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RESEARCH ARTICLE

Synthesis of a novel series of *L*-isoserine derivatives as aminopeptidase N inhibitors

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Abstract

A series of novel L-isoserine derivatives were synthesised and evaluated for their ability to inhibit aminopeptidase N (APN)/CD13. In our preliminary biological results, some of these compounds possessed a potent inhibitory activity against the APN. Within this series, compound **14b** not only showed similar enzyme inhibition (IC_{50} of 12.2 μM) compared with the positive control bestatin (half maximal inhibitory concentration (IC_{50}) of 7.3 μM), but also had a potent antiproliferative activity against human cancer cell lines cells.

Keywords: Aminopeptidase N inhibitor, *L*-isoserine, synthesis

Introduction

Aminopeptidase N (APN EC 3.4.11.2), also known as CD13, is a zinc-dependent membrane metalloprotease which belongs to the M1 family of ectoenzymes [1]. It usually forms a noncovalently bound homodimer on the cell membrane of monocytes, myeloid, epithelial cells of the intestine and kidney, fibroblasts and tumour cells [2–3]. This enzyme is able to cleave the *N*-terminal residues from polypeptide chains, as well as play an important role in modulating bioactive peptides or protein responses such as modification, activation, and degradation. APN is also involved in various physiological processes, such as cell proliferation, secretion, invasion and angiogenesis [4–5]. For example, APN is over-expressed on the tumour cell surface for regulating ECM degradation and the invasion of tumour cells [6]. Therefore, inhibition of APN/CD13 could prevent the spread and metastasis of cancer cells since several APN inhibitors have been well studied clinically over the last two decades. Among the APN inhibitors, bestatin is a representative molecule for the treatment of adult acute nonlymphocytic leukemia [7].

So far, X-ray crystal structures for APN for both the *holo*- and *apo*-forms [8] have been resolved along with a number of different ligands, such as bestatin [9–10], arginine and lysine [11]. According to the literature for binding modes, our group has developed various APN inhibitors, including *L*-lysine derivates [12] and AHP A(α-Amino-β-Hydroxyl-phenylbutanoic acid) derivates, [13] which both showed potent APN inhibitory activities. In our recent screening studies, *L*-isoserine showed the ability to inhibit APN at an half maximal inhibitory concentration (IC_{50}) value of 563 μM, which could serve as a new lead compound for further chemical modification and optimisation.

It has been reported that *L*-isoserine-*L*-leucine dipeptide shows moderate aminopeptidase B inhibitory activity (IC_{50} of 140 μM) [14]. This evidence indicates that the incorporation of an amino acid to *L*-isoserine may contribute to its inhibitory activity against APN. Therefore in our current work, different amino acids have been coupled with *L*-isoserine by forming dipeptides or tripeptides. This article will describe the synthesis, preliminary biological evaluation and structure-activity relationship (SAR) study of such a novel series of *L*-isoserine derivatives as potent APN inhibitors.

Materials and methods

Chemistry

The starting material *L*-Isoserine (**7**), is a white solid, (mp 199-201°C, $[\alpha]^{25}_{D} = -32.5°$ (*c* 1, H_2O)), was purchased

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from JiaDing District, Shanghai, China. Unless otherwise specified, all reagents and buffer salts were purchased from commercial vendors and used without further purification. The solvents were distilled prior to use and flash chromatography was performed using silica gel (60 Å, 200 ± 300 mesh). Melting points were determined on an electrothermal melting point apparatus in an uncorrected form. Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined on a Brucker Avance 600 spectrometer using TMS as an internal standard in DMSO-*d*6 solutions. Chemical shifts were reported in delta (δ) units, parts per million (ppm) downfield from trimethylsilane. High-resolution mass spectral (HRMS) data are reported as m/e (relative intensity). The optical rotation was determined on a GYROMAT-HP Digital Automatic Polarimeter. All reported yields correspond to purified products.

General procedure for the synthesis of 2a, 2b, 4a and 4b

The title compound *L*-leucine methyl ester hydrochloride (**2a**) and *L*-phenylalanine methyl ester hydrochloride (**2b**) were prepared from *L*-leucine and *L*-phenylalanine according to the methods described in the literature [15]. Using the same materials, the title compound *L*-leucine benzyl ester hydrochloride (**4a**) and *L*-phenylalanine benzyl ester hydrochloride (**4b**) were prepared as described by Kim et al. [16].

General procedure for the synthesis of 6a, 6b, 6c and 6d

To a 200 mL solution of compound **3a** (0.92 g, 4 mmol), **2b** (0.88 g, 4.4 mmol), and hydroxybenzotriazole (HOBt) (0.65 g, 4.8 mmol), 4-Dimethylaminopyridine (DMAP) (0.09 g, 0.8 mmol) in dry DCM, was added TEA (1.33 g, 13.2 mmol). The reaction mixture was gently cooled to 0°C in an ice bath. To the reaction mixture was added dropwise a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (1.53 g, 8 mmol) in DCM for 1 h. After removal of the ice bath, the reaction mixture was stirred at room temperature for 12h and then filtered to remove the precipitate. The filtrate was washed with a 1 mol/L citric acid solution, saturated $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified by flash column chromatography (EtOAc/PE, 1:3(V/V)) to give the desired compound **5a** as a white solid (1.48 g), yield: 94.2%, mp 70–73°C. ^{1}H-NMR: (DMSO-d_6) δ 7.919 (d, J = 7.8 Hz, 1H, -CONH-), 7.287–7.214 (m, 5H, Ar-H), 5.807 (d, J = 5.4 Hz, 1H, -CONH-), 4.474–4.459 (m, 1H, CH), 4.346–4.311 (m, 1H, CH), 3.568 (s, 3H, -OCH_3), 3.102–3.089 (m, 1H, CH_2), 2.787–2.75 (m, 1H, CH_2), 1.526–1.501 (m, 1H, CH), 1.435 (s, 9H, CH_3), 1.475–1.328 (m, 2H, CH_2), 0.863 (d, J = 6.6 Hz, 3H, CH_3), 0.828 (d, J = 6.6 Hz, 3H, CH_3); ESI-MS *m/z*: 393.2 $[M+H]^+$.

