Bioorganic & Medicinal Chemistry 19 (2011) 900–906

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design, synthesis and biological evaluation of novel L-lysine ureido derivatives as aminopeptidase N inhibitors

Li Su, Hao Fang *, Kanghui Yang, Yingying Xu, Wenfang Xu *

Department of Medicinal Chemistry, School of Pharmacy, Shandong University, 44 West Culture Road, 250012 Jinan, Shandong, PR China

ARTICLE INFO

Article history:
Received 21 October 2010
Revised 29 November 2010
Accepted 30 November 2010
Available online 4 December 2010

Keywords:
Aminopeptidase N inhibitor
L-Lysine ureido derivatives
Synthesis

ABSTRACT

As the exopeptidase over-expressed in the cell surface of endothelial cells, aminopeptidase N (APN/CD13) is an essential target for tumor angiogenesis and metastasis. Based on the previous work of L-lysine amide derivatives in our laboratory, we designed and synthesized two series of L-lysine ureido derivatives as APN inhibitors. Within these compounds, one compound, **5d** (IC_{50} = 4.51 μM), showed similar inhibitory effect compared with Bestatin (IC_{50} = 5.87 μM).

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Aminopeptidase N (APN/CD13) (EC 3.4.11.2) is a zinc dependent exopeptidase, which is selectively expressed in endothelial cells, and usually over-expressed in the process of tumor angiogenesis and metastasis.[1] Regulated by angiogenic factors such as VEGF and bFGF,[2] the over-expression of APN results in the degradation of extra cellular matrix (ECM) and then lead to the formation of capillary tube-like structures of vascular endothelial cells. This process could promote angiogenesis and has been regarded as the first step of tumor proliferation and metastasis. Therefore, APN inhibitors and anti-APN antibodies have been applied to suppress the invasion and metastasis of tumor cells.[3]

Up to date, a number of APN inhibitors have been reported, which include Bestatin[4], probestin,[5] RB38A[6] and so on. Among them, zinc-binding group (ZBG) should be required in the structure of APN inhibitors such as AHPA derivatives, hydroxamic acids,[7] and organophosphorus compounds.[8] In our previous studies, a series of N^6-Cbz substituted L-lysine amide derivatives have been synthesized and screened for the inhibitory activities of APN. The results suggest that one compound **B6** exhibited potent APN inhibition.[9] However, most of the derivatives with substituted aromatic carbonyl in N2 position could not afford good APN inhibitory effect. In our on-going studies, N2 position of L-lysine was optimized with following modification: (i) replacement of the amide bond with the ureido bioisostere group; (ii) introducing the amino acids or alkyl groups in the part of ureido group substitution because the aromatic ring substitution didn't show good inhibition in this part; (iii) hydroxamic acid was kept or converted to other derivatives (Fig 1).

2. Chemistry

The target compounds were synthesized efficiently following the procedures shown in Schemes 1 and 2. In Scheme 1, benzylamine and phenethylamine were refluxed with triphosgene to generate isocyanates, and then converted to compound **1a** and **1b** with the reaction of N^6-cbz-L-lysine methyl ester hydrochloride. Finally, methyl ester groups of **1a** and **1b** were transformed to hydroxamic acids (**2a** and **2b**) to give another two target products. However, compounds **1c** and **2c** had to be prepared with another synthetic route in Scheme 2 because relevant isocyanate can not easily be generated due to steric hindrance of *t*-butylamine. In Scheme 2, N^6-cbz-L-lysine methyl ester hydrochloride was transformed into its isocyanate according to the literature,[10] then the isocyanate reacted with *t*-butylamine or L-amino acid methyl esters to yield **1c** or **3a–3g**. The methyl groups were finally transformed to carboxylic acids (**4a–4g**) or hydroxamic acids (**2c, 5a–5g**), respectively. It should be noted that **1a** and **1b** cannot be obtained with the synthetic method in Scheme 2 because of the occurrence of the by-products such as 1,3-dibenzylamine urea and 1,3-diphenethylamine.[11]

3. Results and discussion

All the target compounds were evaluated for their inhibitory activities against APN and the results listed in Tables 1 and 2. According to the data in Table 1, compound **2a** is more potent than the others in this series, which suggested that hydroxamate group

* Corresponding authors. Tel./fax: +86 531 88382264 (W.X.).
E-mail addresses: haofangcn@sdu.edu.cn (H. Fang), xuwenf@sdu.edu.cn (W. Xu).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.11.066

