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ARTICLE *in* BIOPOLYMERS · JANUARY 2007

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Fmoc-Based Solid-Phase Synthesis of GPR54-Agonistic Pentapeptide Derivatives Containing Alkene- and Fluoroalkene-Dipeptide Isosteres

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Received 8 November 2006; revised 12 December 2006; accepted 19 December 2006
Published online 10 January 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bip.20676

ABSTRACT:

Fmoc-protected Phe-Gly-type (Z)-alkene dipeptide isostere (ADI) and (E)-fluoroalkene dipeptide isostere (FADI) were synthesized and applied to Fmoc-based solid-phase peptide synthesis (SPPS). These cis-peptide bond mimetics were introduced into a bioactive pentapeptide [H-Amb-Phe-Gly-Leu-Arg-Trp-NH_2; Amb = 4-(aminomethyl) benzoic acid], which has potent GPR54 agonistic activity. The resulting pentapeptide derivatives showed low GPR54 agonistic activity, as compared with the parent peptide and (E)-ADI-containing derivative. This suggests that the trans-amide conformer of Phe-Gly peptide bond of the parent peptide would be significantly important for bioactivity. Contrary to our expectations, a (Z)-FADI-containing derivative exhibited essentially no activity, revealing the necessity of critical validation of FADI-bioisosterism. © 2007 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 88: 272–278, 2007.

Keywords: (Z)-alkene dipeptide isostere; (E)-fluoroalkene dipeptide isostere; Fmoc-based SPPS; GPR54 agonist

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Contract grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan
Contract grant sponsor: Health and Labour Sciences Research Grants
Contract grant sponsor: 21th COE Program "Knowledge Information Infrastructure for Genome Science"
This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/0006-3525/suppmat

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

INTRODUCTION

Many endogenous bioactive substances such as hormones and cytokines are involved in physiological and pathogenic processes through specific interactions with their receptors. The minimum sequences can be considered as "lead compounds" in rational drug design of small molecule agonists, antagonists, and inhibitors. Since the establishment of solid-phase peptide synthesis (SPPS) protocol, a great number of artificial bioactive peptides have been developed through structure-activity relationship (SAR) studies using commercially available natural and unnatural amino acids.[1–4] However, only a few peptide compounds have been placed on the market as therapeutics. This may be partly due to unfavorable characteristics of peptide therapeutics such as low oral bioavailability and in vivo instability caused by endogenous peptidases.

To overcome these intrinsic drawbacks of peptides, a number of peptide-bond mimetics have been developed and applied to bioactive peptides. Alkene-type dipeptide isosteres are included in the nonhydrolyzable peptide-bond mimetics, which were designed based on the planar structure of peptide bonds. Because of restricted *cis*/*trans* isomerization, alkene isosteres can be also used as molecular probes to investigate peptide bond (ω-angle) rotations.[5] Among a variety of reported alkene dipeptide isosteres including (*E*/*Z*)-alkene dipeptide isosteres (ADIs), (*E*/*Z*)-fluoroalkene dipeptide isosteres (FADIs), multisubstituted alkene dipeptide isosteres,[6,7] and trifluoromethylalkene dipeptide isosteres,[8] FADIs represent promising isosteric units because of the presence of an electronegative fluorine atom on the alkene carbon (γ-position) as a mimic of carbonyl oxygen (Figure 1).[9]

