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Synthesis and structure–activity relationships of potent 3- or 4-substituted-2-cyanopyrrolidine dipeptidyl peptidase IV inhibitors

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Abstract—Dipeptidyl peptidase IV (DPP-IV) inhibitors have attracted attention as potential drugs for use in the treatment of type 2 diabetes because they prevent degradation of glucagon-like peptide-1 (GLP-1) and extend its duration of action. A series of 2-cyanopyrrolidines are among the most potent of DPP-IV inhibitors. We focused our attention on substitutions at the 3- or 4-position of 2-cyanopyrrolidines and synthesized and evaluated various derivatives. Among them, the 4-fluoro derivative was found to exhibit better DPP-IV inhibitory activity and higher plasma drug concentrations after oral administration to rats than the 4-unsubstituted derivative. We report here on the synthesis and biological data of the aforementioned derivatives.

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] The natural substrates of DPP-IV have been described, and it is recognized that inhibition of DPP-IV is of potential therapeutic use in modulation of glucagon-like peptide processing and in attenuation of the immune response.[3] The role of DPP-IV in glucagon-like peptide (GLP) processing is well known.[4] Given the importance of the regulation of this process, DPP-IV inhibition has an obvious application to the treatment of diabetes.[5] Since the blood glucose-lowering effects of GLP-1 are dependent on elevated blood glucose and abate as glucose levels return to normal, the incidence of hypoglycemia during treatment with a DPP-IV inhibitor is expected to be very low.[6]

Many DPP-IV inhibitors are shaped like dipeptides, which contain a basic nitrogen and a proline mimic. Among proline mimics, 2-cyanopyrrolidine is one of the most potent moieties.[7]

We focused on substitutions of 2-cyanopyrrolidine, because the affinity between DPP-IV and its inhibitors appeared to be increased by hydrogen bonding or hydrophobic interaction or by change in the conformation around the pyrrolidine ring of the inhibitor. Since DPP-IV specifically recognizes a proline residue at the P1 site, we suspected that there would be affinity between DPP-IV and the pyrrolidine ring. We therefore attempted to introduce substitutions at the 3- or 4-position on the pyrrolidine ring to obtain more potent inhibition.

2. Chemistry

Carboxylic acids **2a**–**h**, which were commercially available or prepared by methylation[8] or fluorination[9] of hydroxyprolines and difluorination of 4-oxoproline, were coupled with ammonia using 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenztriazole (HOBt) as coupling reagents

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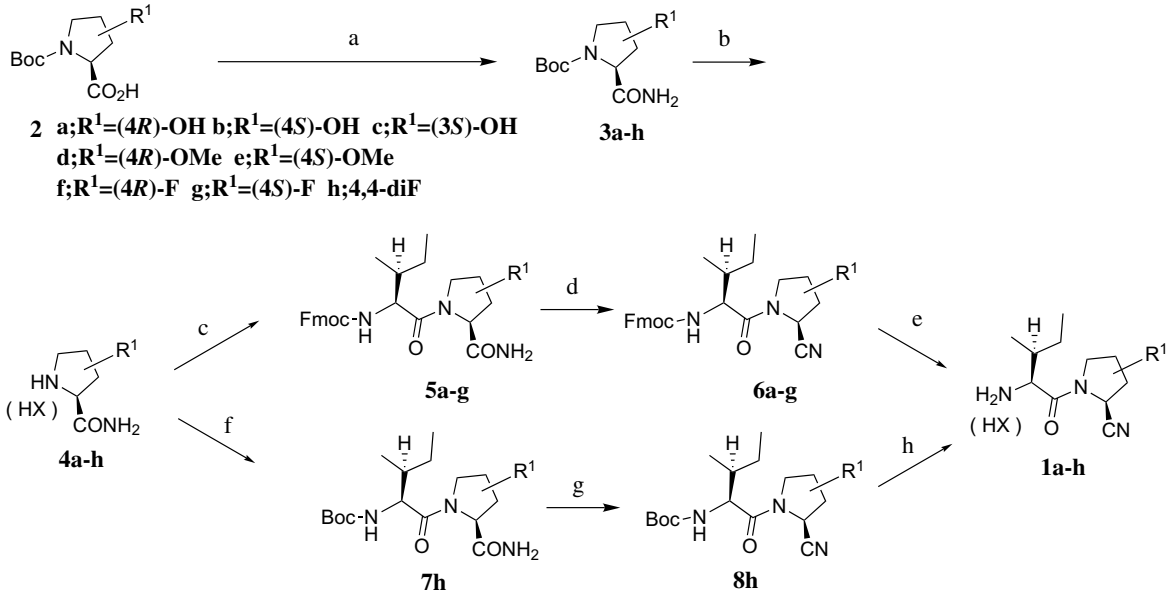
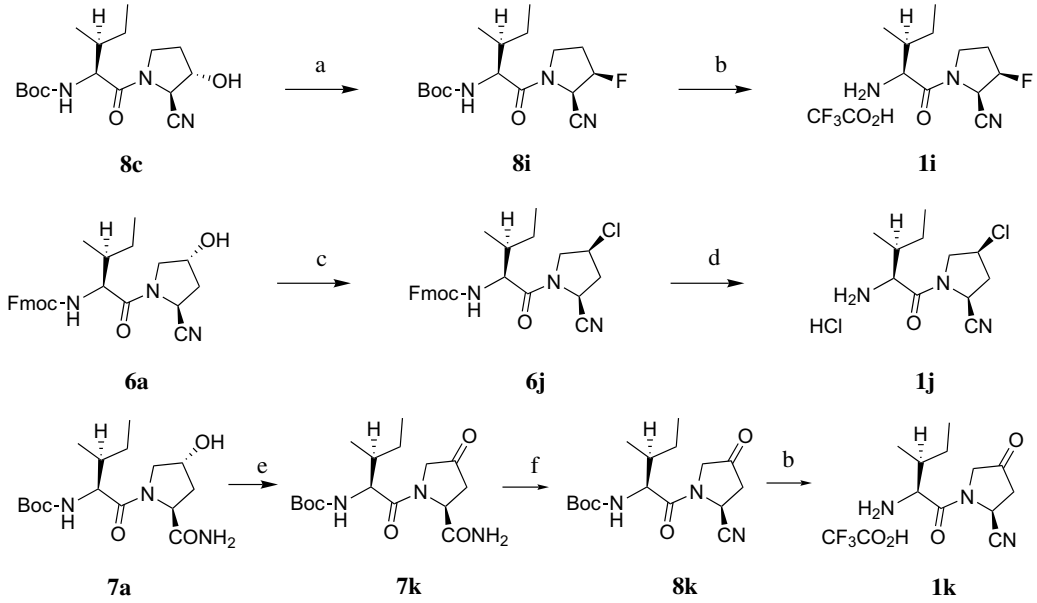


Scheme 1. Reagents: (a) EDC, HOBt, NH_3, DMF or CH_3CN; (b) 4 M HCl/AcOEt or 4 M HCl/1,4-dioxane; (c) Fmoc-L-isoleucine, EDC, HOBt, *N*,*N*-diisopropylethylamine, DMF or CH_3CN; (d) $(CF_3CO)_2O$, THF; (e) Et_2NH, 1,2-dichloroethane; then HCl; (f) Boc-L-isoleucine, EDC, HOBt, *N*,*N*-diisopropylethylamine, THF–DMF; (g) cyanuric chloride, DMF; (h) 2 M HCl.