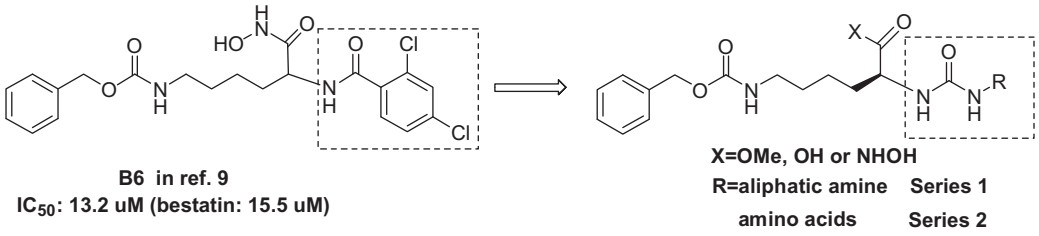
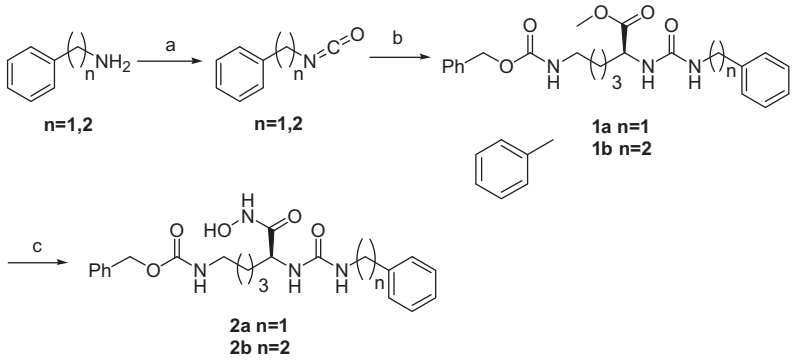
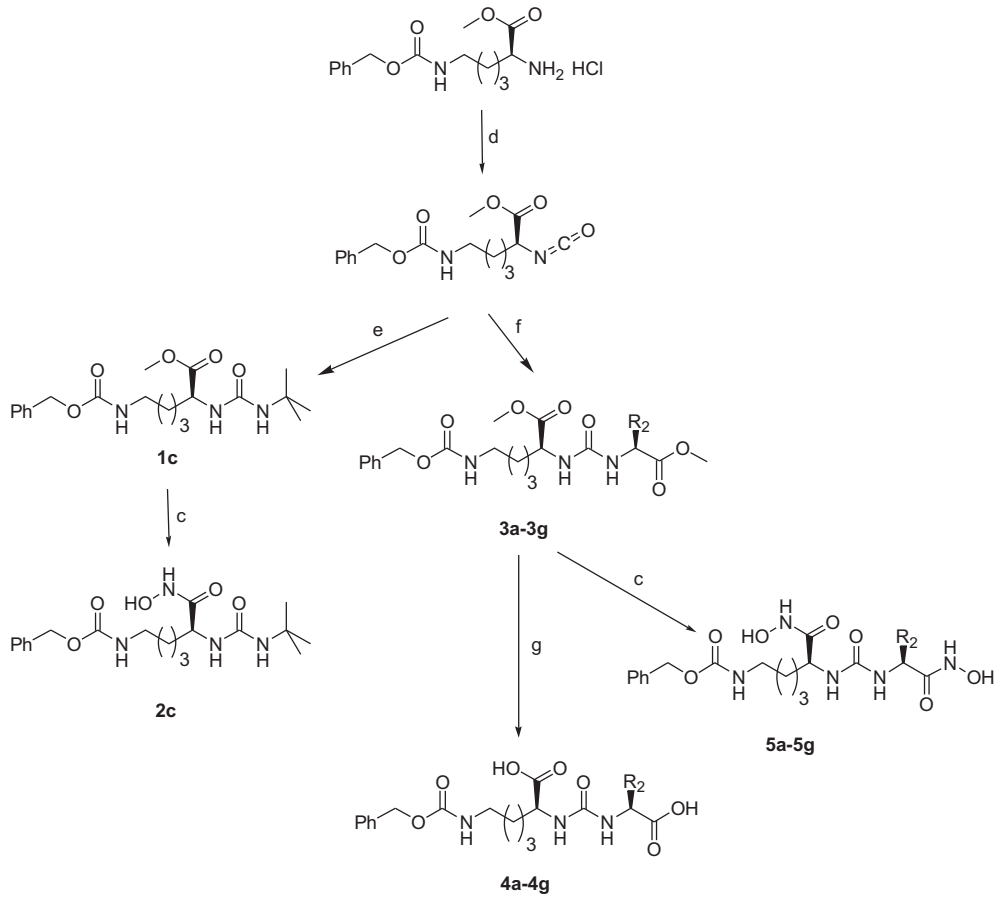


Figure 1. Target compounds: series 1 and 2.

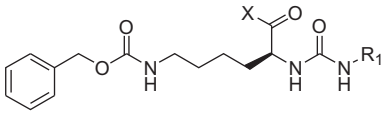


Scheme 1. Reagents and conditions: (a) triphosgene, toluene, reflux; (b) N^6-cbz-L-lysine methyl ester hydrochloride, TEA/DCM, rt; (c) NH_2OK/MeOH.



Scheme 2. Reagents and conditions: (d) triphosgene, $NaHCO_3$/DCM, 0 °C; (e) *t*-butylamine, TEA/DCM, rt; (c) NH_2OK/MeOH; (g) NaOH/H_2O; (f) methyl esters of L-Gly, L-Ala, L-Val, L-leu, L-Ile, L-Phe, or L-Met, TEA/DCM, rt.

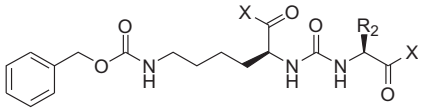
Table 1
The structures and IC_{50} values of the target compounds in series 1



Compounds	R_1	X	IC_{50} (μM)[a]
1a	$-CH_2Ph$	–OMe	>50
1b	$-CH_2CH_2Ph$	–OMe	>50
1c	$-C(CH_3)_3$	–OMe	>50
2a	$-CH_2Ph$	–NHOH	16.4 ± 0.9
2b	$-CH_2CH_2Ph$	–NHOH	>50
2c	$-C(CH_3)_3$	–NHOH	>50
Bestatin	—		5.87 ± 0.2

[a] All of the compounds were assayed three times, and their inhibition results are means of the three independent assays and expressed with standard deviations.

Table 2
The structures and IC_{50} values of the target compounds in series 2



Compounds	R_2	X	IC_{50} (μM)[a]
3a	–H	–OMe	>50
3b	$-CH_3$	–OMe	>50
3c	$-CH(CH_3)_2$	–OMe	>50
3d	$-CH_2CH(CH_3)_2$	–OMe	>50
3e	$-CH(CH_3)CH_2CH_3$	–OMe	>50
3f	$-CH_2Ph$	–OMe	>50
3g	$-CH_2CH_2SCH_3$	–OMe	>50
4a	–H	–OH	>50
4b	$-CH_3$	–OH	>50
4c	$-CH(CH_3)_2$	–OH	>50
4d	$-CH_2CH(CH_3)_2$	–OH	>50
4e	$-CH(CH_3)CH_2CH_3$	–OH	>50
4f	$-CH_2Ph$	–OH	>50
4g	$-CH_2CH_2SCH_3$	–OH	>50
5a	–H	–NHOH	20.4 ± 2.1
5b	$-CH_3$	–NHOH	19.9 ± 2.6
5d	$-CH_2CH(CH_3)_2$	–NHOH	4.51 ± 0.5
5e	$-CH(CH_3)CH_2CH_3$	–NHOH	>50
5f	$-CH_2Ph$	–NHOH	14.5 ± 1.8
5g	$-CH_2CH_2SCH_3$	–NHOH	14.4 ± 1.7
Bestatin	—	—	5.87 ± 0.2

[a] All of the compounds were assayed three times, and their inhibition results are means of the three independent assays and expressed with standard deviations.

is better than methyl ester. In addition, the benzyl group in R_1 position would be much better than phenethyl and *t*-butyl group for increasing the activity. It seems that the bulky alkyl groups and longer alkyl chain with phenyl group in the R_1 substitution could not show benefit for APN inhibitory activities.

