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Synthesis and structure–activity relationships of potent 4-fluoro-2-cyanopyrrolidine dipeptidyl peptidase IV inhibitors

Hiroshi Fukushima,[a,*] Akira Hiratate,[a] Masato Takahashi,[a] Ayako Mikami,[a] Masako Saito-Hori,[b] Eiji Munetomo,[b] Kiyokazu Kitano,[b] Sumi Chonan,[b] Hidetaka Saito,[c] Akio Suzuki,[d] Yuji Takaoka[e] and Koji Yamamoto[b]

[a]*Medicinal Chemistry Laboratories, Taisho Pharmaceutical Co., Ltd, 1-403, Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan*
[b]*Molecular Function and Pharmacology Laboratories, Taisho Pharmaceutical Co., Ltd, 1-403, Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan*
[c]*Non-clinical Quality Assurance Section, Taisho Pharmaceutical Co., Ltd, 1-403, Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan*
[d]*Center for Quality Evaluation, Taisho Pharmaceutical Co., Ltd, 1-403, Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan*
[e]*Research Computer System, Taisho Pharmaceutical Co., Ltd, 1-403, Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan*

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Abstract—Dipeptidyl peptidase IV (DPP-IV) inhibitors are promising antidiabetic drugs, and several drugs are in the developmental stage. We previously reported that the introduction of fluorine to the 4-position of 2-cyanopyrrolidine enhanced the DPP-IV inhibitory effect. In the present report, we examined the structure–activity relationship (SAR) of 2-cyano-4-fluoropyrrolidine with N-substituted glycine at the 1-position. We report the identification of a potent and stable DPP-IV inhibitor (TS-021) with a long-term persistent plasma drug concentration and a potent antihyperglycemic activity.

1. Introduction

Dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5, CD26)[1] is a highly specific serine protease that cleaves N-terminal dipeptides from polypeptides with L-proline or L-alanine at the penultimate position.[2] Inhibitors of DPP-IV have been shown to elevate levels of the active glucagon-like peptide-1 (GLP-1) and promote insulin secretion and thereby regulate blood glucose levels. Several DPP-IV inhibitors have entered clinical development, including NVP-DPP728 (Fig. 1).[3]

We previously reported that 2-cyano-4-fluoropyrrolidine **1** (Fig. 1) had a greater DPP-IV inhibitory activity and a higher plasma drug concentration after oral administration in rats than a 4-unsubstituted derivative.[4] However, as compound **1** has still failed in chemical stability and persistence effect, we continued converting the side chain at the 1-position.

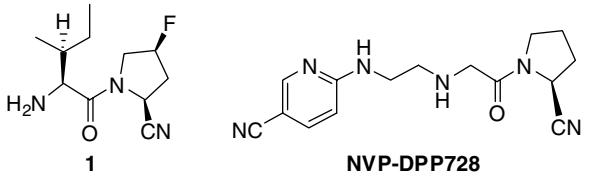

Figure 1.

Two types of the side chain at the 1-position are known in 2-cyanopyrrolidine DPP-IV inhibitor. One is α-branched amino acid type and the other is N-substituted glycine type. NVP-DPP728 contains an N-substituted glycine side chain at the 1-position of pyrrolidine; its efficacy was reported in a clinical trial for diabetic patients.[5] In order to obtain a more potent compound, the side chain of NVP-DPP728 was introduced to the 1-position

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* Corresponding author. Tel.: +81 (0) 48 669 3108; fax: +81 (0) 48 652 7254; e-mail: hiroshi.fukushima@po.rd.taisho.co.jp

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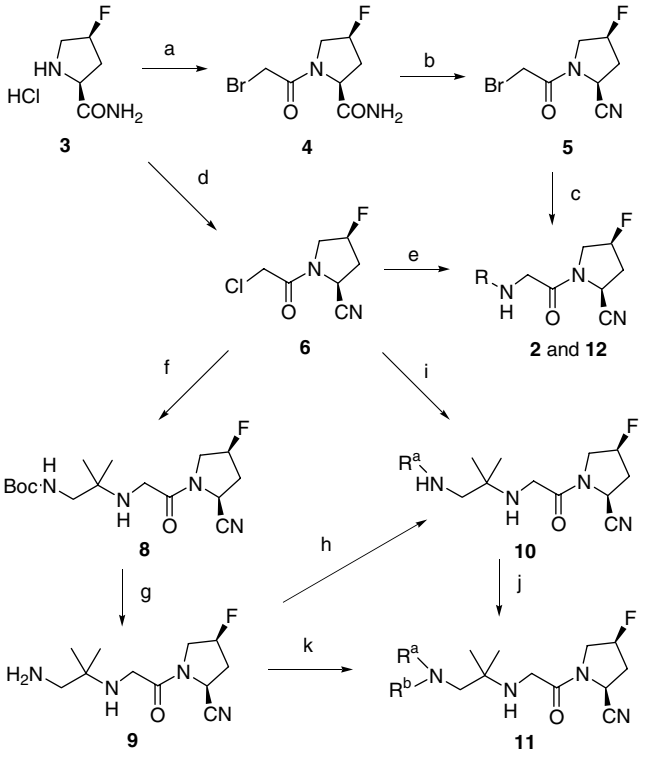
of the 2-cyano-4-fluoropyrrolidine to produce compound **2a**. Compound **2a** exhibited a potent inhibitory activity, but it was not sufficient profile of its chemical stability and duration of activity. Therefore, we continued developing 2-cyano-4-fluoropyrrolidines with N-substituted glycine side chains in an attempt to identify superior DPP-IV inhibitors.

2. Chemistry

First, we selected bromoacetae **5** or chloroacetate **6** as an intermediate to synthesize N-substituted glycine derivatives. Amine hydrochloride **3**[4] was treated with potassium 2-ethylhexanoate and was reacted with bromoacetyl bromide to give bromoacetate **4** (Scheme 1). Then, bromoacetate **4** was dehydrated with trifluoroacetic anhydride to yield cyanide **5**, which was reacted with primary amines to give **2a**–**2d**, **12f**–**12k**, and **12m**–**12v** (Method A). At this time, 2–5 molar equivalents of the primary amines were used to avoid producing byproducts, which seemed to be dialkylated compounds.

As an alternative route, amine hydrochloride **3** was reacted with chloroacetyl chloride and triethylamine in DMF and was then continuously reacted with cyanuric chloride to yield chloroacetate **6**. As the reaction of chloroacetate **6** and primary amine was very slow at room temperature, potassium iodide was added to accelerate the reaction, yielding **12a**–**12e** and **12l** (Method B).



Scheme 1. Reagents: (a) potassium 2-ethylhexanoate, toluene/$BrCH_2COBr$, THF; (b) $(CF_3CO)_2O$, THF; (c) R-NH_2, THF (Method A); (d) i—$ClCH_2COCl$, Et_3N, DMF, ii—cyanuric chloride; (e) R-NH_2, KI, MeOH, THF (Method B); (f) $BocNHCH_2C(CH_3)_2NH_2$ **7**, KI, MeOH; (g) 4 M HCl/AcOEt; (h) acylation (Method C), sulfonylation (Method D), or reductive amination (Method E); (i) $R^aR^bNCH_2C(CH_3)_2NH_2$, KI, MeOH (Method F); (j) methylation (Method G) or acylation (Method H); (k) dialkylation (Method I).

Next, we focused on the ethylenediamine part of **2a**, to which we planned to introduce a dimethyl group after the bulkiness near the amino group of the side chain at the 1-position of pyrrolidine was found to be important for chemical stability. Diamine intermediate **9** was selected and used to synthesize the designed compounds (Scheme 1). Boc-protected diamine derivative **7** was reacted with chloroacetate **6** to yield **8**, but **8** was difficult to separate from unreacted **7**. Di-*tert*-butyl dicarbonate was added to the reaction mixture to convert **7** to a diBoc derivative, and then **8** was easily purified using column chromatography. Boc-protected amine **8** was treated with hydrochloric acid to yield **9**.

Diamine **9** was used to produce **10** through acylation (Method C), sulfonylation (Method D), and reductive amination (Method E). Acid chlorides or carboxylic acids with coupling reagents were used during the acylation step to produce **10a**–**10h**. Sulfonyl chlorides and triethylamine were used during the sulfonylation step to yield **10j**. Aldehyde and sodium triacetoxyborohydride were used during the reductive amination step to yield **10k** and **10l**. The primary amine of diamine **9** was selectively reacted during the acylation, sulfonylation, and reductive amination steps because of steric crowding from the dimethyl group.

On the other hand, chloroacetate **6** and an amine with Ra as an N-substitution was used to synthesize **10i** (Method F). N-Methylation of phenyl sulfonamide **10j** was performed using diethylazodicarboxylate, triphenylphosphine, and methanol to yield **11a** (Method G). When the Ra was isobutyl or a 4-cyanobenzyl group (**10k** or **10l**), acylation with benzoyl chloride yielded **11c** or **11d**, respectively (Method H). In these cases, although two secondary amino groups are present in **10k** or **10l**, the amino group attached to Ra was selectively reacted because of the vacancy. *N*,*N*-Diethyl derivative **11b** was synthesized using reductive amination with **9** and acetaldehyde (Method I).

3. Results and discussion

The synthesized compounds were evaluated for DPP-IV-inhibitory activity in human plasma by fluorescence assay using Gly-Pro-4-methylcoumaryl-7-amide.

We previously reported that the introduction of a fluorine atom to 2-cyanopyrrolidine increased the DPP-IV inhibitory activity and that treatment with **1** improved impaired glucose tolerance.[4] NVP-DPP728, which is a 2-cyanopyrrolidine derivative with an N-substituted glycine side chain, was also shown to improve hyperglycemia in a clinical trial.[5] We synthesized **2a**, which is a 4-fluoropyrrolidine derivative with the same side chain as NVP-DPP728 (lit. IC_{50} = 7.0 nM[3a]), and found that **2a** exhibited a several-fold more potent DPP-IV inhibitory activity (IC_{50} = 1.1 nM) than NVP-DPP728 (Table 1).

Commercial drugs generally require not only in vitro potency, but also good physicochemical, pharmacokinetic, and safety profiles. Though **2a** exhibited a potent inhibitory activity, we continued to search for a more efficient drug. First, we modified the cyano group on the pyridine to yield an unsubstituted derivative **2b**, chloride **2c**, and carbamoyl compound **2d**; however, the activities of these compounds was less potent than that of **2a** (IC_{50} = 2.8, 2.7, and 2.4 nM, respectively) (Table 2).

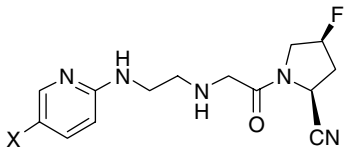
Amides **10a**–**10c**, which were synthesized from diamine intermediate **9**, pyridine derivative **10a**, benzoate **10b**, and thiophene derivative **10c**, exhibited decent potencies (IC_{50} = 8.2, 4.5, and 5.4 nM, respectively). Bicyclic benzodihydrofuran derivative **10d** exhibited a potent activity (IC_{50} = 2.9 nM), and benzotriazole derivative **10f** exhibited a very potent activity (IC_{50} = 1.5 nM). Aliphatic acyl derivatives **10g**, **10h**, and **10i** did not exhibit better activities than **10b** (IC_{50} = 5.8, 13, and 109 nM, respectively). Especially, **10i** exhibited a much lower potency, possibly because of its large side chain. And benzenesulfonamide **10j** exhibited a 17-fold less potent activity (IC_{50} = 75 nM) than benzamide **10b**. The reason for the sulfonamide's low activity did not seem to be the acidic proton because the *N*-methyl derivative **11a** also exhibited a low activity (IC_{50} = 31 nM).

