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Research Article

Design, Synthesis, Biological Evaluation, and Docking Studies of (*S*)-Phenylalanine Derivatives with a 2-Cyanopyrrolidine Moiety as Potent Dipeptidyl Peptidase 4 Inhibitors

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A novel series of (*S*)-phenylalanine derivatives with a 2-cyanopyrrolidine moiety were designed and synthesized through a rational drug design strategy. Biological evaluation revealed that most tested compounds were potent dipeptidyl peptidase 4 (DPP-4) inhibitors; among them, the cyclopropyl-substituted phenylalanine derivative 11h displayed the most potent DPP-4 inhibitory activity with an IC_{50} value of 0.247 μM. In addition, molecular docking analysis of the representative compounds 11h, 11k, and 15a were performed, which not only revealed the impact of binding modes on DPP-4 inhibitory activity but also provided additional methodological values for design and optimization.

Key words: (*S*)-phenylalanine, 1,2,3-triazole, 2-cyanopyrrolidine, Dipeptidyl peptidase 4 inhibitors, type 2 diabetes

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Type 2 diabetes mellitus, which is expected to affect 366 million people by 2030 according to an estimate made by World Health Organization (WHO), has become a major concern for human health worldwide.[a] Current available treatment options including insulin and several oral antidiabetic agents have been found with drawbacks, such as hypoglycemia, edema, gastrointestinal disturbances, and weight gain (1,2). Therefore, the development of novel antidiabetic agents with improved safety and tolerability profile is of significant importance. The incretin glucagon-like peptide-1 (GLP-1)-based therapy appears to be a promising strategy because GLP-1 regulates blood glucose homeostasis in a dose-dependent manner, which significantly reduces the risk of appearance of hypoglycemia (3). In addition, GLP-1 stimulates insulin secretion, improves β-cells functions, and inhibits glucagon secretion; all these biological effects help to prevent or reverse progression of diabetes (4). While the short half-life of GLP-1, which is attributed to the rapid degradation through a serine protease dipeptidyl peptidase 4 (DPP-4), limits its application in practical therapy (5). Thus, small-molecule DPP-4 inhibitors offer a new therapeutic approach for the treatment of type 2 diabetes because DPP-4 inhibition leads to the extension of the half-life of GLP-1, enhances insulin secretion and improves the glucose tolerance (6). Efforts in this area have already led to the discovery of a number of potent DPP-4 inhibitors, such as marketed drugs, sitagliptin (**1**), vildagliptin (**2**), saxagliptin (**3**), alogliptin (**4**), and linagliptin (**5**) (Figure 1). Several others promising DPP-4 inhibitors are currently under clinical trials of different phases (7–11).

Due to the important role of glycinyl-2-cyanopyrrolidine moiety in contributing to potency, selectivity, and pharmacokinetic properties, it has been widely used as a key pharmacophore for many reported DPP-4 inhibitors, such as marketed drugs vildagliptin and saxagliptin (12). Although the phenylalanine derivative **6** containing this moiety showed only weak DPP-4 inhibition (K_i = 1.2 μM) in previous studies (13), it bore advantageous facets including small molecular weight, low logP value, and flexible positions for chemical modifications (14). Thus, compound **6** was selected as the starting point for our work reported herein. Docking study of compound **6** with DPP-4 was first performed using the FlexiDock module of Sybyl 6.9 version. It was discovered that the benzyl group of **6** was in close proximity to Phe357 and Arg358 residues, two important binding sites, in S2 pocket of DPP-4 (15). But the benzyl group of **6** did not form interaction with Phe357 and Arg358 residues. So, Phe357 and Arg358 were selected as potential target amino acid residues for forming additional interaction. We carried out structure-based drug design based on the docking simulated interaction mode of lead **6** with DPP-4. A series of phenylalanine derivatives with 1,2,3-triazole substituent were designed, synthesized, and evaluated for their DPP-4 inhibitory activity.

