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Design, synthesis and primary activity evaluation of L-arginine derivatives as amino-peptidase N/CD13 inhibitors

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ABSTRACT

A series of L-arginine derivatives were designed, synthesized and assayed for their activities against amino-peptidase N (APN)/CD13 and metalloproteinase-2 (MMP-2). The results showed that most compounds exhibited high inhibitory activities against APN and low activities against MMP-2. Within this series, two compounds **5q** and **5s** (IC_{50} = 5.3 and 5.1 μM) showed similar inhibitory activities compared with bestatin (IC_{50} = 3.8 μM), which could be used as novel lead compounds for the future APN inhibitors development as anticancer agents.

1. Introduction

Amino-peptidase N (APN/CD13; EC 3.4.11.2) is a zinc-dependent metalloproteinase that cleaves neutral or basic amino acids from the N-terminus of oligopeptides. Scientist found that outside the hematopoietic system, APN was widely expressed on various kinds of cells. For example, epithelial cells of the intestine and kidney, hepatocytes, osteoclasts, endometrial cells, fibroblasts, endothelial cells, bone marrow stromal cells and on neuronal synaptic membranes. APN is involved in many physiology and pathology processes such as hydrolysis of nutrients, inactivation of bioactive peptides, binding of corona viruses, mediating cytomegalovirus infection and antigen presentation. Furthermore, APN has been a target for anti-tumor agents due to its important functions in ECM degradation, tumor cells invasion and tumor angiogenesis.[1–3]

In the past two decades, several APN inhibitors have been found. For example, Bestatin, Probestin, Amastatin, Actinonin, Phebestin, Lapstatin, AHPA-Val, Leuhistin, Curcumin and Pasmmaplin A.[4–13] Among them, bestatin has been launched and is now widely employed clinically as an anti-tumor agent.[14] In 2006, the 3D structure of APN has been studied according to the co-crystal complex of APN and bestatin by Kiyoshi.[15] Our group has studied the binding site and catalytic domain of APN based on this co-crystal complex. The binding sites of APN with bestatin can be divided to three parts. Part 1 is a hydrophobic pocket (S_1); part 2 is the zinc binding group (ZBG); part 3 is another hydrophobic pocket in the other side (S_1').

With the help of computer-aided molecular design, in 2008, our group designed and synthesized a series of L-lysine derivatives and found that some compounds exhibited potential APN inhibitory activities[16] (Fig. 1). Also in 2008, the crystal structures of *Escherichia coli* amino-peptidase N (e PepN) in complex with L-arginine, L-lysine, L-phenylalanine, L-tyrosine and L-tryptophan have been determined to understand the structural basis for APN's hydrolysis pattern. The result showed that all these amino acids bind with their backbone atoms close to the active-site zinc ion and their side chain occupying the S_1 pocket of e PepN. And the specificity of the S_1-binding pocket suggest that the preferred amino acid is, in decreasing order, arginine, lysine, tyrosine and phenylalanine.[17] Additionally, arginine is similar with lysine in structure except the guanidinium group and the longer carbon chain. According to the former reasons, we choose L-arginine as the starting material in order to get more efficient and potential APN inhibitors. The compounds designed are showed in Figure 1.

2. Chemistry

All the target compounds were designed and synthesized via the route shown in Scheme 1. The guanidinium group of

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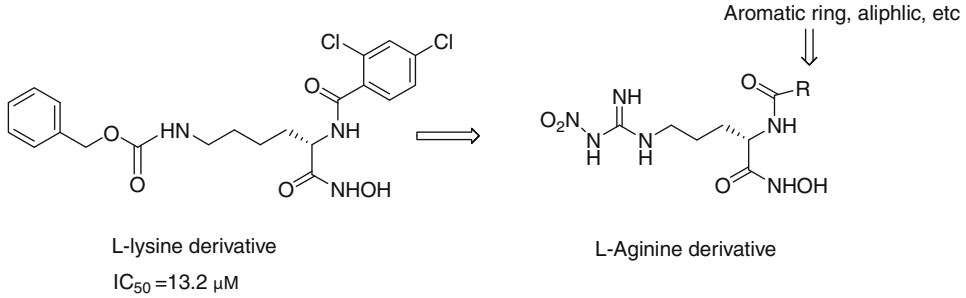


Figure 1. The structure of L-lysine derivative and L-aginine derivative.

compound **1** was protected by nitro group to get compound **2**. Compound **2** was then esterificated with methanol under HCl atmosphere to get compound **3**. The acylation of compound **3** with acyl chloride, carboxylic acid or sulfochloride led to compounds **4a–w**, **6a,b**. Finally the ester groups of **4a–w**, **6a,b** were treated with NHOK in anhydrous methanol to get the target compounds **5a–w**, **7a,b**.

3. Results and discussion

All the inhibition results were listed in Table 1. Similar to APN, MMP-2 is also a zinc-dependant metalloproteinase that involved in tumor invasion and metastasis. Thus the assay was performed on both of APN and MMP-2 so as to identify the compounds selectivity. Bestatin was used as the positive control.

Almost all the compounds except **5a**, **5c**, **5i**, **5v** and **7b** showed better activities against APN than MMP-2. For example, **5q** with an IC_{50} (MMP-2)/ IC_{50} (APN) ratio equals to 65.8, while **5s** equals to 46.3. The result, to a certain extent, confirmed our strategy for designing APN inhibitors. This possibly descript from the differences between the structures of two enzymes, leading to different requirements for their respective inhibitors. APN is a membrane-bound zinc exopeptidase that catalyzed the removal of NH_2-terminal amino acid from the peptide, while MMP-2 is a zinc-dependent endopeptidase that could cut the peptide to parts from the specific amino acid residue of the peptide. The former L-lysine derivatives and 3-phenylpropane-1, 2-diamine derivatives designed by our group to inhibit APN all showed better activities against MMP-2.[16,18] So L-arginine derivatives are more suitable for APN inhibitors.

