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Original article

Synthesis and biological evaluation of pyrazoline analogues with β-amino acyl group as dipeptidyl peptidase IV inhibitors

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Abstract

A series of pyrazoline derivatives with β-amino acyl group were synthesized and evaluated for their ability to inhibit dipeptidyl peptidase IV. Several pyrazoline derivatives exhibited submicromolar inhibitory activities against DPP-IV. X-ray co-crystal structure of initial hit compound **1h** was determined. Among this series, carboxylic acid substituted pyrazoline derivative **2u** was the most active and greatly decreased the inhibitory activity toward CYP3A4 enzyme.

Keywords: Dipeptidyl peptidase IV inhibitior; Pyrazoline; Anti-diabetic agent

1. Introduction

Glucagon-like peptide-1 (GLP-1) [1] is an incretin hormone secreted from the L cells of the small intestine in response to food intake. This hormone plays several biological roles including the stimulation of insulin secretion, inhibition of glucagon secretion, retardation of gastric emptying, induction of satiety and stimulating the regeneration and differentiation of islet β cells [2]. However, GLP-1 (GLP-1[7-36]amide) is rapidly degraded in vivo (lifetime: about 1 min) through the action of dipeptidyl peptidase IV (DPP-IV), which cleaves a dipeptide from the N-terminus to give the inactive GLP[9-36]amide [3]. DPP-IV is a serine protease cleaving the N-terminal dipeptide with a preference for L-proline or L-alanine at the penultimate position [4]. This protease is expressed in many tissues and body fluids, and exists as either a membrane-bound or a soluble enzyme. Inhibition of DPP-IV increases the level of circulating GLP-1 and thus increases insulin secretion [5], which can ameliorate hyperglycemia in type 2 diabetes. A number of small molecule inhibitors of DPP-IV have been described [6] and several of these, including Vildagliptin (LAF237) [7a], Saxagliptin (BMS477118) [7b] and Sitagliptin (MK-0431) [8] are in late-stage of clinical development or approved by the U.S. Food and Drug Administration (Fig. 1).

Since DPP-IV is a dipeptidase that selectively binds substrates with proline at the P1 position, many of its inhibitors investigated to date possess 5-membered heterocyclic rings (e.g., pyrrolidine, thiazolidine, cyano-pyrrolidine, and cyano-thiazolidine) that serve as proline mimics. A number of DPP-IV inhibitors contain cyano-pyrrolidine ring [6]. We have recently described several novel 5-membered proline surrogates that are based on pyrazolidine [9,10] and pyrazoline

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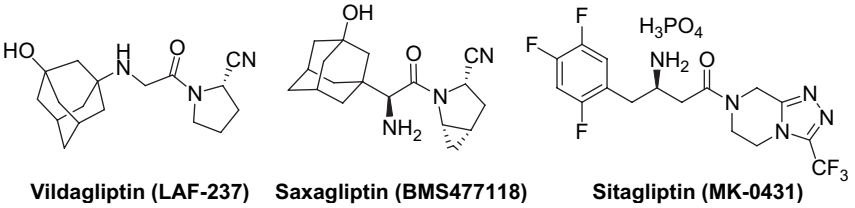
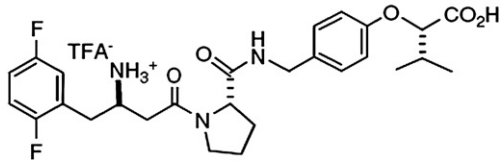


Fig. 1. Molecular structures of representative DPP-IV inhibitors.

[11]. Our compounds containing the pyrazoline template showed a good in vivo efficacy as well as in vitro activity against DPP-IV [11]. In the meantime, a group at Merck recently described a series of structurally novel β-amino amide derivatives that have good in vitro potency and in vivo efficacy [8a]. Among these, MK-0431, which is approved by FDA, shows excellent selectivity and in vivo efficacy. Also, Merck's researcher identified proline derived homophenylalanine derivatives, which were potent and highly selective [8b].



This observation prompted us to explore the properties of new DPP-IV inhibitors that possess our basic pyrazoline structure and contain appended β-amino acid moiety (Fig. 2). We now wish to report the synthesis of pyrazoline analogues with β-amino acyl group and their biological evaluation as DPP-IV inhibitors (Fig. 2).

2. Synthesis

Compounds selected for biological evaluation were prepared as described in Scheme 1. Compound **4** was prepared based on our previous paper [12]. Acryloyl chloride was esterificated with benzyl alcohol to produce benzyl ester, followed by cyclization with 2 M trimethylsilyl diazomethane and removal of TMS group under acidic condition to afford the pyrazoline ester (**4**). Pyrazoline ester (**4**) was coupled with β-amino acid (**5**) in the presence of dimethylpropyl ethyl carbodiimide hydrochloride (EDCI) to produce the coupled product (**6**), which upon reaction with 10% palladium on charcoal in MeOH yielded the corresponding acid (**7**). (*R*)-2,4,5-Trifluorophenylbutyryl pyrazoline carboxylic acid **7** could be converted to ester and amide derivatives (**8**) in the presence of EDCI with diverse amine and alcohol derivatives which were commercially available or synthesized according to the procedures in Section 5. Compound **8** was deprotected by 4 M HCl to afford compound **1**. Among compound **8**, amide derivatives (X = NH) were hydrolyzed with $LiOH{\cdot}H_2O$ to give the acid (**9**), followed by the deprotection under acidic condition to produce the final acid compound **2**.

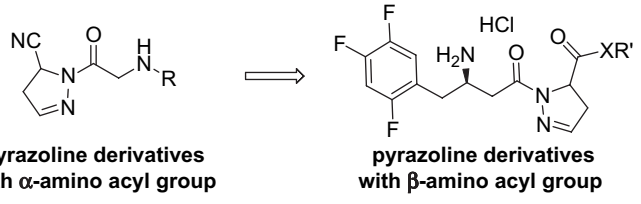


Fig. 2. The design of new DPP-IV inhibitor, β-amino pyrazoline derivatives.

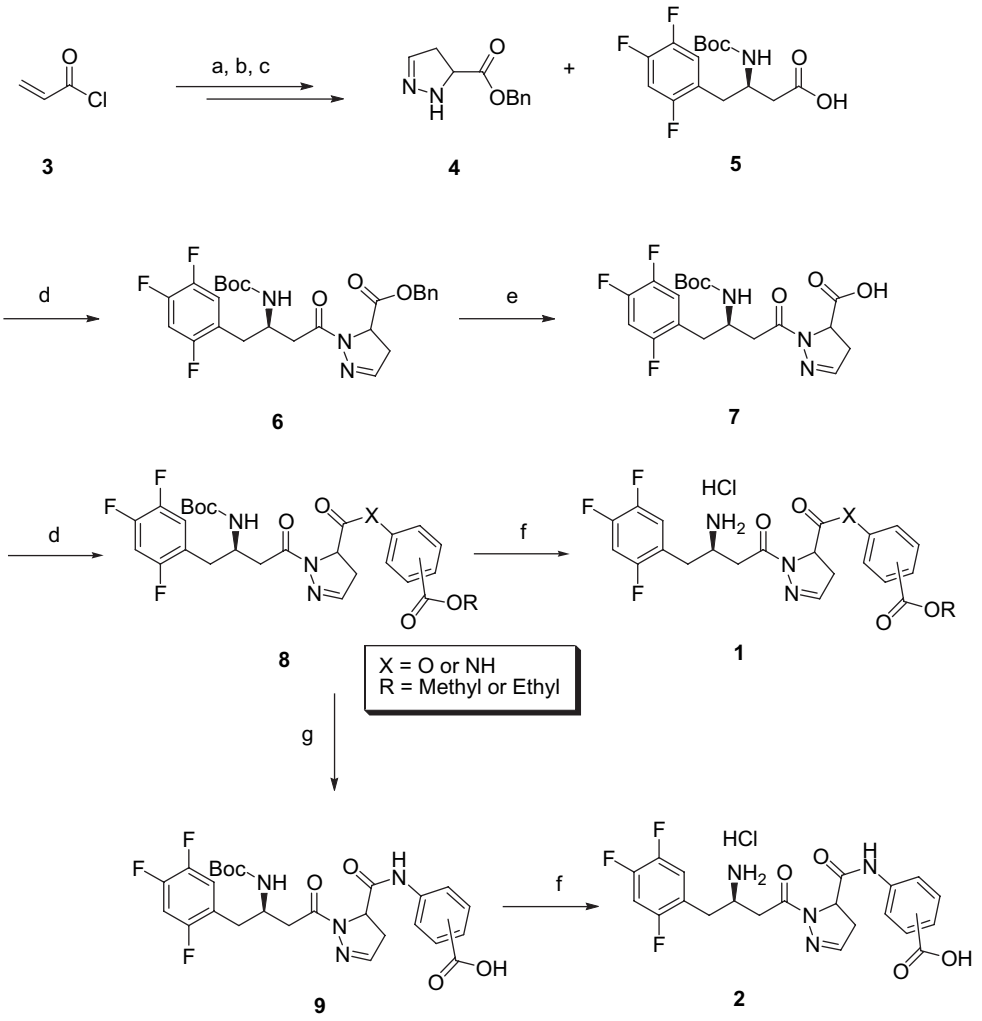
3. Results and discussion

Pyrazoline derivatives **1** were tested in vitro against DPP-IV and the results are summarized in Table 1. MK-0431 was used as a reference compound [8]. The benzyl substituted ester analogue **1b** showed a submicromolar activity with an IC_{50} value of 0.71 μM and was 2-fold more active than the unsubstituted acid compound **1a**. But other ester derivatives (**1c**–**1f**) showed decreases of the in vitro activities compared to benzyl ester **1b**.

