European Journal of Medicinal Chemistry 45 (2010) 1618–1626

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Novel cyclic-imide peptidomimetics as aminopeptidase N inhibitors. Structure-based design, chemistry and activity evaluation. II

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ARTICLE INFO

Article history:
Received 25 June 2009
Accepted 29 December 2009
Available online 14 January 2010

Keywords:
Aminopeptidase N
Peptidomimetics
Anti-tumor
Cyclic-imide

ABSTRACT

A novel class of potent aminopeptidase N inhibitors with 3-amino-cyclic-imide scaffold is described. The preliminary biological test revealed that all the compounds displayed high specific inhibitory activity against aminopeptidase N compared with previous work because of the existence of free amino group. Compounds containing hydroxamate group are more potent than carboxyl and ester derivatives. Compound **13f** potentially inhibited APN activity with IC_{50} value of 1.8 μM and displayed specific activity profiles in cells assay and in vivo anti-metastasis assay.

1. Introduction

A structure-based approach to design potent and selective inhibitors is an important component of the modern drug development process [1,2]. Aminopeptidase N (EC3.4.11.2, APN, CD13), expressed as an ectopeptidase with one zinc ion in active site, is of significant biological and medical importance because of its key role in protein modification, activation, and degradation as well as in the metabolism of biologically active peptides in tumor metastasis and leukemia [3–6]. The design and synthesis of inhibitors against aminopeptidase N may result in potential therapeutic agents [7,8].

Since 2006, the availability of X-ray crystal structures for APN and complexes with various inhibitors have been investigated [9–11]. Both Yoshimoto's and Matthews's had reported the binding site and catalytic domain of APN based on the co-crystal complex of *Escherichia coli* APN and bestatin, the only APN inhibitors applied in clinic [10,11]. The binding site of the APN with bestatin has several characteristics: part I is a hydrophobic pocket interacting with phenyl group of bestatin; part II is one zinc binding group (ZBG) which can coordinate with zinc ion in enzyme active site; part III is another hydrophobic pocket in deeper cavity which can be divided into two subsites [8,12,13].

In our previous work, we have reported a series of piperidinedione-N-acetamide derivatives [14]. The biological characterization for this kind of piperidinedione analogues revealed that the most potent compound **1** displayed potent inhibitory effect and can be used as a leading compound for further structure optimization.

Herein, we report the design and synthesis of a novel cyclic-imide peptidomimetics. In order to find better APN inhibitors, we modified the structure as the following, Fig. 1: (i) R_1 group corresponding to the phenyl group of bestatin can be the side chains of different amino acids. (ii) Keeping the amino group freely so that it can interact with the anion binding site of enzyme that contain a Glu355 (Human) or Glu264 (*E. coli*), as this residue is of great importance during the first step for the recognition of substrates or inhibitors by enzyme [15]. (iii) R_2 group can be OH, OC_2H_5 or NHOH, which play a role in the strong interaction with the active site of enzyme. The in vitro inhibitory activity was measured against APN enzyme. Additionally, potent compound are also evaluated on HL-60 cell and K562 cell lines, and in vivo anti-metastasis assay as well.

2. Chemistry

Compounds **5a–l** were synthesized efficiently following the procedures as shown in Scheme 1. The starting material (*S*)-tert-butyl 2,6-dioxopiperidin-3-ylcarbamate (**2**) was prepared from L-Glutamine following the reference [16]. The amino group of cyclic-imide scaffold was alkylated by ethyl bromoacetate followed by deprotection of Boc group in the existence of TFA to the key free amine intermediate **4**. Compound **4** was combined with different

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doi:10.1016/j.ejmech.2009.12.071

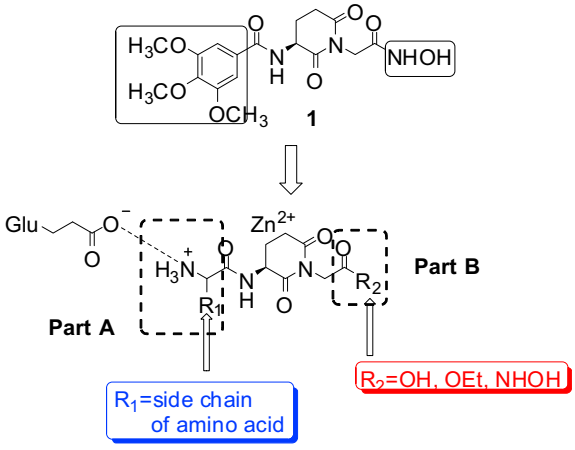


Fig. 1. The strategy applied to design cyclic-imide peptidomimetics inhibitors with free amino group.

Boc-amino acid derivatives to provide ethyl ester derivatives **5a–l** using DCC and HOBt as coupling reagents.

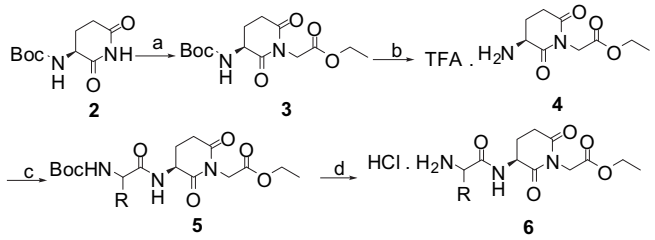
The critical intermediate **10a–i** were synthesized following the procedures as shown in Scheme 2. Starting from the same material **2** to Scheme 1, the NH was alkylated instead by benzyl bromoacetate to obtain benzyl ester intermediate **7**. The carboxyl group of different amino acid can be activated by isobutyl chloroformate and N-methylmorpholine and then coupled with compound **8** to yield **9a–i** whose benzyl ester can be easily deprotected by catalyzed transfer hydrogenation to form carboxyl derivatives **10a–z**. In addition, the carboxyl group in **10a–i** was transferred to its hydroxamate acid derivatives **12a–i** using the same method and NH_2OH as the base.

The Boc-protecting group can be easily removed by 3N HCl in ethyl acetate to give the target compounds **6a–l**, **11a–i** and **13a–i** as hydrochloride salts.

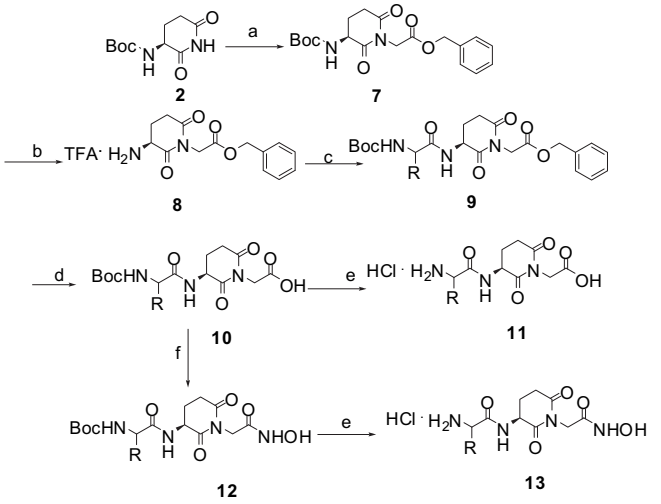
3. Result and discussion

The enzymatic inhibition assay of APN was monitored by the spectrophotometric method as described previously [17]. All the inhibition results are summarized in Table 1.

Bioassay results indicate that most compounds exhibited a better inhibitory activity against APN than previous work. This result possibly resulted from the introduction of free amine group for the requirement of active site of APN. Of the 30 compounds, most compounds showed strong APN inhibitory activity with IC_{50} values of 1.8–30 μM in Table 1. The activity of the most potent compound **13f** (IC_{50} = 1.8 μM) increased 46 times. Structurally, all compounds have similar chemical scaffolds with the C-3 free amino group, indicating that the free amine is the most optimized



Scheme 1. a. Ethyl bromoacetate, TBAI, acetone, 56 °C; b. TFA, DCM; c. Et_3N, THF, DCC, HOBt; d. 3N EtOAc–HCl.



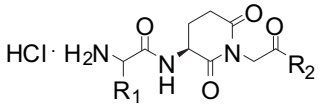
Scheme 2. a. benzyl bromoacetate, TBAI, acetone, 56 °C; b. TFA, DCM; c. N-methylmorphiline, THF, isobutyl Chloroformate, Boc-AA-OH; d. cyclohexene, C_2H_5OH, 5% Pd/C, 40 °C; e. 3N EtOAc–HCl; f. Et_3N, THF, isobutyl Chloroformate.

fragment for part A. According to reference, the free group is of great importance for the recognition of substrate by aminopeptidases [15]. Fragments for R_1 are structurally more diverse than fragments for part A. Fragments R_2 are the building blocks of part B for the strong inhibitors. Interestingly, the tendency of inhibitory profile of target compounds with different substituents in R_2 is CONHOH > COOH > COOEt.