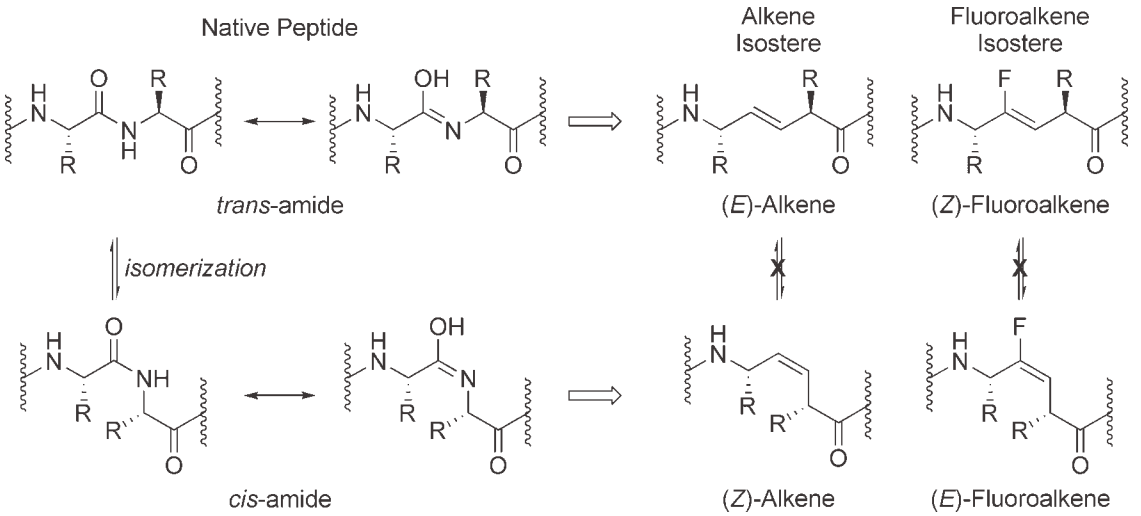


FIGURE 1 Structures of alkene and fluoroalkene dipeptide isosteres.

The synthetic methods reported for ADIs preferentially provide *trans*-amide bond mimics such as (*E*)-ADIs[10–14] and (*Z*)-FADIs.[15–20] These isosteres have been applied to bioactive peptides and peptidomimetics by employing liquid phase as well as SPPS methodologies.[21–26] For instance, we previously reported the synthesis and application of Phe-Gly-type (*E*)-ADI and (*Z*)-FADI for investigating substrate recognition by a peptide transporter, PEPT1. The *trans*-amide mimetics such as (*E*)-ADI and (*Z*)-FADI showed more potent inhibition of [^{3}H]Gly-Sar uptake in PEPT1-expressing Caco-2 cell than the *cis*-amide counterpart (*Z*)-ADI **1** and (*E*)-FADI **2**, suggesting that PEPT1 predominantly recognizes *trans*-amide conformations of dipeptides.[27]

On the other hand, a limited number of applications of *cis*-amide bond mimics possessing (*Z*)-alkene or (*E*)-fluoroalkene units into functional peptides have been reported,[28] because an efficient (*E*/*Z*)-selective synthetic method has not been established. Recently, we reported the synthesis of Phe-Gly-type (*Z*)-ADI **1** and (*E*)-FADI **2** via organocopper-mediated reduction of α,β-unsaturated-δ-lactams **5** and **6** possessing a leaving group(s) at the γ-position, which were prepared from phenylalanine-derived allylalcohol **3** and from chiral β-amino acid **4**,[16,29] respectively (Figure 2).[27] In this study, we describe the synthesis of Fmoc-protected Phe-Gly-type (*Z*)-ADI and (*E*)-FADI, and the application to the Fmoc-based SPPS of pentapeptide derivatives of a bioactive pentapeptide **9** [H-Amb-Phe-Gly-Leu-Arg-Trp-NH_2; Amb = 4-(aminomethyl)benzoic acid], which has potent GPR54 agonistic activity, to probe the conformational requirement of its Phe-Gly peptide bond.

