

Synthesis and biological evaluation of pyrrolidine-2-carbonitrile and 4-fluoropyrrolidine-2-carbonitrile derivatives as dipeptidyl peptidase-4 inhibitors for the treatment of type 2 diabetes



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ABSTRACT

A novel series of pyrrolidine-2-carbonitrile and 4-fluoropyrrolidine-2-carbonitrile derivatives was designed, synthesized, and found to act as dipeptidyl peptidase-4 (DPP-4) inhibitors. From this series of compounds, compound **17a** was identified as an efficacious, safe, and selective inhibitor of DPP-4. In vivo studies in ICR and KKAY mice showed that administration of this compound resulted in decreased blood glucose in these mice after an oral glucose challenge. Compound **17a** showed high DPP-4 inhibitory activity ($IC_{50} = 0.017 \mu M$), moderate selectivity against DPP-4 (selective ratio: DPP-8/DPP-4 = 1324; DPP-9/DPP-4 = 1164), and good efficacy in oral glucose tolerance tests in ICR and KKAY mice. These in vivo anti-diabetic properties and its desirable pharmacokinetic profile in Sprague–Dawley rats demonstrate that compound **17a** is a promising candidate for development as an anti-diabetic agent.

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1. Introduction

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes. At the start of the current millennium, the global number of patients with diabetes was estimated to be 171 million, and this number is predicted to increase to be 366 million by 2030.¹ The main goal of T2DM treatment is glycemic control, to obtain a blood glucose level as close to the non-diabetic range as possible, which reduces the risk of complications associated with late-stage T2DM. The incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are released by gut endocrine cells in response to food intake, and play important roles in glucose homeostasis.² GLP-1 has a range of physiological effects, such as increasing insulin secretion, β -cell mass and insulin gene expression, inhibiting acid secretion and gastric emptying in the stomach, decreasing food intake by increasing satiety in brain, and promoting insulin sensitivity.^{3,4} However, GLP-1 and GIP are rapidly degraded and inactivated in vivo by the serine protease dipeptidyl peptidase-4 (DPP-4), which limits their ability to normalize blood glucose levels. Therefore, compounds that inhibit DPP-4 are used in the treatment of type 2 diabetes.^{5–8} DPP-4 inhibitors are a new class of anti-hyperglycemic agents, and the follow-

ing drugs have recently been approved for use in T2DM treatment: Sitagliptin (Januvia[®], MK-0431),^{9–11} Vidagliptin (Glavus[®], LAF-237),^{12–14} Saxagliptin (Onglyza[®], BMS-477118),¹⁵ Alogliptin (Nesina[®], SYR-322),¹⁶ and Linagliptin (Tradjenta[®], BI-1356)¹⁷ (Fig. 1).

DPP-4 is structurally similar to other serine proteases, thus, to avoid unwanted side effects, it is important to ensure that any newly synthesized DPP-4 inhibitor acts selectively against DPP-4, and does not affect other serine proteases. It is especially important that DPP-4 inhibitors do not inhibit DPP-8 or DPP-9, as inhibition of these enzymes has been associated with multi-organ toxicity in rats and dogs, and the in vitro attenuation of human T-cell activation.¹⁸ Our interest in DPP-4 inhibition as a T2DM therapy prompted our investigation into a novel class of DPP-4 inhibitors. Here, we report the design, synthesis, structure-activity relationships, and biological evaluation studies of pyrrolidine-2-carbonitrile and 4-fluoropyrrolidine-2-carbonitrile derivatives as novel DPP-4 inhibitors.

2. Results and discussion

2.1. Design

Fluorinated compounds comprise a substantial proportion of therapeutic drugs,¹⁹ it is an important strategy to introduce fluorine or fluorinated substitutes into small molecules in structure-based medicinal chemistry.²⁰ Fluorination can modulate the

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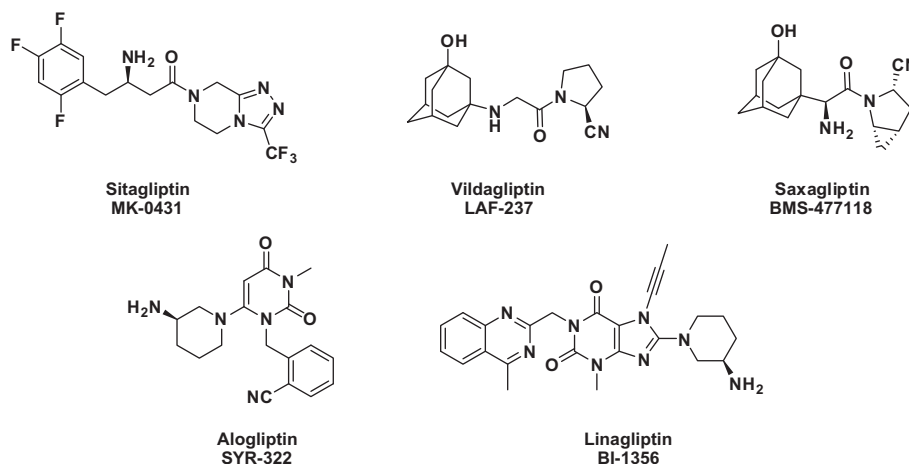


Figure 1. DPP-4 inhibitors on the market.

physicochemical and pharmacokinetic properties of compounds by improving bioavailability, improving binding affinity and target selectivity by alteration of their molecular conformations, or increasing their stability by blocking metabolic site.^{21–24} Our studies of the structure-activity relationships (SARs) of Vildagliptin and Saxagliptin have led to the design of pyrrolidine-2-carbonitrile derivatives, which showed that the nitrile group effectively formed a covalent adduct with the catalytic Ser630 residue and binded to DPP-4 in the S1 pocket (Fig. 2). Furthermore, introducing the fluorine atom in Denagliptin²⁵ demonstrated that the inhibitory activity was improvement compared to pyrrole-2-carbonitrile derivatives. The reason might be due to the fluorine atom interactions with certain amino acid residues via hydrogen bonding. A series of pyrrolidine-2-carbonitrile and 4-fluoropyrrolidine-2-carbonitrile derivatives, all of which retained the pyrrolidine-2-carbonitrile fragment, was designed, synthesized, and evaluated as potential DPP-4 inhibitors.

2.2. Chemistry

The general synthetic procedures used in the synthesis of the series of target compounds **7a–r** are outlined in Scheme 1. A straightforward, six-step, synthetic route enabled us to vary position R. (*S*)-Proline was the starting material for the synthesis of compound **1**. Compound **4** was obtained from compound **1** via amidation, dehydration, and deprotection, followed by individual coupling reactions with *N*-Boc- α -amino acids (**5a–r**) to yield the compounds **6a–r**. Deprotection of compounds **6a–r** with trifluoroacetic acid (TFA) produced the target compounds **7a–r**.

Compounds **17a–o** were synthesized according to Scheme 2. Compound **8**, which is commercially available, was protected and then fluorinated with diethylaminosulfur trifluoride (DAST) to generate compound **10**. Compound **14** was obtained from compound **10** via demethylation, amidation, dehydration, and deprotection. The intermediate **14** coupled with the *N*-Boc- α -amino acids

individually (**15a–o**), under standard conditions, to yield the compounds **16a–o**. Deprotection of compounds **16a–o** with TFA produced the target compounds **17a–o**.

2.3. In vitro enzyme inhibition studies and structure-activity relationships

All the synthesized compounds (**7a–r** and **17a–o**) were evaluated in vitro for their capacity to inhibit human recombinant DPP-4. Inhibitory potency was measured by monitoring the hydrolysis of Ala-Pro-aminomethylcoumarin (Ala-Pro-AMC) by human DPP-4, using the reaction with Vildagliptin as the positive control; these results are reported as the concentrations required for 50% inhibition (IC₅₀) of DPP-4. Data on the inhibitory effects of the synthesized compounds on the enzymes DPP-7, DPP-8, DPP-9, and fibroblast activation protein (FAP) are also presented because the specificity of inhibition on DPP-4 is critical, as any in vivo inhibition of the related enzymes DPP-8 and DPP-9 may be associated with profound toxic effects.¹⁸ Structure-activity relationships (SARs) for these compounds are summarized in Tables 1 and 2.

Our SAR analysis started with phenyl-substituted α -amino pyrrolidine-2-carbonitrile compounds, and explored the effects of regiochemical substitutions and substitutions of electron-donating or withdrawing groups on the benzene ring. As shown in Table 1, most of the synthesized compounds were good in vitro DPP-4 inhibitors, and selectively inhibited DPP-4 over DPP-8 and DPP-9. Additionally, all of these inhibitors occupied a narrow potency range, indicating tolerability for a wide variety of substituents on the benzene ring. Compound **7a**, which was non-substituted at the benzene ring, was found to be a potent and selective inhibitor of DPP-4 (IC₅₀ = 0.027 μ M, SR: DPP-8/DPP-4 = 7.8; DPP-9/DPP-4 = 12.2). The mono-fluoro substituted compounds demonstrated regiochemical preferences of *para* > *ortho* > *meta* (**7d** = 0.011 μ M, **7b** = 0.018 μ M, and **7c** = 0.248 μ M). A fluorine group (**7d**) at the *para*-position improved DPP-4 inhibitory potency and selectivity (SR: DPP-8/DPP-4 = 600; DPP-9/DPP-4 = 369). Both electron-donating and electron-withdrawing groups on the benzene ring tolerated DPP-4 inhibition. For example, compounds **7e** (4-Me), **7f** (4-OMe), **7g** (4-NH₂), **7h** (4-NO₂), **7i** (4-CN), and **7j** (4-CF₃) were desirable inhibitors of DPP-4, with IC₅₀ values of 0.017, 0.029, 0.075, 0.020, 0.021, and 0.031 μ M, respectively. The halogen-substituted compounds, **7k** (4-Cl) and **7l** (4-Br) also displayed good potency against DPP-4. However, large substitution, for example **7m** (a phenyl at the *para*-position) reduced the DPP-4 inhibitory potency and selectivity. Electron-donating and electron-withdrawing groups at the 2-position of the benzene ring (**7n**, **7o**, and **7p**) also