The *N*-benzyl amide analogue **1h** was 3-fold more potent than the unsubstituted amide **1g** with an IC_{50} value of 1.6 μM. Therefore, we determined the crystal structure of human DPP-IV complexed with **1h** (Fig. 3, we ran the co-crystallization of diastereomer mixtures of pyrazoline ring with human DPP-IV). Chiral (*S*) pyrazoline-derived co-crystal structure was obtained from the (*R*) and (*S*) mixtures. This seemed that (*S*) pyrazoline isomer was more effective for binding with DPP-IV. The 2,4,5-trifluorophenyl moiety fully occupies a catalytic pocket including S630 and (*R*)-β-amino group forms an ionic interaction with carboxylate anion of Glu205 and Glu206 residues as well known in other crystal structures [8]. The carbonyl group of pyrazoline interacts with side chain of Tyr547 via a water molecule. But benzyl amide part doesn't show a special interaction with DPP-IV, so these areas could be more modified to increase potency.

We further modified this amide derivative **1h** by adoption of diverse substituents. Phenyl amide **1j** was similar to benzyl amide **1h**. *p*-Methoxy carbonyl substituted phenyl amide **1k** showed a submicromolar inhibitory activity with an IC_{50} value of 0.77 μM. Heteroaryl compounds (**1l** and **1m**) exhibited weak activities. Also, other amino, morpholino, sulfonamide, sulfonic acid derivatives (**1n**, **1o**, and **1s** and **1t**) exhibited moderate IC_{50} values in the range 1.3–8.9 μM. The *para*-2-ethoxyl-2-oxoethoxy substituted phenyl amide analogue **1u** showed the most potent activity (IC_{50} = 0.69 μM), whereas, *meta* or *ortho* substitutent showed a decrease of the in vitro activity compared to **1u**.

We turned our attention to replace the ester moiety with a carboxylic acid. So, various acid derivatives were evaluated the inhibitory activities as shown in Table 2. Notably, *para*



Scheme 1. Synthesis of pyrazoline derivatives. (a) Benzyl alcohol, Et_3N, CH_2Cl_2, 83%; (b) 2 M TMS–CHN_2 in Et_2O, toluene; (c) TFA, CH_2Cl_2, 83% (for 2 steps); (d) EDCI, Et_3N, CH_2Cl_2, 15–99%; (e) 10% Pd/C, MeOH, H_2, 95%; (f) 4 M HCl/1,4-dioxane, EtOAc, 69–99%; (g) LiOH·H_2O, THF, H_2O, 43–99%.

alkoxy acid substituted phenyl amide **2u** showed the most active in this series with an IC_{50} value of 0.51 μM.

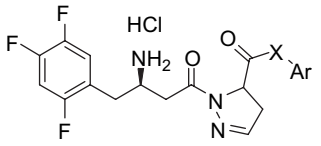
Cytochrome P450 (CYP) enzymes play a major role in metabolizing drug molecules. Many lead candidate molecules in pharmaceutical development fail due to the potent inhibition of one or more isozymic forms of CYP enzymes. Cytochrome P450 3A4 (CYP3A4), a heme–thiolate protein, is one of the most important P450s in human liver [13]. The ability of CYP3A4 to metabolize >50% of administered therapeutic agents accounts for the large number of documented drug–drug interactions associated with CYP3A4 inhibition [14]. It is therefore of great importance to know as early as possible the affinity of new drug candidates to CYP3A4, in order to determine their potential as inhibitors and to evaluate their influence on the metabolism of co-administered drugs. Several pyrazoline derivatives **1** and **2** were evaluated for their inhibitory activity toward cytochrome P450 3A4 (CYP3A4) and the results are summarized in Table 3. Hydrogen, amino, and ester substituted derivatives (**1h**, **1n**, and **1u**) showed strong inhibitions toward P450 3A4 (CYP3A4). But, carboxylic acid substituted pyrazoline derivatives **2i** and **2u** greatly decrease the inhibitory activities toward CYP3A4 (62 and 59%, respectively).

We carried out the systematic study for developing new DPP-IV inhibitor which was considered for SAR study, X-ray structure determination, and CYP inhibition. We found compound **2u** as a pro-type of alternative DPP-IV inhibitor. Therefore we are under further investigation to find out more active and safe drug candidate.

4. Conclusion

In conclusion, we have synthesized a series of pyrazoline derivatives with β-amino acyl group and evaluated their ability to inhibit dipeptidyl peptidase IV. Several pyrazoline derivatives exhibited submicromolar inhibitory activities against DPP-IV; X-ray co-crystal structure of initial hit compound **1h** was determined. Among this series, carboxylic acid substituted pyrazoline derivative **2u** was the most active and overcomed the CYP3A4 enzyme inhibition.

Table 1
In vitro activity against DPP-IV of pyrazoline derivatives



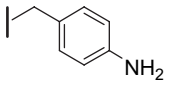
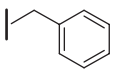
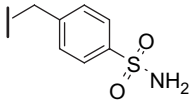
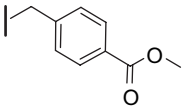
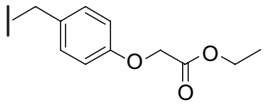
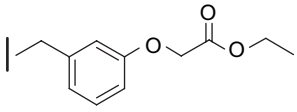
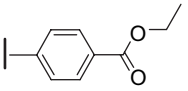
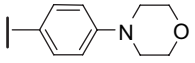
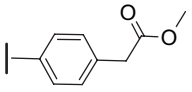
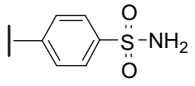
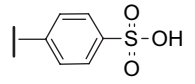
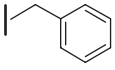
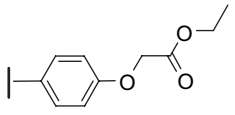
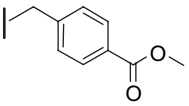
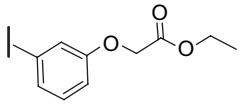
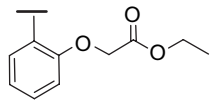
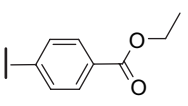
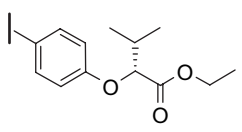
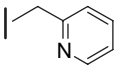
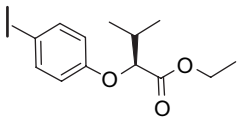
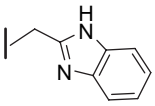
Entry	x	Ar	IC_{50} (μM)	Entry	x	Ar	IC_{50} (μM)
1a	o	H	1.4	**1n**	NH		1.3
1b	o		0.71	**1o**	NH		3.1
1c	o		2.7	**1p**	NH		2.1
1d	o		2.2	**1q**	NH		2.0
1e	o		1.6	**1r**	NH		1.6
1f	o		1.9	**1s**	NH		9.1
1g	NH	H	5.6	**1t**	NH		8.9
1h	NH		1.6	**1u**	NH		0.69
1i	NH		3.0	**1v**	NH		1.9
1j	NH		1.6	**1w**	NH		4.8
1k	NH		0.77	**1x**	NH		2.6

Table 1 (*continued*)

Entry	x	Ar	IC_{50} (μM)	Entry	x	Ar	IC_{50} (μM)
1l	NH		7.4	**1y**	NH		3.6
1m	NH		3.7		MK-0431		0.11

IC_{50} values were determined from direct regression curve analysis.

5. Experimental procedure

5.1. Chemistry

5.1.1. 3,4-Dihydro-2H-pyrazole-3-carboxylic acid benzyl ester (*4*)

To a solution of acryloyl chloride **3** (12.7 ml, 123.08 mmol) in CH_2Cl_2 (400 ml) was added dropwise benzyl alcohol (10.0 ml, 123.08 mmol) and Et_3N (25.7 ml, 184.62 mmol) at 0 °C. The reaction mixture was stirred for 12 h at room temperature under N_2 atmosphere, quenched with H_2O, diluted with CH_2Cl_2, washed with brine and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* and the residue was purified by flash chromatography (EtOAc:hexane = 1:19) to give acrylic acid benzyl ester (16.5 g, 83%) as a colorless oil. ^1H NMR ($CDCl_3$, 300 MHz) δ 7.39–7.30 (m, 5H), 6.45 (dd, $J = 1.5$, 17.4 Hz, 1H), 6.17 (dd, $J = 10.2$, 17.4 Hz, 1H), 5.85 (dd, $J = 1.5$, 10.2 Hz, 1H), 5.20 (s, 2H). EI-MS *m/z* (relative intensity) 162 (M^+, 10), 117 (26), 105 (32), 91 (100), 77 (82), 55 (75), 51 (70).

