Bioorganic & Medicinal Chemistry 20 (2012) 5705–5719

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Discovery and preclinical profile of teneligliptin (3-[(2*S*,4*S*)-4-[4-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl]thiazolidine): A highly potent, selective, long-lasting and orally active dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes

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ARTICLE INFO

Article history:
Received 19 July 2012
Revised 9 August 2012
Accepted 9 August 2012
Available online 17 August 2012

Keywords:
DPP-4 inhibitor
Thiazolidine
Bicyclic heteroarylpiperazine

ABSTRACT

Dipeptidyl peptidase IV (DPP-4) inhibition is suitable mechanism for once daily oral dosing regimen because of its low risk of hypoglycemia. We explored linked bicyclic heteroarylpiperazines substituted at the γ-position of the proline structure in the course of the investigation of L-prolylthiazolidines. The efforts led to the discovery of a highly potent, selective, long-lasting and orally active DPP-4 inhibitor, 3-[(2*S*,4*S*)-4-[4-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl]thiazolidine (**8g**), which has a unique structure characterized by five consecutive rings. An X-ray co-crystal structure of **8g** in DPP-4 demonstrated that the key interaction between the phenyl ring on the pyrazole and the S_2 extensive subsite of DPP-4 not only boosted potency, but also increased selectivity. Compound **8g**, at 0.03 mg/kg or higher doses, significantly inhibited the increase of plasma glucose levels after an oral glucose load in Zucker fatty rats. Compound **8g** (teneligliptin) has been approved for the treatment of type 2 diabetes in Japan.

1. Introduction

Type 2 diabetes is a rapidly growing metabolic disease that affects millions of people worldwide,[1] and is characterized by elevated fasting plasma glucose, insulin resistance, abnormally elevated hepatic glucose production, and reduced glucose-stimulated insulin secretion. Moreover, long-term lack of glycemic control increases the risk of micro- and macrovascular complications, such as coronary artery disease, stroke, hypertension, nephropathy, peripheral vascular disease, neuropathy and retinopathy.[2] Current treatment strategies include reducing insulin resistance, supplementing the insulin deficiency with exogenous insulin, enhancing endogenous insulin secretion, reducing hepatic glucose output, and limiting glucose absorption.[3] Among them, insulin secretagogues such as glinides (e.g., repaglinide), which are widely used, need to be taken before each meal for avoiding a risk of hypoglycemia. Therefore antihyperglycemic agents which are orally available in a once-daily regimen without causing hypoglycemia have been much awaited in medical settings.

An incretin hormone, glucagon-like peptide-1 (GLP-1) stimulates insulin biosynthesis and secretion in response to meal ingestion, inhibits glucagon secretion, and promotes proliferation of pancreatic β cells.[4] In contrast to other insulinotropic agents, for

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0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.08.012

example, sulfonylureas, the insulinotropic effect of GLP-1 depends even more closely on the actual plasma glucose concentration providing the possibility of glucose normalization with minimizing the risk of hypoglycemia. Continuous infusion of GLP-1 for 6 weeks to patients with type 2 diabetes increased insulin secretion and normalized both fasting and post-prandial blood glucose levels without serious side-effects.[5] On the basis of the above evidence, GLP-1 itself is one of the logical candidates for therapeutic agent in the treatment of diabetes.[6] However, active GLP-1 (GLP-1[7–36]amide) is rapidly degraded by dipeptidyl peptidase IV (DPP-4),[7] which is a serine protease to produce a dipeptide and an inactive GLP-1.[8] DPP-4 inhibition increases the plasma concentration of active GLP-1 and causes the secretion of insulin in response to increased blood glucose level.[9] Therefore, DPP-4 inhibitors are expected to be glucose-lowering, safer and once daily agents.[10]

Several DPP-4 inhibitors have already been approved, including sitagliptin (**1**), vildagliptin (**2**), alogliptin (**3**), saxagliptin (**4**) and linagliptin (**5**) (Fig. 1).[11] Several other candidates have been reported to be in an advanced stage in clinical trials for type 2 diabetes.[10b]

DPP-4 is a dipeptidase that recognizes an amino acid sequence having proline or alanine at the N-terminal penultimate (P_1) position,[12] and many DPP-4 inhibitors therefore have substituted pyrrolidines or thiazolidines as a proline mimetic in the P_1 part.[13] In particular, pioneering inhibitors possess an electrophilic trap such as a nitrile group to form a covalent bond with the Ser630 of the catalytic triad in the active site. DPP-4 inhibitors possessing the electrophilic trap have two main problems: (i) general chemical instability, and (ii) low selectivity against other related prolyl peptidases, DPP-8 and DPP-9.[14] Their chemical instability is due to intramolecular cyclization between the electrophilic nitrile and the amine of the P_2 part,[15] and the resulting lack of durability makes it difficult to control plasma glucose all day long for once-daily treatment. The low selectivity is probably due to covalent bond formation with the Ser630 which is a common amino acid conserved in the S_1 subsite of DPP-4, DPP-8 and DPP-9.[16] Since DPP-8 and DPP-9 inhibition are reported to be associated with multiorgan toxicities in rats and dogs and inhibition of T cell activation/proliferation,[17] DPP-4 selectivity is one of the key issues for clinical use.

We started our research on novel DPP-4 inhibitors without the electrophilic nitrile moiety to increase chemical stability and focused on the substituent at the γ-position of the proline moiety of the prolylthiazolidine core structure to increase affinity with the S_2 subsite.[18] We previously reported that substitution of arylpiperazine (**6**) or fused bicyclic heteroarylpiperazine (**7**) resulted in highly potent and long-lasting inhibitors.[19] The SAR study of fused bicyclic heteroarylpiperazine parts revealed that the nonlinear (L-shaped) structure such as a 4-quinolylpiperazine substituent was more suitable than the linear (I-shaped) one such as a 2-quinolylpiperazine substituent in DPP-4 inhibitory activity.[19b] Moreover, the X-ray crystal structure determination of compound **7a** in complex with human DPP-4 indicated that interactions generated between the quinolyl ring and the S_2 extensive subsite enhanced the DPP-4 inhibitory activity and selectivity.[19b]

Here we addressed the introduction of another nonlinear structure, a linked bicyclic heteroaryl group on the piperazine or piperidine moiety, instead of a fused bicyclic heteroaryl group (Fig. 2), and discovered highly efficacious long-lasting DPP-4 inhibitors that have led to the identification of compound **8g** (teneligliptin), which has been approved for the treatment of type 2 diabetes in Japan.

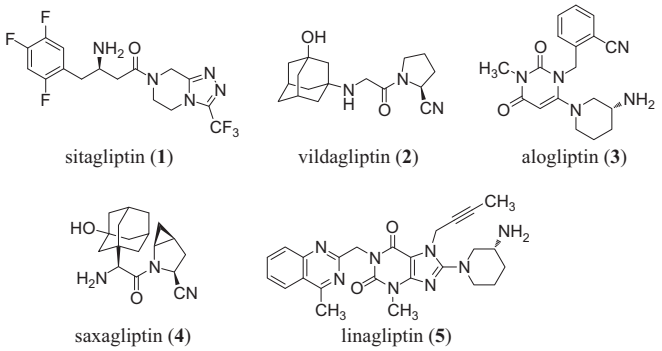


Figure 1. DPP-4 inhibitors.

2. Results and discussion

2.1. Rational design

To explore a new type of substituent that can interact with the S_2 extensive subsite more effectively, we focused on the introduction of a nonlinear, linked, bicyclic group on the piperazine or piperidine moiety (Fig. 2). A docking study was carried out to predict the difference between compounds having a six-membered ring and those having a five-membered ring as a linker connecting the terminal phenyl group and the piperazine or piperidine group. We selected *ortho*-substituted biphenyl and 1-phenylimidazol-2-yl groups (**8a**, **8b**) as a nonlinear linked bicycle. Figure 3 shows docking models of compounds **8a** and **8b** both of which are superimposed on the cocrystal structure of **7a** in the DPP-4 active site (PDB code: 3VJM).[19b] While the phenyl group on compound **8b** faced Arg358 in the S_2 extensive subsite, that of compound **8a** faced the opposite direction of Arg358 presumably due to steric repulsion. Thus, compound **8b** was expected to have similar potency to compound **7a** and we decided to synthesize 1-arylimizaol-2-yl, 1-arylterazol-5-yl, and 1-arylpyrazol-5-yl derivatives to optimize the substituent for the S_2 extensive subsite.

2.2. Synthesis of derivatives having a linked bicyclic group

4-Heterocycle-substituted piperazinyl intermediates **12** were synthesized following the routes illustrated in Scheme 1. Reaction of N-Cbz-protected piperazine **10a** and phenyl isothiocyanate followed by methylation afforded methylthioimidate **11a**, which was cyclized with aminoacetaldehyde dimethyl acetal by heating and then deprotected by hydrogenolysis to afford phenylimidazole **12a**. After treatment of the piperazine **10a** with cyclohexyl isocyanate, compound **11b** was cyclized into the corresponding tetrazole according to the literature procedure,[20] and then the Cbz group was removed to afford cyclohexyltetrazole **12b**. Pyrazole derivatives **12c–12n** were synthesized by dehydration of ß-ketoamides **11c**–**11e** with various arylhydrazines followed by cyclodehydration with phosphorus oxychloride in pyridine[21] and subsequent deprotection. The synthesis of ß-ketoamides **11c**-**11e** is as follows. Piperazine **10a** was condensed with 3,3-diethoxypropionic acid in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide/3-hydroxybenztriazole followed by treatment with 50% trifluoroacetic acid/water to afford formylacetamide **11c**, which was used in the next pyrazole synthesis step without purification. Treatment of *N*-Boc-protected piperazine **10b** with diketene gave acetoacetamide **11d**. Acetylation of piperazine **10a** followed by aldol reaction with trifluoroacetate yielded trifluoroacetoacetamide **11e**.

The procedure for the synthesis of piperidinyl intermediates **15** and **20** is summarized in Scheme 2. Coupling of *N*-Cbz-protected isonipecotic acid (**13**) with aniline followed by tetrazole-forming reaction using trimethylsilylazide under Mitsunobu conditions[22] and deprotection gave phenyltetrazole **15**. Aldol reaction of 4-acetylpyridine **16** with ethyl trifluoroacetate and subsequent

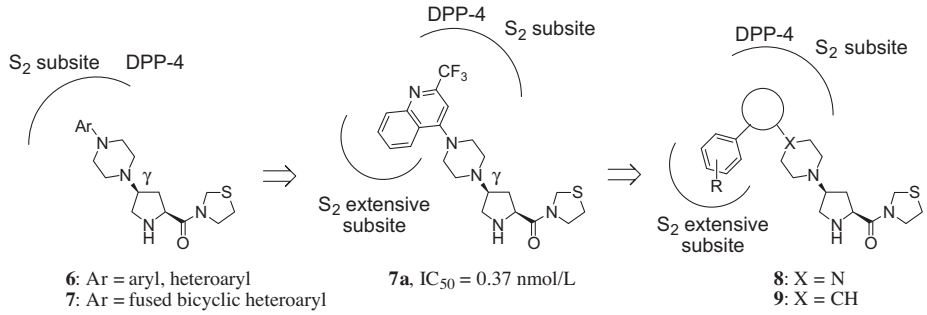


Figure 2. (S)-γ-substituted L-prolythiazolidines as DPP-4 inhibitors.

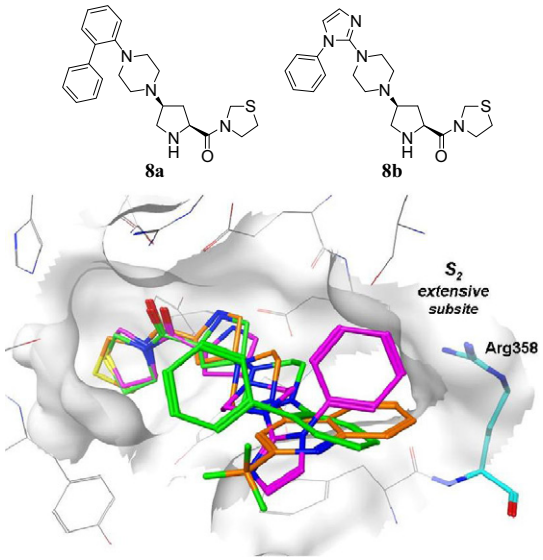


Figure 3. Docking models of linked bicyclic compounds **8a** and **8b** superimposed on the X-ray co-crystal structure of compound **7a** in the DPP-4 active site. Compound **7a** is shown as orange stick. Compounds **8a** and **8b** are shown as green and pink sticks, respectively.

cyclization with phenylhydrazine afforded pyridylpyrazole **18**. N-benzylation of **18** followed by reduction of pyridine core with sodium borohydride gave *N*-benzyl tetrahydropyridine **19**. Simultaneous deprotection and saturation of the compound **19** in the presence of a palladium catalyst and ammonium formate afforded phenylpyrazole **20**.

