Synthesis and Structure–Activity Relationships of Potent 1-(2-Substituted-aminoacetyl)-4-fluoro-2-cyanopyrrolidine Dipeptidyl Peptidase IV Inhibitors

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Dipeptidyl peptidase IV (DPP-IV) inhibitors have attracted attention as potential drugs for use in the treatment of type 2 diabetes because they prevent the degradation of glucagon-like peptide-1 (GLP-1) and extend its duration of action. We previously reported that 2-cyano-4-fluoropyrrolidines act as potent DPP-IV inhibitors and have been modifying the 1-position of pyrrolidine to obtain more useful inhibitors. An L-tert-butyglycine derivative was found to be a stable and potent DPP-IV inhibitor that exhibits a glucose lowering effect in vivo. Here, we report the synthesis of and biological data on the aforementioned derivatives.

Key words  
Dipeptidyl peptidase IV; inhibitor; fluoropyrrolidine; diabetes

Fig. 1. DPP-IV Inhibitors

Table 1. Chemical Stability of 1a

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Residual amount (%)</th>
</tr>
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<tbody>
<tr>
<td>pH 1.2</td>
<td>pH 6.8</td>
</tr>
<tr>
<td>1a</td>
<td>&gt;95</td>
</tr>
</tbody>
</table>

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cyano group to give cyclic amidine 2, which was subsequently transformed into diketopiperadine 3. Cyclic amidine 2 (retention time = 4.7 min) was observed as a 67% peak area using reverse phase high-performance liquid chromatography (HPLC) after 2 h of the incubation of 1a in a pH 6.8 aqueous buffer solution. After 10 h of incubation, compound 2 changed to diketopiperadine 3 (retention time = 7.4 min), which was observed as an 86% peak area (Table 2).

Cyclic amidine 2 was formed during the decomposition of 1a in neutral solution, but it was difficult to isolate from aqueous solution. Since the cyclization of 4, a free base of 1a, was accelerated by the presence of carboxylic acid in an organic solvent, 4 was treated with a 1.1 molar equivalent of acetic acid in ethanol–n-hexane to obtain cyclic amidine 5 (acetic acid salt) as a precipitate. Diketopiperadine 3 was obtained by heating 5 in a pH 6.8 buffer solution at 80 °C (Chart 1). Spectral data for 5 and 3 supported their structures, and the HPLC retention times of 5 and 3 corresponded with those of the degradation products of 1a in buffer solution.

Next, conversion at the P2 site was conducted in order to find a potent, stable and highly efficacious inhibitor. Magnin reported that the changes in stability caused by the conversion at the P2 site were affected by the substituent of the pyrrolidine ring. Several derivatives converted at the P2 site were then synthesized, as in the case of 2-cyano-4-fluoropyrrolidine. First, 2-aminocarbonyl-4-fluoropyrrolidine 7 was obtained in three steps from 4-hydroxide 6 and was then converted to amine hydrochloride 8 (Chart 2).

Compound 9 was obtained via the dehydration of compound 7 with cyanuric chloride in N,N-dimethylformamide (DMF) and was treated with hydrochloric acid to obtain intermediate 10. Compound 8 was useful as a common intermediate, and cyano derivative 10 seemed to be more useful for the synthesis of a variety of derivatives.

Compound 8 was coupled with N-Boc or N-Fmoc α-amino acids using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenztriazole (HOBt) as coupling reagents to yield the dipeptides 11a—f, 11k, 11l, 13i and 13j. Either the Fmoc group or the Boc group was used as an N-protecting group for α-amino acids. In the same manner, cyanide 10 was converted to dipeptides 12g and 14h (Chart 3).

Natural amino acids (valine and proline), artificial amino acids (alloisoleucine, tert-butylglycine and cyclohexylglycine) and modified amino acids (N-benzyloxycarbonyl-ly-
sine, O-methylthreonine, O-benzylthreonine and O-tert-butylthreonine) were used at the P2 site.

The cyano compounds 12a—f, 14i and 14j were obtained from carbamoyl compounds 11a—f, 13i and 13j using cyanuric chloride in DMF or trifluoroacetic anhydride and N,N-diisopropylethylamine.