In order to obtain further insight into the interaction of target compound with APN, the most active compound **13f** was built and docked into the active site of APN (PDB code: 2DQM) using Gold4.0, Fig. 2. The binding studies showed that two carbonyl groups (C=O from C-2 on the cyclic-imide ring and L-Phe) bind to the zinc ion and form a five-membered ring in the active pocket with distance of 2.34 Å and 2.27 Å, respectively. In addition, the C-2 carbonyl group, C-6 carbonyl group and NH from L-Phe also interact with the hydroxyl group of Tyr381, Tyr275 and Glu298 by hydrogen bond, respectively, which would be benefit to stabilize of the reaction intermediate with the zinc ion. The free amino group in part A can bind to anion binding site Glu121 (2.59 Å) and Glu264 (2.08 Å) which can anchor the inhibitor to the active site of the enzyme. The aromatic ring of L-Phe is more favorable as it can form a π–π interaction with the Tyr376 located in the active site, which is the same to that of bestatin, Fig. 2. The similar phenomenon can also be seen from aromatic ring-containing compounds (**6g**, **6h** and **6j**, **11f**, **11j**, **13j**). The hydroxamate acid group in part B can form hydrogen bond interaction with His297 (2.34 Å), that are the essential amino acids of the conserved sequence (HEXXHX18E) in the catalytic domain that is well conserved in peptidase M1 family, and Asp327 and Arg293 as well.

The effects of the active compounds on HL-60 cell (with high APN expression) and K562 cell (with low APN expression) proliferation and survival were tested using MTT assay method. A screen of six test compounds showed that compound **13f** and **13i** exhibited the inhibitory effect of HL-60 cell proliferation with IC_{50} values of 105 ± 12 μM and 147 ± 10 μM, respectively, which showed better potency than that of **1** (IC_{50} = 136 ± 14 μM) and Bestatin (IC_{50} = 245 ± 8 μM), Fig. 3a. Furthermore, **13f** showed less cytotoxicity toward K562 cell than bestatin even the dose is much higher than 400 μg/mL, Fig. 3b. Another interesting result showed that within lower concentration range (0.5 μM and 5 μM) **13f** is more active than that of bestatin on HL-60 cell proliferation. The

Table 1
In vitro enzyme assay results for compounds **6, 11, 13** and Bestatin against APN



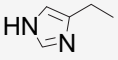
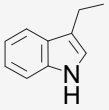
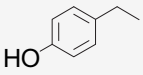
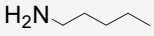
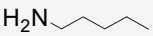
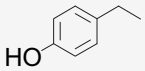
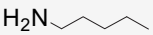
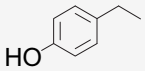
Compds	Substituents		$IC_{50}/\mu M$[a]
	R_1	R_2	APN
6a	H	OC_2H_5	23.7 ± 1.2
6b	CH_3	OC_2H_5	64.3 ± 1.3
6c		OC_2H_5	28.8 ± 1.6
6d		OC_2H_5	30.8 ± 1.3
6e		OC_2H_5	23.3 ± 1.6
6f		OC_2H_5	38.1 ± 2.8
6g		OC_2H_5	18.9 ± 4.4
6h		OC_2H_5	42.0 ± 7.6
6i		OC_2H_5	20.7 ± 2.7
6j		OC_2H_5	16.9 ± 1.5
6k		OC_2H_5	41.0 ± 0.4
6l		OC_2H_5	32.1 ± 1.4
11a	H	OH	15.4 ± 1.4
11b	CH_3	OH	21.5 ± 1.6
11c		OH	15.8 ± 1.4
11d		OH	22.1 ± 1.5
11e		OH	16.6 ± 2.3

Table 1 (*continued*)

Compds	Substituents		$IC_{50}/\mu M$[a]
	R_1	R_2	APN
11f		OH	16.9 ± 2.7
11g		OH	14.1 ± 1.7
11h		OH	13.2 ± 1.9
11i		OH	11.3 ± 1.4
13a	H	NHOH	9.4 ± 1.6
13b	CH_3	NHOH	10.9 ± 1.4
13c		NHOH	12.5 ± 0.1
13d		NHOH	5.3 ± 1.2
13e		NHOH	9.2 ± 0.7
13f		NHOH	1.8 ± 0.2
13g		NHOH	9.4 ± 2.1
13h		NHOH	9.7 ± 0.3
13i		NHOH	7.3 ± 1.4
	1		3.1 ± 0.7
	Bestatin		2.4 ± 0.5

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

results will be published elsewhere. These results suggested that compound **13f** exhibit the most specific inhibitory activity toward APN with the IC_{50} value in sub-micromolar range.

To further examine effects of **13f**, we measured its ability to inhibit H22 cell's location to lung tissue in vivo. As shown in Fig. 4, the inhibitory rate for compound **13f** and **13i** is higher than that of Bestatin (45.37%) with the value of 72.91% and 62.71%, respectively. This activity profile for **13f** can also be demonstrated by another experiment carried on ES-2 tumor cell bearing nude mice, in which **13f** displays equal potency at the dosage of 10 mg/kg to bestatin at 50 mg/kg, with the inhibitory rate value against tumor cell growth of 30.9% and 36.2%, respectively, the results in detail will be published elsewhere. All of these results indicate that compound **13f** is

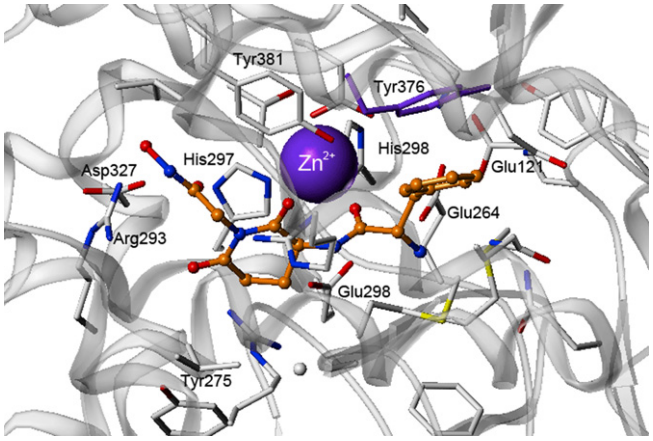


Fig. 2. Docking mode for **13f** with APN (PDB code: 2DQM).

a potent molecule which can be used for further pre-clinical studies and is promising for the application of invasion-preventing therapy.

4. Conclusion

In conclusion, our studies have shown that cyclic-imide peptidomimetics is a novel scaffold for the design of aminopeptidase N inhibitors. Accurate structure knowledge of the inhibitors bound to the target made it clear that at least four regions of the active site of APN are needed to be occupied to establish a consistent binding orientation: one anion binding site and two hydrophobic pocket and a zinc binding site.

In this effort, a specific activity profile against APN has been discovered when 3-amino-cyclic-imide scaffold was introduced in the structure compared with the previous work. Most of the compounds possess potent activity toward APN and the most potent compound, **13f**, exhibited good in vitro and in vivo activity. This feature may also guide the design of new, specific inhibitors of aminopeptidases with 'one-zinc' active site.

5. Experimental section

5.1. Chemistry

Unless otherwise stated, materials were obtained from commercial suppliers and purified according to the methods of chemical reagents. Melting points were determined on Boetius apparatus and were not corrected. Column chromatography was carried out on silica gel (200–300 mesh). TLCs were performed on precoated silica gel plates, and the resulting chromatograms were visualized under UV light at 254 nm or stained by ny. Proton NMR spectra were recorded on a Bruker DRX spectrometer, measurements were made in $CDCl_3$ or DMSO-d_6 solutions. Proton chemical shifts are reported in relation to tetramethylsilane (TMS) with the unit of δ in ppm and J in Hertz. ESI-MS was measured on an API 4000 spectrometer. Anhydrous reactions were carried out in over-dried glassware under a nitrogen atmosphere.

5.1.1. (S)-tert-Butyl 2,6-dioxopiperidin-3-ylcarbamate **(2)** [16]

To the stirring mixture of Boc-L-Gln (41.6 g, 0.169 mol) and *N*-hydroxy-succinimide (NHSu, 19.5 g, 0.170 mol) in 200 mL of THF was added in dropwise DCC (35.1 g, 0.170 mol) in 100 mL of THF at 0 °C on ice-water bath and keep stirring at room temperature for 2 h. The mixture was heated and refluxed for 12 h and then evaporated. To the residues was added 50 mL of EtOAc and 1 mL acetic acid and evaporate. The residues were added 100 mL of EtOAc and cooled in fridge overnight. The mixture was filtered by 4.0 g of Celite and evaporated to obtain yellow oil. To the residues was added 100 mL of DCM. The organic phase was washed in turn with saturated citric acid (10 mL × 3), 0.5% Na_2CO_3 (20 mL × 4), and brine and dried over anhydrous Na_2SO_4. Filter and evaporate to 1/3 volume to obtain white crystal solid.

