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Novel aminopeptidase N (APN/CD13) inhibitors derived from 3-phenylalanyl-N′-substituted-2,6-piperidinedione

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ABSTRACT

Aminopeptidase N (APN/CD13) over expressed on tumor cells, plays a critical role in tumor invasion, metastasis, and tumor angiogenesis. Here we described the design, synthesis and preliminary activity studies of novel APN inhibitors with 3-phenylalanyl-N′-substituted-2,6-piperidinedione scaffold. The results showed that compound **7c** had the most potent inhibitory activity against APN with the IC_{50} value to 5.00 ± 3.17 μM, which could be used as the lead compound in the future for anticancer agent research.

1. Introduction

Aminopeptidase N (APN; EC 3.4.11.2), also known as CD13 is a zinc-dependent type II membrane-bond ectopeptidase, and belongs to the M1 family of MA clan of peptidase.[1,2] This enzyme consists of 967 amino acids, and can preferentially release neutral or basic amino acids from the N-terminal of peptides. APN is widely distributed in the body of mammalian. It can be expressed on the surface of different cells such as myeloid progenitors and monocytes, epithelial cells of the intestine and kidney, synaptic membranes in the central nervous system, fibroblasts, endothelial cells, epithelial cells,[3–6] and so on. APN is highly expressed on tumor cells which play a critical role in tumor invasion, metastasis and angiogenesis. Therefore, the design and synthesis of APN inhibitors may be a clinically significance for the discovery of anti-cancer agents.

Since 1976, the first APN inhibitor Bestatin was discovered, many APN inhibitors have been developed such as Probestin,[7] Amatatin,[8] Prebestin,[9] Lapstatin,[10] AHPA-Val,[11] and so on. The X-ray crystal structures of APN and its complexes with varies inhibitors have been studied in recently years.[12–14]

The binding site of APN with Bestatin could be divided into three parts (Fig. 1): part A (S1 pocket) is a hydrophobic pocket which can interact with the phenyl group of Bestatin; part B contains a zinc ion and can interact with the zinc binding group (ZBG) such as hydroxyl group and carbonyl group in Bestatin; and part C is another hydrophobic pocket in deep cavity which can be divided into two subsites (S1′ pocket and S2′ pocket). The free amino group of Bestatin can interact with Glu 355.[15,16]

In our previous work, a series of piperidinedione derivatives was reported as potent APN inhibitors.[17,18] Compound **13f** showed even better APN inhibitory effect with the IC_{50} value to 1.8 μM. Compound **13f** can also significantly inhibit the growth and migration of human ovarian carcinoma cell ES-2[19] and hepatocellular carcinoma (HCC) cell line HuH-7.[20] Herein, in order to find better APN inhibitors, we used compound **13f** as the leading compound and modified the structures as follows (Fig. 2): (1) due to the conformationally constrained effect, we maintained the cyclic-imide scaffold; (2) we maintained the phenylalanine moiety in order to mimic the 3-amino-2-hydroxyl-4-phenylbutanoic acid (AHPA) skeleton in Bestatin to interact with S1 pocket (part A) of APN; (3) we replaced the hydroxamate group with different amino acid benzyl ester, amino acid or organic amino in order to interact with S1′ pocket (part C) of APN.

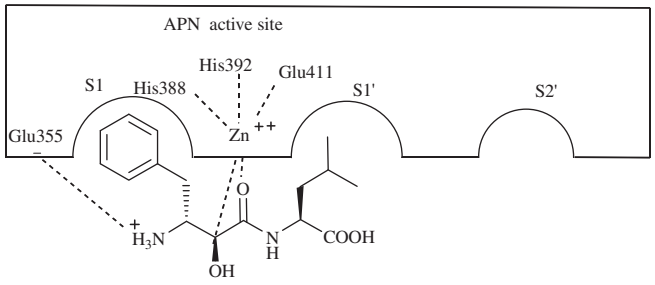

Figure 1. The binding mode of Bestatin with APN.

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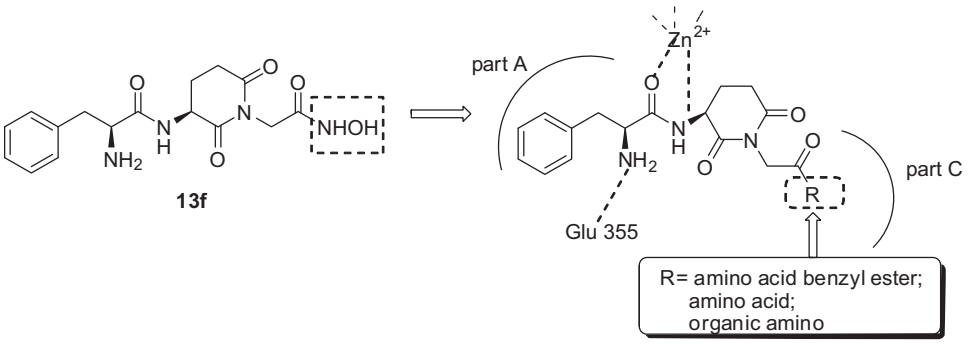
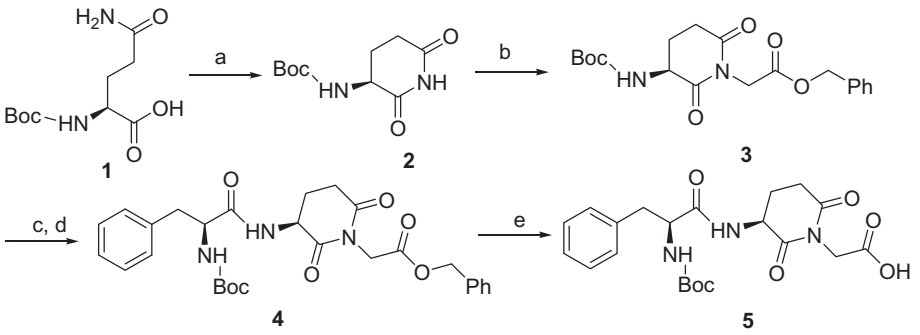
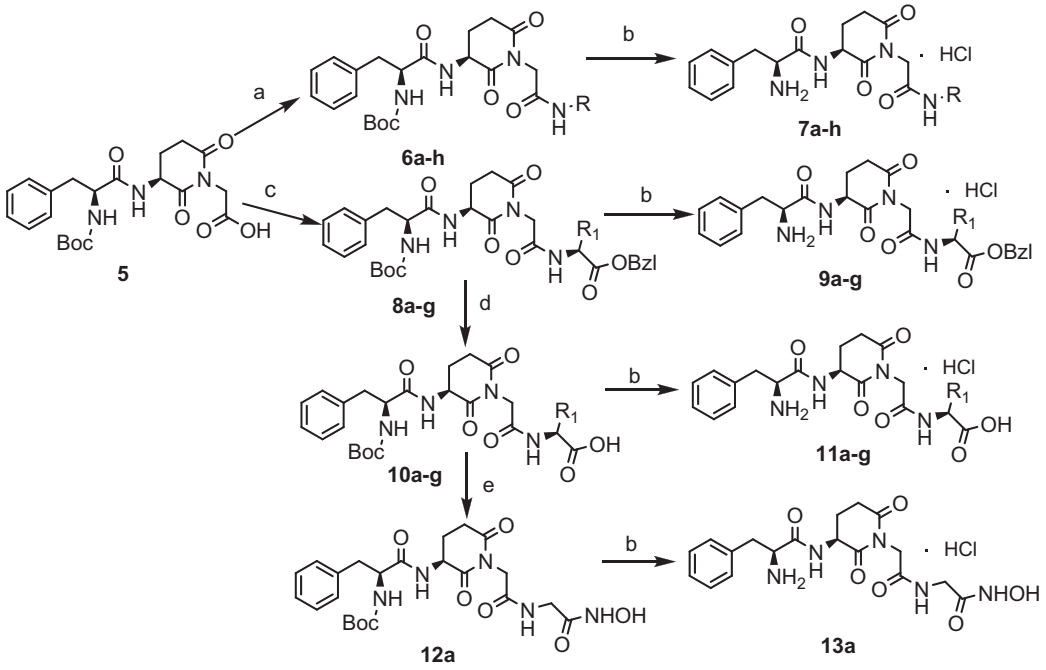


