 106.2, 117.3, 120.0, 127.3, 128.7, 129.2, 135.5, 147.2, 152.3, 156.0, 160.8, 171.5; ^{19}F

NMR (375 MHz, $CDCl_3$) δ −140.8, −127.0, −114.5. Anal. Calcd for $C_{17}H_{14}F_3NO_3$: H, 4.18; C, 60.54; N, 4.15. Found: H, 4.18; C, 60.38; N, 4.19.

Methyl (S)-2-[N-(2,4,6-trifluorobenzoyl)amino]-3-phenylpropionate (23) [Bz(2,4,6-F)-Phe-OMe]. The title compound **23** was obtained from 2,4,6-trifluorobenzoic acid (352 mg, 2.00 mmol) as colorless crystals (413 mg, 61% yield): mp 89–91°C; $[\alpha]_D^{21}$ +82.8 (c 0.67, CH_2Cl_2); IR (ATR) n 3286 (CONH), 1742 (C=O), 1639 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.21 (dd, J = 13.9, 5.6 Hz, 1H), 3.30 (dd, J = 13.9, 5.6 Hz, 1H), 3.77 (s, 3H), 5.09 (ddd, J = 7.6, 5.6, 5.6 Hz, 1H), 6.45 (d, J = 7.6 Hz,1H), 6.66–6.74 (m, 2H), 7.11–7.17 (m, 2H), 7.21–7.32 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.7, 52.5, 53.8, 101.0, 110.4, 127.2, 128.6, 129.4, 135.4, 159.0, 160.7, 163.6, 171.3; ^{19}F NMR (375 MHz, $CDCl_3$) δ −108.7, −103.9. Anal. Calcd for $C_{17}H_{14}F_3NO_3$: H, 4.18; C, 60.54; N, 4.15. Found: H, 4.15; C, 60.39; N, 4.12.

Methyl (S)-2-[N-(pentafluorobenzoyl)amino]-3-phenylpropionate (24) [Bz(2,3,4,5,6-F)-Phe-OMe]. The title compound **24** was obtained from pentafluorobenzoic acid (1.59 g, 7.50 mmol) as colorless crystals (1.05 g, 38% yield): mp 114–116°C; $[\alpha]_D^{21}$ +75.2 (c 0.75, CH_2Cl_2); FTIR (ATR) n 3314 (CONH), 1736 (C=O), 1656 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.21 (dd, J = 13.9, 5.6 Hz, 1H), 3.31 (dd, J = 13.9, 5.6 Hz, 1H), 3.78 (s, 3H), 5.09 (ddd, J = 7.6, 5.6, 5.6 Hz, 1H), 6.53 (d, J = 7.6 Hz,1H), 7.10–7.15 (m, 2H), 7.22–7.33 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.7, 52.7, 54.0, 111.0, 127.4, 128.7, 129.3, 135.1, 137.7, 141.2, 144.3, 156.9, 171.1; ^{19}F NMR (400 MHz, $CDCl_3$) δ −160.4, −150.7, −140.5. Anal. Calcd for $C_{17}H_{12}F_5NO_3$: H, 3.24; C, 54.70; N, 3.75. Found: H, 3.36; C, 54.77; N, 3.73.

Measurement of Binding Affinity

Assays were performed as previously described.[2,37] Membrane fraction was prepared using homogenizing buffer (10 m*M* $NaHCO_3$, 2 m*M* EGTA, 0.2 m*M* $MgCl_2$ protease inhibitors, pH 7.4) and stored in 50% glycerol-50% homogenizing buffer at −20°C. Kisspeptin-15 was labeled with ^{125}I-Na using lactoperoxidase, and purified to a carrier-free single peak.

Measurement of $[Ca^{2+}]_i$ Using Flipr Technology

The ability of peptides to activate human GPR54 was evaluated in CHO/dhfr cells according to the method described previously.[32]

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