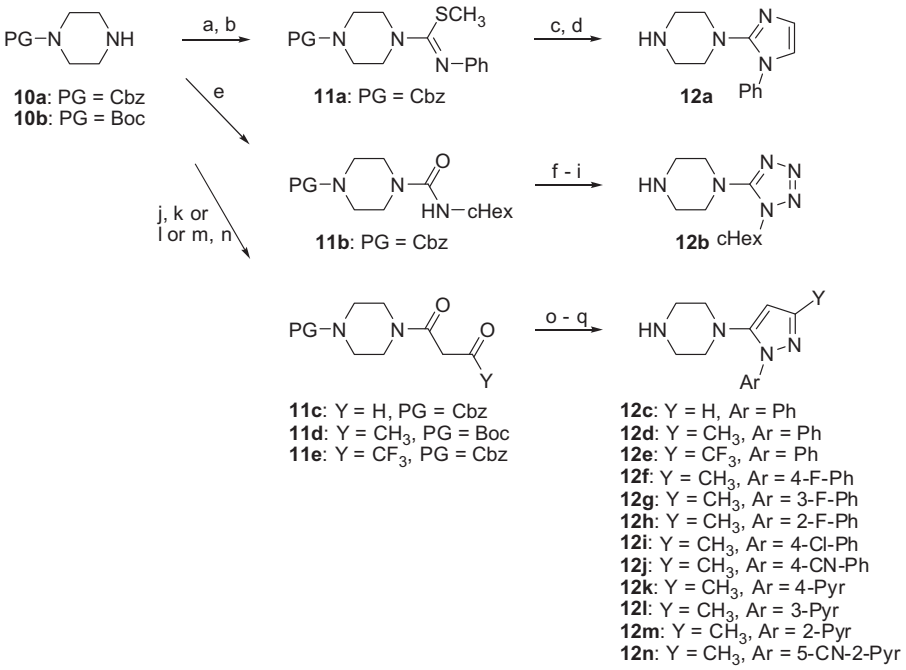
A series of piperazine- or piperidine-substituted prolylthiazolidines (**8**, **9**) was synthesized as previously reported (Scheme 3).[18a] The key intermediate **22**[18a] was prepared by coupling *N*-Boc-*trans*-4-hydroxy-L-proline (**21**) with thiazolidine followed by oxidation with dimethyl sulfoxide and sulfur trioxide.[23] Reductive amination of ketone **22** with commercially available amines or the above-described intermediates **12**, **15**, **20** afforded only the *cis*-isomers. Subsequent removal of the Boc group yielded analogs **8** and **9**. Diastereomer **8r** (2*S*,4*R*) and enantiomer **8s** (2*R*,4*R*) of compound **8g** (2*S*,4*S*) were prepared as follows. Silylation of the hydroxyl group on *N*-Boc-*cis*-4-hydroxy-L-proline (**23**) with *tert*-butyldimethylsilyl chloride followed by coupling with thiazolidine and subsequent removal of the protective group with tetrabutylammonium fluoride gave *N*-Boc-*cis*-4-hydroxy-L-prolylthiazolidide (**24**), as previously described.[18b] Mesylation of the intermediate **24** followed by S_N2 nucleophilic substitution with phenylpyrazole **12d** in *N*,*N*-dimethylformamide at high temperature afforded the diastereomer **8r**. The enantiomer **8s** was prepared by the same procedure as that for the synthesis of compound **8g** except that *N*-Boc-*trans*-4-hydroxy-D-proline was used instead of the L-proline **21**.

2.3. In vitro and in vivo DPP-4 inhibition

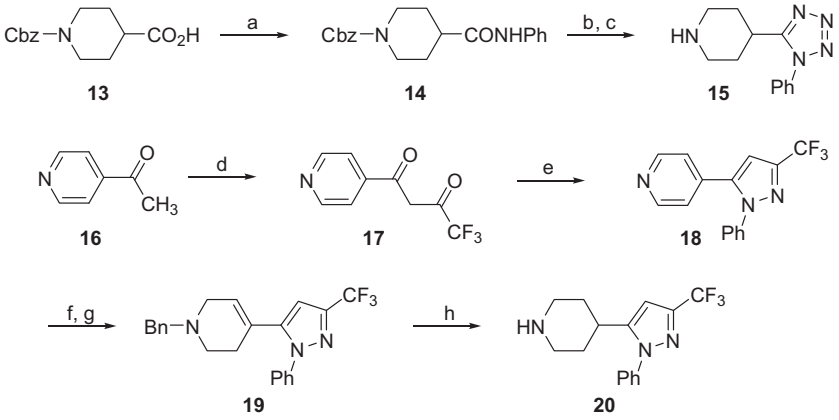
A series of the prolylthiazolidine analogs **6**, **7**, **8** and **9** was evaluated for DPP-4 inhibitory activity in human and rat plasma in vitro by fluorescence assay using Gly-Pro-MCA and the results are listed in Tables 1 and 2. Since evaluation of ex vivo plasma DPP-4 inhibition can be utilized to predict the efficacy of antihyperglycemic activity as a surrogate biomarker and provide information of pharmacokinetic–pharmacodynamic relationships, the plasma DPP-4 activity was measured at 0.5 h and 9 h after oral dosing (3 μmol/kg) of each compound in rats (Tables 1 and 2).

We previously reported that 2- and 4-pyridyl analogs **6a** and **6b** had a moderate DPP-4 inhibitory activity[19a] and that 4-quinolyl compound **7c** having a nonlinear structure in the piperazine-aryl part was more potent than 2-quinolyl compound **7b** having a linear one.[19b] The comparisons of the activity between compounds **6a**, **6b**, **7b** and **7c** suggested that the nonlinear structure on the piperazine such as compound **7c** was suitable for potent DPP-4 inhibition. X-ray crystal structure determination of compound **7a** indicated that CH–π interactions between the quinolyl ring and the guanidinyl group of Arg358 of the S_2 extensive subsite enhanced the DPP-4 inhibitory activity.[19b] To explore a linked bicyclic group on the piperazine or piperidine moiety instead of the fused bicyclic ones as another nonlinear structure, we attempted the introduction of 1-arylimizaol-2-yl, 1-arylterazol-5-yl, and 1-arylpyrazol-5-yl groups on the piperazine or piperidine moiety.

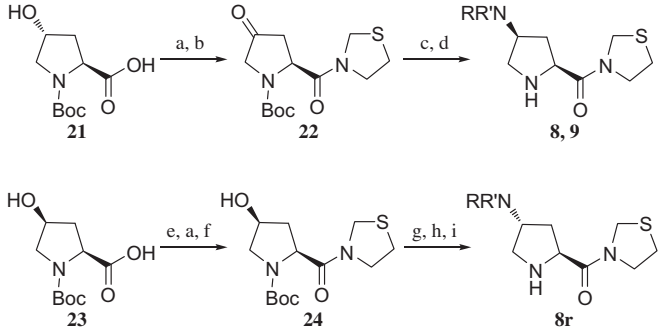
While introduction of a 4-phenylthiazol-2-yl group (**8c**), which has a linear structure, led to a decrease in DPP-4 inhibitory activity (4.5 nmol/L), introduction of a nonlinear structure such as a 1-phenylimidazol-2-yl or 1-phenyltetrazol-5-yl groups (**8b**, **8d**) led to an increase in activity. On the other hand, substitution of a cyclohexyl group for the phenyl ring led to a decrease in activity (**8e**). The results suggested that the phenyl group of compounds **8b** and **8d** forms CH–π interactions with the S_2 extensive subsite. Although pyrazole compound **8f** had less potent in vitro activity than imidazolyl or tetrazolyl compounds (**8b**, **8d**), these compounds showed similar ex vivo DPP-4 inhibitory activity. The results suggested that compound **8f** had better pharmacokinetic property than compounds **8b** and **8d**. Furthermore, introduction of a methyl group into the 3-position of the pyrazolyl ring led to a threefold increase in DPP-4 inhibitory activity, while maintaining duration of ex vivo activity (**8g**). 3-Trifluoromethylpyrazole analog **8h** also exhibited in vitro and ex vivo potency. On the other side, tetrazolyl and pyrazolyl piperidine analogs (**9a**, **9b**) remarkably decreased in vitro inhibitory activity compared with the corresponding piperazine analogs (**8d**, **8h**). These findings suggested that piperidine analogs would not have any interaction with the S_2 extensive subsite.



Scheme 1. Reagents and conditions: (a) Ph-NCS, acetone, rt; (b) CH_3I, CH_3OH, CH_2C1_2, rt; (c) $N_2NCH_2CH(OCH_3)_2$, pyridine, 100 °C; (d) 10%Pd/C, H_2, CH_3OH, r.t; (e) cHex-NCO, CH_2C1_2, rt; (f) $POCl_3$, THF, 70 °C; (g) triazole, CH_3CN, rt; (h) NaN_3, H_2O, CH_3OH, 70 °C; (i) 10%PdVC, H_2, EtOH, AcOEt; (j) $(EtO)_2CHCH_2CO_2H$, EDC, HOBt, DMF, rt; (k) 50%TFA/H_2O, $CHC1_3$, 0 °C to rt; (l) diketene, DMF, rt; (m) Ac_2O, pyridine, rt; (n) LHMDS, THF, −78 °C, then CF_3CO_2Et, −78 °C to rt; (o) $ArNHNH_2$, MsOH or MS3A, EtOH, rt; (p) $POCl_3$, pyridine, rt; (q) TFA, CH_2C1_2 rt, or 10%Pd/C, H_2, CH_3OH, rt.



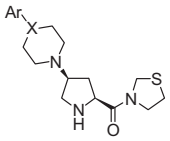
Scheme 2. Reagents and conditions: (a) $PhNH_2$, EDC, HOBt, THF, rt; (b) PPh_3, *i*-PrO_2CNNCO_2-*i*-Pr, $TMSN_3$, 0 °C to rt; (c) 10% Pd/C, H_2, CH_3OH, rt; (d) CF_3CO_2Et $NaOCH_3/CH_3OH$, MTBE, rt; (e) $PhNHNH_2$, EtOH, rt, (f) BnCl, CH_3CN, rt; (g) $NaBH_4$, EtOH, rt; (h) 10%Pd/C, HCO_2NH_4, MeOH, rt.

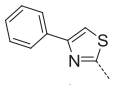
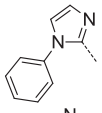
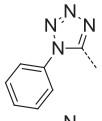
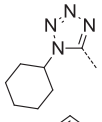
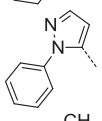
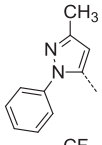
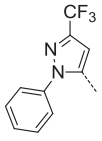


Scheme 3. Reagents and conditions: (a) Thiazolidine, HOBt, EDC, DMF, rt; (b) DMSO, SO_3–pyridine, Et_3N, CH_2C1_2, 0 °C; (c) RR′NH, $NaBH(OAc)_3$, AcOH, 1,2-dichloroethane, rt; (d) HCl, AcOEt, rt; (e) TBDMSCl, imidazole DMF, rt; (f) TBAF, THF, 0 °C; (g) MsCl, Et_3N, CH_2Cl_2, 0 °C; (h) **12d**, DMF, 120 °C; (i) TFA, CH_2C1_2, rt.