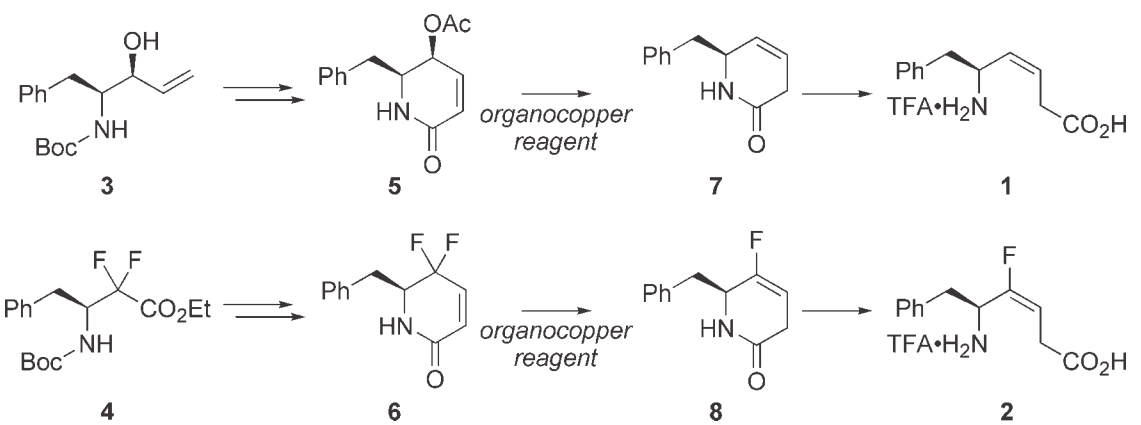
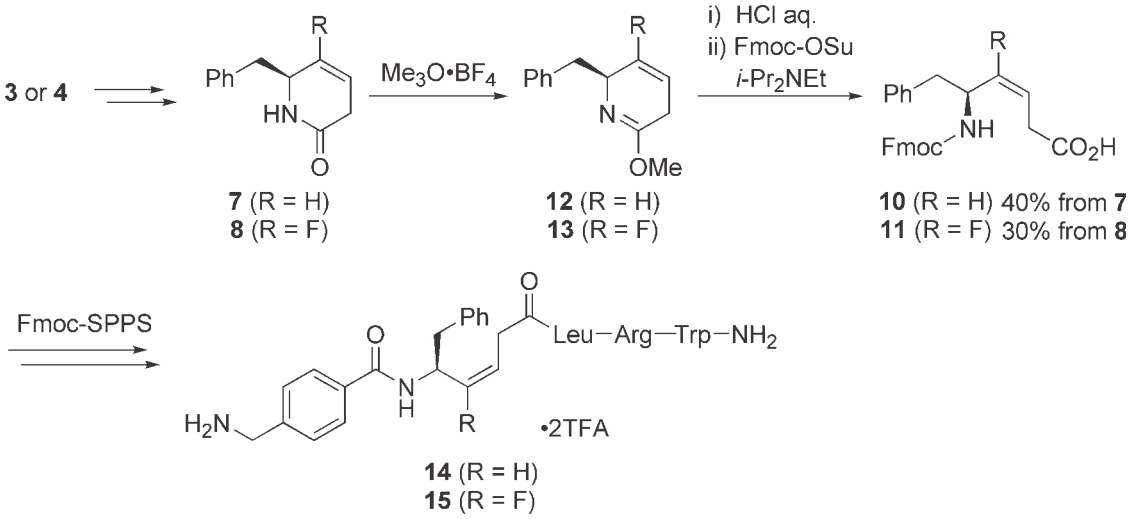


FIGURE 2 Synthesis of Phe-Gly-type (*Z*)-ADI and (*E*)-FADI.



SCHEME 1 Synthesis of Fmoc-protected Phe-Gly-type isosteres **10**, **11** and peptides **14**, **15**.

RESULTS AND DISCUSSION

Synthesis of Fmoc-Protected (*Z*)-Alkene and (*E*)-Fluoroalkene Dipeptide Isosteres

Phe-Gly dipeptide is one of the key units observed in several G-protein-coupled receptor (GPCR) ligands and their precursors. For example, C-termini of bioactive RF-amide peptides are generated from post-translational processing of the precursors containing R*F*/*G*K and R*F*/*G*R motifs,[30] while the N-terminal *FG*GF sequence of nociceptin induces the receptor agonistic signal as a message domain.[31] In addition, peptide bonds within Phe-Gly dipeptide might be hydrolyzed by endogenous enzymes,[32] leading to loss of the function or activity. Thus, Phe-Gly dipeptide mimetics could be applicable to a variety of structure-activity/function relationship studies. For this model study, application of a combination of Phe-Gly-type ADIs was attempted onto kisspeptin-derived pentapeptide carboxamide **9**, which possesses GPR54 agonistic activity.[33,34] Kisspeptin/GPR54 axis has been reported to be involved in metastasis of carcinomas[35–37] as well as secretion of gonadotropin-releasing hormones.[38–40]

To use isosteres for Fmoc-based SPPS, Fmoc-protected Phe-Gly-type (*Z*)-ADI **10** (Fmoc-Phe-ψ[(*Z*)-CH=CH]-Gly-OH) and (*E*)-FADI **11** (Fmoc-Phe-ψ[(*E*)-CF=CH]-Gly-OH) were synthesized (Scheme 1). The key intermediates, β,γ-unsaturated-δ-lactams **7** and **8**, were prepared from amino alcohol **3** and amino ester **4**, respectively.[27] Lactam **7** was converted into lactim ether **12** with $Me_3O \cdot BF_4$, which was used for the next reaction without purification. Hydrolysis of lactim ether **12** under acidic conditions[41] followed by protection of the amino group using Fmoc-OSu yielded the desired Fmoc-protected (*Z*)-ADI **10** after purification by flash chromatography (40% from **7**). Fmoc-protected (*E*)-FADI **11** was also prepared via lactim ether **13** in the same manner (30% from **8**).