On the other hands, the derivatives with different amino acids or their methyl esters in N2 position could not enhance the inhibitory activities compared with Bestatin. For example, both compounds **3a–3g** and **4a–4g** showed IC_{50} values over 50 μM (Table 2). However, the APN inhibitory activity was significantly improved when the methyl ester or carboxylic acid groups were converted to their corresponding hydroxamic acid derivatives (**5a–5g**) except compound **5e**. Considering hydroxamic acids belong to zinc binding group (ZBG), this phenomena indicated that derivatives of methyl esters (**3a–3g**) and carboxylic acids (**4a**–**4g**) cannot efficiently bind the zinc ion in the active site of APN and the introduction of an efficient ZBG (such as hydroxamic acid) in N2 position will be a favor to enhance the inhibitory activities.

According to the results listed in Tables 1 and 2, most of the amino acid derivatives **5a–5g** showed better potency (IC_{50} 4.51–20 μM) than the ones with aliphatic amine substitutions (**2a–2c**). Furthermore, different R_2 substitution also affected the inhibitory activities of compound **5a–5g**. For example, compound **5d**, (IC_{50} = 4.51 μM) with isobutyl in R_2 position is the most potent inhibitors among all the target compounds and has the similar potency compared with Bestatin (IC_{50} = 5.87 μM). But compound **5e** with *sec*-butyl group substitution has very poor inhibition (IC_{50} >50 μM).

Furthermore, compounds **5b**, **5d**, **5f** and **5g** were selected to be assayed on their anti-tumor inhibitory activity against ES-2 human ovary clear cell carcinoma cell proliferation by MTT method. The IC_{50} values of those compounds (**5b** IC_{50} = 415 ± 35 μM, **5d** IC_{50} = 291 ± 16 μM, **5f** IC_{50} = 395 ± 31 μM and **5g** IC_{50} = 356 ± 23 μM) confirmed that their anti-tumor inhibitory activities are similar with Bestatin (IC_{50} = 332 ± 20 μM) (Fig. 2).

In order to investigate the interaction between the enzyme and the active compounds, compound **5d** was docked into the active site of APN (PDB code: 2DQM) with Sybyl 7.0. The result suggests that the binding mode of compound **5d** is similar with Bestatin and include the subsite of S1 and S1′. For Bestatin, the benzyl group can interact with the S1 subsite, and the iso-butyl group from Leu residue inserts into the S1′ subsite. The 1-carbonyl oxygen and the 2-hydroxy group chelate the zinc ion of APN (Fig. 3a). In addition, Bestatin can form hydrogen bonds with Gly^{261}, Glu^{121}, His^{301} and Glu^{264} with the distance of 3.00, 2.82, 2.63 and 2.73 Å. According to the docking studies, N^6-cbz substitution of compound **5d** could deeply insert to the S_1 subsite, while the Leu residue in N2 position occupied the S1' subsite (Fig. 3a and b). The zinc ion of APN is coordinated with hydroxamate part of compound **5d** (Fig. 3b). Furthermore, compound **5d** also can form hydrogen bonds with

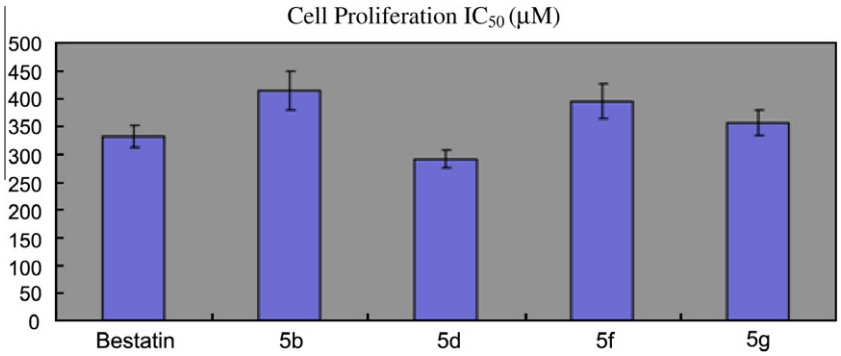


Figure 2. Effects of Bestatin and compounds **5b**, **5d**, **5f** and **5g** on ES-2 cell line proliferation. Each column represents the mean values with SE values for three independent experiments.