To a solution of compound **5a** (0.78 g, 2 mmol) in dry EtOAc at 0°C was added dropwise a solution of EtOAc (10 mL) saturated by dry HCl gas. The reaction solution was stirred at 0°C for 2 h, and then the temperature was raised to room temperature and the reaction proceeded for 5 h before being concentrated *in vacuo*. The residue was recrystallised with MeOH and ether to give **6a** (0.56 g) as a white crystals, yield: 85.3%, mp 190–193°C, $[\alpha]^{25}_D$ = +14.2° (*c* 1, MeOH). ^{1}H-NMR: (DMSO-d_6) δ 8.403 (d, J = 7.8 Hz, 1H, -CONH-), 8.054 (s, 3H, NH_3), 7.285–7.202 (m, 5H, Ar-H), 4.482–4.472 (m, 1H, CH), 4.351–4.318 (m, 1H, CH), 3.573 (s, 3H, -OCH_3), 3.11–3.092 (m, 1H, CH_2), 2.791–2.751 (m, 1H, CH_2), 1.527–1.495 (m, 1H, CH), 1.486–1.351 (m, 2H, CH_2), 0.861 (d, J = 6.6 Hz, 3H, CH_3), 0.831 (d, J = 6.6 Hz, 3H, CH_3); ESI-MS *m/z*: 293.1 $[M+H]^+$.

Compounds **6b**, **6c** and **6d** were prepared following the general procedure as described above.

L-phenylalanyl-L-leucine methyl ester hydrochloride (6b)

White crystals, yield: 79.4%, mp 141–143°C; $[\alpha]^{25}_D$ = +17.5° (*c* 1, MeOH); ^{1}H-NMR: (DMSO-d_6) δ 8.578 (d, J = 7.8 Hz, 1H, -CONH-), 8.108 (s, 3H, NH_3), 7.28–7.182 (m, 5H, Ar-H), 4.622–4.587 (m, 1H, CH), 4.325–4.198 (m, 1H, CH), 3.628 (s, 3H, -OCH_3), 2.793–2.605 (m, 1H, CH_2), 2.787–2.52 (m, 1H, CH_2), 1.652–1.604 (m, 1H, CH), 1.541–1.479 (m, 2H, CH_2), 0.893 (d, J = 6.6 Hz, 3H, CH_3), 0.842 (d, J = 6.6 Hz, 3H, CH_3); ESI-MS *m/z*: 293.2 $[M+H]^+$.

L-leucyl-L-phenylalanine benzyl ester hydrochloride (6c)

A white solid, yield: 83.9%, mp 160–162°C; $[\alpha]^{25}_D$ = +6.3° (*c* 1, H_2O); ^{1}H-NMR: (DMSO-d_6) δ 9.203 (d, J = 7.8 Hz, 1H, -CONH-), 8.345 (s, 3H, NH_3), 7.363–7.226 (m, 10H, Ar-H), 5.09–5.04 (m, 2H, Ar-CH_2-O-), 4.612–4.576 (m, 1H, CH), 3.805–3.781 (m, 1H, CH), 3.102–3.032 (m, 2H, Ar-CH_2-), 1.671–1.627 (m, 1H, CH), 1.522–1.499 (m, 2H, CH_2), 0.85 (d, J = 6.6 Hz, 3H, CH_3), 0.829 (d, J = 6.6 Hz, 3H, CH_3); ESI-MS *m/z*: 369.3 $[M+H]^+$.

L-phenylalanyl-L-leucine benzyl ester hydrochloride (6d)

A white solid, yield: 81.8%, mp 161–163°C; $[\alpha]^{25}_D$ = +5.6° (*c* 1, H_2O); ^{1}H-NMR: (DMSO-d_6) δ 9.187 (d, J = 7.8, 1H, -CONH-), 8.32 (s, 3H, NH_3), 7.389–7.24 (m, 10H, Ar-H), 5.168–5.105 (m, 2H, Ar-CH_2-O-), 4.398–4.36 (m, 1H, CH), 4.133–4.111 (m, 1H, CH), 3.162–3.13 (m, 1H, Ar-CH_2-), 2.953–2.917 (m, 1H, Ar-CH_2-), 1.72–1.674 (m, 1H, CH), 1.678–1.526 (m, 2H, CH_2), 0.896 (d, J = 6.6 Hz, 3H, CH_3), 0.848 (d, J = 6.6 Hz, 3H, CH_3); ESI-MS *m/z*: 369.2 $[M+H]^+$.

Synthesis of (S)-3-(tert-butoxycarbonylamino)-2-hydroxypropanoic acid (8)

Compound **8** was synthesised from *L*-Isoserine following the general procedure as described above (preparation of **3a**). a colourless solid, yield: 93.5%, mp 85–88°C; $[\alpha]^{25}_D$ = +6.7° (*c* 1, MeOH); ^{1}H-NMR: (DMSO-d_6) δ 7.934 (d, J = 7.8 Hz, 1H, -CONH-), 6.54 (s, 1H, OH), 4.674–4.651 (m, 1H, CH), 3.457 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 3.241 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 1.435 (s, 9H, CH_3); ESI-MS *m/z*: 206.1 $[M+H]^+$.

General procedure for the synthesis of 11a–11d and 13a–13d

Compound **9a** was synthesised from *N* protected *L*-Isoserine (**8**) and *L*-phenylalanine methyl ester

hydrochloride (**2b**) following the general procedure as described above (preparation of **5a**), yield: 84.5%, mp 95–97°C; $[\alpha]^{25}_{D}$ = -9.72° (*c* 1, MeOH); ^{1}H-NMR (DMSO-d_6) δ 7.921 (d, *J* = 7.8 Hz, 1H, -CONH-), 7.287–7.362 (m, 2H, Ar-H), 7.177–7.166 (m, 3H, NH_3), 6.526 (s, 1H, OH), 5.801 (d, *J* = 5.4 Hz, 1H, -CONH-), 4.604–4.567 (m, 1H, CH), 3.889–3.869 (m, 1H, CH), 3.627 (s, 3H, -OCH_3), 3.173–3.135 (m, 1H, Ar-CH_2-), 3.075 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 3.032 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 2.817–2.77 (m, 1H, Ar-CH_2-),1.435 (s, 9m, CH_3); HRMS (FAB) calcd for $C_{18}H_{26}N_2O_6$ 389.1683; found: 389.167 $[M+Na]^+$.