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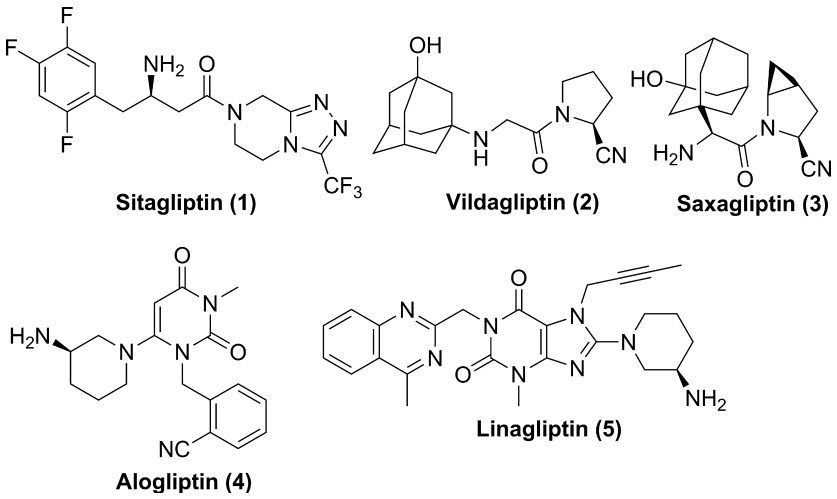


Figure 1: Approved DPP-4 inhibitors.

Methods and Materials

Chemistry

Standard Schlenk techniques were employed for air and water sensitive reaction. THF and Et_2O were dried over and distilled from Na/benzophenone under a dry nitrogen atmosphere. CH_2Cl_2 and Et_3N were dried over calcium hydride and KOH pellets then distilled for use, respectively. Other commercially available chemicals and solvents are reagent grade and were used without further purification. Melting points were determined on a Büchi B-540 apparatus (Büchi Labortechnik, Flawil, Switzerland) and were uncorrected. ^{1}H NMR spectra were recorded on Bruker 500 MHz instruments (Bruker Bioscience, Billerica, MA, USA) with $CDCl_3$ or DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard. ESI Mass spectral data were obtained by Esquire-LC-00075 spectrometer (Bruker Bioscience). Flash column chromatography was performed using silica gel (200-300 mesh). All yields are unoptimized and only represent one experiment's result.

Detailed synthetic procedures and characterization data for all new compounds **7–9**, **10a–k**, **11a–k**, **12**, **14a–c**, **and 15a–c** are provided in the Supporting Information (Appendix S1) of this article.

DPP-4 inhibitory assay

Porcine kidney DPP-4 (0.8 U/cm^3, Sigma) and Gly-Pro-*p*NA (Sigma) were used as the enzyme source and substrate, respectively. Sitagliptin, the first approved DPP-4 inhibitor, was selected as positive control. The assay principle was based on the cleavage of substrate by DPP-4 to release the chromogenic *p*-nitroaniline, which has a characteristic absorption peak at 405 nm. The absorption intensity change compared with solvent control was taken as the DPP-4 percent inhibition rate (16). Each tested compound was dissolved in dimethyl sulfoxide (DMSO) and diluted into a series of concentrations. DPP-4 was diluted using the assay buffer solution (Tris–HCl, pH 7.5) and was incubated for 10 min at 37 °C with different concentrations of the test compound solutions in a 96-well flat-bottomed microtiter plate. Subsequently, the reaction was initiated by the addition of Gly-Pro-*p*NA substrate solution. Absorbance at 405 nm of each reaction mixture was measured using a microplate reader at the start and end time of reaction. The initial rate of DPP-4 activity was calculated over the first 15-min of the reaction. According to these inhibitory rates in different concentrations, IC_{50} values were determined by using the GraphPad Prism software with three independent determinations.