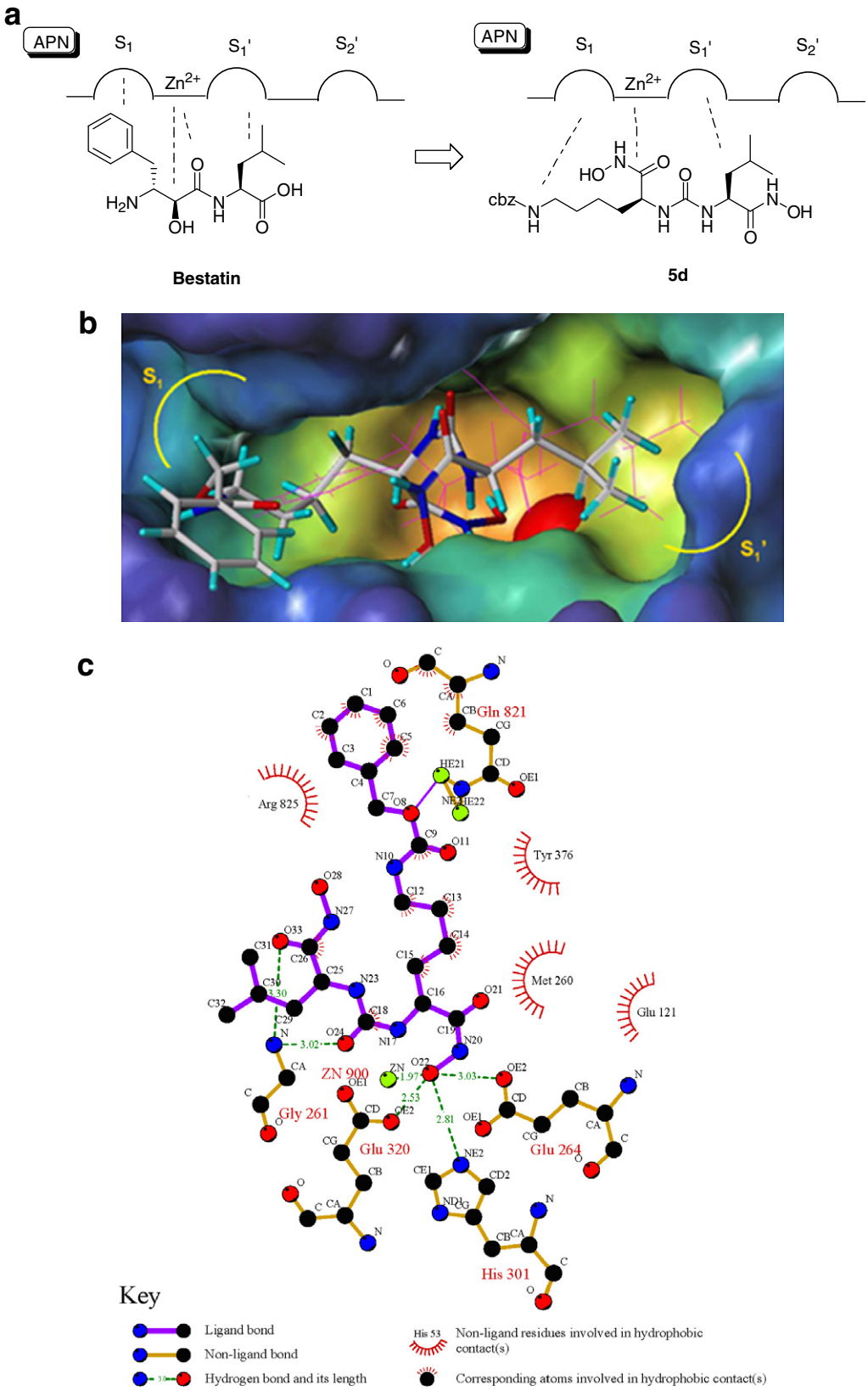


Figure 3. (a) Binding mode diagram of Bestatin and **5d**; (b) The docking result of **5d** (Bestatin in the X-ray crystal diffraction is shown in purple); (c) Ligplot diagram of **5d**.

Gly^{261}, Glu^{320}, His^{301} and Glu^{264} at the distance of 3.02, 2.53, 2.81 and 3.03Å (Fig. 3c). Therefore, both Bestatin and **5d** can interact with Gly^{261}, His^{301} and Glu^{264} through hydrogen bonds, but angles of these hydrogen bonds are different (Table 3).

Table 3
Hydrogen bonds between APN active site and Bestatin or compound **5d**

	Bestatin		**5d**	
	Distance (Å)	Angle (°)	Distance (Å)	Angle (°)
Gly^{261}	3.00	158.0	3.02	156.7
His^{301}	2.63	154.6	2.81	120.7
Glu^{264}	2.73	161.1	3.03	169.3

4. Conclusion

In summary, we designed and synthesized two series of novel L-lysine ureido derivatives as APN inhibitors. The preliminary results showed that compound **5d** was the most potent compound and had the similar inhibition compared with Bestatin. These results suggest that L-lysine ureido derivatives could possess good inhibitory activities after hydroxamic acid group introduced, which could be used as new lead compounds to develop potent APN inhibitors.

5. Experimental

5.1. Chemistry

5.1.1. General procedure

The chemical materials were purchased from commercial suppliers and used without further purification. Solvents were dried over $MgSO_4$ or distilled prior to use and flash chromatography was performed using silica gel (60 Å, 200 ± 300 mesh). Melting points are uncorrected. NMR spectra were recorded on a Bruker DRX-300 spectrometer. Chemical shifts are in parts per million (ppm). ESI-MS were determined on an API 4000 spectrometer.

5.1.1.1. 2-(3-Benzyl-ureido)-4-(*N*-benzyloxycarbonyl)-aminobutyl-acetic acid methyl ester (1a). Benzylamine (1.61 g, 15 mmol).-was added to a solution of triphosgene (2.22 g, 7.5 mmol) in dry toluene (80 mL) in room temperature. The reaction mixture was refluxed for 4 h and then solvents removed under low pressure. The residue was dissolved in DCM (20 mL) and this solution was added to the mixture of N^6-cbz-L-lyscine methyl ester hydrochloride (3.96 g, 15 mmol) and triethylamine (2.12 g, 21 mmol) in DCM (80 mL) under ice-bath. After stirred at room temperature for 30 min, the reaction mixture was concentrated under vaccum and then ethyl acetate (20 mL) was added to the residue. The organic phase was washed with 1 N HCl (10 mL) and saturated brine (10 mL) and dried with $MgSO_4$. After the solvent removed under low pressure, Compound **1a** was obtained as yellow oil and separated by silica gel column chromatography (petroleum ether/ethyl acetate = 2:1) as white solid (4.22 g), yield 63.5%, mp = 45–47 °C, ESI-MS *m/z*: 427.5 $[M+H]^+$; ^1H NMR (300 MHz, DMSO-d_6) δ 7.36–7.24 (m, 10H), 7.22–7.19 (m, 1H), 6.46–6.43 (m, 1H), 6.37–6.27 (m, 1H), 5.00 (s, 2H), 4.24–4.18 (m, 2H), 4.15–4.11(m, 1H), 3.62, (s, 3H), 2.98–2.94 (m, 2H), 1.66–1.62 (m, 6H).

5.1.1.2. 2-(3-Benzyl-ureido)-*N*-hydroxy-4-(*N*-benzyloxycarbonyl)-aminobutyl-acetmaide (2a). Compound **1a** (3.84 g, 9 mmol) was added to a solution of potassium hydroxylamine (8.37 g, 56 mmol) in methanol (20 mL). The reaction mixture was stirred at room temperature for 5 h and then removed methanol under low pressure. The residue was taken up with 1 N HCl and extracted with ethyl acetate. The organic phase was washed with brine and dried with $MgSO_4$. After the solvent removed under low pressure, Compound **2a** was obtained as yellow oil and separated by silica gel column chromatography (petroleum ether/ethyl acetate = 1:1) as white solid (2.63 g), yield 41.3%, mp = 165–167 °C; ESI-MS: *m/z* $[M+H]^+$ 428.5; ^1H NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H), 8.80 (s, 1H), 7.40–7.21 (m, 10H), 6.45 (s, 1H), 6.14 (s, 1H), 5.00 (s, 2H), 4.19 (s, 1H), 4.05–3.98 (m, 1H), 2.99–2.93 (m, 2H), 1.44–1.21 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.2, 157.4, 156.0, 140.7, 137.2, 128.3, 128.2, 127.7, 126.9, 126.5, 65.1, 50.5, 42.8, 33.1, 29.1, 22.5.