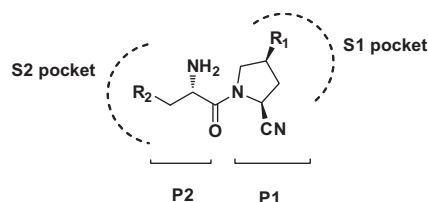
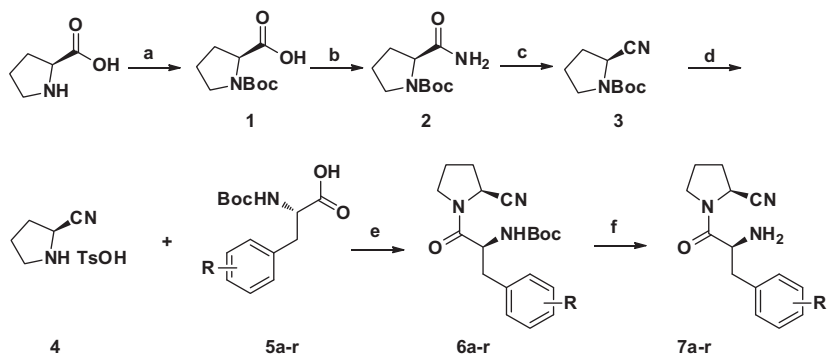
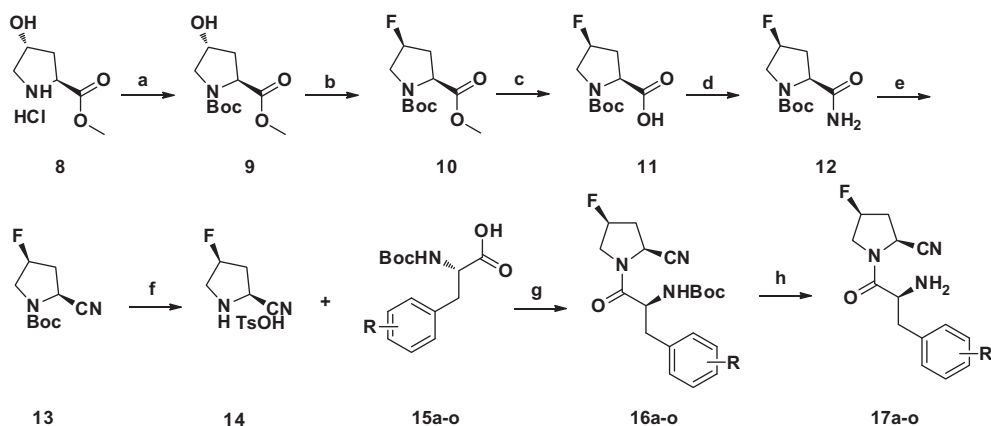


Figure 2. Design of novel pyrrole-2-carbonitrile derivatives.



Scheme 1. Reagents and conditions: (a) $(\text{Boc})_2\text{O}$, NaHCO_3 , dioxane, 24 h; (b) $(\text{Boc})_2\text{O}$, NH_4HCO_3 , pyridine, dioxane, 6 h; (c) cyanuric chloride, DMF, 1 h; (d) TsOH, CH_3CN , rt, 24 h; (e) EDCI, HOBT, TEA, DMF, 20 h; (f) CH_2Cl_2 , TFA, 0 °C to rt, 1 h.



Scheme 2. Reagents and conditions: (a) $(\text{Boc})_2\text{O}$, NaHCO_3 , dioxane, 24 h; (b) DAST, CH_2Cl_2 , -78 °C to rt, 24 h; (c) LiOH, dioxane, H_2O , overnight; (d) $(\text{Boc})_2\text{O}$, NH_4HCO_3 , pyridine, dioxane, 6 h; (e) cyanuric chloride, DMF, 1 h; (f) TsOH, CH_3CN , rt, 24 h; (g) EDCI, HOBT, TEA, DMF, 20 h; (h) CH_2Cl_2 , TFA, 0 °C to rt, 1 h.

provided potent DPP-4 inhibitory activities, among which **7o** (2-CN) or **7p** (2- CF_3), led to good selectivity. In addition, presence of a cyano group (**7q**) or trifluoromethyl group (**7r**) at the *meta*-position reduced inhibitory potency (by 2.3 and 7.7-fold, respectively). Fluorine group at the *ortho*-position of the benzene ring (**7b**) provided excellent DPP-7 selectivity (SR: DPP-7/DPP-4 = 16494.4), replacement of the **7b** (*o*-fluorine) with electron-withdrawing group at the *meta*-position, **7q** (3-CN) or **7r** (3- CF_3) led to poor selectivity against DPP-7 (**7q**, SR: DPP-7/DPP-4 = 20.95; **7r**, SR: DPP-7/DPP-4 = 8.14). The **7i** (4-CN, SR: FAP/DPP-4 = 38.09) or **7m** (4-Ph, SR: FAP/DPP-4 = 9.72) showed low FAP selectivity. While, replacing **7i** (4-CN) with **7o** (2-CN) led to good selectivity over FAP (**7o**, SR: FAP/DPP-4 = 1023.3).

Finally, an investigation of the inhibitory activity and DPP-4 selectivity of these α -amino pyrrolidine-2-carbonitrile derivatives showed that compound **7d** exhibited both desirable inhibitory activity and enzyme selectivity.

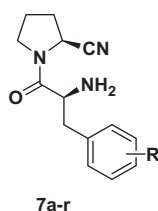
To identify a compound with enhanced inhibitory activity and enzyme selectivity, 4-fluoropyrrolidine-2-carbonitrile derivatives (**17a–o**) were designed, synthesized, and evaluated (Table 2). Compound **17a** exhibited excellent inhibitory activity (IC_{50} = 0.017 μM) and good selectivity against other serine proteases (SR: DPP-8/DPP-4 = 1324; DPP-9/DPP-4 = 1164). In order to improve the inhibitory activity of DPP-4 in vitro, electron-withdrawing and electron-donating groups were introduced at the *para*-position of the benzene ring. Introduction of a fluorine group at this position (**17b**) showed slightly improved DPP-4 activity, but low enzyme selectivity (IC_{50} = 0.003 μM , SR: DPP-8/DPP-4 = 970; DPP-9/DPP-4 = 463.3). Other compounds with electron-withdrawing or donating groups

exhibited satisfactory inhibition against DPP-4: compounds **17c** (4-Me), **17d** (4- OCH_3), **17e** (4- NO_2), **17f** (4-CN), and **17g** (4- CF_3) gave IC_{50} values of 0.004, 0.015, 0.292, 0.005, and 0.006 μM , respectively. However, sterically hindered substitutes at the *para*-position, such as **17h** (4-*tert*-butyl) or **17i** (4-benzyloxy), resulted in a dramatic decrease in activity; the **17j** (2-cyano) or **17k** (2-trifluoromethyl), resulted in lower DPP-4 inhibition activity. The 2,4-difluorophenyl analog (**17l**), 2,4,5-trifluorophenyl analog (**17m**), 2,3,4-trifluorophenyl analog (**17n**), and 2,3,5-trifluorophenyl analog (**17o**) showed decreased DPP-4 potencies compared with the 4-fluorophenyl analog (**17b**). Comparing compound **17a** with compounds **17b–o**, the substitution on the benzene ring played an important role in the DPP-7 and FAP potency activity of compounds. Generally, **17j** (2-CN) and **17k** (2- CF_3) are favorable to the selectivities. However, large aromatic **17i** (4-OBn) or **17l–o** (multi fluorine substituents) are improper.

Compound **17a** was the most potent compound of this type, it displayed excellent DPP-4 inhibition, with an IC_{50} value of 0.017 μM , and moderate enzyme selectivity for DPP-4 over DPP-8 and DPP-9 which is 13.5 and 116.4 times more selective than the positive control Vidagliptin.

According to the above results (Tables 1 and 2), we can draw some noteworthy conclusions: (1) introduction of the fluorine atom at the *para*-position of the benzene ring, the DPP4 inhibitory activity of compounds **7d** and **17b** was showed 2.5-fold and 5.6-fold improvement compared to compounds **7a** and **17a**. The probable reason was that the fluorine atom formed a hydrogen bond with residue Phe357. (2) Through rational design, compound **17a** was identified as an effective DPP-4 inhibitor. Compared to

Table 1
Potency and selectivity of pyrrolidine-2-carbonitrile derivatives



Compd	R	IC ₅₀ ^a (μM)					SR ^b	
		DPP-4	DPP-7	DPP-8	DPP-9	FAP	DPP-8/DPP-4	DPP-9/DPP-4
7a	H	0.027	84.43	0.21	0.33	N.D. ^c	7.8	12.2
7b	2-F	0.018	296.9	9.4	6.5	2.22	522	361.1
7c	3-F	0.248	5.44	0.63	2.0	N.D.	2.54	8.06
7d	4-F	0.011	5.88	6.6	4.06	1.58	600	369
7e	4-Me	0.017	8.78	6.22	20.15	1.46	366	1185
7f	4-OMe	0.029	4.67	46.82	8.09	2.14	1614	278.9
7g	4-NH ₂	0.075	1.7	0.52	2.03	N.D.	6.9	27.1
7h	4-NO ₂	0.020	2.14	3.04	1.39	N.D.	152	69.5
7i	4-CN	0.021	1.57	7.91	8.14	0.8	377	388
7j	4-CF ₃	0.031	7.01	52.12	11.14	7.87	1681	359
7k	4-Cl	0.004	6.22	0.96	4.72	3.01	240	1180
7l	4-Br	0.004	4.11	0.90	1.95	2.69	225	487.5
7m	4-Ph	0.145	4.13	3.44	9.42	1.41	23.7	64.9
7n	2-Me	0.042	N.I. ^d	2.72	9.72	5.81	64.8	231.4
7o	2-CN	0.027	N.I.	40.77	15.97	27.63	1510	591.5
7p	2-CF ₃	0.046	N.I.	17.56	22.9	2.6	381.7	497.8
7q	3-CN	0.063	1.32	29.6	9.46	1.58	469.8	150.2
7r	3-CF ₃	0.209	1.7	0.52	2.03	N.D.	2.49	9.71
Vidagliptin	—	0.020	N.I.	1.96	0.20	3.72	98	10

^a In vitro activity.

^b Selectivity Ratio.

^c N.D.: not detected.

^d N.I.: no inhibition.

compound **7a**, the inhibitory activity of compound **17a** was increased 1.6-fold against DPP-4, and the selectivity was increased 169.7-fold (to DPP-8/DPP-4) and 95.4-fold (to DPP-9/DPP-4). The probable reason was that the 4-fluoropyrrolidine-2-carbonitrile moiety occupied S1 pocket formed by residues of Ser630, His740, and Tyr547, the fluorine atom interactions with certain amino acid residues via hydrogen bonding. (3) Introduction of large groups such as 4-Ph and 4-*t*Bu on the benzene ring cannot be tolerated, nearly leading to a loss of activity.

2.4. In vivo studies

2.4.1. Effects of **17a** on glucose excursion after an oral glucose tolerance test (OGTT) in ICR mice

Compound **17a** was chosen for in vivo evaluation as it showed excellent in vitro potency and selectivity. Following an oral glucose challenge in ICR mice, compound **17a** had an acute effect on glucose excursion: compound **17a** (1, 3, 10, and 30 mg/kg), Vidagliptin (**LAF237**) (1, 3, and 10 mg/kg) or vehicle (distilled water) was orally administered to 6 h-fasted ICR mice ($n = 6$ in each group) 30 min prior to an oral glucose load (2.5 g/kg). Blood glucose values in the mice were measured at 0, 30, 60, and 120 min after glucose loading.