Preparation of (*Z*)-ADI and (*E*)-FADI-Containing Peptides by Fmoc-Based Solid-Phase Synthesis

Next, we introduced Fmoc-protected (*Z*)-ADI **10** and (*E*)-FADI **11** into the pentapeptide carboxamide **9** according to the Fmoc-based peptide synthesis method. Protected tripeptide chain [Leu-Arg(Pbf)-Trp] of the C-terminus was constructed on Rink-amide resin by standard Fmoc-based SPPS. The resulting peptide resins were condensed with Fmoc-protected Phe-Gly-type (*Z*)-ADI **10** or (*E*)-FADI **11** using *N*,*N*′-diisopropylcarbodiimide (DIC)/*N*-hydroxy-7-azabenzotriazole (HOAt). After coupling of Amb by the standard protocol, final deprotection and cleavage from the resins were conducted with 1*M* thioanisole/TFA cocktail in the presence of H_2O, *m*-cresol, and 1,2-ethanedithiol as scavengers. HPLC purification of crude peptides yielded the desired pentapeptide analogues **14** (H-Amb-Phe-ψ[(*Z*)-CH=CH]-Gly-Leu-Arg-Trp-NH_2) as a main product. In the case of **15** (H-Amb-Phe-ψ[(*E*)-CF=CH]-Gly-Leu-Arg-Trp-NH_2), a significant amount of isostere-deficient peptide **16** was observed which resulted from incomplete coupling of the Fmoc-protected isostere **11** on resin (Figure 3). The resulting peptides **14** and **15** were fully characterized by ion-spray mass spectrometry as well as ^{1}H NMR spectra. Possible olefin isomerization of the isostere β,γ-unsaturated units to α,β-unsaturated ones[42] was not observed on the NMR spectra, in which couplings between β-alkenyl proton and two α-protons was detected. This indicates that these isosteres tolerate the standard Fmoc-based SPPS conditions.