To a solution of acrylic acid benzyl ester (5.0 g, 30.83 mmol) in toluene (50 ml) was added 2 M trimethylsilyl diazomethane in Et_2O (23.0 ml, 46.24 mmol). The reaction mixture was stirred for 3 h at room temperature under N_2 atmosphere. The solvent was removed *in vacuo* and the obtained residue **5** was diluted with CH_2Cl_2 (50 ml) and added dropwise trifluoroacetic acid (6.9 ml, 92.48 mmol) at 0 °C. The resulting mixture was stirred for 1 h at room temperature under N_2 atmosphere. The solvent was removed *in vacuo* and the residue was purified by flash chromatography (EtOAc:hexane = 1:2) to give 3,4-dihydro-2*H*-pyrazole-3-carboxylic acid benzyl ester **4** (5.2 g, 83%) as a colorless oil. ^1H NMR ($CDCl_3$, 300 MHz) δ 7.36–7.27 (m, 5H), 6.86 (s, 1H), 5.18 (s, 2H), 4.28 (m, 1H), 3.03 (m, 2H). EI-MS *m/z* (relative intensity)

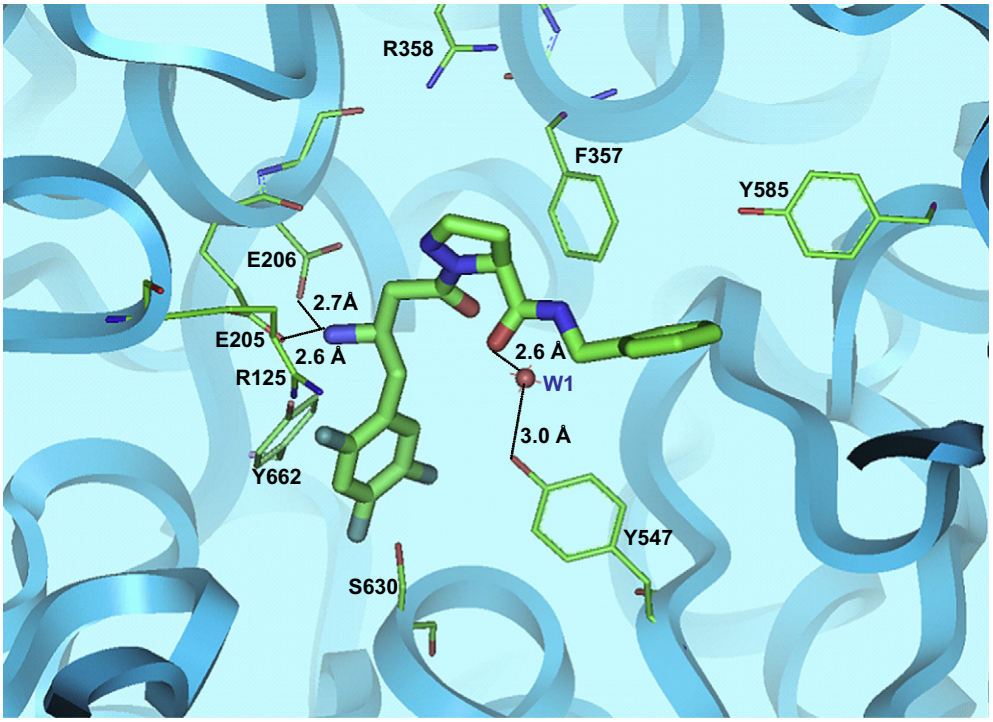
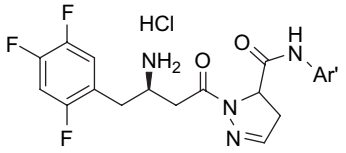


Fig. 3. X-ray co-crystal structure of pyrazoline derivative **1h**. **1h** is represented by thick stick model.

Table 2
In vitro activity against DPP-IV of pyrazoline derivatives



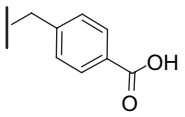
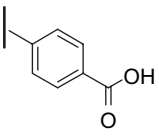
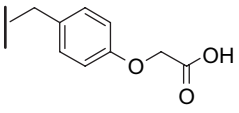
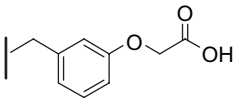
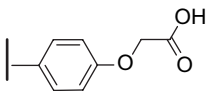
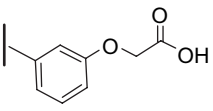
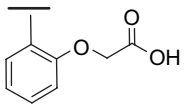
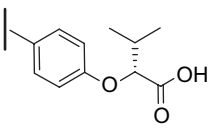
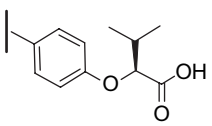
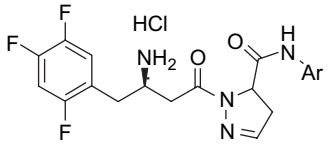
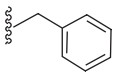
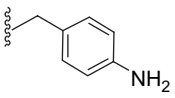
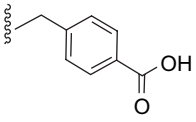
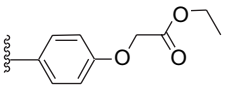
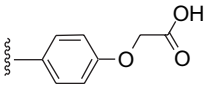
Entry	Ar′	IC_{50} (μM)
2i		1.4
2k		5.9
2p		2.5
2q		2.8
2u		0.51
2v		4.3
2w		8.5
2x		2.7
2y		2.2
	MK-0431	0.11

Table 3
In vitro activity against CYP of pyrazoline derivatives



Entry	Ar	CYP3A4 activity at 10 μM (%)
1h		18.8
1n		4.5
2i		62.0
1u		6.9
2u		59.3

203 (M^+, 1), 165 (1), 149 (1), 112 (1), 91 (35), 69 (76), 58 (16), 43 (100).

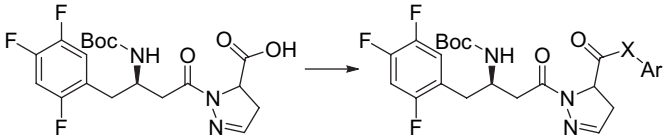
*5.1.2. (R)-2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid benzyl ester (**6**)*

To a solution of (*R*)-3-*tert*-butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyric acid **5** (500 mg, 1.50 mmol) in CH_2Cl_2 (5 ml) was added 3,4-dihydro-2*H*-pyrazole-3-carboxylic acid benzyl ester **4** (305 mg, 4.50 mmol), EDCI (863 mg, 4.50 mmol), and Et_3N (1.5 ml, 10.50 mmol). The reaction mixture was stirred for 12 h at room temperature, quenched with H_2O, diluted with CH_2Cl_2, washed with brine and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* and the residue was purified by flash chromatography (EtOAc:hexane = 4:1) to give an (*R*)-2-[3-*tert*-butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2*H*-pyrazole-3-carboxylic acid benzyl ester **6** (848 mg, 54%) as a white solid. ^1H NMR ($CDCl_3$, 300 MHz) δ 7.35–7.30 (m, 5H), 7.07–7.02 (m, 1H), 6.91–6.82 (m, 1H + s, 1H), 5.20 (s, 2H), 4.90–4.85 (m, 1H), 4.21–4.20 (m, 1H), 3.30–3.20 (m, 1H), 2.96–2.72 (m, 5H), 1.37 (s, 9H). LC–MS *m/z* 520 (MH^+).

*5.1.3. (R)-2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid (**7**)*

To a solution of (*R*)-2-[3-*tert*-butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2*H*-pyrazole-3-carboxylic acid benzyl ester **6** (760 mg, 1.46 mmol) in MeOH (5 ml) was added 10 wt.% Pd/C (142 mg). The reaction mixture was stirred for 3 h under H_2 pressure, filtered with Celite, removed the solvent and dried *in vacuo* to give an (*R*)-2-[3-*tert*-butoxycarbonylamino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2*H*-pyrazole-3-carboxylic acid **7** (595 mg, 95%) as a white solid. ^{1}H NMR (acetone-d_6, 300 MHz) δ 7.22–7.15 (m, 1H), 7.13–7.00 (m, 1H), 6.96 (s, 1H), 6.00–5.80 (br s, 1H), 4.71–4.64 (m, 1H), 4.17–4.15 (m, 1H), 3.27–2.82 (m, 6H), 1.19 (s, 9H). LC–MS *m/z* 429 (MH^+).

*5.2. General acylation procedure of **7***



7 **8**

To a solution of (*R*)-2-[3-*tert*-butoxycarbonylamino-4-(2,4,5-trifluorophenyl)-butyryl]-3,4-dihydro-2*H*-pyrazole-3-carboxylic acid **7** (30 mg, 0.07 mmol) in CH_2Cl_2 (1 ml) was added various amine/alcohol (0.14 mmol), EDCI (40 mg, 0.21 mmol), Et_3N (49μl, 0.35 mmol). The reaction mixture was stirred for 12 h at room temperature, quenched with H_2O, diluted with CH_2Cl_2, washed with brine and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* and the residue was purified by flash chromatography to give the desired product **8**.

*5.2.1. (R)-2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid 4-methoxycarbonyl benzyl ester (**8c**)*

Yield: 53%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.03 (d, $J = 8.4$ Hz, 2H), 7.42 (d, $J = 8.4$ Hz, 2H), 7.20–7.00 (m, 1H), 6.89–6.86 (m, 1H), 6.88 (s, 1H), 5.45–5.40 (br s, 1H), 5.25 (s, 2H), 4.89 (q, $J = 6.0$ Hz, 2H), 4.30–4.20 (m, 1H), 3.91 (s, 3H), 3.27–3.22 (m, 1H), 2.97–2.83 (m, 5H), 1.37 (s, 9H).

*5.2.2. (R)-2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid phenyl ester (**8d**)*

Yield: 15%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39 (t, $J = 7.8$ Hz, 3H), 7.13 (d, $J = 7.8$ Hz, 2H), 7.07–7.04 (m, 1H), 6.95 (s, 1H), 6.91–6.85 (m, 1H), 5.35 (br s, 1H), 5.03 (q, $J = 6.3$ Hz, 1H), 4.24–4.22 (m, 1H), 3.41 (dd, $J = 13.8$, 18.6 Hz, 1H), 3.21–3.12 (m, 1H), 2.99–2.88 (m, 4H), 1.35 (s, 9H).