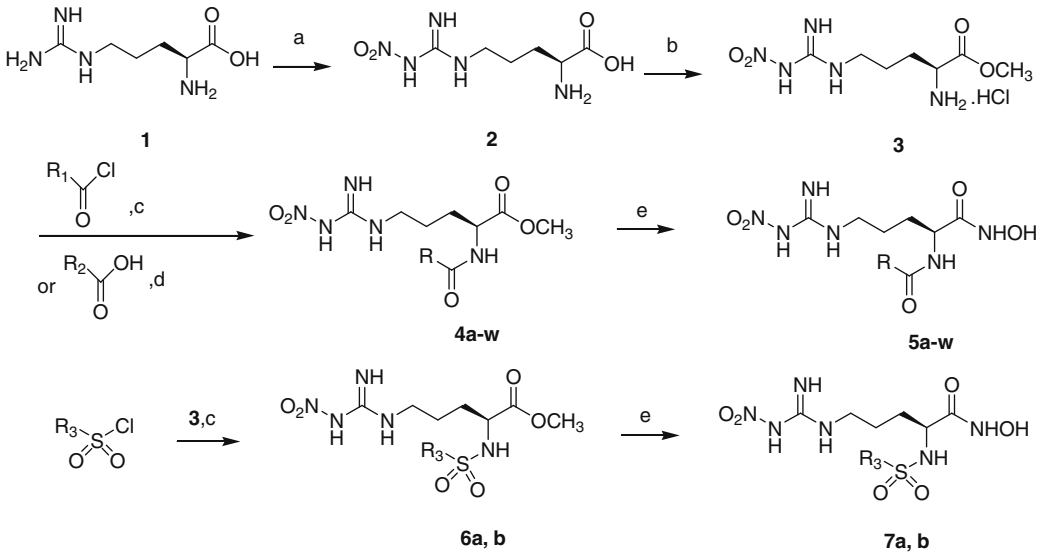
Comparing **5a–d** and **5e–w**, we could find that the compounds contained substituted phenyl groups have better activities than those contained unsubstituted phenyl groups.

Among compounds **5e–w**, we can conclude that the compounds contained two substituted groups on phenyl group have more potent activities than others. The most active compounds **5q** and **5s** both contain bi-substituted phenyl group. It is worth mentioning that the 2,4-dichlorobenzyl moiety of **5q** was also effective in the precious L-lysine derivatives.[16] The possible reason might be that the 2,4-dichloro substituted benzene ring could accommodate the hydrophobic site of APN suitably, suggesting it is a potential moiety for APN inhibitors.

From compounds **7a,b**, we could confirm that the compounds contained sulfonyl groups showed equal activities against APN and MMP-2. That coincides with the fact that some sulfonyl groups containing compounds our group had synthesized before showed better MMP-2 inhibitory activities.[19,20]

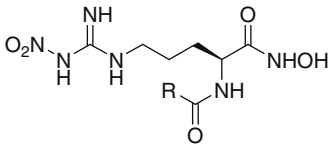
The most active compounds are **5q** and **5s** (IC_{50} = 5.3 and 5.1 μM), which have comparable activity with bestatin (IC_{50} = 3.8 μM). **5o**, **5t** and **5l** also have considerable activities (IC_{50} = 15.9, 14.6 and 16.5 μM, respectively).

In order to investigate the interaction of our compounds with APN, the most active compound **5s** 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



Scheme 1. Reagents and conditions: (a) fuming nitric acid, fuming sulfuric acid; (b) MeOH, HCl; (c) Et_3N, THF, 0 °C; (d) Et_3N, TBTU, CH_2Cl_2; (e) NHOK, MeOH.

Table 1
The structure and inhibitory activities of compounds against APN and MMP-2



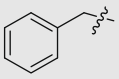
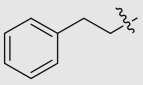
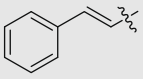
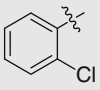
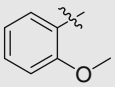
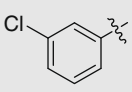
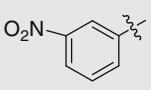
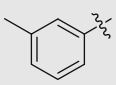
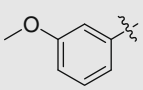
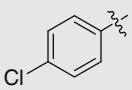
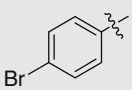
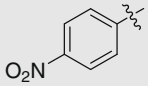
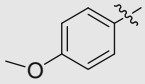
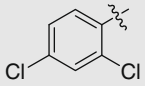
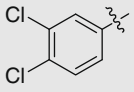
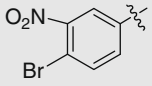
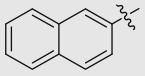
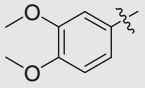
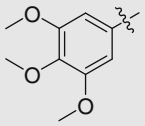
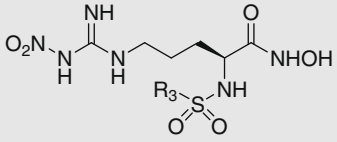
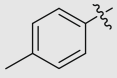
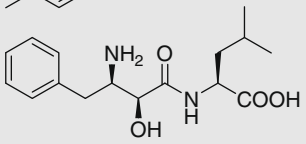
Compounds	R	IC_{50}[a] (μM)		IC_{50}(MMP-2)/IC_{50}(APN)
		APN	MMP-2	
5a		1662.6 ± 4.9	8.7 ± 3.9	0.05
5b		331.8 ± 2.8	1642.9 ± 8.4	4.9
5c		1008.0 ± 5.9	36.7 ± 1.6	0.04
5d		80.6 ± 1.2	692.8 ± 5.7	8.6
5e		20.4 ± 1.1	149.6 ± 2.8	7.3
5f		21.9 ± 1.5	176.0 ± 2.4	8.0
5g		221.5 ± 2.6	2067.2 ± 23.5	9.3
5h		78.4 ± 2.5	253.2 ± 3.1	3.2
5i		202.2 ± 2.9	89.2 ± 2.9	0.4
5j		43.4 ± 1.3	101.6 ± 2.1	2.3
5k		42.8 ± 1.9	623.1 ± 4.5	14.6
5l		16.5 ± 2.2	150.8 ± 3.0	9.1
5m		215.1 ± 3.3	237.6 ± 3.6	1.1
5n		443.4 ± 2.7	483.6 ± 3.9	1.1

