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[(*S*)-γ-(Arylamino)prolyl]thiazolidine compounds as a novel series of potent and stable DPP-IV inhibitors

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Abstract—Dipeptidyl peptidase-IV (DPP-IV) inhibitors, or glucagon-like peptide-1 (GLP-1) enhancers, are looked to as a potential new class of antidiabetic agents. In particular, potent and long-acting inhibitors might offer advantages in exploiting DPP-IV inhibition. The series of [(*S*)-γ-(arylamino)prolyl]-(*S*)-2-cyanopyrrolidine compounds on which we reported previously has a highly potent inhibitory activity but seemed to be unstable in neutral aqueous solution. Here, we describe [(*S*)-γ-(arylamino)prolyl]thiazolidine compounds as a novel series of potent and stable DPP-IV inhibitors. They are the thiazolidine analogs of [(*S*)-γ-(arylamino)prolyl]-(*S*)-2-cyanopyrrolidine but with the electrophilic nitrile removed to improve chemical stability in aqueous solution. Of the compounds investigated in the present study, the [((*S*)-γ-3,4-dicyanophenylamino)prolyl]thiazolidine **12m** was the most potent. The structure–activity relationship (SAR) of the γ-substituent in the proline moiety of the thiazolidide was similar to that obtained with the (*S*)-2-cyanopyrrolidide. The γ-substituent in the proline moiety of both the (*S*)-2-cyanopyrrolidide and the thiazolidide may engage with the S_2 binding pocket of DPP-IV and thereby achieve hydrophobic interaction in the same manner. Based on pharmacokinetic experiments in rats, the representative compound **11**, which displayed high oral bioavailability (BA = 83.9%) and long half-life in plasma ($t_{1/2}$ = 5.27 h), was found to have an excellent pharmacokinetic profile.

1. Introduction

Dipeptidyl peptidase-IV (EC 3.4.14.5, DPP-IV) is a member of the serine protease family that recognizes an amino acid sequence having proline or alanine at the second position from the N-terminal and produces dipeptide.[1] DPP-IV is widely distributed in mammalian tissues and plays several physiological roles; in particular, its role as a peptidase that rapidly inactivates glucagon-like peptide-1 (GLP-1) has drawn interest.[2] GLP-1 is secreted in response to meal ingestion and stimulates insulin secretion.[3] It has been suggested that potentiation and extension of the action of GLP-1 by DPP-IV inhibition would stimulate insulin secretion after meal ingestion only,[4] and DPP-IV inhibitors have therefore come to be seen as a potential new type of antidiabetic agent free of side effects such as hypoglycemia and exhaustion of pancreatic β-cells. In particular, a potent and long-acting inhibitor might offer advantages in exploiting DPP-IV inhibition.

Although a number of DPP-IV inhibitors have been reported and classified into structural types, most of them are substrate analogs composed of the P_2–P_1 fragment (Fig. 1).[5] (*S*)-2-Cyanopyrrolidine and thiazolidine structures are used as proline mimic in the P_1 part. Dipeptide-type inhibitors containing an (*S*)-2-cyanopyrrolidine structure, for example, NVP-DPP728 (**1**)[6–8] and NVP-LAF237 (**2**),[8,9] have been reported as potent inhibitors. The latter is under clinical trials as an antidiabetic agent.[9]

We previously reported that a series of [(*S*)-γ-(arylamino)prolyl]-(*S*)-2-cyanopyrrolidine compounds had a potent inhibitory activity (Fig. 2b and Table 1).[10] The

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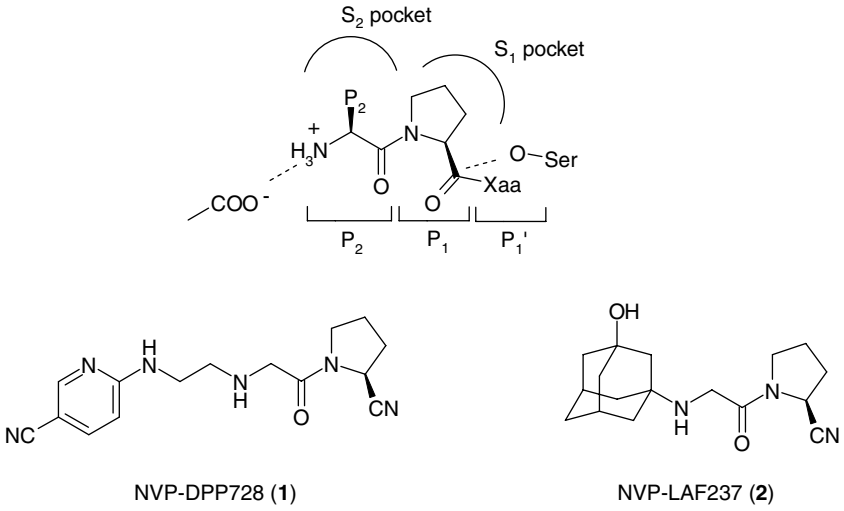


Figure 1.

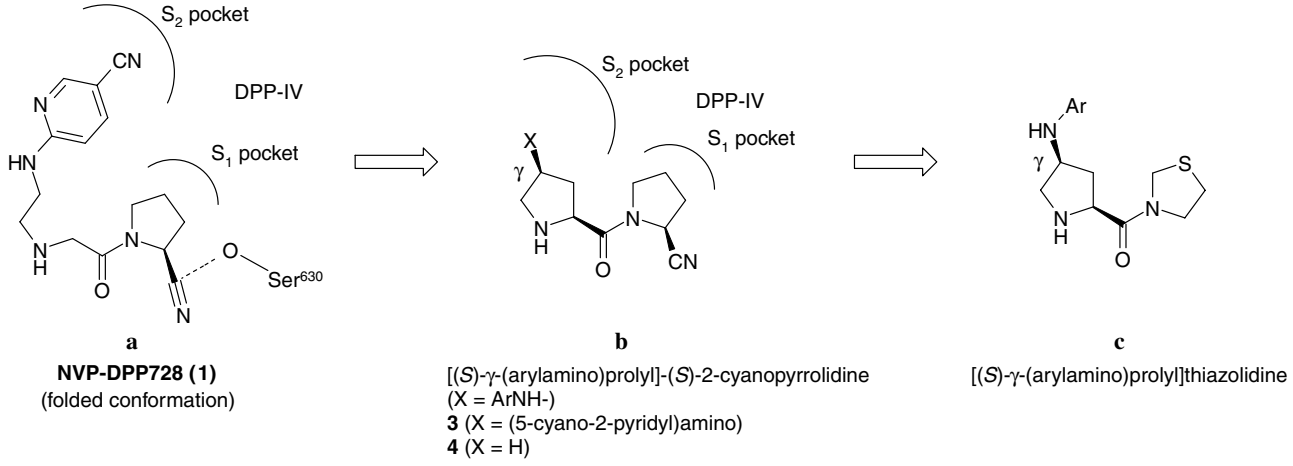
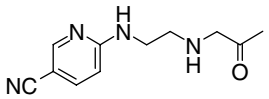
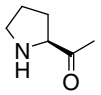
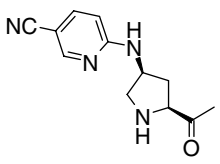


Figure 2. Design of [(*S*)-(γ-arylamino)prolyl]-(*S*)-2-cyanopyrrolidine and thiazolidine.

