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Design, synthesis and primary activity assay of bi- or tri-peptide analogues with the scaffold L-arginine as amino-peptidase N/CD13 inhibitors

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ABSTRACT

A series of bi- or tri-peptide analogues with the scaffold L-arginine were designed, synthesized and evaluated for their inhibitory activities against amino-peptidase N (APN) and metalloproteinase-2 (MMP-2). The primary activity assay showed that all the compounds exhibited higher inhibitory activities against APN than MMP-2. Within this series, compounds C6 and **C7** (IC_{50} = 4.2 and 4.3 μM) showed comparable APN inhibitory activities with the positive control bestatin (IC_{50} = 3.8 μM).

1. Introduction

Amino-peptidase N (APN/CD13; EC 3.4.11.2) is a monomeric or homodimeric type II membrane-bound zinc-dependent metalloproteinase, which can release neutral and basic amino acid from the N-terminal of peptides. It is particularly noticeable that overexpression of APN is highly correlated with various pathological disorders, such as inflammatory bowel diseases, encephalomyelitis, multiple sclerosis, ulcerative colitis, Crohn's disease, rheumatoid arthritis and cancer.[1–5] Because it plays key roles in extracellular matrix degradation, tumor cell invasion and tumor angiogenesis, APN was regarded as an attractive target for anti-cancer drug design.

The matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependant endoproteinases that degrade and remodel the structural protein in the extracellular matrix. MMP gene family consists of at least 28 members, among which MMP-2 and MMP-9 are proved to be highly correlated with cancer. Because similar to APN, MMP-2 is also a zinc-dependant metalloproteinase that involved in tumor invasion and metastasis. Thus in the enzyme activity assay of our lab, both APN and MMP-2 were performed so as to identify the compounds selectivity.

Up to now, several excellent reviews on natural and small molecule inhibitors of APN have been published.[6–8] Among these inhibitors, bestatin is a representative one, which has been launched and is now widely employed clinically as an anti-tumor agent.[9] In 2006, the 3D structure of APN has been studied according to the co-crystal complex of APN and bestatin by Ito.[10] Accordingly, the interactions of bestatin with the active sites of APN can be illustrated by a simplified diagram (see Fig. 1). It revealed that beside the catalytic center zinc(II) ion of APN, there were three hydrophobic binding domains, which were called S_1 pocket, S_1' pocket and S_2' pocket, respectively. The side chains of bestatin occupied S_1 and S_1' pocket.

In our previous work, we reported a series of L-arginine derivatives as APN inhibitors.[11] Some of them showed potential inhibitory activities against APN. Especially, compound **5s**, whose IC_{50} was 5.1 μM compared with 3.1 μM of bestatin. Considering the side chains of this series of compounds were all aromatic acid substituents and not long enough, we designed a new series of bi- or tri-peptide analogues, which contained amino acid and bi-peptide

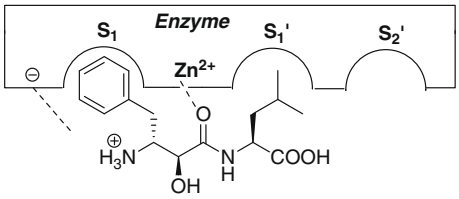

Figure 1. Schematic diagram of the interaction of bestatin with APN.[6]

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analogues as side chains. Within these compounds, tri-peptide analogues are much longer than the former compounds. We hope that the long side chains can simultaneously occupy S_1' and S_2' pockets of APN and enhance the activities towards APN (Fig. 2).

2. Chemistry

All the target compounds were designed and synthesized via the route shown in Scheme 1. The synthesis of compound **3** has been described in the previous paper. Boc protection of compound **4** led to compound **5**. Compound **10** was obtained by the condensation of the methyl ester **9** and Boc protected amino acids **5**. Hydrolysis of compound **10** with NaOH/MeOH led to the bi-peptide **11**. Compound **17** was prepared as described for compound **11**. The acylation of compound **3** with Boc protected amino acids **5** or compounds **11** or compound **17** yielded compound **6**, compound **12** and compound **18**. Then the ester groups of compound **6**, compound **12** and compound **18** were treated with NHOK in anhydrous methanol to get compounds **7**, **13** and **19**. The Boc group of compound **7** and compound **13** can be easily removed by 2 N HCl in ethyl acetate to get the target compounds.