*5.2.3. (R)-2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid 4-ethoxycarbonyl phenyl ester (**8e**)*

Yield: 32%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.08 (d, $J = 8.7$ Hz, 2H), 7.21 (d, $J = 8.7$ Hz, 2H), 7.12–7.03 (m, 1H), 6.96 (s, 1H), 6.91–6.82 (m, 1H), 5.42 (br, 1H), 4.83 (q, $J = 6.0$ Hz, 1H), 4.37 (q, $J = 7.2$ Hz, 2H), 4.22 (m, 1H), 3.32–2.86 (m, 6H), 1.39 (t, $J = 7.2$ Hz, 3H), 1.37 (s, 9H).

*5.2.4. (R)-2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid 4-methoxycarbonyl methyl phenyl ester (**8f**)*

Yield: 31%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.30 (d, $J = 8.4$ Hz, 2H), 7.09 (d, $J = 8.4$ Hz, 2H), 7.08–7.01 (m, 1H), 6.95 (s, 1H), 6.91–6.82 (m, 1H), 5.38–5.35 (br, 1H), 5.01 (q, $J = 6.0$ Hz, 1H), 4.23–4.22 (m, 1H), 3.69 (s, 3H), 3.61 (s, 2H), 3.46–2.87 (m, 6H), 1.35 (s, 9H).

*5.2.5. (R)-[3-(5-Carbamoyl-4,5-dihydropyrazol-1-yl)-3-oxo-1-(2,4,5-trifluorobenzyl)propyl]carbamic acid tert-butyl ester (**8g**)*

Yield: 84%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.08–7.01 (m, 1H), 7.00 (s, 1H), 6.93–6.85 (m, 1H), 5.46 (br, 1H), 5.25–5.10 (br, 1H), 4.94–4.86 (m, 1H), 4.45–4.20 (m, 1H), 3.69–3.60 (m, 1H), 3.09–2.81 (m, 5H), 1.37 (s, 9H).

*5.2.6. (R)-[3-(5-Benzylcarbamoyl-4,5-dihydropyrazol-1-yl)-3-oxo-1-(2,4,5-trifluoro benzyl)propyl]carbamic acid tert-butyl ester (**8h**)*

Yield: 47%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.72–7.23 (m, 5H), 7.01 (s, 1H), 7.00–6.85 (m, 2H), 5.30–5.10 (br, 1H), 4.97–4.89 (m, 1H), 4.44 (s, 2H), 4.32–4.20 (m, 1H), 3.70–3.66 (m, 1H), 3.09–2.75 (m, 5H), 1.36 (s, 9H). LC–MS *m/z* 519 (MH^+).

*5.2.7. (R)-4-[({2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)methyl]benzoic acid methyl ester (**8i**)*

Yield: 62%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.97 (d, $J = 8.1$ Hz, 2H), 7.85 (br s, 1H), 7.32 (d, $J = 8.1$ Hz, 2H), 7.01 (s, 1H), 7.01–6.83 (m, 2H), 5.08 (br s, 1H), 4.95 (dd, $J = 4.8$, 11.7 Hz, 1H), 4.50 (d, $J = 5.4$ Hz, 2H), 4.28–4.24 (m, 1H), 3.88 (s, 3H), 3.69–3.64 (m, 1H), 3.06 (dd, $J = 11.7$, 18.9 Hz, 1H), 2.90–2.81 (m, 4H), 1.34 (s, 9H).

*5.2.8. (R)-[3-(5-Phenylcarbamoyl-4,5-dihydropyrazol-1-yl)-3-oxo-1-(2,4,5-trifluoro benzyl)propyl]carbamic acid tert-butyl ester (**8j**)*

Yield: 52%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.60 (s, 1H), 7.52 (d, $J = 7.8$ Hz, 2H), 7.33–7.26 (m, 2H), 7.12–7.04 (m, 3H), 6.92–6.83 (m, 1H), 5.13–5.11 (m, 1H), 5.09–5.07 (m, 1H), 4.27–4.20 (m, 1H), 3.91–3.80 (m, 1H), 3.10–2.89 (m, 4H), 1.33 (s, 9H). LC–MS *m/z* 505 (MH^+).

*5.2.9. (R)-4-({2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)benzoic acid ethyl ester (**8k**)*

Yield: 23%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.00 (d, $J = 8.7$ Hz, 2H), 7.61 (d, $J = 8.7$ Hz, 2H), 7.07 (s, 1H), 7.07–7.06 (m, 1H), 6.91–6.87 (m, 1H), 5.15–5.09 (m, 2H), 4.35 (q, $J = 7.2$ Hz, 1H), 4.40–4.20 (m, 1H), 3.90–3.80 (m, 1H), 3.11–2.89 (m, 4H), 1.38 (t, $J = 7.2$ Hz, 3H), 1.33 (s, 9H).

*5.2.10. (R)-[3-{5-(Pyridin-2-ylmethyl)carbamoyl-4,5-dihydropyrazol-1-yl)-3-oxo-1-(2,4,5-trifluorobenzyl) propyl]carbamic acid tert-butyl ester (**8l**)*

Yield: 40%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.51 (d, $J = 4.5$ Hz, 1H), 8.07 (br s, 1H), 7.65 (t, $J = 7.5$ Hz, 1H), 7.26–7.15 (m, 2H), 7.00–6.98 (m, 2H), 6.88–6.85 (m, 1H), 5.65–5.50 (br, 1H), 5.01–4.94 (m, 1H), 4.58 (s, 2H), 4.30–4.15 (m, 1H), 3.70–3.55 (m, 1H), 3.14–2.77 (m, 5H), 1.35 (s, 9H). LC–MS *m/z* 520 (MH^+).

*5.2.11. (R)-[3-{5-[(1H-Benzoimidazol-2-ylmethyl) carbamoyl]-4,5-dihydro-pyrazol-1-yl}-3-oxo-1-(2,4,5-trifluorobenzyl)propyl]carbamic acid tert-butyl ester (**8m**)*

Yield: 28%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.15 (br s, 3H), 7.55–7.53 (m, 2H), 7.24–7.20 (m, 2H), 7.05–6.84 (m, 3H), 5.75–5.55 (m, 1H), 4.76 (s, 2H), 4.57–4.49 (m, 1H), 4.32–4.28 (m, 1H), 3.10–2.81 (m, 5H), 1.35 (s, 9H). LC–MS *m/z* 559 (MH^+).

*5.2.12. (R)-[3-[5-(4-Amino-benzylcarbamoyl)-4,5-dihydropyrazol-1-yl]-3-oxo-1-(2,4,5-trifluorobenzyl) propyl]carbamic acid tert-butyl ester (**8n**)*

Yield: 58%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.48 (br s, 1H), 7.05 (d, $J = 8.4$ Hz, 2H), 6.99 (s, 1H), 6.99–6.85 (m, 2H), 6.61 (d, $J = 8.4$ Hz, 2H), 5.30 (br s, 1H), 4.89 (dd, $J = 4.8$, 11.7 Hz, 1H), 4.31 (d, $J = 5.7$ Hz, 2H), 4.29–4.23 (m, 1H), 3.71–3.70 (m, 1H), 3.63–3.61 (br, 2H), 3.03 (dd, $J = 11.7$, 18.9 Hz, 1H), 2.91–2.78 (m, 4H), 1.37 (s, 9H).

*5.2.13. (R)-[3-Oxo-3-[5-(4-sulfamoyl-benzylcarbamoyl)-4,5-dihydropyrazol-1-yl]-1-(2,4,5-trifluorobenzyl)propyl] carbamic acid tert-butyl ester (**8o**)*

Yield: 42%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.93 (br, 1H), 7.84 (d, $J = 8.1$ Hz, 2H), 7.67 (br s, 1H), 7.39 (d, $J = 8.1$ Hz, 2H), 7.10–6.99 (m, 1H), 7.01 (s, 1H), 6.93–6.85 (m, 1H), 5.10–5.08 (br, 1H), 4.97–4.87 (m, 1H), 4.49 (s, 2H), 4.40–4.21 (m, 2H), 3.65–3.49 (m, 1H), 2.89–2.83 (m, 1H), 1.35 (s, 9H).

*5.2.14. (R)-{4-[({2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)methyl]phenoxy}acetic acid ethyl ester (**8p**)*

Yield: 33%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.61 (br s, 1H), 7.19 (d, $J = 8.7$ Hz, 2H), 7.05–6.98 (m, 1H), 6.99 (s, 1H), 6.92–6.88 (m, 1H), 6.84 (d, $J = 8.7$ Hz, 2H), 5.18 (br, 1H), 4.90 (dd, $J = 4.8$, 11.7 Hz, 1H), 4.57 (s, 2H), 4.37 (s, 2H), 4.26 (q, $J = 7.2$ Hz, 2H), 4.27–4.22 (m, 1H), 3.70–3.64 (m, 1H), 3.03 (dd, $J = 11.7$, 20.1 Hz, 1H), 2.89 (d, $J = 6.0$ Hz, 2H), 2.80 (d, $J = 8.1$ Hz, 2H), 1.36 (s, 9H), 1.30 (t, $J = 7.2$ Hz, 3H).