Compound **11a** was obtained through the deprotection of compound **9a** in the saturated HCl/EtOAc solution as the preparation of **6a**, yield: 77.7%, mp 138–140°C; $[\alpha]^{25}_{D}$ = -19.8° (*c* 1, H_2O). ^{1}H-NMR (DMSO-d_6) δ 8.25 (d, *J* = 7.8 Hz, 1H, -CONH-), 8.094 (s, 3H, NH_3), 7.294–7.27 (m, 2H, Ar-H), 7.23–7.197 (m, 3H, Ar-H), 6.541 (s, 1H, OH), 4.6–4.563 (m, 1H, CH), 4.203–4.173 (m, 1H, CH), 3.107 (s, 3H, -OCH_3), 3.101 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 3.05 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 2.927 (dd, *J* = 13.2 Hz, *J* = 9 Hz, 1H, Ar-CH_2-), 2.569 (dd, *J* = 13.2 Hz, *J* = 9 Hz, 1H, Ar-CH_2-); HRMS (FAB) calcd for $C_{13}H_{18}N_2O_4$ 267.1339; found: 267.1335 $[M+H]^+$.

Compounds **9c**–**9d** were prepared from *N* protected *L*-Isoserine and amino acid methyl/benzyl ester hydrochlorides (**2a**,**4a** and **4b**) and compounds **10a**–**10d** were prepared from *N* protected *L*-Isoserine and dipeptide methyl/benzyl ester hydrochlorides(**6a**, **6b**, **6c** and **6d**). Compounds **11c**–**11d** and **13a**–**13d** were obtained through the deprotection of compounds **9c**–**9d** and **10a**–**10d**.

L-Isoseryl-L-leucine methyl ester hydrochloride (11b)

A white solid, yield: 63.1%, mp 120–123°C; $[\alpha]^{25}_{D}$ = -36.1° (*c* 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 8.054 (d, *J* = 7.8 Hz, 1H, -CONH-), 7.903 (s, 3H, NH_3), 6.427 (s, 1H, OH), 4.607–4.593 (m, 1H, CH), 4.186–4.152 (m, 1H, CH), 3.218 (s, 3H, -OCH_3), 3.106 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 3.023 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 1.523–1.704 (m, 3H, CH_2CH), 0.926–0.947 (m, 6H, CH_3); HRMS (FAB) calcd for $C_{10}H_{20}N_2O_4$ 233.1496; found: 233.1493 $[M+H]^+$.

L-Isoseryl-L- phenylalanine benzyl ester hydrochloride (11c)

A white solid, yield: 76.6%, mp 177–179°C; $[\alpha]^{25}_{D}$ = –23.3° (*c* 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 8.285 (d, *J* = 7.8 Hz, 1H, -CONH-), 8.032 (s, 3H, NH_3), 7.391–7.303 (m, 5H, Ar-H), 7.274–7.177 (m, 5H, Ar-H), 6.511 (s, 1H, OH), 5.14–5.097 (m, 2H, Ar-CH_2-O-), 4.644–4.607 (m, 1H, CH), 4.195–4.164 (m, 1H, CH), 3.126 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 3.028 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 2.95–2.93 (m, 1H, Ar-CH_2-), 2.601–2.566 (m, 1H, Ar-CH_2-); HRMS (FAB) calcd for $C_{19}H_{22}N_2O_4$ 343.1652; found: 343.1650 $(M+H)^+$.

L-Isoseryl-L-leucine benzyl ester hydrochloride (11d)

A white solid, yield: 84.6%, mp 98–103°C; $[\alpha]^{25}_{D}$ = -25.7° (*c* 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 8.387 (d, *J* = 7.8 Hz, 1H, -CONH-), 8.189 (s, 3H, NH_3), 7.405–7.324 (m, 5H, Ar-H), 6.48 (s, 1H, OH), 5.187–5.13 (m, 2H, Ar-CH_2-O-), 4.372–4.335 (m, 1H, CH), 4.287–4.256 (m, 1H, CH), 3.088 (dd, *J* = 12.6 Hz, *J* = 8.4 Hz, 1H, CH), 2.778 (dd, *J* = 12.6 Hz, *J* = 8.4 Hz, 1H, CH), 1.7–1.594 (m, 2H, CH_2), 1.567–1.522 (m, 1H, CH), 0.882 (d, *J* = 6.6 Hz, 3H, CH_3), 0.839 (d, *J* = 6.6 Hz, 3H, CH_3); HRMS (FAB) calcd for $C_{16}H_{24}N_2O_4$ 309.1809; found: 309.1807 $(M+H)^+$.

L-Isoseryl-L-leucyl-L-phenylalanine methyl ester hydrochloride (13a)

A white solid, yield: 64.3%, mp 65–68°C; $[\alpha]^{25}_{D}$ = -33.2° (*c* 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 8.588 (d, *J* = 7.8 Hz, 1H, -CONH-), 8.12 (s, 3H, NH_3), 7.843 (d, *J* = 7.8 Hz, 1H, -CONH-), 7.298–7.2 (m, 5H, Ar-H), 6.485 (s, 1H, OH), 4.486–4.499 (m, 1H, CH), 4.368–4.329 (m, 1H, CH), 4.12–4.199 (m, 1H, CH), 3.579 (s, 3H, -OCH_3), 3.102–3.093 (m, 1H, Ar-CH_2-), 3.055 (dd, *J* = 13.80 Hz, *J* = 8.4 Hz, 1H, CH_2), 2.977 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 2.798–2.752 (m, 1H, Ar-CH_2-), 1.528–1.494 (m, 1H, CH), 1.483–1.359 (m, 2H, CH_2), 0.861 (d, *J* = 6.6 Hz, 3H, CH_3), 0.831 (d, *J* = 6.6 Hz, 3H, CH_3); HRMS (FAB) calcd for $C_{19}H_{29}N_3O_5$ 380.2180; found: 380.2174 $(M+H)^+$.