Next, *N*-alkyl derivatives, *N*-isobutyl derivative **10k**, and *N*,*N*-diethyl derivative **11b**, both exhibited weak activities (IC_{50} = >316 and 252 nM, respectively). The basicity of the amino group seemed to reduce the potency. *N*-(4-Cyanobenzyl) derivative **10l** exhibited a better activity (IC_{50} = 39 nM) than **10k** or **11b**, possibly because of the affinity of the phenyl group.

N,*N*-Dialkylbenzamide **11c** and **11d**, synthesized from **10e** or **10f**, exhibited improved potencies (IC_{50} = 7.3 and 6.2 nM, respectively). In other words, the inhibitory potency was not influenced by the introduction of an *N*-alkyl group to benzamide **10b**.

As described above, we modified the ethylenediamine part of the side chain and obtained a potent compound, **10f** (IC_{50} = 1.5 nM); however, the potency of **10f** did not exceed that of **2a**. We next examined the introduction of alkyl side chains other than ethylenediamine. To increase the affinity using aromatic ring, several aromatic

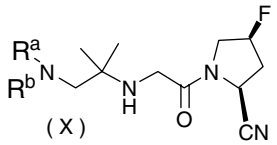
Table 1. DPP-IV inhibitory activity



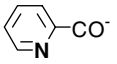
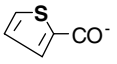
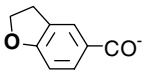
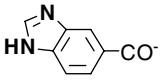
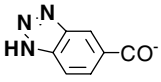
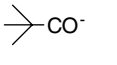
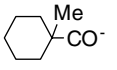
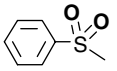
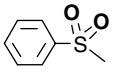
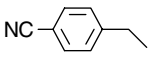
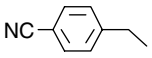
Compound	X	IC_{50}[a] (nM)
2a	CN	1.1
2b	H	2.8
2c	Cl	2.7
2d	$CONH_2$	2.4

[a] DPP-IV inhibitory activity.

Table 2. DPP-IV inhibitory activity



10 or **11**

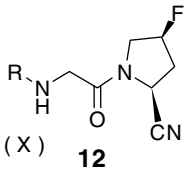
Compound	R^a	R^b	Synthetic method	IC_{50}[a] (nM)
10a		H	C	8.2
10b	PhCO–	H	C	4.5
10c		H	C	5.4
10d		H	C	2.9
10e		H	C	5.7
10f		H	C	1.5
10g		H	C	5.8
10h		H	C	13
10i	$(4\text{-Cl-Ph})_2CHCO$–	H	F	109
10j		H	D	75
11a		Me	G	31
10k		H	E	>316
10l		H	E	39
11b	Et	Et	I	252
11c		PhCO–	H	7.3
11d		PhCO–	H	6.2

[a] DPP-IV inhibitory activity.

derivatives (**12a**–**12f**) were synthesized; however, the potencies of these compounds were lower (IC_{50} from 8.6 to 88 nM) than that of **2a** (Table 3).

The introduction of a simple alkyl side chain, as found in *tert*-butyl derivative **12g** (IC_{50} = 2.9 nM) and isopropyl derivative **12h** (IC_{50} = 7.8 nM), was found to produce a potent inhibitory activity. Compounds **12i** and **12j**, containing an alkyloxy group on their side chains,

Table 3. DPP-IV inhibitory activity



12

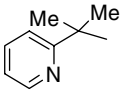
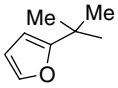
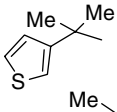
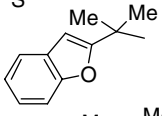
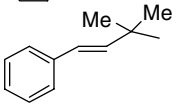
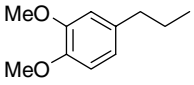
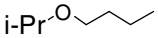
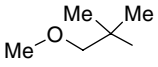
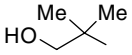
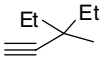
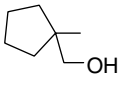
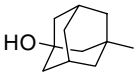
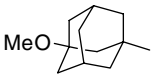
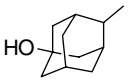
Compound	R	Synthetic method	IC_{50}[a] (nM)
12a		B	22
12b		B	8.6
12c		B	12
12d		B	88
12e		B	16
12f		A	27
12g	*t*-Bu–	A	2.9
12h	*i*-Pr–	A	7.8
12i		A	8.7
12j		A	13
12k		A	4.6
12l		B	>316
12m		A	33
12n		A	3.1
12o		A	2.6
12p		A	3.3
12q		A	3.3
12r		A	4.1

Table 3 (*continued*)

Compound	R	Synthetic method	IC_{50}[a] (nM)
12s		A	3.1
12t		A	3.1
12u		A	8.3
12v		A	5.3

[a] DPP-IV inhibitory activity.

exhibited IC_{50} values of 8.7 and 13 nM, respectively, while alcohol **12k** exhibited a potent activity (IC_{50} = 4.6 nM). Acetylene derivative **12l**, which contains a diethyl group next to an amino group, did not exhibit a potent activity (IC_{50} > 316 nM), possibly because of its bulkiness.

Cycloalkyl groups were introduced to yield potent compounds, the 4-to-8-membered ring derivatives **12n**, **12o**, and **12q** (IC_{50} = 3.1, 2.6, and 3.3 nM, respectively). Compound **12p**, containing a hydroxymethyl group, exhibited a potency (IC_{50} = 3.3 nM) similar to that of **12o**. But the cyclopropyl derivative **12m** (IC_{50} = 33 nM) exhibited a lower potent activity, possibly because of the short spread of its branch. Adamantyl derivatives **12r**–**12v** exhibited potent activities (IC_{50} = 3.1–8.3 nM), regardless of whether a substitution was present.

We synthesized and estimated the 2-cyano-4-fluoropyrrolidine derivatives described above. Although a derivative that was more potent than **2a** was not found, many potent compounds, like **10f** (IC_{50} = 1.5 nM), were obtained. The chemical stabilities and plasma drug concentrations after oral administration of these compounds were estimated, and several compounds were found to have favorable profiles.

Among these derivatives, **12k** was selected and compared with **1** and **2a** (Table 4). The residual amount of

Table 4. Chemical stability and plasma drug concentrations after oral administration of **1**, **2a**, and **12k**

Compound	Residual amount (%)[a]	Plasma drug concentrations[b] (ng/mL)			
		0.5 (h)	1 (h)	2 (h)	6 (h)
1	70	195	24	5	1
2a	86[c]	157	62	8	6
12k	96	355	274	168	31

[a] HPLC determination after 6 h of incubation at 37 °C in pH 6.8 aqueous buffer solution.
[b] LC–MS determination after oral administration at a dose of 1 mg/kg to Wistar rats.
[c] Dihydrochloride salt of **2a** was used in the chemical stability test instead of **2a**.

12k after 6 h of incubation at 37 °C in pH 6.8 aqueous buffer solution was drastically higher than that of **1** and **2a**, as determined using reverse phase high-performance liquid chromatography (HPLC). A long-term, persistent drug effect is an important pharmacological characteristic, and this parameter was investigated by measuring the plasma drug concentrations after oral administration to rats. The concentrations of **12k** at 2 and 6 h were markedly higher than those of **1** or **2a**. Thus, compound **12k** likely has a persistent drug effect.

The binding mode of compound **12k** was predicted using an X-ray crystallographic structure of DPP-IV (PDB code: 1ORW, Fig. 2).[6] A 2-hydroxy-1,1-dimethylethyl side chain fits very well to shape of the pocket created by Phe357, Arg358, Ser201, His126, and Arg358, which is thought to contribute to a high affinity.

The effect of **12k** on glucose tolerance was examined in Zucker fatty rats, a model of obesity and impaired glucose tolerance. The oral administration of **12k** at doses of 0.1 and 0.3 mg/kg reduced the increase in plasma glucose beginning 30 min after glucose loading (Fig. 3A), and the 0.3 mg/kg dose significantly suppressed hyperglycemia (Fig. 3B). DPP-IV activity was almost completely inhibited at 15 min after glucose loading at both doses, with the inhibitory effect continuing until 120 min after glucose loading (Fig. 3D). These results suggest that the efficacy of **12k** on hyperglycemia is based on its inhibitory effect on plasma DPP-IV activity. Insulin secretion was enhanced in both of the 0.1 and 0.3 mg/kg dose groups (Fig. 3C). This finding supports the proposed mechanism that **12k** might prevent the inactivation of active GLP-1 via DPP-IV inhibition and that an increased GLP-1 activity level might stimulate insulin secretion by acting upon β-cells in the pancreas, resulting in the suppression of hyperglycemia after glucose loading.

4. Conclusion

We designed and synthesized derivatives with N-substituted glycine side chains at the 1-position of 2-cyano-4-fluoropyrrolidine. We found that 2-aminopyridine derivative **2a** and benzotriazole derivative **10f** exhibited potent DPP-IV inhibitory activities. After further alterations to the side chain, we found that **12k** exhibited a superior chemical stability a long-term, persistent plasma drug concentration and a potent antihyperglycemic activity. After examining the salt of **12k**, TS-021[7] (Fig. 4), the benzenesulfonic acid salt of **12k** was selected as a candidate for clinical drug development. TS-021 is a potent and long-acting DPP-IV inhibitor and effective with single daily dosing. Subsequent reports will describe the results of further investigations of TS-021.

5. Experimental

5.1. Chemistry

^{1}H NMR spectroscopy was performed using a Varian VXR-300 or a JEOL GX500 spectrometer. Chemical shifts were reported in parts per million relative to tetramethylsilane as an internal standard (in NMR descriptions: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad peak). ^{13}C NMR spectroscopy was performed using a JEOL GX500 spectrometer. ^{19}F NMR spectroscopy was performed using a Varian VXR-300 spectrometer. ESI-Mass spectra were recorded using a Shimadzu/Kratos HV-300. Melting points were

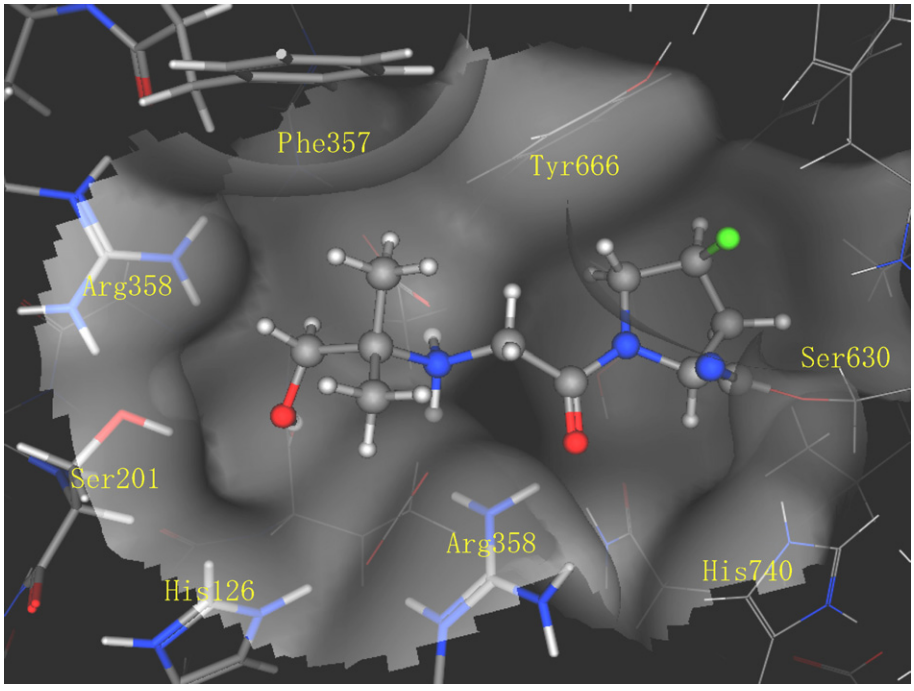


Figure 2. Predicted binding model of compound **12k** in DPP-IV active site.

