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Research Article

Synthesis of Aminoacyl-2-Cyanopyrrolidine Compounds and Their Inhibitory Effect on the DPP-4 Enzyme

Liyuan Guo[1], Man Di[1], Zhiyan Dong[1], Da Zhang[1], Xiaoqing Zhu[1], Shi Wu[1,2,*], Chao Liu[2,*]

[1]School of Pharmacy, Xianning Medical College, Hubei University of Science and Technology, 437100 Xianning, Hubei, China
[2]Hubei Key Laboratory of Diabetes and Angiopathy, Hubei University of Science and Technology, 437100 Xianning, Hubei, China
*Correspondence: wushi2544@163.com (Shi Wu); 94031240@qq.com (Chao Liu)
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Abstract

Background: Diabetes mellitus is a major global health concern. DPP-4 (dipeptidyl peptidase-4) inhibitors containing a 2-cyanopyrrolidine scaffold are in clinical use; however, systematic evaluation of amides derived from amino acids adjacent to the *N*-2 position of GLP-1 (glucagon-like peptide-1) has not been reported. **Methods:** A total of eight 2-cyanopyrrolidine derivatives, grouped into three series, were synthesized from *N*-Boc-protected amino acids via condensation with 2-cyanopyrrolidine followed by deprotection. The compounds were characterized by nuclear magnetic resonance (NMR), elemental analysis, and mass spectrometry (MS). DPP-4 inhibitory activity was evaluated *in vitro* using a DPP-IV Glo™ assay, with sitagliptin as a control. IC_{50} (Half Maximal Inhibitory Concentration) values were determined from three independent replicates (n=3) and calculated by nonlinear regression. **Results:** Compounds **4d**, **4g**, and **4h** exhibited excellent DPP-4 inhibition, with IC_{50} values of 1.36 ± 0.09, 5.24 ± 0.31, and 4.83 ± 0.22 nM, respectively. Compound **4d** (histidine) was more potent than sitagliptin (6.13 ± 0.42 nM). The bis-cyanopyrrolidine derivatives **4g** and **4h** also showed strong activity. The remaining compounds (**4a**–**4c**, **4e**, **4f**) displayed moderate inhibition (IC_{50} 112–181 nM). **Conclusions:** Aminoacyl-2-cyanopyrrolidine derivatives bearing an imidazole or bis-cyanopyrrolidine structure are potent DPP-4 inhibitors. These findings highlight the importance of neighboring GLP-1 amino acid residues (positions 1 and 3) in inhibitor design. Further cellular and *in vivo* studies are warranted to evaluate the therapeutic potential of these compounds.

Keywords: dipeptidyl peptidase 4; enzyme inhibitors; pyrrolidines; hypoglycemic agents; structure–activity relationship

1. Introduction

Diabetes mellitus (DM) encompasses a range of metabolic disorders whose hallmark is chronic hyperglycemia. The 11th edition of the International Diabetes Federation (IDF) Diabetes Atlas (2025) estimates that in 2024, 589 million adults aged 20–79 years—accounting for 11.1% of the world's adult population—were affected by diabetes. By 2050, this figure is expected to reach 853 million, corresponding to 13.0% of all adults [1]. Sustained hyperglycemia damages blood vessels throughout the body, involving the heart, eyes, kidneys, and nervous system. This vascular injury greatly elevates the risk of severe, potentially fatal health complications [2,3]. The ensuing consequences include escalated healthcare expenditures, diminished quality of life, and increased mortality [4]. A wide array of pharmacological options exists for glycemic control. These include biguanides, sulfonylureas, thiazolidinediones, incretin-based therapies, α-glucosidase inhibitors, peroxisome proliferator-activated receptor (PPAR) agonists, sodium-glucose cotransporter-2 (SGLT-2) inhibitors, and GLP-1 receptor agonists. These agents can be administered as monotherapy or in combination [5]. Despite this armamentarium, glycemic control remains suboptimal for approximately half of individuals with type 2 diabetes [1,6]. Moreover, many of these agents carry notable risks, including hypoglycemia, adverse cardiovascular events, and β-cell dysfunction. Even metformin, a first-line mainstay, has been linked to uncommon but serious adverse effects [7,8].

DPP-4 enzyme inhibitors are second-line therapies for patients with type 2 diabetes and can be used alone or in combination. Sitagliptin and Vildagliptin have demonstrated favorable glucose-lowering effects in clinical practice, offering advantages such as protection of pancreatic islet cells, a low risk of hypoglycemia, and ease of administration [9,10]. These agents have also shown efficacy in managing diabetic complications [9], improving cardiovascular outcomes [11], exerting beneficial effects against cancer [12] and autoimmune diseases [9], and serve as potential therapeutic targets for fibrosis in systemic sclerosis [13], among others. However, gliptins have also been associated with adverse effects, including severe acute hemorrhagic pancreatitis and worsening heart failure [14,15]. Further research on DPP-4 enzyme inhibitors is therefore warranted.