Table 1. DPP-IV inhibition of (*S*)-2-cyanopyrrolidines and thiazolidines

Compound	U	V	W	DPP-IV inhibition, IC_{50} (nmol/L)	
				Human	Rat
NVP-DPP728 (**1**)		CH_2	CN	1.4	2.3
15		S	H	270	500
4		CH_2	CN	2.9	2.9
14		S	H	538	607
3		CH_2	CN	0.25	0.27
11		S	H	25.4	30.4

representative compound **3** is an analog conformationally restricted using a proline structure and which mimics the folded conformation of the conformationally flexible compound **1** to allow interaction of the (5-cyano-2-pyridyl)amino moiety with the S_2 binding pocket as shown in Figure 2a.[6] As a result, compound **3** showed 5-fold more potent inhibitory activity than the flexible compound **1**. In addition, compound **3**, with an arylamino group introduced at the γ-position, has 10-fold more potent inhibitory activity than the γ-unsubstituted compound **4**.[11]

The (*S*)-2-cyanopyrrolidide class of compounds thus includes many potent DPP-IV inhibitors. It suffers, however, from chemical instability whereby the N-terminal amine intramolecularly attacks the electrophilic nitrile of the (*S*)-2-cyanopyrrolidine moiety, affording cyclic products (Fig. 3).[8] Ashworth and coworkers reported on the DPP-IV-inhibitory activity of dipeptide-type analogs having (*S*)-2-cyanopyrrolidide and their chemical stability in aqueous solution (pH 7.4).[11] They found that, although most of the tested dipeptide analogs exhibited excellent half-lives ($T_{1/2decomp} > 48$ h or $T_{1/2decomp} = 48$ h), the prolyl-(*S*)-2-cyanopyrrolidine **4** has a relatively short half-life ($T_{1/2decomp} = 7.5$ h). [(*S*)-γ-(Arylamino)prolyl]-(*S*)-2-cyanopyrrolidines, bearing the same core structure, seemed to cyclize relatively rapidly in neutral aqueous solution in the same way as shown in Figure 3, and their instability may result in the short half-life in plasma.

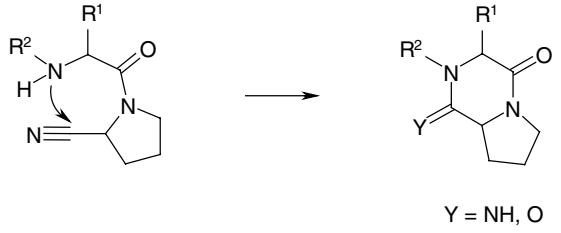


Figure 3.

Meanwhile, dipeptide-type analogs of pyrrolidide (**5**) and thiazolidide (**6**) lacking the electrophilic nitrile are known as DPP-IV inhibitors; in general, the thiazolidide **6** is more effective than the corresponding pyrrolidide **5** (Fig. 4).[12,13] Free from intramolecular cyclization, they are chemically stable. P32/98 (**7**), one example of this compound class, produced improved glucose tolerance in diabetic patients and healthy volunteers in clinical trial,[14,15] but exhibited only modest inhibitory activity ($IC_{50} = 75$ nmol/L for human plasma[16]). To identify a DPP-IV inhibitor with more potent and favorable pharmacokinetic action, we focused on the [(*S*)-γ-(arylamino)prolyl]thiazolidine compounds (Fig. 2c). It was expected that, even in the thiazolidide, the introduction at the γ-position of a proline moiety would bring more potent inhibitory activity.

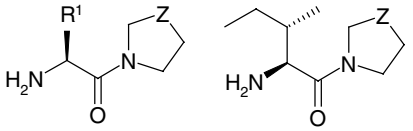
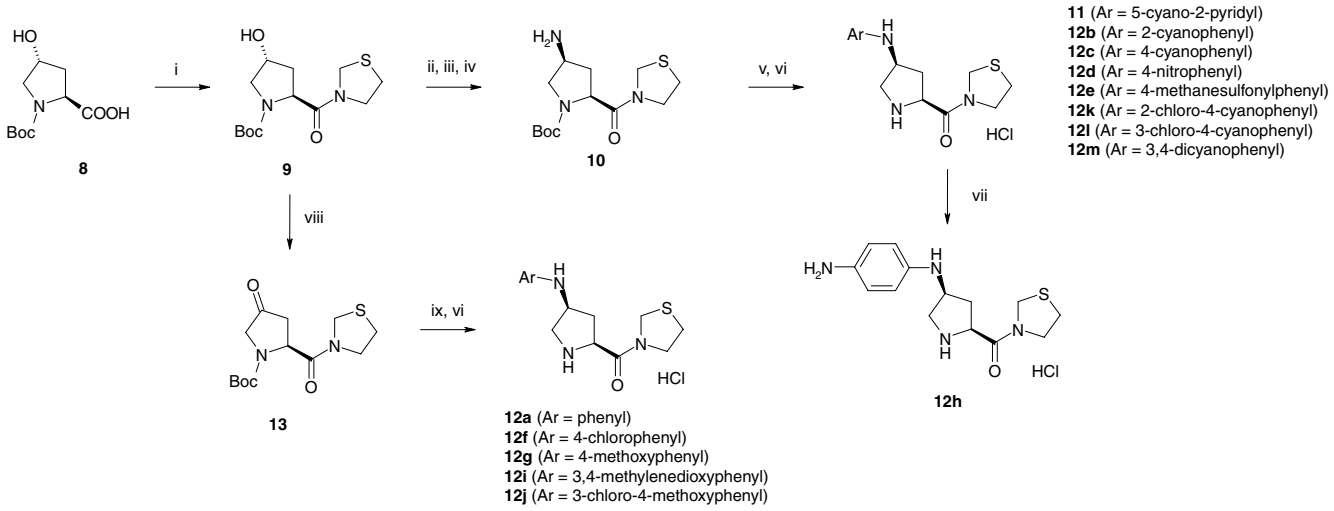


Figure 4.

The inhibitory activity of the most potent of the compounds resulting from this approach, **12m**, is single nanomolar, which is ca. 10-fold that of P32/98 (**7**). The representative compound **11** was found to have an excellent pharmacokinetic profile and improved chemical stability. Herein we report on [(*S*)-γ-(arylamino)prolyl]thiazolidine as a novel series of potent and stable DPP-IV inhibitors.