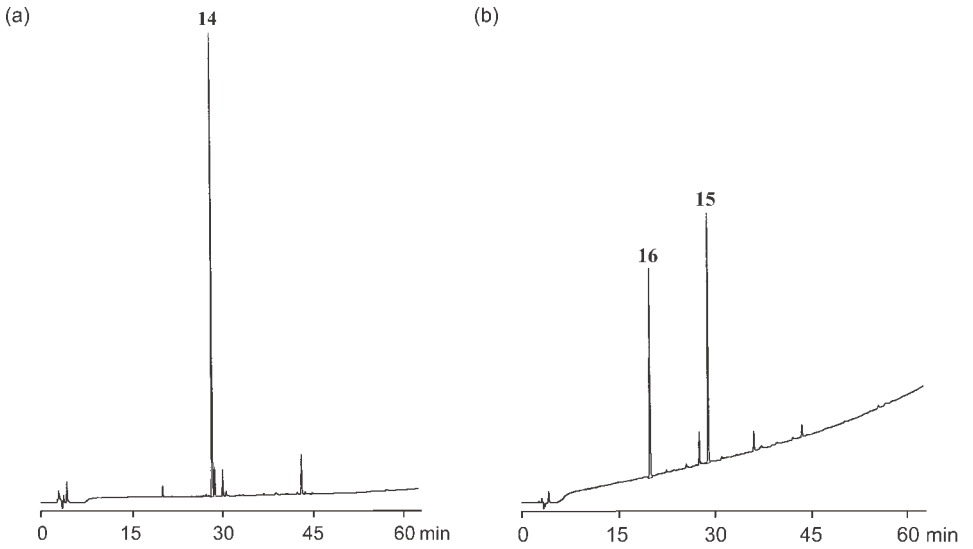


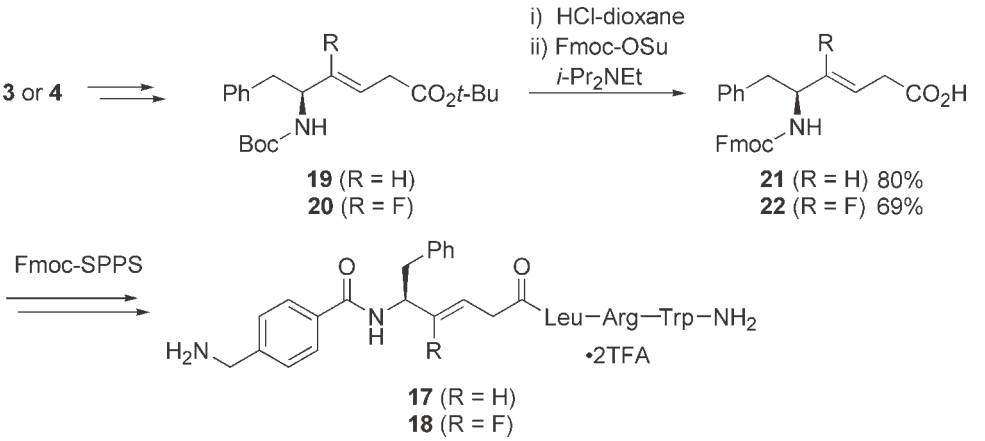
FIGURE 3 HPLC diagrams of crude products **14** (a) and **15** (b) resulting from resin cleavage.

We also prepared pentapeptide analogs **17** and **18** containing Phe-Gly-type (*E*)-ADI and (*Z*)-FADI[23] as *trans*-amide equivalents. Deprotection of Boc-Phe-ψ[(*E*)-CH=CH]-Gly-O*t*-Bu **19** and Boc-Phe-ψ[(*Z*)-CF=CH]-Gly-O*t*-Bu **20**,[27] and successive Fmoc protection provided Fmoc-protected Phe-Gly-type (*E*)-ADI **21** (80% from **19**) and (*Z*)-FADI **22** (69% from **20**), respectively, after purification by flash chromatography (Scheme 2). These isosteres **21** and **22** were applied to Fmoc-based SPPS using the identical procedure to afford the expected peptidomimetics **17** and **18**.

Structure–Activity Relationships of GPR54 Agonists

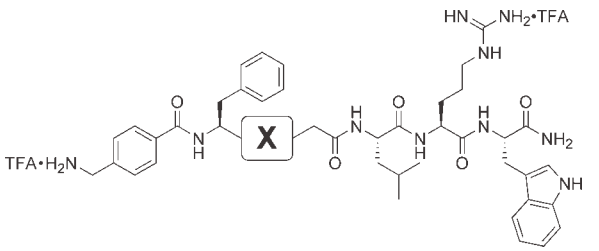
GPR54 agonistic activity of peptides **14**, **15**, **17**, and **18**, along with the parent compound **9**, was evaluated by Flipr assay system. We defined 100% activity as the fluorescence induced by 1 μM of kisspeptin-10, also known as metastin (45–54), which is a potent GPR54 agonist.[35] Biological activities were assessed by comparing the fluorescence induced by 10 n*M* of pentapeptide analogs **9**, **14**, **15**, **17**, or **18** with the activity of kisspeptin-10. The results are summarized in Table I.

Among isostere-containing peptides **14**, **15**, **17**, and **18**, peptide **17** having (*E*)-ADI exerted strong GPR54 agonistic activity, comparable to that of the parent peptide **9** [EC_{50}(**9**) = 3.1 n*M*; EC_{50}(**17**) = 7.6 n*M*]. In contrast, *cis*-amide mimetic-containing peptides **14** and **15** induced extremely low receptor activation at 10 n*M*, indicating that *trans*-amide conformation within Phe-Gly peptide bond is favorable for the agonistic activity. It is of note that replacement of Phe-Gly dipeptide with the (*Z*)-FADI led to a significant loss in GPR54 activity [**18**, 2.6% agonistic activity at 10 n*M*], although the isostere unit should restrict Phe-Gly peptide bond rotation within *trans*-conformation. This suggests that mimicking of carbonyl oxygen by fluorine was inappropriate to design a GPR54-activating agent. Previously, we reported that (*Z*)-FADI does not exceed (*E*)-ADI as a *trans*-amide surrogate in terms of PEPT1 substrate recognition, in [^{3}H]Gly-



SCHEME 2 Synthesis of Fmoc-protected Phe-Gly-type isosteres **21**, **22** and peptides **17**, **18**.

Table I Structures and Bioactivities of Pentapeptide Analogues 9, 14, 15, 17, and 18



Compound	X	Activity (%)[a]	EC_{50} (n*M*)[b]
9	—CO—NH—	96.5 ± 0.3	3.1
14	—ψ[(*Z*)-CH=CH]—	2.0 ± 0.3	—[c]
15	—ψ[(*E*)-CF=CH]—	5.4 ± 0.1	—[c]
17	—ψ[(*E*)-CH=CH] —	75.8 ± 1.6	7.6
18	—ψ[(*Z*)-CF=CH]—	2.6 ± 0.1	—[c]

[a] % Activity is based on the relative maximum agonistic activity at 10 n*M* of a compound to 1 μ*M* kisspeptin-10 (%). 100% = maximum agonistic activity signal at 1 μ*M* kisspeptin-10.