L-Isoseryl-L-phenylalanyl-L-leucine methyl ester hydrochloride (13b)

A white solid, yield: 50.9%, mp 77–80°C; $[\alpha]^{25}_{D}$ = -26.7° (*c* 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 8.631(d, *J* = 7.8 Hz, 1H, -CONH-), 8.1 (s, 3H, NH_3), 7.862 (d, *J* = 7.8 Hz, 1H, -CONH-), 7.298–7.195 (m, 5H, Ar-H), 6.512 (s, 1H, OH), 4.633–4.596 (m, 1H, CH), 4.33–4.269 (m, 1H, CH), 4.166–4.145 (m, 1H, CH), 3.639 (s, 3H, -OCH_3), 3.077 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 2.918 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 2.895–2.55 (m, 1H, Ar-CH_2-), 2.568–2.521 (m, 1H, Ar-CH_2-), 1.656–1.597 (m, 2H, CH_2), 1.544–1.497 (m, 1H, CH), 0.894 (d, *J* = 6.6 Hz, 3H, CH_3), 0.847 (d, *J* = 6.6 Hz, 3H, CH_3); HRMS (FAB) calcd for $C_{19}H_{29}N_3O_5$ 380.2180; found: 380.2179 $(M+H)^+$.

L-Isoseryl-L-leucyl-L-phenylalanine benzyl ester hydrochloride (13c)

A white solid, yield: 52.2%, mp 135–137°C; $[\alpha]^{25}_{D}$ = -30.5° (*c* 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 8.631(d, *J* = 7.8 Hz, 1H, -CONH-), 8.134 (s, 3H, NH_3), 7.827 (d, *J* = 7.8 Hz, 1H, -CONH-), 7.368–7.311 (m, 3H, Ar-H), 7.27–7.202 (m, 7H, Ar-H), 6.493 (s, 1H, -OH), 5.088–5.033 (m, 2H, Ar-CH_2-O-), 4.547–4.51 (m, 1H, CH), 4.381–4.341 (m, 1H, CH), 4.216–4.201 (m, 1H, CH), 3.084–3.074 (m, 1H, Ar-CH_2-), 3.067 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 2.993 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H, CH_2), 2.782–2.63 (m, 1H, Ar-CH_2-),1.512-1.35 (m, 3H, CH_2CH), 0.844 (d, *J* = 6.6 Hz, 3H, CH_3), 0.797 (d, *J* = 6.6 Hz, 3H, CH_3); HRMS (FAB) calcd for $C_{25}H_{33}N_3O_5$ 456.2493; found: 456.2490 $(M+H)^+$.

L-Isoseryl-L-phenylalanyl-L-leucine benzyl ester hydrochloride (13d)

A white solid, yield: 58.1%, mp 140–142°C; $[\alpha]^{25}_{D}$ = -28.6° (*c* 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 8.644 (d, *J* = 7.8 Hz, 1H, -CONH-), 8.037 (s, 3H, NH_3), 7.852 (d, *J* = 7.8 Hz, 1H, -CONH-), 7.377–7.319 (m, 3H, Ar-H), 7.242–7.171 (m, 5H,

Ar-H), 6.494 (s, 1H, OH), 5.166–5.121 (m, 2H, Ar-CH_2-O-), 4.64–4.603 (m, 1H, CH), 4.385–4.348 (m, 1H, CH), 4.142–4.126 (m, 1H, CH), 3.028 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 2.912–2.892 (m, 1H, Ar-CH_2-), 2.851 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 2.54–2.504 (m, 1H, Ar-CH_2-),1.657–1.529 (m, 3H, CH_2CH), 0.906 (d, J = 6.6 Hz, 3H, CH_3), 0.846 (d, J = 6.6 Hz, 3H, CH_3); HRMS (FAB) calcd for $C_{25}H_{33}N_3O_5$ 456.2493; found: 456.2496 $(M+H)^+$.

General procedure for the synthesis of 12a–12b and 14a–14b

Compound **9c** (1.77 g, 4 mmol) was hydrogenated in absolute MeOH (30 mL) with a catalytic quantity of 10% Pd/C (0.17 g). The mixture was stirred for 10 h under normal pressure then filtered. The filtrate was evaporated to give a white solid. The solid was dissolved in dry EtOAc (5 mL) at 0°C and added dropwise to a solution of EtOAc (10 mL) saturated by dry HCl gas. The reaction solution was stirred at 0°C for 12 h to provide a white solid. The solution was removed by filtration, and the remaining solid was recrystallised with MeOH and Et_2O to give **12a** white crystals (0.91 g), yield: 78.9%, mp 187–189°C; $[\alpha]^{25}_D$ = -13.4° (c 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 12.964 (s, 1H, COOH), 8.081 (s, 3H, NH_3), 8.07 (d, J = 8.4 Hz, 1H, -CONH-), 7.275 (t, J = 7.8 Hz, 2H, Ar-H), 7.223–7201 (m, 3H, Ar-H), 6.527 (s, 1H, OH), 4.543–4.507 (m, 1H, CH), 4.183–4.169 (m, 1H, CH), 3.113 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 3.031 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 2.957–2.938 (m, 1H, Ar-CH_2-), 2.62–2.561 (m, 1H, Ar-CH_2-); HRMS (FAB) calcd for $C_{12}H_{16}N_2O_4$ 253.1183; found: 253.1180 $(M+H)^+$.

L-Isoseryl-L-leucine hydrochloride (12b)

A white solid, yield: 88.3%, mp 213–215°C; $[\alpha]^{25}_D$ = -44.5° (c 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 12.713 (s, 1H, COOH), 8.102 (s, 3H, NH_3), 8.088 (d, J = 8.4 Hz, 1H, -CONH-), 6.441 (s, 1H, OH), 4.236–4.229 (m, 1H, CH), 4.274–4.251 (m, 1H, CH), 3.086 (dd, J = 12.6 Hz, J = 7.2 Hz, 1H, CH_2), 2.784 (dd, J = 12.6 Hz, J = 7.2 Hz, 1H, CH_2), 1.681–1.585 (m, 2H, CH_2), 1.556–1.513 (m, 1H, CH), 0.893 (d, J = 6.6 Hz, 3H, CH_3), 0.854 (d, J = 6.6 Hz, 3H, CH_3); HRMS (FAB) calcd for $C_9H_{18}N_2O_4$ 219.1339; found: 219.1340 $(M+H)^+$.