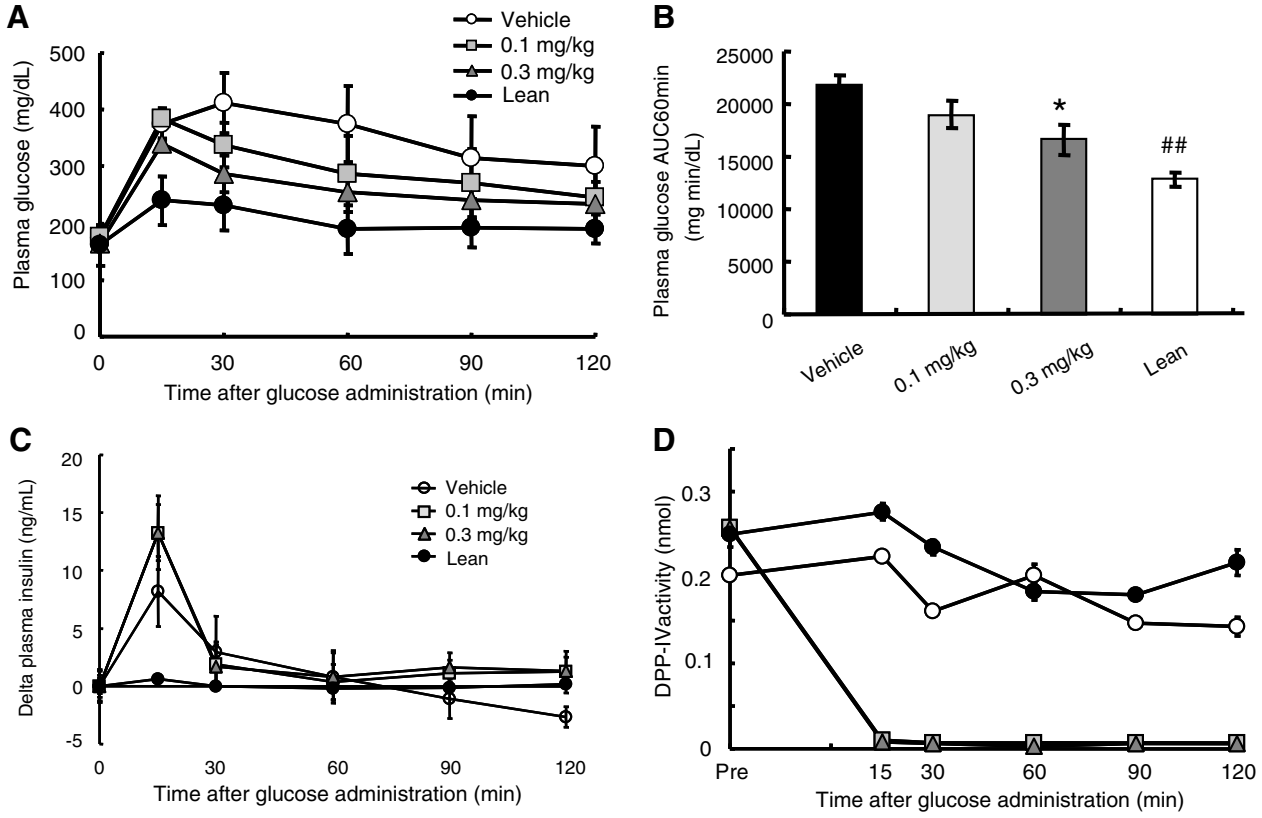
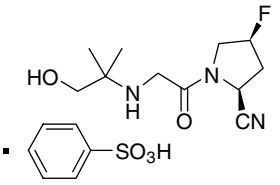


Figure 3. Effects of oral administration of **12k** (0.1 or 0.3 mg/kg) on plasma glucose, plasma insulin, and plasma DPP-IV activity during OGTT in Zucker fatty rats. Each point represents mean ± SE ($n = 6$). $^{*}p < 0.05$ versus Vehicle, Dunnett's test. $^{\#\#}p < 0.01$ versus Vehicle, Student's t-test.



TS-021

Figure 4.

measured using a Buchi 535 melting point apparatus without correction. Infrared spectra were recorded using a Perkin-Elmer 1760 spectrometer. Elemental analyses were performed using a Perkin-Elmer 240C analyzer (for carbon, hydrogen, and nitrogen) or a Yokokawa-Denki IC7000P analyzer (for halogens and sulfur).

Analytical thin-layer chromatography was conducted on precoated silica gel 60 F254 plates (Merck). Chromatography was performed on 100- to 200-mesh silica gel C-200 (Wako Pure Chemical) using the solvent systems (volume ratios) indicated below.

5.1.1. (4*S*)-1-(Bromoacetyl)-4-fluoro-L-prolinamide (4). (4*S*)-4-Fluoro-L-prolinamide hydrochloride **3** (650 mg, 3.9 mmol)[4] was suspended in THF (10 mL), and potassium 2-ethylhexanoate (90% purity, 1.6 g, 7.9 mmol) was added while cooling on ice, followed by stirring for 1 h. Bromoacetyl bromide (0.37 mL, 4.3 mmol) was added while cooling on ice, and the solution was stirred on ice for 30 min and at room temperature for 1 h. $CHCl_3$/MeOH (10:1, 50 mL) was then added to the reaction solution, followed by stirring at room temperature for 15 min. The precipitated salt was separated using filtration, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH = 40:1–25:1) to give the desired product (570 mg, 58%) as a colorless amorphous substance. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.56 and 7.19 (0.3H each, br s each), 7.23 and 7.03 (0.7H each, br s each), 5.35 (0.7H, br d, $J = 51.9$ Hz, H-4 of major isomer), 5.26 (0.3H, br d, $J = 52.9$ Hz, H-4 of minor isomer), 4.58–4.53 (0.3H, m, H-2 of minor isomer), 4.36–4.31 (0.7H, m, H-2 of major isomer), 4.21 and 4.09 (1.4H, ABq, $J = 12.1$ Hz, $COCH_2$), 3.92–3.55 (2H, m, H-5), 2.48–2.16 (2H, m, H-3). MS(ESI pos.) m/z 275 and 277 ($[M+Na]^+$).

5.1.2. (2*S*,4*S*)-1-(Bromoacetyl)-4-fluoropyrrolidine-2-carbonitrile (5). Compound **4** (560 mg, 2.2 mmol) was dissolved in THF (6 mL), and trifluoroacetic anhydride (0.62 mL, 4.4 mmol) was added while cooling on ice; the solution was then stirred on ice for 1 h. The reaction solution was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH = 50:1–30:1) to give the desired product (540 mg, quant) as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.52 (1H,

br d, J = 52.5 Hz, H-4), 5.02–4.96 (1H, m, H-2), 4.23 and 4.19 (2H, ABq, J = 12.3 Hz, $COCH_2$), 4.00 (1H, dd like, J = 23.9, 12.5 Hz, H-5), 3.78 (1H, ddd, J = 39.6, 12.5, 3.4 Hz, H-5), 2.46–2.32 (2H, m, H-3). MS(ESI pos.) m/z 257 and 259 ($[M+Na]^+$).

5.1.3. (2*S*,4*S*)-1-(Chloroacetyl)-4-fluoropyrrolidine-2-carbonitrile (6). Compound **5** (43.0 g, 255 mmol) was suspended in DMF (255 mL) and cooled with ice-salt; chloroacetyl chloride (22.3 mL, 281 mmol) was then added, followed by stirring for 10 min. Triethylamine (74.7 mL, 536 mmol) was added and cooled with ice-salt for 1 h. After stirring for 1 h on ice, cyanuric chloride (28.2 g, 153 mmol) was added and the reaction solution was stirred while increasing the temperature to 18 °C. After stirring for 50 min, the solution solidified and was poured into ice-water. The precipitated solid was collected and washed with water to give the desired product (41.9 g, 86%) as a colorless solid. Mp 140–141 °C (decomp.). ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.50 (1H, d, J = 51.8 Hz, H-4), 5.02–4.96 (1H, m, H-2), 4.51 and 4.39 (2H, ABq, J = 14.2 Hz, $COCH_2$), 3.97 (1H, dd like, J = 23.6, 12.4 Hz, H-5), 3.76 (1H, ddd, J = 39.3, 12.4, 3.4 Hz, H-5), 2.6–2.3 (2H, m, H-3). MS(ESI pos.) m/z 213 ($[M+Na]^+$). HRMS calcd for $C_7H_8ClFN_2ONa$ $[M+Na]^+$ 213.0207; found: (m/z) 213.0201. Anal. Calcd for $C_7H_8ClFN_2O$: C, 44.11; H, 4.23; N, 14.70; Cl, 18.60. Found: C, 43.91; H, 4.21; N, 14.56; Cl, 18.47. $[\alpha]_D^{25}$ −125 (c 0.3, MeOH).

5.1.4. (2*S*,4*S*)-4-Fluoro-1-(*N*-{2-[(5-cyanopyridin-2-yl)-amino]ethyl}glycyl)pyrrolidine-2-carbonitrile maleate (2a). (Method A). 6-(2-Amino-ethylamino)-nicotinonitrile (520 mg, 3.2 mmol) was dissolved in THF (10 mL), and a THF solution (5 mL) of (2*S*,4*S*)-1-bromoacetyl-2-cyano-4-fluoropyrrolidine **5** (250 mg, 1.1 mmol) was then added while cooling on ice. The temperature was gradually raised, and the mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure, and the resulting residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH = 50:1–25:1). The resulting residue (80 mg, 0.25 mmol) was dissolved in EtOH (1 mL), and then an EtOH solution (1 mL) of maleic acid (30 mg, 0.26 mmol) was added. Diethyl ether was added to the solution, the supernatant was removed, and the precipitate was washed with diethyl ether. The residue was dried under reduced pressure to give the desired product (70 mg, 15%) as a colorless powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.44 (1 H, d, J = 1.7 Hz), 7.80–7.70 (2H, m), 6.62 (1H, d, J = 8.9 Hz), 6.03 (2H, s, maleic acid), 5.55 (1H, br d, J = 52.2 Hz, H-4), 5.09–5.03 (1H, m, H-2), 4.23 and 3.98 (2H, ABq, J = 16.5 Hz, $COCH_2$), 4.00–3.87 (1H, m, H-5), 3.80–3.58 (3H, m, H-5 and NCH_2), 3.20–3.06 (2H, m, NCH_2), 2.67–2.32 (2H, m, H-3). MS(ESI pos.) m/z 339 ($[M+Na]^+$), 317 ($[M+H]^+$); (ESI neg.) m/z 315 ($[M-H]^-$). HRMS calcd for $C_{15}H_{18}FN_6O[M+H]^+$ 317.152613.0207; found: (m/z) 317.1536.