3. Result 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.

As shown in Table 1, all the compounds displayed better inhibitory activities towards APN as compared with MMP-2. The results, to a certain extent, validated our strategy for rational drug design for potential APNIs. And almost all the compounds showed better APN inhibitory activities than the previous compounds.

It is worthy to mention that compound **C6** and **C7** (IC_{50} = 4.2 and 4.3 μM) exhibited similar activities with bestatin (IC_{50} = 3.8 μM). That may be due to the two aromatic groups of **C6** and **C7** occupying S_1' and S_2' hydrophobic pockets of APN suitably. And compound **B4**, **C3**, **C4** and **C5** (IC_{50} = 8.4, 6.7, 4.8 and 7.2 μM, respectively) also showed potential activities.

In another way, the data shown in Table 1 suggested that the preferred compounds against APN were, in decreasing order, **C** series of compounds, **B** series of compounds and **A** series of compounds. The possible reason may be due to the hydrophobic chains of **C** series of compounds occupying the three hydrophobic pockets of APN comfortably.

Among **A** and **B** series of compounds, generally speaking, compounds with Boc protected amino groups showed better activities than those with free amino group. For example, **B4** and **B11** (IC_{50} = 8.4 and 35.4 μM). This activity difference might be explained by the FlexX docking results of **B4** and **B11** with APN (Fig. 3). In Figure 3, the backbone of **B4** inserted into S_1 pocket, the hydroxymate group chelated with the zinc ion of APN, the isobutyl group occupied S_1' pocket and the Boc group extended into S_2' pocket. While the free amino group of **B11** can not insert into S_2' pocket at all.

When it comes to **C** series, we can see that almost all this series of compounds exhibited good activities. But, to some extent, the bulk of R_6 group was positively relative with APN inhibition. Compound **C6** and **C7** with benzyl group displayed better APN inhibitory activities than compound **C1** and **C2**, which only contained methyl group in the R_6 position. This suggested that it was favorable to increase the bulk of R_6 substituent to get better APN inhibitory activity.