We subsequently turned our attention to substitution on the phenyl ring of compound **8g**, which had shown a favorable effect on in vitro and ex vivo DPP-4 inhibitory activity. The results are summarized in Table 2. *para*-Substitution of the phenyl ring decreased activity (**8i**, **8l**, **8m**), suggesting that the S_2 extensive subsite may be spatially rather restricted. Comparison of activity in terms of the substituted position on the phenyl ring indicated that 3-fluoro analog (**8j**) was superior to 2- or 4-fluoro one (**8k**, **8i**), and compound **8j** had similar potency and efficacy compared with non-substituted analog **8g**. While 4-pyridyl analog **8n** was also about 13-fold weaker than the phenyl analog **8g**, the 2- and 3-pyridyl analogs (**8p**, **8o**) relatively retained the activity. Substitution of a cyano group at the 5-position of the 2-pyridyl ring (**8q**), however, led to decreased activity in vitro. The plasma DPP-4 inhibitory activity of the 2-pyridyl analog **8p** in Wister rats was stronger than that of 3-pyridyl analog **8o**. Regarding the

Table 1
Inhibition of derivatives having a linked bicyclic group



Compd	Ar	X	In vitro DPP-4 inhiition IC_{50} (nmol/L)		Ex vivo DPP-4 inhibition at 3 μmol/kg po, Wistar rats[a] (%)	
			Human	Rat	0.5 h	9 h
6a		N	2.7	3.5	62.7 ± 2.7	19.7 ± 8.7
6b		N	3.1	3.9	N.T.	N.T.
7b		N	2.2	3.0	N.T.	N.T.
7c		N	0.95	1.0	29.7 ± 6.1	12.9 ± 4.5
8c		N	4.5	5.4	N.T.	N.T.
8b		N	0.26	0.19	59.3 ± 3.1	58.0 ± 2.2
8d		N	0.20	0.18	85.1 ± 1.5	70.7 ± 1.5
9a		CH	4.0	2.9	N.T.	N.T.
8e		N	4.3	3.1	25.6 ± 2.9	10.5 ± 3.3
8f		N	0.94	0.69	90.6 ± 1.3	50.8 ± 8.2
8g		N	0.37	0.29	94.8 ± 0.1	78.3 ± 1.6
8h		N	0.32	0.31	87.4 ± 3.2	81.8 ± 3.6
9b		CH	5.6	2.5	N.T.	N.T.

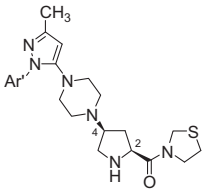
N.T.: not test.
[a] Data are expressed as means ± SEM ($n = 3$).

stereochemistry, a diastereomer **8r** (2*S*,4*R*) of **8g** showed decreased inhibitory activity, and enantiomer **8s** (2*R*,4*R*) resulted in a complete loss of activity.

2.4. Selectivity against DPP-8 and DPP-9

The enzymes most closely related to DPP-4 are fibroblast activation protein (FAP), DPP-II, DPP-8 and DPP-9.[14] Although the precise physiological functions of these enzymes have not been elucidated, DPP-8 and DPP-9 are widely distributed cytosolic enzymes, and their inhibition would induce the toxicity of DPP-4 inhibitors identified to date, including alopecia, thrombocytopenia, anemia, enlarged spleen, multiple histological pathologies, and animal mortality.[17] Thus selectivity of representative compounds against DPP-8 and DPP-9[17] was measured and the results are summarized in Table 3. The arylpyrazolyl analogs **8g**, **8j** and **8p** showed more potent DPP-4 inhibitory activity than phenyl analog **6c** and pyridinyl analog **6d**, and a marked decrease in inhibitory activities against DPP-8 and DPP-9. The results indicated that compounds **8g**, **8j** and **8p** showed at least 290-fold selectivity, while compounds **6c** and **6d** had at most 70-fold selectivity over DPP-8 and DPP-9. Especially, **8g** had about 700-and 1500-fold selectivity over DPP-8 and DPP-9, respectively. Therefore the interaction of the aryl group on the pyrazole with the S_2 extensive subsite in DPP-4 and steric crashes with the corresponding site in DPP-8 and DPP-9 would contribute to the high selectivity of **8g** as discussed in more detail in the next section. Compound **8g** showed high potency and off-target selectivity and was selected for further evaluation of X-ray crystal structure, pharmacokinetic profiles and in vivo pharmacology.

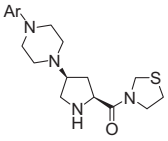
Table 2
Inhibition of 1-arylpyrazolyl derivatives

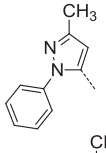
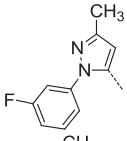
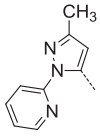


Compd	Ar′	In vitro DPP-4 inhibition IC_{50} (nmol/L)		Ex vivo DPP-4 inhibition at 3 μmol/kg po, Wistar rats[a] (%)	
		Human	Rat	0.5 h	9 h
8g	Ph	0.37	0.29	94.8 ± 0.1	78.3 ± 1.6
8i	4-FPh	2.9	2.3	N.T.	N.T.
8j	3-FPh	0.30	0.49	96.1 ± 0.1	75.1 ± 3.9
8k	2-FPh	0.85	0.53	75.9 ± 3.1	34.7 ± 2.5
8l	4-ClPh	3.4	2.9	N.T.	N.T.
8m	4-CNPh	12	13	N.T.	N.T.
8n	4-Pyr	5.0	4.7	N.T.	N.T.
8o	3-Pyr	0.62	0.77	66.9 ± 8.4	40.8 ± 4.6
8p	2-Pyr	0.63	0.72	83.0 ± 5.5	53.7 ± 3.1
8q	5-CN-2-Pyr	15	14	N.T.	N.T.
8r	Ph (2*S*,4*R*)	6.3	5.7	N.T.	N.T.
8s	Ph (2*R*,4*R*)	>1000	>1000	N.T.	N.T.

N.T.: not test.
[a] Data are expressed as means ± SEM ($n = 3$)

Table 3
Selectivity of DPP-4 inhibitors against DPP-8 and DPP-9



Compound	Ar	Inhibitory activity IC_{50} (nmol/L) on human enzymes		
		DPP-4	DPP-8	DPP-9
6c		1.6	12	30
6d		1.6	40	110
8g		0.37	260	540
8j		0.30	86	330
8p		0.63	220	610

2.5. X-ray crystal structure determination

The X-ray crystal structure shows that the characteristic five rings of **8g** fit into the active site of DPP-4 (Fig. 4). The thiazolidine moiety fully occupies the S_1 hydrophobic subsite. The secondary amino group of the proline moiety forms salt bridges to Glu205 and Glu206, and the carbonyl oxygen forms a hydrogen bond with Asn710. The pyrazolyl ring is stacked with the side chain of Phe357 and the piperazinyl ring forms CH–π interaction with Phe357. In addition, the phenyl substituent on the pyrazolyl ring is oriented favorably for hydrophobic interactions with the side chains of Ser209 and Arg358. In this S_2 extensive subsite, the 4-position

carbon of the phenyl ring of **8g** is so close to the carbonyl oxygen of the main chain of Val207 (distance$_{C\cdots O}$ = 3.3 Å) and a weak hydrogen bond could be formed between them. This subsite that accommodates the phenyl ring is quite tight, and the fact explains a loss in activity by replacement of the phenyl with a cyclohexyl (**8e**) or introduction of a *para*-substituent into the phenyl ring (**8i**, **8l**, **8m**, **8q**), in agreement with the SAR results (Tables 1 and 2). Moreover, the electric repulsion between the nitrogen atom of 4-pyridyl analog **8n** and the carbonyl oxygen of Val207 seems to decline the inhibitory activity. This interaction with the S_2 extensive subsite not only increased potency, but also improved selectivity against DPP-8 and DPP-9 as discussed above. It has been reported that neither DPP-8 nor DPP-9 has a residue corresponding to Ser209 of DPP-4 and that Asp435 of DPP-8 and Asp425 of DPP-9 correspond to Arg358 of DPP-4.[16] Homology models show that DPP-8 and DPP-9 have unique P2-loop structures quite different from that of DPP-4, which comprises 16 amino acids (Ser349-Phe362 in DPP-4).[16] Moreover, Arg358 and Ser209 have been suggested to play a crucial role for selectivity on the basis of each docking model of sitagliptin (**1**) with DPP-8 and DPP-9.[16] The phenyl group of compound **8g**, as in the case of the trifluoromethyl group on sitagliptin, would not interact with Asp435 (DPP-8) or Asp425 (DPP-9) by induced fit because of their low flexibility to form hydrogen bonds with Arg508 and Arg498 in DPP-8 and DPP-9, respectively.[16] Therefore compound **8g** showed weak activities in DPP-8 and DPP-9. Besides, steric clashes between the phenyl group of **8g** and the P2-loops of DPP-8 and DPP-9 caused a drastic reduction of the activity against them. These findings accentuate that the S_2 extensive subsite is unique to DPP-4.

Figure 5 shows the X-ray crystal structure of piperidinyl analog **9b** in complex with human DPP-4. Compound **9b** has critical interactions with the S_1 hydrophobic subsite and hydrogen bonding interactions with the secondary amino group in the proline moiety; however, the orientation of the 1-phenylpyrazol-2-yl moiety is completely different from that of the piperazinyl analog **8g** (inclined at about 45 degrees). This difference may be caused by the generally planar conformation to conjugate a lone pair of the nitrogen atom at the piperazinyl ring with π-electron of pyrazole in the compound **8g**. The phenyl group of **8g** pushes the side chain of Arg358 away, and then the S_2 extensive subsite becomes available. As a result, the piperidinyl analogs (**9a**, **9b**) earn no hydrophobic interactions with the S_2 extensive subsite, and exhibit almost 20-fold reduction in activity as compared to the corresponding piperazine analogs **8d** and **8h**. These results suggested that the interaction with the S_2 extensive subsite played an important role for DPP-4 inhibition.

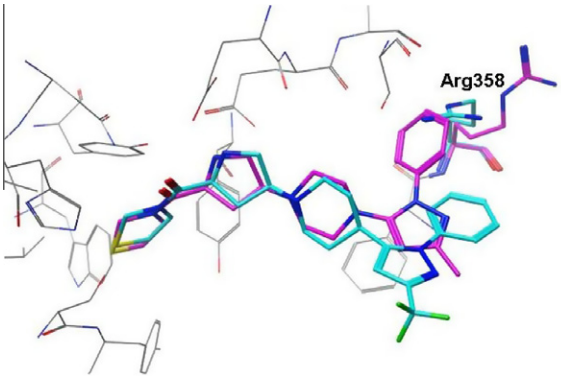


Figure 5. X-ray structure of compound **9b** (cyan carbon atoms) bound to DPP-4 (Arg 358 and key residues are shown as cyan and gray carbon atoms, respectively. Superimposed is the bound conformation of compound **8g** and Arg358 of DPP-4 (purple carbon atoms).

2.6. Pharmacokinetic profile of compound 8g

The plasma concentration profiles and pharmacokinetic parameters of compound **8g** are shown in Figure 6 and Table 4, respectively. After oral administration of compound **8g** (HBr salt) to rats and monkeys at each dose of 0.1, 0.3, 1.0 mg/kg, unchanged **8g** reached maximal concentrations (C_{max}) at 0.75–0.88 h in rats, and 0.50–1.38 h in monkeys. At the same time, long terminal half-lives (8–16 h in rats, and 15–19 h in monkeys) were observed after oral dosing, which was suitable for long-lasting plasma DPP-4 inhibitory activity in vivo. In the range of 0.1–1.0 mg/kg, dose-proportionality of area under curve was confirmed in rats. Compound **8g** showed excellent bioavailability (BA) ranging from 63% to 86% in rats, and 44% to 83% in monkeys, and was mainly excreted into feces via bile in both rats and monkeys. This profile may be useful for diabetes patients having impaired renal functions.

2.7. In vivo pharmacological evaluation of compound 8g

On the basis of its excellent in vitro potency, selectivity, and pharmacokinetic profiles, compound **8g** was chosen for in vivo evaluation. Compound **8g** (HBr salt) was administered orally to Wistar rats at a dose of 0.03, 0.1, 0.3 or 1 mg/kg and the plasma DPP-4 activity was evaluated ex vivo. As shown in Figure 7, **8g** indicated dose-dependent, fast-onset and long-lasting DPP-4 inhibitory activity. It is most noteworthy that more than 50% inhibition of plasma DPP-4 level was sustained at a single oral dose of

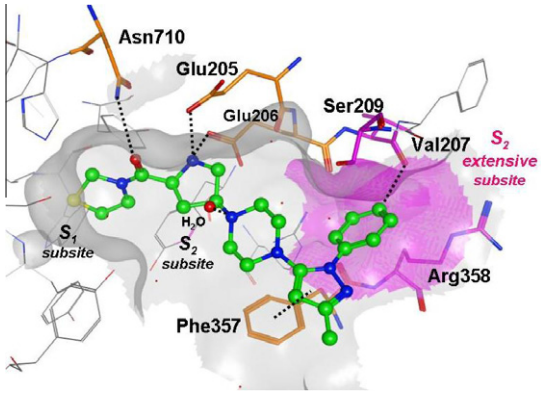


Figure 4. Binding interactions in active site. Co-crystallization of compound **8g** and human DPP-4. The surface in pink represents S_2 extensive subsite.