As shown in Table 3 and Figure 3, blood glucose levels reached a peak value 30 min after glucose loading. Administration of **17a**, at the doses of 1, 3, 10, and 30 mg/kg caused a significant reduction in the blood glucose peak values of 18.75%, 27.36%, 29.13%, and 27.57%, respectively ($P < 0.05$). **LAF237** showed a stronger glucose lowering effect, with reductions of 24.10%, 33.48%, and 35.51% at the doses of 1, 3, and 10 mg/kg, respectively ($P < 0.01$). Blood

glucose excursion profiles, from 0 to 120 min, were drawn and used to calculate the area under the curve (AUC_{0–120 min Glu}) for each treatment. Both compound **17a** and **LAF237** caused significantly decreases in the AUC_{0–120 min Glu}.

Therefore, in ICR mice, a single oral dose of compound **17a** can reduce blood glucose levels, and the AUC_{0–120 min Glu} following an OGTT in a dose-dependent manner.

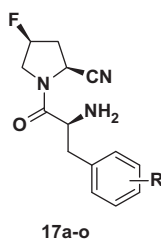
2.4.2. Effects of **17a** on glucose excursion after an OGTT in type 2 diabetic KKAY mice

Male KKAY mice were divided into 4 groups ($n = 5$ in each group) based on overnight fasting blood glucose levels. Compound **17a** (10 and 30 mg/kg), **LAF237** (10 mg/kg) or vehicle (distilled water) were orally administered to overnight fasted KKAY mice 30 min prior to an oral glucose load (2.5 g/kg).

Blood glucose levels were measured at 0, 30, 60, and 120 min after the glucose load. As shown in Table 4 and Figure 4, KKAY mice showed hyperglycemia and impaired glucose tolerance in response to oral glucose challenges. In these type 2 diabetic mice, following an OGTT, a single oral dose of **17a** reduced blood glucose levels and the area under the glucose profile curve, AUC_{0–120 min} in a dose-dependent manner. At 60 min after glucose loading, **17a** (at a dose of 10 or 30 mg/kg) caused a significant reduction in blood glucose (32.1% and 35.8%, respectively, $P < 0.01$). **LAF237** (10 mg/kg) showed a similar glucose lowering effect, with a reduction of 40.9%.

Compound **17a** (10 and 30 mg/kg) reduced the area under the glucose curve (AUC_{0–120 min Glu}) by 19.2% and 26.4%, respectively ($P < 0.05$), while **LAF-237** (10 mg/kg) showed a decrease of 30.0% ($P < 0.01$). Therefore, in KKAY mice, a single oral dose of **17a** reduces

Table 2
Potency and selectivity of 4-fluoropyrrolidine-2-carbonitrile derivatives



Compd	R	IC ₅₀ ^a (μM)					SR ^b	
		DPP-4	DPP-7	DPP-8	DPP-9	FAP	DPP-8/DPP-4	DPP-9/DPP-4
17a	H	0.017	14.52	22.51	19.79	1.53	1324	1164
17b	4-F	0.003	1.44	2.91	1.39	0.082	970	463.3
17c	4-Me	0.004	1.72	2.55	1.76	0.47	637.5	440
17d	4-OMe	0.015	1.44	1.34	2.867	0.14	89.3	191.1
17e	4-NO ₂	0.292	39.09	>58	>58	3.65	>198.6	>198.6
17f	4-CN	0.005	0.80	3.11	1.29	0.47	622	258
17g	4-CF ₃	0.006	N.D. ^c	0.24	2.49	0.61	40	622.5
17h	4-tBu	0.125	1.08	7.19	9.19	1.70	57.5	73.5
17i	4-OBn	0.094	0.12	3.42	7.74	0.14	36.4	82.3
17j	2-CN	0.022	100	4.94	5.60	3.91	225.9	254.5
17k	2-CF ₃	0.020	102	8.97	3.14	6.10	448.5	157
17l	2,4-F ₂	0.006	1.81	0.96	0.87	0.15	160	145
17m	2,4,5-F ₃	0.017	0.90	3.35	2.74	0.35	197.1	161.2
17n	2,3,4-F ₃	0.023	2.54	1.07	1.00	0.22	46.52	43.47
17o	2,3,5-F ₃	0.060	1.29	2.35	7.36	0.90	39.2	122.7
Vidagliptin	—	0.020	N.I. ^d	1.96	0.20	3.72	98	10

^a In vitro activity.

^b Selectivity ratio.

^c N.D.: not detected.

^d N.I.: no inhibition.

Table 3
Glucose responses during an OGTT in ICR mice following treatment with **17a** or **LAF237** (mmol/L, Mean ± SD, n = 6)

Group	Dose (mg/kg)	Post-dose (min)				AUC _{0-120 min} Glu (mmol/L·h)
		0	30	60	120	
Control	—	6.84 ± 1.12	13.42 ± 1.12	11.07 ± 1.09	8.04 ± 0.79	20.74 ± 1.25
LAF237	1	6.84 ± 1.03	10.18 ± 1.58**	10.05 ± 0.72	7.29 ± 0.98	17.99 ± 1.99**
	3	6.74 ± 1.58	8.92 ± 0.85***	9.55 ± 0.54*	7.29 ± 0.79	16.96 ± 1.40***
	10	6.85 ± 1.90	8.65 ± 0.91***	8.91 ± 1.62*	7.21 ± 1.99	16.33 ± 2.91**
17a	1	6.66 ± 1.34	10.90 ± 1.78*	10.88 ± 1.12	8.79 ± 0.62	19.67 ± 1.78
	3	6.54 ± 1.82	9.75 ± 1.23***	9.54 ± 1.69	7.40 ± 1.57	17.37 ± 2.76*
	10	7.08 ± 0.51	9.51 ± 0.83***	9.50 ± 0.72*	8.07 ± 0.71	17.68 ± 1.20**
	30	7.00 ± 0.86	9.72 ± 0.74***	9.64 ± 0.45*	8.02 ± 0.38	17.84 ± 0.83***

* P < 0.05.

** P < 0.01.

*** P < 0.001, versus control.

blood glucose levels, following an OGTT in a dose-dependent manner.

2.5. Pharmacokinetic evaluation of compound **17a**

The pharmacokinetic (PK) profile of the compound **17a** was assessed in Sprague–Dawley (SD) rats. Compound **17a** showed a high AUC_{0-t} and a high maximal concentration (C_{max}) when dosed orally. The half-life and the absolute oral bioavailability of compound **17a** were 5.4 h and 21.8%, respectively (Table 5).

3. Conclusions

We described the design and synthesis of a novel series of pyrrolidine-2-carbonitrile and 4-fluoropyrrolidine-2-carbonitrile

derivatives, which selectively inhibit DPP-4, but not the related enzymes DPP-8 and DPP-9. Moreover, of these derivatives, compound **17a** possessed satisfactory in vitro DPP-4 inhibitory activity, moderate selectivity, good in vivo efficacy in an OGTT in ICR and KKAY mice, and has a pharmacokinetic profiles suitable for clinical use. Further studies are underway to optimize this class of compounds for use in the treatment of T2DM.

4. Experiments

4.1. Chemistry

The reagents (chemicals) were purchased and used without further purification. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker AMX-400 and AMX-300 NMR (1S as

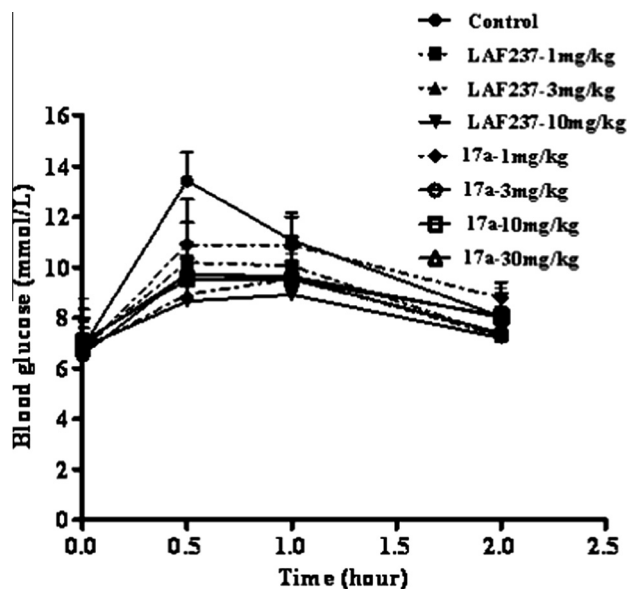


Figure 3. Glucose responses during an OGTT in ICR mice following treatment with **17a** or **LAF237** (Mean \pm SD, $n = 6$).

TMS). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). Low- and high-resolution mass spectra (LRMS and HRMS) were given with electric, electrospray, and matrix-assisted laser desorption ionization (EI and ESI) produced by a Finnigan MAT-95, LCQ-DECA spectrometer and IonSpec 4.7 T.

4.1.1. (2S)-1-(tert-Butoxycarbonyl)pyrrolidine-2-carboxylic acid (**1**)

A solution of (2S)-pyrrolidine-2-carboxylic acid (0.5 g, 4.34 mmol) in dioxane (15 mL) was added (Boc)₂O (1.14 g, 5.21 mmol) and saturated NaHCO₃ (10.5 mL). The reaction was stirred at room temperature overnight. The solvent was then removed in vacuo and CH₂Cl₂ was added. The organics were washed with H₂O twice and saturated NaCl once, then dried, filtered and concentrated. The residue was purified by flash chromatography on silica gel, eluted with a mixture of EA/PE (1:5, v/v), to afford **8** (0.8 g, 86%) as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 4.37–4.24 (m, 1H), 3.58–3.32 (m, 2H), 2.41–1.85 (m, 4H), 1.46 (s, 9H). MS (ESI) m/z 216 [M+H]⁺.

4.1.2. tert-Butyl (2S)-2-carbamoylpyrrolidine-1-carboxylate (**2**)

A mixture of compound **1** (1.00 g, 4.65 mmol), (Boc)₂O (1.52 g, 6.97 mmol), NH₄HCO₃ (0.55 g, 6.97 mmol) and pyridine (1.0 mL) in dioxane (20 mL) was stirred at room temperature for 6 h. The product was extracted with CH₂Cl₂, washed with 1 M HCl and

saturated NaCl, dried, filtrated, and concentrated. *n*-Hexane (100 mL) was added and the product **2** (0.85 g, 85%) began to precipitate using the ultrasound as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 4.37–4.34 (m, 1H), 3.47–3.36 (m, 2H), 2.07–1.85 (m, 4H), 1.49 (s, 9H). MS (ESI) m/z 215 [M+H]⁺.

4.1.3. tert-Butyl (2S)-2-cyanopyrrolidine-1-carboxylate (**3**)

A mixture of compound **2** (5 g, 23.3 mmol) and cyanuric chloride (2.58 g, 14.0 mmol) in DMF (10 mL) was stirred at room temperature for 1 h (monitored by TLC). After the reaction completed, the solution was extracted with EtOAc, washed, dried, concentrated, and purified by flash chromatography on silica gel, eluted with a mixture of PE/EA (1/1, v/v), to afford **3** (3.48 g, 76%) as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 4.76 (s, 1H), 3.51 (s, 2H), 2.34–2.31 (m, 1H), 2.17–2.10 (m, 2H), 1.90–1.87 (m, 1H), 1.49 (s, 9H). MS (ESI) m/z 197 [M+H]⁺.