L-Isoseryl-L-leucyl-L-phenylalanine hydrochloride (14a)

A white solid, yield: 56.9%, mp 90–92°C; $[\alpha]^{25}_D$ = -34.7° (c 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 12.69 (s, 1H, COOH), 8.468 (d, J = 7.8 Hz, 1H, -CONH-), 8.119 (s, 3H, NH_3), 7.836 (d, J = 7.8 Hz, 1H, -CONH-), 7.245–7.168 (m, 5H, Ar-H), 6.52 (s, 1H, OH), 4.652–4.573 (m, 1H, CH), 4.263–4.225 (m, 1H, CH), 4.176–4.084 (m, 1H, CH), 3.986 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 2.887 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 2.812–2.798 (m, 1H, Ar-CH_2), 2.51–2.439 (m, 1H, Ar-CH_2), 1.502–1.414 (m, 3H, CH_2CH), 0.862 (d, J = 6.6 Hz, 3H, CH_3), 0.813 (d, J = 6.6 Hz, 3H, CH_3); HRMS (FAB) calcd for $C_{18}H_{27}N_3O_5$ 366.2023; found: 366.2026 $(M+H)^+$.

L-Isoseryl-L-phenylalanyl-L-leucine hydrochloride (14b)

A white solid, yield: 53.6%, mp 87–89°C; $[\alpha]^{25}_D$ = -35.1° (c 1, H_2O); ^{1}H-NMR (DMSO-d_6) δ 12.647 (s, 1H, COOH), 8.475 (d, J = 7.8 Hz, 1H, -CONH-), 8.109 (s, 3H, NH_3), 7.847 (d, J = 7.8 Hz, 1H, -CONH-), 7.265–7.176 (m, 5H, Ar-H), 6.524 (s, 1H, OH), 4.637–4.6 (m, 1H, CH), 4.274–4.236 (m, 1H, CH), 4.159–4.095 (m, 1H, CH), 3.09 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 2.905 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H, CH_2), 2.896–2.882 (m, 1H, Ar-CH_2), 2.539–2.505 (m, 1H, Ar-CH_2), 1.72–1.526 (m, 3H, CH_2CH), 0.907 (d, J = 6.6 Hz, 3H, CH_3), 0.853 (d, J = 6.6 Hz, 3H, CH_3); HRMS (FAB) calcd for $C_{18}H_{27}N_3O_5$ 366.2023; found: 366.2027 $(M+H)^+$.

In vitro APN inhibition assay

The IC_{50} values against APN were determined using L-Leu-p-nitroanilide as the substrate and microsomal aminopeptidase from porcine kidney microsomes (Sigma) in 50 mM PBS, pH 7.2, at 37°C. The hydrolysis of the substrate was monitored by following the change in the absorbance measured at 405 nm with a UV-Vis spectrophotometer Pharmacia LKB, Biochrom 4060. All solutions of inhibitors were prepared in the assay buffer, and the pH was neutralised to 7.5 by the addition of either 0.1 M HCl or 0.1 M NaOH.

The samples and positive controls were serial diluted to various concentrations: 1280 μg/mL, 320 μg/mL, 80 μg/mL, and 20 μg/mL, 5 μg/mL and 1.25 μg/mL. All compounds were pre-incubated with APN on a 96-well plate for 30 min. The assay mixture, including the compound solution, the enzyme solution (5 μg/mL final concentration) and the assay buffer, was adjusted to give a total volume of 200 μL. Fifteen minutes later, the absorbance (OD405) of the wells was recorded on a microplate reader. The percentage inhibition was calculated according to the absorbance of the assay wells relative to those of the control wells. Finally, the IC_{50} values were determined using a linear regression analysis of the concentration/inhibition data.

In vitro MMP-2 inhibition assay

Gelatinase A (MMP-2) and picrylsulphonic acid (TNBS) were purchased from Sigma. Succinylated gelatin was synthesised as described by Baragi et al. [17]. The compound samples were assayed for inhibitory activity against MMP-2 in 96-well microplates using succinylated gelatin as the substrate. The compounds and gelatinase were dissolved in sodium borate buffer (pH 8.5, 50 mM), and incubated at 37°C for 30 min. The substrate was added and incubated at 37°C for another 60 min. Then 0.03% picrylsulphonic acid solution was added and incubated at room temperature for an additional 20 min. The resulting solutions were detected under 450 nm wavelength to gain absorbance (OD_{450}). MMP-2 inhibition activity was measured by the following formula [17]: Inhibition ration (%) = [OD_{450}(100%)– OD_{450}(compound)]/[OD_{450}(100%)–OD_{450}(blank)]×100%. The IC_{50} values were obtained from the above inhibitory rates using the OriginPro 7.5 software.

In vitro HL-60 and SKOV3 cells viability assay

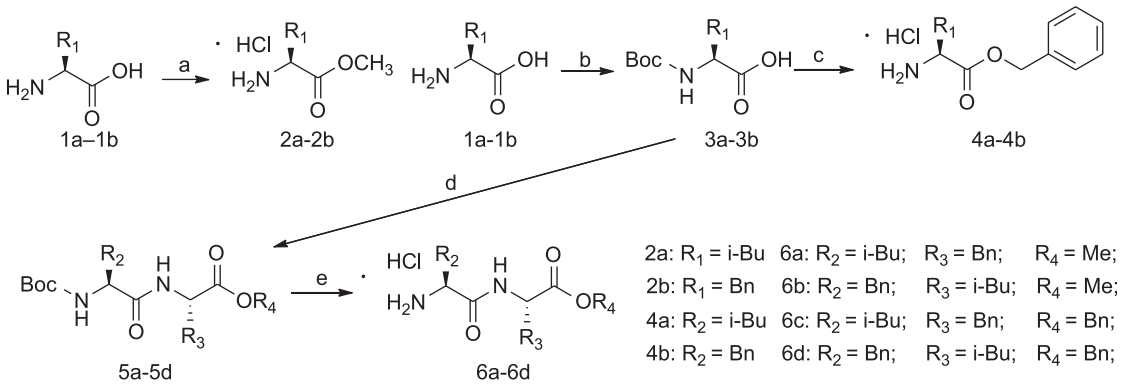
The HL-60 and SKOV3 Cells were grown in RPMI1640 medium with 10% fetal bovine serum at 37°C in a