(Scheme 1). Acid-catalyzed cleavage of the *N*-Boc-protecting group in **3a**–**h** then yielded the amines **4a**–**h**.

Isoleucine was selected for use at the P2 site because it is one of the most potent P2 site residues among natural amino acids.[7] It was possible to use either the fluorenylmethoxycarbonyl (Fmoc) group or the *t*-butoxycarbonyl (Boc) group as the *N*-protecting group of isoleucine. Compounds **4a**–**h** were coupled with Fmoc-L-isoleucine or Boc-L-isoleucine using EDC/HOBt as coupling reagents to yield the dipeptides **5a**–**g** or **7h** quantitatively.

Fmoc-protected prolinamides **5a**–**g** were treated with trifluoroacetic anhydride to obtain 2-cyanopyrrolidines **6a**–**g** and Boc derivative **7h** was dehydrated with cyanuric chloride in dimethylformamide[10] to yield cyano derivative **8h**. The dehydrating reaction was performed in mild acidic conditions in order to avoid deprotection of the *N*-Boc-protecting group. Removal of the *N*-Fmoc



Scheme 2. Reagents: (a) DAST, CH_2Cl_2; (b) TFA; (c) Ph_3P, CCl_4; (d) Et_2NH, 1,2-dichloroethane; then HCl; (e) PCC, MS4A, AcOH, CH_2Cl_2; (f) $(CF_3CO)_2O$, *N*,*N*-diisopropylethylamine, THF.

group with diethylamine or the *N*-Boc group with acids, and in some cases producing salts with hydrochloric acid, yielded **1a**–**h**.

(3*R*)-Fluoro derivative **8i** was obtained via fluorination of (3*S*)-hydroxy derivative **8c** with (diethylamino)sulfur trifluoride (DAST) and the *N*-Boc group of **8i** was deprotected using trifluoroacetic acid (TFA) to obtain **1i** (Scheme 2). (4*R*)-Hydroxy derivatives **6a** and **7a** were converted to the corresponding 4(*S*)-chloride **6j** using Ph_3P/CCl_4 and to 4-oxo derivative **7k** using pyridinium chlorochromate (PCC). Compound **6j** was deprotected using diethylamine and treated with hydrochloric acid to yield hydrochloric acid salt **1j**. Compound **7k** was treated with trifluoroacetic anhydride and *N*,*N*-diisopropylethylamine to yield **8k** quantitatively without producing a Boc-deprotection product. The *N*-Boc group of **8k** was then deprotected using TFA to obtain **1k**.

It is well known that *N*-acylproline derivatives exist as mixtures of *cis*- and *trans*-amide rotamers in solution. It has been reported that *N*-acyl-2-cyanopyrrolidines exist as mixtures of rotamers as well.[11] NMR revealed that some compounds among **1a**–**k** occurred as mixtures of conformers in solution. The protons at the 2- and 4-position of the pyrrolidine ring of **1e** and **1g** tended to appear as separate peaks on the NMR spectra. The protons of the compounds for which the minor conformers were clearly observed are described in the NMR data.

3. Results and discussion

The hydroxy compounds **1a**–**c** and the methoxy compounds **1d** and **1e** were all several orders of magnitude less potent than the non-substituted analogue **9**.[7] Stereochemically, the 4*S* derivatives **1b** and **1e** were more potent than the 4*R* derivatives **1a** and **1d** (Table 1). Introduction of a ketone to the 4-position slightly decreased inhibitory potency (**1k**, $IC_{50} = 21$ nM).

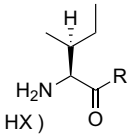
The (4*S*)-fluoride analogue **1g** had a very potent inhibitory effect ($IC_{50} = 0.6$ nM) and was one of the most potent DPP-IV inhibitors found. The 4,4-difluoride analogue **1h** also had a potent inhibitory effect ($IC_{50} = 0.8$ nM). However, the (4*R*)-fluoride analogue **1f** had 480-fold less potency ($IC_{50} = 290$ nM) than **1g**.

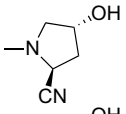
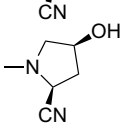
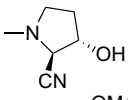
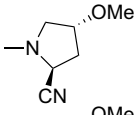
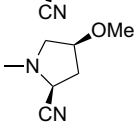
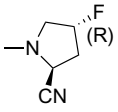
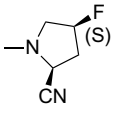
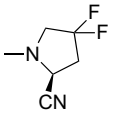
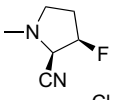
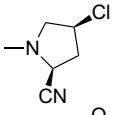
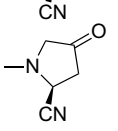
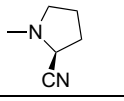
The (3*R*)-fluoride analogue **1i** and the (4*S*)-chloride analogue **1j** demonstrated slightly decreases in inhibitory potency.

The introduction of substituents to the 3- or 4-position on the pyrrolidine ring did not lead to increased potency with the exception of the incorporation of (4*S*)-fluoro substitution. The narrow space of the pyrrolidine-binding site of DPP-IV may not permit substituents greater in size than fluorine. It is unclear why the introduction of (4*S*)-fluorine augmented DPP-IV inhibitory activity. However, the 4*S* position is surrounded by hydrogen atoms from Tyr and Trp that have slight positive charges, while the 4*R* position is adjacent to the π-cloud of another Tyr that has a slight negative charge, which might have caused the variation in potency seen with incorporation of fluorine at the 4-position. Reports on X-ray crystallographic analysis of DPP-IV[12] were used to aid in speculation regarding bonding patterns.