Table 1 (*continued*)

Compounds	R	IC_{50}[a] (μM)		IC_{50}(MMP-2)/IC_{50}(APN)
		APN	MMP-2	
5o		15.9 ± 2.1	203.6 ± 4.6	12.8
5p		60.2 ± 2.2	322.5 ± 3.2	5.4
5q		5.3 ± 1.2	348.5 ± 3.6	65.8
5r		33.5 ± 1.7	193.6 ± 2.9	5.8
5s		5.1 ± 0.6	236.3 ± 4.5	46.3
5t		14.6 ± 0.8	85.5 ± 2.6	5.9
5u		26.9 ± 1.3	345.5 ± 3.8	12.8
5v		270.6 ± 3.4	41.1 ± 1.8	0.2
5w		85.7 ± 2.1	226.4 ± 2.6	2.6



Compounds	R_3	IC_{50}[a] (μM)		IC_{50}(MMP-2)/IC_{50}(APN)
		APN	MMP-2	
7a		60.2 ± 1.8	100.4 ± 3.9	1.7
7b		108.8 ± 2.3	28.9 ± 2.4	0.3
Bestatin		3.8 ± 0.1	162.0 ± 4.8	42.6

[a] Mean value of three experiments and standard deviation are given.

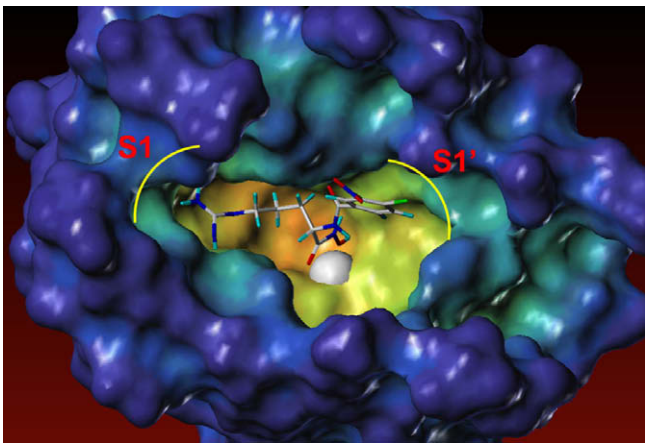


Figure 2. The docking mode of compound **5s** with APN. Zinc ion is shown as pale sphere.

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. From Figure 2, we can see the backbone of **5s** inserted to S_1 pocket, the hydroxymate of **5s** interacted with the zinc ion of APN and the 4-bromo-3-nitro benzamide side chain extended to S_1' pocket.

For a further and detail understanding of the binding mode of **5s** with APN, a 2D picture was also created with the program LIGPLOT. In Figure 3, we can see the backbone of **5s** could form hydrophobic contacts with Glu^{121}, Met^{260} and Tyr^{376} of S_1 pocket and form hydrogen bond with Glu^{121} by the imine of guanidinium group. The two oxygen atoms of hydroxymate chelated with the zinc ion of APN. The carbonyl of amide in R position could form hydrogen bond with Gly^{261} and Ala^{262} of S_1' pocket. The R substituted side chain of **5s** could form hydrophobic contact with Gly^{261} of S_1' pocket. While, the nitro group at the aromatic ring could form hydrogen bonds with $Arg^{783}$and Arg^{825}.

Although the computed information partially supported our assumption, the exact binding mode of the L-arginine derivatives with APN should be obtained from further X-ray crystal studies.

4. Conclusion

In all, we have synthesized a new series of L-aginine derivatives as APN inhibitors. Most of the compounds showed potent activity and selectivity against APN, in which **5q** and **5s** were comparable to bestatin and could be used as lead compounds for the develop-