Figure 2. Chemical structures of the new designed cyclic-imide APN inhibitors.



Scheme 1. Reagents and conditions: (a) DCC, HOSu, THF, 65 °C; (b) K_2CO_3, KI, acetone, $BrCH_2COOCH_2Ph$, 56 °C; (c) TFA, DCM; (d) Boc-L-Phe, isobutyl chloroformate, NMM, THF, −20 °C; (e) 10% Pb/C, H_2, MeOH.



Scheme 2. Reagents and conditions: (a) RNH_2, isobutyl chloroformate, NMM, THF, −20 °C; (b) 3 N HCl–EtOAc; (c) benzyl ester of amino acids, isobutyl chloroformate, NMM, THF, −20 °C; (d) 10% Pb/C, H_2, MeOH; (e) $H_2NOH{\cdot}HCl$, isobutyl chloroformate, NMM, THF, −20 °C.

2. Chemistry

The target compounds were synthesized following the procedures as shown in Schemes 1 and 2. The key intermediate compound (**5**) was easily prepared as our early report.[18] Starting from optically pure Boc-L-Gln (**1**), by using DCC and *N*-hydroxysuccinimide (HOSu) in THF at elevated temperature gives 2,6-dioxopyridine derivative (**2**), which is on treatment with benzyl bromoacetate gives compound (**3**). And then remove the Boc protecting group of compound (**3**) and coupled with Boc-L-Phe gives

compound (**4**), which was then catalytically hydrogenated using 10% Pd/C to afford the key intermediate (**5**).

Subsequently compound (**5**) was activated with isobutyl chloroformate and *N*-methylmorpholine (NMM) and then coupled with different substituted aromatic amine to yield **6a**–**h**. The Boc group can be easily removed by 3 N HCl in anhydrous ethyl acetate to give hydrochloride salts **7a**–**h**. Using the same procedure, compound (**5**) was coupled with different benzyl ester of amino acids to provide **8a**–**g** which can be easily converted to **9a**–**g** after removing the Boc protecting groups. Catalytically hydrogenating **8a**–**g**, and then deprotecting the Boc group gives compounds **11a**–**g**. In addition, the carboxyl group in compound **10a** can be easily converted to its hydroxamate acid derivative (compound **13a**) by using the same method with NH_2OH as the base.

3. Results and discussion

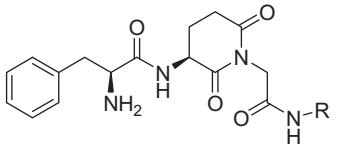
The newly synthesized piperidinedione derivatives were assayed for the enzymatic inhibitions on both APN and MMP-2 and the results are listed in Table 1. Similar to APN, MMP-2 is also a zinc-dependent metalloproteinase which is involved in the process of tumor invasion and metastasis. The difference between them is that MMP-2 is an endopeptidase while APN is an exopeptidase. In order to observe the selectivity of the target compounds against the two enzymes, all the target compounds were assayed for the inhibitory activities on APN and MMP-2.

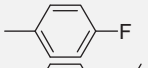
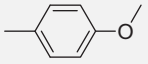
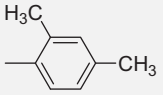
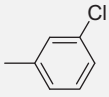
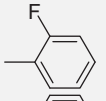
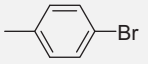
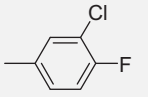
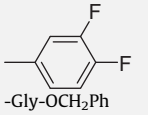
As shown in the results, most compounds exhibited a better APN inhibitory activity than that of MMP-2, with the IC_{50} values lying in micromolar level. The results to a certain extent conformed our strategy for designing APN special inhibitors. The results may be explained by the difference between the structures of the activity site of APN and MMP-2. The activity site of APN is deeper than that of MMP-2, which may be suitable for most of the compounds. On the other hand, APN is an exopeptidase which can release the N-terminal amino acid from the peptide, and the N-terminal amino acid is the neutral or basic amino acids such as Phe, Tyr, Ala, and Leu. The target compounds we designed all have the L-Phe residue and could be well recognized by APN, so the compounds may be suitable for APN inhibitors. And so the following structure–activity relationships were mainly focused on APN inhibition.

Comparing compounds **7a**–**7h** with aromatic side chains as the hydrophobic side chain, the APN inhibitory activities were different with the substitutions on the aromatic ring. General speaking, compounds with di-substitution on the aromatic ring (compounds **7c**, **7h** except **7g**) showed better APN inhibitory activities than compounds with mono-substitution on the aromatic ring. Comparing compounds with di-substitution on the aromatic ring, compound **7c** with two methyl groups on the aromatic ring showed better APN inhibitory activity (IC_{50} = 5.00 ± 3.17 μM) than compounds with two halogens such as compounds **7h** (IC_{50} = 169.85 ± 13.03 μM) and **7g** (IC_{50} = 53.43 ± 8.03 μM). When it comes to compounds with mono-substitution on the aromatic ring, to some extent, the substitution at the meta-position showed better activity such as compound **7d** with the IC_{50} value to 6.92 ± 0.67 μM.