Scheme 1. Reagents and conditions: (i) thiazolidine, HOBT, EDC, DMF, rt; (ii) MsCl, Et_3N, CH_2Cl_2, 0 °C; (iii) NaN_3, DMF, 80 °C; (iv) H_2, Pd/C, EtOH, rt; (v) Ar-Cl or Ar-F, *i*-PrNEt$_2$, *N*-methyl-2-pyrrolidone, 80–100 °C; (vi) HCl, AcOEt; (vii) H_2, Pd/C, HCl, dioxane, AcOEt; (viii) DMSO, SO_3–pyridine complex; (ix) aniline, $NaBH_3CN$, AcOH, MeOH; or Ar-NH_2, $NaBH(OAc)_3$, 1,2-dichloroethane.

2. Chemistry

The synthesis of [(*S*)-γ-(arylamino)prolyl]thiazolidine compounds is shown in Scheme 1. The *trans*-hydroxy group of the amide **9**, which was prepared by coupling the *trans*-Boc-hydroxyproline **8** with thiazolidine, was converted to its mesylate. Treatment of the mesylate with sodium azide, followed by catalytic hydrogenation, gave the *cis*-amino compound **10**. Reaction of the amine **10** with 2-chloro-5-cyanopyridine or other electron-deficient aromatic halides and subsequent removal of the Boc group afforded the (*S*)-γ-substituted derivatives **11** and **12b–e** and **k–m**. The aniline derivative **12h** was prepared from the nitrophenyl derivative **12d** by catalytic hydrogenation. The non-electron-deficient phenyl derivatives **12a**, **f**, **g**, **i**, and **j** were prepared by reductive amination of the ketone **13** with anilines and subsequent removal of the Boc group.

3. Results and discussion

The thiazolidide and related compounds were evaluated for DPP-IV-inhibitory activity in human and rat plasma by fluorescence assay using Gly-Pro-MCA (Tables 1 and 2).

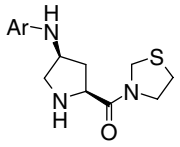
First of all, we examined the effect of the conversion of the (*S*)-2-cyanopyrrolidine moiety into a thiazolidine structure and conformational restriction using a proline structure on the thiazolidide (Table 1).

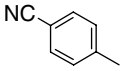
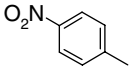
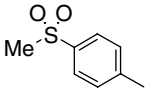
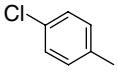
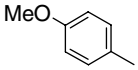
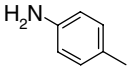
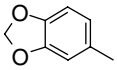
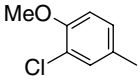
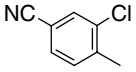
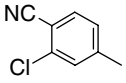
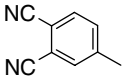
As a result of the conversion, the thiazolidide **15**[17] showed 200-fold decreased activity compared to the (*S*)-2-cyanopyrrolidide **1**. In the same way, the prolyl-thiazolidine **14** was 200-fold less active than the propyl-(*S*)-2-cyanopyrrolidine **4**.[11] These results indicate that interaction of the nitrile group and Ser^{630} at the active site of the DPP-IV is a dominant factor for exhibition of inhibitory activity; an (*S*)-2-cyanopyrrolidine structure might therefore be widely used for DPP-IV inhibitors despite decomposition by intramolecular cyclization.

In a previous study of (*S*)-2-cyanopyrrolidides, we found that compound **3** has 5-fold more potent inhibitory activity than NVP-DPP728 (**1**). Compound **3** is an analog of the flexible compound **1** conformationally restricted using a proline structure to produce interaction of the (5-cyano-2-pyridyl) moiety with the S_2 binding site as shown in Figure 2. As a result of the present research on thiazolidides, compound **11** was found to be 10-fold more potent than the flexible compound **15**.[17] Restriction using a proline structure thus produces more potent inhibitory activity in the thiazolidides in the same way as in (*S*)-2-cyanopyrrolidides.

Moreover, compared to the γ-unsubstituted compound **14**, compound **11**, as a result of the introduction of a γ-substituent, was 20-fold more potent. This result indicates that the γ-substituent of compound **11** plays a significant part in the binding with DPP-IV. Modification of the γ-substituent was therefore undertaken in the next step designed to bring about a recovery of the decreased activity by conversion of the (*S*)-2-cyanopyrrolidine moiety into a thiazolidine structure.

Table 2. DPP-IV inhibition of [(*S*)-γ-(arylamino)prolyl]thiazolidines



Compound	Ar	DPP-IV inhibition, IC_{50} (nmol/L)	
		Human	Rat
12a		147	190
12b		24.1	41.3
12c		25.2	33.6
12d		19.2	21.0
12e		52.3	43.8
12f		45.4	55.1
12g		51.5	54.9
12h		105	125
12i		29.0	34.5
12j		19.9	14.8
12k		18.2	21.8
12l		11.1	13.0
12m		8.4	10.8

In the next step, as the phenylamino-derivatives of (*S*)-2-cyanopyrrolidide had shown potent inhibitory activity comparable to that of (5-cyano-2-pyridyl)amino-derivative **3**,[10] we examined the inhibitory activity of (*S*)-γ-phenylamino-substituted derivatives of thiazolidide (**12a–m**). Table 2 summarizes their inhibitory activity. The non-substituted phenylamino-compound **12a**

showed 6-fold less activity than the (5-cyano-2-pyridyl)amino compound **11**, but introduction of a substitution on the benzene ring of **12a** produced potent activity (**12b–m**). Di-substituted phenylamino compounds tended to be more potent than mono-substituted phenylamino-compounds, for example, **12c** versus **12l** or **m**; or **12g** versus **12i** or **j**. However, no clear-cut electronic influence of the substituent emerged in these compounds; for example, substitution of an electron-donating methoxy group (**12g**) or an electron-withdrawing cyano or nitro group (**12b–d**) both resulted in 3- to 9-fold more potent activity than the non-substituted compound **12a**. These results of SAR study of the γ-substituent in the thiazolidide are similar to those obtained in the (*S*)-2-cyanopyrrolidide. The γ-substituent in both the (*S*)-2-cyanopyrrolidide and the thiazolidide may engage with the S_2 binding pocket of DPP-IV and thereby achieve hydrophobic interaction in the same manner. Of the compounds investigated in the present study, compound **12m**, bearing a 3,4-dicyanophenylamino-group, showed the highest inhibitory activity, which, despite the lack of the electrophilic nitrile, was single nanomolar (IC_{50} = 8.4 nmol/L for human plasma) or ca. 10-fold more potent than that of P32/98 (**7**).