Yield: 75.0%. Mp 211.5–213.8 °C; ESI-MS m/z $[M + H]^+$ 229.6; ^1H NMR (400 MHz, $CDCl_3$): δ 11.06 (s, 1H), 7.39 (d, 1H), 4.53 (d, 1H), 2.10 (m, 4H), 1.42 (s, 9H).

5.1.2. (S)-Ethyl-2-(3-(tert-butoxycarbonylamino)-2,6-dioxopiperidin-1-yl)acetate **(3)**

To a 1L of flask charged with **2** (14.6 g, 0.064 mol), K_2CO_3 (10.6 g, 1.2 eq), tetrabutylammonium iodide (TBAI, 2.5 g, 0.1 eq) and 500 mL dry ketone was added ethyl 2-bromoacetate (11.0 mL, 1.5 eq). The mixture was then refluxed for 12 h and filtered and evaporated to obtain yellow oil, which was added 100 mL of EtOAc and then washed in turn with 0.5% Na_2CO_3 (10 mL × 3), saturated citric acid (10 mL × 2) and brine. The organic phase was dried over anhydrous Na_2SO_4. Filter and evaporate, and then crystallize with EtOAc–Ethyl ether to obtain 16.0 g of **3** in white crystals.

Yield: 80.0%. Mp 134.5–136.3 °C; ESI-MS m/z $[M + H]^+$ 315.4; ^1H NMR (400 MHz, $CDCl_3$): δ 5.36 (br s, 1H), 4.51 (dd, 2 H), 4.39 (m, 1 H), 4.19 (q, 2H, J = 6.90), 2.3 2(m, 4H), 1.46 (s, 9 H), 1.27 (t, 3 H, J = 6.90).

5.2. General procedure for the preparation of compound (**5**)

To the solution of **3** (10 mmol) in 40 mL of DCM was added 10 mL of TFA and stirred at room temperature for 3 h and **4** was obtained as yellow oil after evaporation. To the stirring mixture of **4** (1.5 g,

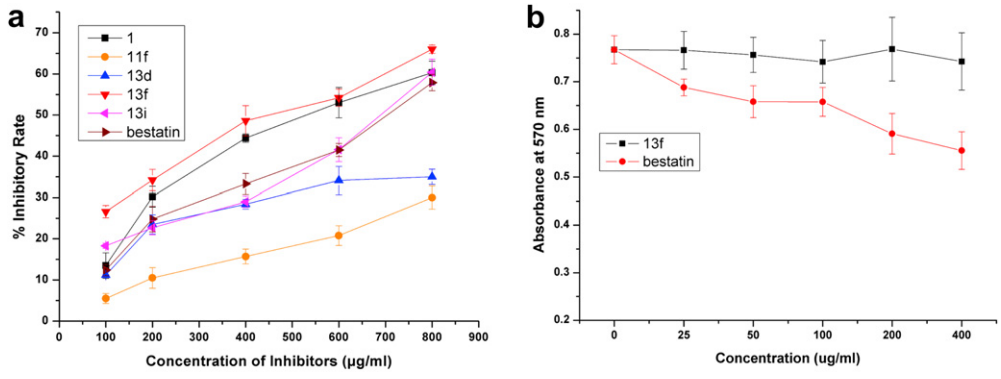


Fig. 3. In vitro HL-60 cell (a) and K562 cell (b) assay for active compounds.

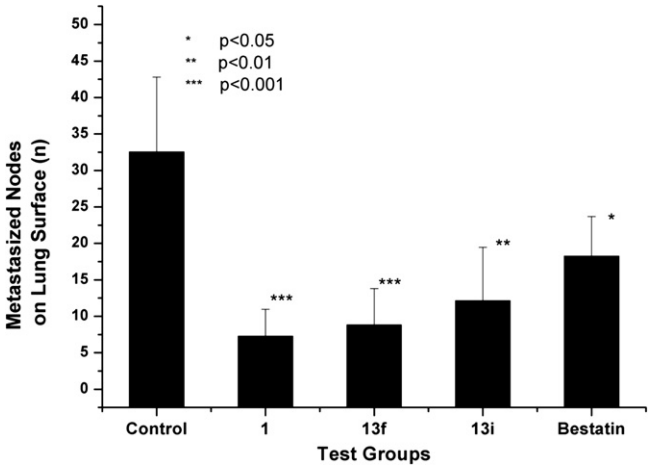


Fig. 4. In vivo H22 cell metastasis assay for active compounds.

7 mmol) in 50 mL anhydrous DCM at 0 °C was added dropwise Et_3N (2.86 mL, 20 mmol) under N_2, and then active ester of Boc-amino acid derivative (1.18 mL, 10 mmol) prepared in advance (Boc-amino acid and 1.1 eq of DCC and 1.2 eq of HOBt) was added in dropwise. After 1.5 h, remove the ice bath and keep for another 3–12 h at room temperature. The reaction was detected by TLC (EtOAc:cyclohexane, 2:1(V/V)) or (DCM:CH_3OH-10:1).The mixture was washed by 0.5% Na_2CO_3, brine to neutral and dried over Na_2SO_4. Filter and condense by rotary evaporation to obtain crude target compounds which were purified by recrystallization with anhydrous C_2H_5OH or chromatography column.

The following intermediates are prepared according to the general procedure.

*5.2.1. (S)-Ethyl-2-(3-(2-(tert-butoxycarbonyl-amino)acetamido)-2,6-dioxopiperidin-1-yl)acetate (**5a**)*

Yield: 95.8%. Mp 149.2–152.8 °C; ESI-MS *m/z* $[M + H]^+$ 372.4; ^1H NMR (300 MHz, $CDCl_3$): δ 6.93(br s, 1H), 4.65 (m, 1H), 4.51 (dd, 2H, $J = 30.3, 16.8$), 4.17 (q, 2H, $J = 7.2$), 3.87 (m, 2H), 2.65 (m, 3H), 1.90 (m, 1H), 1.46 (s, 9H), 1.27 (t, 3H, $J = 7.2$).

*5.2.2. Ethyl-2-((S)-3-((S)-2-(tert-butoxycar bonylamino) propanamido)-2,6-dioxopiperidin-1-yl)acetate (**5b**)*

Yield: 93.3%. Mp 198.2–199.9 °C; ESI-MS *m/z* $[M + H]^+$ 386.7; ^1H NMR: (300 MHz, $CDCl_3$): δ 6.92 (br s, 1H), 4.97 (br s, 1H), 4.63 (m, 1H), 4.50 (dd, 2H, $J = 30.9, 16.8$), 4.18 (q, 3H, $J = 7.2$), 2.87 (m, 2H), 2.52(m, 1H), 1.90 (dq, 1H, $J = 12.9, 5.4$), 1.45 (s, 9H), 1.39 (d, 3H, $J = 6.9$), 1.27 (t, 3H, $J = 7.2$).

*5.2.3. Ethyl-2-((S)-3-((S)-2-(tert-butoxy-carbonylamino)-3-methyl butanamido)-2,6-dioxopiperidin-1-yl)acetate (**5c**)*

Yield: 89.0%. Mp 199.3–201.8 °C; ESI-MS *m/z* $[M + H]^+$ 414.7; ^1H NMR (300 MHz, $CDCl_3$): δ 6.72 (s, 1H), 5.01 (br s, 1H), 4.64 (m, 1H), 4.50 (dd, 2H, $J = 33.6, 16.8$), 4.18 (q, 2H, $J = 7.2$), 3.98 (m, 1H), 2.87 (m, 2H), 2.54 (m, 1H), 2.18 (m, 1H), 1.92 (m, 1H), 1.45 (s, 9H), 1.27 (t, 3H, $J = 7.2$), 0.96 (dd, 6H).

*5.2.4. Ethyl-2-((S)-3-((S)-2-(tert-butoxy-carbonylamino)-4-methyl pentanamido)-2,6-dioxopiperidin-1-yl) acetate (**5d**)*

Yield: 86.3%. Mp 176.6–179.2 °C; ESI-MS *m/z* $[M + H]^+$ 428.7; ^1H NMR (300 MHz, $CDCl_3$): δ 6.84 (br s, 1H), 4.85 ι(br s, 1H), 4.63 (m, 1H), 4.50 (dd, 2H, $J = 31.5, 16.8$), 4.19 (q, 3H, $J = 7.2$), 2.84 (m, 2H), 2.53 (m, 1H), 1.92 (dq, 1H, $J = 12.9, 4.8$), 1.67 (m, 2H), 1.51 (q, 1H, $J = 9.1$), 1.45(s, 9H), 1.28 (t, 3H, $J = 7.2$), 0.95 (dd, 6H).