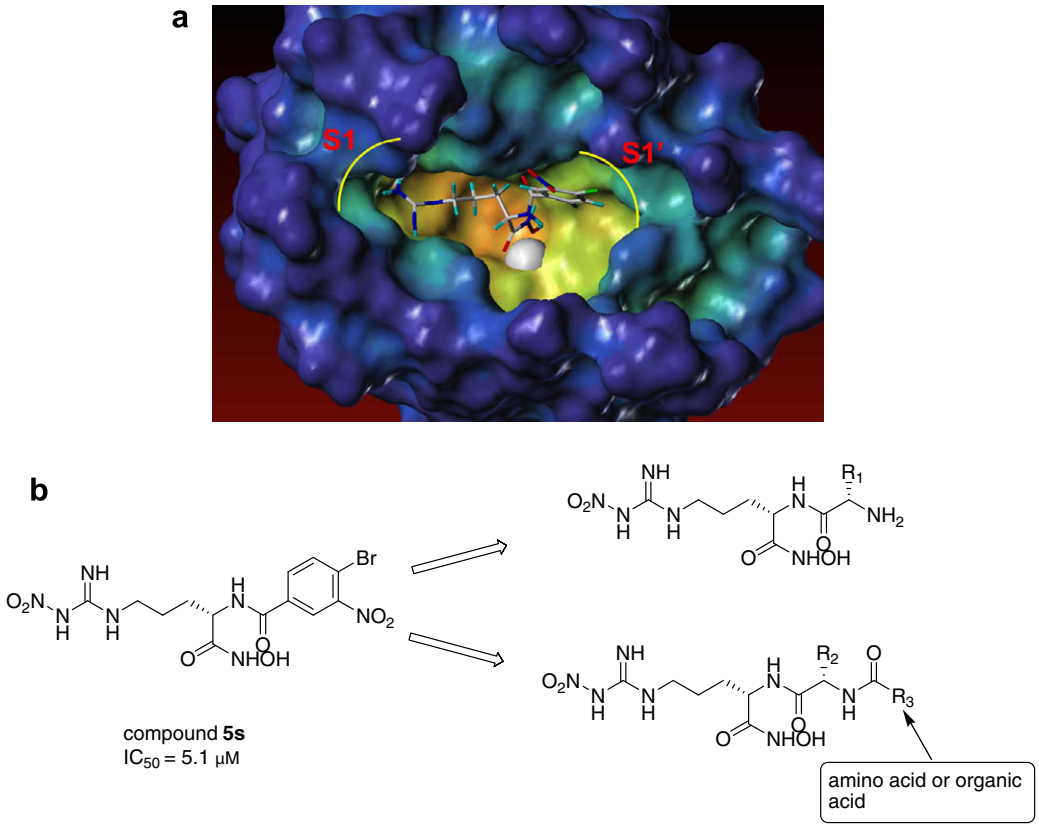
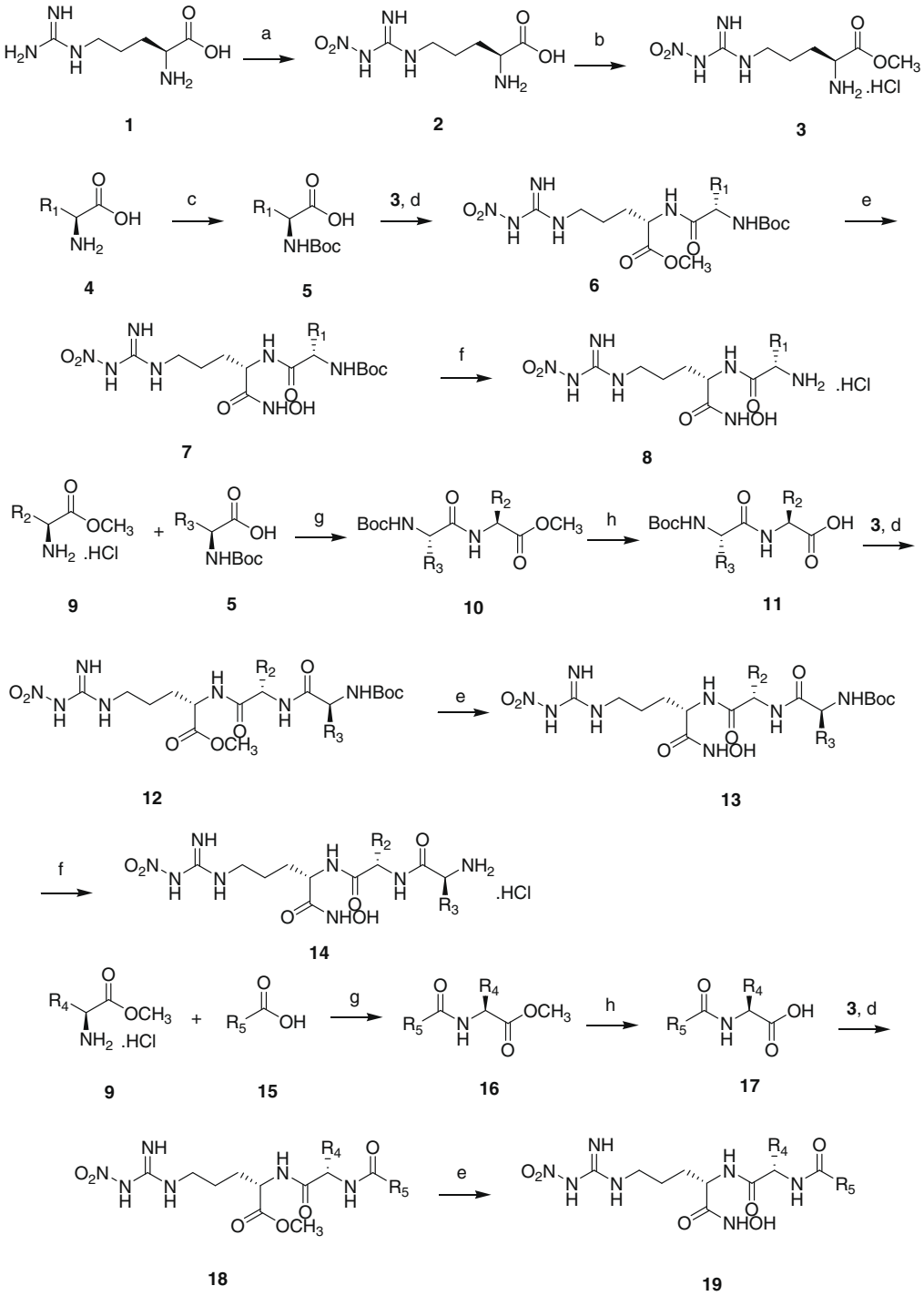


Figure 2. (a) The docking mode of compound **5s** with APN. (b) Chemical structure of compound **5s** and the newly designed compounds.