Introduction of a fluorine atom affected the drug plasma concentration present after oral administration. Following oral administration of 1 mg/kg to rats, the C_{max} for **1g** (372 ng/mL at 10 min) was 2.5-fold that for **9** (147 ng/mL at 10 min) (Fig. 1). The reason for the

Table 1. DPP-IV inhibitory activity



Compd	R	IC_{50} (nM)
1a		5700
1b		370
1c		>10,000
1d		>30,000
1e		850
1f		290
1g		0.6
1h		0.8
1i		65
1j		23
1k		21
9		1.5

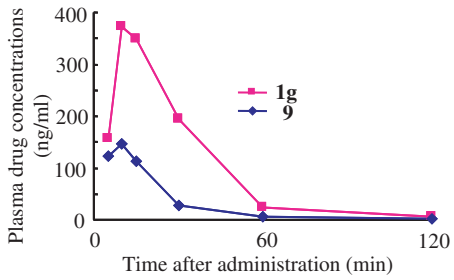


Figure 1. Plasma drug concentrations after oral administration of **1g** or **9** at a dose of 1 mg/kg to Wistar rats.

increase in C_{max} with the introduction of fluorine was not clear, but may have been the increase in absorption and reduction of distribution volume.

The effect of **1g** on glucose tolerance was examined in Zucker fatty rats, a model of obesity and impaired glucose tolerance. Oral administration of **1g** at the doses of 1 and 4 mg/kg reduced the increase in plasma glucose beginning 30 min after glucose loading (Fig. 2A) and at 4 mg/kg significantly suppressed hyperglycemia (Fig. 2B). DPP-IV activity was almost completely inhibited at 15 min after glucose loading at both doses, with the inhibitory effect gradually declining beginning 90 min after glucose loading in the 1 mg/kg dose group (Fig. 2D). These results indicate that the efficacy of **1g** on hyperglycemia is based on its inhibitory effect on plasma DPP-IV activity. Insulin secretion was significantly enhanced in the 1 and 4 mg/kg dose groups (Fig. 2C). This finding provides support for the proposed mechanism that **1g** might prevent the inactivation of active GLP-1 via DPP-IV inhibition and an increased active GLP-1 level might stimulate insulin secretion by acting upon β-cells in the pancreas, resulting in suppression of hyperglycemia after glucose loading.

4. Conclusion

We have designed and synthesized a series of 3- or 4-substituted-2-cyanopyrrolidines as DPP-IV inhibitors. Among these compounds, the (4*S*)-fluoro derivative **1g** exhibited greater DPP-IV inhibitory activity and higher plasma drug concentrations after oral administration to rats than the 4-unsubstituted derivative **9**. Based on these pharmacology efficacy data, the 4-fluoro derivative of 2-cyanopyrrolidine and potent DPP-IV inhibitor **1g** is expected to have potential as a therapeutic agent for use in lowering postprandial hyperglycemia and treatment of type 2 diabetes mellitus. Subsequent reports will describe the results of further investigation of 4-fluoro-2-cyanopyrrolidines.

5. Experimental

5.1. Chemistry

^{1}H NMR spectroscopy was performed using a Varian VXR-300 or JEOL GX500 spectrometer. Chemical shifts are 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

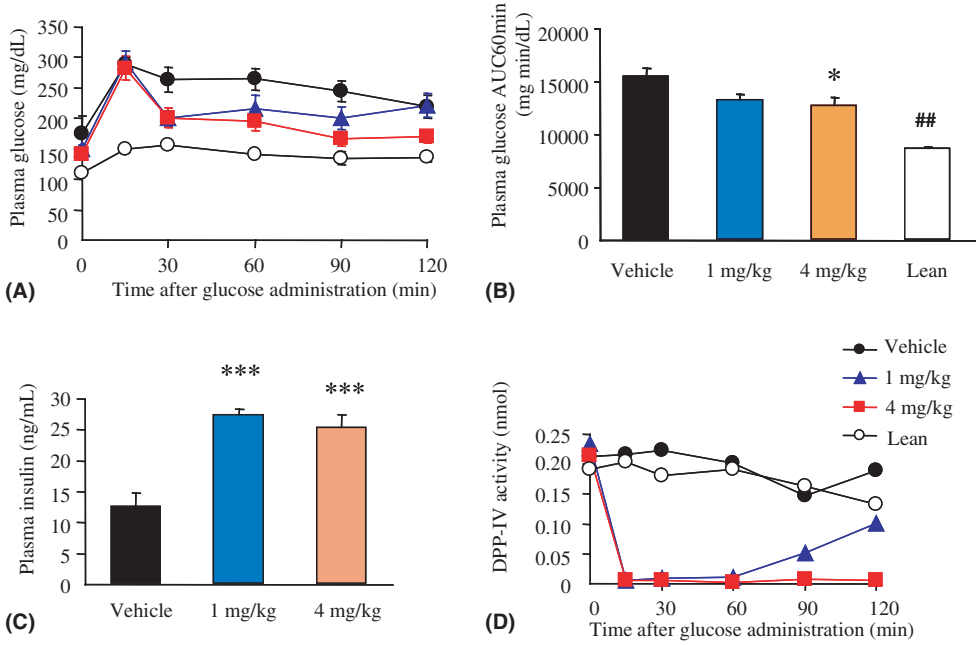


Figure 2. Effects of oral administration of **1g** (1 or 4 mg/kg) on plasma glucose, plasma insulin and plasma DPP-IV activity during OGTT in Zucker fatty rats. Each point represents the mean ± SE ($n = 6$). *$p < 0.05$, ***$p < 0.001$ versus Vehicle, Dunnett's test. $^{\#\#}p < 0.01$ versus Vehicle, Student's *t*-test.

spectra were recorded with a Shimadzu/Kratos HV-300. Melting points were measured with a Buchi 535 melting point apparatus without correction. Infrared spectra were recorded with a Perkin–Elmer 1760 spectrometer. Elemental analyses were performed using a Perkin–Elmer 240C (for carbon, hydrogen, and nitrogen) or Yokokawa–Denki IC7000P (for halogens and sulfur).

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

5.1.1. *tert*-Butyl (2*S*,4*S*)-2-(aminocarbonyl)-4-fluoropyrrolidine-1-carboxylate (3g). In acetonitrile (50 mL), (4*S*)-1-(*tert*-butoxycarbonyl)-4-fluoro-L-proline **2g**[9] (4.5 g, 19.3 mmol) was dissolved, and 1-hydroxybenzotriazole monohydrate (3.6 g, 23.5 mmol) and 1-(3,3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (4.5 g, 23.5 mmol) were then added with ice-cooling. The temperature was gradually increased, and the mixture was then stirred at room temperature overnight. The reaction solution was again ice-cooled, 25% aqueous ammonia (5 mL) was added, and stirring was continued with ice-cooling for 30 min and then at room temperature for 30 min. Acetonitrile (50 mL) was added to the reaction mixture, and an insoluble substance was removed by filtration. The filtrate was concentrated in vacuo and purified by silica gel column chromatography (developing solvent; hexane–EtOAc = 4:1–1:5). Hexane was added to the resulting residue to yield the desired product (4.2 g, 94%) as a colorless powder. Mp 169–170 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.21, 7.15, and 6.95 (2H, each br, $CONH_2$), 5.21 (1H, br d, J = 54.0 Hz, H-4), 4.13 (1H, d, J = 9.6 Hz, H-2), 3.47–3.68 (2H, m, H-5), 2.26–2.55 (1H, m, H-3), 2.09–2.22 (1H, m, H-3), 1.41 and 1.36 (9H, each s, $(CH_3)_3CO$). MS (ESI pos.) m/z 255 ([M+Na]$^+$). Anal. Calcd for $C_{10}H_{17}FN_2O_3$: C, 51.71; H, 7.38; F, 8.18; N, 12.06. Found: C, 51.72; H, 7.46; F, 8.14; N, 11.90. IR (KBr) 3474, 3283, 3218, 3162, 2990, 1696, 1649, 1397, 1378, 1360, 1223, 1167, 1119, 1072, 848 cm^{-1}. $[\alpha]_D^{22}$ −56.3 (c 0.5, MeOH).