Boc-protected compounds 12a—g and 12k—o were deprotected using hydrochloric acid to produce 1a—g and 1k. On the other hand, Fmoc-protected compounds 14h—j were converted to 1h—j using diethylamine.

7-Methoxytetrahydroquinoline derivative 12p and 7-carbamoylmethoxy derivative 12q were obtained by alkylating the 7-hydroxy derivative 12h; 12p and 12q were then treated with hydrochloric acid to produce 1p and 1q.

As the activities of stereoisomers appeared interesting, stereoisomers of 1d were synthesized. The synthetic route was similar to that for 1d. Four different hydroxy prolines (L-trans, L-cis, D-trans and D-cis forms) and L- and D-N-Boc-tert-butylglycine were used to produce seven stereoisomers 15—21.

Results and Discussion

The synthesized compounds were evaluated for DPP-IV inhibitory activity in human plasma using a fluorescence assay with Gly-Pro-4-methylcoumaryl-7-amide. Various sorts of α-amino acids were introduced at the P2 site. Valine derivative 1b and tert-butylglycine derivative 1d exerted potent DPP-IV inhibitory activities (IC50 < 1 nM), similar to that of isoleucine derivative 1a (IC50 = 0.6 nM). Cyclohexylglycine derivative 1j also maintained an inhibitory activity (IC50 = 1.1 nM) (Table 3). On the other hand, threonine derivatives 1f, 1g and 1h exhibited less potent activities (IC50 = 4.7, 2.1, 4.9 nM, respectively).

X-ray crystallographic analysis of DPP-IV 21,22) has suggested that the pocket to which the P2 site binds is lipophilic. The oxygen atom on the threonine side chain seemed to have difficulty binding tightly to the pocket. However, a benzyl ether side chain (1g) resulted in notable activity, possibly because of the affinity of the phenyl group. Compounds 1e and 1i exhibited potent activities (IC50 = 1.4, 0.7 nM, respectively), and the long side chains did not reduce the compounds’ affinities.

Among the secondary amine derivatives, proline derivative 1l had a potent inhibitory activity (IC50 = 0.8 nM) and tetrahydroisoquinoline derivatives 1m—q also had good potencies (IC50 = 1.2—3.3 nM) (Table 4). However, the N-methyl isoleucine derivative 1k had a very weak activity (IC50 > 100 nM).

Table 3. DPP-IV Inhibitory Activity and Chemical Stability of 1a—k

<table>
<thead>
<tr>
<th>Compd.</th>
<th>R1</th>
<th>R2</th>
<th>IC50 (nM)</th>
<th>Remaining amount (%)</th>
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<tbody>
<tr>
<td>1a</td>
<td>H</td>
<td>H</td>
<td>0.6</td>
<td>70</td>
</tr>
<tr>
<td>1b</td>
<td>H</td>
<td>H</td>
<td>0.7</td>
<td>74</td>
</tr>
<tr>
<td>1c</td>
<td>H</td>
<td>H</td>
<td>1.2</td>
<td>65</td>
</tr>
<tr>
<td>1d</td>
<td>H</td>
<td>H</td>
<td>0.6</td>
<td>93</td>
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<tr>
<td>1e</td>
<td>H</td>
<td>H</td>
<td>1.4</td>
<td>52</td>
</tr>
<tr>
<td>1f</td>
<td>H</td>
<td>H</td>
<td>4.7</td>
<td>67</td>
</tr>
<tr>
<td>1g</td>
<td>H</td>
<td>H</td>
<td>2.1</td>
<td>73</td>
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<tr>
<td>1h</td>
<td>H</td>
<td>H</td>
<td>4.9</td>
<td>92</td>
</tr>
<tr>
<td>1i</td>
<td>H</td>
<td>H</td>
<td>0.7</td>
<td>41</td>
</tr>
<tr>
<td>1j</td>
<td>H</td>
<td>H</td>
<td>1.1</td>
<td>68</td>
</tr>
<tr>
<td>1k</td>
<td>Me</td>
<td>H</td>
<td>&gt;100</td>
<td>—</td>
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</table>

(a) DPP-IV inhibitory activity.  (b) Residual amount was measured after incubation at 37 °C for 6 h in pH 6.8 aqueous buffer solution.