*5.2.5. Ethyl-2-((S)-3-((2S,3R)-2-(tert-butoxycarbonylamino)-3-methylpentanamido)-2,6-dioxopiperidin-1-yl) acetate (**5e**)*

Yield: 89.0%. Mp 164.6–165.1 °C; ESI-MS *m/z* $[M + H]^+$ 428.5; ^1H NMR (300 MHz, $CDCl_3$): δ 6.70 (br s, 1H), 4.98 (br s, 1H), 4.64 (m, 1H), 4.51 (dd, 2H, $J = 34.2, 16.8$), 4.19 (q, 2H, $J = 7.2$), 4.01 (m, 1H), 2.86 (m, 2H), 2.55 (m, 1H), 1.90 (m, 2H), 1.45 (s, 9H), 1.27 (t, 3H, $J = 7.2$), 1.18 (m, 2H), 0.96 (dd, 6H).

*5.2.6. Ethyl-2-((S)-3-((S)-2-(tert-butoxycarbonyl-amino)-3-phenyl propanamido)-2,6-dioxopiperidin-1-yl)acetate (**5f**)*

Yield: 91.3%. Mp 164.1–164.2 °C; ESI-MS *m/z* $[M + H]^+$ 462.6; ^1H NMR (300 MHz, $CDCl_3$): δ 7.25 (m, 5H), 6.84 (br d, 1H), 4.94 (br s, 1H), 4.48 (m, 4H), 4.17 (q, 2H, $J = 7.2$), 3.11 (d, 2H, $J = 6.3$), 2.86 (m, 2H), 2.53 (m, 1H), 1.80 (m, 2H), 1.45 (s, 9H), 1.28 (t, 3H, $J = 7.2$).

*5.2.7. Ethyl-2-((S)-3-((S)-2-(tert-butoxycarbony lamino)-3-(1H-imidazol-4-yl)propanamido)-2,6-dioxopiperidin-1-yl)acetate (**5g**)*

Yield: 64.5%. Mp 153.2–155.7 °C; ESI-MS *m/z* $[M + H]^+$ 462.6; ^1H NMR (300 MHz, CDCl3): δ 8.00 (s, 1H), 7.1 7 (s, 1H), 4.47 (m, 4H), 4.18 (q, 2H, $J = 0.2$), 3.00 (m, 4H), 2.50 (m, 1H), 1.97 (m, 1H), 1.60 (s, 9H), 1.45 (s, 9H), 1.28 (t, 3H, $J = 7.2$).

*5.2.8. Ethyl-2-((S)-3-((S)-2-(tert-butoxycarbonylamino)-4-(methylthio)butanamido)-2,6-dioxopiperidin-1-yl)acetate (**5h**)*

Yield: 82.4%. Mp 147.9–148.5 °C; ESI-MS *m/z* $[M + H]^+$ 446.6; ^1H NMR (300 MHz, CDCl3): δ 7.03 (br d, 1H), 5.20 (br s, 1H), 4.67 (m, 1H), 4.40 (dd, 2H), 4.18 (q, 2H, $J = 7.2$), 2.85 (m, 2H), 2.60 (m, 2H), 2.49 (m, 1H), 2.12 (s, 3H), 2.08 (m, 1H), 1.94 (m, 2H), 1.45(s, 9H), 1.28 (t, 3H, $J = 7.2$).

*5.2.9. Ethyl-2-((S)-3-((S)-2-(tert-butoxycarbonyl-amino)-3-(1H-indol-3-yl)propanamido)-2,6-dioxopiperidin-1-yl)acetate (**5i**)*

Yield: 82.6%. Mp 133.1–134.2 °C; ESI-MS *m/z* $[M + H]^+$ 501.5; ^1H NMR (300 MHz, $CDCl_3$): δ 8.16(s, 1H), 7.65 (d, 1H), 7.35 (d, 1H), 7.15 (m, 3H), 6.67 (s, 1H), 5.13 (s, 1H), 4.37 (m, 4H), 4.15 (q, 2H, $J = 7.2$), 3.20 (m, 1H), 2.79 (m, 2H), 2.39(m, 1H), 1.67 (m, 2H), 1.43 (s, 9H), 1.25 (t, 3H, $J = 7.2$).

*5.2.10. Ethyl-2-((S)-3-((S)-2-(tert-butoxycarbonyl-amino)-3-(4-(tert-butoxycarbonyloxy)-phenyl)propanamido)-2,6-dioxopiperidin-1-yl)acetate (**5j**)*

Yield: 93.6%. Mp 179.3–180.8 °C; ESI-MS *m/z* $[M + H]^+$ 578.5; ^1H NMR (300 MHz, $CDCl_3$): δ 7.20 (d, 2H, $J = 8.4$), 7.10 (d, 2H, $J = 8.4$), 6.77 (d, 1H), 4.94 (m, 1H), 4.48 (m, 3H), 4.18 (q, 2H, $J = 7.2$), 3.10 (d, 2H, $J = 6.3$), 2.83 (m, 2H), 2.52 (m, 1H), 1.82 (m, 1H), 1.55 (s, 9H), 1.42 (s, 9H), 1.26 (t, 3H, $J = 7.2$).

*5.2.11. Ethyl-2-((S)-3-((S)-2,6-bis(tert-butoxy-carbonyl-amino)hexanamido)-2,6-dioxopi peridin-1-yl)acetate (**5k**)*

Yield: 86.6%. Mp 115.8–117.3 °C; ESI-MS *m/z* $[M + H]^+$ 543.7; ^1H NMR (300 MHz, $CDCl_3$): δ 6.96 (br s, 1H), 5.15 (br s, 1H), 4.62 (m, 1H), 4.40 (dd, 2H, $J = 30.3, 16.8$), 4.17 (q, 2H, $J = 7.2$), 3.11 (m, 2H), 2.85 (m, 2H), 2.50 (m, 1H), 1.89 (m, 3H), 1.68 (m, 2H), 1.50 (m, 2H), 1.44 (s, 18H), 1.28 (t, 3H, $J = 7.2$).

*5.2.12. (S)-tert-Butyl-2-((S)-1-(2-ethoxy-2-oxoethyl)-2,6-dioxopiperidin-3-ylcarbamoyl)pyrrolidine-1-carboxylate (**5l**)*

Yield: 83.7%. Mp 151.9–152.7 °C; ESI-MS *m/z* $[M + H]^+$ 412.6; ^1H NMR (300 MHz, $CDCl_3$): δ 4.53 (m, 3H), 4.15 (m, 3H), 3.46 (m, 2H), 2.86 (m, 2H), 2.50 (m, 1H), 1.89 (m, 4H), 1.46 (s, 9H), 1.26 (t, 3H, $J = 7.2$).

*5.3. General procedure for the preparation of compound (**6**)*

To the stirring solution of **5** (0.5 g) in 10 mL anhydrous ethyl acetate was added dropwise 5 mL 3N EtOAc–HCl and after 2–3 h,

target compounds **6** were obtained in the form of hydrochloride salt. Filter quickly and dry the cake in vacuum to obtain dried white solid with high yield.

The following compounds are prepared according to the general procedure:

*5.3.1. (S)-Ethyl-2-(3-(2-aminoacetamido)-2,6-dioxopiper idin-1-yl) acetate hydrochloride (**6a**)*

Yield: 89.7%. Mp 218.5–220.4 °C; ESI-MS *m/z* $[M + H]^+$ 272.6; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.97 (d, 1H), 8.18 (br s, 3H), 4.82 (m, 1H), 4.38 (dd, 2H, J = 20.7, 16.8), 4.10 (q, 2H, J = 6.9), 3.62 (s, 2H), 3.96 (m, 1H), 2.72 (m, 1H), 1.98(m, 2H), 1.18 (t, 3H, J = 6.9).

*5.3.2. Ethyl-2-((S)-3-((S)-2-aminopropanamido)-2,6-dioxopiperidin-1-yl)acetate hydrochloride (**6b**)*

Yield: 82.3%. Mp 224.8–225.4 °C; ESI-MS *m/z* $[M + H]^+$ 286.4; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.95 (d, 1H), 8.27 (br s, 3H), 4.83 (m, 1H), 4.38 (dd, 2H, J = 20.7, 16.8), 4.11 (q, 2H, J = 7.2), 3.87 (q, 1H, J = 6.9), 2.99 (m, 1H), 2.78 (m, 1H), 2.01 (m, 2H), 1.42 (d, 3H, J = 6.9), 1.18 (t, 3H, J = 6.9).