DPP-4 specifically removes dipeptides from the N-terminus of its peptide substrates, preferentially cleaving at bonds where the penultimate residue is either proline or alanine (i.e., Xaa-Pro or Xaa-Ala, with Xaa representing any amino acid). This cleavage typically results

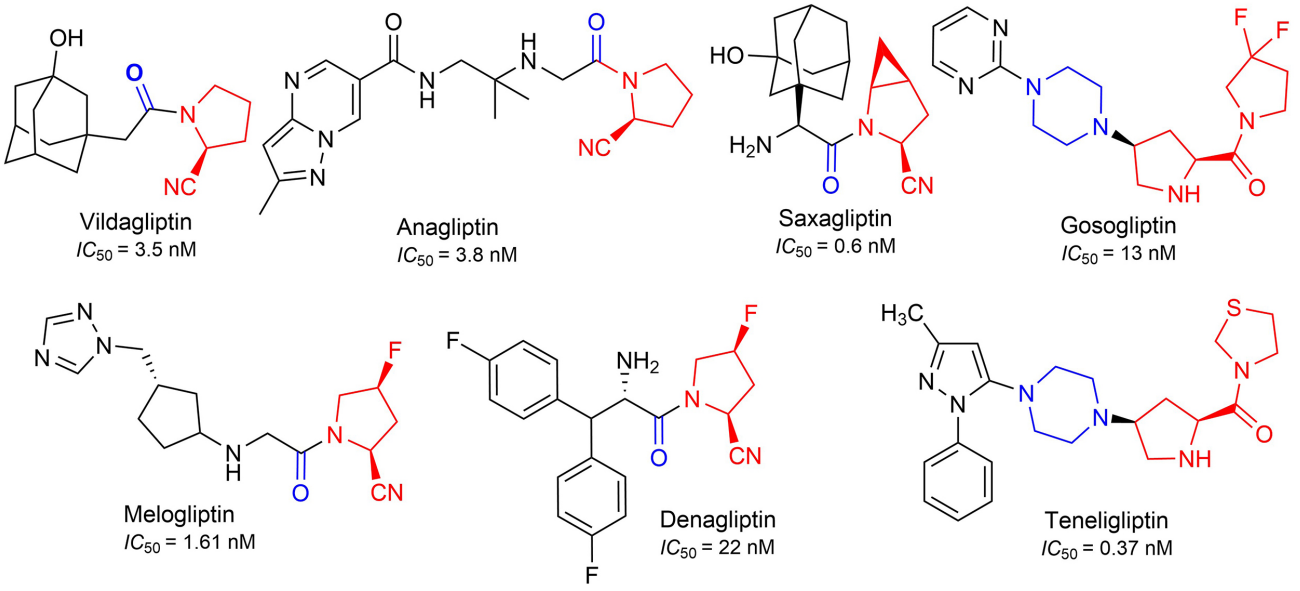


Fig. 1. Structures of DPP-4 enzyme inhibitors with pyrrolidine derivatives.

in a partial or total loss of the peptide's biological function. GLP-1 acts as an intestinal incretin hormone. It exerts its glucose-lowering effects through multiple mechanisms: it supports β-cell proliferation and survival, enhances insulin biosynthesis and release, and dampens glucagon secretion. Furthermore, GLP-1 reduces food intake, slows gastric emptying, lowers hepatic glucose production, and promotes glucose uptake by peripheral tissues. DPP-4 is involved in blood glucose regulation by rapidly removing two amino acids from GLP-1(7-36), converting it to GLP-1(9-36). Therefore, the preference of DPP-4 for substrates bearing alanine (Ala) or proline (Pro) at the second position from the N-terminus has guided the rational design of DPP-4 inhibitors. This has led to the successful discovery of hypoglycemic drugs with 2-cyanopyrrolidine structures as important pharmacophores, such as vildagliptin, saxagliptin, and anagliptin [16,17]. The structures of some DPP-4 enzyme inhibitors with pyrrolidine derivatives are shown in Fig. 1.

As part of research on DPP-4 enzyme inhibitors, various studies have screened hypoglycemic agents using amides derived from the reaction of selected natural amino acids with 2-cyanopyrrolidine [18]. (*S*)-1-(*L*-histidyl)-2-cyanopyrrolidine, (*S*)-1-(*L*-phenylalanyl)-2-cyanopyrrolidine and (*S*)-1-(*L*-glutamyl)-2-cyanopyrrolidine were reported to exhibit promising activity [18,19]. Nevertheless, no systematic screening has been performed for this class of DPP-4 inhibitors, especially for amides formed from amino acids near the *N*-2 position of the GLP-1 substrate in combination with 2-cyanopyrrolidine. The rationale for selecting the specific amino acids (Ser, Cys, Lys, His, Trp, Tyr, Glu, and Asp) in the present study is as follows: (1) Ser, Cys, and Lys represent small polar side chains capable of hydrogen bonding; (2) His, Trp, and Tyr possess aromatic or heteroaromatic side chains that may engage in π-π stacking or hydrophobic interactions within the DPP-4 S2 subsite; (3) Glu and Asp were selected to synthesize bis-cyanopyrrolidine derivatives, thereby exploring the effect of a second cyanopyrrolidine moiety on inhibitory potency. This systematic selection was intended to probe the structure–activity relationship around the substrate-binding region. We hypothesize that other functional groups near the *N*-2 position may also play specific roles at certain degradation sites. Therefore, the present study was undertaken to synthesize and evaluate the activity of a set of compounds formed by coupling various amino acids with 2-cyanopyrrolidine. As a result, new compounds were synthesized and their biological activity was reassessed.