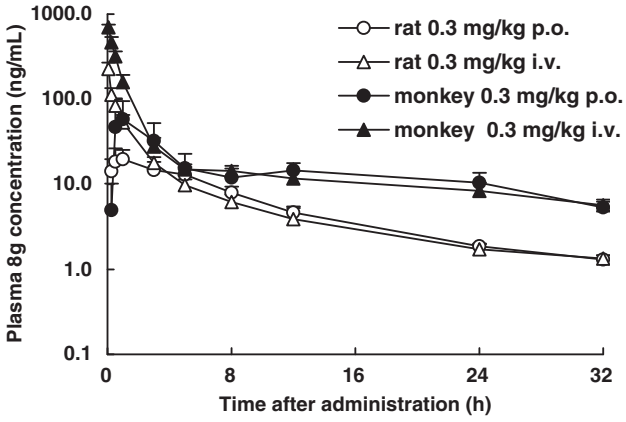


Figure 6. Plasma concentration–time profiles of compound **8g** (HBr salt) after oral and intravenous administration to rats and monkeys (Mean ± S.D., n = 4).

Table 4
Pharmacokinetics properties of compound **8g** (HBr salt)

Animal	Dose (mg/kg)	Phamacokinetic parameter[a]				
		t_{max} (h)	C_{max} (ng/mL)	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (ng h/mL)	BA (%)
Rat	0.1	0.88 ± 0.25	5.48 ± 2.94	15.84 ± 2.32	91.81 ± 24.07	85.9[b]
	0.3	0.75 ± 0.29	20.65 ± 6.58	8.97 ± 1.64	202.12 ± 29.59	63.0[b]
	1.0	0.75 ± 0.29	152.41 ± 16.60	8.43 ± 1.75	672.94 ± 99.32	62.9[b]
Monkey	0.1	0.50 ± 0.00	28.27 ± 5.30	18.86 ± 2.46	295.44 ± 66.17	83.2 ± 16.7
	0.3	1.38 ± 1.11	85.13 ± 28.61	15.24 ± 4.59	613.16 ± 75.83	57.6 ± 5.3
	1.0	0.88 ± 0.25	273.54 ± 70.41	16.11 ± 1.77	1571.64 ± 250.84	44.1 ± 2.6

[a] Data are expressed as the mean ± S.D. of 4 animals.
[b] BA was calculated using the mean $AUC_{0-\infty}$ of each group.

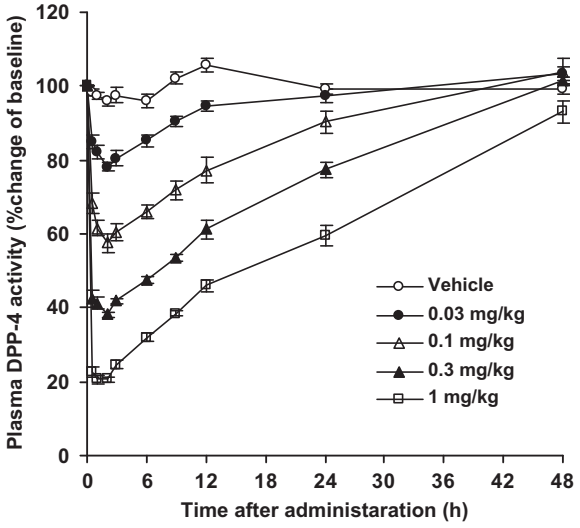


Figure 7. Effects of compound **8g** (HBr salt) on plasma DPP-4 activity (% change of baseline) in Wistar rat. Compound **8g** (HBr salt) was orally administrated at a dose of 0.03, 0.1, 0.3 or 1 mg/kg at 0 h. Data are expressed as means ± SEM (n = 3).

1 mg/kg until 24 h. Compound **8g** was assessed for its ability to improve glucose tolerance in Zucker fatty rats. Pretreatment with compound **8g** at the doses from 0.03 mg/kg to 1 mg/kg 30 min before glucose challenge (1 g/kg) inhibited the increase in glucose levels in an oral glucose tolerance test (Fig. 8). The treatment with compound **8g** significantly lowered the delta $AUC_{(0-60min)}$ and the maximum increase in glucose levels in Zucker fatty rats. Near normalization of the glucose excursion relative to lean controls was seen following a 0.03 mg/kg oral dose of compound **8g** and the significant improvements were observed at more than 35% of DPP-4 inhibition in compared to pre-dosing. These ex vivo and in vivo studies in rats suggest that once-daily and lower dosing of compound **8g** is expected to keep proper blood glucose without causing hypoglycemia in humans.

3. Conclusion

A culmination of work focused on the optimization at the γ-position of the proline moiety of L-prolylthiazolidine series has led to the discovery of the compound **8g**, which proved to be a highly potent, selective and long-lasting DPP-4 inhibitor possessing a unique structure of five consecutive rings. X-ray crystal structure determination of **8g** demonstrated that the key interaction between the phenyl ring on the pyrazole and the S_2 extensive subsite of DPP-4 not only boosted potency but also increased selectivity against DPP-8 and DPP-9. Compound **8g** (HBr salt) at 0.03 mg/kg or higher doses, significantly inhibited the increase of plasma glucose levels after an oral glucose load in Zucker fatty rats. Teneligliptin, 3-[(2*S*,4*S*)-4-[4-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl) piperazin-1-yl]pyrrolidin-2-ylcarbonyl]thiazolidine (**8g**) has been approved for the treatment of type 2 diabetes in Japan.

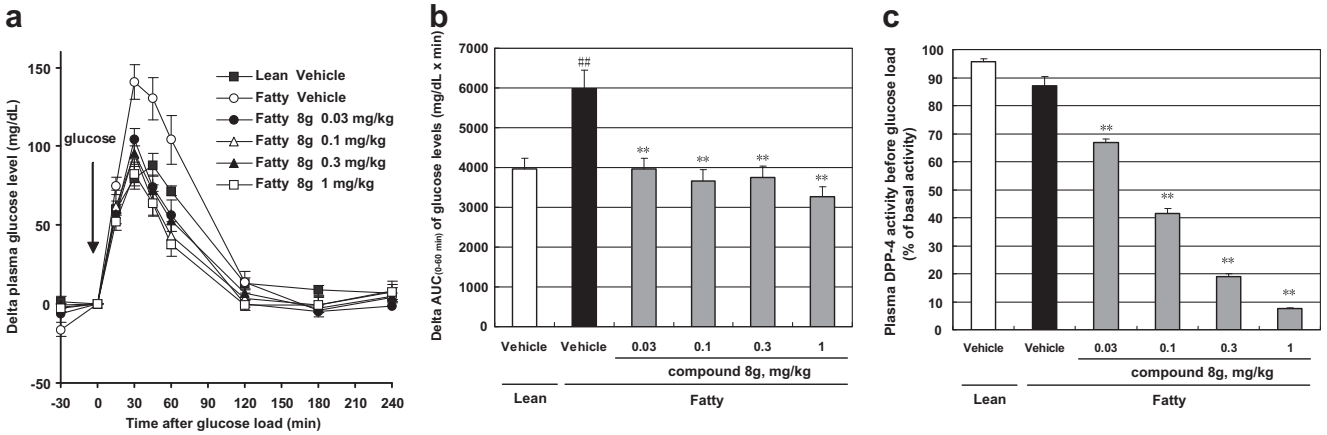


Figure 8. (a) Effects of compound **8g** (HBr salt) on delta glucose levels after an oral glucose tolerance test in Zucker lean and fatty rats. Compound **8g** (HBr salt) or 0.5% HPMC solution (vehicle) was administered 30 min prior to an oral glucose challenge (1 g/kg). Values are mean ± SEM (n = 10) as the delta glucose levels adjusted from the pre-glucose levels at 0 min. (b) The incremental areas under the curves of glucose levels from 0 min to 60 min after glucose load during an oral glucose tolerance test in Zucker rats treated with vehicle or **8g** (HBr salt). (c) The plasma DPP-4 activities before glucose load in Zucker rats treated with vehicle or compound **8g** (HBr salt). Values are means ± SEM (n = 10). $^{\#\#}P$ <0.01 versus 'Zucker lean + Vehicle' by Student's t-test. $^{**}P$ <0.01 versus 'Zucker fatty + Vehicle' by Dunnett's multiple comparison test.

Table 5
Data collection and refinement statistics

	8g	9b
PDB entry code	3VJK	3VJL
Crystal		
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell parameters: a (Å)	117.96	117.81
b (Å)	126.41	125.83
c (Å)	138.01	137.13
Data		
Resolution (Å)	50.00–2.49 (2.58–2.49)	50.00–2.39 (2.48–2.39)
Unique reflections	72,839 (6849)	80,861 (7811)
Redundancy	3.8 (3.1)	5.0 (4.7)
Completeness (%)	90.2 (94.7)	99.0 (97.9)
R_{merge}[a]	0.092 (0.491)	0.051 (0.173)
I/σ (I)	15.6	20.9
Refinement		
Resolution (Å)	30.00–2.49 (2.55–2.49)	30.00–2.39 (2.46–2.39)
Unique reflections	61,379 (4707)	76,090 (5399)
Completeness (%)	88.9 (93.6)	99.1 (96.4)
Data in the test set	3211 (243)	4025 (264)
R-work	0.225 (0.281)	0.184 (0.210)
R-free	0.279 (0.333)	0.230 (0.291)
Structure		
Protein non-H atoms/B (Å^2)	12,180/37.7	12,166/25.7
Ligand atoms/B (Å^2)	60/32.5	66/35.4
Water oxygen atoms/B (Å^2)	456/32.1	894/27.2
Rmsd		
Bond lengths (Å)	0.011	0.010
Bond angles (°)	1.435	1.343
Ramachandran plot		
Most favored regions (%)	87.3	88.5
Additionally allowed regions (%)	12.0	11.0
Generously allowed regions (%)	0.7	0.5

Values in parentheses are for highest-resolution shell.
[a] $R_{merge} = \Sigma|(I - \langle I \rangle)|/\Sigma(I)$, where I is the observed intensity.

4. Experimental

4.1. Chemistry

4.1.1. General

^{1}H NMR spectra were measured on a Bruker DPX-300 instrument or on a Bruker AMX-500 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 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, dimethyl sulfoxide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; HOBT, 3-hydroxybenztriazole hydrate.

4.1.2. 3-{(2*S*,4*S*)-4-[4-(4-Phenylthiazol-2-yl) piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (8c)

To a solution of **22**[18a] (901 mg, 3.00 mmol), 4-phenyl-2-(piperazin-1-yl)thiazole (810 mg, 3.30 mmol) and acetic acid (0.17 mL, 3.0 mmol) in 1,2-dichloroethane (15 mL) was added sodium triacetoxyborohydride (1.27 g, 6.00 mmol) and the mixture stirred at room temperature for 13 h. The reaction mixture was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with brine, dried and concentrated under reduced pressure. The residue purified by silica gel chromatography with chloroform/methanol (30:1, v/v) to give 3-{(2*S*,4*S*)-1-*tert*-butoxycarbonyl-4-[4-(4-phenylthiazol-2-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine (1.59 g, 100%) as a pale yellow amorphous. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.41, 1.46 (9H, s), 1.84–1.97 (1H, m), 2.40–2.73 (4H, m), 2.82–3.21 (4H, m), 3.34 (1H, t, J = 9.9 Hz), 3.47–4.13 (7H, m), 4.38–4.82 (3H, m), 6.78 (1H, s), 7.25 (1H, t, J = 7.1 Hz), 7.37 (2H, t, J = 7.1 Hz), 7.82 (2H, d, J = 7.1 Hz).

To a solution of the above compound (1.59 g, 3.00 mmol) in ethyl acetate (6 mL) was added 4 mol/L hydrochloric acid in ethanol (7 mL), and the mixture stirred at room temperature for 12 h. The precipitated solid was collected by filtration to give the title compound (1.41 g, 83%) as a pale-brown powder. Mp: >135 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.27–2.42 (1H, m), 2.95–3.18 (3H, m), 3.37–4.18 (16H, m), 4.47–4.78 (3H, m), 7.30 (1H, t, J = 7.3 Hz), 7.37–7.45 (3H, s), 7.87 (2H, d, J = 7.1 Hz), 9.17 (1H, br s), 10.93 (1H, br s); Anal. Calcd for $C_{21}H_{27}N_5OS_2 \cdot 3/10HCl \cdot 2/5C_2H_6O$: C, 46.07; H,5.80; N, 12.32. Found: C, 45.84; H, 6.14; N, 11.93; LC–MS (ESI) m/z 430.4 $[M+H]^+$.

4.1.3. 1-Benzyloxycarbonyl-4-[(methylthio)phenyliminom ethyl]piperazine (11a)

To a solution of **10a** (5.00 g, 22.7 mmol) in acetone (50 mL) was added phenyl isothiocyanate (2.9 mL, 24 mmol). The mixture was stirred for 1 h at room temperature. The precipitate was collected by filtration and washed with acetone to give 1-(anilinocarbothioyl)-4-(benzyloxycarbonyl)piperazine (5.08 g, 63%) as a white powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.52 (4H, br s), 3.93 (4H, br s), 5.12 (2H, s), 7.11 (1H, t, J = 8.5 Hz), 7.27–7.45 (9H, m), 9.37 (1H, br s).