5.1.1.3. 2-(3-Phenethyl-ureido)-*N*-hydroxy-4-(*N*-benzyloxycarbonyl)-aminobutyl-acetic acid methyl ester (1b). Yield 65.8%, mp = 46–48 °C; ESI-MS: *m/z* $[M+H]^+$ 441.5; ^1H NMR (300 MHz, DMSO-d_6): δ 7.39–7.18 (m, 11H), 6.30–6.28 (m, 1H), 5.97–5.93 (m, 1H), 5.00 (s, 2H), 4.15–4.08 (m, 1H), 3.61 (s, 3H), 3.32–3.18 (m, 2H), 3.00–2.89 (m, 2H), 2.69–2.64 (m, 2H), 1.63–1.25 (m, 6H).

5.1.1.4. 2-(3-Phenethyl-ureido)-*N*-hydroxy-4-(*N*-benzyloxycarbonyl)-aminobutyl-acetmaide (2b). Yield 38.8%, mp = 123–125 °C; ESI-MS: *m/z* $[M+H]^+$ 442.5; ^1H NMR (300 MHz, DMSO-d_6): δ 10.63 (s, 1H), 8.82 (s, 1H), 7.37–7.18 (m, 10H), 6.12 (s, 1H), 6.00 (s, 1H), 4.99 (s, 2H), 4.01 (s, 1H), 3.64–3.48 (m, 2H), 2.98–2.92 (m, 2H), 2.85–2.80 (m, 2H), 1.59–1.17 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.2, 158.0, 156.1, 139.8, 137.2, 128.6, 128.3, 127.7, 126.3, 125.9, 65.1, 56.0, 50.4, 40.9, 40.8, 33.1, 29.1, 21.2.

5.1.1.5. 2-(3-Tert-butyl-ureido)-4-(*N*-benzyloxycarbonyl)-aminobutyl-acetic acid methyl ester (1c). To a mixture of N^6-cbz-L-lyscine methyl ester hydrochloride (6.94 g, 21 mmol) in saturated $NaHCO_3$ (80 mL) and DCM (80 mL) was added triphosgene (2.08 g, 7 mmol). The reaction mixture was vigorously stirred under ice-water bath for 15 min and the organic layer was collected. The water layer was extracted with DCM for three times and the organic phase was combined and dried with $MgSO_4$. After the solvent removed under vaccum, the residue was dissolved in DCM (20 mL). This solution was then added to the mixture of *t*-butylamine (2.08 g, 21 mmol) and triethylamine (2.12 g, 21 mmol) in DCM (80 mL) under ice bath. The reaction mixture was stirred at room temperature for 30 min and then the solvent was removed under low pressure. The residue was taken up with ethyl acetate (40 mL) and washed with 1 N HCl (10 mL) and brine (20 mL). The organic phase was dried with $MgSO_4$ and Compound **1c** was obtained as yellow oil and separated by silica gel column chromatography (petroleum ether/ethyl acetate = 2:1) as white solid (3.00 g), yield 49.3%, mp = 45–47 °C; ESI-MS: *m/z* $[M+H]^+$ 408.5; ^1H NMR (300 MHz, DMSO-d_6): δ 7.37–7.28 (m, 5H), 7.20 (t, 1H, J = 5.4 Hz), 5.99 (d, 1H, J = 6.0 Hz), 5.79 (s, 1H), 4.98 (s, 2H), 4.23–4.04 (m, 1H), 3.59 (s, 3H), 2.98–2.92 (m, 2H), 1.65–1.43 (m, 2H), 1.39–1.32 (m, 2H), 1.27–1.22 (m, 2H), 1.18 (s, 9H).

5.1.1.6. 2-(3-Tert-butyl-ureido)-*N*-hydroxy-acetamide (2c). Yield 62.2%, mp = 80–83 °C; ESI-MS: *m/z* $[M+H]^+$ 394.5; ^1H NMR (300 MHz, DMSO-d_6): δ 10.60 (s, 1H), 8.80 (s, 1H), 7.36–7.21 (m, 5H), 5.89–5.86 (m, 2H), 5.00 (s, 2H), 3.94 (dd, 1H, J = 6 Hz, 10 Hz), 2.99–2.92 (m, 2H), 1.49–1.34 (m, 6H), 1.19 (s, 9H); ^{13}C NMR (300 MHz, DMSO-d_6): δ169.2, 157.4, 156.0, 140.7, 137.2, 128.3, 128.2, 127.7, 126.9, 126.5, 65.1, 45.1, 33.1, 29.2, 29.1, 28.9.

5.1.1.7. 6-Benzyloxycarbonylamino-2-(3-methoxycarbonyl-methyl-ureido)-hexanoic acid methyl ester (3a). To a mixture of N^6-cbz-L-lyscine methyl ester hydrochloride (6.94 g, 21 mmol) in saturated $NaHCO_3$ (80 mL) and DCM (80 mL) was added triphosgene (2.08 g, 7 mmol), and the reaction mixture was vigorously stirred under ice-water bath for 15 min and the organic layer was collected. The water layer was extracted with DCM for three times and the organic phase was combined and dried with $MgSO_4$. After the solvent removed under vaccum, the residue was

dissolved in DCM (20 mL). This solution was then added to the mixture of L-glycine methyl ester hydrochloride (2.64 g, 21 mmol) and triethylamine (2.12 g, 21 mmol). The reaction mixture was stirred at room temperature for 30 min and then the solvent was removed under low pressure. The residue was taken up with ethyl acetate (40 mL) and washed with 1 N HCl (10 mL) and brine (20 mL). The organic phase was dried with $MgSO_4$ and compound **3a** was obtained as yellow oil and separated by silica gel column chromatography (petroleum ether/ethyl acetate = 1:1) as white solid (4.55 g), yield 53.2%, mp = 48–50 °C; ESI-MS: *m/z* $[M+H]^+$ 409.2; ^1H NMR (300 MHz, $CDCl_3$): δ 7.36–7.28 (m, 5H), 5.68 (br, 2H), 5.15 (s, 1H), 5.09 (s, 2H), 4.46 (s, 1H), 4.04–3.87 (m, 2H), 3.73 (s, 3H), 3.71 (s, 3H), 3.20–3.14 (m, 2H), 1.78–1.26 (m, 6H).