*5.3.3. Ethyl-2-((S)-3-((S)-2-amino-3-methylbutanamido)-2,6-dioxopiperidin-1-yl) acetate hydrochloride (**6c**)*

Yield: 79.6%. Mp 136.0–138.3 °C; ESI-MS *m/z* $[M + H]^+$ 314.5; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.90 (d, 1H), 8.19 (br s, 3H), 4.90 (m, 1H), 4.38 (dd, 2H, J = 20.7, 16.8), 4.10 (q, 2H, J = 7.2), 3.62 (d, 1H, J = 5.4), 3.00 (m, 1H), 2.78 (m, 1H), 2.14 (m, 1H), 2.00 (m, 2H), 1.17 (t, 3H, J = 6.9), 1.01 (d, 3H, J = 6.3).

*5.3.4. Ethyl-2-((S)-3-((S)-2-amino-4-methylpentanamido)-2,6-dioxopiperidin-1-yl) acetate hydrochloride (**6d**)*

Yield: 73.4%. Mp 141.4–142.6 °C; ESI-MS *m/z* $[M + H]^+$ 328.7; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.05 (d, 1H), 8.34 (br s, 3H), 4.86 (m, 1H), 4.38 (dd, 2H, J = 20.7, 16.8), 4.08 (q, 2H, J = 6.9), 3.79 (t, 1H, J = 6.9), 3.00 (m, 1H), 2.78 (m, 1H), 2.02 (m, 2H), 1.79 (m, 1H), 1.62 (m, 2H), 1.19 (t, 3H, J = 7.2), 0.92 (d, 6H, J = 6.6).

*5.3.5. Ethyl-2-((S)-3-((2S,3R)-2-amino-3-methylpentan amido)-2,6-dioxopiperidin-1-yl)acetate hydrochloride (**6e**)*

Yield: 79.4%. Mp 171.1–172.3 °C; ESI-MS *m/z* $[M + H]^+$ 328.7; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.95 (d, 1H), 8.29 (br s, 3H), 4.88 (m, 1H), 4.38 (dd, 2H, J = 20.7, 16.8), 4.10 (q, 2H, J = 6.9), 3.67 (d, 1H, J = 5.4), 2.99 (m, 1H), 2.78 (m, 1H), 2.03 (m, 2H), 1.87 (m, 1H), 1.59 (m, 1H), 1.21 (m, 1H), 1.18 (t, 3H, J = 7.2), 0.98 (d, 3H, J = 6.9), 0.88 (t, 3H, J = 6.6).

*5.3.6. Ethyl-2-((S)-3-((S)-2-amino-3-phenylpropan amido)-2,6-dioxopiperidin-1-yl) acetate hydrochloride (**6f**)*

Yield: 86.4%. Mp 156.3–158.0 °C; ESI-MS *m/z* $[M + H]^+$ 362.4; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.18 (d, 1H), 8.23 (br s, 3H), 7.31 (m, 5H), 4.83 (m, 1H), 4.40 (dd, 2H), 4.10 (m, 3H), 3.21 (m, 1H), 2.99 (m, 2H), 2.78 (m, 1H), 2.02 (m, 2H), 1.20 (t, 3H, J = 7.2).

*5.3.7. Ethyl-2-((S)-3-((S)-2-amino-3-(1H-imidazol-4-yl)-propanamido)-2,6-dioxopiperidin-1-yl)acetate hydrochloride (**6g**)*

Yield: 80.4%. Mp 126.6–127.8 °C; ESI-MS *m/z* $[M + H]^+$ 352.5; ^{1}H NMR (300 MHz, DMSO-d_6): δ 14.60 (br s, 1H), 9.30 (d, 1H), 9.06 (s, 1H), 8.63 (br s, 3H), 7.53 (s, 1H), 4.79 (m, 1H), 4.41 (m, 3H), 4.11 (m, 2H), 2.97 (m, 1H), 2.22 (m, 1H), 2.03 (m, 2H), 1.19 (t, 3H, J = 7.2).

*5.3.8. Ethyl-2-((S)-3-((S)-2-amino-4-(methylthio)butan amido)-2,6-dioxopiperidin-1-yl)acetate hydrochloride (**6h**)*

Yield: 65.7%. Mp 139.2–142.1 °C; ESI-MS *m/z* $[M + H]^+$ 346.4; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.19 (dd, 1H), 8.47 (br s, 3H), 4.81 (m, 1H), 4.37 (dd, 2H, J = 20.7, 16.8), 4.11 (q, 2H, J = 7.2), 3.92 (m, 1H), 2.98 (m, 1H), 2.78 (m, 1H), 2.60 (m, 2H), 2.05 (m, 6H), 1.66 (m, 1H), 1.18 (t, 3H, J = 6.9).

*5.3.9. Ethyl-2-((S)-3-((S)-2-amino-3-(1H-indol-3-yl) propanamido)-2,6-dioxopiperidin-1-yl)acetate hydrochloride (**6i**)*

Yield: 80.2%. Mp 158.6–159.7 °C; ESI-MS *m/z* $[M + H]^+$ 401.6; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.08 (s, 1H), 9.29 (d, 1H), 8.25 (br s, 3H), 7.78 (d, 1H), 7.37 (d, 1H), 7.27 (s, 1H), 7.08 (t, 1H), 7.00 (t, 1H), 4.86 (m, 1H), 4.38 (m, 2H), 4.08 (m, 3H), 3.19 (m, 2H), 3.00 (m, 1H), 2.78 (m, 1H), 2.01 (m, 2H), 1.19 (t, 3H, J = 7.2).

*5.3.10. Ethyl-2-((S)-3-((S)-2-amino-3-(4-hydroxyphenyl)-propanamido)-2,6-dioxopiperidin-1-yl)acetate hydrochloride (**6j**)*

Yield: 83.4%. Mp 179.9–181.2 °C; ESI-MS *m/z* $[M + H]^+$: 378.6; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.39 (s, 1H), 9.16 (d, 1H), 8.18 (br s, 3H), 7.14 (d, 2H, J = 8.4), 6.71 (d, 2H, J = 8.1), 4.82 (m, 1H), 4.40 (s, 2H), 4.11 (m, 2H), 3.99 (t, 1H, J = 6.3), 3.10 (m, 1H), 2.96 (m, 2H), 2.78 (m, 1H), 2.00 (m, 2H).

*5.3.11. Ethyl-2-((S)-3-((S)-2,6-diaminohexanamido)-2,6-dioxopiperidin-1-yl)acetate hydrochloride (**6k**)*

Yield: 78.5%. Mp 133.5–136.2 °C; ESI-MS *m/z* $[M + H]^+$: 343.6; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.05 (s, 1H), 8.08 (br s, 6H), 4.86 (m, 1H), 4.38 (dd, 2H), 4.11 (q, 2H, J = 6.9), 3.80 (t, 1H, J = 6.9), 3.00 (m, 1H), 2.77 (m, 3H), 2.03 (m, 2H), 1.78 (m, 2H), 1.54 (m, 4H), 1.20 (t, 3H, J = 7.2).

*5.3.12. Ethyl-2-((S)-2,6-dioxo-3-((S)-pyrrolidine-2-carboxamido)piperidin-1-yl)acetate hydrochloride (**6l**)*

Yield: 64.8%. Mp 179.0–181.1 °C; ESI-MS *m/z* $[M + H]^+$ 312.6; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.04 (m, 1H), 4.83 (m, 1H), 4.37 (dd, 2H), 4.22 (m, 1H), 4.10 (q, 2H, J = 6.9), 3.20 (m, 2H), 3.00 (m, 1H), 2.78 (m, 1H), 2.34 (m, 1H), 1.98(m, 5H), 1.19 (t, 3H, J = 7.2).

*5.3.13. (S)-Benzyl-2-(3-(tert-butoxycarbonylamino)-2,6-dioxopiperidin-1-yl)acetate (**7**)*

The procedure is similar to that of **3** using benzyl-2-bromoacetate instead of ethyl 2-bromoacetate to obtain white crystal.

Yield: 67%. Mp 78.0–81.0 °C; ESI-MS *m/z* $[M + H]^+$ 377.6; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.37 (m, 5H), 5.14 (s, 2H), 4.39 (m, 3H), 2.93 (m, 1H), 2.72 (m, 1H), 1.97 (m, 2H), 1.40 (s, 9H).