*5.2.15. (R)-{3-[({2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)methyl]phenoxy}acetic acid ethyl ester (**8q**)*

Yield: 62%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.64 (br s, 1H), 7.22 (t, $J = 7.8$ Hz, 1H), 7.05–6.99 (m, 1H), 6.99 (s, 1H), 6.92–6.87 (m, 1H), 6.88 (d, $J = 7.8$ Hz, 1H), 6.85 (s, 1H), 6.83 (d, $J = 7.8$ Hz, 1H), 5.10 (br, 1H), 4.92 (dd, $J = 4.8$, 11.2 Hz, 1H), 4.58 (s, 2H), 4.42 (s, 2H), 4.26 (q, $J = 7.2$ Hz, 2H), 4.25–4.23 (m, 1H), 3.72–3.62 (m, 1H), 3.10–2.80 (m, 5H), 1.35 (s, 9H), 1.26 (t, $J = 7.2$ Hz, 3H).

*5.2.16. (R)-[3-[5-(4-Morpholin-4-ylphenylcarbamoyl)-4,5-dihydropyrazol-1-yl]-3-oxo-1-(2,4,5-trifluorobenzyl) propyl]carbamic acid tert-butyl ester (**8r**)*

Yield: 69%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.43 (d, $J = 9.0$ Hz, 2H), 7.20–7.00 (m, 1H), 7.04 (s, 1H), 6.90–6.87 (m, 1H), 6.85 (d, $J = 9.0$ Hz, 2H), 5.08 (dd, $J = 4.8$, 11.7 Hz, 1H), 4.35–4.20 (m, 1H), 3.87–3.79 (m, 6H), 3.12–3.09 (m, 4H), 2.94–2.88 (m, 4H), 1.30 (s, 9H).

*5.2.17. (R)-[3-Oxo-3-[5-(4-sulfamoylphenylcarbamoyl) -4,5-dihydropyrazol-1-yl]-1-(2,4,5-trifluorobenzyl) propyl]carbamic acid tert-butyl ester (**8s**)*

Yield: 83%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.93 (br, 1H), 8.21 (br s, 3H), 7.11–7.08 (m, 1H), 6.91–9.79 (m, 2H), 6.58–6.51 (m, 4H), 5.50–5.30 (br, 1H), 5.30–5.23 (m, 1H), 4.21–4.10 (m, 1H), 4.09–3.56 (m, 1H), 3.42–3.22 (m, 2H), 2.83–2.81 (m, 1H), 1.80–1.60 (m, 2H), 1.36 (s, 9H).

*5.2.18. (R)-4-({2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)benzenesulfonic acid (**8t**)*

Yield: 100%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.13–7.07 (m, 1H), 6.90–6.82 (m, 3H), 6.55 (s, 1H), 6.54 (d, $J = 5.1$ Hz, 2H), 5.30–5.23 (m, 1H), 4.21–4.05 (m, 2H), 3.43–3.05 (m, 2H), 2.88–2.60 (m, 2H), 1.80–1.60 (m, 1H), 1.37 (s, 9H).

*5.2.19. (R)-{4-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}acetic acid ethyl ester (**8u**)*

Yield: 76%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.45 (d, $J = 9.0$ Hz, 2H), 7.20–7.05 (m, 1H), 7.04 (s, 1H), 6.92–6.84 (m, 1H), 6.86 (d, $J = 9.0$ Hz, 2H), 5.07 (dd, $J = 4.8$, 11.7 Hz, 1H), 4.58 (s, 2H), 4.30–4.22 (m, 1H), 4.26 (q, $J = 7.2$ Hz, 2H), 3.90–3.80 (m, 1H), 3.11–2.89 (m, 5H), 1.33 (s, 9H), 1.26 (t, $J = 7.2$ Hz, 3H).

*5.2.20. (R)-{3-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}acetic acid ethyl ester (**8v**)*

Yield: 77%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.63 (s, 1H), 7.34 (s, 1H), 7.21 (t, $J = 8.1$ Hz, 1H), 7.05–7.03 (m, 3H), 6.88 (d, $J = 8.1$ Hz, 1H), 6.67 (d, $J = 8.1$ Hz, 1H), 5.20–5.00 (br s, 1H), 5.08 (dd, $J = 4.8$, 11.7 Hz, 1H), 4.60

(s, 2H), 4.30–4.23 (m, 1H), 4.26 (q, $J = 7.2$ Hz, 2H), 3.87–3.80 (m, 1H), 3.10–2.86 (m, 5H), 1.34 (s, 9H), 1.27 (t, $J = 7.2$ Hz, 3H).

*5.2.21. (R)-{2-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}acetic acid ethyl ester (**8w**)*

Yield: 32%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.62 (br s, 1H), 8.33 (d, $J = 9.0$ Hz, 1H), 7.06–7.01 (m, 3H), 7.01 (s, 1H), 6.83 (d, $J = 9.0$ Hz, 2H), 5.30 (br, 1H), 5.11 (dd, $J = 4.8$, 11.7 Hz, 1H), 4.71 (s, 2H), 4.26 (q, $J = 7.2$ Hz, 2H), 3.64–3.62 (m, 1H), 3.19–2.90 (m, 5H), 1.32 (s, 9H), 1.26 (t, $J = 7.2$ Hz, 3H).

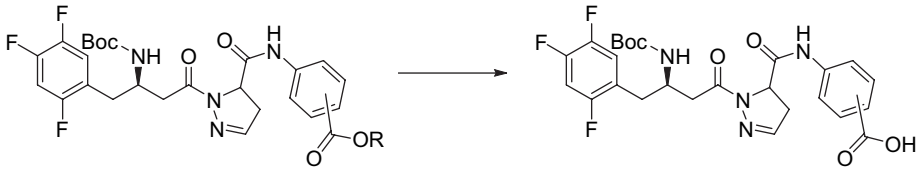
*5.2.22. (R)-{4-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}-(S)-isopropyl acetic acid ethyl ester (**8x**)*

Yield: 83%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.41 (d, $J = 8.9$ Hz, 2H), 7.10–7.03 (m, 1H), 7.04 (s, 1H), 6.92–6.83 (m, 1H), 6.83 (d, $J = 8.9$ Hz, 2H), 5.14 (br, 1H), 5.07 (dd, $J = 4.7$, 11.7 Hz, 1H), 4.30 (d, $J = 5.5$ Hz, 1H), 4.20 (q, $J = 7.2$ Hz, 2H), 3.86–3.80 (m, 1H), 3.10–2.88 (m, 6H), 2.26 (q, $J = 6.4$ Hz, 1H), 1.33 (s, 9H), 1.24 (t, $J = 7.2$ Hz, 3H), 1.06 (d, $J = 7.2$ Hz, 6H).

*5.2.23. (R)-{4-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}-(R)-isopropyl acetic acid ethyl ester (**8y**)*

Yield: 69%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.41 (d, $J = 8.9$ Hz, 2H), 7.10–7.00 (m, 1H), 7.04 (s, 1H), 6.92–6.85 (m, 1H), 6.84 (d, $J = 8.9$ Hz, 2H), 5.14 (br, 1H), 5.07 (dd, $J = 4.7$, 11.7 Hz, 1H), 4.30 (d, $J = 5.6$ Hz, 1H), 4.20 (q, $J = 7.2$ Hz, 2H), 3.86–3.80 (m, 1H), 3.10–2.88 (m, 6H), 2.26 (q, $J = 6.6$ Hz, 1H), 1.33 (s, 9H), 1.24 (t, $J = 7.2$ Hz, 3H), 1.06 (d, $J = 7.2$ Hz, 6H).

*5.3. General hydrolysis procedure of ester **8***



To a solution of ester **8** in THF/H_2O (0.15 M) was added $LiOH \cdot H_2O$ (5 eq) at 0 °C and stirred for 12 h at room temperature. The solvent was removed *in vacuo* and the residue was acidified with 1 N HCl to pH 3–4, extracted with EtOAc, and washed with brine. The combined organic layer was dried over $MgSO_4$, evaporated, and solidified with pet. ether to give an acid **9**.

*5.3.1. (R)-4-[({2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)methyl]benzoic acid (**9i**)*

Yield: 69%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.73 (br, 1H), 7.86 (d, $J = 8.1$ Hz, 2H), 7.50–7.40 (m, 1H), 7.39 (d, $J = 8.1$ Hz, 2H), 7.35–7.20 (m, 1H), 7.15 (s, 1H), 4.68 (dd, $J = 5.7$, 11.4 Hz, 1H), 4.35 (dd, $J = 5.7$, 11.4 Hz, 2H), 4.20–4.05 (m, 1H), 2.89–2.82 (m, 6H), 1.26 (s, 9H).

*5.3.2. (R)-4-({2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)benzoic acid (**9k**)*

Yield: 76%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.93 (br, 1H), 8.21 (br s, 3H), 7.11–7.08 (m, 1H), 6.91–9.79 (m, 2H), 6.58–6.51 (m, 4H), 5.50–5.30 (br, 1H), 5.30–5.23 (m, 1H), 4.21–4.10 (m, 1H), 4.09–3.56 (m, 1H), 3.42–3.22 (m, 2H), 2.83–2.81 (m, 1H), 1.80–1.60 (m, 2H), 1.36 (s, 9H).

*5.3.3. (R)-{4-[({2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)methyl]phenoxy}acetic acid (**9p**)*

Yield: 78%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.60 (br s, 1H), 7.12 (d, $J = 8.7$ Hz, 2H), 7.00–6.97 (m, 1H), 6.91 (s, 1H), 6.85–6.79 (m, 1H), 6.78 (d, $J = 8.7$ Hz, 2H), 5.29 (br, 1H), 4.81 (dd, $J = 5.1$, 11.2 Hz, 1H), 4.50 (s, 2H), 4.29 (s, 2H), 4.22–4.12 (m, 1H), 3.45–3.38 (m, 1H), 3.02 (dd, $J = 12.0$, 20.1 Hz, 1H), 2.83–2.49 (m, 4H), 1.27 (s, 9H).