4.1.4. (2S)-Pyrrolidine-2-carbonitrile 4-methylbenzene-1-sulfonic acid (**4**)

A solution of compound **3** (10.0 g, 50.96 mmol) in CH₃CN (50 mL) was added 4-methylbenzenesulfonic acid hydrate (14.54 g, 76.43 mmol) and stirred at room temperature for 24 h. After the reaction completed, the solution was removed in vacuo. The residual white solid was dissolved in EtOAc (100 mL) and put into fridge overnight, the product **4** (10.3 g, 75%) was precipitated as a white needle crystal. ¹H NMR (300 MHz, CD₃OD) δ 7.77 (d, $J = 7.8$ Hz, 2H), 7.20 (d, $J = 8.1$ Hz, 2H), 4.76–4.75 (m, 1H), 3.51–3.50 (m, 2H), 2.38 (s, 3H), 2.34–2.31 (m, 1H), 2.17–2.09 (m, 2H), 1.90–1.87 (m, 1H). MS (ESI) m/z 97 [M+H]⁺.

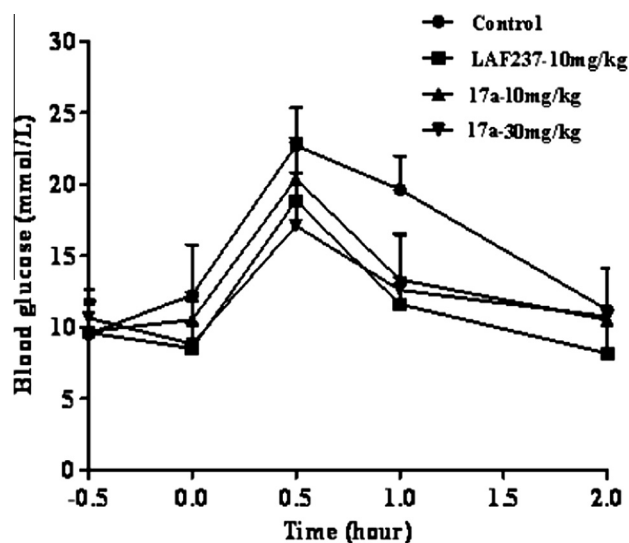


Figure 4. Glucose responses during an OGTT in KKAY mice following treatment with **17a** or **LAF237** (Mean \pm SD, $n = 5$).

Table 4

Glucose responses during an OGTT in KKAY mice following treatment with **17a** or **LAF237** (mmol/L, Mean \pm SD, $n = 5$)

Group	Dose (mg/kg)	Post-dose (min)					AUC _{0–120 min} Glu (mmol/L·h)
		–30	0	30	60	120	
Control	–	9.52 \pm 2.06	12.26 \pm 3.52	22.74 \pm 2.62	19.64 \pm 2.35	11.22 \pm 2.94	34.78 \pm 3.62
LAF237	10	9.62 \pm 2.28	8.52 \pm 1.71	18.86 \pm 4.37	11.60 \pm 1.87 ^{***}	8.16 \pm 2.01	24.34 \pm 3.87 ^{**}
17a	10	9.70 \pm 2.04	10.52 \pm .34	20.38 \pm 2.58	13.34 \pm 3.25 ^{**}	10.52 \pm 3.62	28.09 \pm 5.36 [*]
17a	30	10.64 \pm 2.02	8.84 \pm 3.58	17.10 \pm 3.74 [*]	12.60 \pm 3.87 ^{**}	10.74 \pm 3.34	25.58 \pm 6.86 [*]

^{*} $P < 0.05$.

^{**} $P < 0.01$.

^{***} $P < 0.001$, versus control.

Table 5
Pharmacokinetic properties of **17a** in SD rats

Compd	Admin	Dose (mg/kg)	T_{\max} (h)	C_{\max} (ng/mL)	AUC _{0-t} (ng/mL·h)	AUC _{0-∞} (ng/mL·h)	$t_{1/2}$ (h)	F (%)
17a	p.o.	10	0.5	442	455	458	5.4	21.8
	i.v.	10	—	—	2082	2083	5.4	—

p.o., oral administration; i.v., intravenous injection.

4.1.5. *tert*-Butyl ((*S*)-1-((*S*)-2-cyanopyrrolidin-1-yl)-1-oxo-3-phenylpropan-2-yl) carbamate (**6a**)

(*S*)-2-((*tert*-Butoxycarbonyl)amino)-3-phenylpropanoic acid (compound **5a**, 203.6 mg, 0.768 mmol) in DMF (5 mL) was added HOBt (283.2 mg, 2.10 mmol) and EDCI (257.8 mg, 1.396 mmol). After stirring for 30 min compound **4** (187.2 mg, 0.698 mmol) and additional TEA (0.30 mL, 2.10 mmol) were added. This solution was allowed to stir at room temperature for 20 h and then the saturated NaHCO₃ was added. The mixture was extracted with EtOAc and washed with saturated NaCl, dried over Na₂SO₄ and concentrated. The residue was purified with flash chromatography on silica gel, eluted with a mixture of PE/EA (4/1, v/v) to afford **6a** (130 mg, 54%) as a white solid.

4.1.6. (*S*)-1-((*S*)-2-Amino-3-phenylpropanoyl)pyrrolidine-2-carbonitrile (**7a**)

A solution of **6a** (120 mg) in dry CH₂Cl₂ (2 mL) was added CF₃-COOH (1 mL) at ice-bathe and warmed to room temperature. After 1 h, the mixture was concentrated, and the residue was added 10 mL Et₂O. The white solid was precipitated, filtered and to afford **7a** as TFA salt. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.25 (br, 2H), 7.35–7.33 (m, 3H), 7.25–7.23 (m, 2H), 4.78 (q, *J* = 4.0 Hz, 1H), 4.33–4.30 (m, 1H), 3.41–3.37 (m, 1H), 3.13–.08 (m, 1H), 3.02–2.97 (m, 1H), 2.59–2.56 (m, 1H), 2.11–2.10 (m, 1H), 2.03–2.00 (m, 1H), 1.83–1.80 (m, 1H), 1.66–1.62 (m, 1H). MS (EI, *m/z*): 244 [M]⁺.

4.1.7. (*S*)-1-((*S*)-2-Amino-3-(2-fluorophenyl)propanoyl)pyrrolidine-2-carbonitrile (**7b**)

A similar procedure to that described above for **7a** gave the desired product **7b**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.27 (br, 2H), 7.40–7.37 (m, 1H), 7.30–7.15 (m, 3H), 4.79 (q, *J* = 4.8 Hz, 1H), 4.32 (t, *J* = 7.6 Hz, 1H), 3.52 (q, *J* = 7.6 Hz, 1H), 3.16–3.05 (m, 2H), 2.78–2.75 (m, 1H), 2.16–2.13 (m, 1H), 2.04–1.98 (m, 1H), 1.88–1.85 (m, 1H), 1.75–1.70 (m, 1H). MS (EI, *m/z*): 262 [M]⁺.

4.1.8. (*S*)-1-((*S*)-2-Amino-3-(3-fluorophenyl)propanoyl)pyrrolidine-2-carbonitrile (**7c**)

A similar procedure to that described above for **7a** gave the desired product **7c**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.26 (br, 2H), 7.70–7.65 (m, 2H), 7.59–7.53 (m, 2H), 4.79 (q, *J* = 4.4 Hz, 1H), 4.45 (t, *J* = 7.2 Hz, 1H), 3.56 (q, *J* = 6.8 Hz, 1H), 3.18–3.16 (m, 2H), 3.04–3.02 (m, 1H), 2.19–2.15 (m, 1H), 2.07–2.04 (m, 1H), 1.92–1.88 (m, 1H), 1.82–1.78 (m, 1H). MS (EI, *m/z*): 262 [M]⁺.

4.1.9. (*S*)-1-((*S*)-2-Amino-3-(4-fluorophenyl)propanoyl)pyrrolidine-2-carbonitrile (**7d**)

A similar procedure to that described above for **7a** gave the desired product **7d**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.19 (br, 2H), 7.28 (t, *J* = 6.0 Hz, 2H), 7.16 (t, *J* = 8.8 Hz, 2H), 4.79 (q, *J* = 3.2 Hz, 1H), 4.34 (t, *J* = 6.4 Hz, 1H), 3.49 (q, *J* = 2.4 Hz, 1H), 3.07–3.02 (m, 2H), 2.87 (q, *J* = 9.6 Hz, 1H), 2.19–2.05 (m, 2H), 1.89–1.86 (m, 1H), 1.76–1.72 (m, 1H). MS (EI, *m/z*): 262 [M]⁺.

4.1.10. (*S*)-1-((*S*)-2-Amino-3-*p*-tolylpropanoyl)pyrrolidine-2-carbonitrile (**7e**)

A similar procedure to that described above for **7a** gave the desired product **7e**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.18 (br, 2H),

7.15–7.10 (m, 4H), 4.80–4.76 (m, 1H), 4.27 (t, *J* = 8.0 Hz, 1H), 3.07–2.93 (m, 2H), 2.66 (t, *J* = 0.8 Hz, 1H), 2.29 (s, 3H), 2.14–2.02 (m, 2H), 1.86–1.82 (m, 1H), 1.69–1.63 (m, 1H). MS (EI, *m/z*): 258 [M]⁺.

4.1.11. (*S*)-1-((*S*)-2-Amino-3-(4-methoxyphenyl)propanoyl)pyrrolidine-2-carbonitrile (**7f**)

A similar procedure to that described above for **7a** gave the desired product **7f**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.17 (br, 2H), 7.16–7.14 (m, 2H), 6.89–6.87 (m, 2H), 4.79 (q, *J* = 4.4 Hz, 1H), 4.26 (t, *J* = 8.0 Hz, 1H), 3.46–3.40 (m, 1H), 3.05–2.91 (m, 2H), 2.74–2.67 (m, 1H), 2.17–2.03 (m, 2H), 1.88–1.83 (m, 1H), 1.72–1.67 (m, 1H). MS (EI, *m/z*): 274 [M]⁺.

4.1.12. (*S*)-1-((*S*)-2-Amino-3-(4-aminophenyl)propanoyl)pyrrolidine-2-carbonitrile (**7g**)

A similar procedure to that described above for **7a** gave the desired product **7g**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.22 (br, 2H), 7.38–7.36 (m, 2H), 7.28–7.25 (m, 2H), 4.79–4.75 (m, 1H), 4.33–4.30 (m, 2H), 3.14–3.09 (m, 2H), 2.41–2.37 (m, 1H), 2.11–2.10 (m, 2H), 2.03–2.00 (m, 2H). MS (EI, *m/z*): 259 [M]⁺.

4.1.13. (*S*)-1-((*S*)-2-Amino-3-(4-nitrophenyl)propanoyl)pyrrolidine-2-carbonitrile (**7h**)

A similar procedure to that described above for **7a** gave the desired product **7h**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.28–8.18 (m, 4H), 7.55–7.53 (m, 2H), 4.81 (q, *J* = 8.0 Hz, 1H), 4.47 (t, *J* = 6.4 Hz, 1H), 3.57 (q, *J* = 10.0 Hz, 1H), 3.22 (d, *J* = 6.8 Hz, 2H), 3.14–3.11 (m, 1H), 2.21–2.06 (m, 2H), 1.92–1.81 (m, 2H). MS (EI, *m/z*): 289 [M]⁺.