*5.4. General procedure for the preparation of compound **10***

To the solution of **7** (5.5 mmol) in 22 mL of DCM was added 5.5 mL of TFA and stirred at room temperature for 3 h and **8** was obtained as yellow oil, which was dissolved in 20 mL of anhydrous THF and cooled to −20 °C. N-methylmorphiline (about 1.3 mL) was added slowly to control the pH of the mixture at 6.0. To the flask charged with Boc-amino acid derivatives (5.5 mmol) and 20 mL of THF at −20 °C was added dropwise N-methylmorphiline 5.5 mmol (0.62 mL), and then 5.5 mmol (0.72 mL isobutyl chloroformate after 5 min). The mixture was stirred at −20 °C for another 10 min and to the cold mixture was added **8** in THF dropwise and keep stirring for another 15 min at −20 °C and then 0 °C for 3 h. The reaction mixture was filtered with the aid of 2.0 g of Celite. The filtrate was condensed and to the residues was added 80 mL of EtOAc and then washed in turn by 0.5% Na_2CO_3, 5% citric acid and brine. The organic phase was dried over Na_2SO_4. After filter and condense to obtain intermediates **9** with high yield without further purification. To the clear solution of benzyl ester derivatives **9** (4.0 mmol) in 20 mL anhydrous EtOH was added 20 mL of cyclohexene as hydrogen donor and equivalent amount of 5% Pd/C as catalyst. The mixture

was stirred at 45 °C for 1 h and filtered with the aid of 4.0 g of Celite. The filtrate was condensed and dissolved in 60 mL EtOAc and then washed in turn by 5% citric acid and brine. The organic phase was dried over Na_2SO_4. Compound **10** was further purified by column to obtain white solid.

The following intermediates are prepared according to the general procedure.

*5.4.1. (S)-2-(3-(2-(tert-Butoxycarbonylamino)acetamido)-2,6-dioxopiperidin-1-yl)acetic acid (**10a**)*

Yield: 98.3%. Mp 171–172.9 °C; ESI-MS *m/z* $[M + H]^+$ 344.3; ^1H NMR (300 MHz, DMSO-d_6): δ 8.90 (d, 1H), 8.09 (br s, 3H), 4.82 (m, 1H), 4.30 (dd, 2H, $J = 21.0, 16.8$), 3.63 (m, 2H), 2.85 (m, 2H), 1.99 (m, 2H), 1.43 (s, 9H).

*5.4.2. ((S)-3-((S)-2-(tert-Butoxycarbonylamino)propanamido)-2,6-dioxopiperidin-1-yl)acetic acid (**10b**)*

Yield: 73.8%. Mp 177.7–178.9 °C; ESI-MS *m/z* $[M + H]^+$ 428.7; ^1H NMR (300 MHz, DMSO-d_6): δ 12.96 (br s, 1H), 8.89 (d, 1H), 8.22 (br s, 3H), 4.83 (m, 1H), 4.29 (dd, 2H, $J = 20.4, 17.1$), 3.87 (m, 1H), 2.86 (m, 2H), 2.10 (m, 2H), 1.44 (s, 9H), 1.41 (d, 3H, $J = 6.9$).

*5.4.3. 2-((S)-3-((S)-2-(tert-Butoxycarbonylamino)-3-methylbutanamido)-2,6-dioxopiperidin-1-yl)acetic acid (**10c**)*

Yield: 89.5%. Mp 95.8–96.8 °C; ESI-MS *m/z* $[M + H]^+$ 386.4; ^1H NMR (300 MHz, $CDCl_3$): δ 7.22 (d, 1H), 5.35 (s, 1H), 4.79 (m, 1H), 4.54 (dd, 2H, $J = 30.0, 17.1$), 3.97 (m, 1H), 2.85 (m, 2H), 2.36 (m, 1H), 2.00 (m, 2H), 1.43 (s, 9H), 0.96 (t, 6H).

*5.4.4. 2-((S)-3-((S)-2-(tert-Butoxycarbonylamino)-4-methylpentanamido)-2,6-dioxopiperidin-1-yl)acetic acid (**10d**)*

Yield: 91.4%. Mp 91.2–92.0 °C; ESI-MS *m/z* $[M + H]^+$ 400.4; ^1H NMR (300 MHz, DMSO-d_6): δ 9.03 (d, 1H), 8.41 (br s, 3H), 4.84 (m, 1H), 4.29 (dd, 2H, $J = 20.4, 17.1$), 3.78 (t, 1H), 2.89 (m, 2H), 2.02 (m, 2H), 1.78 (m, 1H), 1.43 (s, 9H), 0.97 (d, 6H, $J = 6.9$).

*5.4.5((S)-3-((2S,3R)-2-(tert-Butoxycarbonylamino)-3-methylpentanamido)-2,6-dioxopiperidin-1-yl)acetic acid (**10e**)*

Yield: 84.5%. Mp 73.5–75.3 °C; ESI-MS *m/z* $[M + H]^+$ 400.4; ^1H NMR (300 MHz, $CDCl_3$): δ 7.32 (d, 1H), 5.37 (s, 1H), 4.79 (m, 1H), 4.53 (dd, 2H, $J = 35.1, 17.1$), 3.99 (m, 1H), 2.84 (m, 2H), 2.34 (m, 1H), 1.95 (m, 1H), 1.74 (m, 1H), 1.54 (m, 1H), 1.43 (s, 9H), 1.15 (m, 1H), 0.91 (dt, 6H).

*5.4.6. 2-((S)-3-((S)-2-(tert-Butoxycarbonylamino)-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetic acid (**10f**)*

Yield: 73.8%. Mp 89.2–91.5 °C; ESI-MS *m/z* $[M + H]^+$ 434.5; ^1H NMR (300 MHz, CDCl3): δ 7.23 (m, 5H), 5.26 (s, 1H), 4.54 (m, 4H), 2.90 (m, 4H), 2.35 (m, 1H), 1.89 (m, 1H), 1.36 (s, 9H).

*5.4.7. 2-((S)-3-((S)-2,6-Bis(tert-butoxycarbonylamino)hexanamido)-2,6-dioxopiperidin-1-yl)acetic acid (**10g**)*

Yield: 79.7%. Mp 93.2–93.7 °C; ESI-MS *m/z* $[M + H]^+$ 515.6; ^1H NMR (300 MHz, $CDCl_3$): δ 7.53 (d, 1H), 5.55 (s, 1H), 4.93 (m, 1H), 4.77 (m, 1H), 4.53 (dd, 2H, $J = 35.1, 17.1$), 4.21 (m, 1H), 3.08 (m, 2H), 2.97–2.87 (m, 2H), 2.32 (m, 1H), 2.06 (m, 1H), 1.79 (m, 1H), 1.63 (m, 1H), 1.43 (s, 18H).

*5.4.8. 2-((S)-3-((S)-1-(tert-Butoxycarbonyl)pyrrolidine-2-carboxamido)-2,6-dioxopiperidin-1-yl)acetic acid (**10h**)*

Yield: 71.7%. Mp 91.7–92.5 °C; ESI-MS *m/z* $[M + H]^+$ 384.4; ^1H NMR (300 MHz, $CDCl_3$): δ 7.66 (d, 1H), 5.55 (s, 1H), 4.94 (m, 1H), 4.49 (s, 2H), 4.38 (m, 1H), 3.50 (m, 1H), 3.37 (m, 1H), 2.78 (s, 2H), 2.27 (m, 1H), 2.05 (m, 3H), 1.66 (m, 2H), 1.43 (s, 9H).

*5.4.9. 2-((S)-3-((S)-2-(tert-Butoxycarbonylamino)-3-(4-(tert-butoxycarbonyloxy)phenyl)propanamido)-2,6-dioxopiperidin-1-yl)acetic acid (**10i**)*

Yield: 91.4%. Mp 89.7–90.4 °C; ESI-MS *m/z* $[M + H]^+$ 550.6; ^1H NMR (300 MHz, $CDCl_3$): δ 7.29 (d, 1H), 7.20 (d, 2H, $J = 8.4$), 7.08 (d, 2H, $J = 8.4$), 5.30 (d, 1H) 4.51 (m, 4H), 2.88 (m, 4H), 2.34 (m, 1H), 1.74 (m, 1H), 1.54 (s, 9H), 1.37 (s, 9H).

*5.5. General procedure for the preparation of compound **11***

The carboxyl acid derivatives **10** (0.3–0.5 g) were dissolved in 5 mL 3N HCl–EtOAc and stirred at room temperature for 2–3 h to obtain target compound **11** in the form of hydrochloride salt. Filter quickly and dry the cake in vacuum to obtain dried white solid with high yield.

The following compounds are prepared according to the general procedure.