To a solution of the above compound (5.07 g, 14.3 mmol) in methanol (100 mL) was added methyl iodide (1.1 mL, 18 mmol) under ice-cooling. The mixture stirred at room temperature for 17 h, and then concentrated under reduced pressure. The residue was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with dichloromethane. The extract was washed with brine, dried and concentrated under reduced pressure to give the title compound (5.71 g, 100%) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.03 (3H, s), 3.59 (8H, br s), 5.17 (2H, s), 6.87 (2H, d, J = 7.3 Hz), 7.00 (1H, t, J = 7.3 Hz), 7.26 (2H, t, J = 7.3 Hz), 7.32–7.43 (5H, m).

4.1.4. 1-(1-Phenyl-1*H*-imidazol-2-yl)piperazine (12a)

A mixture of **11a** (3.00 g, 8.12 mmol) and aminoacetaldehyde dimethyl acetal (1.8 mL, 17 mmol) in pyridine (15 mL) was stirred at 100 °C for 2 days, and then concentrated under reduced pressure. The residue was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with brine, dried and concentrated under reduced pressure. A solution of the residue in 2 mol/L hydrochloric acid (30 mL) was stirred for 2 h at 100 °C. After cooling to room temperature, the reaction mixture was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with chloroform/methanol (30:1, v/v) to give 1-benzyloxycarbonyl-4-(1-phenyl-1*H*-imidazol-2-yl)piperazine (1.16 g, 39%) as a brown oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.96–3.03 (4H, m), 3.44–3.52 (4H, m), 5.12 (2H, s), 6.86 (1H, d, J = 1.6 Hz), 6.88 (1H, d, J = 1.6 Hz), 7.27–7.54 (10H, m).

To the above compound (1.16 g, 3.20 mmol) in methanol (30 mL) was added 10% palladium on carbon (232 mg). The mixture was stirred at room temperature for 20 h under a hydrogen atmosphere (1 atm). Palladium on carbon was removed by filtration. The filtrate was concentrated under reduced pressure to give the title compound (0.742 g, 100%) as a white amorphous. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.04–3.28 (8H, m), 3.49 (1H, s), 6.85 (1H, d, J = 1.6 Hz), 6.88 (1H, d, J = 1.6 Hz), 7.34–7.52 (5H, m).

4.1.5. 3-{(2*S*,4*S*)-4-[4-(1-Phenyl-1*H*-imidazol-1-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (8b)

The title compound was prepared in 58% yield using **12a** in the procedures outlined for **8c**. Mp: >182 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.10–2.26 (1H, m), 2.83–4.05 (16H, m), 4.43–4.77 (3H, m), 7.48 (1H, d, J = 2.3 Hz), 7.53 (1H, d, J = 2.3 Hz), 7.54–7.72 (5H, m), 9.07 (1H, br s), 10.98 (1H, br s); Anal. Calcd for $C_{21}H_{28}N_6OS{\cdot}3HCl{\cdot}3/5C_4H_8O_2{\cdot}5/2H_2O$: C, 45.34; H,6.63; N, 13.56. Found: C, 45.16; H, 6.86; N, 13.66; LC–MS (ESI) m/z 413.4 $[M+H]^+$.

4.1.6. 3-{(2*S*,4*S*)-4-[4-(1-Phenyl-1*H*-tetrazol-5-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (8d)

The title compound was prepared in 68% yield using 1-(1-phenyl-1*H*-tetrazol-5-yl)piperazine in the procedures outlined for **8c**. Mp: 184 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.02–2.22 (1H, m), 2.80–3.95 (16H, m), 4.45–4.73 (3H, m), 7.57–7.73 (5H, m), 9.04 (1H, br s), 10.61 (1H, br s); Anal. Calcd for $C_{19}H_{26}N_8OS{\cdot}5/2HCl{\cdot}1/4C_4H_8O_2{\cdot}1/2H_2O$: C, 44.76; H,5.92; N, 20.88. Found: C, 44.82; H, 6.31; N, 20.94; LC–MS (ESI) m/z 415.4 $[M+H]^+$.

4.1.7. 1-(1-Cyclohexyl-1*H*-tetrazol-5-yl)piperazine (12b)

To a solution of **10a** (2.07 g, 9.40 mmol) in dichloromethane (50 mL) was added cyclohexyl isocyanate (1.2 mL, 9.4 mmol). The mixture was stirred for 1 h at room temperature, and then concentrated under reduced pressure. To a solution of the residue in tetrahydrofuran (100 mL) was added phosphorus oxychloride (8.8 mL, 94 mmol), and the mixture was refluxed for 18 h. The reaction mixture was concentrated under reduced pressure, and a 0.5 mol/L triazole in acetonitrile (100 mL, 50 mmol) was added to the residue. The mixture was stirred at room temperature for 3 h, and then concentrated under reduced pressure. The residue was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure. To the residue in methanol (100 mL) was added an aqueous solution (20 mL) of sodium azide (6.50 g, 100 mmol). The mixture was stirred at 70 °C for 3 h, and then concentrated under reduced pressure. Water was added to the residue, and the mixture was extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with *n*-hexane/ethyl acetate (7:3, v/v) to give 1-benzyloxycarbonyl-4-(1-cyclohexyl-1*H*-tetrazol-5-yl)piperazine (390 mg, 11%) as a white powder.

The title compound was prepared quantitatively using the above compound in the procedures outlined for **12a**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.28–1.47 (3H, m), 1.68–2.07 (7H, m), 3.04–3.08 (4H, m), 3.18–3.23 (4H, m), 3.37–3.57 (1H, m), 3.95–4.08 (1H, m).

4.1.8. 3-{(2*S*,4*S*)-4-[4-(1-Cyclohexyl-1*H*-tetrazol-5-yl)piperazin-1-yl]pyrrolidin-2-ylcarbonyl}thiazolidine trihydrochloride (8e)

The title compound was prepared in 56% yield using **12b** in the procedures outlined for **8c**. Mp: 178 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.20–1.34 (1H, m), 1.40–1.50(2H, m), 1.64–1.88 (5H, m), 1.97–2.03 (2H, m), 2.12–2.32 (1H, m), 2.90–4.05 (16H, m), 4.25 (1H, m), 4.48–4.75 (3H, m), 9.10 (1H, br s), 10.67 (1H, br s); Anal. Calcd for $C_{19}H_{32}N_8OS{\cdot}5/2HCl{\cdot}1/10C_4H_8O_2{\cdot}1/2H_2O$: C, 44.00; H,6.91; N, 21.16. Found: C, 44.02; H, 7.29; N, 20.90; LC–MS (ESI) m/z 421.2 $[M+H]^+$.

4.1.9. 1-(1-Phenyl-5-pyrazolyl)piperazine (12c)

To a solution of ethyl 3,3-diethoxypropionate (5.34 g, 28.1 mmol) in tetrahydrofuran (60 mL) was added a 1 mol/L aqueous sodium hydroxide solution (29 mL, 29 mmol). The mixture was stirred for 12 h at room temperature, and then concentrated under reduced pressure. To a solution of the residue in DMF (60 mL) were added HOBT (5.16 g, 33.7 mmol), EDC hydrochloride (6.46 g, 33.7 mmol) and **10a** (6.20 g, 28.1 mmol) at room temperature. The mixture was stirred at room temperature for 6 h, and then concentrated under reduced pressure. The residue was poured into water and extracted with ethyl acetate. The extract solution was washed with a saturated aqueous sodium hydrogen carbonate solution and brine, dried and concentrated under reduced pressure. The residue was purified by silica gel with *n*-hexane/ethyl acetate (3:7, v/v) to give 1-benzyloxycarbonyl-4-(3,3-diethoxypropionyl)piperazine (10.1 g, 98%) as an oil.

To a solution of the above compound (3.28 g, 9.00 mol) in chloroform (30 mL) was added a 50% aqueous trifluoroacetic acid solution (20 mL) under ice-cooling. The mixture was stirred at room temperature for 24 h. The reaction solution was extracted with chloroform. The extract was washed with water and brine, dried and concentrated under reduced pressure to give a mixture including **11c**. To a solution of the mixture in ethanol (60 mL) was added phenylhydrazine (0.89 mL, 9.0 mmol) and methanesulfonic acid (0.060 mL, 0.90 mmol), and the solution was stirred at room temperature for 3 h. Pyridine (1.0 mL) was added, and then the mixture was concentrated under reduced pressure. To a solution of the residue in pyridine (50 mL), was added phosphorus oxychloride (1.68 mL, 18.0 mmol). The mixture was stirred at room temperature for 18 h, and then concentrated under reduced pressure. The residue was poured into water and extracted with ethyl acetate. The extract solution was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with *n*-hexane/ethyl acetate (7:3, v/v) to give 1-benzyloxycarbonyl-4-(1-phenylpyrazol-5-yl)piperazine (0.218 g, 11%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.06–3.18 (8H, m), 5.93 (1H, d, J = 1.8 Hz), 7.32 (1H, t, J = 7.5 Hz), 7.45 (2H, t, J = 7.5 Hz), 7.54 (1H, d, J = 1.8 Hz), 7.72 (1H, d, J = 7.5 Hz).

The title compound was prepared quantitatively using the above compound in the procedures outlined for **12a**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.83 (4H, br s), 3.49–3.48 (4H, m), 5.12 (2H, s), 5.85 (1H, d, J = 1.9 Hz), 7.24–7.45 (8H, m), 7.51 (1H, d, J = 1.9 Hz), 7.78 (2H, d, J = 8.2 Hz).

4.1.10. 3-{(2*S*,4*S*)-4-[4-(1-Phenyl-1*H*-pyrazol-5-yl)piperazin-1-yl]pyrrolizin-2-ylcarbonyl}thiazolidine trihydrochloride (8f)

The title compound was prepared in 50% yield using **12c** in the procedures outlined for **8c**. Mp: 187 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.10–2.30 (1H, m), 2.80–4.10 (16H, m), 4.46–4.74 (3H, m), 6.10 (1H, d, J = 1.7 Hz), 7.34–7.37 (1H, m), 7.49–7.52 (2H, m), 7.56 (1H, d, J = 1.7 Hz), 7.79–7.81 (2H, m), 9.07 (1H, br s), 10.65 (1H, br s); Anal. Calcd for $C_{21}H_{28}N_6OS{\cdot}5/2HCl{\cdot}1/2C_4H_8O_2{\cdot}3/4H_2O$: C, 49.22; H,6.46; N, 14.97. Found: C, 49.25; H, 6.59; N, 15.20; LC–MS (ESI) m/z 413.4 $[M+H]^+$.

4.1.11. 1-Acetoacetyl-4-*tert*-butoxycarbonylpiperazine (11d)

A solution of **10b** (5.02 g, 27.0 mmol) and diketene (2.50 mL 32.6 mmol) n DMF (90 mL) was stirred for 1.5 h at room temperature, and then concentrated under reduced pressure. The residue was poured into water and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure to give the title compound (6.26 g, 86%) as a

pale-brown powder. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.41 (9H, s), 2.15 (3H, s), 3.27–3.35 (6H, m), 3.43 (2H, m), 3.66 (2H, s).

4.1.12. 1-(3-Methyl-1-phenyl-5-pyrazolyl)piperazine (12d)

To a solution of **11d** (6.24 g, 23.1 mmol) in ethanol (500 mL) was added phenylhydrazine (2.27 mL, 23.1 mmol) and methanesulfonic acid (0.35 mL, 5.4 mmol) at room temperature. The mixture was stirred for 14 h, and then pyridine (6 mL) was added. The mixture was concentrated under reduced pressure. A solution of the residue and phosphorus oxychloride (5.0 mL, 54 mmol) in pyridine (250 mL) was stirred for 20 h at room temperature, and then concentrated under reduced pressure. The residue was acidified with dilute hydrochloric acid to pH 3. The mixture was extracted with ethyl acetate. The extract was washed with a saturated aqueous sodium hydrogen carbonate solution and brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with *n*-hexane/ethyl acetate (7:3, v/v) to give 1-*tert*-butoxycarbonyl-4-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl)piperazine (935 mg, 12%) as an oil. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.39 (9H, s), 2.15 (3H, s), 2.73 (4H, m), 3.37 (4H, m), 5.83 (1H, s), 7.28 (1H, t, J = 7.4 Hz), 7.46 (2H, t, J = 7.4 Hz), 7.76 (2H, d, J = 7.4 Hz).