5.1.1.8. 6-Benzyloxycarbonylamino-2-(3-carboxymethyl-ureido)-hexanoic acid (4a). Compound **3a** (4.09 g, 10 mmol) was added to a solution of sodium hydroxide (0.80 g, 20 mmol) in water (10 mL)and methanol (10 mL). The reaction mixture was stirred at room temperature for 5 h and then methanol removed under low pressure. The residue was acidified with 1 N HCl and extracted with ethyl acetate. The organic phase was washed with brine and dried with $MgSO_4$. After the solvent removed under low pressure, Compound **4a** was obtained as yellow oil and separated by silica gel column chromatography (petrol ether/ethyl acetate = 1:2) as white solid (1.25 g), yield 32.8%, mp = 111–113 °C; ESI-MS: *m/z* $[M+H]^+$ 381.4; ^1H NMR (300 MHz, DMSO-d_6): δ 12.48 (s, 2H), 7.40–7.30 (m, 5H), 7.29–7.23 (m, 1H), 6.46 (s, 1H), 6.24 (s, 1H), 5.02 (s, 2H), 4.10–3.99 (m, 1H), 3.71 (s, 2H), 3.00–2.94 (m, 2H), 1.99–1.24 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.3, 169.0, 156.6, 156.0, 137.2, 128.3, 127.7, 65.1, 50.4, 42.4, 33.1, 29.2, 22.5.

5.1.1.9. [5-Hydroxycarbamoyl-5-(3-hydroxycarbamoylmethy-ureido)-pentyl]-carbamic acid benzyl ester (5a). Yield 29.8%, mp = 72–75 °C; ESI-MS: *m/z* $[M+H]^+$ 411.4; ^1H NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H), 10.50 (s, 1H), 7.39–7.28 (m, 5H), 7.24 (s, 1H), 6.39 (s, 1H), 6.25 (s, 1H), 5.02 (s, 2H), 4.08–4.06 (m, 1H), 3.89 (s, 2H), 2.96–2.94 (m, 2H), 1.36–1.15 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 172.4, 169.0, 157.2, 156.0, 137.2, 128.3, 127.7, 65.1, 50.4, 41.3, 33.1, 29.1, 22.4.

5.1.1.10. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-ethyl)-ureido]-hexanoic acid methyl ester (3b). Yield 53.2%, mp = 50–52 °C; ESI-MS: *m/z* $[M+H]^+$ 423.2; ^1H NMR (600 MHz,$CDCl_3$): δ 7.36–7.30 (m, 5H), 5.24 (br, 2H), 5.13–5.08 (m, 2H), 5.04 (br, 1H), 4.48–4.44 (m, 2H), 3.73 (s, 3H), 3.71 (s, 3H), 3.21–3.17 (m, 2H), 1.81–1.36 (m, 9H).

5.1.1.11. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-ethyl)-ureido]-hexanoic acid (4b). Yield 32.8%, mp = 110–112 °C; ESI-MS: *m/z* $[M+H]^+$ 395.4; ^1H NMR (600 MHz, DMSO-d_6): δ 12.50 (s, 2H), 7.38–7.23 (m, 5H), 6.36–6.28 (m, 3H), 5.02 (s, 2H), 4.10–4.02 (m, 2H), 3.35 (s, 2H), 1.64–0.85 (m, 9H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 170.2, 166.9, 157.0, 156.0, 137.3, 128.3, 127.6, 65.1, 52.1, 47.9, 32.0, 29.1, 22.4, 14.0.

5.1.1.12. {5-Hydroxycarbamoyl-5-[3-(1-hydroxycarbamoyl-ethy)-ureido]-pentyl}-carbamic acid benzyl ester (5b). Yield 40.0%, mp = 75–77 °C; ESI-MS: *m/z* $[M+H]^+$ 425.4; ^1H NMR (300 MHz, DMSO-d_6): δ 10.68 (s, 1H), 10.60 (s, 1H), 8.90 (s, 1H), 8.76 (s, 1H), 7.48–7.28 (m, 5H), 6.30–6.25 (m, 3H), 5.00 (s, 2H), 4.12–4.06 (m, 2H), 3.29 (s, 2H), 1.66–0.95 (m, 9H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 172.4, 169.0, 157.2, 156.0, 137.3, 128.3, 127.7, 65.1, 51.4, 46.3, 33.0, 29.1, 22.4, 18.3.

5.1.1.13. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-2-methyl-propyl)-ureido]-hexanoic acid methyl ester (3c). Yield 52.3%, oil; ESI-MS: *m/z* $[M+H]^+$ 451.7; ^1H NMR (600 MHz, $CDCl_3$): δ 7.36–7.30 (m, 5H), 5.45–5.39 (m, 2H), 5.26–5.24 (m, 1H), 5.14–5.07 (m, 2H), 4.45–4.41 (m, 2H), 3.72 (s, 3H), 3.71 (s, 3H), 3.20–3.14 (m, 2H), 1.80–1.30 (m, 7H), 0.93–0.85 (m, 6H).

5.1.1.14. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-2-methyl-propyl)-ureido]-hexanoic acid (4c). Yield 49.6%, mp = 70–73 °C; ESI-MS: *m/z* $[M+H]^+$ 423.4; ^1H NMR (600 MHz, $CDCl_3$+D_2O): δ 7.32–7.30 (m, 5H), 5.09 (s, 2H), 4.33–4.31 (m, 1H), 4.07–3.94 (m, 1H), 3.13–3.12 (m, 2H), 1.69–0.88 (m, 10H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 172.4, 169.0, 157.2, 156.0, 137.3, 128.3, 127.7, 65.1, 51.4, 46.3, 33.0, 29.1, 23.5, 22.4, 14.2, 14.1.

5.1.1.15. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-3-methyl-butyl)-ureido]-hexanoic acid methyl ester (3d). Yield 51.2%, mp = 50–52 °C; ESI-MS: *m/z* $[M+H]^+$ 465.5; ^1H NMR (600 MHz,$CDCl_3$): δ 7.36–7.30 (m, 5H), 5.32–5.30 (m, 1H), 5.15–5.12 (m, 2H), 5.10–5.07 (m, 2H), 4.47–4.44 (m, 2H), 3.72 (s, 3H), 3.69 (s, 3H), 3.20–3.16 (m, 2H), 1.81–1.35 (m, 9H), 0.92 (d, 6H).

5.1.1.16. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-3-methyl-butyl)-ureido]-hexanoic acid (4d). Yield 44.6%, mp = 60–63 °C; ESI-MS: *m/z* $[M+H]^+$ 437.4; ^1H NMR (300 MHz, DMSO-d_6): δ 8.91 (s, 2H), 7.53–7.30 (m, 5H), 6.24 (m, 3H), 5.06 (s, 2H), 4.11–4.00 (m, 2H), 3.13–3.12 (m, 2H), 2.27–1.28 (m, 9H), 0.93 (d, 6H, *J* = 8.4 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 170.8, 170.3, 156.2, 156.0, 137.2, 128.3, 127.7, 65.1, 51.4, 49.9, 40.3, 36.2, 29.9, 23.1, 23.0, 21.4.