Molecular docking

The molecular docking study was performed using the FlexiDock module of Sybyl 6.9 version.[b] The three-dimensional structures of all ligands were constructed manually and then energy minimized under a Tripos force field. The docked ligands were allowed to rotate on all single bonds, and Gasteiger–Hückel charges were assigned to the ligand atoms. The X-ray co-crystal structure of DPP-4 in complex with its inhibitor has been available from the RCSB Protein Data Bank (PDB code: 1ORW). The protein receptor was preprocessed by removing the initial ligand, and then, the free protein was modified by removing all solvents and adding hydrogen atoms. Kollman all-atom charges were loaded to the DPP-4 protein. Before the molecular docking process, a binding pocket was defined to cover all of the amino acid residues within 4.0 Å radius sphere centered by the docked compound in the initial compound and DPP-4 complex. All the single bonds of residues side chains inside the defined DPP-4 binding pocket were regarded as rotatable or flexible bonds. The structure optimization was performed for 100 000 generation using a genetic algorithm, and the 20 best scoring ligand–protein complexes were kept for further analyses. The lowest energy conformation was selected as a final model to analyze the composition of key amino acid residues of DPP-4 involved in the interac-

tion with its inhibitor. The pictures were prepared using Pymol (http://pymol.sourceforge.net/).

Results and Discussion

Structure-based drug design

Firstly, docking study of compound **6** with DPP-4 was performed using the FlexiDock module of Sybyl 6.9 version (Figure 2). The docking results revealed that the glycinyl-2-cyanopyrrolidine moiety functioned as a key pharmacophore that could form crucial interaction with DPP-4, which was consistent with literature reports (15). For example, 2-cyanopyrrolidine ring was buried in a hydrophobic S1 pocket, the carbonyl group formed a hydrogen bond with Asn710, and the amino group formed two another hydrogen bonds with Glu205 and Glu206. It was noteworthy to mention that the benzyl group at α-C position was adjacent to Phe357 and Arg358 residues, two important binding sites, in S2 pocket of DPP-4. But the benzyl group of **6** did not form interaction with Phe357 and Arg358 residues. Based on these observations, it was speculated that the potency of DPP-4 inhibitors could be improved through the optimization of the substituents on benzyl group. Considering the structural features of the S2 pocket, we intend to make further modifications at 4-position of the benzyl ring. 1,2,3-Triazole generated *via* click chemistry was given priority to the introduction of 4-position of the benzyl ring, because it was usually used in drug design with a good tolerance *in vivo* (17). More importantly, as a hydrogen bonding acceptor, 1,2,3-triazole may form hydrogen bonds with Arg358 residue of this pocket, which could improve the potency of DPP-4 inhibition. Therefore, a series of phenylalanine derivatives containing 1,2,3-triazole substituent were designed and synthesized in this study. Moreover, 1,2,3-triazole-based alanine derivatives were also prepared and evaluated to investigate the impact of the relative positions of phenyl ring and the 1,2,3-triazole on DPP-4 inhibitory activity (Figure 3).

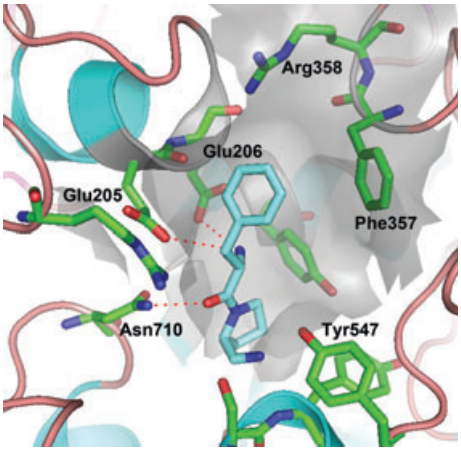


Figure 2: Docking study of compound **6** (cyan) with DPP-4. Hydrogen bonds are shown as dashed red lines. For clarity, only key amino acid residues are shown.