Comparing compounds **9a**–**9g**, of which the R group was different amino acid benzyl ester. These compounds (except compound **9f**) showed moderate APN inhibitory activity with IC_{50} values from 13.45 ± 3.33 μM to 91.61 ± 1.48 μM. The APN inhibitory activity is associated with the volume of hydrophobic side chain of the amino acid. General speaking, compounds with bigger volume hydrophobic side chain of amino acid had better APN inhibitory activities. To compound **9f**, the R group is phenylalanine benzyl ester and the IC_{50} value is 6.48 ± 0.33 μM, the possible reason may be due to the system of the aromatic ring enhancing the interaction with the hydrophobic region of the enzyme.

Table 1
The structures and in vitro APN inhibitory activities of the target compounds and positive control Bestatin



Compound	R	IC_{50}[a] (μM)	
		APN	MMP-2
7a		252.14 ± 2.60	122 ± 28.2
7b		178.21 ± 2.10	>1000
7c		5.00 ± 3.17	8.27 ± 0.63
7d		6.92 ± 0.67	60.4 ± 0.88
7e		245.36 ± 1.21	62.7 ± 9.6
7f		74.00 ± 1.54	71.6 ± 4.97
7g		169.85 ± 13.03	131 ± 14.2
7h		53.43 ± 8.03	46.9 ± 0.73
9a	-Gly-OCH_2Ph	13.45 ± 3.33	454 ± 51
9b	-L-Ala-OCH_2Ph	91.61 ± 1.48	129 ± 12.7
9c	-L-Val-OCH_2Ph	30.14 ± 3.08	18.5 ± 2.38
9d	-L-Leu-OCH_2Ph	30.17 ± 3.31	121 ± 22.7
9e	-β-Ala-OCH_2Ph	49.70 ± 1.23	127 ± 5.65
9f	-L-Phe-OCH_2Ph	6.48 ± 0.33	12 ± 0.12
9g	-L-Glu-$(OCH_2Ph)_2$	91.36 ± 10.91	272 ± 50.82
11a	-Gly-OH	38.74 ± 2.03	107 ± 11.7
11b	-L-Ala-OH	109.84 ± 7.46	155 ± 0.75
11c	-L-Val-OH	13.33 ± 0.03	>1000
11d	-L-Leu-OH	8.42 ± 0.56	>1000
11e	-β-Ala-OH	12.75 ± 0.42	22.7 ± 1.36
11f	-L-Phe-OH	66.40 ± 1.35	315.65 ± 27.08
11g	-L-Glu(OH)-OH	12.61 ± 1.24	>1000
13a	-Gly-NHOH	304.21	141 ± 28.3
Bestatin		4.18	156.98 ± 2.4

[a] Values are means of three experiments, standard derivation is given.

Compounds **11a**–**11g**, of which the R group was different amino acid. Similar to compounds **9a**–**9g**, these compounds also showed moderate APN inhibitory activity with IC_{50} values from 8.42 ± 0.56 μM to109.84 ± 7.46 μM. The APN inhibitory activity is also associated with the volume of hydrophobic side chain of amino acid. Compounds with bigger volume hydrophobic side chain of amino acid had better APN inhibitory activities (**11d** > **11c** > **11a**). In contrast to compounds **9a**–**9g**, the best APN inhibitor was **11d** (IC_{50} = 8.42 ± 0.56 μM) of which the R group is Leucine.

Comparing all the target compounds (compounds **7**, **9**, **11**, and **13**), we can see that compounds with different organic amines in part C (compound **7**) showed less activities than compounds with different amino acid benzyl esters (compound **9**) and amino acids (compound **11**). Compound **13** with a hydroxamate group in part C showed the least activity.

Additionally, the effects of compounds **7c**, **7d**, **9f**, and **11d** on the proliferation of two tumor cell lines (HL-60 and MDA-MB-231) compared with Bestatin were further assessed by using MTT assay, which were shown in Figure 3. The results indicated that the anti-proliferative effects of the four compounds (**7c**, **7d**, **9f**, and **11d**) against HL-60 cell and MDA-MB-231 cell were significantly lower than the positive control Bestatin, which is consistent with the results of enzyme inhibition. For each compound, the anti-proliferative effect against HL-60 cell is better than MDA-MB-231, which may due to the APN expression level on HL-60 cell is higher than that of MDA-MB-231. Compound **7c** showed the best anti- proliferative effect against HL-60 cell with the IC_{50} value of 1.97 ± 0.11 mM.

Compound **7c** showed the most activity towards APN. In order to obtain further insight into the interaction of **7c** with APN, the docking studies were carried out via Sybyl/Sketch module and optimized using Powell's method with the Tripos force field with convergence criterion set at 0.05 kcal/(Å mol), and assigned with Gasteiger–Hückel method. The docking study performed using Sybyl/FlexX module, the residues in a radius of 7.0 Å around Bestatin in the co-crystal structure (PDB code: 2DQM) were selected as the active-site. Other docking parameters implied in the program were kept default. The docking result was shown in Figure 4. As diagramed in Figure 4, the carbonyl group of L-Phe can interact with the zinc ion. The phenyl group of L-Phe moiety can insert into the S1 pocket as the same with Bestatin; the methyl group in the 4th position of 2,4-dimethylaniline can plunge into the S1′ pocket of APN and the other methyl group can interact with the S2′ pocket of APN.

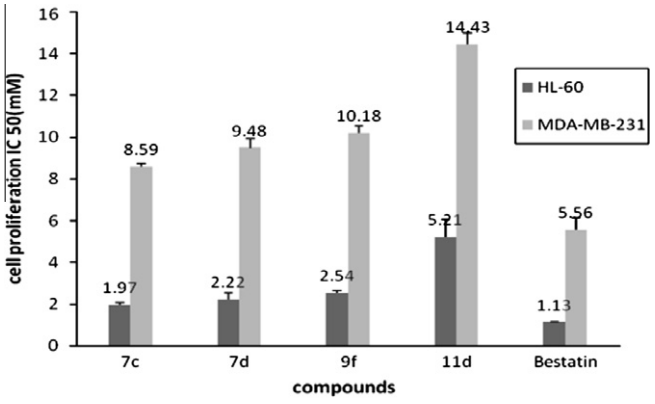


Figure 3. Anti-proliferative activities of compounds **7c**, **7d**, **9f**, **11d**, and Bestatin against two tumor cell lines (HL-60 cell and MDA-MB-231 cell). The columns represent the mean values of three independent experiments.