5.1.2. (4*S*)-4-Fluoro-L-prolinamide hydrochloride (4g). In 4 M HCl/dioxane (45 mL), **3g** (4.2 g, 18.1 mmol) was suspended, and after stirring at room temperature for 2 h, the reaction solution was concentrated in vacuo. Toluene (50 mL) was added to the residue, followed by further concentration in vacuo. This was repeated three times to obtain the desired product (3.1 g, 100%) as a colorless powder. This intermediate was used for the next reaction without purification. Mp 230–231 °C (decomp.). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.72 (2H, br s, H_2N^+), 8.10 (1H, br s, $CONH_2$), 7.70 (1H, br s, $CONH_2$), 5.38 (1H, dt like, J = 52.5, 3.6 Hz, H-4), 4.31 (1H, dd, J = 10.4, 3.7 Hz, H-2), 3.56 (1H, ddd, J = 19.9, 13.4, 1.7 Hz, H-5), 3.39 (1H, ddd, J = 36.5, 13.4, 3.6 Hz, H-5), 2.48–2.72 (1H, m, H-3), 2.24–2.40 (1H, m, H-3). MS (ESI pos.) m/z 155 ([M+Na]$^+$). Anal. Calcd for $C_5H_9FN_2O$·HCl: C, 35.62; H, 5.98; Cl, 21.03; F, 11.27; N, 16.62. Found: C, 35.57; H, 5.95; Cl, 21.03; F, 11.26; N, 16.46. IR (KBr) 3319, 3174, 3063, 2902, 2778, 2731, 1707, 1626, 1583, 1399, 1340, 1322, 1207, 1162, 1055, 1026, 965, 863, 635 cm^{-1}. $[\alpha]_D^{26}$ −48.8 (c 0.5, MeOH).

5.1.3. *N*-[(9*H*-Fluoren-9-ylmethoxy)carbonyl]-L-isoleucyl-(4*S*)-4-fluoro-L-prolinamide (5g). In THF (40 mL)–DMF (10 mL), **4g** (2.4 g, 14.2 mmol), and *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-isoleucine (5.1 g, 14.4 mmol) were dissolved, and then 1-hydroxybenzotriazole monohydrate (2.6 g, 17.0 mmol), 1-(3,3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (3.3 g, 17.2 mmol), and *N*,*N*-diisopropylethylamine (2.5 mL) were added with ice-cooling, and the temperature was gradually increased, followed by stirring at room temperature overnight. The solution was concentrated in vacuo, and water was added to the resulting residue. The resulting powder was collected by filtration and purified by silica gel column chromatography (developing solvent; hexane–EtOAc = 4:1–1:4) to yield the desired product (6.9 g, quant) as a pale yellow amorphous substance. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.89 (2H, d, J = 7.5 Hz, aromatic H), 7.72 (2H, d, J = 7.5 Hz, aromatic H), 7.41 (2H, t like, J = 7.5 Hz, aromatic H), 7.32 (2H, t like, J = 7.5 Hz, aromatic H), 7.05 (1H, br s, NH), 6.91 (1H, br s, NH), 5.31 (1H, br d, J = 52.5 Hz, H-4), 4.45 (1H, dd, J = 8.8, 2.2 Hz, H-2), 4.32–3.96 (5H, m), 3.89–3.74 (1H, dd, J = 25.6, 12.6 Hz, H-5), 2.45–2.12 (2H, m, H-3), 1.88–1.76 (1H, m), 1.61–1.49 (1H, m), 1.20–1.10 (1H, m), 0.95 (3H, d, J = 6.7 Hz, Me), 0.84 (3H, dd, J = 7.5, 7.3 Hz, Me).

5.1.4. 9*H*-Fluoren-9-ylmethyl ((1*S*,2*S*)-1-{[(2*S*,4*S*)-2-cyano-4-fluoropyrrolidin-1-yl]carbonyl}-2-methylbutyl)carbamate (6g). In THF (70 mL), **5g** (6.9 g, 14.8 mmol) was dissolved, and trifluoroacetic anhydride (4.0 mL, 28.3 mmol) was added with ice-cooling, followed by stirring with ice-cooling for 1.5 h. The reaction solution was concentrated in vacuo, and the residue was purified by silica gel column chromatography (developing solvent; hexane–EtOAc = 8:1–3:2) to yield the desired product (6.2 g, 97%) as a pale yellow amorphous substance. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.91 (1H, d, J = 7.9 Hz, NH), 7.89 (2H, d, J = 7.3 Hz, aromatic H), 7.72 (2H, d, J = 7.5 Hz, aromatic H), 7.42 (2H, t like, J = 7.3 Hz, aromatic H), 7.35–7.29 (2H, m, aromatic H), 5.49 (1H, br d, J = 50.5 Hz, H-4), 5.05–4.98 (1H, m, H-2), 4.34–3.80 (6H, m), 2.60–2.30 (2H, m, H-3), 1.90–1.74 (1H, m), 1.65–1.48 (1H, m), 1.20–1.13 (1H, m), 0.87 (3H, d, J = 6.8 Hz, Me), 0.86 (3H, t, J = 7.3 Hz, Me). MS (ESI pos.) m/z 472 ([M+Na]$^+$).