5.1.5. (2*S*,4*S*)-4-Fluoro-1-{*N*-[2-(pyridin-2-ylamino)ethyl]-glycyl}pyrrolidine-2-carbonitrile dihydrochloride (2b). (Method A′). N^1-(Pyridin-2-yl)ethane-1,2-diamine (823 mg, 6.0 mmol) was dissolved in THF (10 mL), and then a suspension of (2*S*,4*S*)-1-bromoacetyl-2-cyano-4-fluoropyrrolidine **5** (705 mg, 3.0 mmol) in a mixed solution of THF (5 mL) and EtOH(5 mL) was then added while cooling on ice. The reaction mixture was stirred on ice for 0.5 h and then at room temperature for 1 h. The solution was concentrated under reduced pressure, and the resulting residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH/25% aqueous ammonia = 30:1:0–20:1:0 to 20:1:0.1). The resulting mixture, which consisted of the desired compound and N^1-(pyridin-2-yl)ethane-1,2-diamine, was treated with di-*tert*-butyl dicarbonate (1.31 g, 6.0 mmol) in THF (20 mL) at room temperature for 0.5 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was purified by silica gel column chromatography (developing solvent: *n*-hexane/EtOAc = 1:1–1:3 to EtOAc/MeOH = 20:1) to give *tert*-butyl 2-((2*S*,4*S*)-2-cyano-4-fluoropyrrolidin-1-yl)-2-oxoethyl(2-(pyridin-2-ylamino)ethyl)carbamate (600 mg, 51%). This compound (540 mg, 1.38 mmol) was treated with 4 M HCl/dioxane (7.5 mL) and dioxane (2.5 mL) on ice for 1 h. The supernatant was decanted off and the residue was washed with dioxane. Isopropanol (2 mL) and diisopropyl ether (20 mL) were then added to the residue. The resulting powder was obtained using filtration to afford the desired crude product (416 mg, 43% from **5**). This crude product (334 mg) was purified by silica gel column chromatography (developing solvent: $CHCl_3$/ammonia in MeOH = 100:3–100:7 to $CHCl_3$/MeOH = 100:5–100:7) and the residue was treated with 4 M HCl/EtOAc (0.55 mL) and diisopropyl ether (15 mL). The resulting powder was obtained using filtration to afford the desired product (266 mg) as a colorless powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.50 (2H, br s, NH_2^+), 7.96 (1H, br d, J = 7 Hz), 7.82 (1H, br t, J = 8 Hz), 7.03 (1H, br d, J = 8 Hz), 6.83 (1H, br t, J = 7 Hz), 5.55 (1H, br d, J = 52.7 Hz, H-4), 5.10–5.03 (1H, m, H-2), 4.32 and 4.09 (2H, ABq, J = 16.7 Hz, $COCH_2$), 4.04–3.66 (2H, m, H-5), 3.80 (2H, br s, NCH_2), 3.21 (2H, br s, NCH_2), 2.7–2.3 (2H, m, H-3). MS(ESI pos.) m/z 292 ($[M+H]^+$), 314 ($[M+Na]^+$); (ESI neg.) m/z 326 ($[M+Cl]^-$). HRMS calcd for $C_{14}H_{18}FN_5O[M]^+$ 291.1495; found: (m/z) 291.1503.

5.1.6. (2*S*,4*S*)-1-(*N*-{2-[(5-Chloropyridin-2-yl)amino]ethyl}-glycyl)-4-fluoropyrrolidine-2-carbonitrile dihydrochloride (2c). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.47 (2H, br s, NH_2^+), 8.04 (1H, d, J = 2.4 Hz), 7.72 (1H, dd, J = 9.3, 2.4 Hz), 6.87 (1H, d, J = 9.3 Hz), 5.55 (1H, br d, J = 52.7 Hz, H-4), 5.09–5.03 (1H, m, H-2), 4.29 and 4.05 (2H, ABq, J = 16.8 Hz, $COCH_2$), 4.05–3.60 (4H, m, H-5 and NCH_2), 3.18 (2H, br s, NCH_2), 2.67–2.32 (2H, m, H-3). MS(ESI pos.) m/z 326 ($[M+H]^+$), 348 ($[M+Na]^+$); (ESI neg.) m/z 324 ($[M-H]^-$), 360 ($[M+Cl]^-$). HRMS calcd for $C_{14}H_{17}ClFN_5O[M]^+$ 325.1106; found: (m/z) 325.1112.

5.1.7. 6-{[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)ethyl]amino}nicotinamide dihydrochloride (2d). The title compound was obtained as a pale

pink powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.42 (2H, br s, NH_2^+), 8.52 (1H, s), 8.17 (1H, d, J = 10.1 Hz), 8.10 (1H, br s, CONH), 7.42 (1H, br s, CONH), 6.99 (1H, d, J = 10.1 Hz), 5.55 (1H, br d, J = 52.5 Hz, H-4), 5.10–5.04 (1H, m, H-2), 4.44–3.50 (4H, m, H-5 and $COCH_2$), 3.83 (2H, br s, NCH_2), 3.23 (2H, br s, NCH_2), 2.68–2.32 (2H, m, H-3). MS(ESI pos.) m/z 357 ($[M+Na]^+$); (ESI neg.) m/z 369 ($[M+Cl]^-$). HRMS calcd for $C_{15}H_{20}FN_6O_2[M+H]^+$ 335.1632; found: (m/z) 335.1638.

5.1.8. *tert*-Butyl [2-({2-[(2*S*,4*S*)-2-cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]carbamate (8). Compound **6** (0.95 g, 5.0 mmol) and *tert*-butyl (2-amino-2-methylpropyl) carbamate **7** (1.88 g, 10 mmol) were dissolved in MeOH (20 mL), and potassium iodide (0.83 g, 5.0 mmol) was added; the mixture was then stirred at room temperature overnight. After the solution was heated at 50 °C for 2 h, the reaction mixture was concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH/25% aqueous ammonia = 100:2:0.2). The resulting mixture, which consisted of compounds **7** and **8**, was dissolved in THF (30 mL); di-*tert*-butyl dicarbonate (1.09 g, 5.0 mmol), 4-dimethylaminopyridine (12 mg, 0.1 mmol) and 0.5 M NaOH aqueous solution (10 mL) were then added to the solution. After the mixture was stirred for 2 h at room temperature, brine was added and the mixture was extracted with EtOAc. The organic phase was dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH/25% aqueous ammonia = 100:2:0.2) to give the desired product (1.08 g, 63%) as a pale yellow amorphous substance. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.64 (1H, br t, J = 6.1 Hz), 5.48 (1H, br d, J = 53.5 Hz, H-4), 4.97–4.91 (1H, m, H-2), 3.91 (1H, dd, J = 24.6, 12.5 Hz, H-5), 3.71 (1H, ddd, J = 39.6, 12.5, 3.5 Hz, H-5), 3.38 and 3.23 (2H, ABq, J = 16.5 Hz, $COCH_2$), 2.87 (2H, d, J = 6.1 Hz, CH_2), 2.60–2.25 (2H, m, H-3), 1.76 (1H, br s, NH), 1.38 (9H, s, CH_3), 0.94 (6H, s, CH_3). MS(ESI pos.) m/z 343 ($[M+H]^+$), 365 ($[M+Na]^+$); (ESI neg.) m/z 341 ($[M-H]^-$). HRMS calcd for $C_{16}H_{28}FN_4O_3[M+H]^+$ 343.2145; found: (m/z) 343.2134.

5.1.9. (2*S*,4*S*)-1-[*N*-(2-Amino-1,1-dimethylethyl)glycyl]-4-fluoropyrrolidine-2-carbonitrile dihydrochloride (9). Compound **8** (100 mg, 0.29 mmol) was dissolved in EtOAc (0.5 mL), and 4 M HCl/EtOAc (0.5 mL) was added. After stirring at room temperature for 4 h, the precipitated solid was filtrated and dried to yield the desired product (88 mg, 96%) as a colorless powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.61 (3H, br s, NH_2^+), 5.57 (1H, br d, J = 50.7 Hz, H-4), 5.11–5.04 (1H, m, H-2), 4.32–3.72 (4H, m, H-5 and $COCH_2$), 3.21 (2H, s, CH_2), 2.58–2.33 (2H, m, H-3), 1.43 (6H, s, CH_3). MS(ESI pos.) m/z 243 ($[M+H]^+$); (ESI neg.) m/z 277 ($[M+Cl]^-$). HRMS calcd for $C_{11}H_{20}FN_4O[M+H]^+$ 243.1621; found: (m/z) 243.1639.

5.1.10. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]pyridine-2-carboxamide (10a). (Method C). Compound **9** (100 mg, 0.32 mmol) and triethylamine (0.177 mL, 1.3 mmol) were dissolved in DMF (0.5 mL), then nicotinoyl chloride hydrochloride (51 mg, 0.29 mmol) and DMF (0.5 mL) were added while cooling on ice and the mixture was stirred for 10 min and at room temperature overnight. A 10% aqueous $NaHCO_3$ solution (10 mL) and a saturated aqueous NaCl solution (10 mL) were added, and the mixture was extracted with $CHCl_3$ (3× 25 mL). The organic phase was dried over $MgSO_4$, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH/28% aqueous ammonia = 20:1:0.1) to yield the desired product (31 mg, 31%) as a yellow oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.69–8.63 (1H,m), 8.61–8.52 (1H, m), 8.05 (1H, m), 8.00 (1H, td, J = 7.3, 1.7 Hz), 7.65–7.57 (1H, m), 5.46 (1H, br d, J = 52.8 Hz, H-4), 5.01–4.94 (1H, m, H-2), 3.96 (1H, dd, J = 23.9, 11.8 Hz, H-5), 3.86–3.64 (1H, m, H-5), 3.54–3.24 (4H, m, CH_2), 2.62–2.25 (2H, m, H-3), 1.04 (6H, s, CH_3). MS(ESI pos.) m/z 348 ($[M+H]^+$), 370 ($[M+Na]^+$); (ESI neg.) m/z 346 ($[M-H]^-$). HRMS calcd for $C_{17}H_{23}FN_5O_2[M+H]^+$ 348.1836; found: (m/z) 348.1831.

5.1.11. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]benzamide (10b). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **10a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.23 (1H, m), 7.86 (1H, br d), 7.83 (1H, br d), 7.55–7.43 (3H, m), 5.50 (1H, br d, J = 52.8 Hz, H-4), 4.98–4.95 (1H, m, H-2), 3.96 (1H, dd, J = 23.8, 12.4 Hz, H-5), 3.74 (1H, ddd, J = 39.7, 12.5, 3.3 Hz, H-5), 3.55–3.30 (2H, m, CH_2), 3.27–3.20 (2H, m, CH_2), 2.59–2.28 (2H, m, H-3), 1.98 (1H, br s, NH), 1.02 (6H, br s, CH_3).

5.1.12. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]thiophene-2-carboxamide (10c). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **10a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.30–8.21 (1H, m), 7.80 (1H, d, J = 3.7 Hz), 7.74 (1H, d, J = 4.8 Hz), 7.15 (1H, dd, J = 4.8, 3.7 Hz), 5.45 (1H, br d, J = 50.4 Hz, H-4), 5.00–4.93 (1H, m, H-2), 3.96 (1H, dd, J = 23.8, 12.4 Hz, H-5), 3.73 (1H, ddd, J = 39.5, 12.4, 3.4 Hz, H-5), 3.52–3.28 (2H, m, CH_2), 3.26–3.12 (2H, m, CH_2), 2.62–2.25 (2H, m, H-3), 1.98–1.86 (1H, br s, NH), 1.02 (3H, s, CH_3), 1.01 (3H, s, CH_3). MS(ESI pos.) m/z 353 ($[M+H]^+$), 375 ($[M+Na]^+$); (ESI neg.) m/z 351 ($[M-H]^-$).