Synthesis

The general synthetic route for compounds **11a–k** is outlined in Scheme 1. (*S*)-*N*-Boc-4-iodophenylalanine derivative **7** was obtained in three steps from the starting material (*S*)-phenylalanine through halogenation, Boc protection, and condensation reaction (14). Sonogashira coupling of **7** with trimethylsilylacetylene (TMSA) in the presence of $Pd(PPh_3)_2Cl_2$ and CuI gave trimethylsilyl-protected compound **8**, whose following desilylation with tetra-*n*-butylammonium fluoride (TBAF) in THF at room temperature afforded (*S*)-*N*-Boc-4-ethynylphenylalanine derivative **9** in good yield. Subsequent click reaction of **9** with different alkyl azides (18,19) in EtOH at room temperature in the presence of $CuSO_4{\cdot}5H_2O$ and sodium ascorbate led to the formation of 1,2,3-triazole derivatives **10a–k** in excellent yields. Ultimate removal of the Boc-protecting group in HCl/Et_2O solution furnished target compounds **11a–k** in the form of hydrochloride salt.

The synthesis of compounds **15a–c** is outlined in Scheme 2. The (*S*)-*N*-Boc-azidoalanine **12** was easily accessible in two steps starting from the commercially available (*S*)-*N*-Boc-serine following a reported procedure (20,21). Click reaction of (*S*)-*N*-Boc-azidoalanine **12** with phenylacetylene or substituted phenylacetylene in the presence of catalytic amount of $CuSO_4{\cdot}5H_2O$ and sodium ascorbate in EtOH afforded the corresponding triazolyl amino acid compounds **13a–c** in good yields (22). The triazolyl amino acid compounds **13a–c** were then condensed with (*S*)-pyrrolidine-2-carbonitrile hydrochloride utilizing EDC and HOBt in CH_2Cl_2 to afford compounds **14a–c**. Removal of the Boc-protecting group of compounds **14a–c** in HCl/Et_2O solution provided target compounds **15a–c**.

DPP-4 inhibitory activity

All synthesized target compounds were evaluated for their DPP-4 inhibitory activity *in vitro*, and sitagliptin (MK-0431) was used as the reference drug. The results were summarized in Table 1. Most of the tested compounds showed potent DPP-4 inhibitory activity with IC_{50} values in the submicromolar range. On the whole, the inhibitory activity of phenylalanine derivatives was significantly better than that of alanine derivatives. Among the phenylalanine derivatives, the substituents at the $N-1$ position of 1,2,3-triazole ring had a major impact on activity. When methyl group was introduced into the $N-1$ position, compound **11a** displayed potent DPP-4 inhibitory activity (IC_{50} = 0.265 μM). Increasing the number of methylene groups was found to be unfavorable for activity (ethyl-substituted **11b**, IC_{50} = 0.339 μM; *n*-propyl-substituted **11c**, IC_{50} = 0.374 μM; *n*-butyl-substituted **11d,** IC_{50} = 0.331 μM). Accordingly, the branched alkyl groups were detrimental to activity