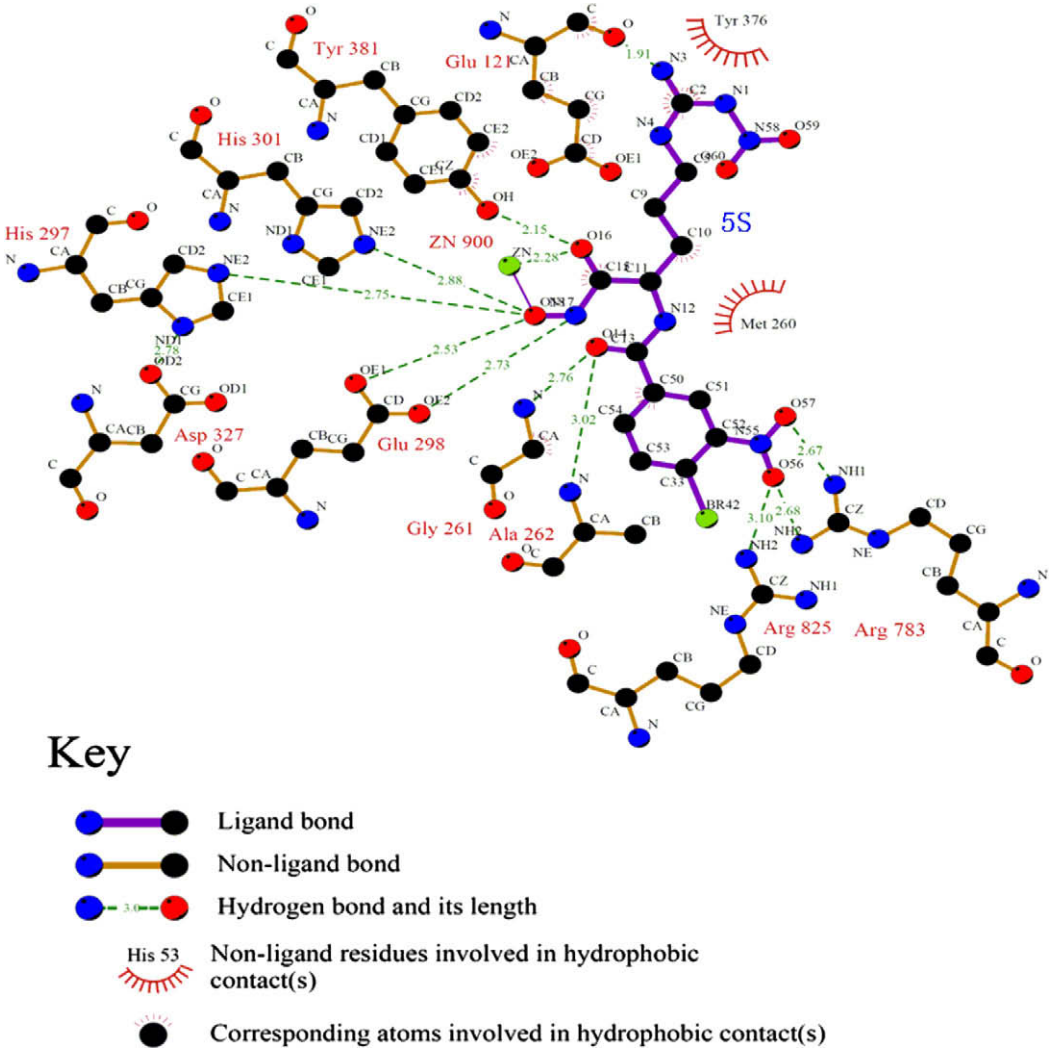


Figure 3. The docking result of **5s** with APN showed by LIGPLOT. Compound **5s** is shown in violet.

ment of future low molecular-weight peptidomimetic APN inhibitors as anticancer agents.

5. Experimental

5.1. Chemistry: general procedures

All the material were commercial available. All the solvents except fuming nitric acid and fuming sulfuric acid were distilled before use. All the 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. Proton NMR spectra were determined on a Brucker DRX spectrometer (300 MHz), δ in parts per million and J in hertz and TMS was used as an internal standard. Measurements were made in D_2O solutions. ESI-MS were determined on an API 4000 spectrometer. Elemental analysis for compound was performed using an elementar vario EL III CN analyzer (Germany). Melting points were determined on an electrothermal melting point apparatus (uncorrected).

5.1.1. 2-Amino-5-(3-nitroguanidino)pentanoic acid (2)

The title compound was prepared as described by Hashimoto et al.[21] from compound **1**.

5.1.2. Methyl 2-amino-5-(3-nitroguanidino)pentanoate hydrochloride (3)

The title compound was prepared as described by Jordis[22] from compound **2**.

5.1.3. Methyl 5-(3-nitroguanidino)-2-(2-phenylacetamido)-pentanoate (4a)

Phenylacetic acid (0.68 g, 5 mmol) and trimethylamine (3 equiv) were dissolved in 30 ml anhydrous dichloromethane (DCM). To this stirring solution was added TBTU (1.3 equiv) followed by compound **3**. The resulting solution was stirred for 6 h and then washed with saturated Na_2CO_3, 10% citric acid and brine. Lying for a while, the white solid precipitated in DCM was filtered and washed with DCM, then dried in vacuum drying oven. Finally, 1.60 g product was obtained, yield 90.8%, mp 115–117 °C. ESI-MS m/z: 352.4 $(M+H)^+$; ^1H NMR (D_2O): 1.58–1.65 (m, 2H), 1.71–1.76 (m, 2H), 3.13–3.14 (m, 2H), 3.47 (s, 2H), 3.62 (s, 3H), 4.22–4.26 (m, 1H), 7.20–7.30 (m, 5H).

Compounds **4c, 4e-n** and **4r-w** were synthesized following the procedure described above.

Methyl 2-cinnamamido-5-(3-nitroguanidino)pentanoate (**4c**): (1.46 g, 80.7%).

Methyl 2-(2-chlorobenzamido)-5-(3-nitroguanidino)pentanoate (**4e**): (1.67 g,89.9%).

Methyl 2-(2-iodobenzamido)-5-(3-nitroguanidino)pentanoate (**4f**): (2.02 g, 87.2%).

Methyl 2-(2-methylbenzamido)-5-(3-nitroguanidino)pentanoate (**4g**): (1.62 g, 92.3%).

Methyl 2-(2-methoxybenzamido)-5-(3-nitroguanidino)pentanoate (**4h**): (1.61 g, 87.7%).

Methyl 2-(3-chlorobenzamido)-5-(3-nitroguanidino)pentanoate (**4i**): (1.69 g, 91.0%).

Methyl 2-(3-nitrobenzamido)-5-(3-nitroguanidino)pentanoate (**4j**): (1.76 g, 92.1%).

Methyl 2-(3-methylbenzamido)-5-(3-nitroguanidino)pentanoate (**4k**): (1.61 g, 91.5%).

Methyl 2-(3-methoxybenzamido)-5-(3-nitroguanidino)pentanoate (**4l**): (1.65 g, 89.7%).

Methyl 2-(4-chlorobenzamido)-5-(3-nitroguanidino)pentanoate (**4m**): (1.69 g, 91.1%).

Methyl 2-(4-bromobenzamido)-5-(3-nitroguanidino)pentanoate (**4n**): (1.87 g, 90.0%).

Methyl 2-(3,4-dichlorobenzamido)-5-(3-nitroguanidino)pentanoate (**4r**): (1.78 g, 87.5%).

Methyl 2-(4-bromo-3-nitrobenzamido)-5-(3-nitroguanidino)pentanoate (**4s**): (1.85 g, 80.1%).

Methyl 2-(2-naphthamido)-5-(3-nitroguanidino)pentanoate (**4t**): (1.61 g, 83.4%).

Methyl 2-(3,4-dimethoxybenzamido)-5-(3-nitroguanidino)pentanoate (**4u**): (1.67 g,84.4%).