2. Experiments

2.1 Materials and Methods

The reagents (chemicals) were analytically pure, and the amino acids, di-tert-butyl dicarbonate [$(Boc)_2O$], 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), Hydroxybenzotriazole (HOBT), trifluoroacetic acid (TFA) and trifluoroacetic anhydride were purchased from Wuhan Geao Chemical Technology Co. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz or 600 MHz as indicated on a Bruker AV-400 (Bruker Corporation, Billerica, MA, USA) (tetramethylsilane as an internal standard). The infrared band was characterized using an IR Affinity-1 infrared spectrometer (SHIMADZU). Elementary analyses were performed on an Elementar UNICUBE elementary analysis instrument. Purity of final compounds (**4a**–**4h**) was determined by high-performance liquid chromatography (HPLC) using an Agilent 1260 Infinity system (Agilent Technologies, Santa Clara, CA, USA) with a C_{18} column

(4.6 × 150 mm, 5 μm), eluting with acetonitrile/water (0.1% TFA) at 1.0 mL/min, UV detection at 220 nm. All tested compounds had purity ≥95%.

2.2 Synthesis of Compound 3

Synthesis protocol: *N*-Boc-L-amino acids (1.1 mmol), EDCI (0.351 g, 1.8 mmol), HOBT (0.291 g, 2.1 mmol), and *N*,*N*-dimethylformamide (DMF) (5 mL) were added successively to the reaction flask under a nitrogen atmosphere and stirred at 0 ℃ for 4 h. Then, 2-cyanopyrrolidine *p*-toluenesulfonate (0.268 g, 1.0 mmol) and diisopropylethylamine (0.35 mL, 2.1 mmol) in DMF were added. The reaction was monitored by thin layer chromatography (TLC) [V_{PE}:V_{EA} = 1:1] for approximately 16 h. After the reaction was completed, the solvent was removed under reduced pressure to give a yellow oily residue. Then, 50 mL of dichloromethane was added, and the sample was washed with saturated Na_2CO_3 (5 mL × 2) and NaCl (5 mL × 2), dried over anhydrous Na_2SO_4 and concentrated. The residue was purified via column chromatography on silica gel and eluted with a mixture of petroleum ether/ethyl acetate (PE/EA).

3a (tert-butyl ((S)-1-((S)-2-cyanopyrrolidin-1-yl)-3-hydroxy-1-oxopropan-2-yl) carbamate): White crystals of 0.167 g were obtained in 59.0% yield, m.p. 125~129 ℃. ^{1}H NMR (400 MHz, CD_3OD): δ 5.57 (d, 1H, *NH*) 4.77~4.76 (t, 1H, *CH*-NH), 4.54~4.52 (q, 1H, *CH*-CN), 3.95~3.91 (q, 1H, CH_2-OH), 3.83~3.74 (m, 3H, CH_2-OH, CH_2), 2.34~2.16 (m, 4H, CH_2-CH_2), 1.46 (s, 9H, 3CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 170.*59 (C=O)*, 155.*69 (C=O)*, 118.09 (*CN*), 80.46 (C), 53.47 (*CH*NH_2), 53.01 (*CH*-CN), 46.69 (CH_2), 46.58 (CH_2), 29.85 (CH_2), 28.30 (CH_3), 25.52 (CH_2). Calcd for $C_{13}H_{21}N_3O_4$: C, 55.11; H, 7.47; N, 14.83. Found: C, 55.68; H, 7.67; N, 14.01.

3b White crystals of 0.167 g were obtained in 59.2% yield, m.p. 105~114 ℃. ^{1}H NMR (400 MHz, CD_3OD): δ 5.06 (d, 1H, *NH*) 4.74~4.59 (m, 2H, *CH*-NH, *CH*-CN), 3.70~3.42 (m, 4H, CH_2-SH, CH_2), 2.16~1.92 (m, 4H, CH_2-CH_2), 1.40 (s, 9H, 3CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 165.*92 (C=O)*, 152.*95 (C=O)*, 119.60 (*CN*), 80.02 (C), 48.79 (*CH*NH_2), 48.55 (*CH*-CN), 48.55 (CH_2), 46.58 (CH_2), 30.32 (CH_2), 28.38 (CH_3), 25.46 (CH_2). Calcd for $C_{13}H_{21}N_3O_3S$: C, 52.15; H, 7.07; N, 14.04; S, 10.71. Found: C, 52.19; H, 7.06; N, 14.01; S, 10.78.

3c White crystals (0.297 g) were obtained in 50.2% yield. Further purification can be achieved by recrystallization in a mixture of dichloromethane and hexane. m.p. 97.0~98.8 ℃. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.33 (d, 1H, *NH*), 4.79~4.78 (t, 1H, *CH*-NH), 4.68 (b, 1H, *NH*), 4.39~4.34 (q, 1H, *CH*-CN), 3.71~3.65 (m, 2H, CH_2), 3.12~3.11 (m, 2H, CH_2), 2.27~2.18 (m, 4H, CH_2-CH_2), 1.66~1.43 (m, 24H, $CH_2CH_2CH_2$, 6 CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.37 (*C=O*), 155.59 (*C=O*), 155.45 (*C=O*), 119.27 (*CN*), 78.05 (C), 77.34 (C), 52.10 (*CH*NH_2), 52.10 (*CH*-CN), 46.16 (CH_2), 46.03 (CH_2), 30.27 (CH_2), 29.28 (CH_2), 29.15 (CH_2), 28.26 (CH_3), 28.19 (CH_3), 24.99 (CH_2), 22.59 (CH_2). Calcd for $C_{16}H_{28}N_4O_3$: C, 59.24; H, 8.70; N, 17.27. Found: C, 59.19; H, 8.60; N, 17.15.