5% CO_2 humidified incubator. Cell proliferation was determined by the MTT (3-[4,5-dimethyl-2-thiazolyl]-2.5-diphenyl-2H-tetrazolium bromide) assay. Briefly, 10,000 HL-60 cells (or 5,000 SKOV3 cells) per well were plated in a 96-well plate, cultured for 4 h in complete growth medium, then treated with 400 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL and 25 µg/mL of compounds for 48 h, respectively. An aliquot of 0.5% MTT (10 µL/well) was then added to each well. After further incubation for 4 h, the formazan formed from the MTT was extracted by adding DMSO and mixing for 10 min. The optical absorbance was read with an micoplate reader at 550 nm, and the IC_{50} values were calculated according to a regression analysis of the concentration/inhibition data.

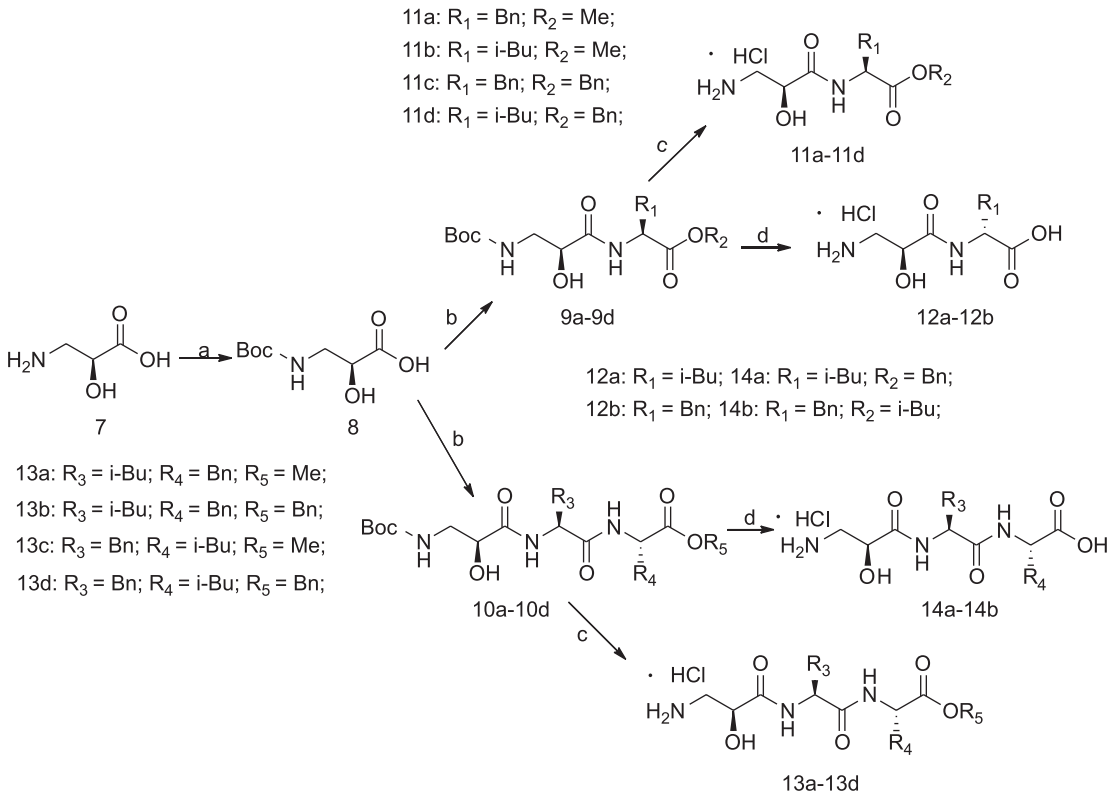
Results and discussion

Chemistry

L-leucine (**1a**) and *L*-phenylalanine (**1b**) were used as the starting materials to yield their methyl esters **2a** and **2b**, and then protected by $(Boc)_2O$ to obtain **3a** and **3b**. The carboxylic acids of **3a**–**3b** were protected using benzyl chloride, and then the Boc group was deprotected with 3 N HCl in ethyl acetate to give **4a** and **4b**. *N*-Boc-protected amino acids (**3a** and **3b**) were coupled with

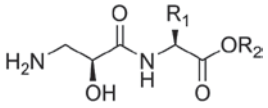


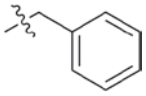
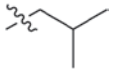
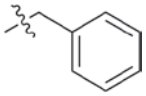
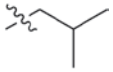
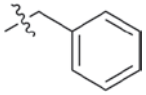
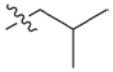
Scheme 1. *Reagents and conditions*: a. CH_3OH, HCl; b. $(Boc)_2O$, CH_3OH, NaOH; c. i) K_2CO_3, KI, TBAI, benzyl chloride, CH_3COCH_3; ii) HCl/anhydrous EtOAc; d. EDCI, HOBt/anhydrous, DCM, **2a~2b**,**4a~4b**, 0°C to room temperature; e. HCl/anhydrous EtOAc.

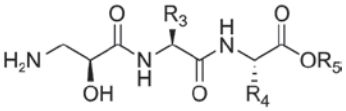


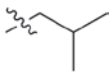
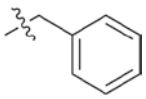
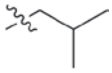
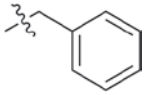
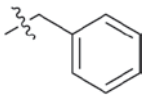
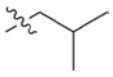
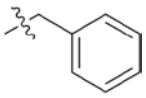
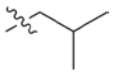
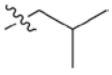
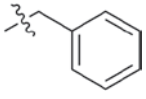
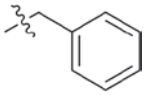
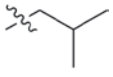
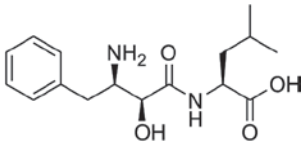
Scheme 2. *Reagents and conditions*: a. $(Boc)_2O$, CH_3OH, NaOH; b. EDCI, HOBt/anhydrous, DCM, **2a~2b**,**4a~4b**,**6a~6d**, 0°C to room temperature; c. HCl/anhydrous EtOAc; d. i) 15% Pd/C, CH_3OH. ii) HCl/anhydrous EtOAc.