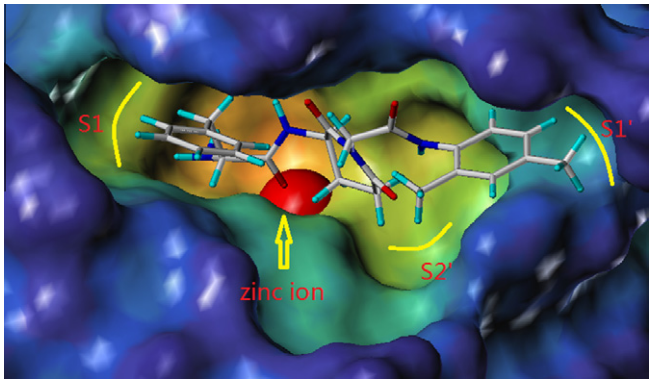


Figure 4. The FlexX docking of compound **7c** with the active-site in *Escherichia coli* APN (PDB: 2DQM). The protein is represented by molecular surface. Zinc ion is indicated (atom types: polar H, sky-blue; N, dark-blue; O, red.).

Although the computed information partially supported our assumption, the exact binding model of **7c** with APN should be obtained from further X-ray crystal studies, which is under research in our lab (Fig. 4).

4. Conclusion

In summary, we have described the synthesis and properties of a series of novel 3-phenylalanyl-N′-substituted-2,6-piperidinedione derivates as APN inhibitors. Most of the target compounds showed potent inhibitory activities towards APN, but comparing with the lead compound **13f**, the APN inhibitory activities were not improved apparently. The most effective compound **7c**, exhibited excellent enzymatic inhibition activity which could be used as a lead compound for further development of small molecular peptidomimetic APN inhibitors.

5. Experiment

5.1. Chemistry: general procedures

All the material we used were commercial available. All the solvents were distilled before use. All reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visualized with UV light, or chloride ferric. 200–300 mesh silica gel was used in column chromatography to depurate the end products. Proton NMR spectra were determined on a Brucker DRX spectrometer (600 MHz), δ in parts per million and J in Hertz, and TMS was used as an internal standard. Measurements were made in DMSO-d_6 solutions. ESI-MS were determined on an API 4000 spectrometer. HRMS spectrums were conducted by Shandong Analysis and Test Center. Melting points were determined on an electrothermal melting point apparatus and were uncorrected.

The purity of the final products was analyzed using a reverse-phase Zorbax Eclipse XDB C18 (5 μm, 100 Å) column (4.6 × 150 mm) with CH_3OH (solvent A)/H_2O (solvent B) as mobile phase, using isocratic conditions at a flow rate of 1.0 ml/min on an Agilent 1200 apparatus (detector DAD G1312, pumps G1312). The eluted peaks were monitored at 254 nm.

5.1.1. 2-((*S*)-3-((*S*)-2-(*tert*-Butoxycarbonylamino)-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl) acetic acid (5)

The title compound was prepared as described by Li et al.[18]

5.1.2. (*S*)-2-Amino-*N*-((*S*)-1-(2-(4-fluorophenylamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide (7a)

To a solution of compound (**5**) (0.87 g, 2 mmol) and *N*-methylmorpholine (0.26 ml, 2.2 mmol) in 30 ml anhydrous THF was added isobutyl chloroformate (0.30 ml, 2.2 mmol) at −15 °C. The mixture was stirred for 30 min at the same temperature. A solution of 4-fluorophenylamine (0.23 g, 2 mmol) in THF (5 ml) was added dropwise to the reaction mixture. Keep the reaction for 1 h at −15 °C and then remove the cooling bath. The reaction was continued for 4 h at room temperature. Filtrated and concentrated with a rotary evaporator. The residue was dissolved in 50 ml EtOAc and washed with saturated $NaHCO_3$ (10 ml × 3), saturated citric acid (10 ml × 3) and brine (10 ml × 2) in turn. The organic phase was dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator to afford crude product. The crude product was recrystallized by EtOAc to give compound (**6a**) (0.56 g, yield 53.2%), mp = 165–167 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.23–1.28 (s, 9H), 2.00–2.02 (m, 2H), 2.75–2.79 (m, 2H), 2.93–2.99 (m, 1H), 3.04–3.07 (m, 1H), 4.20–4.23 (m, 1H), 4.40–4.48 (m, 2H), 4.78–4.82 (m, 1H), 6.91 (m, J = 7.8 Hz, 1H), 7.14 (t, J = 9.0 Hz, 2H), 7.17–7.19 (m, 1H), 7.25–7.30 (m, 4H), 7.55–7.57 (m, 2H), 8.46 (s, 1H), 10.22 (s, 1H); ESI-MS: m/z: 527.4 $[M+H]^+$.

Compound (**6a**) (0.3 g, 0.56 mmol) was dissolved in 10 ml HCl–EtOAc (3 mol/L). After 3 h, the solvent was filtrated and the precipitate was washed with EtOAc to get compound (**7a**) as a white solid (0.21 g, yield 80.8%), mp = 195–198 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.02–2.06 (m, 2H), 2.77–2.81 (m, 1H), 2.95–3.05 (m, 2H), 3.22 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.10 (s, 1H), 4.43–4.51 (m, 2H), 4.82–4.87 (m, 1H), 7.14 (t, J = 8.4 Hz, 2H), 7.25–7.28 (m, 1H), 7.32 (t, J = 7.2 Hz, 1H), 7.37 (d, J = 7.2 Hz, 1H), 7.58–7.61 (m, 2H), 8.30 (s, 2H), 9.25 (s, 1H), 10.47 (s, 1H); HPLC t_R = 17.5 min (89.40 area%); HRMS(AP-ESI) m/z: calcd for $C_{22}H_{24}FN_4O_4$ $[M+H]^+$ 427.2001, found 427.2009.

The other compounds (**7b**–**7i**) were synthesized in the same procedure as described above.

5.1.3. (2*S*)-2-Amino-*N*-(1-(2-(4-methoxyphenylamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide (7b)

White solid (0.21 g, yield 81%), mp = 179–182 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.02–2.06 (m, 2H), 2.77–2.81 (m, 1H), 2.95–3.04 (m, 2H), 3.22 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 3.71 (s, 3H), 4.10 (s, 1H), 4.40–4.49 (m, 2H), 4.83–4.87 (m, 1H), 6.87 (d, J = 7.2 Hz, 2H), 7.27 (t, J = 7.2 Hz, 1H), 7.33 (t, J = 7.2 Hz, 2H), 7.37 (d, J = 7.2 Hz, 2H), 7.47 (d, J = 7.2 Hz, 2H), 8.27 (s, 2H), 9.22 (s, 1H), 10.17 (s, 1H); HPLC t_R = 17.04 min (95.16 area%); HRMS(AP-ESI) m/z: calcd for $C_{23}H_{27}N_4O_5$ $[M+H]^+$ 440.2119, found 440.2128.