Table 4. DPP-IV Inhibitory Activity of 1l—q

<table>
<thead>
<tr>
<th>Compd.</th>
<th>IC50 (nM)</th>
</tr>
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<tbody>
<tr>
<td>1l</td>
<td>0.8</td>
</tr>
<tr>
<td>1m</td>
<td>2.2</td>
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<tr>
<td>1n</td>
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<td>1o</td>
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<tr>
<td>1p</td>
<td>1.7</td>
</tr>
<tr>
<td>1q</td>
<td>3.3</td>
</tr>
</tbody>
</table>

(a) DPP-IV inhibitory activity.
Chemical stability is an important characteristic in the development of 2-cyanopyrrolidine DPP-IV inhibitors. Our lead compound 1a was considerably stable in acidic solution, but the residual amount of compound 1a decreased in neutral solution. The stabilities of the compounds listed in Table 3 varied widely in a manner that was correlated with their structures.

The degradation of compound 1a under neutral conditions might result from the attachment of a basic nitrogen to the carbon atom of the cyano group, yielding a cyclic amidine. By constructing a sterically hindered environment near the nitrogen, this kind of degradation should be prevented.

tert-Butylglycine derivative 1d and D-tert-butylthreonine 1h were comparatively stable, as shown by their residual amounts (93%, 92%, respectively) after 6 h of incubation at 37 °C in a pH 6.8 buffer solution. Magnin reported that a tert-butylglycine derivative exhibited the same chemical stability as an isoleucine derivative in a cis-3,4-methanoprolinonitrile series and better chemical stability than an isoleucine derivative in a cis-4,5-methanoprolinonitrile series. Our result for fluoropyrrolidine was similar to that for the cis-4,5-methanoprolinonitrile series.

In contrast, compounds 1e or 1i, which are not branched at the carbon next to the α-position, yielded low residual amounts (52%, 41%, respectively). Proline derivative 11 and tetrahydroisoquinoline derivatives 1m—o also showed low residual amounts, with the compounds listed in Table 4 showing the residual amounts of less than 20% under the same condition. These compounds with cyclic side chains easily undergo cyclization, since their nitrogen atoms can come into proximity with the cyano group.

To investigate the correlation between stereochemistry and inhibitory activity, all the stereoisomers of 1d were synthesized. Compound 1d was selected for this investigation because it exhibited the best balance between stability and inhibitory activity.

We previously reported the structure–activity relationship (SAR) for the stereochemistry of 4-fluoropyrrolidine 15; here, we report the SAR for that of 2-cyanide and α-amino acids at the P2 site as well as 4-fluoride. (4R)-Fluoride analogue 15 had a 400-fold lower potency (IC_{50}=246 nM) than 1d, and (2R)-cyanide analogue 16 had a more than 500-fold lower potency (IC_{50}>300 nM) (Table 5). D-tert-Butylglycine analogue 18 had a 70-fold lower potency (IC_{50}=42 nM) than 1d, but the genuine activity level of 18 was uncertain because the amount of commingled L-form 1d could not be determined. The other isomers did not exhibit inhibitory activity. We concluded that all three chiral centers were important for activity because of the dramatic reductions in the activities of the stereoisomers of 1d.

We obtained a crystal of 22, the free base of 1d, for X-ray crystallographic analysis and observed that the configuration of the cyano group and the fluorine atom was cis, while the conformation of the amide bond was trans (Fig. 2).

As 1d exhibited the most favorable inhibitory activity and chemical stability profiles, the in vivo effect of 1d was examined in Zucker fatty rats, a model of obesity and impaired glucose tolerance. Compound 1d was orally administered at a dose of 1 mg/kg body weight at 30 min before glucose loading; the plasma glucose, insulin and DPP-IV activity levels were then monitored over time (Fig. 3).

![Fig. 2. X-Ray Crystal Structure of Compound 22 (Free Base of 1d)](image)

The plasma glucose level after glucose loading was significantly lower in the 1d-treated group than in the vehicle-treated control group (Figs. 3A, B). DPP-IV activity was almost completely inhibited at 15 min after glucose loading, and this inhibitory effect was retained for 2 h (Fig. 3D). These results indicate that 1d may be useful for the treatment of hyperglycemia, based on its inhibitory effect on plasma DPP-IV activity. Insulin secretion was significantly enhanced at 15 min after glucose loading (Fig. 3C). This finding supports the proposed mechanism that 1d might prevent the inactivation of active GLP-1 via DPP-IV inhibition and that an increase in GLP-1 activity might stimulate insulin secretion by acting upon β-cells in the pancreas, resulting in the suppression of hyperglycemia after glucose loading.