4.1.14. (*S*)-1-((*S*)-2-Amino-3-(4-cyanophenyl)propanoyl)pyrrolidine-2-carbonitrile (**7i**)

A similar procedure to that described above for **7a** gave the desired product **7i**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.01 (br, 2H), 7.82–7.80 (m, 2H), 7.47–7.45 (m, 2H), 4.81–4.78 (m, 1H), 4.41–4.37 (m, 1H), 3.58–3.52 (m, 1H), 3.14–3.08 (m, 3H), 2.20–2.07 (m, 2H), 1.92–1.79 (m, 2H). MS (EI, *m/z*): 269 [M]⁺.

4.1.15. (*S*)-1-((*S*)-2-Amino-3-(4-(trifluoromethyl)phenyl)propanoyl)pyrrolidine-2-carbonitrile (**7j**)

A similar procedure to that described above for **7a** gave the desired product **7j**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.16 (br, 2H), 7.69 (d, *J* = 8.0 Hz, 2H), 7.49 (d, *J* = 8.0 Hz, 1H), 4.81 (q, *J* = 4.0 Hz, 1H), 4.41 (t, *J* = 6.8 Hz, 1H), 3.54 (q, *J* = 7.6 Hz, 1H), 3.16 (d, *J* = 7.2 Hz, 2H), 3.07–.04 (m, 1H), 2.16–1.97 (m, 3H), 1.79–1.74 (m, 1H). MS (EI, *m/z*): 312 [M]⁺.

4.1.16. (*S*)-1-((*S*)-2-Amino-3-(4-chlorophenyl)propanoyl)pyrrolidine-2-carbonitrile (**7k**)

A similar procedure to that described above for **7a** gave the desired product **7k**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.09 (br, 2H), 7.40–7.38 (m, 2H), 7.29–7.26 (m, 2H), 4.80 (q, *J* = 4.8 Hz, 1H), 4.34 (t, *J* = 7.2 Hz, 1H), 3.52 (q, *J* = 6.4 Hz, 1H), 3.06–2.99 (m, 3H), 2.16–2.08 (m, 2H), 1.90–1.88 (m, 2H). MS (EI, *m/z*): 278 [M]⁺.

4.1.17. (*S*)-1-((*S*)-2-Amino-3-(4-bromophenyl)propanoyl)pyrrolidine-2-carbonitrile (**7l**)

A similar procedure to that described above for **7a** gave the desired product **7l**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.17 (br, 2H),

7.53–7.51 (m, 2H), 7.23–7.20 (m, 2H), 4.80 (q, $J = 4.8$ Hz, 1H), 4.35 (t, $J = 7.2$ Hz, 1H), 3.52 (q, $J = 7.6$ Hz, 1H), 3.05–3.00 (m, 3H), 2.51–2.49 (m, 2H), 2.18–2.07 (m, 1H), 1.93–1.90 (m, 1H). MS (EI, m/z): 322 [M]⁺.

4.1.18. (S)-1-((S)-2-Amino-3-(biphenyl-4-yl)propanoyl)pyrrolidine-2-carbonitrile (7m)

A similar procedure to that described above for **7a** gave the desired product **7m**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.06 (br, 5H), 7.74 (s, 1H), 7.53–7.51 (m, 2H), 7.41–7.39 (m, 1H), 4.80 (q, $J = 4.8$ Hz, 1H), 4.40 (t, $J = 6.8$ Hz, 1H), 3.50–3.44 (m, 1H), 3.23–3.17 (m, 3H), 2.83–2.77 (m, 1H), 2.17–2.08 (m, 1H), 2.00–1.98 (m, 1H), 1.81–1.75 (m, 1H), 1.60–1.51 (m, 1H). MS (EI, m/z): 320 [M]⁺.

4.1.19. (S)-1-((S)-2-Amino-3-*o*-tolylpropanoyl)pyrrolidine-2-carbonitrile (7n)

A similar procedure to that described above for **7a** gave the desired product **7n**. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.04 (br, 2H), 7.20–7.18 (m, 2H), 7.12–7.07 (m, 2H), 4.75 (q, $J = 3.6$ Hz, 1H), 4.20 (q, $J = 5.1$ Hz, 1H), 3.17–3.11 (m, 1H), 2.99–2.90 (m, 1H), 2.35 (s, 3H), 2.13–2.04 (m, 2H), 1.96–1.93 (m, 1H), 1.76–1.72 (m, 1H), 1.52–1.45 (m, 1H). MS (EI, m/z): 258 [M]⁺.

4.1.20. (S)-1-((S)-2-Amino-3-(2-cyanophenyl)propanoyl)pyrrolidine-2-carbonitrile (7o)

A similar procedure to that described above for **7a** gave the desired product **7o**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.39 (br, 2H), 7.88–7.86 (m, 1H), 7.70–7.66 (m, 1H), 7.56–7.49 (m, 2H), 4.79 (q, $J = 4.4$ Hz, 1H), 4.44 (t, $J = 7.6$ Hz, 1H), 3.58 (q, $J = 6.8$ Hz, 1H), 3.33–3.29 (m, 2H), 2.95–2.92 (m, 1H), 2.51–2.49 (m, 1H), 2.20–2.13 (m, 1H), 1.92–1.89 (m, 1H), 1.77–1.72 (m, 1H). MS (EI, m/z): 269 [M]⁺.

4.1.21. (S)-1-((S)-2-Amino-3-(2-(trifluoromethyl)phenyl)propanoyl)pyrrolidine-2-carbonitrile (7p)

A similar procedure to that described above for **7a** gave the desired product **7p**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.32 (br, 2H), 7.80–7.78 (m, 1H), 7.64–7.56 (m, 2H), 7.38 (d, $J = 7.2$ Hz, 1H), 4.79 (q, $J = 4.4$ Hz, 1H), 4.21 (q, $J = 5.6$ Hz, 1H), 3.19–3.13 (m, 2H), 2.13–2.06 (m, 2H), 1.98–1.94 (m, 2H), 1.79–1.75 (m, 1H), 1.56–1.48 (m, 1H). MS (EI, m/z): 312 [M]⁺.

4.1.22. (S)-1-((S)-2-Amino-3-(3-cyanophenyl)propanoyl)pyrrolidine-2-carbonitrile (7q)

A similar procedure to that described above for **7a** gave the desired product **7q**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.25 (br, 2H), 7.81–7.79 (m, 1H), 7.73 (m, 1H), 7.60–7.52 (m, 2H), 4.79 (q, $J = 3.6$ Hz, 1H), 4.50 (t, $J = 6.4$ Hz, 1H), 3.59 (q, $J = 7.2$ Hz, 1H), 3.15–3.13 (m, 2H), 2.22–2.17 (m, 1H), 2.10–2.06 (m, 1H), 1.92–1.84 (m, 2H). MS (EI, m/z): 269 [M]⁺.

4.1.23. (S)-1-((S)-2-Amino-3-(3-(trifluoromethyl)phenyl)propanoyl)pyrrolidine-2-carbonitrile (7r)

A similar procedure to that described above for **7a** gave the desired product **7r**. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.19 (br, 2H), 7.70–7.65 (m, 2H), 7.59–7.53 (m, 2H), 4.79 (q, $J = 4.8$ Hz, 1H), 4.44 (t, $J = 6.8$ Hz, 1H), 3.56 (q, $J = 7.2$ Hz, 1H), 3.18–3.15 (m, 2H), 3.08–3.02 (m, 1H), 2.21–2.16 (m, 1H), 2.07–2.05 (m, 1H), 1.92–1.89 (m, 1H), 1.81–1.76 (m, 1H). MS (EI, m/z): 312 [M]⁺.

4.1.24. 1-*tert*-Butyl 2-methyl (2S,4R)-4-hydroxypyrrolidine-1,2-dicarboxylate (9)

In the same manner as described for **1**, **9** was prepared from (2S,4R)-methyl-4-hydroxypyrrolidine-2-carboxylate hydrochloride (**8**). ¹H NMR (300 MHz, CDCl₃) δ 4.35–4.48 (m, 2H), 3.72 (s,

3H), 3.46–3.65 (m, 2H), 2.03–2.29 (m, 2H), 1.39 (s, 9H). MS (ESI) m/z 246 [M+H]⁺.

4.1.25. 1-*tert*-Butyl 2-methyl (2S,4S)-4-fluoropyrrolidine-1,2-dicarboxylate (10)

Under the Nitrogen protected, a solution of compound **9** (2 g, 8.2 mmol) in dry CH₂Cl₂ cooled to –78 °C, was added DAST (1.97 g, 12.2 mmol). After stirring 3 h, the reaction slowly warmed to room temperature overnight. Then the reaction solution was poured into 200 mL ice and NaHCO₃ mixture solution and stirred acutely until no CO₂ evolution. The organic layer was separated and the aqueous layer extracted with CH₂Cl₂, dried, filtered, and concentrated. The residue was purified by flash chromatography on silica gel, eluted with a mixture of EA/PE (1:4, v/v), to afford **10** (1.6 g, 78%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃) δ 5.18–5.34 (m, 2H), 3.72 (s, 3H), 3.46–3.65 (m, 2H), 2.03–2.29 (m, 2H), 1.40 (s, 9H). MS (ESI) m/z 248 [M+H]⁺.

4.1.26. (2S,4S)-1-[(*tert*-Butoxy)carbonyl]-4-fluoropyrrolidine-2-carboxylic acid (11)

A solution of compound **10** (2.46 g, 9.96 mmol) in dioxane (20 mL), was added 10 mL of H₂O followed by lithium hydroxide hydrate (2.09 g, 49.8 mmol) at room temperature, the reaction was stirred for 3 h (monitored by TLC). Then the solution was filtered removing the excess lithium hydroxide, the filtrate was removed the solvent in vacuo, the residue was added 10 mL of H₂O and acidified with concentrated HCl to pH 3–4, the product began to precipitate, filtered and dried to afford **11** (2.2 g, 95%) as a white solid. ¹H NMR (300 MHz, CD₃OD) δ 5.12–5.30 (m, 1H), 4.40 (m, 1H), 3.59–3.72 (m, 2H), 2.39–2.46 (m, 2H), 1.47 (s, 9H). MS (ESI) m/z 232 [M-H][–].

4.1.27. *tert*-Butyl (2S,4S)-2-carbamoyl-4-fluoropyrrolidine-1-carboxylate (12)

In the same manner as described for **2**, **12** was prepared from (2S,4S)-1-[(*tert*-butoxy)carbonyl]-4-fluoropyrrolidine-2-carboxylic acid (**11**). ¹H NMR (300 MHz, CDCl₃) δ 5.59 (br s, 1H), 5.13–5.31 (m, 1H), 4.36 (br s, 1H), 3.52–3.81 (m, 2H), 2.31–2.78 (m, 2H), 1.48 (s, 9H). MS (ESI) m/z 233 [M+H]⁺.