5.1.4. (2*S*)-2-Amino-*N*-(1-(2-(2,4-dimethylphenylamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide (7c)

White solid (0.22 g, yield 81.5%), mp = 175–177 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.01–2.06 (m, 2H), 2.14 (s, 3H), 2.24 (s, 3H), 2.76–2.80 (m, 1H), 2.94–3.05 (m, 2H), 3.23 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.10 (s, 1H), 4.44–4.51 (m, 2H), 4.84–4.89 (m, 1H), 6.95 (d, J = 7.8 Hz, 1H), 7.02 (s, 1H), 7.18 (d, J = 7.8 Hz, 1H), 7.28 (t, J = 7.2 Hz, 1H), 7.33 (t, J = 7.2 Hz, 2H), 7.38 (m, 2H), 8.26 (s, 2H), 9.19 (s, 1H), 9.48 (s, 1H); HPLC t_R = 18.74 min (95.06 area%); HRMS(AP-ESI) m/z: calcd for $C_{24}H_{29}N_4O_4$ $[M+H]^+$ 437.2565, found 437.2575.

5.1.5. (2*S*)-2-Amino-*N*-(1-(2-(3-chlorophenylamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide (7d)

White solid (0.21 g, yield 78.3%), mp = 186–188 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.02–2.06 (m, 2H), 2.77–2.81 (m, 1H), 2.96–3.02 (m, 2H), 3.20 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.07 (s, 1H), 4.44–4.53 (m, 2H), 4.83–4.87 (m, 1H), 7.12 (d, J = 7.8 Hz, 1H), 7.27 (t, J = 7.2 Hz, 1H), 7.31–7.36 (m, 2H), 7.40 (d, J = 7.8 Hz, 1H), 7.78 (s, 1H), 8.09 (br s, 2H), 9.16 (s, 1H), 10.61 (s, 1H); HPLC t_R = 17.59 min (90.73 area%); HRMS(AP-ESI) m/z: calcd for $C_{22}H_{24}ClN_4O_4$ $[M+H]^+$ 443.1486, found 443.1495.

5.1.6. (2*S*)-2-Amino-*N*-(1-(2-(2-fluorophenylamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide (7e)

White solid (0.23 g, yield 88.7%), mp = 172–175 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.02–2.06 (m, 2H), 2.77–2.81 (m, 1H), 2.95–3.05 (m, 2H), 3.23 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.11 (s, 1H), 4.51–4.58 (m, 2H), 4.83–4.87 (m, 1H), 7.13–7.17 (m, 2H), 7.25–7.28 (m, 2H), 7.32 (t, J = 7.2 Hz, 1H), 7.38 (d, J = 7.2 Hz, 1H), 7.86 (s, 1H), 8.27 (s, 2H), 9.27 (s, 1H), 10.10 (s, 1H); HPLC t_R = 18.87 min (90.82 area%); HRMS(AP-ESI) m/z: calcd for $C_{22}H_{24}FN_4O_4$ $[M+H]^+$ 427.2001, found 427.2009.

5.1.7. (2*S*)-2-Amino-*N*-(1-(2-(4-bromophenylamino)-2-oxoethyl)-2,6-dioxopiperidin-3- yl)-3-phenylpropanamide (7f)

White solid (0.22 g, yield 74.9%), mp = 195–197 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.02–2.06 (m, 2H), 2.77–2.81 (m, 1H), 2.96–3.04 (m, 2H), 3.22 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.09 (s, 1H), 4.43–4.53 (m, 2H), 4.83–4.88 (m, 1H), 7.27 (t, J = 7.2 Hz, 1H), 7.32 (t, J = 7.2 Hz, 2H), 7.36 (d, J = 7.2 Hz, 2H), 7.49 (d, J = 7.2 Hz, 2H), 7.55 (d, J = 7.2 Hz, 2H), 8.27 (s, 2H), 9.22 (s, 1H), 10.55 (s, 1H); HPLC t_R = 17.05 min (92.58 area%); HRMS(AP-ESI) m/z: calcd for $C_{22}H_{24}BrN_4O_4$ $[M+H]^+$ 488.3544, found 488.3535.

5.1.8. (2*S*)-2-Amino-*N*-(1-(2-(3-chloro-4-fluorophenylamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide (7g)

White solid (0.21 g, yield 75.5%), mp = 192–195 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.02–2.06 (m, 2H), 2.77–2.81 (m, 1H), 2.96–3.04 (m, 2H), 3.22 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.11 (s, 1H), 4.44–4.53 (m, 2H), 4.83–4.87 (m, 1H), 7.27–7.29 (m, 1H), 7.30–7.32 (m, 2H), 7.33–7.39 (m, 3H), 7.46–7.49 (m, 1H), 7.90–7.92 (m, 1H), 8.31 (s, 2H), 9.22 (s, 1H), 10.71 (s, 1H); HPLC t_R = 16.20 min (92.60 area%); HRMS(AP-ESI) m/z: calcd for $C_{22}H_{23}ClFN_4O_4$ $[M+H]^+$ 461.8938, found 461.8920.

5.1.9. (2*S*)-2-Amino-*N*-(1-(2-(3,4-difluorophenylamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide (7h)

White solid (0.23 g, yield 85.4%), mp = 183–185 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.03–2.06 (m, 2H), 2.77–2.81 (m, 1H), 2.96–3.04 (m, 2H), 3.22 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.11 (s, 1H), 4.44–4.53 (m, 2H), 4.83–4.88 (m, 1H), 7.26–7.41 (m, 8H), 8.26 (s, 2H), 9.22 (s, 1H), 10.71 (s, 1H); HPLC t_R = 16.81 min (93.83 area%); HRMS(AP-ESI) m/z: calcd for $C_{22}H_{23}F_2N_4O_4$ $[M+H]^+$ 445.4392, found 45.4382.