3d White crystals (0.250 g) were obtained in 57.7% yield, m.p. 105~110 ℃. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.05 (s, 1H, N-*CH*), 7.19 (s, 1H, NH-*CH*), 5.40~5.39 (m, 1H, CN-*CH*), 4.74~4.71 (m, 1H, NH_2-*CH*), 3.72~3.67 (m, 2H, N-CH_2), 3.57~3.53 (m, 1H, CH_2), 2.97~2.89 (m, 2H, CH_2), 2.21~2.20 (m, 1H, CH_2), 2.13~2.08 (m, 2H, CH_2), 1.60 (s, 9H, 3CH_3), 1.43 (s, 9H, 3CH_3). Calcd for $C_{16}H_{23}N_5O_3$: C, 57.64; H, 6.95; N, 21.01. Found: C, 57.58; H, 7.02; N, 21.09.

3e White crystals of 0.190 g were obtained in 62.3% yield. m.p. 143~149 ℃. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56~7.53 (m, 1H, =*CH*-CH), 7.35~7.33 (m, 1H, =*CH*-CH), 7.12~7.08 (m, 2H, =*CH*-CH), 6.98 (m, 1H, =*CH*-CH), 4.73~4.69 (m, 1H, CN-*CH*), 4.34~4.29 (m, 1H, NH_2-*CH*), 3.12~3.09 (m, 2H, CH_2), 2.80~2.78 (m, 1H, CH_2), 2.12~2.04 (m, 2H, CH_2), 1.78~1.75 (m, 1H, CH_2), 1.68~1.66 (m, 2H, CH_2), 1.39 (s, 9H). Calcd for $C_{21}H_{26}N_4O_3$: C, 65.95; H, 6.85; N, 14.65. Found: C, 65.88; H, 7.02; N, 14.78.

3f White crystals of 0.220 g were obtained in 59.3% yield. m.p. 102~105 ℃. ^{1}H NMR (600 MHz, $CDCl_3$): δ7.00~6.93 (d, 2H, 2 =*CH*-CH), 6.71~6.68 (d, 2H, 2 =*CH*-CH), 4.78~4.70 (m, 1H, CN-*CH*), 4.44~4.41 (m, 1H, NH_2-*CH*), 3.38~3.24 (m, 2H, CH_2), 2.90~2.58 (m, 2H, CH_2), 2.22~2.12 (m, 2H, CH_2), 1.80~1.63 (m, 2H, CH_2), 1.40 (s, 9H, 3 CH_3). Calcd for $C_{19}H_{25}N_5O_4$: C, 63.49; H, 7.01; N, 11.69. Found: C, 63.57; H, 7.02; N, 11.57.

3g A total of colorless oil (0.205 g) was obtained, yielding 51.3%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.83 (t, *J* = 7.7 Hz, 1H, *CH*-CN), 4.69 (d, *J* = 5.9 Hz, 1H, *CH*-CN), 4.62 (dd, *J* = 7.3, 2.4 Hz, 1H, *CH*-NH_2), 3.66 (m, *J* = 23.9, 17.2, 7.7 Hz, 4H, 2CH_2), 2.86~2.60 (m, 2H, CH_2), 2.37~2.04 (m, 8H, 4CH_2), 1.45 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.*60 (C=O)*, 169.*55 (C=O)*, 155.*09 (C=O)*, 118.36 (*CN*), 118.18 (*CN*), 80.32 (C), 49.93 (*CH*NH_2), 47.56 (*CH*-CN), 47.13 (*CH*-CN), 46.88 (CH_2), 46.40 (CH_2), 29.96 (CH_2), 29.69 (CH_2), 28.32 (CH_3), 25.36 (CH_2), 25.04 (CH_2), 23.20 (CH_2). Calcd for $C_{19}H_{27}N_5O_4$: C, 58.60; H, 6.99; N, 17.98. Found: C, 58.55; H, 7.05; N, 18.02.

3h A total of 0.317 g of colorless oil was obtained, yielding 51.2% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 5.46~5.44 (d, 1H, *NH*), 4.80~4.78 (q, 1H, *CH*-CN), 4.76~4.73 (q, 1H, *CH*-CN), 4.50~4.47 (t, 1H, *CH*-NH_2), 4.02~4.00 (m, 1H, CH_2), 3.69~3.65 (m, 1H, CH_2), 3.59~3.56 (m, 1H, CH_2), 3.45~3.41 (m, 1H, CH_2), 2.47~2.43 (m, 1H, CH_2), 2.36~2.34 (m, 1H, CH_2), 2.26~2.11 (m, 10H, 5CH_2), 1.40 (s, 9H, 3CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.*44 (C=O)*, 170.*91 (C=O)*, 155.*82 (C=O)*, 118.55 (*CN*), 118.26 (*CN*), 79.70 (C), 50.83 (*CH*NH_2), 46.59 (*CH*-CN), 46.42 (*CH*-CN), 46.30 (CH_2),

29.94 (CH_2), 29.73 (CH_2), 29.24 (CH_2), 28.22 (CH_2), 25.28 (CH_2), 25.03 (CH_2), 20.96 (CH_2), 20.64 (CH_2). Calcd for $C_{20}H_{29}N_5O_4$: C, 59.54; H, 7.24; N, 17.36. Found: C, 59.47; H, 7.22; N, 17.45.