*5.3.4. (R)-{3-[({2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)methyl]phenoxy}acetic acid (**9q**)*

Yield: 93%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.53 (br s, 1H), 7.23 (t, $J = 7.8$ Hz, 1H), 7.04–6.98 (m, 1H), 6.98 (s, 1H), 6.91–6.83 (m, 4H), 4.86 (m, 1H), 4.66 (s, 2H), 4.38 (s, 2H), 4.20–4.00 (m, 1H), 3.62–3.61 (m, 1H), 3.04–2.59 (m, 5H), 1.34 (s, 9H).

*5.3.5. (R)-{4-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}acetic acid (**9u**)*

Yield: 100%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.48 (d, $J = 9.0$ Hz, 2H), 7.15–7.09 (m, 1H), 7.00 (s, 1H), 6.93–6.85 (m, 1H), 6.88 (d, $J = 9.0$ Hz, 2H), 5.70–5.50 (br, 1H),

5.01 (q, $J = 6.0$ Hz, 1H), 4.56 (s, 2H), 4.25–4.24 (m, 1H), 3.50–2.86 (m, 6H), 1.34 (s, 9H).

*5.3.6. (R)-{3-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}acetic acid (**9v**)*

Yield: 92%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.23–6.89 (m, 4H), 6.86 (s, 1H), 6.74–6.70 (m, 1H), 6.58–6.56 (m, 1H), 5.10–5.06 (m, 1H), 4.64 (s, 2H), 3.82–3.74 (m, 1H), 3.06–2.60 (m, 6H), 1.31 (s, 9H).

*5.3.7. (R)-{2-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}acetic acid (**9w**)*

Yield: 93%. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.97 (d, $J = 9.0$ Hz, 1H), 7.20–7.05 (m, 1H), 7.00–6.90 (m, 1H), 6.99 (d, $J = 9.0$ Hz, 1H), 6.89 (s, 1H), 6.87 (t, $J = 9.0$ Hz, 2H), 5.00–4.96 (m, 1H), 4.63 (s, 2H), 4.20–4.10 (m, 1H), 3.15–3.09 (m, 2H), 2.90–2.58 (m, 4H), 1.21 (s, 9H).

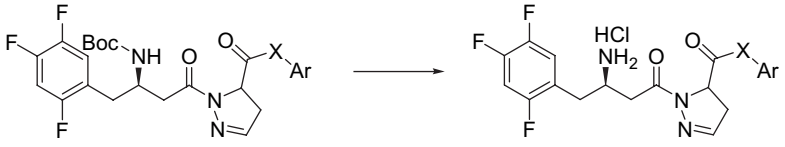
*5.3.8. (R)-{4-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}-(S)-isopropyl acetic acid (**9x**)*

Yield: 100%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39 (d, $J = 7.2$ Hz, 2H), 7.03 (d, $J = 7.2$ Hz, 2H), 6.93–6.81 (m, 2H + s, 1H), 5.17 (br, 1H), 5.01 (m, 1H), 4.36 (d, $J = 4.8$ Hz, 1H), 4.29–4.19 (m, 2H), 3.08–2.86 (m, 6H), 1.33 (s, 9H), 1.08 (d, $J = 7.2$ Hz, 6H).

*5.3.9. (R)-{4-[{2-[3-tert-Butoxycarbonylamino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}-(R)-isopropyl acetic acid (**9y**)*

Yield: 70%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.39 (d, $J = 7.2$ Hz, 2H), 7.03 (d, $J = 7.2$ Hz, 2H), 6.93–6.81 (m, 2H + s, 1H), 5.17 (br, 1H), 5.01 (m, 1H), 4.36 (d, $J = 4.8$ Hz, 1H), 4.29–4.19 (m, 2H), 3.08–2.86 (m, 6H), 1.33 (s, 9H), 1.08 (d, $J = 7.2$ Hz, 6H).

*5.4. General deprotection procedure of **8**/**9***



8 and **9** → **1** and **2**

To a solution of **9/10** EtOAc (0.2 M) was added 4 M HCl in 1,4-dioxane (10 eq). The reaction mixture was stirred for 12 h at room temperature under N_2 atmosphere, evaporated to remove solvent, and recrystallized with Et_2O. A recrystallized solid was filtered, washed with pet. ether and dried *in vacuo* to obtain the desired product **1/2**.

*5.4.1. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid HCl (**1a**)*

Yield: 51%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.05 (br s, 3H), 7.74–7.39 (m, 2H), 7.16 (s, 1H), 4.58–4.54 (m, 1H), 4.05 (m, 1H), 2.96–2.81 (m, 6H). LC–MS *m/z* 329 (MH^+).

*5.4.2. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid benzyl ester HCl (**1b**)*

Yield: 46%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.33 (br s, 3H), 7.18–7.65 (m, 2H), 7.72–7.65 (m, 2H), 7.61–7.45 (m, 5H), 7.40 (s, 1H), 5.28 (s, 2H), 4.98–4.92 (m, 1H), 3.91–3.89 (m, 1H), 3.66–3.48 (m, 1H), 3.19–3.03 (m, 5H). LC–MS *m/z* 420 (MH^+).

*5.4.3. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid 4-methoxycarbonyl benzyl ester HCl (**1c**)*

Yield: 90%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.88 (d, $J = 8.1$ Hz, 2H), 7.37 (d, $J = 8.1$ Hz, 2H), 7.25–7.07 (m, 2H), 7.03 (s, 1H), 5.16 (s, 2H), 4.83 (q, $J = 6.0$ Hz, 1H), 3.80 (s, 3H), 3.78–3.75 (m, 1H), 3.01–2.83 (m, 6H).

*5.4.4. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid phenyl ester (**1d**)*

Yield: 99%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.40 (t, $J = 8.1$ Hz, 3H), 7.35–7.24 (m, 2H), 7.20 (s, 1H), 7.14 (d, $J = 8.1$ Hz, 2H), 5.09 (q, $J = 6.0$ Hz, 1H), 3.94–3.92 (m, 1H), 3.56–3.49 (m, 1H), 3.27–3.00 (m, 5H).

*5.4.5. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid 4-ethoxycarbonyl phenyl ester HCl (**1e**)*

Yield: 100%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.96 (d, $J = 8.4$ Hz, 2H), 7.20 (d, $J = 8.4$ Hz, 2H), 7.14–7.11 (m, 2H), 7.00 (s, 1H), 5.20–4.90 (m, 1H), 4.26 (q, $J = 6.9$ Hz, 2H), 3.80–3.70 (m, 2H), 3.07–2.84 (m, 5H), 1.28 (t, $J = 6.9$ Hz, 3H).

*5.4.6. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid 4-methoxycarbonyl methyl phenyl ester HCl (**1f**)*

Yield: 100%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.21–6.97 (m, 7H), 5.10–4.90 (m, 1H), 4.05 (m, 1H), 3.80 (m, 2H), 3.64 (s, 2H), 3.41–2.96 (m, 4H).

*5.4.7. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid amide HCl (**1g**)*

Yield: 74%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.16–8.14 (br s, 3H), 7.62–7.50 (m, 2H), 7.16 (s, 1H), 4.60–4.52 (m, 1H), 3.85–3.75 (m, 1H), 2.98–2.79 (m, 6H).

*5.4.8. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid benzyl amide HCl (**1h**)*

Yield: 73%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.74–8.71 (br, 1H), 8.14–8.11 (br s, 3H), 7.59–7.50 (m, 2H), 7.35–7.23 (m, 5H), 7.21 (s, 1H), 4.72–4.64 (m, 1H), 4.28 (s, 2H), 3.80–3.70 (m, 1H), 3.04–2.82 (m, 6H). LC–MS *m/z* 455 (MH^+).

*5.4.9. (R)-4-[({2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino) methyl]benzoic acid methyl ester HCl (**1i**)*

Yield: 71%. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.17 (d, $J = 8.1$ Hz, 2H), 7.46 (d, $J = 8.1$ Hz, 2H), 7.58–7.38 (m, 2H), 7.32 (s, 1H), 5.12–5.00 (m, 1H), 4.75, 4.60 (d, $J = 15.6$ Hz, 2H), 4.09 (s, 3H), 4.13–4.07 (m, 1H), 3.57–3.37 (m, 2H), 3.24–3.07 (m, 4H).

*5.4.10. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid phenyl amide HCl (**1j**)*

Yield: 73%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.05 (br s, 3H), 7.59–7.49 (m, 4H), 7.31 (t, $J = 7.8$ Hz, 2H), 7.24 (s, 1H), 7.07 (t, $J = 7.2$ Hz, 1H), 4.87–4.81 (m, 1H), 3.77–3.75 (m, 1H), 3.37–3.33 (m, 1H), 3.02–2.96 (m, 5H). LC–MS *m/z* 442 (MH^+).

*5.4.11. (R)-4-({2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino) benzoic acid ethyl ester HCl (**1k**)*

Yield: 74%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.88 (d, $J = 8.7$ Hz, 2H), 7.60 (d, $J = 8.7$ Hz, 2H), 7.30–7.17 (m, 1H), 7.15–7.08 (m, 1H), 7.04 (s, 1H), 4.88–4.82 (m, 1H), 4.25 (q, $J = 7.2$ Hz, 2H), 3.90–3.80 (m, 1H), 3.36–2.78 (m, 6H), 1.28 (t, $J = 7.2$ Hz, 3H).