5.1.5. (2*S*,4*S*)-4-Fluoro-1-L-isoleucylpyrrolidine-2-carbonitrile hydrochloride (1g). In 1,2-dichloroethane (90 mL), **6g** (6. 2 g, 13.8 mmol) was dissolved, and after addition of diethylamine (10 mL) with ice-cooling, stirring was continued with ice-cooling for 30 min and then at room temperature for 5 h. The solution was concentrated in vacuo, the residue was dissolved in a mixture of diethyl ether (100 mL), THF (50 mL) and $CHCl_3$ (50 mL), and then 4 M HCl/dioxane (4.0 mL) was added with ice-cooling. The resulting salt was collected by filtration and washed with diethyl ether. The resulting powder was

purified by silica gel column chromatography (developing solvent; $CHCl_3$–MeOH–25% aqueous ammonia = 40:1:0.1–25:1:0.1). The resulting residue was dissolved in $CHCl_3$ and, after addition of 4 M HCl/dioxane (4.0 mL) with ice-cooling, the resulting salt was collected by filtration, washed with $CHCl_3$, and dried in vacuo to yield the desired product (2.9 g, 80%) as a colorless powder. Mp 245–248 °C (decomp.). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.59 (3H, br s, H_3N^+), 5.54 (0.9H, br d, J = 52.1 Hz, H-4 of the major conformer), 5.45 (0.1H, br d, J = 53.3 Hz, H-4 of the minor conformer), 5.39 (0.1H, d, J = 6.7 Hz, H-2 of the minor conformer), 5.06 (0.9H, d, J = 9.4 Hz, H-2 of the major conformer), 4.07–3.77 (3H, m, H-5 and NCHCO), 2.55–2.34 (2H, m, H-3), 1.94–1.83 (1H, m), 1.66–1.53 (1H, m), 1.22–1.13 (1H, m), 0.94 (3H, d, J = 6.7 Hz, Me), 0.88 (3H, t, J = 7.3 Hz, Me); ^{13}C NMR (125.4 MHz, DMSO-d_6): δ 168.2, 118.3, 92.9 (d, J_{C-F} = 175.7 Hz), 54.4, 53.6 (d, J_{C-F} = 21.7 Hz), 44.7, 36.3, 35.4 (d, J_{C-F} = 20.7 Hz), 23.7, 13.9, 10.9; ^{19}F NMR (282.2 MHz, DMSO-d_6): δ −175.1. MS (ESI pos.) m/z 250 ([M+Na]$^+$); (ESI neg.) m/z 262 ([M+Cl]$^-$). HRMS calcd for $C_{11}H_{19}FN_3O$ [M+H]$^+$ 228.1512, found (m/z) 228.1508. Anal. Calcd for $C_{11}H_{18}FN_3O{\cdot}HCl{\cdot}0.5H_2O$: C, 48.44; H, 7.39; N, 15.41. Found: C, 48.20; H, 7.37; N, 15.23. $[\alpha]_D^{25}$ −62.7 (c 0.3, MeOH).

5.1.6. (2*S*,4*R*)-4-Hydroxy-1-L-isoleucylpyrrolidine-2-carbonitrile hydrochloride (1a). The title compound was obtained as a colorless powder in a manner similar to the preparation of **1g**. Mp 173–176 °C (decomp.). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.32 (3H, br s, H_3N^+), 5.45 (1H, d, J = 3.7 Hz, OH), 4.75 (1H, t like, J = 9 Hz, H-2), 4.38 (1H, br s, H-4), 4.08 (1H, d, J = 5.3 Hz, NCHCO), 3.74 (1H, d, J = 10.8 Hz, H-5), 3.64 (1H, dd, J = 10.8, 3.8 Hz, H-5), 2.37–2.21 (2H, m, H-3), 1.95–1.81 (1H, m), 1.55–1.40 (1H, m), 1.26–1.05 (1H, m), 0.97 (3H, d, J = 7.0 Hz, Me), 0.88 (3H, t, J = 7.3 Hz, Me). MS (ESI pos.) m/z 248 ([M+Na]$^+$), 226 ([M+H]$^+$); (ESI neg.) m/z 260 ([M+Cl]$^-$). HRMS calcd for $C_{11}H_{20}N_3O_2$ [M+H]$^+$ 226.1556, found (m/z) 226.1570.

5.1.7. (2*S*,4*S*)-4-Hydroxy-1-L-isoleucylpyrrolidine-2-carbonitrile hydrochloride (1b). The title compound was obtained as a colorless amorphous powder in a manner similar to the preparation of **1g**. ^{1}H NMR (300 MHz, DMSO-d_6): 8.40 (3H, br s, H_3N^+), 5.44 (1H, br s, OH), 4.88 (1H, dd, J = 9.0, 2.5 Hz, H-2), 4.45–4.38 (1H, m, H-4), 3.94–3.82 (1H, m, NCHCO), 3.75 (1H, dd, J = 10.8, 4.3 Hz, H-5), 3.53 (1H, d, J = 10.8 Hz, H-5), 2.25 (1H, ddd, J = 13.2, 9.2, 4.1 Hz, H-3), 2.10 (1H, br d, J = 13.2 Hz, H-3), 2.01–1.80 (1H, m), 1.65–1.47 (1H, m), 1.26–1.08 (1H, m), 0.96 (3H, d, J = 7.0 Hz, Me), 0.88 (3H, t, J = 7.4 Hz, Me). MS (ESI pos.) m/z 248 ([M+Na]$^+$), 226 ([M+H]$^+$); (ESI neg.) m/z 260 ([M+Cl]$^-$). HRMS calcd for $C_{11}H_{20}N_3O_2$ [M+H]$^+$ 226.1556, found (m/z) 226.1565.

5.1.8. (2*R*,3*S*)-3-Hydroxy-1-L-isoleucylpyrrolidine-2-carbonitrile (1c). In a manner similar to the preparation of **1g**, except for hydrochloride salt formation, the title compound was obtained as a colorless oily substance. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.76 (1H, br s, H-2), 4.60 (1H, br d, J = 3.7 Hz, H-3), 3.83–3.67 (2H, m, H-5), 3.33 (1H, d, J = 6.4 Hz, NCHCO), 2.40–2.26 (1H, m, H-3), 2.19–2.09 (1H, m, H-3), 1.70–1.54 (2H, m), 1.27–1.05 (1H, m), 0.96 (3H, d, J = 6.8 Hz, Me), 0.92 (3H, t, J = 7.3 Hz, Me). MS (ESI pos.) m/z 248 ([M+Na]$^+$). HRMS calcd for $C_{11}H_{20}N_3O_2$ [M+H]$^+$ 226.1556, found (m/z) 226.1545.

5.1.9. (2*S*,4*R*)-1-L-Isoleucyl-4-methoxypyrrolidine-2-carbonitrile hydrochloride (1d). The title compound was obtained as a colorless amorphous powder in a manner similar to the preparation of **1g**. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.37 (3H, br s, H_3N^+), 4.70 (1H, dd, J = 9.1, 7.9 Hz, H-2), 4.20 (1H, br d, J = 4.3 Hz, NCHCO), 4.07 (1H, br s, H-4), 3.99 (1H, d, J = 11.5 Hz, H-5), 3.63 (1H, dd, J = 11.5, 3.6 Hz, H-5), 3.25 (3H, s, Ome), 2.54–2.47 (1H, m, H-3), 2.34–2.27 (1H, m, H-3), 1.95–1.86 (1H, m), 1.53–1.44 (1H, m), 1.26–1.12 (1H, m), 0.98 (3H, d, J = 7.0 Hz, Me), 0.88 (3H, t, J = 7.3 Hz, Me). MS (ESI pos.) m/z 262 ([M+Na]$^+$), 240 ([M+H]$^+$); (ESI neg.) m/z 274 ([M+Cl]$^-$). HRMS calcd for $C_{12}H_{22}N_3O_2$ [M+H]$^+$ 240.1712, found (m/z) 240.1723.