2.3 Synthesis of Compound 4

Synthesis protocol: TFA (1 mL) was slowly added to a solution of **3** (0.6 mmol) in dry CH_2Cl_2 (6 mL) dropwise on ice in a N_2 atmosphere. The reaction mixture was stirred for 1 h at room temperature, the mixture was concentrated to 0.5 mL, and 10 mL of Et_2O was added to the residue. After complete precipitation, the solvent was poured off, and the precipitate was washed twice with anhydrous ether (5 mL × 2) and dried under vacuum at 40 °C for 2 h to afford a white powder. **Supplementary Figs. 1–23** provide partial raw spectral data (including NMR, IR and MS) for structural elucidation of the synthesized compounds.

4a ((*S*)-1-(*L*-seryl)pyrrolidine-2-carbonitrile): A white solid powder (0.154 g) was obtained in 91.6% yield. Purity (HPLC): 97.2%. ^{1}H NMR (400 MHz, CD_3OD): δ 4.84~4.82 (t, 1H, *CH*-CN), 4.27~4.25 (q, 1H, *CH*-NH_2), 4.00~3.96 (q, 1H, *CH*$_2$-OH), 3.83~3.79 (q, 1H, *CH*$_2$-OH), 3.73~3.66 (m, 2H, *CH*$_2$), 2.32~2.11 (m, 4H, CH_2-CH_2). ^{13}C NMR (100 MHz, CD_3OD): δ 166.01 (*C*=*O*), 117.82 (*CN*), 59.09 (*CH*NH_2), 54.12 (*CH*-CN), 47.17 (*CH*$_2$), 46.96 (*CH*$_2$), 29.46 (*CH*$_2$), 24.85 (*CH*$_2$). Mass spectrometry (MS): m/z[M+1]$^+$ 183.1.

4b ((*S*)-1-(*L*-cysteinyl) pyrrolidine-2-carbonitrile): A white solid powder of 0.166 g was obtained in 93.4% yield. Purity (HPLC): 96.5%. ^{1}H NMR (400 MHz, CD_3OD): δ 4.85~4.83 (t, 1H, *CH*-CN), 4.29~4.27 (q, 1H, *CH*-NH_2), 3.75~3.68 (m, 2H, *CH*$_2$), 3.02~2.98 (m, 2H, *CH*$_2$-SH), 2.35~2.12 (m, 4H, *CH*$_2$-*CH*$_2$). ^{13}C NMR (100 MHz, CD_3OD): δ 166.*20 (C*=*O*), 118.43 (*CN*), 60.89 (*CH*NH_2), 50.07 (*CH*-CN), 48.90 (*CH*$_2$), 46.51 (*CH*$_2$), 30.26 (*CH*$_2$), 24.44 (*CH*$_2$). MS: m/z[M+1]$^+$ 200.2.

4c ((*S*)-1-(*L*-lysyl) pyrrolidine-2-carbonitrile): A white solid powder of 0.177 g was obtained in 91.9% yield. Purity (HPLC): 95.8%. When washing with anhydrous ether, care should be taken not to absorb moisture, and the sample should work under nitrogen protection. ^{1}H NMR (400 MHz, CD_3OD): δ 8.35 (b, 2H, *NH*$_2$), 7.90 (b, 2H, *NH*$_2$), 4.80~4.77 (q, 1H, *CH*-CN), 4.13 (b, 1H, *CH*-NH_2), 3.66~3.54 (m, 2H, *CH*$_2$), 2.77~2.75 (m, 2H, *CH*$_2$), 2.23~2.001 (m, 4H, *CH*$_2$-*CH*$_2$), 1.78~1.73 (m, 2H, *CH*$_2$), 1.58~1.55 (m, 2H, *CH*$_2$), 1.39~1.35 (m, 2H, *CH*$_2$). ^{13}C NMR (100 MHz, DMSO-*d*$_6$): δ 168.17 (*C*=*O*), 159.23~158.23 (*C*=*O, CF*$_3$*COOH*), 118.95 (*CN*), 50.86 (*CH*NH_2), 46.58 (*CH*-CN), 38.60 (*CH*$_2$), 29.52 (*CH*$_2$), 29.45 (*CH*$_2$), 28.56 (*CH*$_2$), 26.59 (*CH*$_2$), 25.03 (*CH*$_2$), 21.11 (*CH*$_2$). MS: m/z[M/2+1]$^+$ 106.1.