*5.5.1. (S)-2-(3-(2-Aminoacetamido)-2,6-dioxopiperidin-1-yl)acetic acid hydrochloride (**11a**)*

Yield: 85.9%. Mp 170.1–172.1 °C; ESI-MS *m/z* $[M + H]^+$ 244.2; ^1H NMR (300 MHz, DMSO-d_6): δ 8.90 (d, 1H), 8.09 (br s, 3H), 4.82 (m, 1H), 4.30 (dd, 2H, $J = 21.0, 16.8$), 3.63 (m, 2H), 2.85 (m, 2H), 1.99 (m, 2H).

*5.5.2. 2-((S)-3-((S)-2-Aminopropanamido)-2,6-dioxopiperidin-1-yl) acetic acid hydrochloride (**11b**)*

Yield: 96.9%. Mp 121.5–122.0 °C; ESI-MS *m/z* $[M + H]^+$258.2; ^1H NMR (300 MHz, DMSO-d_6): δ 12.96 (br s, 1H), 8.89 (d, 1H), 8.22 (br s, 3H), 4.83 (m, 1H), 4.29 (dd, 2H, $J = 20.4, 17.1$), 3.87 (m, 1H), 2.86 (m, 2H), 2.10 (m, 2H), 1.41 (d, 3H, $J = 6.9$).

*5.5.3. 2-((S)-3-((S)-2-amino-3-methylbutanamido)-2,6-dioxopiperidin-1-yl)acetic acid hydrochloride (**11c**)*

Yield: 91.8%. Mp 119.6–121.4 °C; ESI-MS *m/z* $[M + H]^+$ 286.3; ^1H NMR (300 MHz, DMSO-d_6): δ 8.84 (s, 1H), 8.22 (br s, 3H), 4.83 (m, 1H), 4.29 (dd, 2H, $J = 20.4, 17.1$), 3.87 (m, 1H), 2.87 (m, 2H), 2.10 (m, 4H), 1.00 (d, 3H, $J = 6.6$).

*5.5.4. 2-((S)-3-((S)-2-Amino-4-methylpentanamido)-2,6-dioxopiperidin-1-yl)acetic acid hydrochloride (**11d**)*

Yield: 95.2%. Mp 156.5–158.1 °C; ESI-MS *m/z* $[M + H]^+$ 300.3; ^1H NMR (300 MHz, DMSO-d_6): δ 9.05 (d, 1H), 8.40 (br s, 3H), 4.85 (m, 1H), 4.29 (dd, 2H, $J = 20.4, 17.1$), 3.78 (t, 1H), 2.89 (m, 2H), 2.02 (m, 2H), 1.78 (m, 1H), 1.01 (d, 6H, $J = 6.9$).

*5.5.5. 2-((S)-3-((2S,3R)-2-Amino-3-methylpentanamido)-2,6-dioxopiperidin-1-yl)acetic acid hydrochloride (**11e**)*

Yield: 83.3%. Mp 143.3–145.7 °C; ESI-MS *m/z* $[M + H]^+$ 300.3; ^1H NMR (300 MHz, DMSO-d_6): δ 12.96 (br s, 1H), 8.92 (d, 1H), 8.26 (br s, 3H), 4.87 (m, 1H), 4.29 (dd, 2H, $J = 20.4, 17.1$), 3.66 (d, 1H), 2.84 (m, 2H), 2.04 (m, 2H), 1.88 (m, 1H), 1.59 (m, 1H), 1.16 (m, 1H), 0.98 (d, 3H, $J = 6.9$), 0.83 (d, 3H, $J = 7.2$).

*5.5.6. 2-((S)-3-((S)-2-Amino-3-phenylpropanamido)-2,6-dioxopiperidin-1-yl)acetic acid hydrochloride (**11f**)*

Yield: 93.8%. Mp 135.6–138.2 °C; ESI-MS *m/z* $[M + H]^+$ 334.3; ^1H NMR (300 MHz, DMSO-d_6): δ 9.15 (s, 1H), 8.24 (br s, 3H), 7.31 (s, 5H), 4.83 (m, 1H), 4.31 (dd, 2H, $J = 19.2, 16.8$), 4.03 (m, 1H), 3.21 (m, 1H), 2.98 (m, 2H), 2.76 (m, 1H), 2.00 (m, 2H).

*5.5.7. 2-((S)-3-((S)-2,6-Diaminohexanamido)-2,6-dioxopiperidin-1-yl)acetic acid dihydrochloride (**11g**)*

Yield: 96.3%. Mp 126.8–127.3 °C; ESI-MS *m/z* $[M + H]^+$ 315.3; ^1H NMR (300 MHz, DMSO-d_6): δ 12.96 (br s, 1H), 9.11 (d, 1H), 8.37 (br s, 3H), 8.02 (br s, 3H), 4.85 (m, 1H), 4.30 (dd, 2H, $J = 20.4, 17.1$), 3.83 (m, 1H), 2.99 (m, 1H), 2.77 (m, 3H), 2.00 (m, 2H), 1.80 (m, 2H), 1.61 (m, 2H), 1.43 (m, 2H).

*5.5.8. 2-((S)-2,6-Dioxo-3-((S)-pyrrolidine-2-carboxamido)piperidin-1-yl)acetic acid hydrochloride (**11h**)*

Yield: 53.9%. Mp 132.4–135.4 °C; ESI-MS *m/z* $[M + H]^+$ 284.3; ^1H NMR (300 MHz, DMSO-d_6): δ 12.96 (br s, 1H), 9.49 (br s, 1H), 8.97 (br s, 1H), 8.59 (br s, 1H), 7.32 (s, 5H), 4.83 (m, 1H), 4.31 (dd, 2H, *J* = 19.2, 16.8), 4.23 (m, 1H), 3.22 (m, 2H), 2.97 (m, 1H), 2.77 (m, 1H), 2.30 (m, 1H), 1.98 (m, 5H).

*5.5.9. 2-((S)-3-((S)-2-Amino-3-(4-hydroxyphenyl)propanamido)-2,6-dioxopiperidin-1-yl)acetic acid hydrochloride (**11i**)*

Yield: 96.9%. Mp 146.6–148.1 °C; ESI-MS *m/z* $[M + H]^+$ 350.3; ^1H NMR (300 MHz, DMSO-d_6): δ 12.94 (br s, 1H), 9.39 (s, 1H), 9.11 (s, 1H), 8.14 (br s, 3H), 7.13 (d, 2H, *J* = 8.4), 6.72 (d, 2H, *J* = 8.4), 4.82 (m, 1H), 4.31 (dd, 2H, *J* = 19.2, 16.8), 4.03 (m, 1H), 3.09 (m, 1H), 2.94 (m, 2H), 2.75 (m, 1H), 1.95 (m, 2H).

*5.6. General procedure for the preparation of compound **13***

To the clear solution of $HCl{\cdot}NH_2OH$ (0.2 g) in 1.5 mL anhydrous MeOH was added 0.5 mL of Et_3N to control the pH value at 7.0. The carboxyl acid derivatives **10** (2.0 mmol) were dissolved in 20 mL THF and cooled to −20 °C. To the mixture was added dropwise 2.0 eq of Et_3N, and then 1.01 eq of isobutyl chloroformate after 5 min. The mixture was stirred at −20 °C for another 10 min and the MeOH solution with NH_2OH was added dropwise, after which the mixture was stirred at −20 °C for 15 min and then 0 °C for 2 h. The reaction mixture was filtered with the aid of 2.0 g of Celite. The filtrate was condensed and added 30 mL of EtOAc and washed in turn with 0.05%$NaHCO_3$, 5% citric acid and brine. The organic phase was dried over Na_2SO_4. Remove the solvents to obtain **12** and purified by column.

To the stirring solution of **12** (0.5 g) in 10 mL anhydrous ethyl acetate was added dropwise 5 mL 3N EtOAc–HCl and after 2–3 h, target compounds **13** were obtained in the form of hydrochloride salt. Filter quickly and dry the cake in vacuum to obtain dried white solid with high yield.

The following compounds are prepared according to the general procedure.

*5.6.1. (S)-2-Amino-N-(1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)acetamide hydrochloride (**13a**)*

Yield: 56.6%. Mp 121.6–123.2 °C; ESI-MS *m/z* $[M + H]^+$ 259.2; ^1H NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H), 8.90 (d, 1H), 8.12 (br s, 3H), 4.82 (m, 1H), 4.16 (dd, 2H, *J* = 20.4, 17.1), 3.60 (m, 2H), 2.92 (m, 1H), 2.70 (m, 1H), 2.00 (m, 2H).

*5.6.2. (S)-2-Amino-N-((S)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)propanamide hydrochloride (**13b**)*

Yield: 82.0%. Mp 154.3–155.1 °C; ESI-MS *m/z* $[M + H]^+$273.3; ^1H NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H), 8.88 (s, 1H), 8.22 (s, 3H), 4.83 (m, 1H), 4.20 (m, 2H), 3.87 (m, 1H), 2.95 (m, 1H), 2.74 (m, 1H), 1.94 (m, 2H), 1.40 (d, 3H).