Methyl 5-(3-nitroguanidino)-2-(3,4,5-trimethoxybenzamido)pentanoate (**4v**): (1.80 g, 84.3%).

Methyl 5-(3-nitroguanidino)-2-(thiophene-2-carboxamido)pentanoate (**4w**): (1.44 g, 84.0%).

5.1.4. Methyl 5-(3-nitroguanidino)-2-(3-phenylpropanamido)pentanoate (4b)

Compound **3** (1.35 g, 5 mmol) and triethylamine (1 equiv) were mixed in 30 ml anhydrous DCM under ice-bath. 3-Phenylpropionyl chloride (1.2 equiv) dissolved in DCM was added dropwise. The resulting solution was stirred for 2 h and then washed with saturated Na_2CO_3, 10% citric acid and brine. After lying for a while, white solid would precipitate in DCM. The solid was filtered and washed with DCM, then dried in vacuum drying oven. Finally 1.69 g product was obtained, yield 92.7%.

Compounds **4d, 4o, 4p** and **4q** were synthesized following the procedure described above.

Methyl 2-benzamido-5-(3-nitroguanidino)pentanoate (**4d**): (1.43 g, 85.1%).

Methyl 2-(4-nitrobenzamido)-5-(3-nitroguanidino)pentanoate (**4o**): (1.74 g, 91.3%).

Methyl 2-(4-methoxybenzamido)-5-(3-nitroguanidino)pentanoate (**4p**): (1.65 g, 89.9%).

Methyl 2-(2,4-dichlorobenzamido)-5-(3-nitroguanidino)pentanoate (**4q**): (1.68 g, 90.3%).

5.1.5. Methyl 2-(4-methylphenylsulfonamido)-5-(3-nitroguanidino)pentanoate (6a)

Compound **6a** was synthesized following the procedure of compound **4b**. Finally, 1.72 g product was got, yield 88.9%.

Compound **6b** was synthesized following the procedure of compound **6a**.

Methyl 5-(3-nitroguanidino)-2-(phenylsulfonamido)pentanoate (**6b**): (1.62 g, 87.0%).

5.1.6. *N*-Hydroxy-5-(3-nitroguanidino)-2-(2-phenylacetamido)-pentanamide (5a)

To a solution of compound **4a** (0.71 g, 2 mmol) in 7 ml anhydrous methanol at room temperature was added dropwise a solution of NHOK (6 mmol) in methanol (3.4 ml). The mixture was stirred for 12 h and the solvent was evaporated in vacuum. The residue was purified by column chromatography (dichloromethane/methanol = 20/1–5/1). Finally, 059 g **5a** was obtained. White solid, yield 83.4%, mp 76–79 °C. ESI-MS m/z: 353.5 $(M+H)^+$; ^1H NMR (D_2O): 1.51–1.77 (m, 4H), 3.00–3.13 (m, 2H), 3.41–3.50 (m, 2H), 4.09–4.17 (m, 1H), 7.18–7.32 (m, 5H). Anal. Calcd for $C_{14}H_{20}N_6O_5$: C, 47.72; H, 5.72; N, 23.85. Found: C, 47.62; H, 5.65; N, 23.75.

Compounds **5b–w** and **7a,b** were synthesized following the procedure described above.

5.1.6.1. *N*-Hydroxy-5-(3-nitroguanidino)-2-(3-phenylpropanamido)pentanamide (5b). White solid, yield 78.8%, mp 161–162 °C. ESI-MS m/z: 367.3 $(M+H)^+$; ^1H NMR (D_2O):1.31–1.62 (m, 4H), 2.42–2.50 (m, 2H), 2.76–2.79 (t, J = 7.5 Hz, 2H), 3.12–3.16 (m, 2H), 4.12–4.18 (m, 1H), 7.16–7.29 (m, 5H). Anal. Calcd for

$C_{15}H_{22}N_6O_5$: C, 49.19; H, 6.05; N, 22.94. Found: C, 49.01; H, 5.99; N, 22.88.

5.1.6.2. 2-Cinnamamido-*N*-hydroxy-5-(3-nitroguanidino)pentanamide (5c). White solid, yield 70.4%, mp 296–298 °C. ESI-MS *m*/*z*: 365.3 $(M+H)^+$; ^1H NMR (D_2O):1.53–1.57 (m, 2H), 1.73–1.77 (m, 2H), 3.28–3.35 (m, 2H), 4.01–4.04 (m, 1H), 6.76–6.84 (d, *J* = 16 Hz, 1H), 7.38–7.48 (m, 4H), 7.52–7.58 (m, 2H). Anal. Calcd for $C_{15}H_{20}N_6O_5$: C, 49.45; H, 5.53; N, 23.07. Found: C, 49.26; H, 5.49; N, 23.15.

5.1.6.3. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)benzamide (5d). White solid, yield 73.5%, mp 76–79 °C. ESI-MS *m*/*z*: 339.5 $(M+H)^+$; ^1H NMR (D_2O): 1.54–1.58 (m, 2H), 1.66–1.75 (m, 2H), 3.15–3.18 (m, 2H), 4.31–4.39 (m, 1H), 7.44–7.56 (m, 5H). Anal. Calcd for $C_{13}H_{18}N_6O_5$: C, 46.15; H, 5.36; N, 24.84. Found: C, 46.03; H, 5.22; N, 24.68.

5.1.6.4. 2-Chloro-*N*-(1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)benzamide (5e). White solid, yield 85.8%, mp 105–108 °C. ESI-MS *m*/*z*: 373.3 $(M+H)^+$; ^1H NMR (D_2O): 1.52–1.69 (m, 4H), 3.14–3.17 (m, 2H), 4.30–4.34 (m, 1H), 7.38–7.47 (m, 4H). Anal. Calcd for $C_{13}H_{17}ClN_6O_5$: C, 41.89; H, 4.60; N, 22.55. Found: C, 41.72; H, 4.49; N, 22.63.