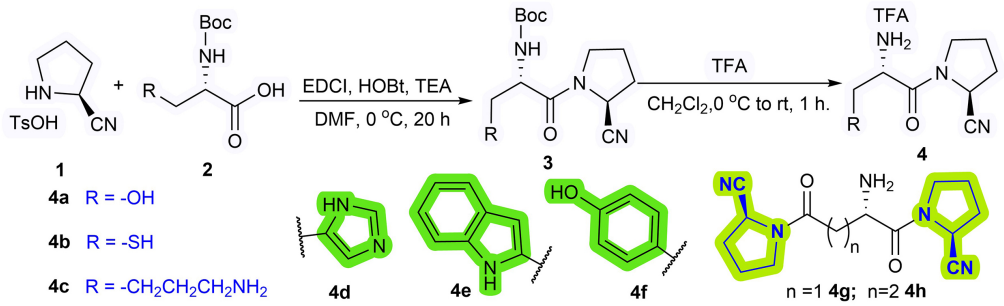
4d ((*S*)-1-(*L*-histidyl) pyrrolidine-2-carbonitrile): A 0.170 g white solid powder was obtained in 85.8% yield. Purity (HPLC): 98.4%. ^{1}H NMR (600 MHz, CD_3OD): δ 8.90 (s, 1H, N-*CH*), 7.48 (s, 1H, NH-*CH*), 4.86~4.84 (m, 1H, CN-*CH*), 4.63~4.61 (t, 1H, NH_2-*CH*), 3.72~3.71 (m, 1H, N-*CH*$_2$), 3.58~3.57 (m, 1H, N-*CH*$_2$), 3.43~3.34 (m, 2H, *CH*$_2$), 2.35~2.32 (m, 1H, *CH*$_2$), 2.28~2.25 (m, 1H, *CH*$_2$), 2.15~2.13 (t, 2H, *CH*$_2$). ^{13}C NMR (101 MHz, DMSO-*d*$_6$): δ 167.23 (*C*=*O*), 135.15 (*N*=*C*-*N*-), 126.75 (*N*-*C*=), 118.99 (=*C*-*N*-), 118.98 (*CN*), 50.42 (*C*-NH_2), 47.00 (*CH*-CN), 46.95 (*CH*$_2$), 29.57 (*CH*$_2$), 25.61 (*CH*$_2$), 25.02 (*CH*$_2$). MS: m/z[M+1]$^+$ 234.1.

4e ((*S*)-1-(*L*-tryptophyl) pyrrolidine-2-carbonitrile): A brown solid powder (0.128 g) was obtained in 56.2% yield. Purity (HPLC): 96.1%. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ 7.61~7.59 (m,1H, CH-*CH*=), 7.40~7.38 (m, 1H, -*CH*-CH=), 7.16 (s, 1H, NH-*CH*=), 7.12 (m, 1H, -*CH*-CH=), 7.04 (m, 1H, -CH-*CH*=), 4.79 (m, 1H, CN-*CH*), 4.30 (m, 1H, NH_2-*CH*), 3.22~3.20 (m, 2H, *CH*$_2$), 2.80 (m, 1H, CH-*CH*$_2$), 2.12~2.04 (m, 2H, *CH*$_2$), 1.82~1.81 (m, 1H, CH-*CH*$_2$), 1.68~1.66 (m, 2H, *CH*$_2$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.54 (*C*=*O*), 136.64 (NH-*C*=C), 127.34 (NH-*C*=C), 125.23 (C=*C*-C), 121.84 (C=*C*H-C), 119.37 (C=*C*H-C), 118.98 (C=*C*H-C), 118.51 (*CN*), 112.06 (C=*C*H-C), 106.91 (C=*C*H-C), 51.90 (*C*-NH_2), 46.76 (*CH*-CN), 46.60 (*CH*$_2$), 29.82 (*CH*$_2$), 27.19 (*CH*$_2$), 25.14 (*CH*$_2$). MS: m/z[M+1]$^+$ 283.1.

4f ((*S*)-1-(*L*-tyrosyl) pyrrolidine-2-carbonitrile): A white solid powder of 0.170 g was obtained in 79.6% yield. Purity (HPLC): 97.3%. ^{1}H NMR (600 MHz, D_2O): δ 7.08~7.04 (d, CH-*CH*=C, 2H), 6.79~6.74 (d, CH-*CH*=C, 2H), 4.79~4.74 (m, 1H, CN-*CH*), 4.34~4.31 (m, 1H, NH_2-*CH*), 3.28~3.13 (m, 2H, *CH*$_2$), 2.99~2.55 (m, 2H, *CH*$_2$), 2.14~2.02 (m, 2H, *CH*$_2$), 1.81~1.60 (m, 2H, *CH*$_2$); ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.02 (*C*=*O*), 157.28 (*C*-OH), 131.34 (C=*C*H-C), 124.52 (C=*C*-C), 121.91 (C=*C*H-C), 118.67 (*CN*), 52.83 (*C*-NH_2), 46.67 (*CH*-CN), 46.54 (*CH*$_2$), 36.66 (*CH*$_2$), 29.80 (*CH*$_2$), 25.02 (*CH*$_2$). MS: m/z[M+1]$^+$ 260.2.

4g ((2*S*,2′*S*)-1,1′-((S)-2-aminosuccinyl) bis(pyrrolidine-2-carbonitrile)): A white solid powder of 0.205 g was obtained in 88.6% yield. Purity (HPLC): 98.0%. ^{1}H NMR (400 MHz, D_2O) δ 4.90~4.82 (m, 2H, CN-*CH*), 4.75~4.62 (m, 1H, *CH*-NH_2), 3.81~3.51 (m, 4H, 2*CH*$_2$), 3.22~2.95 (m, 2H, *CH*$_2$), 2.46~2.05 (m, 8H, 4*CH*$_2$). ^{13}C NMR (101 MHz, D_2O): δ 168.*36 (C*=*O*), 167.*63 (C*=*O*), 164.02~162.62 (CF_3COOH), 118.87, 118.43 (*CN*), 116.27 (*CN*), 48.45 (*CH*NH_2), 47.46 (*CH*-CN), 47.33 (*CH*-CN), 47.20 (*CH*$_2$), 46.80 (*CH*$_2$), 34.31 (*CH*$_2$), 29.56 (*CH*$_2$), 29.25 (*CH*$_2$), 24.75 (*CH*$_2$), 24.51 (*CH*$_2$). MS: m/z[M+1]$^+$ 290.3.