*5.4.12. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid (pyridin-2-ylmethyl)amide HCl (**1l**)*

Yield: 43%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.10 (d, $J = 5.5$ Hz, 1H), 8.71 (s, 1H), 8.25 (br s, 3H), 7.70 (d, $J = 7.2$ Hz, 2H), 7.55 (d, $J = 7.7$ Hz, 2H), 7.23 (s, 1H), 4.76–4.67 (m, 1H), 4.57 (s, 2H), 4.06–3.99 (m, 1H), 2.97–2.89 (m, 6H). LC–MS *m/z* 420 (MH^+).

*5.4.13. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid (1H-benzoimidazol-2-ylmethyl)amide HCl (**1m**)*

Yield: 50%. ^{1}H NMR (CD_3OD, 300 MHz) δ 9.43 (s, 1H), 8.38 (br s, 3H), 7.96–7.93 (m, 2H), 7.70–7.68 (m, 4H), 7.42 (s, 1H), 4.95–4.91 (m, 3H), 3.90–3.80 (m, 1H), 3.51–3.12 (m, 5H). LC–MS *m/z* 459 (MH^+).

*5.4.14. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid 4-amino benzyl amide HCl (**1n**)*

Yield: 85%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.40 (d, $J = 8.1$ Hz, 2H), 7.35–7.32 (m, 2H), 7.26 (d, $J = 8.1$ Hz, 2H), 7.19 (s, 1H), 4.41 (dd, $J = 5.4$, 12.3 Hz, 1H), 3.95 (d, $J = 4.8$ Hz, 2H), 3.63–3.54 (m, 1H), 3.10–2.90 (m, 6H).

*5.4.15. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid 4-sulfamoyl benzyl amide HCl (**1o**)*

Yield: 76%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.14 (br s, 3H), 7.76 (q, $J = 4.2$ Hz, 1H), 7.70 (q, $J = 4.2$ Hz, 1H), 7.54–7.42 (m, 4H), 7.22 (s, 1H), 4.68 (br s, 1H), 4.35 (d, $J = 4.8$ Hz, 2H), 4.15–4.12 (m, 1H), 3.78–3.76 (m, 1H), 2.96–2.84 (m, 4H).

*5.4.16. (R)-{4-[({2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino) methyl]phenoxy}acetic acid ethyl ester HCl (**1p**)*

Yield: 95%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.63 (t, $J = 5.7$ Hz, 2H), 8.04 (br s, 1H), 7.60–7.49 (m, 2H), 7.20 (s, 1H), 7.17 (d, $J = 8.4$ Hz, 2H), 6.85 (d, $J = 8.4$ Hz, 2H), 4.73 (s, 2H), 4.70–4.63 (m, 1H), 4.21 (s, 2H), 4.15 (q, $J = 7.2$ Hz, 2H), 3.78–3.74 (m, 1H), 3.32–3.23 (m, 1H), 2.98–2.78 (m, 5H), 1.21 (t, $J = 7.2$ Hz, 3H).

*5.4.17. (R)-{3-[({2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino) methyl]phenoxy}acetic acid ethyl ester HCl (**1q**)*

Yield: 87%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.70 (t, $J = 5.7$ Hz, 2H), 8.09 (br s, 1H), 7.60–7.49 (m, 2H), 7.22 (d, $J = 7.8$ Hz, 1H), 7.21 (s, 1H), 6.86 (d, $J = 7.8$ Hz, 1H), 6.83 (s, 1H), 6.68 (d, $J = 7.8$ Hz, 1H), 4.76 (s, 2H), 4.74–4.70 (m, 1H), 4.25 (s, 2H), 4.15 (q, $J = 7.2$ Hz, 2H), 3.78–3.76 (m, 1H), 3.33–3.18 (m, 1H), 2.99–2.78 (m, 5H), 1.21 (t, $J = 7.2$ Hz, 3H).

*5.4.18. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid (4-morpholin-4-ylphenyl)amide HCl (**1r**)*

Yield: 100%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.92 (br, 1H), 7.73 (d, $J = 8.1$ Hz, 2H), 7.59 (d, $J = 8.1$ Hz, 2H), 7.28–7.25 (m, 1H), 7.17–7.08 (m, 1H), 7.04 (s, 1H), 4.83–4.76 (m, 1H), 4.00 (m, 4H), 3.80 (m, 1H), 3.64–3.56 (m, 6H), 3.09–2.84 (m, 4H).

*5.4.19. (R)-2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carboxylic acid (4-sulfamoyl phenyl)amide HCl (**1s**)*

Yield: 87%. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.13 (d, $J = 6.6$ Hz, 2H), 7.43–7.40 (m, 1H), 7.25–7.23 (m, 1H), 7.12 (s, 1H), 7.02 (d, $J = 6.6$ Hz, 2H), 5.30–5.20 (m, 1H),

3.95–3.68 (m, 2H), 3.54–3.47 (m, 2H), 3.19–3.04 (m, 2H), 2.14–2.04 (m, 1H).

*5.4.20. (R)-4-({2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino)benzenesulfonic acid HCl (**1t**)*

Yield: 80%. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.13 (d, $J = 6.8$ Hz, 2H), 7.45–7.40 (m, 1H), 7.28–7.19 (m, 1H), 7.12 (s, 1H), 7.01 (d, $J = 6.8$ Hz, 2H), 5.35 (br, 1H), 3.92–3.67 (m, 3H), 3.20–3.00 (m, 4H).

*5.4.21. (R)-{4-[{2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl} amino]phenoxy}acetic acid ethyl ester HCl (**1u**)*

Yield: 100%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.16 (br s, 1H), 7.56–7.50 (m, 2H), 7.49 (d, $J = 9.0$ Hz, 2H), 7.23 (s, 1H), 6.88 (d, $J = 9.0$ Hz, 2H), 4.81 (q, $J = 6.0$ Hz, 1H), 4.73 (s, 2H), 4.16 (q, $J = 7.2$ Hz, 2H), 3.76–3.74 (m, 1H), 3.00–2.91 (m, 6H), 1.21 (t, $J = 7.2$ Hz, 3H).

*5.4.22. (R)-{3-[{2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy} acetic acid ethyl ester HCl (**1v**)*

Yield: 67%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.24–7.21 (m, 1H), 7.21 (s, 1H), 7.16–7.11 (m, 1H), 7.12 (d, $J = 8.4$ Hz, 1H), 7.03 (s, 1H), 7.03–7.01 (m, 1H), 6.60 (d, $J = 8.4$ Hz, 1H), 4.81 (q, $J = 6.0$ Hz, 1H), 4.57 (s, 2H), 4.14 (q, $J = 7.2$ Hz, 2H), 3.90–3.80 (m, 1H), 3.29–2.80 (m, 6H), 1.47 (t, $J = 7.2$ Hz, 3H).

*5.4.23. (R)-{2-[{2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl} amino]phenoxy}acetic acid ethyl ester HCl (**1w**)*

Yield: 74%. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.01 (d, $J = 7.5$ Hz, 1H), 7.39–7.20 (m, 2H), 7.17 (s, 1H), 7.11 (d, $J = 7.5$ Hz, 1H), 7.01 (t, $J = 7.5$ Hz, 2H), 5.11 (q, $J = 6.0$ Hz, 1H), 4.93–4.86 (m, 1H), 4.81 (s, 2H), 4.28 (q, $J = 7.2$ Hz, 2H), 4.00–3.80 (m, 1H), 3.36–2.90 (m, 5H), 1.31 (t, $J = 7.2$ Hz, 3H).

*5.4.24. (R)-{4-[{2-[3-Amino-4-(2,4,5-trifluorophenyl)butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino]phenoxy}-(S)-isopropyl acetic acid ethyl ester HCl (**1x**)*

Yield: 80%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.35 (d, $J = 8.9$ Hz, 2H), 7.30–7.21 (m, 1H), 7.17–7.08 (m, 1H), 7.02 (s, 1H), 6.75 (d, $J = 8.9$ Hz, 2H), 4.83–4.79 (m, 1H), 4.36 (d, $J = 5.3$ Hz, 1H), 4.09 (q, $J = 7.2$ Hz, 2H), 3.90–3.80 (m, 1H), 3.36–2.81 (m, 6H), 2.20–2.10 (m, 1H), 1.13 (t, $J = 7.2$ Hz, 3H), 0.97 (d, $J = 6.8$ Hz, 6H).

*5.4.25. (R)-{4-[{2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl} amino]phenoxy}-(R)-isopropyl acetic acid ethyl ester HCl (**1y**)*

Yield: 74%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.35 (d, $J = 8.9$ Hz, 2H), 7.30–7.20 (m, 1H), 7.17–7.08 (m, 1H), 7.02 (s, 1H), 6.75 (d, $J = 8.9$ Hz, 2H), 4.82–4.75 (m, 1H), 4.36 (d, $J = 5.3$ Hz, 1H), 4.09 (q, $J = 7.2$ Hz, 2H), 3.90–3.80 (m, 1H), 3.27–2.75 (m, 6H), 2.16–2.14 (m, 1H), 1.11 (t, $J = 7.2$ Hz, 3H), 0.97 (d, $J = 6.8$ Hz, 3H).