4.1.28. *tert*-Butyl (2S,4S)-2-cyano-4-fluoropyrrolidine-1-carboxylate (13)

In the same manner as described for **3**, **13** was prepared from *tert*-butyl (2S,4S)-2-carbamoyl-4-fluoropyrrolidine-1-carboxylate (**12**). ¹H NMR (300 MHz, CDCl₃) δ 5.21–5.41 (m, 1H), 4.62–4.76 (m, 1H), 3.49–3.93 (m, 2H), 2.63 (d, $J_1 = 15.0$ Hz, $J_2 = 15.3$ Hz, 1H), 2.30–2.44 (m, 1H), 1.49 (s, 9H). MS (ESI) m/z 215 [M+H]⁺.

4.1.29. (2S,4S)-4-Fluoropyrrolidine-2-carbonitrile: 4-methylbenzene-1-sulfonic acid (14)

In the same manner as described for **4**, **14** was prepared from *tert*-butyl (2S,4S)-2-cyano-4-fluoropyrrolidine-1-carboxylate (**13**). ¹H NMR (300 MHz, CD₃OD) δ 7.51 (dd, $J_1 = 12.0$ Hz, $J_2 = 6.0$ Hz, 2H), 7.13 (m, 2H), 5.45–5.62 (m, 1H), 4.99–5.04 (m, 1H), 3.42–3.67 (m, 2H), 2.30–2.70 (m, 3H), 3.17 (s, 3H). ¹³C NMR (CD₃OD, 100 MHz): δ 143.3, 142.0, 130.0, 127.0, 116.4, 93.9, 92.5, 53.8 (d, $J = 19.0$ Hz), 46.8, 38.4 (d, $J = 17.0$ Hz), 21.4. MS (EI) m/z 114 [M]⁺. HRMS (EI) m/z calcd C₅H₇FN₂ 114.0593 [M]⁺, found 114.0668.

4.1.30. *tert*-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidine-1-yl]-1-oxo-3-phenylpropan-2-yl]carbamate (16a)

A similar procedure to that described above for **6a** gave the desired product **16a**. ¹H NMR (300 MHz, CDCl₃) δ 7.24–7.37 (br, 5H), 5.20–5.31 (m, 1H), 4.93 (d, $J = 9.3$ Hz, 1H), 4.43 (q, $J = 6.0$, 1H), 3.61–3.77 (m, 1H), 3.02 (s, 9H), 2.90 (t, $J = 12.0$ Hz, 1H), 2.52 (t, $J = 15.0$ Hz, 1H), 2.24–2.37 (m, 1H). ESI-MS m/z 384, [M+Na]⁺.

4.1.31. (2S,4S)-1-[(2S)-2-Amino-3-phenylpropanoyl]-4-fluoropyrrolidine-2-carbonitrile (17a)

A similar procedure to that described above for **7a** gave the desired product **17a**. $^1\text{H NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ 7.21–7.30 (m, 5H), 5.19–5.36 (m, 1H), 5.00 (d, $J = 8.4$ Hz, 1H), 4.19 (q, $J = 6.9$ Hz, 1H), 3.61–3.78 (m, 1H), 3.16 (dd, $J_1 = 5.1$ Hz, $J_2 = 5.1$ Hz, 1H), 2.93–2.96 (m, 1H), 2.74–2.77 (m, 1H), 2.21–2.38 (m, 2H); ESI-MS m/z 262, $[\text{M}+\text{H}]^+$. HRMS (ESI) m/z calcd $\text{C}_{14}\text{H}_{16}\text{FN}_3\text{ONa}$ $[\text{M}+\text{Na}]^+$ 284.1175, found 284.1173.

4.1.32. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(4-fluorophenyl)-1-oxopropan-2-yl]carbamate (16b)

A similar procedure to that described above for **6a** gave the desired product **16b**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.19–7.26 (m, 2H), 7.03 (t, $J = 6.0$ Hz, 2H), 5.20–5.31 (m, 1H), 4.92 (d, $J = 9.6$ Hz, 1H), 4.42 (q, $J = 6.0$ Hz, 1H), 3.72 (dd, $J_1 = 3.9$ Hz, $J_2 = 12.6$ Hz, 1H), 2.97–3.80 (m, 3H), 2.56 (t, $J = 15.0$ Hz, 1H), 2.16–2.32 (m, 1H), 1.42 (s, 9H). ESI-MS m/z 402, $[\text{M}+\text{H}]^+$.

4.1.33. (2S,4S)-1-[(2S)-2-Amino-3-(4-fluorophenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17b)

A similar procedure to that described above for **7a** gave the desired product **17b**. $^1\text{H NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ 7.19–7.24 (m, 2H), 6.98–7.05 (m, 2H), 5.25–5.42 (m, 1H), 4.92 (q, $J = 6.0$ Hz, 1H), 4.40–4.51 (m, 1H), 3.61–3.80 (m, 1H), 2.91–3.11 (m, 2H), 2.56–2.71 (m, 2H), 2.16–2.33 (m, 1H); ESI-MS m/z 279, $[\text{M}+\text{H}]^+$. HRMS (ESI) m/z calcd $\text{C}_{14}\text{H}_{15}\text{FN}_3\text{ONa}$ $[\text{M}+\text{Na}]^+$ 302.1081, found 302.1083.

4.1.34. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(4-methylphenyl)-1-oxopropan-2-yl]carbamate (16c)

A similar procedure to that described above for **6a** gave the desired product **16c**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.14 (s, 4H), 5.23–5.28 (m, 1H), 4.91 (d, $J = 9.0$ Hz, 1H), 4.38–4.44 (m, 1H), 3.68 (dd, $J_1 = 12.0$ Hz, $J_2 = 12.0$ Hz, 1H), 2.86–3.01 (m, 3H), 2.54 (t, $J = 15.0$ Hz, 1H), 2.31 (s, 3H), 2.11–2.23 (m, 1H), 1.41 (s, 9H). MS (ESI) m/z 398 $[\text{M}+\text{Na}]^+$.

4.1.35. (2S,4S)-1-[(2S)-2-Amino-3-(4-methylphenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17c)

A similar procedure to that described above for **7a** gave the desired product **17c**. $^1\text{H NMR}$ (400 MHz, CD_3OD) δ 7.17 (s, 4H), 5.11–5.25 (m, 1H), 5.01 (d, $J = 12.0$ Hz, 1H), 4.20–4.24 (m, 1H), 3.51–3.65 (m, 1H), 3.07–3.18 (m, 2H), 2.76–2.85 (m, 1H), 2.48 (t, $J = 15.0$ Hz, 1H), 2.23–2.44 (m, 4H). ESI-LRMS: 276 $[\text{M}+\text{H}]^+$. ESI-HRMS calcd for $\text{C}_{15}\text{H}_{18}\text{N}_3\text{OFNa}$ m/z 298.1332 $[\text{M}+\text{Na}]^+$, found 298.1332 $[\text{M}+\text{Na}]^+$.

4.1.36. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(4-methoxyphenyl)-1-oxopropan-2-yl]carbamate (16d)

A similar procedure to that described above for **6a** gave the desired product **16d**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 8.12–8.21 (m, 2H), 8.02 (s, 1H), 7.37–7.49 (m, 2H), 5.25–5.31 (m, 1H), 4.56–4.78 (m, 1H), 4.05–4.14 (m, 1H), 3.73–3.85 (m, 1H), 3.29 (dd, $J_1 = 5.1$ Hz, $J_2 = 5.1$ Hz, 1H), 3.04–3.20 (m, 1H), 2.87–2.98 (m, 1H), 2.57–2.73 (m, 2H). ESI-MS m/z 414, $[\text{M}+\text{Na}]^+$.

4.1.37. (2S,4S)-1-[(2S)-2-Amino-3-(4-methoxyphenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17d)

A similar procedure to that described above for **7a** gave the desired product **17d**. $^1\text{H NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ 8.32 (s, 1H), 7.47 (d, $J = 8.1$ Hz, 2H), 7.12 (d, $J = 7.8$ Hz, 2H), 4.41–4.80 (m, 4H), 4.19–4.24 (m, 1H), 3.62–3.78 (m, 1H), 2.82–3.18 (m, 2H), 2.38–2.43 (m, 1H), 2.30 (s, 3H); ESI-MS m/z 292, $[\text{M}+\text{H}]^+$. HRMS (ESI) m/z calcd $\text{C}_{15}\text{H}_{18}\text{FN}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 292.1461, found 292.1456.

4.1.38. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(4-nitrophenyl)-1-oxopropan-2-yl]carbamate (16e)

A similar procedure to that described above for **6a** gave the desired product **16e**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 8.12–8.21 (m, 2H), 8.02 (s, 1H), 7.37–7.49 (m, 2H), 5.28–5.30 (m, 1H), 4.60–4.87 (m, 1H), 4.05–4.15 (m, 1H), 3.70–3.95 (m, 1H), 3.29 (dd, $J_1 = 5.1$ Hz, $J_2 = 5.1$ Hz, 1H), 3.06–4.16 (m, 1H), 2.85–2.99 (m, 1H), 2.57–2.73 (m, 2H). ESI-MS m/z 429, $[\text{M}+\text{Na}]^+$.

4.1.39. (2S,4S)-1-[(2S)-2-Amino-3-(4-nitrophenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17e)

A similar procedure to that described above for **7a** gave the desired product **17e**. $^1\text{H NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ 8.19 (d, $J = 8.4$ Hz, 2H), 7.53 (d, $J = 8.7$ Hz, 2H), 5.08–5.26 (m, 1H), 4.31–4.38 (m, 1H), 3.95 (q, $J = 6.0$ Hz, 1H), 3.06–3.27 (m, 3H), 2.00–2.25 (m, 2H); ESI-MS m/z 307, $[\text{M}+\text{H}]^+$. HRMS (ESI) m/z calcd $\text{C}_{14}\text{H}_{16}\text{FN}_4\text{O}_3$ $[\text{M}+\text{H}]^+$ 307.1206, found 307.1228.

4.1.40. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(4-cyanophenyl)-1-oxopropan-2-yl]carbamate (16f)

A similar procedure to that described above for **6a** gave the desired product **16f**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.63 (d, $J = 9.0$ Hz, 2H), 7.36 (d, $J = 6.0$ Hz, 2H), 5.12–5.29 (m, 1H), 4.90 (d, $J = 12.0$ Hz, 1H), 4.48 (q, $J = 9.0$ Hz, 1H), 3.73 (dd, $J_1 = 12.0$ Hz, $J_2 = 12.0$ Hz, 1H), 3.02–3.20 (m, 3H), 2.56 (t, $J = 15.0$ Hz, 1H), 2.11–2.36 (m, 1H), 1.39 (s, 9H). MS (ESI) m/z 409 $[\text{M}+\text{Na}]^+$.