As proof of concept, the solution stability of (*S*)-2-cyanopyrrolidide and thiazolidide was examined at 37 °C using a buffer of pH 6.8. The (*S*)-2-cyanopyrrolidide **3** and the thiazolidide **11**, both bearing (5-cyano-2-pyridyl)amino group, the component parts of NVP-DPP728 (**1**), at the γ-position of the proline moiety were selected as representative compounds. As shown in Figure 5, the (*S*)-2-cyanopyrrolidide **3** decomposed rapidly ($T_{1/2decomp}$ = 1.3 h), whereas the thiazolidide **11** was quite stable. We therefore undertook ex vivo evaluation of the plasma DPP-IV-inhibitory activity and the pharmacokinetic study of the thiazolidide **11**. The plasma DPP-IV activity after oral administration of **11** to Wistar rats is shown in Figure 6. Half-inhibition was observed at 9 h after administration of 100 μmol/kg. The plasma drug concentration profiles and pharmacokinetic parameters after intravenous or oral administration (10 mg/kg, 19 μmol/kg) of **11** to Sprague–Dawley rats are shown in Figure 7 and Table 3, respectively. With high oral bioavailability (BA = 83.9%) and long half-life in plasma ($t_{1/2}$ = 5.27 h), the thiazolidide **11** exhibited an excellent pharmacokinetic profile. The pharmacokinetic

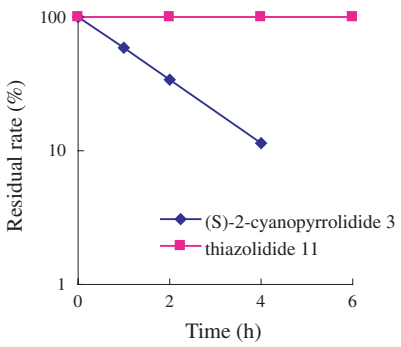


Figure 5. Solution stability of (*S*)-2-cyanopyrrolidide **3** and thiazolidide **11** at pH 6.8 and 37 °C.

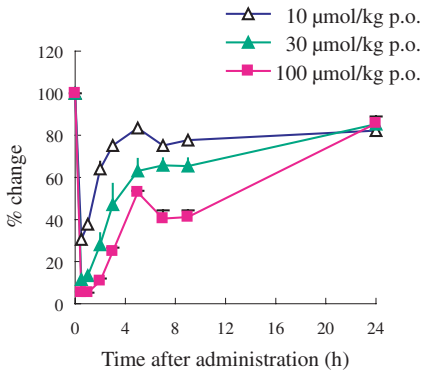


Figure 6. Plasma DPP-IV activities after oral administration of thiazolidide **11** to Wistar rats.

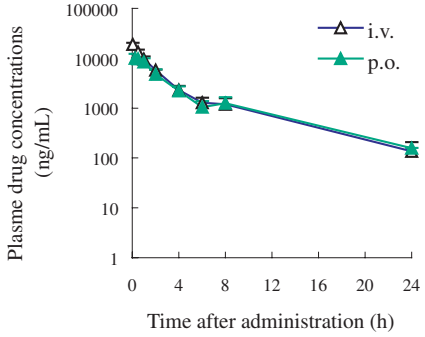


Figure 7. Plasma drug concentration profiles after a intravenous or oral administration of thiazolidide **11** at a dose of 10 mg/kg to Sprague–Dawley rats.

data support the ex vivo experimental finding of long-lasting action.

4. Conclusion

We have described here a novel series of potent DPP-IV inhibitors, the [(*S*)-γ-(arylamino)prolyl]thiazolidine compounds, which are the thiazolidine analogs of the previously reported [(*S*)-γ-(arylamino)prolyl]-(*S*)-2-cyanopyrrolidine compounds.[10] The electrophilic nitrile has been removed in them to improve chemical stability in aqueous solution. Of the compounds investigated in the present study, the (*S*)-γ-3,4-dicyanophenylamino-substituted compound **12m** showed the most potent inhibitory activity. The SAR of the γ-substituent in the proline moiety of the thiazolidide was similar to that obtained in the (*S*)-2-cyanopyrrolidide. The γ-substituent in both the (*S*)-2-cyanopyrrolidide and the thiazolidide may engage with the S_2 binding pocket of DPP-IV and thereby achieve hydrophobic interaction in the same manner. Based on ex vivo and pharmacokinetic experiments in rats, the representative compound **11** exhibited an excellent pharmacokinetic profile. Through conversion of the (*S*)-2-cyanopyrrolidine moiety into a thiazolidine structure, we consider that removal of the electrophilic nitrile is one of the most promising methods for improvement of the chemical stability of prolyl-(*S*)-2-cyanopyrrolidine compounds. Further optimization of these proline-containing inhibitors for

Table 3. Pharmacokinetic parameters of drug in plasma after intravenous or oral administration of thiazolidide **11** to Sprague–Dawley rats

Dosage	n[a]	$t_{1/2}$[b] (h)	AUC[c] (ng h/mL)	C_{max}[d] (ng/mL)	BA[e] (%)
10 mg/kg iv	3	5.27 ± 1.55	46,463 ± 5196		
10 mg/kg po	3		39,937 ± 5461	9939.6 ± 2364.5	83.9 ± 11.5

[a] Number of animals.
[b] Elimination half-life.
[c] Area under plasma concentration–time curve from zero to infinity.
[d] Maximum plasma concentration of unchanged drug.
[e] Oral bioavailability: $(AUC_{po} \times dose_{iv})/(AUC_{iv} \times dose_{po}) \times 100$.

more potent activity, focused on the S_2 binding pocket, will be described in due course.

5. Experimental

5.1. Chemistry

^{1}H NMR spectra were measured on a Bruker DPX-300 instrument with tetramethylsilane as the internal standard; chemical shifts are reported in parts per million (ppm, δ units). Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; br s, broad singlet. Mass spectra (MS) were recorded on a JEOL JMS-700 instrument operating in the electron ionization (EI) or the chemical ionization (CI) mode. Electron analysis for carbon, hydrogen, and nitrogen was performed with a Yanagimoto CHN CORDER MT-6. Chromatography refers to flash chromatography conducted on silica gel BW-300 (Fuji Silysia). All chemicals and solvents were of reagent grade unless otherwise specified. For drying organic solutions in extraction, anhydrous sodium sulfate or anhydrous magnesium sulfate was used unless otherwise indicated. The following abbreviations are used: DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; HOBT, 3-hydroxybenztriazole hydrate.

5.1.1. 3-[(2*S*,4*R*)-1-*tert*-Butoxycarbonyl-4-hydroxy-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (9). To a solution of thiazolidine (29.4 g, 0.33 mmol) in DMF (300 mL) were added *N*-*tert*-butoxycarbonyl-L-*trans*-hydroxyproline (**8**, 69.4 g, 0.3 mol), HOBT (50.5 g, 0.33 mol), and EDC (63.3 g, 0.33 mol). The mixture was stirred at room temperature for 18 h and the reaction mixture concentrated. To the concentrate were added brine and saturated aqueous sodium hydrogen carbonate solution and the mixture was extracted with ethyl acetate. The extract was dried and the solvent evaporated under reduced pressure to give the title compound (56.3 g, 62%) as a colorless transparent oil. ^{1}H NMR ($CDCl_3$) δ 1.41–1.45 (9H, m), 1.95–2.34 (2H, m), 2.62–3.25 (2H, m), 3.40–3.98 (4H, m), 4.40–4.90 (4H, m).