**Conclusion**

We modified the P2 site of 2-cyano-4-fluoropyrrolidine derivative 1a, which we previously reported, to obtain a more useful DPP-IV inhibitor. As a result, we obtained a stable and potent DPP-IV inhibitor, 1d, which is expected to be useful as a therapeutic agent for lowering postprandial hyperglycemia and treating type 2 diabetes mellitus. Subsequent reports will describe the results of further investigations of 4-fluoro-2-cyanopyrrolidines.

**Experimental**

^1H-NMR spectroscopy was performed using a Varian VXR-300 or a JEOL GX500 spectrometer. Chemical shifts were recorded 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). ^13C-NMR spectroscopy was performed using a JEOL GX500 spectrometer. ^19F-NMR spectroscopy was performed using a Varian VXR-300 spectrometer. ESI mass spectra were recorded using a Shimadzu/Kratos HV-300. High resolution spectra were recorded on a Micro-
mass Q-TOF2 instrument. Melting points were 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.

Degradation of 1a and HPLC Analysis Compound 1a (5 mg) was dissolved in pH 6.8 Britton–Robinson buffer solution (5 ml) and incubated at 60 °C. The solution was then analyzed using reverse phase HPLC using a CAPCELL PAK UG120 (5 μm particle size, φ 4.6×150 mm; SHISEIDO) and eluted at 1.0 ml/min with acetonitrile–H2O (15:85 v/v, 10 mm ammonium acetate solution); its UV absorbance was monitored at 210 nm. To examine chemical stability, the residual amount was also analyzed using HPLC in a manner similar to the method described above.

(35,7S)-7-Fluoro-1-imino-3-[(1S)-1-methylpropyl]hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (3) Compound 5 (240 mg, 1.28 mmol) was dissolved in pH 6.8 Britton–Robinson buffer solution (24 ml). The solution was then stirred at 80 °C for 5 h. The reaction solution was extracted with EtOAc and the organic phase was successively washed with a saturated aqueous NaHCO3 solution and a saturated aqueous NaCl solution, successively, and then dried over MgSO4. The drying agent was removed by filtration and dried in vacuo in vacuo. EtOAc and the organic phase was successively washed with a saturated aqueous NaCl solution and extracted with EtOAc. The reaction solution was taken up in water and extracted with EtOAc. The organic phase was washed with a saturated aqueous NaCl solution and dried over MgSO4. The drying agent was removed by filtration, and the filtrate was concentrated under reduced pressure to yield the desired product (680 mg, quant.) as a light brown powder. 1H-NMR (300 MHz, DMSO-d6) δ 5.37 (1H, d, J = 20.6 Hz), 64.1, 55.8, 52.4 (d, Jc = 23.8 Hz), 38.7, 35.9 (d, Jc = 26.6 Hz), 24.6, 15.1, 10.9; 19F-NMR (282.2 MHz, DMSO-d6) δ −172.7. MS (ESI pos.) m/z 227 (M+Na)+; (ESI neg.) m/z 225 (M−H). Anal. Calcd for C11H18FN3O3: C, 57.88; H, 7.51; N, 12.27; F, 8.32. Found: C, 57.86; H, 7.44; N, 12.26; F, 8.35. α: vs. 76.0° (c=0.3, MeOH).

(25,45)-4-Fluoropyrrolidine-2-carbonitrile Hydrochloride (9) Compound 9 (450 mg, 2.10 mmol) was dissolved in MeOH (3 ml), and 4 mL HCl (3 mol) was added, followed by stirring at room temperature for 20 h. The reaction solution was concentrated in vacuo to yield the desired product (320 mg, quant.) as a light brown powder. This intermediate was used in the next reaction without purification. 1H-NMR (300 MHz, DMSO-d6) δ 5.53 (1H, br d, J = 52.4 Hz, H-4), 4.97 (1H, dd, J = 8.2, 4.3 Hz, H-2), 3.59 (1H, dd, J = 22.1, 13.7 Hz, H-3), 3.47 (1H, ddd, J = 35.9, 13.7, 3.7 Hz, H-5), 2.64—2.43 (2H, m), 1.44 (9H, M, H). MS (ESI pos.) m/z 237 (M+Na)+; (ESI neg.) m/z 249 (M+Cl).
amino)acetyl)-4-fluoro-L-prolinamide (13j) into diisopropyl ether (20 ml). The precipitated insoluble substance was collected, dried agent using filtration, the filtrate was concentrated under reduced (M (300 MHz, DMSO-