4.1.41. (2S,4S)-1-[(2S)-2-Amino-3-(4-cyanophenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17f)

A similar procedure to that described above for **7a** gave the desired product **17f**. $^1\text{H NMR}$ (400 MHz, CD_3OD) δ 7.71 (d, $J = 8.0$ Hz, 2H), 7.48 (d, $J = 8.0$ Hz, 2H), 5.17–5.30 (m, 1H), 5.01 (d, $J = 8.0$ Hz, 1H), 4.35 (q, $J = 6.0$ Hz, 1H), 3.56–3.69 (m, 1H), 3.20–3.30 (m, 2H), 2.98–3.07 (m, 1H), 2.53 (t, $J = 12.0$ Hz, 1H), 2.24–2.43 (m, 1H). ESI-LRMS: 287 $[\text{M}+\text{H}]^+$. ESI-HRMS calcd for $\text{C}_{15}\text{H}_{16}\text{N}_4\text{OF}$ m/z 287.1308 $[\text{M}+\text{H}]^+$, found 287.1324 $[\text{M}+\text{H}]^+$.

4.1.42. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-[4-(trifluoromethyl)phenyl]-1-oxopropan-2-yl]carbamate (16g)

A similar procedure to that described above for **6a** gave the desired product **16g**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.63 (d, $J = 6.0$ Hz, 2H), 7.38 (d, $J = 9.0$ Hz, 2H), 5.23–5.27 (m, 1H), 4.91 (d, $J = 9.0$ Hz, 1H), 4.48 (q, $J = 6.0$ Hz, 1H), 3.70 (dd, $J_1 = 12.0$ Hz, $J_2 = 12.0$ Hz, 1H), 3.06–3.12 (m, 3H), 2.56 (t, $J = 15.0$ Hz, 1H), 2.11–2.34 (m, 1H), 1.41 (s, 9H). MS (ESI) m/z 452 $[\text{M}+\text{Na}]^+$.

4.1.43. (2S,4S)-1-[(2S)-2-Amino-3-[4-(trifluoromethyl)phenyl]propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17g)

A similar procedure to that described above for **7a** gave the desired product **17g**. $^1\text{H NMR}$ (400 MHz, CD_3OD) δ 7.65 (d, $J = 8.0$ Hz, 2H), 7.49 (d, $J = 8.0$ Hz, 2H), 5.13–5.28 (m, 1H), 5.01 (d, $J = 8.0$ Hz, 1H), 4.35 (q, $J = 6.0$ Hz, 1H), 3.54–3.68 (m, 1H), 3.21–3.33 (m, 2H), 2.95 (q, $J = 6.0$ Hz, 1H), 2.50 (t, $J = 12.0$ Hz, 1H), 2.24–2.41 (m, 1H). ESI-LRMS: 330 $[\text{M}+\text{H}]^+$. ESI-HRMS calcd for $\text{C}_{15}\text{H}_{16}\text{N}_3\text{OF}_4$ m/z 330.1230 $[\text{M}+\text{H}]^+$, found 330.1233 $[\text{M}+\text{H}]^+$.

4.1.44. tert-Butyl N-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(4-tert-butylphenyl)-1-oxopropan-2-yl]carbamate (16h)

A similar procedure to that described above for **6a** gave the desired product **16h**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.36 (d, $J = 6.0$ Hz, 2H), 7.18 (d, $J = 6.0$ Hz, 2H), 5.18–5.29 (m, 1H), 4.92 (d, $J = 9.0$ Hz, 1H), 4.35–4.42 (m, 1H), 3.60 (dd, $J_1 = 12.0$ Hz, $J_2 = 12.0$ Hz, 1H), 2.72–3.04 (m, 3H), 2.49 (t, $J = 15.0$ Hz, 1H), 2.04–2.29 (m, 1H), 1.41 (s, 9H), 1.28 (s, 9H). MS (ESI) m/z 440 $[\text{M}+\text{Na}]^+$.

4.1.45. (2S,4S)-1-[(2S)-2-Amino-3-(4-*tert*-butylphenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17h)

A similar procedure to that described above for **7a** gave the desired product **17h**. $^1\text{H NMR}$ (400 MHz, CD_3OD) δ 7.40 (d, $J = 8.0$ Hz, 2H), 7.21 (d, $J = 8.0$ Hz, 2H), 5.09–5.22 (m, 1H), 5.01 (d, $J = 8.0$ Hz, 1H), 4.19–4.23 (m, 1H), 3.49–3.61 (m, 1H), 3.06–3.19 (m, 2H), 2.62–2.71 (m, 1H), 2.46 (t, $J = 12.0$ Hz, 1H), 2.19–.35 (m, 1H), 1.29 (s, 9H). ESI-LRMS: 318 $[\text{M}+\text{H}]^+$. ESI-HRMS calcd for $\text{C}_{18}\text{H}_{25}\text{N}_3\text{OF}$ m/z 318.1982 $[\text{M}+\text{H}]^+$, found 318.1984 $[\text{M}+\text{H}]^+$.

4.1.46. *tert*-Butyl *N*-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-[4-(benzyloxy)phenyl]-1-oxopropan-2-yl]carbamate (16i)

A similar procedure to that described above for **6a** gave the desired product **16i**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.98 (s, 1H), 7.25–7.40 (m, 4H), 7.15 (d, $J = 8.4$ Hz, 2H), 6.93 (d, $J = 8.4$ Hz, 2H), 5.00 (s, 2H), 4.88 (d, $J = 9.0$ Hz, 1H), 4.26–4.38 (m, 1H), 3.57–3.74 (m, 1H), 2.84–2.99 (m, 4H), 2.49 (t, $J = 15.0$ Hz, 1H), 2.11–2.27 (m, 1H), 1.39 (s, 9H). ESI-MS m/z 490, $[\text{M}+\text{Na}]^+$.

4.1.47. (2S,4S)-1-[(2S)-2-Amino-3-[4-(benzyloxy)phenyl]propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17i)

A similar procedure to that described above for **7a** gave the desired product **17i**. $^1\text{H NMR}$ (300 MHz, $\text{DMSO}-d_6$) δ 7.58 (s, 1H), 7.27–7.45 (m, 5H), 7.90–7.96 (m, 1H), 6.91–6.70 (m, 2H), 5.24–5.42 (m, 1H), 5.08 (s, 2H), 4.08–4.20 (m, 2H), 3.51–3.58 (m, 1H), 3.36–3.45 (m, 1H), 2.90–2.96 (m, 2H), 2.28–2.41 (m, 1H); ESI-MS m/z 368, $[\text{M}+\text{H}]^+$. HRMS (ESI) m/z calcd $\text{C}_{21}\text{H}_{23}\text{FN}_3\text{O}_2\text{Na}$ $[\text{M}+\text{Na}]^+$ 368.1774, found 368.1754.

4.1.48. *tert*-Butyl *N*-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(2-cyanophenyl)-1-oxopropan-2-yl]carbamate (16j)

A similar procedure to that described above for **6a** gave the desired product **16j**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.56–7.68 (m, 2H), 7.35–7.42 (m, 2H), 5.20–5.40 (m, 1H), 4.96 (d, $J = 9.0$ Hz, 1H), 4.64 (q, $J = 9.0$ Hz, 1H), 3.92 (dd, $J_1 = 12.0$ Hz, $J_2 = 12.0$ Hz, 1H), 3.66 (q, $J = 12.0$ Hz, 1H), 3.12–3.33 (m, 2H), 2.60 (t, $J = 15.0$ Hz, 1H), 2.18–2.40 (m, 1H), 1.33 (s, 9H). MS (ESI) m/z 409 $[\text{M}+\text{Na}]^+$.

4.1.49. (2S,4S)-1-[(2S)-2-Amino-3-(2-cyanophenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17j)

A similar procedure to that described above for **7a** gave the desired product **17j**. $^1\text{H NMR}$ (400 MHz, CD_3OD) δ 7.80 (d, $J = 8.0$ Hz, 1H), 7.67 (t, $J = 6.0$ Hz, 1H), 7.54 (t, $J = 9.0$ Hz, 1H), 7.48 (d, $J = 8.0$ Hz, 1H), 5.19–5.33 (m, 1H), 5.03 (d, $J = 12.0$ Hz, 1H), 4.40 (t, $J = 12.0$ Hz, 1H), 3.67–3.80 (m, 1H), 3.43–3.47 (m, 2H), 3.07–3.16 (m, 1H), 2.50 (t, $J = 12.0$ Hz, 1H), 2.26–2.43 (m, 1H). ESI-LRMS: 287 $[\text{M}+\text{H}]^+$. ESI-HRMS calcd for $\text{C}_{15}\text{H}_{16}\text{N}_4\text{OF}$ m/z 287.1308 $[\text{M}+\text{H}]^+$, found 287.1300 $[\text{M}+\text{H}]^+$.

4.1.50. *tert*-Butyl *N*-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-[2-(trifluoromethyl)phenyl]-1-oxopropan-2-yl]carbamate (16k)

A similar procedure to that described above for **6a** gave the desired product **16k**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.55–7.68 (m, 3H), 7.34–7.43 (m, 2H), 5.08–5.31 (m, 1H), 4.92 (d, $J = 9.0$ Hz, 1H), 4.53 (q, $J = 9.0$ Hz, 1H), 3.72 (dd, $J_1 = 12.0$ Hz, $J_2 = 12.0$ Hz, 1H), 3.10–3.32 (m, 2H), 2.95 (q, $J = 15.0$ Hz, 1H), 2.53 (t, $J = 15.0$ Hz, 1H), 2.11–2.36 (m, 1H), 1.38 (s, 9H). MS (ESI) m/z 452 $[\text{M}+\text{Na}]^+$.

4.1.51. (2S,4S)-1-[(2S)-2-Amino-3-[2-(trifluoromethyl)phenyl]propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17k)

A similar procedure to that described above for **7a** gave the desired product **17k**. $^1\text{H NMR}$ (400 MHz, CD_3OD) δ 7.77 (d, $J = 8.0$ Hz,

1H), 7.52–7.64 (m, 2H), 7.39 (d, $J = 4.0$ Hz, 1H), 5.05–5.20 (m, 1H), 5.01 (d, $J = 12.0$ Hz, 1H), 4.22 (q, $J = 6.0$ Hz, 1H), 3.47–3.65 (m, 2H), 3.24–3.29 (m, 1H), 2.48 (t, $J = 15.0$ Hz, 1H), 2.20–2.39 (m, 2H). ESI-LRMS: 330 $[\text{M}+\text{H}]^+$. ESI-HRMS calcd for $\text{C}_{15}\text{H}_{16}\text{N}_3\text{OF}_4$ m/z 330.1230 $[\text{M}+\text{H}]^+$, found 330.1240 $[\text{M}+\text{H}]^+$.

4.1.52. *tert*-Butyl *N*-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(2,4-difluorophenyl)-1-oxopropan-2-yl]carbamate (16l)

A similar procedure to that described above for **6a** gave the desired product **16l**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.14–7.19 (m, 1H), 6.76–6.84 (m, 2H), 5.22–5.41 (m, 1H), 4.92 (d, $J = 6.0$ Hz, 1H), 4.50 (q, $J = 6.0$ Hz, 1H), 3.85 (dd, $J_1 = 9.0$ Hz, $J_2 = 9.0$ Hz, 1H), 3.57 (q, $J = 6.0$ Hz, 1H), 2.90–3.01 (m, 2H), 2.56 (t, $J = 15.0$ Hz, 1H), 2.22–2.36 (m, 1H), 1.34 (s, 9H). MS (ESI) m/z 420 $[\text{M}+\text{Na}]^+$.