[b] EC_{50} values mean the concentration needed for 50% of the full agonistic activity induced by 1 μ*M* kisspeptin.

[c] Not determined.

Sar uptake inhibition experiments using PEPT1-expressing Caco-2 cell.[27] The presence of fluorine atom on the isostere olefin unit seems to have a significant influence on the bioactivity. In other words, the ligand **18** may still have some interaction with GPR54 without receptor activation, although the binding affinity have not been evaluated.

CONCLUSION

In conclusion, we investigated the applicability of Fmoc-protected Phe-Gly-type (*Z*)-ADI **10** and (*E*)-FADI **11** as *cis*-amide equivalents to standard Fmoc-SPPS. The isostere-containing peptides **14** and **15** were successfully prepared without possible isomerization of the isostere β,γ-unsaturated bond to α,β-counterpart. Furthermore, we utilized the resulting peptides **14** and **15** for SAR study of GPR54 agonist **9**. Peptide **17** containing *trans*-amide mimetics exerted more potent GPR54 agonistic activity compared with **14** and **15**. It was demonstrated that the peptide bond within Phe-Gly of peptide **9** exhibits *trans*-amide conformation in the biologically active conformation.

EXPERIMENTAL

General Synthetic Approach

^{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 $CHCl_3$ or DMSO-d_6 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 flash 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 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.

(5*S*,3*Z*)-5-[(9-Fluorenylmethoxycarbonyl)amino]-6-phenylhex-3-enoic Acid (Fmoc-L-Phe-Ψ[(*Z*)-CH=CH]-Gly-OH, 10)

$Me_3O \cdot BF_4$ (301 mg, 2.04 mmol) was added to a stirred solution of lactam **7** (76.3 mg, 0.407 mmol) in CH_2Cl_2 (5 mL). After stirring for 20 h at room temperature, PBS buffer (pH 7.4, 2 mL) was added to the above mixture. The mixture was extracted with CH_2Cl_2 and dried over $MgSO_4$. Concentration under reduced pressure gave crude *O*-methyl lactim ether **12**, which was dissolved in 0.5*N* HCl (8 mL) and THF (2 mL). After stirring for 24 h at room temperature, the mixture was concentrated under reduced pressure. The resulting residue was dissolved in water (3 mL). Et_3N (170 μL, 1.22 mmol) and Fmoc-OSu (137 mg, 0.407 mmol) in CH_3CN (3 mL) was successively added to the above solution at 0°C. After stirring for 5.5 h at room temperature, the mixture was extracted with EtOAc. The extract was washed with saturated citric acid, brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with *n*-hexane-EtOAc (2:1) gave the title compound **10** (70 mg, 40% yield) as colorless crystals: mp 136–138°C; $[\alpha]_D^{24}$ + 30.0 (*c* 0.138, DMSO); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.60–3.03 (m, 4H), 4.08–4.29 (m, 3H), 4.31–4.43 (m, 1H), 5.35–5.57 (m, 2H), 7.12–7.27 (m, 5H), 7.30 (dd, *J* = 13.7, 6.8 Hz, 2H), 7.40 (t, *J* = 7.6 Hz, 2H), 7.50 (d, *J* = 8.5 Hz, 1H), 7.62 (d, *J* = 7.6 Hz, 2H), 7.87 (d, *J* = 7.6 Hz, 2H), 12.17 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 37.4, 40.4, 46.7, 50.0, 65.2, 120.1, 122.9, 125.1, 126.1, 127.0, 127.6, 128.0, 129.3, 132.6, 138.2, 140.7, 143.8, 155.3, 172.3; Anal. Calcd. for $C_{27}H_{25}NO_4 \cdot 0.25H_2O$: C, 75.07; H, 5.95; N, 3.24. Found: C, 75.08; H, 5.95; N, 3.29.