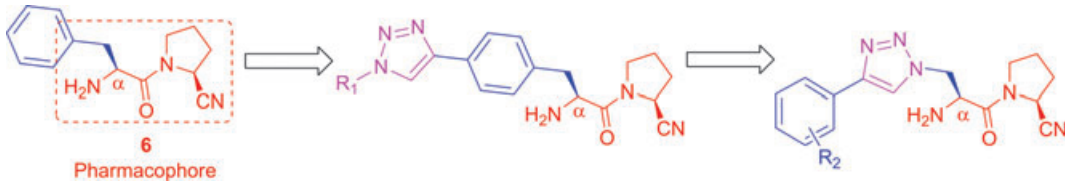
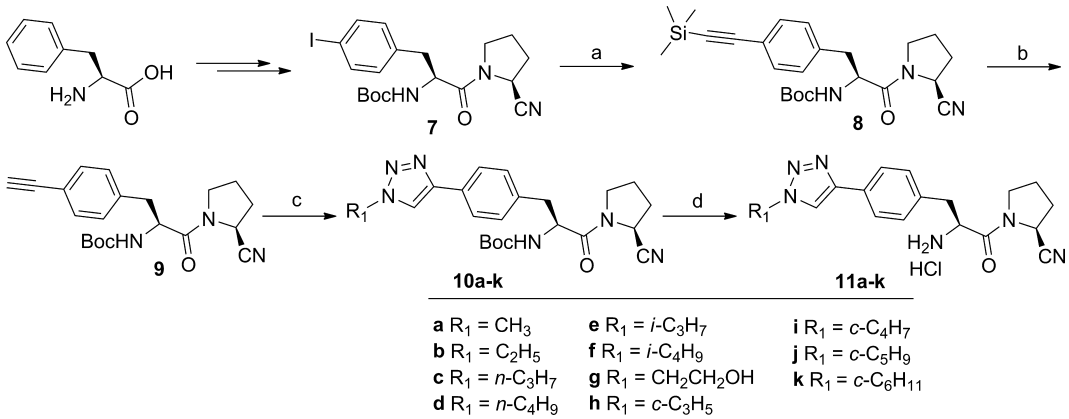
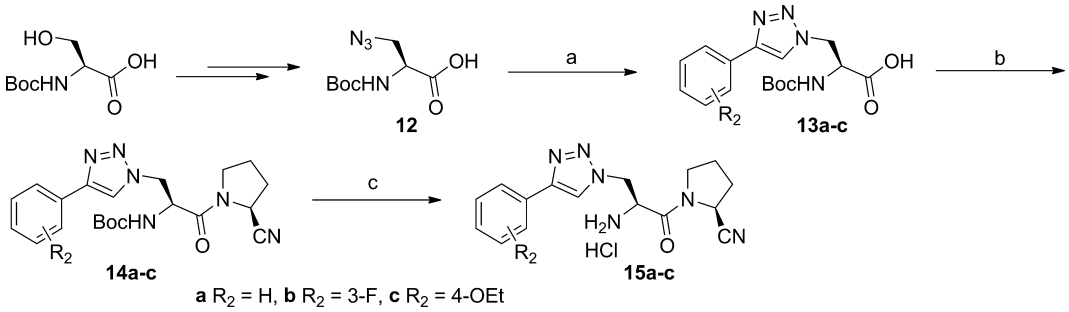


Figure 3: Docking-assisted design of novel DPP-4 inhibitors.



Scheme 1: Reagents and conditions: (a) TMSA, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, rt; (b) TBAF, THF, rt; (c) R_1N_3, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, EtOH, rt; (d) HCl/Et_2O, CH_2Cl_2, rt.



Scheme 2: Reagents and conditions: (a) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, phenylacetylene or substituted phenylacetylene, EtOH, rt; (b) HOBt, EDC, DIPEA, (*S*)-pyrrolidine-2-carbonitrile hydrochloride, CH_2Cl_2, rt; (c) HCl/Et_2O, CH_2Cl_2, rt.

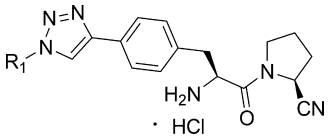
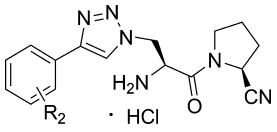
(*i*-propyl-substituted **11e**, IC_{50} = 0.527 μM versus *n*-propyl-substituted **11c**, IC_{50} = 0.374 μM; *i*-butyl-substituted **11f**, IC_{50} = 0.578 μM versus *n*-butyl-substituted **11d**, IC_{50} = 0.331 μM). In contrast, small cyclopropyl group showed the most potent inhibitory activity in the phenylalanine derivatives (**11h**, IC_{50} = 0.247 μM). With the further expansion of ring, the inhibitory activity was decreased (cyclobutyl-substituted **11i**, IC_{50} = 0.342 μM; cyclopentyl-substituted **11j**, IC_{50} = 0.332 μM). In particular, incorporation of a cyclohexyl ring at the $N-1$ position resulted in a dramatic loss of activity (**11k**, IC_{50} = 0.863 μM). In addition, compound **11g** with a 2-hydroxylethyl group at the $N-1$ position showed the moderate inhibitory activity (IC_{50} = 0.478 μM), but it was lower than the activity of compound **11h**. These results suggested that the presence of a small substituent, either a linear alkyl group or a cyclic group, at the $N-1$ position was beneficial to activity.