5.1.13. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-2,3-dihydro-1-benzofuran-5-carboxamide (10d). The title compound was obtained as a colorless gummy substance in a manner similar to method used to prepare **10a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.07–7.95 (1H, m), 7.75–7.73 (1H, m), 7.69–7.61 (1H, m), 6.80 (1H, d, J = 8.4 Hz), 5.46 (1H, br d, J = 51.4 Hz, H-4), 4.99–4.92 (1H, m, H-2), 4.59 (2H, t, J = 8.8 Hz, OCH_2), 3.96 (1H, dd,

J = 23.5, 12.6 Hz, H-5), 3.74 (1H, ddd, J = 39.8, 12.5, 3.3 Hz, H-5), 3.52–3.14 (6H, m, CH_2), 2.62–2.26 (2H, m, H-3), 2.10–1.80 (1H, m, NH), 1.01 (3H, s, CH_3), 1.00 (3H, s, CH_3). MS(ESI pos.) m/z 411 ($[M+Na]^+$); (ESI neg.) m/z 387 ($[M-H]^-$). HRMS calcd for $C_{20}H_{26}FN_4O_3[M+H]^+$ 389.1989; found: (m/z) 389.1977.

5.1.14. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-1*H*-benzimidazole-5-carboxamide (10e). The title compound was obtained as a yellow amorphous substance in a manner similar to method used to prepare **10a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.80–12.50 (1H, m), 8.38–8.18 (3H, m), 7.82–7.70 (1H, m), 5.47 (1H, br d, J = 51.8 Hz, H-4), 5.04–4.90 (1H, m, H-2), 4.10–3.18 (6H, H-5 and CH_2), 2.62–2.25 (2H, H-3), 1.06 (6H, s, CH_3). MS(ESI pos.) m/z 387 ($[M+H]^+$), 409 ($[M+Na]^+$); (ESI neg.) m/z 385 ($[M-H]^-$). HRMS calcd for $C_{19}H_{24}FN_6O_2[M+H]^+$ 387.1945; found: (m/z) 387.1959.

5.1.15. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-1*H*-1,2,3-benzotriazole-5-carboxamide (10f). The title compound was obtained as a pale yellow amorphous substance in a manner similar to method used to prepare **10a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.49–8.38 (2H, m), 7.99–7.82 (2H, m), 5.46 (1H, br d, J = 51.0 Hz, H-4), 5.03–4.90 (1H, m, H-2), 3.97 (1H, dd, J = 24.2, 12.7 Hz, H-5), 3.86–3.00 (5H, H-5 and CH_2), 2.64–2.25 (2H, m, H-3), 1.07 (6H, s, CH_3). MS(ESI pos.) m/z 410 ($[M+Na]^+$); (ESI neg.) m/z 386 ($[M-H]^-$). HRMS calcd for $C_{18}H_{23}FN_7O_2[M+H]^+$ 388.1897; found: (m/z) 388.1881.

5.1.16. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-2,2-dimethylpropanamide (10g). The title compound was obtained as a colorless solid in a manner similar to method used to prepare **10a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.27–7.15 (1H, m), 5.62–5.30 (1H, m, H-4), 4.99–4.92 (1H, m, H-2), 3.94 (1H, dd, J = 24.1, 11.9 Hz, H-5), 3.72 (1H, ddd, J = 39.6, 12.5, 3.5 Hz, H-5), 3.47–3.21 (2H, m, $COCH_2$), 3.08–2.92 (2H, m, CH_2), 2.56–2.26 (2H, m, H-3), 1.12 (9H, s, CH_3), 0.94 (6H, d, J = 2.5 Hz, CH_3). MS(ESI pos.) m/z 349 ($[M+Na]^+$); (ESI neg.) m/z 325 ($[M-H]^-$). HRMS calcd for $C_{16}H_{28}FN_4O_2[M+H]^+$ 327.2196; found: (m/z) 327.2185.

5.1.17. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-1-methylcyclohexanecarboxamide (10h). The title compound was obtained as a pale yellow oil in a manner similar to method used to prepare **10a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.29–7.19 (1H, m), 5.43 (1H, br d, J = 51.5 Hz, H-4), 4.99–4.90 (1H, m, H-2), 3.94 (1H, dd, J = 24.0, 11.6 Hz, H-5), 3.82–3.61 (1H, m, H-5), 3.48–3.22 (2H, m, $COCH_2$), 3.10–2.95 (2H, m, CH_2), 2.62–2.26 (2H, m, H-3), 2.04–1.75 (3H, m), 1.54–1.10 (8H, m), 1.05 (3H, s, CH_3), 0.96 (3H, s, CH_3), 0.95 (3H, s, CH_3). MS(ESI pos.) m/z 367 ($[M+H]^+$), 389 ($[M+Na]^+$); (ESI neg.) m/z 365 ($[M-H]^-$).

5.1.18. 2,2-Bis(4-chlorophenyl)-*N*-[2-({2-[(2*S*,4*S*)-2-cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]acetamide (10i). (Method F). The title compound was obtained as a pale yellow amorphous substance in a manner similar to method used to prepare **10a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.22–8.13 (1H, m), 7.41–7.27 (8H, m), 5.45 (1H, br d, J = 53.2 Hz, H-4), 5.10 (1H, s, CH), 4.97–4.90 (1H, m, H-2), 3.90 (1H, dd, J = 24.2, 12.0 Hz, H-5), 3.78–3.58 (1H, m, H-5), 3.48–3.20 (2H, m, $COCH_2$), 3.15–2.90 (2H, m, CH_2), 2.60–2.25 (2H, m, H-3), 0.94 (6H, s, CH_3).

5.1.19. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]benzenesulfonamide (10j). (Method D). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **10a** using benzenesulfonyl chloride. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.82 (2H, dd, J = 8.1, 1.9 Hz), 7.67–7.55 (4H, m), 5.49 (1H, br d, J = 53.0 Hz, H-4), 4.98–4.92 (1H, m, H-2), 3.87 (1H, dd, J = 24.3, 12.1 Hz, H-5), 3.66 (1H, ddd, J = 39.4, 12.5, 3.4 Hz, H-5), 3.33 and 3.18 (2H, ABq, J = 16.5 Hz, $COCH_2$), 2.63 (2H, s, CH_2), 2.53–2.30 (2H, m, H-3), 0.96 (6H, s, CH_3). MS(ESI pos.) m/z 383 ($[M+H]^+$), 405 ($[M+Na]^+$); (ESI neg.) m/z 381 ($[M-H]^-$). HRMS calcd for $C_{17}H_{24}FN_4O_3S[M+H]^+$ 383.1551; found: (m/z) 383.1551.

5.1.20. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-*N*-methylbenzenesulfonamide (11a). (Method G). Compound **10d** (57 mg, 0.15 mmol) and triphenylphosphine (59 mg, 0.23 mmol) were dissolved in THF (3 mL), and then MeOH (0.009 mL) and diethyl azodicarboxylate (40% toluene solution, 98 mg, 0.23 mmol) were added at room temperature, followed by stirring overnight. The mixture was concentrated under reduced pressure, and the resulting residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH/25% aqueous ammonia = 100:3:0.3–100:5:0.5) to give the desired product (25 mg, 42%) as a colorless amorphous substance. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.79 (2H, br d, J = 8.4 Hz), 7.72–7.60 (3H, m), 5.49 (1H, br d, J = 52.7 Hz, H-4), 4.98–4.92 (1H, m, H-2), 3.94 (1H, dd, J = 24.1, 12.0 Hz, H-5), 3.70 (1H, ddd, J = 39.5, 12.5, 3.2 Hz, H-5), 3.46 and 3.30 (2H, ABq, J = 17.4 Hz, $COCH_2$), 2.92 (2H, s, CH_2), 2.78 (3H, s, CH_3), 2.60–2.26 (2H, m, H-3), 1.07 (6H, s, CH_3). MS(ESI pos.) m/z 397 ($[M+H]^+$), 419 ($[M+Na]^+$); (ESI neg.) m/z 395 ($[M-H]^-$). HRMS calcd for $C_{19}H_{26}FN_4O_3S[M+H]^+$ 397.1710; found: (m/z) 397.1718.

5.1.21. (2*S*,4*S*)-4-Fluoro-1-{*N*-[2-(isobutylamino)-1,1-dimethylethyl]glycyl}pyrrolidine-2-carbonitrile (10k). (Method E). Compound **9** (150 mg, 0.48 mmol) and isobutyraldehyde (34 mg, 0.48 mmol) were suspended in $CHCl_3$ (2 mL), and the mixture was stirred at room temperature for 30 min. Sodium triacetoxyborohydride (202 mg, 0.95 mmol) was then added, followed by stirring at room temperature for 30 min. A 10% aqueous $NaHCO_3$ solution and a saturated aqueous NaCl solution were added, and the mixture was extracted with $CHCl_3$.

The organic phase was dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH/25% aqueous ammonia = 100:3:0.3–100:5:0.5) to yield the desired product (99 mg, 52%) as a colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.60–5.28 (1H, m, H-4), 4.98–4.90 (1H, m, H-2), 3.93 (1H, dd, J = 23.5, 12.4 Hz, H-5), 3.71 (1H, ddd, J = 39.6, 12.4, 3.4 Hz, H-5), 3.41–3.16 (2H, m, $COCH_2$), 2.62–2.28 (6H, m), 1.64 (1H, m), 0.97 (6H, s, CH_3), 0.85 (6H, d, J = 6.7 Hz, CH_3). MS(ESI pos.) m/z 299 ($[M+H]^+$); (ESI neg.) m/z 297 ($[M-H]^-$). HRMS calcd for $C_{15}H_{28}FN_4O$ $[M+H]^+$ 299.2247; found: (m/z) 299.2236.

5.1.22. (2*S*,4*S*)-4-Fluoro-1-(*N*-{2-[(4-cyanobenzyl)amino]-1,1-dimethylethyl}glycyl)pyrrolidine-2-carbonitrile (10l). The title compound was obtained as a colorless gum in a manner similar to method used to prepare **10e** using 4-cyanobenzaldehyde. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.77 (2H, d, J = 8.4 Hz), 7.54 (2H, d, J = 8.2 Hz), 5.49 (1H, br d, J = 52.8 Hz, H-4), 4.99–4.93 (1H, m, H-2), 3.89 (1H, dd, J = 24.1, 11.6 Hz, H-5), 3.68 (1H, ddd, J = 39.6, 12.4, 3.3 Hz, H-5), 3.32 and 3.17 (2H, ABq, J = 16.3 Hz, $COCH_2$), 2.60–2.25 (4H, m, H-3 and CH_2), 0.98 (6H, s, CH_3). MS(ESI pos.) m/z 358 ($[M+H]^+$), 380 ($[M+Na]^+$); (ESI neg.) m/z 356 ($[M-H]^-$). HRMS calcd for $C_{19}H_{25}FN_5O$ $[M+H]^+$ 358.2043; found: (m/z) 358.2044.