4h ((2*S*,2′*S*)-1,1′-((S)-2-aminopentanedioyl) bis(pyrrolidine-2-carbonitrile)): A white solid powder of 0.185 g was obtained in 77.1% yield. Purity (HPLC): 97.6%. ^{1}H NMR (400 MHz, CD_3OD): δ 4.85~4.84 (q, 1H, *CH*-CN), 4.79~4.76 (t, 1H, *CH*-NH_2), 4.42~4.39 (q, 1H, *CH*-CN), 3.824~3.48 (m, 4H, 2*CH*$_2$), 2.63~2.60 (t, 2H, *CH*$_2$), 2.29~2.15 (m, 10H, 5CH_2). ^{13}C NMR (100 MHz, DMSO-*d*$_6$): δ 170.07 (*C*=*O*), 168.0 (*C*=*O*),



Scheme 1. Synthesis of compounds 4a–4h.

Table 1. DPP-4 inhibitory activity of synthesized compounds.

Compound	Inhibition ratio at 64 nM (%)[1]	Mean inhibition (%)	IC_{50} (nM)[2]
Sitagliptin	93.44, 92.35	92.90	6.13 ± 0.42
4a	35.33, 35.47	35.40	156.2 ± 8.1
4b	34.03, 34.65	34.34	181.1 ± 9.3
4c	35.63, 36.13	35.88	146.4 ± 7.5
4d	97.66, 92.19	94.93	1.36 ± 0.09
4e	34.11, 38.43	36.27	112.5 ± 5.6
4f	33.02, 35.72	34.37	128.1 ± 6.3
4g	94.20, 92.31	93.26	5.24 ± 0.31
4h	94.31, 92.39	93.35	4.83 ± 0.22

[1] Inhibition ratios are from two independent determinations; IC_{50} values are from three independent experiments (n = 3).

[2] Values are mean ± SD from three independent experiments.

158.70~157.70 (CF_3COOH), 119.37 (*CN*), 118.85 (*CN*), 50.28 ($CHNH_2$), 46.45 (*CH*-CN), 46.11 (*CH*-CN), 46.11 (CH_2), 46.07 (CH_2), 29.68 (CH_2), 29.35 (CH_2), 28.57 (CH_2), 24.94 (CH_2), 24.74 (CH_2). MS: m/z$[M+1]^+$ 304.2.

2.4 Enzyme-Based Assay of DPP-4

Inhibition of human recombinant DPPIV protease activity was measured using the DPPIV-Glo™ protease assay kit with sitagliptin as the positive control and target compounds **4a-4h** as test compounds, and a blank group. The inhibition rates of the target compounds were determined according to the manufacturer's instructions.

Compound stock solutions were prepared in 100% DMSO and serially diluted 5-fold to obtain 10 concentration gradients. Sitagliptin was diluted from 100 μM. Test compounds **4a–4h** were diluted from the following starting concentrations: **4a**: 3.6 mM, **4b**: 1.5 mM, **4c**: 4.8 mM, **4d**: 80 μM, **4e**: 10 mM, **4f**: 10 mM, **4g**: 100 μM, **4h**: 100 μM. IC_{50} values were determined from three independent replicates (n=3). The IC_{50} values were determined by analyzing the data using GraphPad Prism 5 software (version 9.5.1; GraphPad Software, Inc., San Diego, CA, USA) with nonlinear regression. The inhibition ratios at 64 nM were obtained from two independent determinations. The dose-response curves for all tested compounds are provided in the Supporting Information (**Supplementary Figs. 24–32**).

3. Results and Discussion

3.1 Chemistry

To investigate the impact of the surrounding amino acid residues at the *N*-2 position of the GLP-1 substrate on the inhibitory activity of the DPP-4 enzyme, three compound series were designed: compounds with normal functional side chains (**4a–c**), including -OH, -SH and $-NH_2$; aromatic/heteroaromatic functional groups (**4d–f**), including bis(2-cyanopyrrolidine) structures (**4g, 4h**). The synthetic procedures for accessing target compounds **4a–h** are outlined in Scheme 1. The *p*-toluenesulfonate of the raw material 2-cyanopyrrolidine (**1**) was prepared in the laboratory, and the experimental conditions were optimized. Compound **3** was formed by condensing 1 with *N*-Boc, an amino acid, using a combination of EDCI and HOBT as the condensing agent. However, the synthesis of compounds **3g** and **3h** required two condensation reactions and twice the amounts of chemical reagents. Compounds **4a–h** were obtained by deprotecting compounds **3a–h** with trifluoroacetic acid (TFA). Compound **4** is a highly absorbent salt that requires isolation from moisture. The compounds were characterized through ^{1}H NMR, ^{13}C NMR, elemental analysis, and mass spectrometry. The data obtained from the characterization confirmed the structure of the compounds. HPLC analysis confirmed that all final compounds had purity ≥95%, suitable for biological evaluation.