5.1.10. (2*S*,4*S*)-1-L-Isoleucyl-4-methoxypyrrolidine-2-carbonitrile hydrochloride (1e). The title compound was obtained as a colorless powder in a manner similar to the preparation of **1g**. Mp 175–178 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.46 (3H, br s, H_3N^+), 5.23 (0.05H, d, J = 8.5 Hz, H-2 of the minor conformer), 4.91 (0.95H, dd, J = 9.2, 1.6 Hz, H-2 of the major conformer), 4.14 (0.95H, br s, H-4 of the major conformer), 4.09 (0.05H, m, H-4 of the minor conformer), 3.87 (1H, d, NCHCO), 3.80–3.73 (2H, m, H-5), 3.29 (3H, s, Ome), 2.37 (1H, d, J = 14.0 Hz, H-3), 2.22 (1H, ddd, J = 13.7, 9.5, 4.0 Hz, H-3), 1.91–1.80 (1H, m), 1.64–1.53 (1H, m), 1.23–1.12 (1H, m), 0.94 (3H, d, J = 6.7 Hz, Me), 0.88 (3H, t, J = 7.3 Hz, Me). MS (ESI pos.) m/z 262 ([M+Na]$^+$), 240 ([M+H]$^+$); (ESI neg.) m/z 274 ([M+Cl]$^-$). HRMS calcd for $C_{12}H_{22}N_3O_2$ [M+H]$^+$ 240.1712, found (m/z) 240.1717.

5.1.11. (2*S*,4*R*)-4-Fluoro-1-L-isoleucylpyrrolidine-2-carbonitrile hydrochloride (1f). The title compound was obtained as a colorless powder in a manner similar to the preparation of **1g**. Mp 222–225 °C (decomp.). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.38 (3H, br s, H_3N^+), 5.42 (1H, br d, J = 51.9 Hz, H-4), 4.87 (1H, dd, J = 10.1, 8.0 Hz, H-2), 4.28–4.20 (2H, m, H-5 and NCHCO), 3.81 (1H, ddd, J = 39.0, 12.6, 2.5 Hz, H-5), 2.77–2.67 (1H, m, H-3), 2.60–2.45 (1H, m, H-3), 1.95–1.84 (1H, m), 1.52–1.43 (1H, m), 1.23–1.13 (1H, m), 0.98 (3H, d, J = 6.7 Hz, Me), 0.88 (3H, t, J = 7.3 Hz, Me). MS (ESI pos.) m/z 250 ([M+Na]$^+$), 228 ([M+H]$^+$); (ESI neg.) m/z 262 ([M+Cl]$^-$). HRMS calcd for $C_{11}H_{19}FN_3O$ [M+H]$^+$ 228.1512, found (m/z) 228.1518.

5.1.12. *tert*-Butyl ((1*S*,2*S*)-1-{[(2*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl]carbonyl}-2-methylbutyl)carbamate (8h). In DMF (2.5 mL), *tert*-butyl ((1*S*,2*S*)-1-{[(2*S*)-2-(aminocarbonyl)-4,4-difluoropyrrolidin-1-yl]carbonyl}-2-meth-

ylbutyl)carbamate **7h** (900 mg, 2.48 mmol) was dissolved, and then cyanuric chloride (280 mg, 1.49 mmol) was added, followed by stirring at room temperature for 1 h. The reaction solution was taken up in water, and extracted with EtOAc. The organic phase was washed with a saturated aqueous $NaHCO_3$ solution and a saturated aqueous NaCl solution, successively, and dried over Na_2SO_4. The drying agent was removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (developing solvent; hexane–EtOAc = 20:1–4:1) to yield the desired product (760 mg, 89%) as a colorless amorphous substance. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.29 (1H, d, J = 7.9 Hz, NH), 5.08 (1H, dd, J = 9.1, 4.1 Hz, H-2), 4.34–4.10 (2H, m, H-5), 3.90 (1H, t like, J = 8.7 Hz, NCHCO), 3.03–2.73 (2H, m, H-3), 1.83–1.67 (1H, m), 1.59–1.43 (1H, m), 1.36 (9H, s, Boc), 1.26–1.04 (1H, m), 0.87–0.78 (6H, m, 2Me). MS (ESI pos.) m/z 368 ($[M+Na]^+$); (ESI neg.) m/z 344 ($[M-H]^-$).

5.1.13. (2*S*)-4,4-Difluoro-1-L-isoleucylpyrrolidine-2-carbonitrile hydrochloride (1h). To **8h** (560 mg, 1.62 mmol) was added 2 M aqueous HCl solution (12 mL), followed by stirring at room temperature overnight. An additional 2 M aqueous HCl solution (6 mL) was added to the solution, and after stirring at room temperature overnight the aqueous solution was washed with EtOAc. To the aqueous phase, 1 M aqueous NaOH solution (35 mL) and an excess amount of NaCl were added, and after stirring the mixture was taken up in a saturated aqueous $NaHCO_3$ solution and extracted with EtOAc. The organic phase was washed with a saturated aqueous NaCl solution and dried over Na_2SO_4. After removal of the drying agent by filtration, the filtrate was concentrated under reduced pressure to obtain (2*S*)-4,4-difluoro-1-L-isoleucylpyrrolidine-2-carbonitrile, which was then dissolved in diethyl ether (20 mL), followed by addition of 4 M HCl/EtOAc (0.50 mL) with ice-cooling. The precipitated insoluble substance was collected by filtration to yield the desired product (370 mg, 81%) as a colorless powder. Mp 235–243 °C (decomp.). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.38 (3H, br s, H_3N^+), 5.14 (1H, t like, J = 7.1 Hz, H-2), 4.46–4.12 (2H, m, H-5), 4.00 (1H, d, J = 6.2 Hz, NCHCO), 2.98–2.81 (2H, m, H-3), 1.96–1.78 (1H, m), 1.60–1.44 (1H, m), 1.28–1.06 (1H, m), 0.95 (3H, d, J = 6.8 Hz, Me), 0.88 (3H, t, J = 7.3 Hz, Me). MS (ESI pos.) m/z 268 ($[M+Na]^+$), 246 ($[M+H]^+$); (ESI neg.) m/z 280 ($[M+Cl]^-$). HRMS calcd for $C_{11}H_{17}F_2N_3O$ $[M]^+$ 245.1340, found (m/z) 245.1353.