(5*S*,3*E*)-5-[(9-Fluorenylmethoxycarbonyl)amino]-4-fluoro-6-phenylhex-3-enoic Acid (Fmoc-L-Phe-Ψ[(*E*)-CF=CH]-Gly-OH, 11)

By use of a procedure similar to that described for the preparation of (*Z*)-ADIs **10**, lactam **8** (375 mg, 1.83 mmol) was converted into the title compound **11** (colorless oil, 240 mg, 30% yield): $[\alpha]^{24}_D$ + 4.7 (*c* 0.138, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.55–3.05 (m, 4H), 4.16 (t, *J* = 6.6 Hz, 1H), 4.26–4.46 (m, 2H), 4.60–4.80 (m, 1H), 5.17–5.36 (m, 2H), 7.12–7.32 (m, 7H), 7.37 (t, *J* = 7.3 Hz, 2H), 7.51 (t, *J* = 8.0 Hz, 2H), 7.73 (d, *J* = 7.6 Hz, 2H); ^{13}C NMR

Table II Characterization Data of Peptides 14, 15, 17, and 18

		Optical Rotations				HRMS (FAB)	
Compound	Yield (%)	$[\alpha]_D$ (H_2O)	*C* (g/dL)	Temperature (°C)	Formula	Found	Calculated
14	7	−40.2	0.33	22	$C_{43}H_{57}N_{10}O_5$	793.4503	793.4513
15	4	−132.9	0.20	22	$C_{43}H_{56}FN_{10}O_5$	811.4413	811.4419
17	4	−132.6	0.08	22	$C_{43}H_{57}N_{10}O_5$	793.4517	793.4513
18	6	−26.7	0.41	22	$C_{43}H_{56}FN_{10}O_5$	811.4434	811.4419

(100 MHz, $CDCl_3$) δ 30.0, 38.1, 47.0, 50.2, 67.0, 101.2, 119.9, 124.9, 126.9, 127.0, 127.7, 128.5, 129.1, 136.1, 141.2, 143.6, 155.6, 157.8, 175.6; ^{19}F NMR (376 MHz, DMSO-d_6) δ −120.3 (dd, J = 29.0, 20.7 Hz, 1F); Anal. Calcd. for $C_{27}H_{24}FNO_4$: C, 72.80; H, 5.43; N, 3.14. Found: C, 72.80; H, 5.70; N, 2.97.

(5*S*,3*E*)-5-[(9-Fluorenylmethoxycarbonyl)amino]-6-phenylhex-3-enoic Acid (Fmoc-L-Phe-Ψ[(*E*)-CH=CH]-Gly-OH, 21)

To a solution of enoate **19** (50.0 mg, 0.138 mmol) was added 4*M* HCl in 1,4-dioxane (2 mL), and the mixture was stirred for 4.0 h at room temperature. The resulting residue was dissolved in H_2O (1 mL). Et_3N (57.7 mL, 0.415 mmol) and Fmoc-OSu (46.7 mg, 0.138 mmol) in CH_3CN (1 mL) were successively added to the above solution at 0°C. After stirring for 3.5 h at room temperature, the mixture was extracted with EtOAc. The extract was washed with 1*M* aqueous HCl, brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with *n*-hexane-EtOAc (2:1) gave the title compound **21** (47 mg, 80% yield) as colorless crystals: mp 140–142°C; $[\alpha]_D^{23}$ −20.3 (c 0.820, DMSO); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.65–2.81 (m, 2H), 2.90 (d, J = 4.4 Hz, 2H), 4.08–4.29 (m, 4H), 5.48–5.70 (m, 2H), 7.12–7.25 (m, 5H), 7.30 (dd, J = 17.6, 7.6 Hz, 2H), 7.39 (t, J = 7.3 Hz, 2H), 7.50 (d, J = 8.8 Hz, 1H), 7.62 (t, J = 7.3 Hz, 2H), 7.87 (d, J = 7.3 Hz, 2H), 12.20 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 37.2, 40.5, 46.7, 53.7, 65.2, 120.0, 122.6, 125.2, 126.0, 127.0, 127.5, 128.0, 129.2, 133.9, 138.6, 140.7, 143.8, 155.3, 172.5; Anal. Calcd. for $C_{27}H_{25}NO_4 \cdot 0.25H_2O$: C, 75.07; H, 5.95; N, 3.24. Found: C, 75.03; H, 5.89; N, 3.28.