5.1.10. (*S*)-Benzyl-2-(2-(3-((*S*)-2-amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl))acetate (9a)

To a solution of compound (**5**) (0.87 g, 2 mmol) and *N*-methylmorpholine (0.26 ml, 2.2 mmol) in 30 ml anhydrous THF was added isobutyl chloroformate (0.30 ml, 2.2 mmol) at -15 °C. The mixture was stirred for 30 min at the same temperature. A solution of glycine benzyl ester *p*-toluenesulfonate salt (0.67 g, 2 mmol) in DCM (5 ml) (which was previous neutralized with *N*-methylmorpholine) was added dropwise to the reaction mixture. Keep the reaction for 1 h at −15 °C and then remove the cooling bath. The reaction was continued for another 4 h at room temperature. Filtrated and concentrated with a rotary evaporator. The residue was dissolved in 50 ml EtOAc and washed with saturated $NaHCO_3$ (10 ml × 3), saturated citric acid (10 ml × 3) and brine (10 ml × 2) in turn. The organic phase was dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator to afford crude product. The crude product was recrystallized by EtOAc/diethyl ether to give compound (**8a**) (0.87 g, yield 75.4%), mp = 154–156 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.25 (s, 9H), 1.99 (s, 1H), 2.70–2.81 (m, 2H), 2.86–2.92 (m, 1H), 2.96–3.08 (m, 1H), 3.91 (d, J = 5.7 Hz, 2H), 4.21–4.24 (m, 1H), 4.30 (d, J = 6.6 Hz, 2H), 4.74–4.83 (m, 1H), 5.12 (s, 2H), 6.93 (d, J = 8.7 Hz, 1H), 7.19–7.21 (m, 1H), 7.24–7.29 (m, 4H), 7.31–7.37 (m, 5H), 8.43 (d, J = 8.7 Hz, 1H), 8.52 (t, J = 5.7 Hz, 1H); ESI-MS: m/z: 581.6 $[M+H]^+$.

Compound (**8a**) (0.1 g, 0.17 mmol) was dissolved in 10 ml HCl–EtOAc (3 mol/L). After 3 h, the solvent was filtrated and the precipitate was washed with EtOAc to get compound (**9a**) as a white solid (0.08 g, yield 89.1%), mp = 158–161 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.01 (s, 2H), 2.74–2.77 (d, 1H), 2.91–2.96 (m, 1H), 3.01–3.05 (m, 1H), 3.23 (dd, J = 4.8 Hz, 14.4 Hz, 1H), 3.92 (d, J = 5.4 Hz, 2H), 4.10 (s, 1H), 4.27–4.37 (m, 2H), 4.81–4.85 (m, 1H), 5.12 (s, 2H), 7.27 (t, J = 7.2 Hz, 1H), 7.33 (t, J = 7.2 Hz, 3H), 7.37 (m, 6H), 8.31 (s, 2H), 8.61 (s, 1H), 9.26 (s, 1H); HPLC t_R = 16.32 min (90.67 area%); HRMS(AP-ESI) m/z: calcd for $C_{25}H_{29}N_4O_6$ $[M+H]^+$ 481.5210, found 481.5201.

The other compounds (**9b**–**9g**) were synthesized in the same procedure as described above.

5.1.11. (*S*)-Benzyl-2-(2-(3-((*S*)-2-amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl) acetamido)propanoate (9b)

White solid (0.08 g, yield 88.9%), mp = 128–130 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.27 (s, 3H), 2.01 (s, 2H), 2.75–2.78

(m, 1H), 2.91–2.97 (m, 1H), 3.01–3.05 (m, 1H), 3.23 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.10 (t, J = 6.6 Hz, 1H), 4.21–4.33 (m, 2H), 4.37 (d, J = 14.4 Hz, 1H), 5.08–5.14 (m, 2H), 7.26–7.39 (m, 10H), 8.30 (s, 2H), 8.62 (s, 1H), 9.24 (s, 1H); HPLC t_R = 15.22 min (92.37 area%); HRMS(AP-ESI) m/z: calcd for $C_{26}H_{31}N_4O_6$ $[M+H]^+$ 495.5475, found 495.5465.

5.1.12. (*S*)-Benzyl-2-(2-(3-((*S*)-2-amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl) acetamido)-3-methylbutanoate (9c)

White solid (0.08 g, yield 85.1%), mp = 134–137 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.86 (s, 6H), 1.98–2.07 (m, 3H), 2.75 (d, J = 17.4 Hz, 1H), 2.91–2.97 (m, 1H), 3.01–3.05 (m, 1H), 3.22–3.24 (m, 1H), 4.09 (s, 1H), 4.22 (t, J = 7.2 Hz, 1H), 4.31–4.42 (m, 2H), 4.79–4.84 (m, 1H), 5.10–5.17 (m, 2H), 7.27 (t, J = 7.2 Hz, 1H), 7.32 (t, J = 6.6 Hz, 3H), 7.37 (s, 6H), 8.30 (s, 2H), 8.50 (s, 1H), 9.22 (s, 1H); HPLC t_R = 17.28 min (90.34 area%); HRMS(AP-ESI) m/z: calcd for $C_{28}H_{35}N_4O_6$ $[M+H]^+$ 523.6007, found 523.6002.

5.1.13. (*S*)-Benzyl-2-(2-((*S*)-3-((*S*)-2-amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)-4-methylpentanoate (9d)

White solid (0.08 g, yield 82.5%), mp = 120–121 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.81–0.82 (m, 3H), 0.87–0.88 (m, 3H), 1.48–1.64 (m, 3H), 1.97–2.02 (m, 2H), 2.76 (d, J = 17.4 Hz, 1H), 2.91–2.97 (m, 1H), 3.24 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.10 (t, J = 6.0 Hz, 1H), 4.25–4.37 (m, 3H), 4.80–4.84 (m, 1H), 4.31–4.42 (m, 2H), 4.79–4.84 (m, 1H), 5.09–5.14 (m, 2H), 7.27 (t, J = 7.2 Hz, 1H), 7.31–7.36 (m, 5H), 7.36–7.38 (m, 4H), 8.32 (s, 2H), 8.58 (s, 1H), 9.24 (s, 1H); HPLC t_R = 17.86 min (92.32 area%); HRMS(AP-ESI) m/z: calcd for $C_{29}H_{37}N_4O_6$ $[M+H]^+$ 537.6273, found 537.6264.

5.1.14. Benzyl-3-(2-((*S*)-3-((*S*)-2-amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)propanoate (9e)

White solid (0.083 g, yield 92.2%), mp = 127–128 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.01 (s, 2H), 2.53 (d, J = 6.6 Hz, 2H), 2.74–2.77 (m, 1H), 2.90–2.96 (m, 1H), 3.02–3.05 (m, 1H), 3.23 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 3.30 (dd, J = 6.6 Hz, 12.6 Hz, 2H), 4.10 (m, 1H), 4.11–4.27 (m, 2H), 4.80–4.85 (m, 1H), 5.10 (s, 2H), 7.26 (t, J = 7.2 Hz, 1H), 7.32 (t, J = 7.2 Hz, 3H), 7.37–7.38 (m, 6H), 8.25 (s, 1H), 8.31 (s, 2H), 9.27 (s, 1H); HPLC t_R = 16.61 min (89.23 area%); HRMS(AP-ESI) m/z: calcd for $C_{26}H_{31}N_4O_6$ $[M+H]^+$ 495.5475, found 495.5460.