5.1.2. 3-((2*S*,4*S*)-4-Amino-1-*tert*-butoxycarbonyl-2-pyrrolidinylcarbonyl)-1,3-thiazolidine (10). (1) To a solution of 3-((2*S*,4*R*)-1-*tert*-butoxycarbonyl-4-hydroxy-2-pyrrolidinylcarbonyl)-1,3-thiazolidine (**9**, 56.3 g, 186 mmol) and triethylamine (28.5 mL) in dichloromethane (1 L) was added methanesulfonyl chloride (15.1 mL, 195 mmol) under ice cooling. After stirring under ice cooling for 1 h, the reaction mixture was washed with water and dried. The solvent was evaporated under reduced pressure to give 3-((2*S*,4*R*)-1-*tert*-butoxycarbonyl-4-methanesulfonyloxy-2-pyrrolidinylcarbonyl)-1,3-thiazolidine (70.5 g, 100%) as an oil. (2) The compound thus prepared (70.5 g, 185 mmol) and sodium azide (13.3 g, 204 mmol) were dissolved in DMF (500 mL) and stirred at 80 °C for 5 h before concentration of the reaction mixture under reduced pressure and addition of water. The mixture was then extracted with ethyl acetate and the organic layer dried and concentrated. The residue was purified by silica gel chromatography to give the compound 3-((2*S*,4*S*)-4-azido-1-*tert*-butoxycarbonyl-2-pyrrolidinylcarbonyl)-1,3-thiazolidine (39.5 g, 65%) as a white solid. (3) The compound thus prepared (26.7 g, 81.6 mmol) was dissolved in ethanol (270 mL) and the mixture stirred under a hydrogen atmosphere (1 atm) for 18 h in the presence of 10% palladium carbon catalyst (13.4 g). The reaction mixture was filtered and the filtrate concentrated under reduced pressure to give the title compound (24.5 g, quant) as a black solid. ^{1}H NMR ($CDCl_3$) δ 1.40–1.45 (9H, m), 1.70–1.83 (1H, m), 2.07 (2H, br s), 2.32–2.56 (1H, m), 2.90–3.19 (2H, m), 3.25–3.58 (2H, m), 3.60–4.14 (3H, m), 4.31–4.80 (3H, m).

5.1.3. Representative example of preparation of 11, 12b–e, 12k–m: general method A

5.1.3.1. 3-[(2*S*,4*S*)-4-(5-Cyano-2-pyridyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine dihydrochloride (11). (1) To a solution of compound **10** (904 mg, 3 mmol) in *N*-methyl-2-pyrrolidone (9 mL) were added 2-chloro-5-cyanopyridine (416 mg, 3 mmol) and diisopropylethylamine (9 mmol) and the mixture stirred at 80 °C for 18 h. The reaction mixture was added to saturated aqueous sodium hydrogen carbonate solution and the mixture extracted with ethyl acetate. The extract was dried and the solvent evaporated under reduced pressure. The residue was purified by silica gel chromatography to give 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(5-cyano-2-pyridyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (807 mg, 67%) as a white amorphous substance. (2) The compound thus prepared (711 mg) was dissolved in ethyl acetate (1.76 mL) and 4 mol/L hydrochloric acid–ethyl acetate (2.20 mL) added to the solution. The mixture was stood at room temperature for 18 h and the precipitated solid collected by filtration to give the title compound (709 mg) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.74–1.94 (1H, m), 2.78–2.94 (1H, m), 2.97–3.26 (3H, m), 3.40–3.77 (3H, m), 4.40–4.80 (4H, m), 6.64 (1H, d, J = 9.0 Hz), 7.77 (1H, dd, J = 8.7, 2.4 Hz), 8.08 (1H, br s), 8.46 (1H, d, J = 1.8 Hz), 8.86 (1H, br s), 10.37 (1H, br s). Anal. Calcd

for $C_{14}H_{17}N_5OS{\cdot}2HCl{\cdot}C_4H_8O_2{\cdot}1/4C_4H_9Cl{\cdot}3/2H_2O$: C, 44.35; H, 6.32; N, 13.61. Found: C, 44.19; H, 6.36; N, 13.66. HRMS (CI) calcd. for $C_{14}H_{18}N_5OS$ ($M+H^+$) *m/e* 304.1232, found *m/e* 304.1231.

5.1.3.2. 3-[(2*S*,4*S*)-4-(2-Cyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine hydrochloride (12b). (1) From compound **10** (1810 mg, 6 mmol) with addition of diisopropylethylamine (3.14 mL, 18 mmol) and 2-fluorobenzonitrile (727 mg, 6 mmol), and using a procedure analogous to general method A (1) (*N*-methyl-2-pyrrolidone, 80 °C, 32 h), the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(2-cyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (88 mg, 3.6%) was prepared. (2) The compound thus prepared (88 mg) was deprotected using a procedure analogous to general method A (2) to give the title compound (25 mg, 34%). ^{1}H NMR (DMSO-d_6) δ 1.74–1.95 (1H, m), 2.85–3.16 (3H, m), 3.30–3.92 (4H, m), 4.27–4.79 (4H, m), 6.15–6.27 (1H, m), 6.77 (1H, t, J = 7.5Hz), 6.87 (1H, d, J = 8.4 Hz), 7.38–7.59 (2H, m), 8.90 (1H, br s), 10.80 (1H, br s). HRMS (EI) calcd. for $C_{15}H_{18}N_4OS$ (M^+) *m/e* 302.1201, found *m/e* 302.1196.

5.1.3.3. 3-[(2*S*,4*S*)-4-(4-Cyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine hydrochloride (12c). (1) From compound **10** (1810 mg, 6 mmol) with addition of diisopropylethylamine (3.14 mL, 18 mmol) and 4-fluorobenzonitrile (727 mg, 6 mmol), and using a procedure analogous to general method A (1) (*N*-methyl-2-pyrrolidone, 100 °C, 24 h), the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(4-cyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (170 mg, 7.0%) was prepared. (2) The compound thus prepared (170 mg) was deprotected using a procedure analogous to general method A (2) to give the title compound (69.3 mg, 49%) as a pale-brown powder. ^{1}H NMR (DMSO-d_6) δ 1.64–1.80 (1H, m), 2.84–3.20 (4H, m), 3.45–3.96 (3H, m), 4.15–4.34 (1H, m), 4.39–4.78 (3H, m), 6.70 (2H, d, J = 8.8 Hz), 6.85–7.01 (1H, m), 7.52 (2H, d, J = 8.7 Hz), 9.45 (2H, br s). Anal. Calcd for $C_{15}H_{18}N_4OS{\cdot}HCl{\cdot}3/10C_4H_8O_2{\cdot}5/4H_2O$: C, 50.17; H, 6.21; N, 14.45. Found: C, 50.14; H, 6.21; N, 14.14.