5.1.6.5. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-2-iodobenzamide (5f). Jacinth solid, yield 79.8%, mp 76–80 °C. ESI-MS *m*/*z*: 465.3 $(M+H)^+$; ^1H NMR (D_2O): 1.54–1.69 (m, 4H), 3.15–3.19 (m, 2H), 4.27–4.30 (m, 1H), 7.13–7.46 (m, 4H). Anal. Calcd for $C_{13}H_{17}IN_6O_5$: C,33.64; H, 3.69; N, 18.10. Found: C, 33.48; H, 3.66; N, 18.02.

5.1.6.6. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-2-methylbenzamide (5g). White solid, yield 89.9%, mp 201–203 °C. ESI-MS *m*/*z*: 353.5 $(M+H)^+$; ^1H NMR (D_2O): 1.48–1.52 (m, 2H), 1.68–1.73 (m, 2H), 2.27 (s, 3H), 3.16–3.17 (d, *J* = 5.4 Hz, 2H), 4.12–4.18 (m, 1H), 7.22–7.33 (m, 4H). Anal. Calcd for $C_{14}H_{20}N_6O_5$: C, 47.72; H, 5.72; N, 23.85. Found: C, 47.51; H, 5.60; N, 23.81.

5.1.6.7. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-2-methoxybenzamide (5h). Jacinth solid, yield 85.3%, mp 173–175 °C. ESI-MS *m*/*z*: 369.3 $(M+H)^+$; ^1H NMR (D_2O): 1.52–1.63 (m, 2H), 1.67–1.76 (m, 2H), 3.14–3.22 (m, 2H), 3.92 (S, 3H), 4.38–4.45(m, 1H), 7.04–7.08 (t, *J* = 7.2 Hz, 1H), 7.17–7.20 (d, *J* = 7.8 Hz, 1H),7.48–7.54 (m, 1H), 7.82–7.85 (d, *J* = 7.8 Hz, 1H). Anal. Calcd for $C_{14}H_{20}N_6O_6$: C, 45.65; H, 5.47; N, 22.82. Found: C, 45.48; H, 5.43; N, 22.67.

5.1.6.8. 3-Chloro-*N*-(1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)benzamide (5i). White solid, Yield 80.0%, mp 77–79 °C. ESI-MS *m*/*z*: 373.2 $(M+H)^+$; ^1H NMR (D_2O): 1.55–1.76 (m, 4H), 3.15–3.17 (m, 2H), 4.32–4.34 (m, 1H), 7.47–7.96 (m, 4H). Anal. Calcd for $C_{13}H_{17}ClN_6O_5$: C, 41.89; H, 4.60; N, 22.55. Found: C, 41.63; H, 4.53; N, 22.47.

5.1.6.9. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-3-nitrobenzamide (5j). Yellow solid, yield 83.1%, mp 149–151 °C. ESI-MS *m*/*z*: 384.3 $(M+H)^+$; ^1H NMR (D_2O): 1.52–1.54 (m, 2H), 1.73–1.75 (m, 2H), 3.15–3.17 (m, 2H), 4.35–4.37 (m, 1H), 7.72–7.77 (t, *J* = 7.8 Hz, 1H), 8.32–8.37 (m, 2H), 8.68 (s, 1H). Anal. Calcd for $C_{13}H_{17}N_7O_7$: C, 40.73; H, 4.47; N, 25.58. Found: C, 40.55; H, 4.53; N, 25.61.

5.1.6.10. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-3-methylbenzamide (5k). White solid, yield 86.5%, mp 176–179 °C. ESI-MS *m*/*z*: 353.5 $(M+H)^+$; ^1H NMR (D_2O):1.49–1.54 (m, 2H), 1.71–1.75 (m, 2H), 2.36 (S, 3H), 3.16–3.18 (d, *J* = 5.4 Hz, 2H), 4.30–4.38 (m, 1H), 7.33–7.35 (d, *J* = 4.8 Hz, 2H), 7.66–7.71 (m, 2H). Anal. Calcd for $C_{14}H_{20}N_6O_5$: C, 47.72; H, 5.72; N, 23.85. Found: C, 47.55; H, 5.78; N, 23.66.

5.1.6.11. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-3-methoxybenzamide (5l). White solid, yield 85.6%, mp 68–71 °C. ESI-MS *m*/*z*: 369.3 $(M+H)^+$; ^1H NMR (D_2O): 1.57–1.62 (m, 2H), 1.69–1.75 (m, 2H), 3.16–3.18 (m, 2H), 3.81 (s, 3H), 4.30–4.38 (m, 1H), 7.08–7.11 (m, 1H), 7.34–7.39 (m, 1H), 7.45–7.50 (m, 2H). Anal. Calcd for $C_{14}H_{20}N_6O_6$: C, 45.65; H, 5.47; N, 22.82. Found: C, 45.54; H, 5.38; N, 22.74.

5.1.6.12. 4-Chloro-*N*-(1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)benzamide (5m). White solid, yield 82.1%, mp 67–72 °C. ESI-MS *m*/*z*: 373.3 $(M+H)^+$; ^1H NMR (D_2O): 1.49–1.52 (m, 2H), 1.69–1.73 (m, 2H), 3.16–3.18 (d, *J* = 5.1 Hz, 2H), 4.29–4.36 (m, 1H), 7.53–7.56 (d, *J* = 8.4 Hz, 2H), 7.90–7.93 (d, *J* = 8.4 Hz, 2H). Anal. Calcd for $C_{13}H_{17}ClN_6O_5$: C, 41.89; H, 4.60; N, 22.55. Found: C, 41.48; H, 4.79; N, 22.56.

5.1.6.13. 4-Bromo-*N*-(1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)benzamide (5n). Jacinth solid, yield 84.4%, mp 147–149 °C. ESI-MS *m*/*z*: 417.4 $(M+H)^+$; ^1H NMR (D_2O):1.50–1.52 (m, 2H), 1.68–1.78 (m, 2H), 3.16–3.18 (m, 2H), 4.28–4.36 (m, 1H), 7.67–7.80 (d, *J* = 8.4 Hz, 2H), 7.82–7.85 (d, *J* = 8.4 Hz, 2H). Anal. Calcd for $C_{13}H_{17}BrN_6O_5$: C, 37.42; H, 4.11; N, 20.14. Found: C, 37.18; H, 4.24; N, 19.93.