*5.6.3. (S)-2-Amino-N-((S)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-methylbutanamide hydrochloride (**13c**)*

Yield: 67.3%. Mp 165.6–167.5 °C; ESI-MS *m/z* $[M + H]^+$ 301.3; ^1H NMR (300 MHz, DMSO-d_6): δ 9.90 (s, 1H), 8.89 (br s, 1H), 8.21 (br s, 3H), 4.88 (m, 1H), 4.63 (m, 1H), 4.15 (dd, 2H, *J* = 18.9, 15.9), 2.93 (m, 1H), 2.74 (m, 1H), 2.08 (m, 2H), 1.02 (dd, 6H).

*5.6.4. (S)-2-Amino-N-((S)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-4-methylpentanamide hydrochloride (**13d**)*

Yield: 79.8%. Mp 176.3–176.9 °C; ESI-MS *m/z* $[M + H]^+$ 315.3; ^1H NMR (300 MHz, DMSO-d_6): δ 8.89 (br s, 1H), 8.25 (br s, 3H), 4.87 (br s, 1H), 4.62 (m, 1H), 4.41 (dd, 2H), 3.87 (m, 1H), 3.01 (m, 1H), 2.73 (m, 1H), 2.00(s, 2H), 1.78 (m, 1H), 1.61 (m, 2H), 0.88 (d, 6H).

*5.6.5. (2S,3R)-2-Amino-N-((S)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-methylpentanamide hydrochloride (**13e**)*

Yield: 85.5%. Mp 174.0–175.0 °C; ESI-MS *m/z* $[M + H]^+$ 315.3; ^1H NMR (300 MHz, DMSO-d_6): δ 10.59 (s, 1H), 8.82 (d, 1H), 8.16 (s, 3H), 4.16 (s, 2H), 3.65 (s, 1H), 2.92 (m, 1H), 2.74 (m, 1H), 2.06 (m, 2H), 1.85 (m, 1H), 1.56 (m, 1H), 1.21 (m, 1H), 0.98 (d, 3H, *J* = 6.9), 0.89 (t, 3H, *J* = 7.2).

*5.6.6. (S)-2-Amino-N-((S)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide hydrochloride (**13f**)*

Yield: 84.9%. Mp 171.1–172.6 °C; ESI-MS *m/z* $[M + H]^+$ 349.4; ^1H NMR (300 MHz, DMSO-d_6): δ 10.62 (s, 1H), 8.86 (s, 1H), 8.24 (s, 1H), 7.32 (s, 5H), 4.84 (m, 1H), 4.16 (dd, 2H, *J* = 20.7, 15.6), 3.21 (m, 1H), 2.78 (m, 2H), 2.72 (m, 1H), 2.00(s, 2H).

*5.6.7. (S)-2,6-Diamino-N-((S)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl) hexanamide dihydrochloride (**13g**)*

Yield: 91.1%. Mp 143.5–144.9 °C; ESI-MS *m/z* $[M + H]^+$ 330.4; ^1H NMR (300 MHz, DMSO-d_6): δ 10.66 (s, 1H), 9.09 (d, 1H, *J* = 8.4), 8.37 (s, 3H), 8.03 (s, 3H), 4.86 (m, 1H), 4.17 (dd, 2H, *J* = 18.0, 15.9), 3.82 (m, 1H), 2.94 (m, 1H), 2.71 (m, 3H), 2.04 (m, 2H), 1.80 (m, 2H), 1.51 (m, 4H).

*5.6.8. (S)-N-((S)-1-(2-(Hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl) pyrrolidine-2-carboxamide hydrochloride (**13h**)*

Yield: 45.8%. Mp 138.6–139.4 °C; ESI-MS *m/z* $[M + H]^+$ 299.3; ^1H NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H), 9.84 (d, 1H), 9.00 (d, 1H, *J* = 8.4), 8.58 (s, 1H), 8.03 (s, 3H), 4.82 (m, 1H), 4.45 (m, 1H), 4.17 (dd, 2H, *J* = 19.5, 15.9), 3.20 (m, 2H), 2.92 (m, 1H), 2.72 (m, 1H), 2.32 (m, 1H), 1.96 (m, 5H).

*5.6.9. (S)-2-Amino-N-((S)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-(4-hydroxyphenyl) propanamide hydrochloride (**13i**)*

Yield: 93.1%. Mp 165.5–167.1 °C; ESI-MS *m/z* $[M + H]^+$ 365.4; ^1H NMR (300 MHz, DMSO-d_6): δ 10.70 (s, 1H), 9.40 (s, 1H), 9.31(s, 1H), 8.21(s, 3H), 7.06 (d, 2H, *J* = 8.4), 6.70 (d, 2H, *J* = 8.4), 4.69 (m, 1H), 3.97 (m, 3H), 3.02 (m, 3H), 2.49 (m, 1H), 1.91 (m, 2H).

5.7. Biological materials and methods

5.7.1. Materials

5.7.1.1. Chemicals. Chemicals was synthesized as mentioned above and dissolved in dimethylsulfoxide (DMSO) or PBS and diluted to the required concentration with culture medium when used.

5.7.1.2. Cell line and cell culture. The human leukemia cell line, HL-60 and K562 were obtained from the Institute of National Cancer Research of China (Beijing, China). Cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin–streptomycin (100 IU/ml–100 μg/mL), 2 mM glutamine, and 10 mM Hepes buffer at 37 °C in a humid atmosphere (5% CO_2–95% air). Cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Sigma, USA) assay as described elsewhere.

5.7.2. In vitro APN enzyme assay [17]

IC_{50} values against APN were determined as previously described and using L-Leu-*p*-nitroanilide as substrate and microsomal aminopeptidase from porcine kidney microsomes (Sigma) as the enzyme 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 the UV–vis spectrophotometer Pharmacia LKB, Biochrom 4060. All 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 μg/mL final concentration), and the assay buffer, was adjusted to 200 μL.

5.7.3. In vitro cytostatic activity [18]

The inhibitors were dissolved in culture medium and diluted to the required concentration, and the in vitro cytostatic activity was evaluated by MTT assay. Briefly, the human leukemia cell line HL-60 was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and incubated at 37 °C in a CO_2 incubator. Cells were seeded on a 96-well plate (10^4 cells per well). After 4 h incubation, inhibitors were then added to the wells to achieve final concentration of 800, 600, 400, 200 and 100 μg/mL Control wells were prepared by addition of culture medium. At same time, the bestatin (Apeloa Pharma/Kangyu Pharma, China) was used as positive control. The plate was incubated for 48 h. Upon completion of the incubation, 1% of 0.5 mg/mL MTT was added to each well and incubated for an additional 4 h. After centrifugalization, medium was removed and 100 μL DMSO was added. Absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay reader (Model 680, BIO-RAD), and absorbance at 630 nm was used as a reference. The percent growth inhibitory rate of treated cells was calculated by $(OD_{\text{drug-free control}} - OD_{\text{tested}})/OD_{\text{drug-free control}} \times 100\%$, where OD is the mean value calculated using the data from three replicate tests. The IC50 values were determined by plotting the percentage viability versus concentration on a logarithmic graph and reading off the concentration at which 50% of cells viable relative to the control. Each experiment was repeated at least three times to get the mean values. The curves were defined using Origin 7.5 software.

5.7.4. Anti-metastasis assay in vivo [19]

Mice bearing H22 tumor were injected via the caudal vein and randomly divided into 5 groups. The animals of the control group were treated with the same volume of excipient, while the other groups were given the inhibitors (**1**, **13f**, **13i** and bestatin) by oral administration, at a dose of 50 mg/kg/day, 6 days/week for 2 weeks. The mice were then weighed and sacrificed for autopsy immediately. The lungs with tumor nodes were removed, weighed, and then placed in bouin stationary solution (saturated 2,4,6-trinitrophenol solution/formaldehyde/glacial acetic acid = 15:5:1). One day later, the metastasized nodes on the surface of lungs were counted.

Acknowledgements

We thank Prof. Xianjun Qu and his graduate students (Institute of Pharmacology, School of Pharmacy, Shandong University) for in vivo compounds screenings. We would also like to thank Dr. Philip Mosier (Department of Medicinal Chemistry, Virginia Commonwealth University) for docking software GOLD4.0 and training. This work was supported by National Nature Science Foundation of China (Grant No. 30772654; 36072541) and the Ph.D. Programs Foundation of Ministry of Education of P.R. China (No. 20060422029) and National High Technology Research and Development Program of China (863 project; Grant No 2007AA02Z314).

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