5.1.23. (2*S*,4*S*)-1-{*N*-[2-(Diethylamino)-1,1-dimethylethyl]glycyl}-4-fluoropyrrolidine-2-carbonitrile (11b). (Method I). The title compound was obtained as a colorless oil in a manner similar to method used to prepare **10e** using acetaldehyde. Dialkylated **11b** was a major product. ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.60–5.30 (1H, m, H-4), 4.99–4.90 (1H, m, H-2), 3.93 (1H, dd, J = 23.3, 12.5 Hz, H-5), 3.71 (1H, ddd, J = 39.6, 12.5, 3.4 Hz, H-5), 3.44–3.20 (2H, m, $COCH_2$), 2.62–2.30 (6H, m, H-3 and CH_2), 2.28–2.18 (2H, m, CH_2), 1.01–0.85 (12H, m, CH_3).

5.1.24. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-*N*-isobutylbenzamide (11c). (Method H). The title compound was obtained as a colorless oil in a manner similar to method used to prepare **10a** using benzoyl chloride and **10e**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.50–7.28 (5H, m), 5.46 (1H, br d, J = 51.1 Hz, H-4), 5.01–4.91 (1H, m, H-2), 4.06–3.18 (8H, m, H-5 and CH_2), 2.62–2.35 (2H, m, H-3), 1.90–1.78 (1H, m), 1.13–0.88 (6H, m), 0.86–0.54 (6H, m). MS(ESI pos.) m/z 403 ($[M+H]^+$), 425 ($[M+Na]^+$); (ESI neg.) m/z 401 ($[M-H]^-$). HRMS calcd for $C_{22}H_{32}FN_4O_2$ $[M+H]^+$ 403.2509; found: (m/z) 403.2524.

5.1.25. *N*-[2-({2-[(2*S*,4*S*)-2-Cyano-4-fluoropyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-*N*-(4-cyanobenzyl)benzamide (11d). The title compound was obtained as a colorless amorphous substance in a manner similar to method used to prepare **10a** using benzoyl chloride and **10f**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.88–7.72 (2H, m), 7.60–7.20 (7H, m), 5.61–5.30 (1H, br d, J = 51.8 Hz, H-4), 5.08–4.92 (1H, m, H-2), 4.81 (2H, br s, $PhCH_2$), 3.89 (1H, dd, J = 24.0, 12.5 Hz, H-5), 3.80–3.54 (1H, m, H-5), 3.50–3.20 (4H, m, $COCH_2$ and CH_2), 2.60–2.25 (2H, m, H-3), 1.11 (4.3H, s, CH_3 of major isomer), 0.85 (1.7H, s, CH_3 of minor isomer). NMR revealed that compound **11d** occurred as mixtures of conformers in solution. By heating, two peaks of methyl group tended to converge. MS(ESI pos.) m/z 462 ($[M+H]^+$), 484 ($[M+Na]^+$); (ESI neg.) m/z 460 ($[M-H]^-$). HRMS calcd for $C_{26}H_{29}FN_5O_2$ $[M+H]^+$ 462.2305; found: (m/z) 462.2307.

5.1.26. (2*S*,4*S*)-4-Fluoro-1-[*N*-(1-methyl-1-pyridin-2-yl-ethyl)glycyl]pyrrolidine-2-carbonitrile (12a). (Method B). 1-Methyl-1-pyridin-2-yl-ethylamine (177 mg, 1.3 mmol) was dissolved in MeOH (10 mL), and compound **6** (112 mg, 0.59 mmol) and potassium iodide (98 mg, 0.59 mmol) were added. The mixture was stirred at room temperature for 3 days. The solution was concentrated under reduced pressure, and the resulting residue was purified by silica gel column chromatography (developing solvent: $CHCl_3$/MeOH = 50:1–25:1) to yield the desired product (104 mg, 61%) as a light brown amorphous substance. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.61–8.54 (1H, m), 7.67 (1H, td, J = 7.7, 1.9 Hz), 7.49–7.39 (1H, m), 7.19–7.12 (1H, m), 5.48–5.16 (1H, m, H-4), 4.92 (1H, d, J = 9.2 Hz, H-2), 4.03–3.26 (4H, m, H-5 and CH_2), 2.63 (1H, t, J = 15.8 Hz), 2.40–2.12 (2H, m, H-3), 1.53 (6H, s, CH_3). MS(ESI pos.) m/z 291 ($[M+H]^+$), 313 ($[M+Na]^+$); (ESI neg.) m/z 289 ($[M-H]^-$). HRMS calcd for $C_{15}H_{20}FN_4O$ $[M+H]^+$ 291.1621; found: (m/z) 291.1612.

5.1.27. (2*S*,4*S*)-4-Fluoro-1-{*N*-[1-(2-furyl)-1-methylethyl]-glycyl}pyrrolidine-2-carbonitrile (12b). The title compound was obtained as a brown gum in a manner similar to method used to prepare **12a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.55 (1H, dd, J = 1.8, 0.9 Hz), 6.35 (1H, m), 6.21 (1H, dd, J = 3.2, 0.9 Hz), 5.40 (1H, br d, J = 50.8 Hz, H-4), 4.95–4.87 (1H, m, H-2), 3.84 (1H, dd, J = 23.9, 12.6 Hz, H-5), 3.61 (1H, ddd, J = 39.6, 12.5, 3.3 Hz, H-5), 3.21 and 3.02 (2H, ABq, J = 16.0 Hz, $COCH_2$), 2.60–2.22 (2H, m, H-3), 1.44–1.32 (6H, m, CH_3). MS(ESI pos.) m/z 302 ($[M+Na]^+$); (ESI neg.) m/z 278 ($[M-H]^-$). HRMS calcd for $C_{14}H_{18}FN_3O_2Na$ $[M+Na]^+$ 302.1281; found: (m/z) 302.1277.

5.1.28. (2*S*,4*S*)-4-Fluoro-1-{*N*-[1-methyl-1-(2-thienyl)-ethyl]glycyl}pyrrolidine-2-carbonitrile (12c). The title compound was obtained as a black oil in a manner similar to method used to prepare **12a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.45 (1H, dd, J = 5.0, 3.0 Hz), 7.23 (1H, dd, J = 3.0, 1.4 Hz), 7.13 (1H, dd, J = 5.0, 1.4 Hz), 5.40 (1H, br d, J = 52.1 Hz, H-4), 4.95–4.87 (1H, m, H-2), 3.82 (1H, dd, J = 24.1, 12.4 Hz, H-5), 3.60 (1H, ddd, J = 39.7, 12.4, 3.4 Hz, H-5), 3.18 and 3.00 (2H, ABq, J = 16.1 Hz, $COCH_2$), 2.56–2.22 (2H, m, H-3), 1.40–1.35 (6H, m, CH_3). MS(ESI pos.) m/z 318 ($[M+Na]^+$); (ESI neg.) m/z 294 ($[M-H]^-$). HRMS calcd for $C_{14}H_{18}FN_3ONaS[M+Na]^+$ 318.1052; found: (m/z) 318.1060.

5.1.29. (2*S*,4*S*)-1-{*N*-[1-(1-Benzofuran-2-yl)-1-methylethyl]glycyl}-4-fluoropyrrolidine-2-carbonitrile (12d). The title compound was obtained as a pale yellow solid in a manner similar to method used to prepare **12a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.59–7.48 (2H, m), 7.28–7.26 (2H, m), 6.70 (1H, s), 5.38 (1H, br d, J = 52.2 Hz, H-4), 4.90–4.83 (1H, m, H-2), 3.85 (1H, dd, J = 23.2, 12.3 Hz, H-5), 3.60 (1H, ddd, J = 39.6, 12.5, 3.4 Hz, H-5), 3.38–3.04 (2H, m, $COCH_2$), 2.60–2.18 (2H, m, H-3), 1.48 (6H, s, CH_3). MS(ESI pos.) *m*/*z* 352 ([M+Na]$^+$); (ESI neg.) *m*/*z* 328 ([M−H]$^-$). HRMS calcd for $C_{18}H_{20}FN_3O_2Na$[M+Na]$^+$ 352.1437; found: (*m*/*z*) 352.1454.

5.1.30. (2*S*,4*S*)-1-{*N*-[(2*E*)-1,1-Dimethyl-3-phenylprop-2-en-1-yl]glycyl}-4-fluoropyrrolidine-2-carbonitrile (12e). The title compound was obtained as a colorless amorphous substance in a manner similar to method used to prepare **12a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.44–7.38 (2H, m), 7.35–7.27 (2H, m), 7.25–7.17 (1H, m), 6.40 (1H, d, J = 16.3 Hz, C═CH), 6.21 (1H, d, J = 16.3 Hz, C═CH), 5.42 (1H, br d, J = 51.8 Hz, H-4), 4.91 (1H, d, J = 8.9 Hz, H-2), 3.90 (1H, dd, J = 23.5, 12.4 Hz, H-5), 3.78–3.56 (1H, m, H-5), 3.44–3.14 (2H, m, $COCH_2$), 2.60–2.20 (2H, m, H-3), 2.06–1.98 (1H, m), 1.22 (6H, s, CH_3). MS(ESI pos.) *m*/*z* 338 ([M+Na]$^+$). HRMS calcd for $C_{18}H_{22}FN_3ONa$[M+Na]$^+$ 338.1645; found: (*m*/*z*) 338.1641.

5.1.31. (2*S*,4*S*)-1-{*N*-[2-(3,4-Dimethoxyphenyl)ethyl]glycyl}-4-fluoropyrrolidine-2-carbonitrile hydrochloride (12f). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.89 (1H, d, J = 8.2 Hz), 6.83 (1H, d, J = 1.9 Hz), 6.74 (1H, dd, J = 8.2, 1.9 Hz), 5.52 (1H, br d, J = 53.0 Hz, H-4), 5.05–5.00 (1H, m, H-2), 4.02–3.61 (4H, m, H-5 and $COCH_2$), 3.75 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 3.07–2.92 (2H, m, NCH_2), 2.87–2.77 (2H, m, $PhCH_2$), 2.64–2.30 (2H, m, H-3). MS(ESI pos.) *m*/*z* 336 ([M+H]$^+$), 358 ([M+Na]$^+$); (ESI neg.) *m*/*z* 334 ([M−H]$^-$), 370 ([M+Cl]$^-$). HRMS calcd for $C_{17}H_{22}FN_3O_3Na$ [M+Na]$^+$ 335.1645; found: (*m*/*z*) 335.1670.

5.1.32. (2*S*,4*S*)-1-[*N*-(*tert*-Butyl)glycyl]-4-fluoropyrrolidine-2-carbonitrilehydrochloride (12g). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. Mp 247–250 °C (decomp.). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.10 (2H, br s, NH_2^+), 5.56 (1H, br d, J = 52.9 Hz, H-4), 5.09–5.06 (1H, m, H-2),4.16 (1H, dd, J = 24.4, 12.5 Hz, H-5), 4.12 and 3.88 (2H, ABq, J = 16.5 Hz, $COCH_2$), 3.86 (1H, ddd, J = 39.9, 12.5, 3.3 Hz, H-5), 2.54–2.40 (2H, m, H-3),1.33 (9H, s, CH_3); ^{13}C NMR (125.4 MHz, DMSO-d_6) δ 165.1, 118.4, 93.0 (d, J_{C-F} = 174.7 Hz), 56.4, 52.1 (d, J_{C-F} = 22.7 Hz), 44.8, 42.0, 35.7 (d, J_{C-F} = 19.6 Hz), 25.0; ^{19}F NMR (282.2 MHz, DMSO-d_6) δ −174.5. MS(ESI pos.) *m*/*z* 250 ([M+Na]$^+$); (ESI neg.) *m*/*z* 226 ([M−H]$^-$), 262 ([M+Cl]$^-$). HRMS calcd for $C_{11}H_{19}FN_3O$[M+H]$^+$ 228.1512; found: (*m*/*z*) 228.1515. Anal. Calcd for $C_{11}H_{18}FN_3O$ HCl: C, 50.09; H, 7.26; N, 15.93; Cl, 13.44; F, 7.20. Found: C, 50.11; H, 7.37; N, 15.89; Cl, 13.38; F, 7.19. $[\alpha]_D^{25}$ −101.5 (*c* 0.3, MeOH).