The title compound was obtained as a colorless amorphous powder from 1 (100 mg, 2.1 mmol) and 2 (300 mg, 1.78 mmol) was dissolved in THF (20 ml), and trifluoroacetic anhydride (1.74 mmol) was added. The reaction solution was concentrated to prepare 1. 

The title compound was obtained as a brown powder by similar method used to prepare 1b. mp 265 — 269 °C (decomp.). 

The title compound was obtained as a colorless amorphous powder from 8 (300 mg, 1.78 mmol) and (2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b) The title compound (656 mg, 91%) was obtained as a colorless amorphous powder from 11b (760 mg, 2.29 mmol) in a manner similar to used method to prepare 9. 

The title compound was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-[[(9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b). The title compound (870 mg, 88%) was obtained as a colorless amorphous powder from 10 (301 mg, 2.0 mmol) and (2S)-N-((9S,2S,5R)-4-Cyano-4-fluoropyrrolidin-2-yl-[carboxyl]-t-prolylpyrrolidine (14b).
The title compound was obtained as a colorless amorphous powder in a manner similar to method used to prepare 1b. 1H-NMR (300 MHz, DMSO-d$_6$) δ 8.48 (3H, br s), 5.54 (1H, d, J = 51.6 Hz, H-4), 5.06 (1H, d, J = 8.7 Hz, H-2), 4.10—3.78 (3H, m), 2.60—2.26 (2H, m, H-3), 1.86—1.51 (6H, m), 1.30—1.00 (5H, m). MS (ESI pos.) m/z 276 (M+N$_2$); (ESI neg.) m/z 238 (M$^+$). HR-MS Calcd for C$_{15}$H$_{17}$FN$_3$O$_2$ (M$^+$) 290.1305, Found (m/z) 290.1304.

(2S,4R)-1-{{(3S)-7-methoxy-3,4-dihydroisoquinolin-3-yl}carbonyl}pyrrolidine-2-carbonitrile Hydrochloride (14) The title compound was obtained as a colorless amorphous powder in a manner similar to method used to prepare 1b. 1H-NMR (300 MHz, DMSO-d$_6$) δ 8.48 (3H, br s), 5.54 (1H, d, J = 51.6 Hz, H-4), 5.06 (1H, d, J = 8.7 Hz, H-2), 4.10—3.78 (3H, m), 2.60—2.26 (2H, m, H-3), 1.86—1.51 (6H, m), 1.30—1.00 (5H, m). MS (ESI pos.) m/z 276 (M+N$_2$); (ESI neg.) m/z 238 (M$^+$). HR-MS Calcd for C$_{15}$H$_{17}$FN$_3$O$_2$ (M$^+$) 290.1305, Found (m/z) 290.1304.

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X-Ray Crystal Analysis Single crystals of 22 (free base of 1d) that were suitable for X-ray crystallography were grown by crystallization from chloroform/ethyl acetate. Data were collected at 288 K on a Mac Science/Bruker axs MXC18 four-circle automated diffractometer using CuKα radiation (λ=1.54178). The compound with the chemical formula of C11H18FN3O, crystallized in the monoclinic space group P2₁, a=9.642(2), b=10.662(2), c=6.1103(11) Å, β=106.793(13)°, V=601.4(2) Å³, Z=2. The structure was solved by a direct method using the program SHELXS. All non-hydrogen atoms were refined anisotropically, and all H-atoms were refined isotropically. The final R and Rw values were 0.039 and 0.068, respectively.

DPP-IV Inhibitory Activity The inhibition of DPP-IV activity was tested using a method described by Deacon et al.23 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 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 MgCl₂ 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).

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.24 Food was withheld overnight from male Zucker fatty and lean rats (10 weeks of age; n=6). Compound 1d 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 DPP-IV activity were measured.

References