*5.4.26. (R)-4-[({2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl} amino)methyl]benzoic acid HCl (**2i**)*

Yield: 86%. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.73 (br, 1H), 7.86 (d, $J = 8.1$ Hz, 2H), 7.50–7.40 (m, 1H), 7.39 (d, $J = 8.1$ Hz, 2H), 7.35–7.20 (m, 1H), 7.15 (s, 1H), 4.68 (dd, $J = 5.7$, 11.4 Hz, 1H), 4.35 (dd, $J = 5.7$, 11.4 Hz, 2H), 4.20–4.05 (m, 1H), 2.89–2.82 (m, 6H).

*5.4.27. (R)-4-({2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl} amino)benzoic acid HCl (**2k**)*

Yield: 99%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.88 (d, $J = 7.8$ Hz, 2H), 7.24–7.18 (m, 1H), 7.14–7.09 (m, 1H), 7.02 (d, $J = 7.2$ Hz, 2H), 7.01 (s, 1H), 4.86 (m, 1H), 3.72 (m, 1H), 3.35–3.21 (m, 2H), 3.00–2.90 (m, 3H), 2.60–2.55 (m, 1H).

*5.4.28. (R)-{4-[({2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl} amino)methyl]phenoxy}acetic acid HCl (**2p**)*

Yield: 61%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.63 (br, 1H), 8.15 (br s, 3H), 7.60–7.54 (m, 2H), 7.20 (s, 1H), 7.18 (d, $J = 8.7$ Hz, 2H), 6.85 (d, $J = 8.7$ Hz, 2H), 4.73 (s, 2H), 4.73–4.63 (m, 1H), 4.20 (s, 2H), 3.76–3.74 (m, 1H), 2.98–2.78 (m, 6H).

*5.4.29. (R)-{3-[({2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl} amino)methyl]phenoxy}acetic acid HCl (**2q**)*

Yield: 46%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.83 (br s, 1H), 8.26 (br s, 3H), 7.70–7.60 (m, 2H), 7.36–7.29 (m, 2H), 6.98–6.87 (m, 3H), 4.85 (s, 2H), 4.81–4.74 (m, 1H), 4.77 (s, 2H), 3.89–3.85 (m, 1H), 3.15–2.61 (m, 6H).

*5.4.30. (R)-{4-[{2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl} amino]phenoxy}acetic acid HCl (**2u**)*

Yield: 61%. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.06 (br s, 1H), 7.83–7.47 (m, 2H), 7.50 (d, $J = 8.4$ Hz, 2H), 7.23 (s, 1H), 6.88 (d, $J = 8.4$ Hz, 2H), 4.84–4.73 (m, 1H), 4.68 (s, 2H), 3.74–3.57 (m, 1H), 2.97–2.94 (m, 6H).

*5.4.31. (R)-{3-[{2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl} amino]phenoxy}acetic acid HCl (**2v**)*

Yield: 86%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.21–7.05 (m, 5H), 7.03 (s, 1H), 6.61 (d, $J = 8.1$ Hz, 1H), 4.81 (q, $J = 6.0$ Hz, 2H), 4.54 (s, 2H), 3.90–3.75 (m, 1H), 3.28–2.92 (m, 6H).

*5.4.32. (R)-{2-[{2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino] phenoxy}acetic acid HCl (**2w**)*

Yield: 57%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.89 (d, $J = 8.1$ Hz, 1H), 7.37–7.36 (m, 1H), 7.26–7.19 (m, 1H), 7.14 (s, 1H), 7.09 (d, $J = 8.1$ Hz, 1H), 6.97 (t, $J = 8.1$ Hz, 2H), 5.09 (q, $J = 6.0$ Hz, 1H), 4.70 (s, 2H), 3.90–3.80 (m, 1H), 3.78–2.88 (m, 6H).

*5.4.33. (R)-{4-[{2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino] phenoxy}-(S)-isopropyl acetic acid HCl (**2x**)*

Yield: 65%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.36 (d, $J = 7.2$ Hz, 2H), 7.30–7.27 (m, 1H), 7.17–7.08 (m, 1H), 7.02 (s, 1H), 6.77 (d, $J = 7.2$ Hz, 2H), 4.83–4.76 (m, 1H), 4.32 (d, $J = 5.0$ Hz, 1H), 3.81–3.78 (m, 1H), 3.27–2.91 (m, 6H), 2.17–2.13 (m, 1H), 0.98 (d, $J = 6.8$ Hz, 3H).

*5.4.34. (R)-{4-[{2-[3-Amino-4-(2,4,5-trifluorophenyl) butyryl]-3,4-dihydro-2H-pyrazole-3-carbonyl}amino] phenoxy}-(R)-isopropyl acetic acid HCl (**2y**)*

Yield: 79%. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.48 (d, $J = 7.2$ Hz, 2H), 7.44–7.30 (m, 1H), 7.26–7.22 (m, 1H), 7.14 (s, 1H), 6.89 (d, $J = 7.2$ Hz, 2H), 4.94–4.86 (m, 1H), 4.43 (d, $J = 4.9$ Hz, 1H), 3.90–3.80 (m, 1H), 3.39–2.80 (m, 6H), 2.30–3.15 (m, 1H), 1.09 (d, $J = 6.8$ Hz, 6H).

5.5. In vitro study

Caco-2 cell lysate (10 μl) was suspended in Tris–HCl (pH 7.5) and then 40 μM Ala–Pro–AFC (ICN Biomedicals, Inc) was added. After treatment of compounds, the mixture was incubated for 60 min at 24 °C. AFC as an indicator of DPP-IV activity was detected at 405/510 nm (Ex/Em) by Fluorometer, Synergy HT (Biotek). IC_{50} was calculated by Prism 4.0 software (GarphPad Software, Inc).

5.6. CYP assay

The CYP3A4 enzyme assay was carried out using fluorometric enzyme assays with Vivid CYP3A4 assay kit (PanVera, USA, CA) in a 96-well microtiter plate following the manufacturer's instruction with some modifications. Test compounds including ketoconazole as known as CYP3A4 inhibitor were prepared in acetonitrile to give final concentrations of 10 μM. Briefly, to each well of the microtiter plate was added NADP generating solution (1.0 mM $NADP^+$, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2 \cdot 6H_2O$, and 0.4 U/ml glucose 6-phosphate dehydrogenase in 10 mM KPO_4, pH 8.0) followed by the vehicle acetonitrile (control) and the test samples. The plate was covered and then incubated at 37 °C for 20 min. Enzyme reaction was initiated by the addition of enzyme/substrate (E/S) mixture (0.5 pmol CYP3A4 enzyme and 5 μM dibenzylfluorescein, DBF). The plate was further incubated for 20 min, followed by the addition of the stop solution to terminate the enzyme activity. Background reading was measured in a similar manner except for the E/S mixture which was added after the enzyme reaction was terminated. The fluorescence of DBF metabolite fluorescein was measured on a fluorescence plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The effect of test compounds on CYP3A4 enzyme was calculated as the percentage of the enzyme activity.

5.7. X-ray co-crystal structure determination

DPP-IV with a carboxy terminal 6 histidine tag was expressed in Hi5 insect cells (Invitrogen) using pAcGP67A baculovirus vector (Pharmingen). The secreted DPP-IV was purified with protein-A Sepharose affinity chromatography (AP Biotech), ion exchange chromatography and Superdex 200 gel filtration chromatography (AP Biotech). Purified DPP-IV was incubated with 2 mM compound **1h** for overnight and then concentrated to 10 mg/ml. The DPP-IV complex was crystallized at 22 °C using the hanging-drop vapor diffusion method by mixing 1 μl of the protein solution and 1 μl of crystallization buffer. Final crystallization condition was 0.1 M Tris pH 8.0, 22% (w/v) PEG4000, 0.3 M Na acetate. For data collection, the crystals were flash frozen at −170 °C in the crystallization buffer supplemented with 40% PEG4000. The diffraction data were collected at the 4A and 6B beam lines of the Pohang Accelerator Laboratory (PAL) and processed using the HKL package (Table 1). The structure was determined by molecular replacement method using the previously reported DPP-IV structure (PDB code: 1N1M) as a search model. The program AMoRe placed the DPP-IV dimer in the asymmetric unit. Refinement was carried out using the program CNS [15]. The randomly selected 5% of data were set aside for the R_{free} calculation. Rounds of refinements were performed with manual rebuilding by using the program O [16]. Although extra densities for compound **1h** were

Table 4
Data collection and refinement statistics

Space group	*P*21
Cell parameter (Å)	$a = 65.8$, $b = 126.5$, $c = 112.1$, $\alpha = r = 90°$, $\beta = 99.6°$
Highest resolution (Å)	2.3
Unique reflections (total)	63,339 (69,216)
Completeness[a] (%)	91.5 (81.0)
R_{merge}[b] (%)	8.0 (28.8)
$I/\sigma I$	17.6 (3.1)
Refinement	
Number of reflections	63,185
Number of atoms	12,511
(Protein/inhibitor/water)	(11,912/60/538)
R_{cryst}/R_{free}	21.6/24.1
Rms deviations	
Bond distances (Å)	0.015
Bond angles (°)	1.8
Impropers (°)	1.07
Dihedrals (°)	26.0

[a] The values in parentheses (completeness and R_{merge}) are for the highest resolution bin.

[b] $R_{merge} = \sum_i |I_i - \langle I \rangle| / \sum |\langle I \rangle|$, where I is the intensity for the ith measurement of an equivalent reflection with the indices h, k, l.

apparent, the model was not included until the last stage of refinement. The summary of crystallographic statistics is shown in Table 4.

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