5.1.1.17. {5-Hydroxycarbamoyl-5-[3-(1-hydroxycarbamoyl-3-methyl-butyl)-ureido]-pentyl}-carbamic acid benzyl ester (5d). Yield 63.5%, mp = 144–146 °C; ESI-MS: *m/z* $[M+H]^+$ 467.5; ^1H NMR (300 MHz, DMSO-d_6): δ 10.64 (s, 1H), 10.58 (s, 1H), 8.81 (s, 1H), 8.79 (s, 1H), 7.39–7.28 (m, 5H), 7.23 (s, 1H), 6.21 (s, 1H), 6.18 (s, 1H), 5.00 (s, 2H), 4.06–3.92 (m, 2H), 2.96–2.93 (m, 2H), 1.50–1.15 (m, 9H), 0.87–0.82 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.3, 169.0, 156.6, 156.0, 137.2, 128.3, 127.7, 65.1, 50.5, 49.0, 42.4, 33.1, 29.1, 22.7, 22.5, 22.3.

5.1.1.18. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-2-methyl-butyl)-ureido]-hexanoic acid methyl ester (3e). Yield 50.3%, mp = 50–52 °C; ESI-MS: *m/z* $[M+H]^+$ 465.5; ^1H NMR (300 MHz, DMSO-d_6): δ 7.71–7.30 (m, 5H), 7.24 (t, 1H, *J* = 5.4 Hz), 6.39 (t, 2H, 4.5 Hz), 5.00 (s, 2H), 4.12–4.06 (m, 2H), 3.61 (s, 3H), 3, 60 (s, 3H), 3.01–2.94 (m, 2H), 1.71–0.82 (m, 15H).

5.1.1.19. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-2-methyl-butyl)-ureido]-hexanoic acid (4e). Yield 44.6%, mp = 65–67 °C; ESI-MS: *m/z* $[M+H]^+$ 437.4; ^1H NMR (300 MHz, DMSO-d_6): δ 12.67 (s, 2H), 7.39–7.30 (m, 5H), 7.28–7.23 (m, 1H), 6.34–6.25 (m, 2H), 5.00 (s, 2H), 4.08–4.03 (m, 2H), 3.52–3.42 (m, 2H), 1.39–0.88 (m, 15H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 170.2, 169.9, 156.6, 156.0, 137.3, 128.3, 127.6, 65.1, 55.9, 51.3, 36.4, 31.0, 28.7, 23.0, 14.7, 11.5.

5.1.1.20. {5-Hydroxycarbamoyl-5-[3-(1-hydroxycarbamoyl-2-methyl-butyl)-ureido]-pentyl}-carbamic acid benzyl ester (5e). Yield 44.8%, mp = 75–77 °C; ESI-MS: *m/z* $[M+H]^+$ 467.5; ^1H NMR (300 MHz, DMSO-d_6): δ 10.63 (s, 1H), 10.59 (s, 1H), 8.85 (s, 1H), 8.81 (s, 1H), 7.39–7.30 (m, 5H), 7.24–7.23 (m, 1H), 6.30–6.22 (m, 2H), 5.00 (s, 2H), 4.06–3.79 (m, 2H), 2.95 (s, 2H), 1.91–1.17 (m, 9H), 0.92–0.71 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.0, 168.4, 156.8, 156.0, 137.2, 128.3, 127.7, 65.1, 54.8, 50.5, 37.5, 33.1, 29.2, 22.5, 15.2, 11.1.

5.1.1.21. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-2-benzyl)-ureido]-hexanoic acid methyl ester (3f). Yield: mp = 55–57 °C; ESI-MS: *m*/*z* $[M+H]^+$ 465.5; ^{1}H NMR (600 MHz,$CDCl_3$): δ 7.35–7.09 (m, 10H), 5.29 (br, 1H), 5.12 (br, 1H), 5.03–5.01 (m, 3H), 4.77–4.75 (m, 1H), 4.44–4.43 (m, 1H), 3.71 (s, 3H), 3.66 (s, 3H), 3.19–3.12 (m, 2H), 3.06–3.05 (m, 2H), 1.77–1.33 (m, 6H).

5.1.1.22. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-2-benzyl)-ureido]-hexanoic acid (4f). Yield 40.3%, mp = 67–70 °C; ESI-MS: *m*/*z* $[M+H]^+$ 499.5; ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.58 (s, 2H), 7.22–7.15 (m, 1H), 6.40–6.33 (m, 2H), 5.00 (s, 2H), 4.23–4.15 (m, 1H), 3.95–3.90 (m, 1H), 2.95–2.93 (m, 2H), 2.90–2.83 (m, 1H), 1.47–1.13 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 170.4, 170.2, 156.0, 155.9, 137.3, 135.0, 129.8, 128.9, 128.8, 128.2, 128.0, 127.7, 65.1, 52.6, 51.2, 43.0, 33.4, 28.9, 22.4.

5.1.1.23. {5-Hydroxycarbamoyl-5-[3-(1-hydroxycarbamoyl-2-pheny-ethyl)-ureido]-pentyl}-carbamic acid benzyl ester (5f). Yield 35.0%, mp = 127–130 °C; ESI-MS: *m*/*z* $[M+H]^+$ 501.2; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H), 10.58 (s, 1H), 8.82 (s, 2H), 7.39–7.23 (m, 10H), 7.20–7.15 (m, 1H), 6.39–6.24 (m, 2H), 5.00 (s, 2H), 4.27–4.16 (m, 1H), 3.95–3.90 (m, 1H), 2.95–2.93 (m, 2H), 2.84 (dd, 1H, *J* = 6.0 Hz, 22.8 Hz), 1.46–1.15 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.0, 168.5, 156.6, 156.1, 152.2, 137.6, 137.2, 129.2, 129.1, 128.3, 128.1, 128.0, 127.7, 65.1, 52.2, 50.1, 42.9, 33.0, 29.1, 22.4.