To a solution of the above compound (935 mg, 2.72 mmol) in dichloromethane (10 mL) was added trifluoroacetic acid (5 mL) at room temperature. The mixture was stirred for 1.5 h, and then concentrated under reduced pressure. The residue was poured into water (50 mL) and washed with diethyl ether. The aqueous layer was basified with a aqueous sodium hydrogen carbonate solution, and the mixture was extracted with chloroform. The extract was dried and concentrated under reduced pressure to give the title compound (584 mg, 88%) as a brown powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.14 (3H, s), 2.66–2.76 (8H, m), 5.77 (1H, s), 7.26 (1H, t, J = 7.5 Hz), 7.45 (2H, t, J = 7.5 Hz), 7.76 (2H, d, J = 7.5 Hz);

4.1.13. 3-{(2*S*,4*S*)-4-[4-(3-Methyl-1-phenyl-1*H*-pyrazol-5-yl)piperazin-1-yl]pyrrolidin-2-yl-carbonyl}thiazolidine hemipentahydrobromide (8g)

3-{(2*S*,4*S*)-1-*tert*-Butoxycarbonyl-4-[4-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl)piperazin-1-yl]pyrrolidin-2-yl-carbonyl}thiazolidine was prepared in 50% yield using **12d** in the procedures outlined for **8c**.

To a solution of the above compound (25.45 g) in dichloromethane (200 mL) was added trifluoroacetic acid (50 mL) at room temperature. The mixture was stirred for 19 h, and then concentrated under reduced pressure. The residue was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with, dried and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with chloroform/methanol (9:1, v/v) to give free base of the title compound (19.28 g, 93%) as a solid.

To a solution of the above compound (5.09 g, 11.9 mmol) in ethanol (51 mL) was added 48% hydrobromic acid (5.03 g) at the refluxing temperature, The mixture was cooled to room temperature over 1 h with stirring, and further stirred at room temperature for 1 h. The precipitate was collected by filtration, washed with ethanol (5 mL) to give the title compound as white crystals (6.76 g, 90%). Mp: 201 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.28–2.08 (1H, m), 2.17 (3H, s), 2.95–4.22 (16H, m), 4.43–4.80 (3H, m), 5.95 (1H, s), 7.32 (1H, t, J = 7.5 Hz), 7.48 (2H, t, J = 7.5Hz), 7.78 (2H, d, J = 7.5Hz), 9.18 (1H, br s),9.90 (1H, br s); Anal. Calcd. for $C_{22}H_{30}N_6OS{\cdot}2.5HBr{\cdot}1.5H_2O$: C, 40.29; H, 5.46; N, 12.81. Found: C, 40.23; H, 5.34; N, 12.81; LC–MS (ESI) m/z 427.4 $[M+H]^+$.

4.1.14. (3-Trifluoromethyl-1-phenyl-1*H*-pyrazol-5-yl)piperazine (12e)

To a solution of **10a** (19.0 g, 86.3 mmol) in pyridine (150 mL) was added acetic anhydride (9.0 mL, 95 mmol) at room temperature. The mixture was stirred for 18 h, and then concentrated under reduced pressure. The residue was poured into a 10% aqueous citric acid solution and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure to give 4-acetyl-1-benzyloxycarbonylpiperazine (22.6 g, 100%) as an oil. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.78 (2H, s), 3.35–3.68 (8H, m), 5.10 (2H, s), 7.28–7.42 (5H, m).

To a solution of the above compound (7.12 g, 27.1 mmol) in tetrahydrofuran (150 mL) was added a 1 mol/L lithium bis(trimethylsilyl)amide in tetrahydrofuran (41 mL, 41 mmol) dropwise at −78 °C over 40 min. After stirring at constant temperature for 1 h, a solution of ethyl trifluoroacetate (4.85 mL, 40.1 mmol) in tetrahydrofuran (20 mL) was added to the reaction solution. After warming to room temperature over 18 h, the mixture was poured into a saturated aqueous ammonium chloride solution and extracted with ethyl acetate. The extract solution was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with *n*-hexane/ethyl acetate (7:3, v/v) to give 1-benzyloxycarbonyl-4-(trifluoroacetoacetyl)piperazine (7.35 g, 76%) as a pale-yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.86 (4H, br s), 3.49–3.55 (4H, m), 5.12 (2H, s), 6.12 (1H, s), 7.32–7.43 (6H, m), 7.46 (2H, t, J = 7.5 Hz), 7.74 (2H, d, J = 7.5 Hz).

The title compound was prepared in 13% yield using the above compound in the procedures outlined for **12c**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.22 (8H, br s), 6.21 (1H, s), 7.42 (1H, t, J = 7.5 Hz), 7.49 (2H, t, J = 7.5 Hz), 7.66 (2H, d, J = 7.5 Hz), 9.96 (1H, br s).

4.1.15. 3-{(2*S*,4*S*)-4-[4-(3-Trifluoromethyl-1-phenyl-1*H*-pyrazol-5-yl)piperazin-1-yl]pyrrolizin-2-ylcarbonyl}thiazolidine trihydrochloride (8h)

The title compound was prepared in 52% yield using **12e** in the procedures outlined for **8c.** ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.00–2.28 (1H, m), 2.80–4.00 (16H, m), 4.44–4.74 (3H, m), 6.64 (1H, s), 7.44–7.49 (1H, m), 7.54–7.59 (2H, m), 7.77–7.79 (2H, m), 9.03 (1H, br s), 10.55 (1H, br s); Anal. Calcd for $C_{22}H_{27}F_3N_6OS{\cdot}2.5HCl{\cdot}0.25H_2O$: C, 45.86; H, 5.25; N, 14.59. Found: C, 45.89; H, 5.63; N, 14.23; LC–MS (ESI) m/z 481.4 $[M+H]^+$.

4.1.16. 1-[1-(4-Fluorophenyl)-3-methyl-1*H*-pyrazol-5-yl]piperazine (12f)

The title compound was prepared in 38% yield from **11d** using 4-fluorophenylhydrazine hydrochloride in the procedures outlined for **12d**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.26 (3H, s), 2.75–2.92 (8H, m), 5.68 (1H, s), 7.10 (2H, ddd, J = 2.3, 4.8, 8.4 Hz), 7.72–7.79 (2H, m).

4.1.17. 3-((2*S*,4*S*)-4-{4-[1-(4-Fluorophenyl)-3-methyl-1*H*-pyrazol-5-yl]piperazin-1-yl}pyrrolizin-2-ylcarbonyl) thiazolidine trihydrochloride (8i)

The title compound was prepared in 41% yield using **12f** in the procedures outlined for **8c**. Mp: >145 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.17 (3H, s), 2.20–2.40 (1H, m), 2.90–4.35(16H, m), 4.43–4.82 (3H, m), 5.95 (1H, s), 7.21–7.37 (2H, m), 7.74–7.89 (2H, m), 9.13 (1H, br s), 11.10 (1H, br s); Anal. Calcd for $C_{22}H_{29}FN_6OS{\cdot}3HCl{\cdot}0.5C_4H_8O_2{\cdot}2.2H_2O$: C, 44.80; H, 6.44; N, 13.63. Found: C, 44.67; H, 6.38; N, 13.64; LC–MS (ESI) m/z 445.2 $[M+H]^+$.

4.1.18. 1-[1-(3-Fluorophenyl)-3-methyl-1*H*-pyrazol-5-yl] piperazine (12g)

The title compound was prepared in 23% yield from **11d** using 3-fluorophenylhydrazine hydrochloride in the procedures outlined for **12d**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.27 (3H, s), 2.82–2.97 (8H, m), 5.69 (1H, s), 6.91–6.98 (1H, m), 7.31–7.40 (1H, m), 7.60–7.67 (2H, m).

4.1.19. 3-((2*S*,4*S*)-4-{4-[1-(3-Fluorophenyl)-3-methyl-1*H*-pyrazol-5-yl]piperazin-1-yl}pyrrolizin-2-ylcarbonyl) thiazolidine trihydrochloride (8j)

The title compound was prepared in 72% yield using **12g** in the procedures outlined for **8c**. Mp: 172 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.10–2.35 (1H, m), 2.17 (3H, s), 2.90–4.15 (16H, m), 4.46–4.76 (3H, m), 5.98 (1H, s), 7.11–7.19 (1H, m), 7.47–7.55 (1H, m), 7.59–7.64 (1H, m), 7.70–7.73 (1H, m), 9.09 (1H, br s), 10.79 (1H, br s); Anal. Calcd for $C_{22}H_{29}FN_6OS{\cdot}3HCl{\cdot}0.75H_2O$: C, 46.56; H, 5.95; N, 14.81. Found: C, 46.63; H, 6.29; N, 14.45; LC–MS (ESI) *m*/*z* 445.4 $[M+H]^+$.

4.1.20. 1-[1-(2-Fluorophenyl)-3-methyl-1*H*-pyrazol-5-yl]piperazine (12h)

The title compound was prepared in 23% yield from **11d** using 2-fluorophenylhydrazine hydrochloride in the procedures outlined for **12d**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.28 (3H, s), 2.79 (8H, br s), 5.67 (1H, s), 7.16–7.55 (4H, m).

4.1.21. 3-((2*S*,4*S*)-4-{4-[1-(2-Fluorophenyl)-3-methyl-1*H*-pyrazol-5-yl]piperazin-1-yl}pyrrolizin-2-ylcarbonyl) thiazolidine trihydrochloride (8k)

The title compound was prepared in 67% yield using **12h** in the procedures outlined for **8c**. Mp: 162 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.03–2.25 (1H, m), 2.16 (3H, s), 2.72–4.00 (16H, m), 4.45–4.71 (3H, m), 5.91 (1H, s), 7.32–7.35 (1H, m), 7.40–7.44 (1H, m), 7.51–7.57 (2H, m), 9.02 (1H, br s), 10.41 (1H, br s); Anal. Calcd for $C_{22}H_{29}FN_6OS{\cdot}3HCl{\cdot}1.5H_2O$: C, 45.48; H, 6.07; N, 14.43. Found: C, 45.61; H, 5.96; N, 14.14; LC–MS (ESI) *m*/*z* 445.4 $[M+H]^+$.

4.1.22. 1-[1-(4-Chlorophenyl)-3-methyl-1*H*-pyrazol-5-yl]piperazine (12i)

The title compound was prepared in 32% yield from **11d** using 4-chlorophenylhydrazine hydrochloride in the procedures outlined for **12d**. LC–MS (ESI) *m*/*z* 277.4 $[M+H]^+$.

4.1.23. 3-((2*S*,4*S*)-4-{4-[1-(4-Chlorophenyl)-3-methyl-1*H*-pyrazol-5-yl]piperazin-1-yl}pyrrolizin-2-ylcarbonyl) thiazolidine trihydrochloride (8l)

The title compound was prepared in 73% yield using **12i** in the procedures outlined for **8c**. Mp: 210 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.17 (3H, s), 2.25–2.40 (1H, m), 2.95–4.15 (17H, m), 4.46–4.77 (3H, m), 5.97 (1H, s), 7.48–7.53 (2H, m), 9.13 (1H, br s), 11.01 (1H, br s); Anal. Calcd for $C_{22}H_{29}ClN_6OS{\cdot}2.5HCl{\cdot}1.5H_2O$: C, 45.62; H, 6.00; N, 14.51. Found: C, 45.27; H, 6.29; N, 14.19; LC–MS (ESI) *m*/*z* 461.4 $[M+H]^+$.

4.1.24. 1-[1-(4-Cyanophenyl)-3-methyl-1*H*-pyrazol-5-yl]piperazine (12j)

The title compound was prepared in 40% yield from **11d** using 4-cyanophenylhydrazine hydrochloride in the procedures outlined for **12d**. LC–MS (ESI) *m*/*z* 269.4 $[M+H]^+$.

4.1.25. 3-((2*S*,4*S*)-4-{4-[1-(4-Cyanophenyl)-3-methyl-1*H*-pyrazol-5-yl]piperazin-1-yl}pyrrolizin-2-ylcarbonyl) thiazolidine trihydrochloride (8m)

The title compound was prepared in 67% yield using **12j** in the procedures outlined for **8c**. Mp: 220 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.19 (3H, s), 2.20–2.40 (1H, m), 2.95–4.15 (17H, m), 4.46–4.77 (3H, m), 6.05 (1H, s), 7.91 (2H, d, *J* = 9.0 Hz), 8.08 (2H, d, *J* = 9.0Hz), 9.13 (1H, br s), 10.09 (1H, br s); Anal. Calcd for $C_{23}H_{29}N_7OS{\cdot}2.5HCl{\cdot}0.05C_4H_8O_2{\cdot}1.5H_2O$: C, 48.53; H, 6.13; N, 17.08. Found: C, 48.35; H, 6.42; N, 16.71; LC–MS (ESI) *m*/*z* 452.4 $[M+H]^+$.