Among the alanine derivatives, all compounds showed weak DPP-4 inhibitory activity (**15a**, IC_{50} = 22.5 μM; **15b**, IC_{50} = 9.4 μM; **15c**, IC_{50} = 19.0 μM). Whether the electron-withdrawing fluoro- or electron-donating ethoxyl group was introduced into the phenyl ring of compound **15a**, the activity showed no significant enhancement. These results led us to conclude that the phenylalanine scaffold played a vital role in DPP-4 inhibitory activity.

Molecular docking

Based on results of biological evaluation *in vitro*, three representative compounds, high potent compound **11h**,

Table 1: The DPP-4 inhibitory activity of compounds **11a–k** and **15a–c**

Compound	Structure	R_1 (R_2)	IC_{50} (μM)[a]
11a		CH_3	0.265
11b		C_2H_5	0.339
11c		*n*-C_3H_7	0.374
11d		*n*-C_4H_9	0.331
11e		*i*-C_3H_7	0.527
11f		*i*-C_4H_9	0.578
11g		CH_2CH_2OH	0.478
11h		*c*-C_3H_5	0.247
11i		*c*-C_4H_7	0.342
11j		*c*-C_5H_9	0.332
11k		*c*-C_6H_{11}	0.863
15a		H	22.5
15b		3-F	9.4
15c		4-OEt	19.0
Sitagliptin			0.040

[a]IC_{50} values are an average of three independent determinations.

moderate potent compound **11k**, and low potent compound **15a**, were selected for further docking simulations to analyze their interaction modes with DPP-4 using the FlexiDock module of Sybyl 6.9 version. Figure 4 represented the docking simulated interaction modes of these three compounds binding to the active pocket of DPP-4. At first, the high potent compound **11h** was analyzed of its binding mode with DPP-4 from docking calculation. From the docking results (Figure 4A), 2-cyanopyrrolidine ring of **11h** fully occupied the hydrophobic S1 pocket, which was similar to the same group of lead compound **6**. The carbonyl group of **11h** formed a hydrogen bond with the side chain of Asn710. The amino group of compound **11h** could form hydrogen bond interactions with the oxygen atoms in the carboxyl groups of both residues, Glu205 and Glu206, respectively. The position-1 nitrogen of triazole group formed a hydrogen bond with the side chain of Arg358, which confirmed our design conception. This additional hydrogen bond interaction made compound **11h** more potent DPP-4 inhibition than lead compound **6**. Docking simulation revealed that compound **11h** (−4042.3 kcal/mol) had a lower docking energy with DPP-4 than the lead **6** (−2739.5 kcal/mol). As illustrated in Figure 4B, compound **11k** had a similar interaction mode with compound **11h** binding to DPP-4. However, the amino group of compound **11k** only forms a hydrogen bond with Glu205 due to a little change of its binding conformation, which led compound **11k** to the decrease in activity compared with **11h**. In addition, the docking energy of **11h** was much lower than that of **11k** (−3709.1 kcal/mol). As for the low potent compound **15a** (Figure 4C), docking simulation indicated that it had a different binding mode from compound **11h**. The key hydrogen bond was not formed between its amino group and