5.1.6.14. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-4-nitrobenzamide (5o). White solid, yield 91.3%, mp 148–150 °C. ESI-MS *m*/*z*: 384.3 $(M+H)^+$; ^1H NMR (D_2O):1.51–1.54 (m, 2H), 1.69–1.76 (m, 2H), 3.14–3.16 (m, 2H), 4.31–4.36 (m, 1H), 8.09–8.11 (d, *J* = 8.4 Hz, 2H), 8.29–8.32 (d, *J* = 8.4 Hz, 2H). Anal. Calcd for $C_{13}H_{17}N_7O_7$: C, 40.73; H, 4.47; N, 25.58. Found: C, 40.46; H, 4.39; N, 25.61.

5.1.6.15. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-4-methoxybenzamide (5p). White solid, yield 86.7%, mp 156–159 °C. ESI-MS *m*/*z*: 369.2 $(M+H)^+$; ^1H NMR (D_2O):1.50–1.52 (m, 2H), 1.69–1.71 (m, 2H), 3.16–3.19 (m, 2H), 3.81(s, 3H), 4.31–4.33 (m, 1H), 6.97–7.00 (d, *J* = 8.7 Hz, 2H), 7.86–7.89 (d, *J* = 8.7 Hz, 2H). Anal. Calcd for $C_{14}H_{20}N_6O_6$: C, 45.65; H, 5.47; N, 22.82. Found: C, 45.55; H, 5.42; N, 22.80.

5.1.6.16. 2,4-Dichloro-*N*-(1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)benzamide (5q). White solid, yield 83.4%, mp 154–156 °C. ESI-MS *m*/*z*: 407.4 $(M+H)^+$; ^1H NMR (D_2O):1.61–1.64 (m, 2H), 1.73–1.77 (m, 2H), 3.16–3.18 (m, 2H), 4.26–4.33 (m, 1H), 7.43–7.51 (m, 2H), 7.67–7.68 (d, *J* = 1.8 Hz, 1H). Anal. Calcd for $C_{13}H_{16}Cl_2N_6O_5$: C, 38.34; H, 3.96; N, 20.64. Found: C, 38.23; H, 4.04; N, 20.52.

5.1.6.17. 3,4-Dichloro-*N*-(1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)benzamide (5r). White solid, yield 86.4%, mp 130–134 °C. ESI-MS *m*/*z*: 407.5 $(M+H)^+$; ^1H NMR (D_2O):1.50–1.52 (m, 2H), 1.63–1.76 (m, 2H), 3.16–3.18 (d, *J* = 5.7 Hz, 2H), 4.29–4.36 (m, 1H), 7.75–7.78 (d, *J* = 8.4 Hz, 1H), 7.85–7.88 (dd, J_1 = 8.4 Hz, J_2 = 2.1 Hz, 1H), 8.17–8.18 (s, d, *J* = 2.1 Hz, 1H). Anal. Calcd for $C_{13}H_{16}Cl_2N_6O_5$: C, 38.34; H, 3.96; N, 20.64. Found: C, 38.19; H, 3.94; N, 20.69.

5.1.6.18. 4-Bromo-*N*-(1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-3-nitrobenzamide (5s). Brown solid, yield 69.3%, mp 147–150 °C. ESI-MS *m*/*z*: 462.4 $(M+H)^+$; ^1H NMR (D_2O):1.54–1.56 (m, 2H), 1.73–1.76 (m, 2H), 3.16–3.18 (m, 2H), 4.34–4.36 (m, 1H), 7.42–7.45 (d, *J* = 9 Hz, 1H), 7.93–7.96 (d, *J* = 9 Hz, 1H), 8.52 (s, 1H). Anal. Calcd for $C_{13}H_{16}BrN_7O_7$: C, 33.78; H, 3.49; N, 17.29. Found: C, 33.60; H, 3.42; N, 17.35.

5.1.6.19. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-2-naphthamide (5t). Yellow solid, yield 75.4%, mp 72–74 °C. ESI-MS *m*/*z*: 389.4 $(M+H)^+$; ^1H NMR (D_2O): 1.60–1.73 (m, 2H), 1.77–1.88 (m, 2H), 3.17–3.20 (m, 2H), 4.38–4.43 (m, 1H), 7.58–7.80 (m, 4H), 7.98–8.05 (m, 3H). Anal. Calcd for $C_{17}H_{20}N_6O_5$: C, 52.57; H, 5.19; N, 21.64. Found: C, 52.28; H, 5.07; N, 21.46.

5.1.6.20. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-3,4-dimethoxybenzamide (5u). White solid, yield 90.0%, mp 148–151 °C. ESI-MS *m*/*z*: 399.3 $(M+H)^+$; ^1H NMR (D_2O):1.49–1.53 (m, 2H), 1.71–1.77 (m, 2H), 3.16–3.18 (m, 2H), 3.80 (s, 6H), 4.34–4.36 (m, 1H), 6.99–7.02 (d, *J* = 8.4 Hz, 1H), 7.48–7.54(m, 2H). Anal. Calcd for $C_{15}H_{22}N_6O_7$: C, 45.22; H, 5.57; N, 21.10. Found: C, 45.01; H, 5.49; N, 21.00.

5.1.6.21. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-3,4,5-trimethoxybenzamide (5v). White solid, yield 85.2%, mp 101–104 °C. ESI-MS *m*/*z*: 429.5 $(M+H)^+$; ^1H NMR (D_2O):1.47–1.56 (m, 2H), 1.68–1.72 (m, 2H), 3.14–3.17 (m, 2H), 3.70 (s, 9H), 4.35–4.40 (m, 1H), 7.25 (s, 2H). Anal. Calcd for $C_{16}H_{24}N_6O_8$: C, 44.86; H, 5.65; N, 19.62. Found: C, 44.49; H,5.56; N, 19.46.