5.1.15. (*S*)-Benzyl-2-(2-((*S*)-3-((*S*)-2-amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)-3-phenylpropanoate (9f)

White solid (0.082 g, yield 82%), mp = 143–145 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.01 (s, 2H), 2.53 (d, J = 6.6 Hz, 2H), 2.74–2.77 (m, 1H), 2.90–2.96 (m, 1H), 3.02–3.05 (m, 1H), 3.23 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 3.30 (dd, J = 6.6 Hz, 12.6 Hz, 2H), 4.10 (m, 1H), 4.11–4.27 (m, 2H), 4.80–4.85 (m, 1H), 5.10 (s, 2H), 7.26 (t, J = 7.2 Hz, 1H), 7.32 (t, J = 7.2 Hz, 3H), 7.37–7.38 (m, 6H), 8.25 (s, 1H), 8.31 (s, 2H), 9.27 (s, 1H); HPLC t_R = 16.81 min (93.45 area%); HRMS(AP-ESI) m/z: calcd for $C_{32}H_{35}N_4O_6$ $[M+H]^+$ 571.6435, found 571.6425.

5.1.16. (*S*)-Dibenzyl-2-(2-((*S*)-3-((*S*)-2-amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)pentanedioate (9g)

White solid (0.09 g, yield 78%), mp = 118–122 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.88–1.93 (m, 1H), 2.01–2.05 (m, 3H), 2.42–2.47 (m, 2H), 2.76 (d, J = 18 Hz, 1H), 2.90–2.95 (m, 1H), 3.02–3.05 (m, 1H), 3.23 (dd, J = 4.2 Hz, 13.8 Hz, 1H), 4.09–4.11 (m, 1H), 4.27–4.30 (m, 1H), 4.32–4.38 (m, 2H), 4.80–4.84 (m, 1H), 5.06–5.09 (m, 2H), 5.11–5.14 (m, 2H), 7.25–7.38 (m, 15H), 8.32 (s, 3H), 8.65 (s, 1H), 9.26 (s, 1H); HPLC t_R = 17.23 min (92.78 area%); HRMS(AP-ESI) m/z: calcd for $C_{35}H_{39}N_4O_8$ $[M+H]^+$ 643.7062, found 643.7055.

5.1.17. 2-(2-(3-((*S*)-2-Amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)acetic acid (11a)

Compound (**8a**) (0.4 g, 8.02 mmol) and catalytic amount of 10% Pd/C in methanol (30 ml) was hydrogenated in the presence of H_2 at room temperature. After 16 h, the catalyst was filtered and the solvent was removed under vacuum to give compound (**10a**) in white solid (3.38 g, yield 97.2%). Compound (**10a**) (0.1 g, 0.2 mmol) was then dissolved in 10 ml HCl–EtOAc (3 mol/L). After 3 h, the solvent was filtrated and the prepicitate was washed with EtOAc to get compound (**11a**) as white solid (0.07 g, yield 85%), mp = 178–180 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.01 (s, 2H), 2.73–2.76 (m, 1H), 2.91–2.95 (m, 1H), 3.05 (m, 1H), 3.24 (d, J = 12 Hz, 1H), 3.76 (s, 2H), 4.02–4.10 (m, 1H), 4.25–4.35 (m, 2H), 4.82 (m, 1H), 5.12 (s, 1H), 7.27 (t, J = 7.2 Hz, 1H), 7.32 (t, J = 7.2 Hz, 2H), 7.38 (d, J = 7.2 Hz, 2H), 8.44 (s, 2H), 8.57 (s, 1H), 9.30 (s, 1H); HPLC t_R = 7.51 min (90.86 area%); HRMS(AP-ESI) m/z: calcd for $C_{18}H_{23}N_4O_6$ $[M+H]^+$ 391.3984, found 391.3975.

The other compounds (**11b**–**11g**) were synthesized in the same procedure as described above.

5.1.18. (*S*)-2-(2-(3-((*S*)-2-Amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)propanoic acid (11b)

White solid (0.067 g, yield 86.5%), mp = 195–198 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.27 (s, 3H), 1.96–2.01 (m, 2H), 2.73–2.76 (m, 1H), 2.92–2.97 (m, 1H), 3.00–3.04 (m, 1H), 3.22 (dd, J = 5.4 Hz, 14.4 Hz, 1H), 4.09 (s, 1H), 4.17–4.25 (m, 2H), 4.35 (d, J = 16.2 Hz, 1H), 4.79–4.83 (m, 1H), 7.28 (t, J = 7.2 Hz, 1H), 7.33 (t, J = 7.2 Hz, 2H), 7.37 (t, J = 7.2 Hz, 2H), 8.27 (s, 2H), 8.43 (s, 1H), 9.18 (s, 1H), 12.60 (s, 1H); HPLC t_R = 6.67 min (92.33 area%); HRMS(AP-ESI) m/z: calcd for $C_{19}H_{25}N_4O_6$ $[M+H]^+$ 405.4250, found 405.4240.

5.1.19. (*S*)-2-(2-(3-((*S*)-2-Amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)-3-methylbutanoic acid (11c)

White solid (0.079 g, yield 85%), mp = 178–180 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.87 (s, 6H), 1.96–2.06 (m, 3H), 2.74 (d, J = 18 Hz, 1H), 2.90–2.97 (m, 1H), 3.01–3.05 (m, 1H), 3.21–3.24 (m, 1H), 4.09 (s, 1H), 4.14–4.16 (m, 1H), 4.30–4.40 (m, 2H), 4.78–4.82 (m, 1H), 7.27 (t, J = 7.2 Hz, 1H), 7.32 (t, J = 7.2 Hz, 2H), 7.37 (d, J = 7.2 Hz, 2H), 8.31 (s, 2H), 9.22 (s, 1H), 12.68 (s, 1H); HPLC t_R = 9.45 min (94.85 area%); HRMS(AP-ESI) m/z: calcd for $C_{21}H_{29}N_4O_6$ $[M+H]^+$ 433.4782, found 433.4775.

5.1.20. (*S*)-2-(2-((*S*)-3-((*S*)-2-Amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acet amido)-4-methylpentanoic acid (11d)

White solid (0.083 g, yield 87%), mp = 170–172 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.82–0.83 (m, 3H), 0.88–0.89 (m, 3H), 1.48–1.57 (m, 2H), 1.62–1.64 (m, 1H), 2.00 (s, 2H), 2.75 (d, J = 17.4 Hz, 1H), 2.90–2.96 (m, 1H), 3.02–3.05 (m, 1H), 3.23 (dd, J = 4.8 Hz, 14.4 Hz, 1H), 4.06–4.10 (m, 2H), 4.20–4.27 (m, 2H), 4.34 (d, J = 15.6 Hz, 1H), 4.78–4.82 (m, 1H), 4.31–4.42 (m, 2H), 4.79–4.84 (m, 1H), 7.26–7.39 (m, 5H), 8.38 (s, 2H), 8.50 (s, 1H), 9.24 (s, 1H); HPLC t_R = 11.21 min (95.80 area%); HRMS(AP-ESI) m/z: calcd for $C_{22}H_{31}N_4O_6$ $[M+H]^+$ 447.5047, found 447.5035.