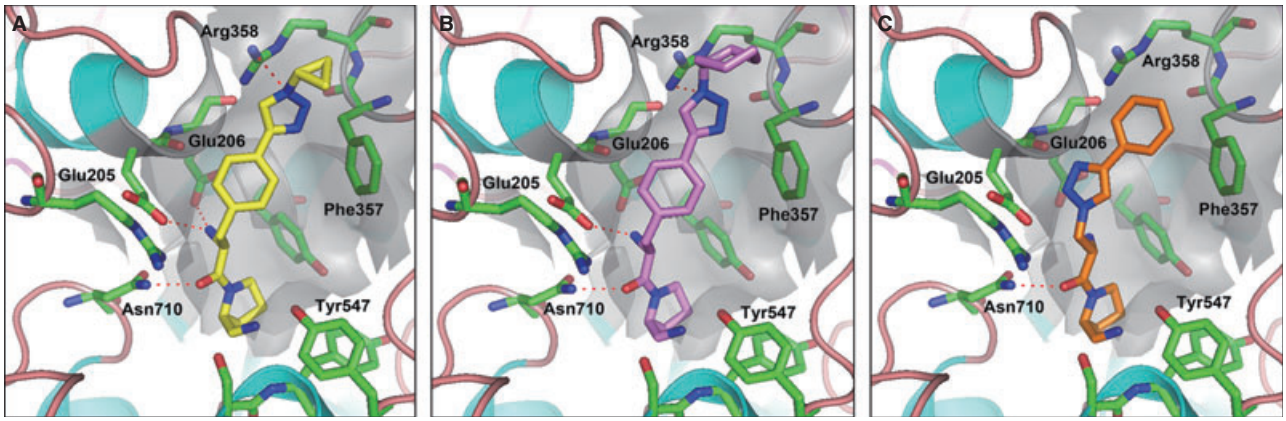


Figure 4: Docking studies of compounds **11h** (yellow), **11k** (magenta), and **15a** (orange) with DPP-4. Hydrogen bonds are shown as dashed red lines. For clarity, only key amino acid residues are shown. (A) the interaction of compound **11h** with the key residues of DPP-4; (B) the interaction of compound **11k** with the key residues of DPP-4; (C) the interaction of compound **15a** with the key residues of DPP-4.

Glu205 or Glu206 of DPP-4, but only a hydrogen bond was formed between the carbonyl group and the side chain of Asn710. Such an unfavorable binding mode of **15a** resulted in loss of its activity, and the docking energy of **15a** (−922.3 kcal/mol) was also significantly higher than that of compound **11h**. These docking results could explain why the potency of compounds **11k** and **15a** was lower than that of compound **11h**. Combining with the results from biological evaluation, it can be hypothesized that both Glu205 and Glu206 play critical roles in the binding of phenylalanine derivatives to the DPP-4.

In addition, it has been reported that Phe357 and Arg358 in S2 pocket of DPP-4 are also two key residues for the inhibitor binding to DPP-4 (15,23). However, as shown in Figure 4, our docking simulations indicate that compounds **11h**, **11k,** and **15a** do not have hydrophobic interaction with side chain of Phe357; only **11h** and **11k** has hydrogen bond interaction with Arg358 of DPP-4. The result induced us to get an idea for further design of novel compound possessing a hydrophobic interaction with Phe357 to improve inhibitory potency.

Conclusions

In conclusion, based on docking results of compound **6** with DPP-4, we carried out structure-based drug design. As a result, a novel series of (*S*)-phenylalanine derivatives with a 2-cyanopyrrolidine moiety were designed and synthesized. Subsequent biological evaluation revealed that most tested compounds showed potent DPP-4 inhibitory activity with submicromolar IC_{50} values. Preliminary SAR studies on (*S*)-phenylalanine derivatives led to the identification of the cyclopropyl-substituted compound **11h**, which was the most potent in phenylalanine derivatives with an IC_{50} value of 0.247 μM. Further docking analysis of the representative compounds **11h**, **11k**, and **15a** not only revealed the impact of binding modes on DPP-4 inhibitory activity but also provided additional methodological values for design and optimization.

Acknowledgments

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Conflict of Interest

The authors have declared no conflict of interest.

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Notes

[a]World Health Organization at http://www.who.int/diabetes/facts/world_figures/en/.
[b]Sybyl molecular modeling software packages. Tripos Associates Inc., St. Louis, MO63144, 2002.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Experimental details and characterization for all new compounds **7**–**9**, **10a**–**k**, **11a**–**k**, **12**, **14a**–**c**, **and 15a**–**c**.