5.1.6.22. *N*-(1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)thiophene-2-carboxamide (5w). White solid, yield 78.6%, mp 160–162 °C. ESI-MS *m*/*z*: 345.4 $(M+H)^+$; ^1H NMR (D_2O):1.50–1.69 (m, 2H), 1.71–1.80 (m, 2H), 3.16–3.18 (d, *J* = 5.4 Hz, 2H), 4.29–4.31 (m, 1H), 7.13–7.16 (t, *J* = 4.8 Hz, 1H), 7.76–7.77 (d, *J* = 5.4 Hz, 1H), 7.82–7.85 (d, *J* = 3.0 Hz, 1H). Anal. Calcd for $C_{11}H_{16}N_6O_5S$: C, 38.37; H, 4.68; N, 24.41. Found: C, 38.12; H, 4.62; N, 24.30.

5.1.6.23. *N*-Hydroxy-5-(3-nitroguanidino)-2-(phenylsulfonamido)pentanamide (7a). White solid, yield 86.6%, mp 59–61 °C. ESI-MS *m*/*z*: 375.3 $(M+H)^+$; ^1H NMR (D_2O): 1.15–1.23 (m, 2H), 1.30–1.49 (m, 2H), 2.98–3.08 (m, 2H), 3.52–3.57 (m, 1H), 7.54–7.61(m, 2H), 7.76–7.89 (m, 3H). Anal. Calcd for $C_{12}H_{18}N_6O_6S$: C, 38.50; H, 4.85; N, 22.45. Found: C, 38.29; H, 4.76; N, 22.63.

5.1.6.24. *N*-Hydroxy-2-(4-methylphenylsulfonamido)-5-(3-nitroguanidino)pentanamide (7b). Orange solid, yield 82.2%, mp 64–66 °C. ESI-MS *m*/*z*: 389.3 $(M+H)^+$; ^1H NMR (D_2O): 1.13–1.35 (m, 2H), 1.38–1.49 (m, 2H), 2.29–2.36 (m, 3H), 2.99–3.06 (q, *J* = 7.2 Hz, 2H), 3.50–3.54 (m, 1H), 7.11–7.13 (d, *J* = 8.1 Hz, 1H), 7.32–7.35 (d, *J* = 8.1 Hz, 1H),7.46–7.49 (d, *J* = 8.1 Hz, 1H), 7.63–7.66 (d, *J* = 8.1 Hz, 1H). Anal. Calcd for $C_{13}H_{20}N_6O_6S$: C, 40.20; H, 5.19; N, 21.64. Found: C, 40.06; H, 5.23; N, 21.58.

5.2. APN inhibition assay

IC_{50} values against APN were determined by using L-Leu-*p*-nitroanilide as substrate and microsomal amino-peptidase from Porcine Kidney Microsomes (Sigma) as 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 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 μg/mL final concentration), and the assay buffer, was adjusted to 200 μL.

5.3. MMP-2 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.

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References and notes

1. Nathalie, L.; Cynthia, M. C.; Emmanuel, R.; Ann, B.; Bernard, P. R.; Marie-Claude, F. Z. *Biochemistry* **1998**, *37*, 686.
2. Florence, N.; Nathalie, L.; Sophie, D. N.; René, L. K.; Laurent, B.; Hui, X. C.; Marie-Claude, F. Z.; Bernard, P. R. *FEBS Lett.* **2000**, *467*, 81.
3. Xu, W. F.; Li, Q. B. *Curr. Med. Chem., Anti-Cancer Agents* **2005**, *5*, 281.
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. Rich, D. H.; Moon, B. J.; Harbeson, S. *J. Med. Chem.* **1984**, *27*, 417.
7. Umezawa, H.; Aoyagi, T.; Tanaka, T.; Suda, H. *Antibiotics* **1985**, *38*, 1629.
8. Nagai, M.; Kojima, F.; Naganawa, H. *Antibiotics* **1997**, *50*, 82.
9. Lampret, B. R.; Kidrie, J.; Kralj, B. *Arch. Microbiol.* **1999**, *171*, 397.
10. Chung, M. C.; Lee, H. J.; Chun, H. K. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 898.
11. Aoyagi, T.; Yoshida, S.; Matsuda, N. *Antibiotics* **1991**, *44*, 573.
12. Egan, M. E.; Pearson, M.; Weiner, S. A., et al *Science* **2004**, *304*, 600.
13. Shim, J. S.; Lee, H.; Shin, J. *Cancer Lett.* **2004**, *203*, 163.
14. Shimazawa, R.; Takayama, H.; Hashimoto, Y., et al *J. Enzyme Inhib.* **1999**, *14*, 259.
15. Ito, K.; Nakajima, Y.; Yoshimoto, T., et al *J. Biol. Chem.* **2006**, *281*, 33664.
16. Wang, Q.; Chen, M. Y.; Zhu, H. W.; Zhang, J.; Fang, H.; Wang, H. B.; Xu, W. F. *Bioorg. Med. Chem.* **2008**, *16*, 5473.
17. Addlagatta, A.; Gay, L.; Matthews, B. W. *Biochemistry* **2008**, *47*, 5303.
18. Shang, L. Q.; Wang, Q.; Fang, H.; Mu, J. J.; Wang, X. J.; Yuan, Y. M.; Wang, B. H.; Xu, W. F. *Bioorg. Med. Chem.* **2008**, *16*, 6663.
19. Li, Y. L.; Xu, W. F. *Bioorg. Med. Chem.* **2004**, *12*, 5171.
20. Li, X.; Li, Y. L.; Xu, W. F. *Bioorg. Med. Chem.* **2006**, *15*, 1287.
21. Hashimoto, S.; Okada, K.; Sakakibara, R.; Fujii, S. U.S. Patent [11] 3, 799, 988 March 26, 1974.
22. Jordis, U.; Sauter, F.; Siddiqi, S. M. *Indian J. Chem. Sec. B* **1989**, 294.