5.1.33. (2*S*,4*S*)-4-Fluoro-1-(*N*-isopropylglycyl)pyrrolidine-2-carbonitrile hydrochloride (12h). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.52 (1H, br d, J = 51.4 Hz, H-4), 5.03–4.97 (1H, m, H-2), 3.98 (1 H, dd, J = 23.2, 12.4 Hz, H-5), 3.84–3.64 (1H, m, H-5), 3.74 and 3.55 (2H, ABq, J = 16.6 Hz, $COCH_2$), 3.02–2.88 (1H, m, C*H*Me_2), 2.62–2.26 (2H, m, H-3), 1.09 (6H, d, J = 6.2 Hz, CH_3). MS(ESI pos.) *m*/*z* 214 ([M+H]$^+$), 236 ([M+Na]$^+$); (ESI neg.) *m*/*z* 212 ([M−H]$^-$), 248 ([M+Cl]$^-$). HRMS calcd for $C_{10}H_{16}FN_3O$ [M]$^+$ 213.1277; found: (*m*/*z*) 213.1297.

5.1.34. (2*S*,4*S*)-4-Fluoro-1-[*N*-(3-isopropoxypropyl)glycyl]pyrrolidine-2-carbonitrile hydrochloride (12i). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.23 (2H, br s, NH_2^+), 5.54 (1H, br d, J = 52.5 Hz, H-4), 5.10–5.03 (1H, m, H-2), 4.21 and 3.96 (2H, ABq, J = 16.6 Hz, $COCH_2$), 4.04–3.90 (1H, m, H-5), 3.84–3.76 (1H, m, H-5), 3.58–3.48 (1H, m, C*H*Me_2), 3.43 (2H, t, J = 5.8 Hz, OCH_2), 3.10–2.85 (2H, m, NCH_2), 2.67–2.31 (2H, m, H-3), 1.95–1.82 (2H, m, CH_2), 1.09 (6H, d, J = 6.2 Hz, CH_3). MS(ESI pos.) *m*/*z* 294 ([M+Na]$^+$); (ESI neg.) *m*/*z* 270 ([M−H]$^-$) 306 ([M+Cl]$^-$). HRMS calcd for $C_{13}H_{23}FN_3O_2$ [M+H]$^+$ 272.1774; found: (*m*/*z*) 272.1785. Anal. Calcd for $C_{13}H_{22}FN_3O_2$ HCl: C, 50.73; H, 7.53; N, 13.65; Cl, 11.52; F, 6.17. Found: C, 50.44; H, 7.49; N, 13.44; Cl, 11.39; F, 5.92.

5.1.35. (2*S*,4*S*)-4-Fluoro-1-[*N*-(2-methoxy-1,1-dimethylethyl)glycyl]pyrrolidine-2-carbonitrile hydrochloride (12j). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.82 (2H, br s, NH_2^+), 5.56 (1H, br d, J = 53.0 Hz, H-4), 5.09–5.04 (1H, m, H-2), 4.18–3.66 (4H, m, H-5 and $COCH_2$), 3.42 (2H, s, OCH_2), 3.32 (3H, s, OCH_3), 2.58–2.32 (2H, m, H-3), 1.27 (6H, s, CH_3). MS(ESI pos.) *m*/*z* 258 ([M+H]$^+$), 280 ([M+Na]$^+$); (ESI neg.) *m*/*z* 292 ([M+Cl]$^-$). HRMS calcd for $C_{12}H_{21}FN_3O_2$ [M+H]$^+$258.1618; found: (*m*/*z*) 258.1603.

5.1.36. (2*S*,4*S*)-4-Fluoro-1-[*N*-(2-hydroxy-1,1-dimethylethyl)glycyl]pyrrolidine-2-carbonitrile hydrochloride (12k). The title compound was obtained as a pale pink powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.75 (2H, br s, NH_2^+), 5.57 (1H, br s, OH), 5.56 (1H, br d, J = 52.7 Hz, H-4), 5.10–5.04 (1H, m, H-2), 4.20–3.70 (4H, m, H-5 and $COCH_2$), 3.48 (2H, s, OCH_2), 2.70–2.30 (2H, m, H-3), 1.24 (6H, s, CH_3). MS(ESI pos.) *m*/*z* 244 ([M+H]$^+$), 266 ([M+Na]$^+$); (ESI neg.) *m*/*z* 242 ([M−H]$^-$), 278 ([M+Cl]$^-$). HRMS calcd for $C_{11}H_{18}FN_3O_2$ [M+H]$^+$ 244.1461; found: (*m*/*z*) 244.1449. Anal. Calcd for $C_{11}H_{18}FN_3O_2$ HCl $0.3H_2O$: C, 46.33; H, 6.93; N, 4.74; Cl, 12.43; F, 6.66. Found: C, 46.68; H, 6.98; N, 14.29; Cl, 12.20; F, 6.43.

5.1.37. (2*S*,4*S*)-4-Fluoro-1-[*N*-(2-hydroxy-1,1-dimethylethyl)glycyl]pyrrolidine-2-carbonitrile benzenesulfonate (TS-021). (2*S*,4*S*)-4-Fluoro-1-[*N*-(2-hydroxy-1,1-dimethylethyl)glycyl]pyrrolidine-2-carbonitrile (free base of **12k**, 20.0 g, 82.2 mmol) was dissolved in MeOH (300 mL), and benzenesulfonic acid mono hydrate (15.2 g, 86.3 mmol) was added, followed by stirring at room temperature for 30 min. Isoporpyl ether (330 mL) was added, followed by stirring at room temperature for 2 h. The resulting powder was collected using filtration and dried in vacuo to yield the desired product (31.5 g, 95%) as a colorless powder. Mp 220–221 °C (decomp.). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.64 (1H, br s), 8.57 (1H, br s), 7.62–7.58 (2H, m), 7.35–7.28 (3H, m), 5.54 (1H, br d, J = 52.1 Hz), 5.52–5.44 (1H, br s), 5.06 (1H, d, J = 8.5 Hz), 4.15–3.70 (4H, m), 3.47 (2H, s), 2.54–2.38 (2H, m), 1.23 (3H, s), 1.22 (3H, s); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 165.1, 148.3, 128.3, 127.6, 125.4, 118.4, 93.0 (d, J_{C-F} = 174.7 Hz), 65.2, 59.8, 52.1 (d, J_{C-F} = 22.7 Hz), 44.8, 42.2, 35.7 (d, J_{C-F} = 20.7 Hz), 20.2, 20.0; ^{19}F NMR (282.2 MHz, DMSO-d_6) δ − 174.5. MS(ESI pos.) m/z 266 ([M+Na]$^+$), 244 ([M+H]$^+$); (ESI neg.) m/z 400 ([M+BsOH−H]$^-$). Anal. Calcd for $C_{17}H_{24}FN_3O_5S$: C, 50.86; H, 6.03; N, 10.47; F, 4.73;S, 7.99. Found: C, 50.80; H, 6.00; N, 10.34; F, 4.73;S, 7.95. $[\alpha]_D^{23}$ −61 (c 0.3, MeOH).

5.1.38. (2*S*,4*S*)-1-[*N*-(1,1-Diethylprop-2-yn-1-yl)glycyl]-4-fluoropyrrolidine-2-carbonitrile hydrochloride (12l). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **10a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.48 (1H, br d, J = 51.5 Hz, H-4), 4.99–4.93 (1H, m, H-2), 3.95 (1H, dd, J = 24.6, 12.6 Hz, H-5), 3.72 (1H, ddd, J = 39.6, 12.6, 3.4 Hz, H-5), 3.50–3.27 (2H, m, $COCH_2$), 3.18 (1H, s, CH), 2.62–2.28 (2H, m, H-3), 2.06 (1H, t, J = 5.9 Hz), 1.53 (4H, q, J = 7.4 Hz), 0.87 (6H, t, J = 7.4 Hz). MS(ESI pos.) m/z 288 ([M+Na]$^+$). HRMS calcd for $C_{14}H_{21}FN_3O$ [M+H]$^+$ 266.1669; found: (m/z) 266.1654.

5.1.39. (2*S*,4*S*)-1-(*N*-Cyclopropylglycyl)-4-fluoropyrrolidine-2-carbonitrile hydrochloride (12m). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.50 and 9.35 (2H, br s each, NH_2^+), 5.55 (1H, br d, J = 53.0 Hz, H-4), 5.10–5.04 (1H, m, H-2), 4.31 and 4.05 (2H, ABq, J = 16.9 Hz, $COCH_2$), 4.12–3.65 (2H, m, H-5), 2.76–2.64 (1H, m, NCH), 2.62–2.32 (2H, m, H-3), 0.96–0.88 (2H, m, CH_2), 0.77–0.69 (2H, m, CH_2). MS(ESI pos.) m/z 212 ([M+H]$^+$), 234 ([M+Na]$^+$); (ESI neg.) m/z 246 ([M+Cl]$^-$). HRMS calcd for $C_{10}H_{15}FN_3O$ [M+H]$^+$ 212.1198; found: (m/z) 212.1208.

5.1.40. (2*S*,4*S*)-1-(*N*-Cyclobutylglycyl)-4-fluoropyrrolidine-2-carbonitrile hydrochloride (12n). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.98 (2H, br s, NH_2^+), 5.52 (1H, br d, J = 53.0 Hz, H-4), 5.05–4.99 (1H, m, H-2), 4.04–3.34 (5H, m, H-5, NCH and $COCH_2$), 2.63–2.30 (2H, m, H-3), 2.18–1.90 (4H, m, CH_2), 1.80–1.57 (2H, m, CH_2). MS(ESI pos.) m/z 226 ([M+H]$^+$), 248 ([M+Na]$^+$); (ESI neg.) m/z 260 ([M+Cl]$^-$). HRMS calcd for $C_{11}H_{17}FN_3O$ [M+H]$^+$ 226.1356; found: (m/z) 226.1352.

5.1.41. (2*S*,4*S*)-1-(*N*-Cyclopentylglycyl)-4-fluoropyrrolidine-2-carbonitrile hydrochloride (12o). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.11 (2H, br s, NH_2^+), 5.55 (1H, br d, J = 52.7 Hz, H-4), 5.09–5.03 (1H, m, H-2), 4.30–3.60 (4H, m, H-5 and $COCH_2$), 3.52–3.36 (1H, m, NCH), 2.66–2.32 (2H, m, H-3), 2.02–1.87 (2H, m, CH_2), 1.78–1.46 (6H, m, CH_2). MS(ESI pos.) m/z 240 ([M+H]$^+$), 262 ([M+Na]$^+$); (ESI neg.) m/z 238 ([M−H]$^-$), 274 ([M+Cl]$^-$). HRMS calcd for $C_{12}H_{19}FN_3O$ [M+H]$^+$ 240.1512; found: (m/z) 240.1499.