5.1.3.4. 3-[(2*S*,4*S*)-4-(4-Nitrophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine hydrochloride (12d). (1) From compound **10** (904 mg, 3 mmol) with addition of diisopropylethylamine (1.57 mL, 9 mmol) and 4-fluoronitrobenzene (423 mg, 3 mmol), and using a procedure analogous to general method A (1) (*N*-methyl-2-pyrrolidone, 80 °C, 24 h), the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(4-nitrophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (919 mg, 73%) was prepared as a yellow amorphous substance. (2) The compound thus prepared (795 mg) was deprotected using a procedure analogous to general method A (2) to give the title compound (647 mg, 96%) as a yellow powder. ^{1}H NMR (DMSO-d_6) δ 1.68–1.87 (1H, m), 2.88–3.30 (4H, m), 3.48–3.98 (3H, m), 4.24–4.80 (4H, m), 6.72 (2H, d, J = 9.3 Hz), 7.40–7.56 (1H, m), 8.04 (2H, d, J = 7.5 Hz), 9.51 (2H, br s). Anal. Calcd for $C_{14}H_{18}N_4O_3S{\cdot}HCl{\cdot}2/5C_4H_8O2{\cdot}3/5H_2O$: C, 46.28; H, 5.83; N, 13.84. Found: C, 46.19; H, 5.80; N, 13.56.

5.1.3.5. 3-[(2*S*,4*S*)-4-(4-Methanesulfonylphenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine hydrochloride (12e). (1) From compound **10** (904 mg, 3 mmol) with addition of diisopropylethylamine (1.57 mL, 9 mmol) and 4-fluorophenyl methyl sulfone (523 mg, 3 mmol), and using a procedure analogous to general method A (1) (*N*-methyl-2-pyrrolidone, 100 °C, 18 h), the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(4-methanesulfonylphenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (27 mg, 2.0%) was prepared. (2) The compound thus prepared (27 mg) was deprotected using a procedure analogous to general method A (2) to give the title compound (19.4 mg, 84%). ^{1}H NMR (DMSO-d_6) δ 1.65–1.82 (1H, m), 2.89–3.23 (7H, m), 3.49–3.98 (3H, m), 4.18–4.78 (4H, m), 6.74 (2H, d, J = 9.0 Hz), 6.80–6.92 (1H, m), 6.63 (2H, d, J = 9.0 Hz), 9.30 (2H, br s). HRMS (EI) calcd. for $C_{15}H_{21}N_3O_3S_2$ (M^+) *m/e* 355.1024, found *m/e* 355.1025.

5.1.3.6. 3-[(2*S*,4*S*)-4-(2-Chloro-4-cyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine hydrochloride (12k). (1) From compound **10** (904 mg, 3 mmol) with addition of diisopropylethylamine (1.57 mL, 9 mmol) and 3-chloro-4-fluorobenzonitrile (467 mg, 3 mmol), and using a procedure analogous to general method A (1) (*N*-methyl-2-pyrrolidone, 80 °C, 8 h), the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(2-chloro-4-cyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (460 mg, 35%) was prepared as a white solid. (2) The compound thus prepared (395 mg) was deprotected using a procedure analogous to general method A (2) to give the title compound (177 mg, 53%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.80–1.99 (1H, m), 2.82–3.17 (3H, m), 3.25–3.94 (4H, m), 4.36–4.54 (2H, m), 4.54–4.80 (2H, m), 6.42 (1H, d, J = 7.8 Hz), 6.93 (1H, d, J = 8.7 Hz), 7.64 (1H, dd, J = 8.4, 1.8 Hz), 7.82 (d, 1H, J = 1.8 Hz). Anal. Calcd for $C_{15}H_{17}ClN_4OS{\cdot}HCl{\cdot}1/5C_4H_8O_2{\cdot}3/5H_2O$: C, 47.24; H, 5.22; N, 13.95. Found: C, 47.32; H, 5.07; N, 13.89.

5.1.3.7. 3-[(2*S*,4*S*)-4-(3-Chloro-4-cyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine hydrochloride (12l). (1) From compound **10** (0.904 g, 3 mmol) with addition of diisopropylethylamine (1.57 mL, 9 mmol) and 2-chloro-4-fluorobenzonitrile (0.467 g, 3 mmol), and using a procedure analogous to general method A (1) (*N*-methyl-2-pyrrolidone, 80 °C, 8 h), the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(3-chloro-4-cyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (0.630 g, 48%) was prepared as a colorless transparent oil. (2) The compound thus prepared (0.630 g) was deprotected using a procedure analogous to general method A (2) to give the title compound (0.465 g, 87%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.65–1.81 (1H, m), 2.84–2.99 (1H, m), 2.99–3.22 (3H, m), 3.48–3.95 (3H, m), 4.16–4.37 (1H, m), 4.39–4.78 (3H, m), 6.68 (1H, dd, J = 8.7, 2.1 Hz), 6.85 (1H, d, J = 1.8 Hz), 7.30–7.45 (1H, m), 7.60 (1H, d, J = 8.7 Hz), 9.60 (2H, br s). Anal. Calcd for $C_{15}H_{17}ClN_4OS{\cdot}HCl{\cdot}2/5C_4H_8O_2{\cdot}3/5H_2O$: C, 47.54; H, 5.38; N, 13.36. Found: C, 47.77; H, 5.27; N, 12.96.

5.1.3.8. 3-[(2*S*,4*S*)-4-(3,4-Dicyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine hydrochloride (12m). (1) From compound **10** (0.904 g, 3 mmol) with addition of diisopropylethylamine (1.57 mL, 9 mmol) and 4-flu-

orophthalonitrile (0.438 g, 3 mmol), and using a procedure analogous to general method A (1) (*N*-methyl-2-pyrrolidone, 80 °C, 4 h), the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(3,4-dicyanophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (1.08 g, 84%) was prepared as a white amorphous substance. (2) The compound thus prepared (0.924 g) was deprotected using a procedure analogous to general method A (2) to give the title compound (0.782 g, 99%) as a yellow powder. ^{1}H NMR (DMSO-d_6) δ 1.66–1.84 (1H, m), 2.90–3.27 (4H, m), 3.49–3.95 (3H, m), 4.20–4.40 (1H, m), 4.40–5.79 (3H, m), 7.00 (1H, dd, J = 8.7, 2.4 Hz), 7.22 (1H, s), 7.52–7.67 (1H, m), 7.76 (1H, d, J = 9.0 Hz). Anal. Calcd for $C_{16}H_{17}N_5OS{\cdot}HCl{\cdot}3/5C_4H_8O_2{\cdot}1/10C_4H_9Cl{\cdot}H_2O$: C, 50.86; H, 5.83; N, 15.77. Found: C, 51.19; H, 5.94; N, 15.57. HRMS (EI) calcd for $C_{16}H_{17}N_5OS$ (M^+) *m/e* 327.1154, found *m/e* 327.1151.