Table 1. The structures and IC_{50} values of target compounds against APN and MMP-2.



Compound	R_1	R_2	APN/IC_{50} [a] (μM)	MMP/IC_{50} [a] (μM)
11a		**-CH3**	301.9 ± 1.3	>1000
11b		**CH3**	229.2 ± 0.6	681.7 ± 2.8
11c		**-Bn**	99.5 ± 2.1	328.5 ± 1.9
11d		**-Bn**	195.1 ± 0.7	>1000
12a		**-H**	249.1 ± 0.9	710.3 ± 2.3
12b		**-H**	199.2 ± 3.1	364.2 ± 4.0



Compound	R_3	R_4	R_5	APN /IC_{50} (μM)	MMP/IC_{50} (μM)
13a			**-CH3**	139.6 ± 0.5	125.6 ± 2.4
13b			**-Bn**	65.1 ± 0.7	225.4 ± 1.1
13c			**-CH3**	69.1 ± 1.4	236..6 ± 3.3
13d			**-Bn**	32.2 ± 2.3	333.7 ± 2.7
14a			**-H**	20.9 ± 0.8	115.0 ± 0.9
14b			**-H**	12.2 ± 1.6	203.4 ± 2.9
Bestatin				7.3 ± 0.2	167 ± 2.8

a. Each value represents the mean of three experiments, standard deviation is given.

intermediates (**2a**, **2b**, **4a** and **4b**) by a classical EDCI/HOBt method, and then converted into **6a**–**6d** by deprotecting the Boc group (Scheme 1). Using the same procedure, Boc-protected *L*-isoserine **8** was coupled with the intermediate (**2a, 2b, 4a** and **4b**) and **6a**–**6d** to yield the dipeptide **9a**–**9d** and tripeptide **10a**–**10d** containing protecting group. Finally, target compounds **11a**–**11d** and **12a**–**12d** were obtained by easily cleaving Boc, while the target compounds **13a**–**13b** and **14a**–**14b** were obtained from **9c**–**9d** and **10c**–**10d** by first reacting in a hydrogen atmosphere with 15% Pd/C and then deprotecting Boc (Scheme 2).

In vitro APN and MMP assays

All the target compounds were tested for their inhibitory potential against APN and MMP-2 and the results are shown in Table 1. The matrix metalloproteinase-2 (MMP-2) also belongs to the zinc-dependent metalloproteinase family and is associated with tumour invasion and metastasis. These inhibitory studies were performed on both APN and MMP-2 so as to identify the selectivity of the target compounds.

From the results of the enzyme assay, compound **14b** (IC_{50} of 12.2 μM) is the most potent APN inhibitor of all the target compounds, and showed an inhibitory activity similar to that of bestatin (IC_{50} of 7.3 μM). In addition, the dipeptides derivatives (**11a**, **11b**, **11c**, **11d**, **12a** and **12b**) showed less inhibition than their tripeptide analogues (**13a**, **13b**, **13c**, **13d**, **14a** and **14b**). This phenomenon suggests that the third amino acid residue helps to increase the interaction with the APN binding site. One the other hand, the tripeptide compounds (**13c**, **13d** and **14b**) with a scaffold of isoser-*L*-Phe-*L*-Leu exhibited a better inhibitory activity than that of the compounds (**13a**, **13b** and **14a**) with an isoser-*L*-Leu-*L*-Phe scaffold. As for the compounds (**13c**, **13d** and **14b**) with a substituted R_5, the hydrogen seems to be the best substitution for this position. A possible reason may be that the free terminal carboxylate group could enhance the hydrogen bond interaction with the active sites of APN.

In our previous studies, most of the compounds tested showed better inhibition on MMP-2 than APN [18]. However, most of these *L*-isoserine dipeptides and tripeptides derivatives exhibited a better inhibitory activity on APN than MMP-2. This result may be due to the differences between the 3D structure of the active sites of APN and MMP-2. According to the binding site of APN (PDB code: 1HS6) and MMP-2 (PDB code: 1HOV, 1CK7, 1QIB), the active site of APN was much deeper than the active site of MMP-2 [17]. The scaffold of our *L*-isoserine derivatives are linear structures, and therefore there is easier access to the APN active site.

Antiproliferative activity assays on HL-60 and SKOV3 cells

The human promyelocytic leukaemia cell line (HL-60) was used to evaluate antiproliferative activity with the MTT assay due to APN being over-expressed. In addition, the human ovarian carcinoma cell line (SKOV3) was chosen as an additional model for MTT assays. According to the results (Table 2), all the test compounds showed cytostatic activity except for compound **13d.** It should also be pointed out that compound **14b** not only displays the most potent inhibition on APN, but also has a higher antiproliferative activity than bestatin.

In order to determine the interaction between our target compounds with APN, the most potent compound, **14b,** was constructed with a Sybyl/Sketch module and optimised using the Tripos force field. The docking study performed with the Sybyl/FlexX module was based on the active site of the APN co-crystal structure with bestatin (PDB code: 2DQM). The docking results showed that the carbonyl group and hydroxyl group of compound **14b** could chelate with the zinc ion in APN (Figures 1 and 2). The terminal amino group of **14b** could form a hydrogen bond with Glu^{264} and the carboxylic group of **14b** is able to interact with Arg^{825}. In addition, the phenylalanine residue of **14b** can insert an S1′ pocket which is surrounded by Gly^{261}, Ala^{262} and Glu^{298}, while the leucine residue in **14b** interacts with the S2′ pocket containing Met^{263} and Tyr^{376}.

Table 2. *In vitro* antiproliferative inhibiting potency of compounds **11c**, **13c**, **13d** and **14b**.

Compound	HL-60 IC_{50}[a] (mM)	SKOV3 IC_{50}[a] (mM)
11c	0.33 ± 0.06	0.28 ± 0.03
13c	0.31 ± 0.05	0.07 ± 0.02
13d	>1000	>1000
14b	0.17 ± 0.03	0.38 ± 0.07
Bestatin	0.87 ± 0.07	1.98 ± 0.09

Each value represents the mean values with S.E values for 5 independent experiments.