5.1.14. *tert*-Butyl ((1*S*,2*S*)-1-{[(2*R*,3*R*)-2-cyano-3-fluoropyrrolidin-1-yl]carbonyl}-2-methylbutyl)carbamate (8i). In CH_2Cl_2 (5 mL), *tert*-butyl ((1*S*,2*S*)-1-{[(2*R*,3*S*)-2-cyano-3-hydroxypyrrolidin-1-yl]carbonyl}-2-methylbutyl)-carbamate **8c** (168 mg, 0.516 mmol) obtained from (3*S*)-1-(*tert*-butoxycarbonyl)-3-hydroxy-L-proline by a synthetic route similar to that for **6g** or **8h** was dissolved, and diethylaminosulfur trifluoride (0.206 mL) was added dropwise with cooling on a dry ice–acetone bath. The temperature was gradually increased to room temperature, and the mixture was then stirred overnight. The reaction mixture was taken up in a saturated aqueous $NaHCO_3$ solution and extracted with EtOAc. The organic phase was washed with a saturated aqueous $NaHCO_3$ solution and a saturated aqueous NaCl solution, successively, and dried over $MgSO_4$. After removal of the drying agent by filtration, the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (developing solvent; hexane–EtOAc = 3:2) to yield the desired product (65 mg, 38%) as a colorless gummy substance. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.32 (1H, br d, J = 51.8 Hz, H-3), 5.09 (1H, br d, J = 9.3 Hz, NH), 4.84 (1H, dd, J = 21.8, 4.4 Hz, H-2), 4.27–4.08 (2H, m, H-5 and NCHCO), 3.81 (1H, td like, J = 10.2, 6.2 Hz, H-5), 2.52–2.36 (1H, m, H-4), 2.31–2.05 (1H, m, H-4), 1.83–1.71 (1H, m), 1.66–1.52 (1H, m), 1.42 (9H, s, Boc), 1.24–1.08 (1H, m), 0.98 (3H, d, J = 6.8 Hz, Me), 0.92 (3H, t, J = 7.4 Hz, Me). MS (ESI pos.) m/z 350 ($[M+Na]^+$).

5.1.15. (2*R*,3*R*)-3-Fluoro-1-L-isoleucylpyrrolidine-2-carbonitrile trifluoroacetate (1i). In ice-cooled trifluoroacetic acid (1.0 mL), **8i** (62 mg, 0.18 mmol) was dissolved, and after stirring at room temperature for 30 min, the solvent was evaporated under reduced pressure. To the residue, diisopropyl ether (10 mL) was added, and the supernatant was removed. To the residue, diisopropyl ether (10 mL) was added again, and an insoluble substance was collected by filtration to yield the title compound (32 mg, 51%) as a yellow gummy solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.22 (3H, br s, H_3N^+), 5.50 (1H, br d, J = 52.8 Hz, H-3), 5.08 (1H, dd, J = 26.1, 4.0 Hz, H-2), 4.19 (1H, d, J = 5.4 Hz, NCHCO), 4.05 (1H, t like, J = 8.9 Hz, H-5), 3.64–3.54 (1H, m, H-5), 2.42–2.00 (2H, m, H-4), 1.99–1.84 (1H, m), 1.54–1.38 (1H, m), 1.26–1.08 (1H, m), 0.96 (3H, d, J = 6.8 Hz, Me), 0.89 (3H, t, J = 7.3 Hz, Me). MS (ESI pos.) m/z 250 ($[M+Na]^+$), 228 ($[M+H]^+$); (ESI neg.) m/z 340 ($[M+C_2F_3O_2]^-$). HRMS calcd for $C_{11}H_{19}FN_3O$ $[M+H]^+$ 228.1512, found (m/z) 228.1525.

5.1.16. 9*H*-Fluoren-9-ylmethyl ((1*S*,2*S*)-1-{[(2*S*,4*S*)-4-chloro-2-cyanopyrrolidin-1-yl]carbonyl}-2-methylbutyl)-carbamate (6j). In CH_2Cl_2 (2 mL)–carbon tetrachloride (2 mL), 9*H*-fluoren-9-ylmethyl ((1*S*,2*S*)-1-{[(2*S*,4*R*)-2-cyano-4-hydroxypyrrolidin-1-yl]carbonyl}-2-methylbutyl)carbamate **6a** (200 mg, 0.45 mmol) obtained from (4*R*)-1-(*tert*-butoxycarbonyl)-4-hydroxy-L-proline by a synthetic route similar to that for **6g** was dissolved, and then triphenylphosphine (234 mg, 0.89 mmol) was added, followed by stirring at room temperature overnight. To the reaction solution, EtOH (0.5 mL) was added, followed by stirring at room temperature for 4 h. The reaction solution was concentrated in vacuo, and the resulting residue was purified by silica gel column chromatography (developing solvent; hexane–EtOAc = 1:1–2:3) to yield the desired product (126 mg, 60%) as a colorless amorphous substance. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (2H, d, J = 7.5 Hz, aromatic H), 7.57 (2H, d, J = 7.5 Hz, aromatic H), 7.40 (2H, t, J = 7.5 Hz, aromatic H), 7.30 (2H, t, J = 7.5 Hz, aromatic H), 5.42 (1H, d, J = 9.2 Hz, NH), 4.88 (1H, dd, J = 8.3, 3.5 Hz, H-2), 4.63–4.56 (1H, m, H-4),

4.44–4.08 (5H, m), 3.96 (1H, dd, $J = 11.5$, 1.9 Hz, H-5), 2.73–2.56 (2H, m, H-3), 1.93–1.79 (1H, m), 1.72–1.56 (1H, m), 1.30–1.13 (1H, m), 1.12 (3H, d, $J = 6.7$ Hz, Me), 0.96 (3H, t, $J = 7.4$ Hz, Me). MS (ESI pos.) *m/z* 488 ($[M+Na]^+$).

5.1.17. (2*S*,4*S*)-4-Chloro-1-L-isoleucylpyrrolidine-2-carbonitrile hydrochloride (1j). The title compound (32 mg, 53%) was obtained as a colorless powder from **6j** (100 mg, 0.22 mmol) in a manner similar to the preparation of **1g**. Mp 189–192 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.46 (3H, br s, H_3N^+), 5.01–4.92 (2H, m, H-2 and H-4), 4.11 (1H, dd, $J = 12.0$, 4.3 Hz, H-5), 3.98 (1H, br d, $J = 12.0$ Hz, H-5), 3.90 (1H, d, $J = 6.8$ Hz, NCHCO), 2.73 (1H, ddd, $J = 14.6$, 9.4, 4.8 Hz, H-3), 2.54–2.43 (1H, m, H-3), 2.00–1.80 (1H, m), 1.66–1.48 (1H, m), 1.27–1.10 (1H, m), 0.97 (3H, d, $J = 6.8$ Hz, Me), 0.88 (3H, t, $J = 7.4$ Hz, Me). MS (ESI pos.) *m/z* 266 ($[M+Na]^+$), 244 ($[M+H]^+$); (ESI neg.) *m/z* 278 ($[M+Cl]^-$), 242 ($[M-H]^-$). HRMS calcd for $C_{11}H_{19}ClN_3O$ $[M+H]^+$ 244.1217, found (*m/z*) 244.1231.