4.1.26. 1-[3-Methyl-1-(pyridin-4-yl)-1*H*-pyrazol-5-yl]piperazine (12k)

The title compound was prepared in 54% yield from **11d** using 4-hydrazinopyridine dihydrochloride in the procedures outlined for **12d**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.27 (3H, s), 2.86–2.99 (8H, m), 5.77 (1H, s), 7.92 (2H, dd, *J* = 1.6, 4.8 Hz), 8.60 (2H, dd, *J* = 1.6, 4.8 Hz).

4.1.27. 3-((2*S*,4*S*)-4-{4-[3-Methyl-1-(pyridin-4-yl)-1*H*-pyrazol-5-yl] piperazin-1-yl}pyrrolizin-2-ylcarbonyl)thiazolidine trimaleate (8n)

A free base of the title compound was prepared in 90% yield using **12k** in the procedures outlined for **8c**. To this free base (3.20 g, 7.49 mmol) in ethanol (200 mL) was added a solution of maleic acid (3.00 g, 25.8 mmol) in ethanol (20 mL) under ice-cooling. The precipitate was collected by filtration to give the title compound (4.30 g, 74%) as a white powder. Mp: 168–171 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.60–1.80 (1H, m), 2.18 (3H, s), 2.55–3.90 (20H, m), 4.43–4.72 (4H, m), 5.98 (1H, s), 6.18 (6H, s), 7.92–7.94 (2H, m), 8.61–8.63 (2H, m); Anal. Calcd for $C_{21}H_{29}N_7OS{\cdot}3C_4H_4O_4$: C, 51.09; H, 5.32; N, 12.63. Found: C, 50.87; H, 5.27; N, 12.58; LC–MS (ESI) *m*/*z* 428.4 $[M+H]^+$.

4.1.28. 1-[3-Methyl-1-(pyridin-3-yl)-1*H*-pyrazol-5-yl]piperazine (12l)

The title compound was prepared in 19% yield from **11d** using 3-hydrazinopyridine dihydrochloride in the procedures outlined for **12d.** ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.28 (3H, s), 2.83–2.96 (8H, m), 5.76 (1H, s), 7.36 (1H, dd, *J* = 4.7, 8.3 Hz), 8.13 (1H, ddd, *J* = 1.4, 2.4, 8.3 Hz), 8.49 (1H, dd, *J* = 1.4, 4.7 Hz), 9.15 (1H, d, *J* = 2.4 Hz).

4.1.29. 3-((2*S*,4*S*)-4-{4-[3-Methyl-1-(pyridin-3-yl)-1*H*-pyrazol-5-yl] piperazin-1-yl}pyrrolizin-2-ylcarbonyl)thiazolidine trimaleate (8o)

The title compound was prepared in 43% yield using **12l** in the procedures outlined for **8n**. Mp: 165–167 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.60–1.78 (1H, s), 2.17 (3H, s), 2.50–3.90 (20H, m), 4.42–4.71 (4H, m), 5.91 (1H, s), 6.19 (6H, s), 7.49–7.53 (1H, m), 8.12–8.16 (1H, m), 8.18–8.50 (1H, m), 8.98–8.99 (1H, m); Anal. Calcd for $C_{21}H_{29}N_7OS{\cdot}3C_4H_4O_4$: C, 51.09; H, 5.32; N, 12.63. Found: C, 50.90; H, 5.37; N, 12.78; LC–MS (ESI) *m*/*z* 428.4 $[M+H]^+$.

4.1.30. 1-[3-Methyl-1-(pyridin-2-yl)-1*H*-pyrazol-5-yl]piperazine (12m)

The title compound was prepared in 43% yield from **11d** using 2-hydrazinopyridine dihydrochloride in the procedures outlined for **12d**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.29 (3H, s), 3.14 (8H, br s), 5.75 (1H, s), 7.19 (1H, m), 7.74–7.86 (2H, m), 8.50 (1H, m).

4.1.31. 3-((2*S*,4*S*)-4-{4-[3-Methyl-1-(pyridin-2-yl)-1*H*-pyrazol-5-yl] piperazin-1-yl}pyrrolizin-2-ylcarbonyl)thiazolidine dimaleate (8p)

The title compound was prepared in 33% yield using **12m** in the procedures outlined for **8n**. Mp: 120–122 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.19 (3H, s), 2.24–2.44 (1H, m), 2.88–4.20 (16H, m), 4.42–4.80 (3H, m), 5.99 (1H, s), 7.30–7.40 (1H, m), 7.77 (1H, d, *J* = 8.3 Hz), 7.92–8.01 (1H, m), 8.46–8.54 (1H, m), 9.14 (1H, br s), 11.05 (1H, br s); Anal. Calcd for $C_{21}H_{29}N_7OS{\cdot}2C_4H_4O_4{\cdot}H_2O$: C, 51.39; H, 5.80; N, 14.47. Found: C, 51.54; H, 5.52; N, 14.47; LC–MS (ESI) *m*/*z* 428.4 $[M+H]^+$.

4.1.32. 1-[1-(5-Cyanopyridin-2-yl)-3-methyl-1*H*-pyrazol-5-yl]piperazine (12n)

The title compound was prepared in 19% yield from **11d** using 2-cyano-5-hydrazinopyridine in the procedures outlined for **12d.** LC–MS (ESI) *m*/*z* 269.4 $[M+H]^+$.

4.1.33. 3-((2*S*,4*S*)-4-{4-[1-(5-Cyanopyridin-2-yl)-3-methyl-1*H*-pyrazol-5-yl]piperazin-1-yl}pyrrolizin-2-ylcarbonyl) thiazolidine trihydrochloride (8q)

The title compound was prepared in 72% yield using **12n** in the procedures outlined for **8c**. Mp: 210 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.21 (3H, s), 2.25–2.45 (1H, m), 2.95–4.19 (17H, m), 4.47–4.77 (3H, m), 6.05 (1H, s), 7.97 (1H, d, J = 8.7 Hz), 8.37 (1H, dd, J = 2.3, 8.7 Hz), 8.93 (1H, d, J = 2.3 Hz), 9.15 (1H, br s), 10.80 (1H, br s); Anal. Calcd for $C_{22}H_{28}N_8OS{\cdot}3HCl{\cdot}0.2C_2H_4O_2{\cdot}1.5H_2O$: C, 44.77; H, 5.84; N, 18.64. Found: C, 44.54; H, 6.12; N, 18.50; LC–MS (ESI) m/z 430.4 $[M+H]^+$.

4.1.34. 3-[(2*S*,4*S*)-1-(*tert*-Butoxycarbonyl)-4-hydroxypyrrolid inylcarbonyl]thiazolidine (24)

To a solution of *N*-(*tert*-Butoxycarbonyl)-*cis*-4-hydroxy-L-proline (**23**) (5.00 g, 21.6 mmol) and imidazole (6.48 g, 95.2 mmol) in DMF (60 mL) was added *tert*-butyldimethylsilyl chloride (7.16 g, 47.5 mmol) at room temperature. After stirring at room temperature for 19 h, to the mixture were added water (60 mL) and a 10% citric acid aqueous solution (200 mL) under ice-cooling. The mixture was extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure to give (2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-(*tert*-butyldimethylsilyloxy)pyrrolidine -2-carboxylic acid (10.9 g) as a light tan solid.

To a solution of the above compound (10.9 g), thiazolidine (1.9 mL, 24 mmol) and HOBt (3.67 g, 24.6 mmol) in DMF (70 mL) was added EDC hydrochloride (4.97 g, 25.9 mmol) under ice-cooling. The reaction mixture was stirred at room temperature for 6 h. The mixture was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with *n*-hexane/ethyl acetate (7:3, v/v) to give 3-[(2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-(*tert*-butyldimethylsilyloxy) pyrrolidinylcarbonyl]thiazolidine (7.28 g, 81%) as a white solid.

To a solution of the above compound (7.27 g, 17.4 mmol) in tetrahydrofuran (110 mL) was added 1 mol/L tetra-*n*-butylammonium fluoride in tetrahydrofuran (19.2 mL) under ice-cooling. After stirring at room temperature for 1.5 h, the mixture was concentrated under reduced pressure. The residue was poured into brine and extracted with chloroform. The extract was dried and concentrated under reduced pressure. The residue was purified The residue was purified by silica gel column chromatography with chloroform/methanol (20:1, v/v) to give the title compound (5.25 g, 100%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.45, 1.43 (9H, s), 1.95–2.35 (2H, m), 2.95–3.23 (2H, m), 3.45–3.98 (3H, m), 4.30–5.77 (6H, m).

4.1.35. 3-{(2*S*,4*R*)-4-[4-(3-Methyl-1-phenyl-1*H*-pyrazol-5-yl)piperazin-1-yl]pyrrolidin-2-yl-carbonyl}thiazolidine dimaleate (8r)

To a solution of **24** (2.88 g, 9.52 mmol) and 2,6-lutidine (1.22 mL, 10.5 mmol) in dichloromethane (50 mL) was added a solution of trifluoromethanesulfonic anhydride (2.95 g, 10.5 mmol) in dichloromethane (5 mL) dropwise under ice-cooling over 10 min. The mixture was stirred for 30 min and **12d** (2.10 g, 8.67 mmol) and diisopropylethylamine (3.6 mL, 21 mmol) were added to the solution. After stirring at room temperature at 21 h, the reaction mixture was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with chloroform/methanol (30:1, v/v) to give 3-{(2*S*,4*R*)-1-(*tert*-butoxycarbonyl)-4-[4-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl)piperazin-1-yl]-2-pyrrolidinylcarbonyl}-1,3-thiazolidine (2.70 g, 54%) as a amorphous. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.40, 1.44 (9H, s), 1.94–2.10 (2H, m), 2.27 (3H, s). 2.18–2.57 (4H, m), 2.82–4.12 (11H, m), 4.41–4.76 (3H, m), 5.67 (1H, s), 7.24 (1H, t, J = 7.3 Hz), 7.40 (2H, t, J = 7.6 Hz), 7.75 (2H, d, J = 7.7 Hz).

To a solution of the above compound (2.67 g, 5.07 mmol) in dichloromethane (15 mL) was added trifluoroacetic acid (30 mL) under ice-cooling. The mixture was stirred for 1 h, and then concentrated under reduced pressure. The residue was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with chloroform/methanol (10:1, v/v) to give free base of the title compound (1.58 g, 73%) as an amorphous.

To a solution of the above compound (1.57 g, 3.68 mmol) in ethanol (20 mL) was added a solution of maleic acid (0.854 g, 7.36 mmol) in ethanol (20 mL) under ice-cooling. The precipitate was collected by filtration to give the title compound (1.77 g, 72%) as a white powder. Mp: 136 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.04–2.15 (1H, m), 2.15 (3H, s), 2.27 (1H, m), 2.43–2.60 (4H, m), 2.73–2.84 (4H, m), 2.95–3.13 (4H, m), 3.42–3.88 (3H, m), 4.40–4.72 (3H, m), 5.81 (1H, s), 6.15 (4H, s), 7.28 (1H, t, J = 7.5 Hz), 7.45 (2H, t, J = 7.5 Hz), 7.73 (d, J = 7.5 Hz, 2H); Anal. Calcd for $C_{22}H_{30}N_6OS{\cdot}2C_4H_4O_4{\cdot}0.8H_2O$: C, 53.53; H, 5.92; N, 12.48. Found: C, 53.56; H, 5.74; N, 12.10; LC–MS (ESI) m/z 427.2 $[M+H]^+$.

4.1.36. 3-{(2*R*,4*R*)-4-[4-(3-Methyl-1-phenyl-1*H*-pyrazol-5-yl)piperazin-1-yl]pyrrolidin-2-yl-carbonyl}thiazolidine dimaleate (8s)

The title compound was prepared in 49% yield using **12d** and 3-((*R*)-1-*tert*-butoxycarbonyl-4-oxopyrrolidin-2-ylcarbonyl)thiazolidine in the procedures outlined for **8n**. Mp: 166–167 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.66 (1H, m), 2.15 (3H, s), 2.51–2.54 (5H, m), 2.64–2.79 (5H, m), 3.02–3.14 (4H, m), 3.41 (1H, m), 3.60–3.90 (2H, m), 4.42–4.71 (3H, m), 5.80 (1H, s), 6.16 (4H, s), 7.28 (1H, t, J = 7.3 Hz), 7.45 (2H, t, J = 7.3 Hz), 7.73 (2H, d, J = 7.3 Hz); Anal. Calcd for $C_{22}H_{30}N_6OS{\cdot}2C_4H_4O_4$: C, 54.70; H, 5.81; N, 12.76. Found: C, 54.61; H, 5.78; N, 12.68.