5.1.4. 3-[(2*S*,4*S*)-4-(4-Aminophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine hydrochloride (12h). To compound **12d** (200 mg, 557 mmol) dissolved in ethanol (10 mL) were added 4 mol/L hydrochloric acid-1,4-dioxane (0.28 mL) and 10% palladium/carbon (100 mg) and the mixture stirred under a hydrogen atmosphere (1 atm) at room temperature for 18 h. The reaction mixture was filtered, the filtrate concentrated under reduced pressure, and the obtained solid washed with ethanol to give the title compound (13 mg, 6.9%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.58–1.80 (1H, m), 2.83–3.00 (1H, m), 3.00–3.20 (3H, m), 3.60–3.90 (3H, m), 4.08–4.25 (1H, m), 4.39–4.79 (3H, m), 6.67 (2H, d, J = 8.7 Hz), 7.15 (2H, d, J = 8.7 Hz), 8.81 (1H, br s), 10.00 (3H, br s), 10.25 (1H, br s). HRMS (EI) calcd. for $C_{14}H_{20}N_4OS$ (M^+) *m/e* 292.1358, found *m/e* 292.1353.

5.1.5. 3-((*S*)-1-*tert*-Butoxycarbonyl-4-oxo-2-pyrrolidinylcarbonyl)-1,3-thiazolidine (13). To a solution of compound **9** (55.4 g, 183 mmol) and triethylamine (46 mL) in dichloromethane (350 mL) was added sulfur trioxide pyridine complex (52.4 g, 329 mmol) in DMSO (150 mL) under ice cooling and the mixture stirred for 2 h. Saturated aqueous sodium hydrogen carbonate solution was added to the reaction mixture and the mixture extracted with ethyl acetate. The extract was washed with brine and dried. The solvent was evaporated under reduced pressure and the residue purified by silica gel chromatography to give the title compound (30.3 g, 55%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 1.47 (9H, s), 2.45–2.57 (1H, m), 2.70–2.93 (1H, m), 2.97–3.22 (2H, m), 3.66–3.78 (0.6H, m), 3.80–4.10 (3H, m), 4.28–4.38 (0.4H, m), 4.45–5.08 (3H, m).

5.1.6. 3-((2*S*,4*S*)-4-Anilino-2-pyrrolidinylcarbonyl)-1,3-thiazolidine dihydrochloride (12a). Compound **13** (501 mg, 1.67 mmol), aniline (0.20 mL, 2.19 mmol), and acetic acid (0.10 mL, 1.75 mmol) were dissolved in methanol (10 mL) and stirred at room temperature for 1.5 h before addition of sodium cyanoborohydride (95%, 145 mg, 2.19 mmol) to the reaction mixture, stirring for a further 2 h, and evaporation under reduced pressure. Saturated aqueous sodium hydrogen carbonate solution was added to the residue, the mixture extracted with ethyl acetate, and the extract washed with brine and dried. The solvent was evaporated under reduced pressure and the residue purified by silica gel chromatography and crystallized from diethyl ether to give 3-((2*S*,4*S*)-4-anilino-1-*tert*-butoxycarbonyl-2-pyrrolidinylcarbonyl)-1,3-thiazolidine (156 mg, 25%) as white crystals. (2) The compound thus prepared (142 mg) was dissolved in ethyl acetate (2 mL), 4 mol/L hydrochloric acid–ethyl acetate (0.5 mL) added, and the mixture stirred at room temperature for 12 h. The precipitated solid was collected by filtration to give the title compound (89 mg, 68%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.64–1.78 (1H, m), 2.84–2.97 (1H, m), 3.00–3.19 (3H, m), 3.43–3.55 (1H, m), 3.60–4.20 (5H, m), 4.41–4.76 (3H, m), 6.56–6.67 (3H, m), 7.13 (2H, t, J = 7.2 Hz), 8.79 (1H, br s), 10.29 (1H, br s). Anal. Calcd for $C_{14}H_{19}N_3OS{\cdot}2HCl$: C, 48.00; H, 6.04; N, 12.00. Found: C, 48.35; H, 6.34; N, 11.83.

5.1.7. Representative example of preparation of 12f, g, i, j: general method B

5.1.7.1. 3-[(2*S*,4*S*)-4-(4-Chlorophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine dihydrochloride (12f). (1) To a solution of compound **13** (450 mg, 1.50 mmol), *p*-chloroaniline (230 mg, 1.80 mmol) and acetic acid (0.09 mL, 1.57 mmol) in 1,2-dichloroethane (8 mL) was added sodium triacetoxyborohydride (636 mg, 3.00 mmol) and the mixture stirred at room temperature for 3 h before addition of saturated aqueous sodium hydrogen carbonate solution to the reaction mixture, which was then extracted with ethyl acetate. The extract was washed with brine and dried. The solvent was evaporated under reduced pressure and the residue purified by silica gel chromatography to give the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(4-chlorophenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (415 mg, 67%) as an oil. (2) The compound thus produced (412 mg) was dissolved in ethyl acetate (5 mL), 4 mol/L hydrochloric acid–ethyl acetate (1.25 mL) added, and the mixture stirred at room temperature for 13 h. The precipitated solid was collected by filtration to give the title compound (297 mg, 80%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.63–1.78 (1H, m), 2.84–2.97 (1H, m), 3.00–3.17 (3H, m), 3.5–3.92 (3H, m), 4.07–4.18 (1H, m), 4.40–4.73 (3H, m), 6.62 (2H, d, J = 8.8 Hz), 7.15 (2H, d, J = 8.8 Hz), 8.86 (1H, br s), 10.23 (1H, br s). Anal. Calcd for $C_{14}H_{18}N_3OS{\cdot}8/5HCl$: C, 45.43; H, 5.34; N, 11.35. Found: C, 45.24; H, 5.58; N, 11.10.

5.1.7.2. 3-[(2*S*,4*S*)-4-(*p*-Anisidino)-2-pyrrolidinylcarbonyl]-1,3-thiazolidine dihydrochloride (12g). (1) From compound **13** (450 mg, 1.50 mmol) with addition of *p*-anisidine (222 mg, 1.80 mmol), and using a procedure analogous to general method B (1), the compound 3-[(2*S*,4*S*)-4-(*p*-anisidino)-1-*tert*-butoxycarbonyl-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (515 mg, 84%) was prepared as a white solid. (2) The compound thus prepared (448 mg) was deprotected using a procedure analogous to general method B (2) to give the title compound (223 mg, 55%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.77–1.90 (1H, m), 2.77–2.89 (1H, m), 3.00–3.14 (3H, m), 3.20–4.20 (1H, m), 3.60–4.20 (6H, m), 4.40–4.72 (3H, m), 6.87 (4H, s), 8.84 (1H, br s),

10.33 (1H, br s). Anal. Calcd for $C_{15}H_{21}N_3O_2S{\cdot}2HCl{\cdot}H_2O$: C, 46.27; H, 6.21; N, 10.79. Found: C, 45.93; H, 6.43; N, 10.49.