5.1.18. *tert*-Butyl ((1*S*,2*S*)-1-{[(2*S*)-2-(aminocarbonyl)-4-oxopyrrolidin-1-yl]carbonyl}-2-methylbutyl)carbamate (7k). In CH_2Cl_2 (10 mL), *tert*-butyl ((1*S*,2*S*)-1-{[(2*S*,4*R*)-2-(aminocarbonyl)-4-hydroxypyrrolidin-1-yl]carbonyl}-2-methylbutyl)carbamate **7a** (250 mg, 0.73 mmol) was dissolved, and then molecular sieves 4 A (1.5 g), pyridinium chlorochromate (235 mg, 1.09 mmol), and acetic acid (0.07 mL) were added, followed by stirring at room temperature for 2 h. The reaction solution was directly purified by silica gel column chromatography (developing solvent; hexane–EtOAc = 1:1–1:3) to yield the desired product (180 mg, 72%) as a brown amorphous substance. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.70 and 5.50 (1H each, br s each, $CONH_2$), 5.25–5.05 (2H, m, BocNH and H-2), 4.41 (1H, d, $J = 17.3$ Hz, H-5), 4.17–4.07 (1H, m, NCHCO), 3.92 (1H, d, $J = 17.3$ Hz), 3.05 (1H, br d, $J = 18.2$ Hz, H-3), 2.76–2.60 (1H, m, H-3), 1.80–1.50 (2H, m), 1.43 (9H, s), 1.27–1.07 (1H, m), 0.98–0.85 (6H, m, 2Me). MS (ESI pos.) *m/z* 364 ($[M+Na]^+$); (ESI neg.) *m/z* 340 ($[M-H]^-$).

5.1.19. *tert*-Butyl ((1*S*,2*S*)-1-{[(2*S*)-2-cyano-4-oxopyrrolidin-1-yl]carbonyl}-2-methylbutyl)carbamate (8k). In THF (10 mL), **7k** (168 mg, 0.49 mmol) was dissolved, and after ice-cooling, trifluoroacetic anhydride (0.21 mL) and *N*,*N*-diisopropylethylamine (0.51 mL) were added, followed by stirring with ice-cooling for 1 h. The reaction solution was diluted with EtOAc (100 mL), and washed with water, 10% aqueous $KHSO_4$ solution, water, a saturated aqueous $NaHCO_3$ solution and a saturated aqueous NaCl solution, successively, and the organic phase was dried over $MgSO_4$. After removal of the drying agent by filtration, the solvent was evaporated in vacuo to yield the desired product (174 mg, quant) as a brown amorphous substance. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.38 (1H, br d, $J = 7.6$ Hz, H-2), 5.07 (1H, br d, $J = 8.1$ Hz, NH), 4.52 (1H, d, $J = 18.0$ Hz, H-5), 4.03–3.95 (1H, m, NCHCO), 4.00 (1H, d, $J = 18.0$ Hz, H-5), 3.10–2.86 (2H, m, H-3), 1.90–1.72 (1H, m), 1.70–1.54 (1H, m), 1.41 (9H, s, Boc), 1.34–1.10 (1H, m), 0.98 (3H, d, $J = 6.5$ Hz, Me), 0.92 (3H, t, $J = 7.3$ Hz, Me). MS (ESI pos.) *m/z* 378 ($[M+MeOH+Na]^+$), 346 ($[M+Na]^+$); (ESI neg.) *m/z* 322 ($[M-H]^-$).

5.1.20. (2*S*)-1-L-Isoleucyl-4-oxopyrrolidine-2-carbonitrile trifluoroacetate (1k). The title compound (76 mg, 80%) was obtained as a colorless powder from **8k** (91 mg, 0.28 mmol) in a manner similar to the preparation of **1i**. Mp 121–124 °C (decomp.). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.21 (3H, br s, H_3N^+), 5.30 (1H, dd, $J = 9.8$, 5.6 Hz, H-2), 4.29 (1H, d, $J = 17.7$ Hz, H-5), 4.18 (1H, d, $J = 17.7$ Hz, H-5), 4.09–3.98 (1H, m, NCHCO), 3.11 (1H, dd, $J = 19.0$, 9.8 Hz, H-3), 3.01 (1H, dd, $J = 19.0$, 5.6 Hz, H-3), 2.00–1.90 (1H, m), 1.54–1.38 (1H, m), 1.34–1.10 (1H, m), 0.98 (3H, d, $J = 7.0$ Hz, Me), 0.88 (3H, t, $J = 7.3$ Hz, Me). MS (ESI pos.) *m/z* 278 ($[M+MeOH+Na]^+$), 246 ($[M+Na]^+$). HRMS calcd for $C_{11}H_{18}N_3O_2$ $[M+H]^+$ 224.1399, found (*m/z*) 224.1381.

5.2. Biological methods

5.2.1. DPP-IV inhibitory activity. Activity inhibition testing of DPP-IV was carried out according to the method by Deacon et al.[13] Plasma including DPP-IV was prepared via centrifugation of blood collected from healthy human volunteers. Enzyme reactions were carried out using 96-flat-bottom-well plates in buffer solution of pH 7.8 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, and 5 μL of the test compound, 12.5 μL of plasma diluted to 1/100 with the above buffer solution was 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 measurement of 1g and 9 in blood following oral administration to rats. Male Wistar rats (eight weeks of age) fasted overnight were used. Aqueous solution of **1g** or **9** was orally administered at a dose of 1 mg/kg. At 5, 10, 15, 30, 60, and 120 min 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 using 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) for MS/MS, ESI as an ionization method. The indicators of compound **1g** were *m/z* 228.0 and *m/z* 86.0.

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 of Balkan et al.[14] Male Zucker

fatty and lean rats (10 weeks of age; $n = 6$) were fasted overnight. Compound **1g** was dissolved in distilled water and administered orally. After 30 min, glucose solution was orally administered at 2 g/kg body weight. Blood samples were collected from the orbital venous sinus under ether anesthesia at the indicated times and plasma samples were prepared. Plasma glucose concentration, plasma insulin concentration, and plasma DPP-IV activity were measured.

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