4.1.37. 1-Benzyloxycarbonylisonipecotic acid anilide (14)

To a solution of 1-benzyloxycarbonylisonipecotic acid (**13**, 13.1 g, 49.8 mmol), aniline (5.15 g, 54.7 mmol) and HOBt (11.4 g, 74.4 mmol) in tetrahydrofuran (200 mL) was added EDC hydrochloride (11.4 g, 59.5 mmol). The reaction mixture was stirred at room temperature for 17 h, and then concentrated under reduced pressure. The residue wad poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure to give the title compound (8.43 g, 50%) as a pale-yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.61–2.06 (4H, m), 2.44–2. 56 (1H, m), 2.77–3.02 (2H, m), 4.03–4.36 (2H, m), 5.14 (2H, s), 7.11 (1H, t, J = 7.4 Hz), 7.20–7.44 (7H, m), 7.50 (2H, d, J = 7.9 Hz).

4.1.38. 4-(1-Phenyl-1*H*-tetrazol-5-yl)piperidine (15)

To a solution of **14** (2.00 g, 5.91 mmol), triphenylphosphine (3.10 g, 11.8 mmol) and a 40% diisopropyl azodicarboxylate–toluene solution (6.00 g, 11.9 mmol) in tetrahydrofuran (50 mL) was added trimethylsilylazide (1.57 mL, 11.8 mmol) under ice-cooling. The mixture was stirred at room temperature for 5 days, and then concentrated under reduced pressure. The residue was purified by silica gel chromatography with *n*-hexane/ethyl acetate (1:1, v/v) to give 1-benzyloxycarbonyl-4-(1-phenyl-1*H*-tetrazol-5-yl)piperidine (4.09 g) as a brown oil.

To a solution of the above compound (4.09 g) in methanol (50 mL) was added 10% palladium/carbon (420 mg). The resulting mixture was stirred at room temperature under a hydrogen

atmosphere (1 atm) for 5 days. Palladium/carbon was removed by filtration. The filtrate was concentrated under reduced pressure to give the title compound (1.42 g, 55%) as a gray solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.55–2.02 (4H, m), 2.63 (2H, m), 3.00 (1H, m), 3.16 (2H, m), 7.41–7.68 (5H, m).

4.1.39. 3-{(2*S*,4*S*)-4-[4-(1-Phenyl-1*H*-tetrazol-5-yl)piperidino] pyrrolizin-2-ylcarbonyl}thiazolidine dihydrochloride (9a)

The title compound was prepared in 43% yield using **15** in the procedures outlined for **8a**. Mp: >186 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.93–2.34 (5H, m), 2.85–3.95 (13H, m), 4.43–4.77 (3H, m), 7.69 (5H, s), 9.12 (1H, br s), 10.74 (1H, br s), 12.04 (1H, br s); Anal. Calcd for $C_{20}H_{27}N_7OS{\cdot}2HCl{\cdot}0.4C_2H_6O{\cdot}2H_2O$: C, 46.18; H, 6.60; N, 18.13. Found: C, 46.39; H, 6.48; N, 18.26; LC–MS (ESI) m/z 414.4 $[M+H]^+$.

4.1.40. 4-Trifluoroacetoacetylpyridine (17)

To a solution of ethyl trifluoroacetate (6.32 g, 44.5 mmol) and a 28% sodium methoxide–methanol solution (9.4 g, 49 mmol) in *tert*-butyl methyl ether (10 mL) was added a solution of 4-acetylpyridine (**16**, 4.90 g, 40.4 mmol) in *tert*-butyl methyl ether (20 mL) at room temperature and the mixture was stirred for 22 h. A 10% citric acid aqueous solution was added until the reaction solution became about pH 4. The precipitate was collected by filtration, washed with water and dried to give the title compound (5.46 g, 62%) as a yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.60 (1H, br s), 7.96 (2H, d, J = 5.5 Hz), 8.82 (2H, d, J = 5.5 Hz).

4.1.41. 4-(3-Trifluoromethyl-1-phenyl-1*H*-pyrazol-5-yl)pyridine (18)

To a suspension of **17** (760 mg, 3.50 mmol) of ethanol (20 mL) was added phenylhydrazine (0.38 mL, 3.9 mmol) at room temperature. After stirring for 23 h, the reaction mixture was concentrated under reduced pressure. The residue was poured into water and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with chloroform/methanol (7:3, v/v) to give the title compound (470 mg, 47%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.89 (1H, s), 7.22 (2H, d, J = 5.9 Hz), 7.30–7.47 (5H, m), 8.59 (2H, d, J = 5.9 Hz).

4.1.42. 1-Benzyl-4-(3-trifluoromethyl-1-phenyl-5-pyrazolyl)-1,2,3,6-tetrahydropyridine (19)

To a solution of **18** (470 mg, 1.62 mmol) in acetonitrile (50 mL) was added benzyl chloride (0.38 mL, 3.3 mmol). After stirring under reflux for 24 h, the reaction mixture was concentrated under reduced pressure, and diethyl ether was added to the residue. The precipitate was collected by filtration. To a solution of this solid in ethanol was added sodium borohydride (130 mg, 3.44 mmol) under ice-cooling. The mixture was stirred at room temperature for 22 h. The reaction solution was poured into water and extracted with ethyl acetate. The extract was washed with brine, dried and concentrated under reduced pressure. The residue was purified by silica gel chromatography with *n*-hexane/ethyl acetate (4:1, v/v) to give the title compound (142 mg, 23%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.15 (2H, m), 2.53 (2H, d, J = 5.6 Hz), 3.03 (2H, m), 3.56 (2H, s), 5.78 (1H, m), 6.53 (1H, s), 7.23–7.51 (10H, m).

4.1.43. 1-(3-Trifluoromethyl-1-phenyl-1*H*-pyrazol-5-yl) piperidine (20)

To a solution of **19** (142 mg, 0.37 mmol) and ammonium formate (240 mg, 3.81 mmol) in methanol (20 mL) was added 10% palladium/carbon (150 mg). The mixture was heated under reflux under a nitrogen atmosphere for 2 h. After removal of the insoluble materials by filtration, the filtrate was concentrated under reduced pressure. The residue was poured into a saturated aqueous sodium hydrogen carbonate solution and extracted with chloroform. The extract was dried and concentrated under reduced pressure to give the title compound (90 mg, 82%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.47–1.93 (4H, m), 2.54 (2H, dt, J = 2.4, 12.3 Hz), 2.76 (1H, m), 3.07 (2H, m), 6.48 (1H, s), 7.36–7.55 (5H, m).

4.1.44. 3-{(2*S*,4*S*)-4-[4-(3-Trifluoromethyl-1-phenyl-1*H*-pyrazol-5-yl)piperidino]pyrrolizin-2-ylcarbonyl}thiazolidine dihydro chloride (9b)

The title compound was prepared in 56% yield using **20** in the procedures outlined for **8a**. Mp: 280 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.90–2.30 (5H, m), 2.83–4.00 (13H, m), 4.46–4.71 (3H, m), 6.78 (1H, s), 7.57–7.62 (5H, m), 9.07 (1H, br s), 10.45 (1H, br s), 11.82 (1H, br s); Anal. Calcd for $C_{23}H_{28}F_3N_5OS{\cdot}2HCl{\cdot}H_2O$: C, 48.43; H, 5.65; N, 12.28. Found: C, 48.34; H, 5.78; N, 12.03; LC–MS (ESI) m/z 480.4 $[M+H]^+$.

4.2. X-Ray crystallographic studies

The protein of human DPP-4 (33−766) secreted from insect cells was purified and crystallized according to the method reported by Hiramatsu et al.[24] The protein–inhibitor complex was obtained by soaking a preformed DPP-4 crystal in the presence of compound **8g** and preserved in liquid nitrogen for data collection at 100 K. X-ray diffraction data were collected at the High Energy Accelerator Research Organization (KEK) beam line BL5 and processed using the program HKL2000.[25] The structure of DPP-4 inhibitor complex was solved by molecular replacement with the program PHASER,[26] utilizing the previously determined coordinates of DPP-4 with Protein Data Bank accession code 1J2E. Data collection and model refinement statistics are summarized in Table 5.

4.3. Docking studies in DPP-4

The X-ray crystal structure of DPP-4 (PDB code: 3VJM) was utilized in the docking calculations. The compounds were docked into DPP-4 using Glide 5.7.[27]

4.4. Pharmacokinetic studies on rats and monkeys

4.4.1. Measurement of compound 8g concentration in plasma

Concentrations of compound **8g** in plasma were determined by liquid chromatography with tandem mass spectrometry (LC–MS/MS). Plasma samples (50 μL) were placed in polypropylene test tubes and the internal standard was added. The solution was loaded into a solid-phase extraction cartridge (OASIS HLB), preconditioned with acetonitrile (1 mL) and water (1 mL). The cartridge was washed twice with water (1 mL), and the analyte and internal standard were eluted with acetonitrile (1 mL). The eluate was evaporated to dryness under a nitrogen stream (40 °C) and the residue was reconstituted with 200 μL of the mixed solvent (water/acetonitrile/formic acid, 95:5:1, v/v). After the mixture was mixed by the vortex-mixer, it was centrifuged and a portion of the supernatant was injected into the LC–MS/MS system. Analyte concentrations were evaluated using the internal standard method. Standard curves were calculated from the peak area ratio (par) of analyte/internal standard. The nominal compound **8g** concentrations were obtained using linear regression $y = a + bx$. The measured peak area ratios of samples were converted into concentrations using the equation: concentration = par (analyte/internal standard) minus intercept of the corresponding standard curve divided by slope of the standard curve.

4.4.2. Pharmacokinetic analysis

The compound **8g** plasma concentration–time profiles were analyzed and its pharmacokinetic parameters were calculated

using non-compartment analysis in the pharmacokinetic analysis program WinNonlin (Pharsight Corporation, Ver. 4.0.1).

4.5. Biological experiments

4.5.1. DPP-4 inhibitory activity

The DPP-4 inhibitory activity of human and rat plasma was measured by fluorescence assay using Gly-Pro-MCA (Peptide Institute Inc.) as a DPP-4-specific fluorescent substrate. Reaction solutions containing 20 μL of human or rat plasma (20-fold diluted solution for human plasma and 10-fold diluted solution for rat plasma), 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-4 activity. The inhibitory rate relative to the solvent addition group was calculated and IC_{50} values were determined by logistic analysis.

4.5.2. DPP-8,9 inhibitory activity

The DPP8 and DPP9 activity were evaluated using the cytoplasmic fractions of cells expressing human DPP8 or DPP9 as enzyme sources. Reaction solutions containing 20 μL of test compounds of various concentrations, 20 μL of enzyme preparations, 140 μL of buffer (0.003% Brij-35 containing PBS), and 20 μL of Gly-Pro-MCA (50 μmol/L, Peptide Institute Inc.) were incubated at 37 °C for 30 min using a 96-well flat-bottomed microtiter plate. The measured fluorescence intensity of 7-amino-4-methylcoumarin was taken as the enzyme.

4.5.3. Plasma DPP-4 activity after oral administration of 8g to Wistar rats

Male Wistar rats (7–9 weeks of age) fasted overnight were used. Compound **8g** (HBr salt) was dissolved in 0.5% hydroxypropyl methylcellulose (HPMC) and administered orally at a dose of 0.03, 0.1, 0.3 and 1 mg/kg. At pre-administration and at 0.5, 1, 2, 3, 6, 9, 12, 24 and 48 h after administration, 0.1 mL of blood was collected from tail vein. After centrifugation, 10 μL of plasma was diluted 10-fold using buffer (0.003% Brij-35 containing PBS). Twenty microliter of the diluted plasma was used instead of 20 μL of test substrate for the determination of DPP-4 inhibitory activity by fluorescence as described above.

4.5.4. 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.[28] Male Zucker fatty and lean rats (13 weeks of age; $n = 10$) were fasted overnight. Compound **8g** (HBr salt) was dissolved in 0.5% HPMC solution and administered orally. After 30 min, glucose solution was orally administered at 1 g/kg body weight. Blood samples were collected from the tail veins at the indicated times and plasma samples were prepared. Plasma glucose concentrations as well as plasma DPP-4 activity were measured.

Acknowledgments

The authors thank Dr. Kunitomo Adachi, Dr. Hitoshi Kubota and Dr. Kousuke Yasuda for their helpful discussions; Dr. Yoshitaka Ikeda and Mr. Masami Yamashita for analytical support; and Dr. Tohru Nakajima and Dr. Takao Kondo for their insight and guidance for course of this work.

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