5.1.7.3. 3-[(2*S*,4*S*)-4-(3,4-Methylenedioxyphenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine dihydrochloride (12i). (1) From compound **13** (450 mg, 1.50 mmol) with addition of 3,4-methylenedioxyaniline (249 mg, 1.80 mmol), and using a procedure analogous to general method B (1), the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(3,4-methylenedioxyphenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (553 mg, 87%) was prepared as a pale-brown-reddish solid. (2) The compound thus prepared (549 mg) was deprotected using a procedure analogous to general method B (2) to give the title compound (457 mg, 89%) as a pale-brown-reddish powder. ^{1}H NMR (DMSO-d_6) δ 1.72–1.85 (1H, m), 2.820–2.93 (1H, m), 3.00–3.28 (3H, m), 3.45–3.57 (1H, m), 3.60–3.95 (2H, m), 4.08–4.20 (1H, m), 4.42–4.75 (3H, m), 5.92 (2H, s), 6.25–6.32 (1H, m), 6.53 (1H, s), 6.76–6.83 (1H, m), 8.89 (1H, br s) 10.36 (1H, br s). Anal. Calcd for $C_{15}H_{19}N_3O_3S{\cdot}2HCl{\cdot}1/5H_2O$: C, 45.28; H, 5.42; N, 10.56. Found: C, 45.18; H, 5.34; N, 10.28.

5.1.7.4. 3-[(2*S*,4*S*)-4-(3-Chloro-4-methoxyphenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine dihydrochloride (12j). (1) From compound **13** (450 mg, 1.50 mmol) with addition of 3-chloro-4-methoxyaniline (284 mg, 1.80 mmol), and using a procedure analogous to general method B (1), the compound 3-[(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-(3-chloro-4-methoxyphenyl)amino-2-pyrrolidinylcarbonyl]-1,3-thiazolidine (569 mg, 86%) was prepared as an oil. (2) The compound thus produced (561 mg) was deprotected using a procedure analogous to general method B (2) to give the title compound (429 mg, 81%) as a pale-brown powder. ^{1}H NMR (DMSO-d_6) δ 1.62–1.76 (1H, m), 2.82–2.95 (1H, m), 3.00–3.18 (3H, m), 3.5–3.92 (6H, m), 4.07–4.18 (1H, m), 4.40–4.73 (3H, m), 6.61 (1H, dd, J = 8.8, 2.7 Hz), 6.75 (1H, d, J = 2.7 Hz), 6.98 (1H, d, J = 8.8 Hz), 8.80 (1H, br s), 10.15 (1H, br s). Anal. Calcd for $C_{15}H_{20}ClN_3O_2S{\cdot}HCl$: C, 43.44; H, 5.35; N, 10.13. Found: C, 43.07; H, 5.41; N, 9.91.

5.1.8. Solution stability of 3 and 11 in a neutral buffer at 37 °C. Compounds **3** and **11** were dissolved in a cool buffer of pH 6.8 (second fluid of Disintegration Test in Japanese Pharmacopoeia) to produce test solutions of 0.05 mg/mL. A 2 mL sample of each test solution was incubated at 37 °C with shaking at 100 times/min for 1, 2, and 4 h (for compound **3**), or for 2, 4, and 6 h (for compound **11**). After incubation, 0.1 mL of 1 mol/L hydrochloric acid and 8 mL of cool mobile phase were added to each test solution and 20 μL was injected into a HPLC instrument. The drug concentrations were measured by HPLC analysis. Chromatographic separation was performed using a Develosil ODS-HG-5 (150 mm × 4.6 mm id, 5 μm, Nomura Chemical Co., Japan), with a mixture of 0.05 mol/L sodium perchlorate (pH 2.5, adjusted with 70% perchloric acid) and acetonitrile (85/15 for **3**, 80/20 for **11**) as the mobile phase. The flow rate was 1 mL/min. The UV detector was set at 270 nm (for **3**) or 200 nm (for **11**).

5.2. Biological methods

5.2.1. DPP-IV-inhibitory activity. The DPP-IV-inhibitory activity of human plasma and rat plasma was measured by fluorescence assay using Gly-Pro-MCA (Peptide Institute Inc.) as a DPP-IV-specific fluorescent substrate. Reaction solutions containing 20 μL of human or rat plasma (10-fold diluted solution), 20 μL of fluorescent substrate (100 μmol/L), 140 μL of buffer (0.003% Brij-35 containing PBS), and 20 μL of test substrate (of various concentrations) were incubated at room temperature for 60 min using a 96-well flat-bottomed microtiter plate. The measured fluorescent intensity (excitation 360 nm/emission 465 nm, SPECTRA FLUOR, TECAN) was taken as the DPP-IV activity. The inhibitory rate relative to the solvent addition group was calculated and IC_{50} values determined by logistic analysis.

5.2.2. Plasma DPP-IV activity after oral administration of 11 to wistar rats. Male Wistar rats (7–9 weeks of age) fasted overnight were used. Compound **11** was dissolved in 0.5% hydroxypropylmethyl-cellulose and administered orally at a dose of 10, 30, and 100 μmol/kg. At pre-administration and at 0.5, 1, 2, 3, 5, 7, 9, and 24 h after administration, 0.1 mL of blood was collected from the jugular vein. After centrifugation, 10 μL of plasma was diluted 10-fold using buffer (0.003% Brij-35 containing PBS). Twenty microliters of the diluted plasma was used instead of 20 μL of test substrate for the determination of DPP-IV-inhibitory activity by fluorescence as described above.

5.2.3. Pharmacokinetic profile of 11 after intravenous or oral administration to rats. Female Sprague–Dawley rats (7 weeks of age) were used. A solution of compound **11** in 0.9% sodium chloride (for iv) or 0.5% hydroxypropylmethylcellulose (for po) was administered at a dose of 10 mg/kg. Rats were fasted overnight before dosing and for 6 h after dosing. At 0.05 (for iv), 0.25 (for po), 0.5, 1, 2, 4, 6, 8, and 24 h after administration, blood was collected from the jugular vein. The blood sample was centrifuged at 10,000 rpm for 5 min and the separated plasma stored at −20 °C until analysis. For determination of unchanged compound **11**, 0.05 mL of water and 0.1 mL of 0.1 mol/L sodium hydroxide were added to 0.1 mL of plasma sample. The mixture was applied to an OASYS HLB cartridge (30MG/1CC) [which was preconditioned with 1 mL of methanol followed by 1 mL of water]. After rinsing the cartridge with 1 mL of water, the targeted compound **11** was eluted from the cartridge using 1 mL of methanol. The eluate was evaporated by centrifugal concentration at 40 °C, the residue reconstituted in 0.2 mL of mobile phase, and 5 μL injected into an LC–MS/MS system. Chromatographic separation was performed using a Develosil ODS-HG-3 (50 mm × 2 mm id, 3 μm, Nomura Chemical Co., Japan), with a mixture of 0.05% trifluoroacetic acid and acetonitrile (85/15) as the mobile phase. The flow rate was 0.25 mL/min. Protonated analyte ions were generated using electron spray ionization. For quantitation, the ion signal was recorded by selective reaction monitoring. Pharmacokinetic parameters were calculated by a non-compartmental analysis.

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