5.1.21. 3-(2-((*S*)-3-((*S*)-2-Amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)propanoic acid (11e)

White solid (0.076 g, yield 86%), mp = 174–176 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.08–1.11 (m, 1H), 1.16–1.19 (m, 1H), 1.99–2.03 (m, 2H), 2.73–2.76 (m, 1H), 2.88–2.95 (m, 1H), 3.01–3.06 (m, 1H), 3.26–3.28 (m, 2H), 3.39–3.40 (m, 1H), 4.09–4.11 (m, 1H), 4.18–4.26 (m, 2H), 4.77–4.83 (m, 1H), 4.78–4.82 (m, 1H), 7.25–7.29 (m, 1H), 7.33 (t, J = 7.2 Hz, 1H), 7.38 (d, J = 7.2 Hz,

2H), 8.15–8.21 (m, 2H), 8.47 (s, 1H), 9.27 (s, 1H); HPLC t_R = 8.46 min (89.85 area%); HRMS(AP-ESI) *m*/*z*: calcd for $C_{19}H_{25}N_4O_6$ $[M+H]^+$ 405.4250, found 405.4242.

5.1.22. (*S*)-2-(2-((*S*)-3-((*S*)-2-Amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)-3-phenylpropanoic acid (11f)

White solid (0.09 g, yield 88%), mp = 119–120 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.96–2.02 (m, 2H), 2.72–2.76 (m, 1H), 2.90–3.05 (m, 4H), 3.23 (dd, *J* = 5.4 Hz, 14.4 Hz, 1H), 4.06–4.11 (m, 1H), 4.22–4.33 (m, 2H), 4.38–4.42 (m, 1H), 4.77–4.82 (m, 1H), 7.18–7.24 (m, 3H), 7.25–7.29 (m, 3H), 7.31–7.33 (m, 2H), 7.38 (d, *J* = 7.2 Hz, 2H), 8.29–8.36 (m, 2H), 8.63 (s, 1H), 9.23 (s, 1H); HPLC t_R = 16.99 min (92.32 area%); HRMS(AP-ESI) *m*/*z*: calcd for $C_{25}H_{29}N_4O_6$ $[M+H]^+$ 481.5210, found 481.5201.

5.1.23. (*S*)-2-(2-((*S*)-3-((*S*)-2-Amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetamido)pentanedioic acid (11g)

White solid (0.086 g, yield 86%), mp = 187–189 °C; 1H NMR (600 MHz, DMSO-d_6) δ 1.74–1.80 (m, 1H), 1.91–2.01 (m, 3H), 2.23–2.30 (m, 2H), 2.75 (d, *J* = 18 Hz, 1H), 2.90–2.97 (m, 1H), 3.00–3.04 (m, 1H), 3.21 (dd, *J* = 4.8 Hz, 14.4 Hz, 1H), 4.08 (s, 1H), 4.19–4.24 (m, 1H), 4.25–4.36 (m, 2H), 4.79–4.83 (m, 1H), 7.28 (t, *J* = 7.2 Hz, 1H), 7.32–7.37 (m, 4H), 8.16 (s, 3H), 8.46 (s, 1H), 9.17 (s, 1H), 12.45 (br s, 2H); HPLC t_R = 9.78 min (92.34 area%); HRMS(AP-ESI) *m*/*z*: calcd for $C_{21}H_{27}N_4O_8$ $[M+H]^+$ 463.4611, found 463.4609.

5.1.24. (2*S*)-2-Amino-*N*-(1-(2-(2-(hydroxyamino)-2-oxoethylamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide (13g)

The title compound was synthesized in the same procedure as described in the preparation of compound (**7a**). White solid (0.075 g, yield 85%), mp = 181–183 °C; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.00 (s, 2H), 2.71–2.81 (m, 1H), 2.91–2.94 (m, 1H), 3.03 (m, 1H), 3.22–3.25 (m, 1H), 4.11 (s, 1H), 4.29–4.37 (m, 2H), 4.81 (m, 1H), 7.20–7.39 (m, 5H), 8.33 (s, 2H), 9.24–9.29 (m, 1H), 10.72 (s, 1H); HPLC t_R = 8.79 min (94.34 area%); HRMS(AP-ESI) *m*/*z*: calcd for $C_{18}H_{24}N_5O_6$ $[M+H]^+$ 406.4131, found 406.4125.

5.2. APN inhibition assay

IC_{50} values against APN were determined as previously described and by using L-Leu-*p*-nitroanilide as a substrate and microsomal aminopeptidase from Porcine Kidney microsomes (Sigma) as the enzyme in 50 μM 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 the UV–vis spectrophotometer Pharmacia LKB, Biochrom 4060. All the solutions of the inhibitors were prepared in the assay buffer, and the pH was adjusted to 7.5 by the addition of 0.1 M HCl or 0.1 M NaOH. All the inhibitors were preincubated with APN for 30 min at room temperature. The assay mixture, which contained the inhibitor solution (concentration dependent on the inhibitor), the enzyme solution (4 mg/ml final concentration), and the assay buffer, was adjusted to 200 μL.

5.3. MMP inhibition assay

Gelatinase A (MMP-2) and TNBS were purchased from Sigma, and the substance was synthesized as described by Vijaykumar et al. The gelatinase, substance, and inhibitor were dissolved in sodium borate (pH 8.5, 50 mmol/L) and incubated for 30 min at 37 °C, and then 0.03% TNBS was added and incubated for another 20 min, the resulting solution was detected under 450 nm wavelength to gain absorption.

5.4. MTT assay

HL-60 cell (with high APN expression) and MDA-MB-231 Cell (with low APN expression) were grown in RPMI1640 medium with 10% fetal bovine serum at 37 °C in 5% CO_2 humidified incubator. Cell proliferation was determined by the MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium bromide) assay. Briefly, cells were plated in a 96-well plate at 10,000 cells per well, cultured for 4 h in complete growth medium, then treated with 2000, 1000, 500, 250, or 125 μg/ml of the compounds for 72 h. Following this, 0.5% MTT solution was added to each well. After further incubation for 4 h, the formazan formed from MTT was extracted by adding DMSO and mixing for 15 min. The optical density was read with ELISA reader at 570 nm.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.078. These data include MOL files and InChiKeys of the most important compounds described in this article.

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