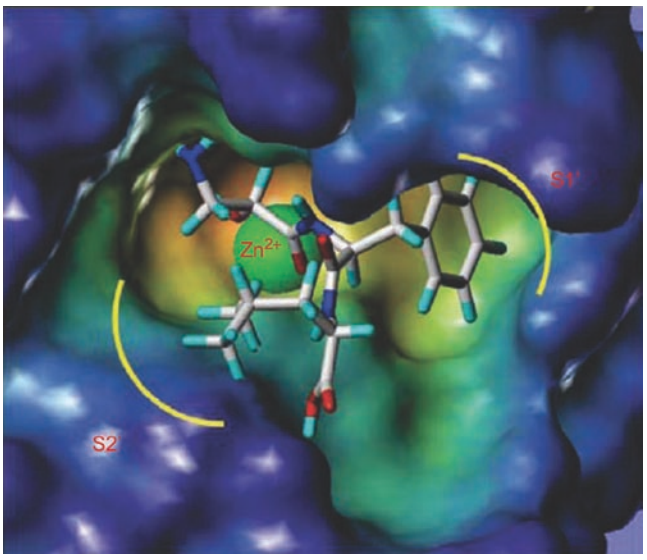


Figure 1. The docking result for **14b** with the active site of APN (PDB: 2DQM).

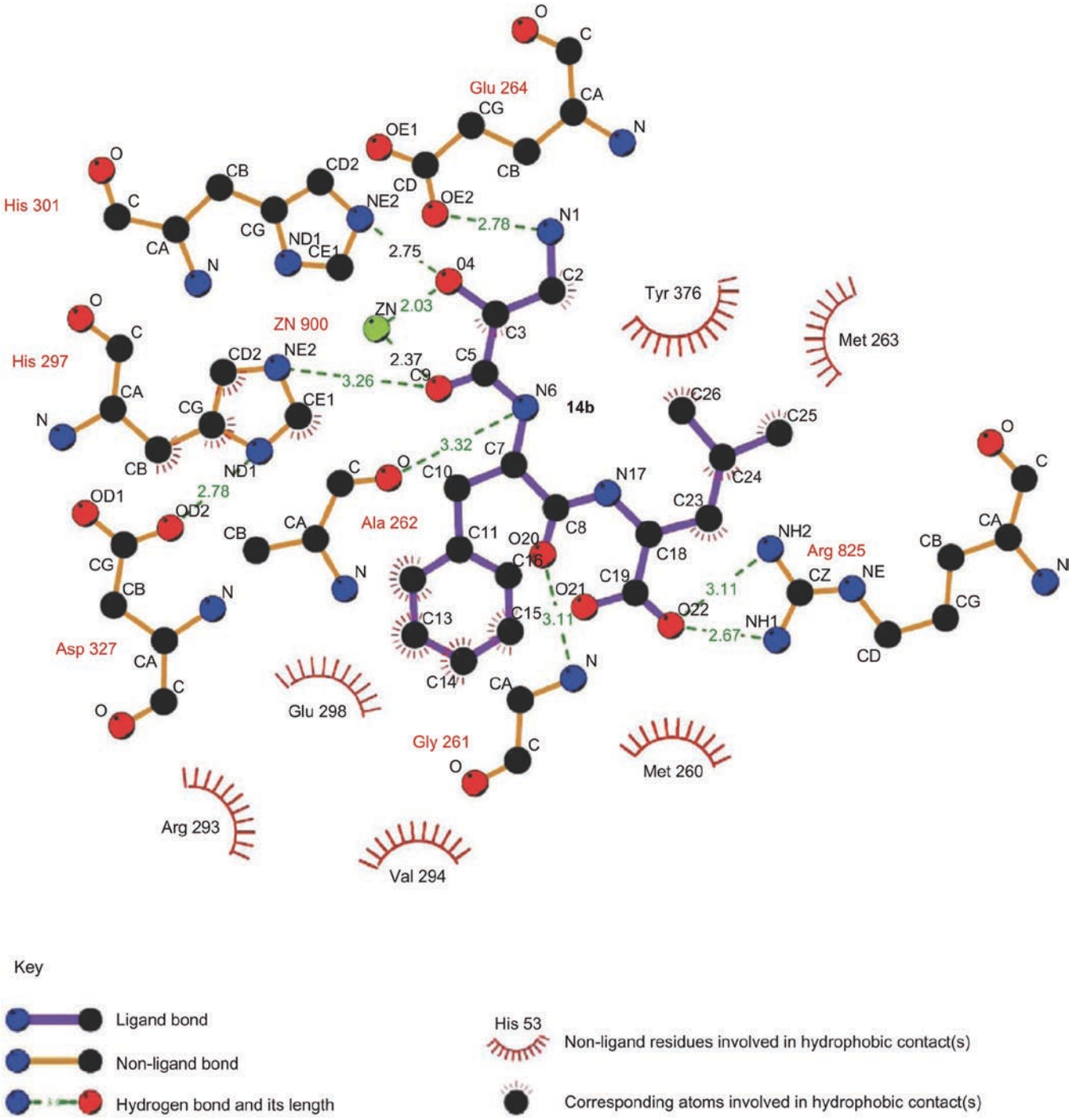


Figure 2. The docking results for **14b** with APN showed by LIGPLOT.

Conclusion

In summary, we have described the synthesis and performed a SAR study for a novel series of *L*-isoserine derivatives as inhibitors of APN. Most of these compounds possessed potent activity toward APN. Among them, compound **14b** exhibited a potent inhibitory activity and a significant selectivity for APN over MMP-2. Furthermore, this compound also showed good cell-based inhibitory activity, and could be used as a potential lead compound for new anti-cancer agents in the future.

Declaration of interest

This work was supported by National Natural Foundation Research Grant (Grant No. 9071304; No 30772654) and National High Technology Research and Development Program of China (863 proJect; Grant No 2007AA02Z314) and Doctoral Foundation of Ministry of Education of the People's Republic of China (Grant No 20060422029).

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