5.1.1.24. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-3-methylsulfanyl-propyl)-ureido]-hexanoic acid methyl ester (3g). Yield 33.6%, mp = 52–55 °C; ESI-MS: *m*/*z* $[M+H]^+$ 469.5; ^{1}H NMR (300 MHz,$CDCl_3$): δ 7.36–7.37 (m, 5H), 5.79 (br, 1H), 5.62 (br, 1H), 5.14 (br, 1H), 5.10 (s, 2H), 4.60–4.54 (m, 1H), 4.48–4.41 (m, 1H), 3.71 (s, 6H), 3.17–3.13 (m, 2H), 2.50 (t, 2H, *J* = 6.0 Hz), 2.14–1.26 (m, 11H).

5.1.1.25. 6-Benzyloxycarbonylamino-2-[3-(1-carboxy-3-methylsulfanyl-propyl)-ureido]-hexanoic acid (4g). Yield 34.7%, mp = 116–119 °C; ESI-MS: *m*/*z* $[M+H]^+$ 455.5; ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.91 (s, 1H), 7.36–7.33 (m, 5H), 7.30–7.24 (m, 1H), 6.46–6.22 (m, 2H), 5.00 (s, 2H), 2.98–2.94 (m, 2H), 2.03 (s, 3H), 1.53–1.15 (m, 10H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 173.5, 173.1, 157.2, 156.1, 137.2, 128.3, 127.7, 127.6, 65.1, 51.8, 51.5, 40.2, 31.5, 31.4, 29.3, 29.0, 22.4.

5.1.1.26. {5-Hydroxycarbamoyl-5-[3-(1-hydroxycarbamoyl-3-methylsulfanyl-propyl)-ureido]-pentyl}-carbamic acid benzyl ester (5g). Yield 39.7%, mp = 138–139 °C; ESI-MS: *m*/*z* $[M+H]^+$ 485.5; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.65 (s, 1H), 10.60 (s, 1H), 8.85 (s, 1H), 8.82 (s, 1H), 7.39–7.29 (m, 5H), 7.28 (s, 1H), 6.34–6.24 (m, 2H), 5.00 (s, 1H), 4.09–3.92 (m, 2H), 2.96–2.94 (m, 2H), 2.40–2.33 (m, 2H), 2.02 (s, 3H), 1.76–1.09 (m, 10H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.0, 168.6, 156.7, 156.0, 137.2, 128.3, 127.7, 65.1, 50.6, 50.0, 40.4, 33.2, 33.0, 29.3, 29.1, 22.5.

5.2. APN inhibition assay

IC_{50} values against APN were determined by using L-leu-*p*-nitroanilide as substrate and Microsomal aminopeptidase from Porcine Kidney Microsomes (Sigma) as lymphilized powder 15–25 units/mg protein. The assay was performed in 96-well plates 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 solutions of inhibitors were prepared in the assay buffer, and pH was neutralized to 7.5 by the addition of 0.1 M HCl or 0.1 M NaOH. All inhibitors were pre-incubated with APN for 10 min at room temperature.[12] The assay mixture, which contained the inhibitor solution (concentration dependent on the inhibitor), the enzyme solution (6 μg/mL final concentration), and the assay buffer, was adjusted to 200 μL.

5.3. MTT assay

Anti-tumor inhibitory activity was determined by MTT (3-[4,5-dimethyl-2-thiazolyl]-2.5-diphenyl-2H-tetrazolium bromide) method. In brief, ES-2 cells were plated in 96-well plates (5000 cells per well) and allowed to adhere and spread for 10 h, then incubated with 2500, 500, 100, 20 and 4 μg/mL of the compounds (Bestatin, **5b**, **5d**, **5f** and **5g**) for 48 h. 0.5% MTT solution was added to each well. After additional incubation for 4 h, DMSO was added and mixed for 10 min. Optical density values were measured at 570 nm.

5.4. Docking study

The representative compound **5d** was constructed with 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.

Acknowledgments

This work was supported by National High Technology Research and Development Program of China (863 project; Grant No. 2007AA02Z314), National Natural Foundation Research Grant (Grant Nos. 9071304; 30772654, 30728031) and Doctoral Foundation of Ministry of Education of the People's Republic of China (Grant No. 20060422029).

References and notes

1. Fukasawa, K.; Fujii, H.; Saitoh, Y.; Koizumi, K.; Aozuka, Y.; Sekine, K.; Yamada, M.; Saiki, I.; Nishikawa, K. *Cancer Lett.* **2006**, *243*, 135.
2. Fontijn, D.; Duyndam, M.; Berkel, M. v.; Yuana, Y.; Shapiro, L.; Pinedo, H.; Broxterman, H.; Boven, E. *Br J Cancer* **2006**, *94*, 1627.
3. Pasqualini, R. K. E.; Kain, R.; Lahdenranta, J.; Sakamoto, M.; Stryhn, A.; AAshmun, R.; HShapiro, L.; Arap, W.; Ruoslahti, E. *Cancer Res.* **2000**, *60*, 722.
4. Umezawa, H.; Aoyagi, T.; Suda, H.; Hamada, M. *Antibiotics* **1976**, *29*, 97.
5. Aoyagi, T.; Yoshida, S.; Nakamura, Y.; Shigihara, Y. *Antibiotics* **1990**, *43*, 143.
6. Roques, B. P.; Noble, F. *NIDA Res. Monogr.* **1995**, *248*, 263.
7. Mou, J.; Fang, H.; Liu, Y.; Shang, L.; Wang, Q.; Zhang, L.; Xu, W. *Bioorg. Med. Chem.* **2010**, *18*, 887.
8. Grembecka, J.; Mucha, A.; Cierpicki, T.; Kafarski, P. *J. Med. Chem.* **2003**, *46*, 2541–2655.
9. Wang, Q.; Chen, M.; Zhu, H.; Zhang, J.; Fang, H.; Wang, B.; Xu, W. *Bioorg. Med. Chem.* **2008**, *16*, 5473.
10. Tsai, J. H.; Takaoka, L. R.; Powell, N. A.; Nowick, J. S. *Org. Synth.* **2002**, *78*, 220.
11. Bennet, W. B.; Saunders, J. H.; Hard, E. E. *J. Am. Chem. Soc.* **1953**, *75*, 2101.
12. (a) Flipo, M.; Beghyn, T.; Charton, J.; Leroux, V. A.; Deprez, B. P.; Deprez-Poulain, R. F. *Bioorg. Med. Chem.* **2007**, *15*, 63; (b) Shang, L.; Wang, Q.; Fang, H.; Mu, J.; Wang, X.; Yuan, Y.; Wang, B.; Xu, W. *Bioorg. Med. Chem.* **2008**, *16*, 9984.