4.1.53. (2S,4S)-1-[(2S)-2-Amino-3-(2,4-difluorophenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17l)

A similar procedure to that described above for **7a** gave the desired product **17l**. $^1\text{H NMR}$ (400 MHz, CD_3OD) δ 7.28–7.34 (m, 1H), 6.94–7.05 (m, 2H), 5.24–5.38 (m, 1H), 5.03 (d, $J = 8$ Hz, 1H), 4.30–4.34 (m, 1H), 3.67–3.80 (m, 1H), 3.20–3.30 (m, 2H), 2.54 (t, $J = 12.0$ Hz, 1H), 2.28–2.46 (m, 1H). ESI-LRMS: 298 $[\text{M}+\text{H}]^+$. ESI-HRMS calcd for $\text{C}_{14}\text{H}_{15}\text{N}_3\text{OF}_3$ m/z 298.1167 $[\text{M}+\text{H}]^+$, found 298.1177 $[\text{M}+\text{H}]^+$.

4.1.54. *tert*-Butyl *N*-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(2,4,5-trifluorophenyl)-1-oxopropan-2-yl]carbamate (16m)

A similar procedure to that described above for **6a** gave the desired product **16m**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 7.02–7.08 (m, 1H), 6.92–6.98 (m, 1H), 5.25–5.42 (m, 1H), 4.94 (d, $J = 6.0$ Hz, 1H), 4.54 (q, $J = 6.0$ Hz, 1H), 3.88 (dd, $J_1 = 9.0$ Hz, $J_2 = 9.0$ Hz, 1H), 3.72 (q, $J = 9.0$ Hz, 1H), 2.88–3.07 (m, 2H), 2.64 (t, $J = 12.0$ Hz, 1H), 2.25–2.39 (m, 1H), 1.36 (s, 9H). MS (ESI) m/z 438 $[\text{M}+\text{Na}]^+$.

4.1.55. (2S,4S)-1-[(2S)-2-Amino-3-(2,4,5-trifluorophenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17m)

A similar procedure to that described above for **7a** gave the desired product **17m**. $^1\text{H NMR}$ (400 MHz, CD_3OD) δ 7.19–7.26 (m, 2H), 5.27–5.40 (m, 1H), 5.02 (d, $J = 12.0$ Hz, 1H), 4.34 (t, $J = 12.0$ Hz, 1H), 3.71–3.82 (m, 1H), 3.15–3.29 (m, 2H), 2.31–2.58 (m, 2H). ESI-LRMS: 316 $[\text{M}+\text{H}]^+$. ESI-HRMS calcd for $\text{C}_{14}\text{H}_{14}\text{N}_3\text{OF}_4$ m/z 316.1073 $[\text{M}+\text{H}]^+$, found 316.1083 $[\text{M}+\text{H}]^+$.

4.1.56. *tert*-Butyl *N*-[(2S)-1-[(2S,4S)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(2,3,4-trifluorophenyl)-1-oxopropan-2-yl]carbamate (16n)

A similar procedure to that described above for **6a** gave the desired product **16n**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 6.89–6.99 (m, 2H), 5.19–5.27 (m, 1H), 4.94 (d, $J = 6.0$ Hz, 1H), 4.53 (q, $J = 6.0$ Hz, 1H), 3.87 (dd, $J_1 = 9.0$ Hz, $J_2 = 9.0$ Hz, 1H), 3.61 (q, $J = 6.0$ Hz, 1H), 3.01–3.07 (m, 2H), 2.63 (t, $J = 12.0$ Hz, 1H), 2.21–2.39 (m, 1H), 1.37 (s, 9H). MS (ESI) m/z 438 $[\text{M}+\text{Na}]^+$.

4.1.57. (2S,4S)-1-[(2S)-2-amino-3-(2,3,4-trifluorophenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (17n)

A similar procedure to that described above for **7a** gave the desired product **17n**. $^1\text{H NMR}$ (400 MHz, CD_3OD) δ 7.08–7.12 (m, 2H), 5.27–5.41 (m, 1H), 5.02 (d, $J = 12.0$ Hz, 1H), 4.37 (t, $J = 12.0$ Hz, 1H), 3.76 (q, $J = 6.0$ Hz, 1H), 3.37 (q, $J = 6.0$ Hz, 1H), 3.17–3.29 (m, 2H), 2.55 (t, $J = 15.0$ Hz, 1H), 2.31–2.46 (m, 1H). ESI-LRMS: 316 $[\text{M}+\text{H}]^+$. ESI-HRMS calcd for $\text{C}_{14}\text{H}_{14}\text{N}_3\text{OF}_4$ m/z 316.1073 $[\text{M}+\text{H}]^+$, found 316.1076 $[\text{M}+\text{H}]^+$.

4.1.58. *tert*-Butyl *N*-[(2*S*)-1-[(2*S*,4*S*)-2-cyano-4-fluoropyrrolidin-1-yl]-3-(2,3,5-trifluorophenyl)-1-oxopropan-2-yl]carbamate (**16o**)

A similar procedure to that described above for **6a** gave the desired product **16o**. ¹H NMR (300 MHz, CDCl₃) δ 6.73–6.91 (m, 2H), 5.20–5.45 (m, 1H), 4.94 (d, *J* = 9.0 Hz, 1H), 4.57 (q, *J* = 9.0 Hz, 1H), 3.70–3.96 (m, 2H), 2.93–3.14 (m, 2H), 2.65 (t, *J* = 15.0 Hz, 1H), 2.16–2.42 (m, 1H), 1.36 (s, 9H). MS (ESI) *m/z* 438 [M+Na]⁺.

4.1.59. (2*S*,4*S*)-1-[(2*S*)-2-amino-3-(2,3,5-trifluorophenyl)propanoyl]-4-fluoropyrrolidine-2-carbonitrile (**17o**)

A similar procedure to that described above for **7a** gave the desired product **17o**. ¹H NMR (400 MHz, CD₃OD) δ 7.15–7.20 (m, 1H), 6.88–6.97 (m, 1H), 5.29–5.43 (m, 1H), 5.03 (d, *J* = 8.0 Hz, 1H), 4.41 (t, *J* = 12.0 Hz, 1H), 3.72–3.85 (m, 1H), 3.42–3.51 (m, 1H), 3.22–3.33 (m, 2H), 2.56 (t, *J* = 12.0 Hz, 1H), 2.32–2.49 (m, 1H). ESI-LRMS: 316[M+H]⁺. ESI-HRMS calcd for C₁₄H₁₄N₃O₄ *m/z* 316.1073[M+H]⁺, found 316.1089 [M+H]⁺.

4.2. In vitro DPP-4, DPP-7, DPP-8, DPP-9, and FAP enzyme assay

4.2.1. Preparation of the DPPs enzyme

The DPP-4, DPP-7, DPP-8, DPP-9, and FAP enzymes were expressed in high five cells using a baculoviral system (Bac-To-Bac; Life Technologies) according to the literature,²⁶ and his6-tagged recombinant proteins were purified by Ni-NTA resin individually.

4.2.2. Enzyme-based assay of DPP-4

To measure the activity of DPP-4, a continuous fluorometric assay was employed using Ala-Pro-AMC, which is cleaved by the enzyme to release the fluorescent aminomethylcoumarin (AMC). Liberation of AMC was monitored using an excitation wavelength of 355 nm and an emission wavelength of 460 nm using Envision microplate reader (PerkinElmer). A typical reaction contained 50 pmol/L enzyme, 10 μmol/L Ala-Pro-AMC, different concentrations of the compounds synthesized in this work, and assay buffer (100 mmol/L HEPES, pH 7.5, 0.1 mg/mL BSA) in a total reaction volume of 50 μL. The DPP-4 enzyme used in these studies was soluble human recombinant protein produced in a baculovirus expression system (Bac-To-Bac; Life Technologies). The dose response of inhibition test was carried out in duplicate. And the IC₅₀ data was calculated using the software GraphPad Prism, and chosen the equation 'sigmoidal dose-response (variable slope)' for curve fitting. The well containing substrate alone was used as a basal control. The well containing the substrate and the enzyme without the compound was used as a total reaction.

4.2.3. Enzyme-based assay of DPP-7, DPP-8, DPP-9, and FAP

DPP-7, DPP-8, DPP-9, and FAP were expressed in high four cells using a baculoviral system, and the activity of DPPs were assayed by continuous fluorometric method. We used Nle-Pro-AMC as substrate to measure the activity of DPP-7 and FAP, and Ala-Pro-AMC for DPP-8 and DPP-9 in the optimized pH (5.5 for DPP-7 and 8.0 for other members) assay system. 1 μL of compound dissolved in DMSO was mixed with 29 μL of distilled water, 10 μL of 1 mol/L Tris-HCl buffer (pH 7.5), and 10 μL of the enzyme fraction. After the mixture was incubated at room temperature for 20 min, the reaction was initiated by adding 50 μL of 2 mmol/L of Gly-PropNA3 Tos for DPP-8 or 4 mmol/L of Gly-Pro-pNA 3 Tos for DPP-9 and run at 37 °C for 90 min. The selective dose response of inhibition test on DPPs and data analysis is the same as DPP4 assay system. The well containing substrate alone was used as a basal control. The well containing the substrate and the enzyme without the compound was used as a total reaction.

4.3. Oral glucose tolerance test on ICR and KKAY mice

KKAY and ICR mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The mice were provided with a normal diet and water ad libitum. The KKAY mice were housed individually. All the animals were kept under conventional conditions of controlled temperature, humidity, and lighting. All procedures were approved by the Animal Care and Use committee, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. The effects of **17a** on blood glucose after an oral glucose challenge were observed on ICR and KKAY mice. Indicated dose of **17a**, **LAF237**, or vehicle (distilled water) was orally administered to 6 h fasting ICR or KKAY mice 0.5 h prior to oral glucose load (2.5 g/kg). Blood glucose values were measured with ONE TOUCH BASIC Plus blood glucose meter (LIFESCAN Inc., USA) at 0, 30, 60, 120 min after the glucose load. The area under the concentration–time curve from 0 to 120 min (AUC_{0–120 min}) of blood glucose after challenge was calculated by the trapezoidal rule. All data were expressed as the mean ± SEM. The statistical analysis between two groups was performed using an unpaired Student's *t* test. *P* < 0.05 was considered to be statistically significance.

4.4. Pharmacokinetic profile in SD rats

Compound **17a** was administered to SD rats. After oral and intravenous administration, blood samples were collected. The blood samples were centrifuged to obtain the plasma fraction. The plasma samples were deproteinized with methanol containing an internal standard. After centrifugation, the supernatant was diluted with methanol and centrifuged again. The compound concentrations in the supernatant were measured by LC/MS/MS.

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