5.1.42. (2*S*,4*S*)-4-Fluoro-1-{*N*-[1-(hydroxymethyl)cyclopentyl]glycyl}pyrrolidine-2-carbonitrile hydrochloride (12p). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. Mp 175–178 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.99 (2H, br s, NH_2^+), 5.68 (1H, br s, OH), 5.55 (1H, br d, J = 52.4 Hz, H-4), 5.08–5.05 (1H, m, H-2), 4.17 and 3.98 (2H, ABq, J = 16.5 Hz, $COCH_2$), 4.09 (1H, dd, J = 23.1, 12.2 Hz, H-5), 3.82 (1H, ddd, J = 39.3, 12.2, 3.1 Hz, H-5), 3.51 and 3.48 (2H, ABq, J = 12.5 Hz, OCH_2), 2.56–2.36 (2H, m, H-3), 1.86–1.68 (6H, m, CH_2), 1.59–1.48 (2H, m, CH_2); ^{13}C NMR (125.4 MHz, DMSO-d_6) δ 165.1, 118.4, 93.0 (d, J_{C-F} = 174.7 Hz), 69.6, 62.7, 52.1 (d, J_{C-F} = 22.7 Hz), 44.8, 43.5, 35.7 (d, J_{C-F} = 20.7 Hz), 31.6, 31.2, 23.9; ^{19}F NMR (282.2 MHz, DMSO-d_6) δ −174.5. MS(ESI pos.) m/z 270 ([M+H]$^+$), 292 ([M+Na]$^+$); (ESI neg.) m/z 304 ([M+Cl]$^-$). HRMS calcd for $C_{13}H_{21}FN_3O_2$[M+H]$^+$270.1618; found: (m/z) 270.1614. Anal. Calcd for $C_{13}H_{20}FN_3O_2$ HCl: C, 51.06; H, 6.92; N, 13.74; Cl, 11.59; F, 6.21. Found: C, 50.85; H, 7.02; N, 13.54; Cl, 11.53; F, 6.16. $[\alpha]_D^{25}$ −89.3 (c 0.3, MeOH).

5.1.43. (2*S*,4*S*)-1-(*N*-Cyclooctylglycyl)-4-fluoropyrrolidine-2-carbonitrile hydrochloride (12q). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.09 and 8.96 (2H, br s each, NH_2^+), 5.56 (1H, br d, J = 52.6 Hz, H-4), 5.10–5.04 (1H, m, H-2), 4.21 and 3.96 (2H, ABq, J = 17.0 Hz, $COCH_2$), 4.04 (1H, dd, J = 23.6, 12.4 Hz, H-5), 3.78 (1H, ddd, J = 39.5, 12.4, 3.3 Hz, H-5), 3.24 (1H, br s, NCH), 2.67–2.33 (2H, m, H-3), 2.00–1.88 (2H, m, CH_2), 1.78–1.34 (12H, m, CH_2). MS(ESI pos.) m/z 282 ([M+H]$^+$), 304 ([M+Na]$^+$); (ESI neg.) m/z 280 ([M−H]$^-$), 316 ([M+Cl]$^-$). HRMS calcd for $C_{15}H_{24}FN_3O$ [M]$^+$ 281.1903; found: (m/z) 281.1911.

5.1.44. (2*S*,4*S*)-1-(*N*-1-Adamantylglycyl)-4-fluoropyrrolidine-2-carbonitrile hydrochloride (12r). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.04 and 8.91 (2H, br s each, NH_2^+), 5.56 (1H, br d, J = 52.4 Hz, H-4), 5.10–5.04

(1H, m, H-2), 4.22–3.72 (4H, m, H-5 and $COCH_2$), 2.68–2.34 (2H, m, H-3), 2.12 (3H, br s), 1.91 (6H, br s), 1.71–1.52 (6H, m). MS(ESI pos.) *m/z* 306 ($[M+H]^+$), 328 ($[M+Na]^+$); (ESI neg.) *m/z* 304 ($[M-H]^-$), 340 ($[M+Cl]^-$). HRMS calcd for $C_{17}H_{25}FN_3O$ $[M+H]^+$ 306.1982; found: (*m/z*) 306.1973.

5.1.45. (2*S*,4*S*)-1-(*N*-2-Adamantylglycyl)-4-fluoropyrrolidine-2-carbonitrile hydrochloride (12s). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^1H NMR (300 MHz, DMSO-d_6) δ 9.01 (2H, br s, NH_2^+), 5.55 (1H, br d, $J = 53.0$ Hz, H-4), 5.10–4.99 (1H, m, H-2), 4.24 and 3.98 (2H, ABq, $J = 16.8$ Hz, $COCH_2$), 4.06 (1H, dd, $J = 24.4$, 12.8 Hz, H-5), 3.78 (1H, ddd, $J = 39.2$, 12.6, 3.3 Hz, H-5), 3.29 (1H, br s, NCH), 2.68–2.32 (2H, m), 2.28–2.08 (4H, m), 1.90–1.78 (4H, m), 1.74–1.64 (4H, m), 1.60–1.49 (2H, m). MS(ESI pos.) *m/z* 306 ($[M+H]^+$), 328 ($[M+Na]^+$); (ESI neg.) *m/z* 304 ($[M-H]^-$), 340 ($[M+Cl]^-$). HRMS calcd for $C_{17}H_{24}FN_3O$ $[M]^+$ 305.1903; found: (*m/z*) 305.1913.

5.1.46. (2*S*,4*S*)-4-Fluoro-1-[*N*-(3-hydroxy-1-adamantyl)-glycyl]pyrrolidine-2-carbonitrile hydrochloride (12t). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^1H NMR (300 MHz, DMSO-d_6) δ 5.55 (1H, br d, $J = 52.7$ Hz, H-4), 5.08–5.02 (1H, m, H-2), 4.75 (1H, s, OH), 4.19–3.70 (4H, m, H-5 and $COCH_2$), 2.65–2.32 (2H, m, H-3), 2.22 (2H, br s), 1.80–1.43 (12H, m). MS(ESI pos.) *m/z* 322 ($[M+H]^+$), 344 ($[M+Na]^+$); (ESI neg.) *m/z* 320 ($[M-H]^-$), 356 ($[M+Cl]^-$). HRMS calcd for $C_{17}H_{25}FN_3O_2$ $[M+H]^+$ 322.1931; found: (*m/z*) 322.1913.

5.1.47. (2*S*,4*S*)-4-Fluoro-1-[*N*-(3-methoxy-1-adamantyl)-glycyl]pyrrolidine-2-carbonitrile hydrochloride (12u). The title compound was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^1H NMR (300 MHz, DMSO-d_6) δ 9.04 (2H, br s, NH_2^+), 5.57 (1H, br d, $J = 52.4$ Hz, H-4), 5.10–5.04 (1H, m, H-2), 4.24–3.66 (4H, m, H-5 and $COCH_2$), 3.14 (3H, s, OCH_3), 2.58–2.38 (2H, m, H-3), 2.36–2.24 (2H, m), 1.88–1.78 (6H, m), 1.72–1.42 (6H, m). MS(ESI pos.) *m/z* 336 ($[M+H]^+$); (ESI neg.) *m/z* 370 ($[M+Cl]^-$). HRMS calcd for $C_{18}H_{27}FN_3O_2$ $[M+H]^+$ 336.2087; found: (*m/z*) 336.2101.

5.1.48. (2*S*,4*S*)-4-Fluoro-1-[*N*-(5-hydroxy-2-adamantyl)-glycyl]pyrrolidine-2-carbonitrile hydrochloride (12v). The title compound, a mixture of stereoisomer, was obtained as a colorless powder in a manner similar to method used to prepare **2a**. ^1H NMR (300 MHz, DMSO-d_6) δ 5.53 (1H, br d, $J = 52.8$ Hz, H-4), 5.06–5.00 (1H, m, H-2), 4.49 (0.3H, s, OH), 4.40 (0.7H, s, OH), 4.08–3.63 (4H, m, H-5 and $COCH_2$), 3.03 (0.3H, br s, NCH), 2.89 (0.7H, br s, NCH), 2.63–2.33 (2H, m, H-3), 2.30–2.15 (2H, m), 2.05–1.89 (3H, m), 1.68–1.48 (6H, m), 1.42–1.27 (2H, m). MS(ESI pos.) *m/z* 322 ($[M+H]^+$), 344 ($[M+Na]^+$); (ESI neg.) *m/z* 356 ($[M+Cl]^-$). HRMS calcd for $C_{17}H_{25}FN_3O_2$ $[M+H]^+$ 322.1931; found: (*m/z*) 322.1949.

5.1.49. HPLC analysis. For the chemical stability test, the residual amount was analyzed using reverse-phase HPLC with a CAPCELL PAK UG120 (5 μm particle size, ϕ 4.6 × 150 mm; SHISEIDO), then eluted at 1.0 mL/min with acetonitrile–H_2O buffer solution and monitored using UV absorbance at 210 nm.

5.2. Biological methods

5.2.1. DPP-IV inhibitory activity. The inhibition of DPP-IV activity was tested using a method described by Deacon et al.[8] Plasma containing DPP-IV was prepared by centrifuging blood collected from healthy human volunteers. Enzyme reactions were carried out using 96-flat-bottom-well plates in a buffer solution of pH7.8 containing 25 mM Hepes, 140 mM NaCl, and 1% BSA. To a mixture of 25 μL of 100 μM Gly-Pro-4-methylcoumaryl-7-amide solution (manufactured by Peptide Institute, Inc.), 7.5 μL of 133 mM $MgCl_2$ solution, 5 μL of the test compound, and 12.5 μL of plasma diluted to 1/100 with the above buffer solution were added. The solution was allowed to react at room temperature for 2 h, and 50 μL of 25% aqueous acetic acid solution was added to stop the reaction. The fluorescence intensity of the liberated 7-amino-4-methylcoumarin was determined using a fluorescence plate reader (1420 ARVO™ Multilabel Counter manufactured by Wallac Oy; Excitation: 390 nm; Emission: 460 nm).

5.2.2. Drug concentration measurements of 1a, 2a, and 12k in blood following oral administration to rats. Food was withheld overnight from 8-week-old male Wistar rats. In the morning, an aqueous solution of **1a**, **2a** or **12k** (1 mg/kg) was orally administered to each rat. At 0.5, 1, 2, and 6 h after administration, 200 μL of blood was collected from the jugular vein. After centrifugation, 50 μL of the resulting plasma was added to 200 μL of acetonitrile. The supernatant was injected into a liquid chromatography system with a CAPCEL PAK C18, UG120 5 μm (150 mm long, 2 mm diameter) column containing a mixture of 10 mM aqueous ammonium acetate solution and 90% aqueous acetonitrile solution (1:9) as an eluent; the Sciex API3000 LC/MS/MS System (Perkin-Elmer Sciex) was utilized for MS/MS, with ESI as the ionization method.

5.2.3. Oral glucose tolerance test (OGTT) in Zucker fatty rats. An OGTT in Zucker fatty rats was carried out based on the method described by Balkan et al.[9] Food was withheld overnight from male Zucker fatty and lean rats (10 weeks of age; $n = 6$). Compound **12k** was then dissolved in distilled water and administered orally. After 30 min, a glucose solution (2 g/kg body weight) was orally administered. Blood samples were collected from the orbital venous sinus under ether anesthesia at the indicated times, and plasma samples were prepared. The plasma glucose concentration, plasma insulin concentration, and plasma DPP-IV activity were measured.

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References and notes

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