(5*S*,3*Z*)-5-[(9-Fluorenylmethoxycarbonyl)amino]-4-fluoro-6-phenylhex-3-enoic Acid (Fmoc-L-Phe-Ψ[(*Z*)-CF=CH]-Gly-OH, 22)

By use of a procedure similar to that described for the preparation of (*E*)-ADIs **21**, enoate **20** (620 mg, 1.63 mmol) was converted into the title compound **22** (500 mg, 69% yield) as colorless crystals: mp 156–158°C; $[\alpha]_D^{24}$ + 34.8 (c 0.138, DMSO); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.75–2.98 (m, 2H), 3.02 (t, J = 6.3 Hz, 2H), 4.08–4.21 (m, 3H), 4.23–4.35 (m, 1H), 5.00 (dt, J = 38.0, 7.1 Hz, 1H), 7.19–7.34 (m, 7H), 7.40 (t, J = 7.3 Hz, 2H), 7.61 (t, J = 7.3 Hz, 2H), 7.87 (t, J = 7.3 Hz, 2H), 7.83–7.90 (m, 1H), 12.35 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 28.9, 37.2, 46.6, 53.1, 65.5, 98.8, 120.1, 125.2, 126.3, 127.0, 127.6, 128.1, 129.1, 137,7, 140.6, 143.7, 155.4, 159.3, 171.8; ^{19}F NMR (376 MHz, DMSO-d_6) δ −117.0 (dd, J = 39.3, 14.5 Hz, 1F); Anal. Calcd. for $C_{27}H_{24}FNO_4$: C, 72.80; H, 5.43; N, 3.14. Found: C, 72.60; H, 5.36; N, 3.14.

General Procedure for the Preparation of Isostere-Containing Peptides

Protected peptide-resins were manually constructed by Fmoc-based solid phase peptide synthesis on Rink-amide resin (0.34 mmol/g, 150 mg, 0.05 mmol). 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg was employed for side-chain protection. Fmoc-amino acids (0.25 mmol, 5.0 equiv.) except for Fmoc-L-Phe-ψ[(*E*/*Z*)-CX=CH]-Gly-OH (X = H or F) were sequentially coupled to a free amino group in DMF for 2 h, using DIC (39 μL, 0.25 mmol, 5.0 equiv.) in the presence of *N*-hydroxybenzotriazole (HOBt) (77 mg, 0.5 mmol, 10.0 equiv.). Fmoc deprotection was performed by 20% piperidine in DMF (2 × 1 min, 1 × 20 min). Fmoc-protected alkene- or fluoroalkene-type dipeptide isostere (0.10 mmol, 2.0 equiv.) was condensed in the presence of DIC (16 μL, 0.10 mmol, 5.0 equiv.) and HOAt (27 mg, 0.20 mmol, 10.0 equiv.). The resulting protected resin was treated with 1*M* thioanisole/TFA (8.5 mL) in the presence of *m*-cresol (500 μL), 1,2-ethanedithiol (500 μL) and H_2O (500 μL) at 4°C for 3 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 washed with ice-cold dry diethyl ether (40 mL). The crude product was purified by preparative HPLC to afford the expected peptides containing alkene- or fluoroalkene-type dipeptide isostere as a colorless powder. Characterization data of synthetic peptides **14**, **15**, **17**, and **18** are summarized in Table II.

Biological Assay

Pigment mixture was prepared by addition of 2 vials of Fluo3-AM (50 μg/vial) in 21 μL dimethylsulfoxide and 21 μL of 20% pluronic acid to 10 mL HANKS/HBSS (prepared from 9.8 g HANKS, 0.35 g sodium hydrogencarbonate and 20 mL of 1*M* HEPES, pH 7.4) containing 2.5 m*M* probenecid and 1% FBS.

GPR54/CHO (3.0×10^4 cells/200 μL/well)[34] was inoculated in 10% dFBS/DMEM on a 96-well black clear-bottom plate (Coster) for FLIPR (Molecular Devices), 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 at 100 μL/well, followed by incubation at 37°C for 1 h in 5% CO_2. Next, 1 m*M* peptide in dimethylsulfoxide was diluted with HANKS/HBSS containing 2.5 m*M* probenecid, 0.2% BSA, and 0.1% CHAPS. The dilution was transferred to a 96-well V-bottom sample plate (Coster) for FLIPR. After completion of the pigment loading onto the cell plate, the cell plate was washed four times with wash buffer (2.5 m*M* Probenecid in HANKS/HBSS) in a plate washer. After washing, 100 μL of wash buffer remained. The cell plate and the sample plate were set in

FLIPR and a 0.05 mL sample from the sample plate was automatically transferred to the cell plate. The change in intracellular calcium ion level over 40 s was measured ($n = 3$).

We thank Takeda Pharmaceutical Company Limited for biological activity evaluation of a series of compounds. A.N. is grateful to Research Fellowships from the JSPS for Young Scientists.

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