3.2 In Vitro Enzyme Inhibition Studies and Structure–Activity Relationships

To the best of our knowledge, this study represents the first systematic evaluation of aminoacyl-2-cyanopyrrolidine derivatives specifically designed based on amino acids near the *N*-2 position of GLP-1. The results of the DPP-4 enzyme inhibition activity of the positive control, sitagliptin, and compounds **4a–h** are shown in Table 1. All data are presented as mean ± standard deviation (SD) from three independent experiments. From the results, it is clear that the synthesized compounds **4a–4h** have a certain inhibitory effect on the DPP-4 enzyme, which is related to the presence of the 2-cyanopyrrolidine structure in the structure of the compounds. Despite containing different functional groups, such as -OH, -SH and NH_2, compounds in the **4a**–**4c** series have moderate inhibitory activity (IC_{50} = 146–181 nM). In the **4d**–**4f** series, the inhibitory activity of **4d** was excellent, with an IC_{50} of 1.36 ± 0.09 nM, which was better than that of the sitagliptin control (6.13 ± 0.42 nM), similar to the results reported in the article [18]. (*S*)-1-(*L*-Histidyl)-2-cyanopyrrolidine (**4d**) has an imidazole group, an aromatic functional group. However, as aromatic substituents, **4e** (tryptophan) and **4f** (tyrosine) had average activities with IC_{50} values of 112.5 ± 5.6 nM and 128.1 ± 6.3 nM, respectively, and (*S*)-1-(*L*-phenylalanyl)-2-cyanopyrrolidine had an IC_{50} of 27 nM [19]. The superior activity of **4d** over **4e** and **4f** suggests that the imidazole side chain may participate in specific hydrogen bonding or coordination with the DPP-4 active site, whereas the larger indole or phenol rings may cause steric hindrance. Compounds **4g**, and **4h** contained a bis-2-cyanopyrrolidine structure and showed very good inhibitory activity, with IC_{50} values of 5.24 ± 0.31 nM and 4.83 ± 0.22 nM, respectively. **4h** was obtained by the reaction of Glu with 2-cyanopyrrolidine. We hypothesize that the second cyanopyrrolidine moiety occupies the S2' subsite of DPP-4, providing additional hydrophobic and hydrogen-bonding interactions that enhance binding affinity.

Of note, the first five amino acid residues of the *N*-terminal primary structure of GLP-1 are His-Ala-Glu-Thr-Phe [20]. DPP-4 is an enzyme that specifically cleaves oligopeptides containing Ala or Pro as the second *N*-terminal amino acid residue [21]. The high inhibitory activity of **4d** (histidine at position 1) and **4h** (glutamate at position 3 linked to a second cyanopyrrolidine) suggests that amino acid residues flanking the scissile bond (positions P1 and P3) also contribute significantly to binding affinity. It has also been reported that **4d** and (*S*)-1-(*L*-glutamyl)-2-cyanopyrrolidine have strong specific selectivity for DPP-4 enzymes [18]. Therefore, it is valuable to design inhibitors that target the amino acid residues surrounding the DPP-4 cleavage site.

4. Limitations of the Study

The present study has several limitations that should be acknowledged. First, the biological evaluation was limited to a single enzyme inhibition assay; no cell-based studies (e.g., DPP-4 activity in intact cells or GLP-1 degradation assays) were performed to confirm the biological relevance of the observed inhibition. Second, selectivity profiling against related proteases such as DPP-8, DPP-9, and fibroblast activation protein (FAP) was not carried out; these enzymes are known to be associated with potential toxicity. Third, no *in vivo* pharmacokinetic or pharmacodynamic studies were conducted, and the metabolic stability of the compounds remains unknown. Fourth, the structure–activity relationship discussion is primarily qualitative, and molecular docking or binding mode analysis was not performed. Consequently, the conclusions regarding the importance of specific amino acid residues are speculative and require further experimental validation. These limitations should be addressed in future studies. Further cellular and *in vivo* studies are warranted to evaluate the therapeutic potential of these compounds.

5. Conclusion

Research on hypoglycemic agents is ongoing. The analysis of some marketed DPP-4 enzyme inhibitors during the research process revealed that insufficient attention has been given to the structural characterization of the GLP-1 substrate. Therefore, these compounds were designed as a class of DPP-4 enzyme inhibitors in which 2-cyanopyrrolidine forms amides with amino acids near the *N*-2 position of GLP-1. Three sets of eight target compounds were successfully synthesized through the condensation of *N*-Boc amino acids with 2-cyanopyrrolidine and subsequent deprotection. The products were identified through NMR, elemental analysis, and mass spectrometry characterization. HPLC analysis confirmed the purity of all final compounds (≥95%). All eight synthesized compounds were tested for DPP-4 enzyme inhibitory activity *in vitro* in triplicate. Compounds **4d**, **4g** and **4h** exhibited excellent DPP-4 enzyme inhibitory activity, with IC_{50} values of 1.36 nM, 5.24 nM and 4.83 nM, respectively. These findings suggest that the amino acid residues adjacent to the DPP-4 cleavage site (positions P1 and P3) play an important role in inhibitor binding. Future work should include selectivity profiling, cellular assays, and *in vivo* studies to further develop these leads.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

CL and SW contributed to the conception and design of the study. DZ contributed to the acquisition of data. LG,

MD, ZD, and XZ carried out the experiments and data collection. CL and SW also drafted the manuscript. All authors reviewed, revised, and approved the final manuscript and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/Pharmazie53112.

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