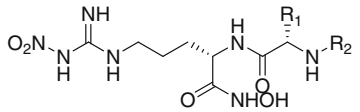


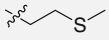
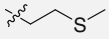
Scheme 1. Reagents and conditions: (a) fuming nitric acid, fuming sulfuric acid; (b) MeOH, HCl; (c) $(Boc)_2O$/DCM, Et_3N, 0 °C; (d) DCC, HOBt, THF, −5 °C; (e) NHOK, MeOH; (f) HCl/EtOAC; (g) IBCF, NMM, THF, −15 °C; (h) NaOH/MeOH.

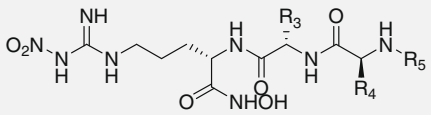
Compound **C6** was the most active compound. In order to investigate the interaction of it with APN, the docking studies were carried out via Sybyl/Sketch module and optimized using Powell's method with the Tripos force field with convergence criterion set at 0.05 kcal/(Å mol), and assigned with Gasteiger–Hückel method. The docking study performed using Sybyl/FlexX module, the residues in a radius of 7.0 Å around bestatin in the co-crystal structure (PDB code: 2DQM) were selected as the active site. Other docking parameters implied in the program were kept default. The docking result was shown in Figure 4. As diagrammed in Figure 4, the backbone of **C6** extended into S_1 pocket, the hydroxymate group chelated with the zinc ion of APN, the benzyl group occupied S_1' pocket and the phenyl group occupied S_2' pocket. In addition, the hydroxyl of hydroxymate group could form hydrogen bond with the carbonyl group of Ala^{262} of APN, the amide of hydroxymate group could form hydrogen bond with the carboxyl group of Glu^{264} and the amide group near hydroxymate group could form hydrogen bond with the carboxyl group of Glu^{298}.

It should be pointed out that although compounds **C6** and **C7** gave comparable APN inhibitory activities with bestatin, none of the compounds demonstrated better potency than the positive control. Therefore, further structural optimization should be carried out towards **C** series.

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



Compound	R_1	R_2	IC_{50}[a] (μM)	
			APN	MMP-2
A1		Boc	200.9 ± 5.8	3464.2 ± 4.6
A2		Boc	334.1 ± 4.1	5684.6 ± 5.2
A3		Boc	132.1 ± 4.0	1935.5 ± 3.7
A4		H	280.0 ± 2.9	428.3 ± 3.1
A5		H	380.3 ± 5.6	5308.9 ± 7.5
A6		H	242.8 ± 4.2	4204.2 ± 6.6



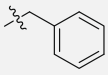
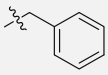
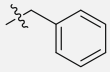
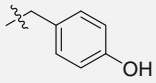
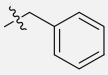
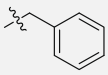
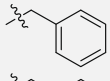
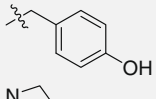
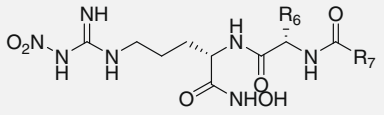
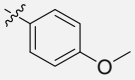
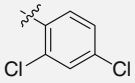
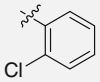
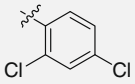
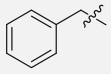
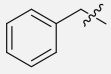
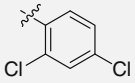
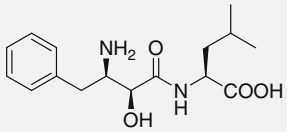
Compound	R_3	R_4	R_5	IC_{50}[a] (μM)	
				APN	MMP-2
B1	H		Boc	25.2 ± 1.4	2976.2 ± 5.0
B2			Boc	39.5 ± 1.7	2895.8 ± 5.4
B3			Boc	33.1 ± 1.8	446.9 ± 3.9
B4			Boc	8.4 ± 1.8	407.4 ± 3.5
B5			Boc	1336.1 ± 6.6	7903.1 ± 5.8
B6			Boc	436.3 ± 4.3	4515.2 ± 5.1
B7			Boc	42.1 ± 2.5	8364.4 ± 6.9
B8	H		H	226.2 ± 5.1	3630.2 ± 4.0
B9			H	157.7 ± 2.6	1710.6 ± 3.8
B10			H	98.6 ± 4.0	3160.1 ± 3.6
B11			H	35.4 ± 1.9	513.6 ± 3.7
B12			H	363.8 ± 6.7	8047.3 ± 7.9
B13			H	248.0 ± 4.5	7586.4 ± 7.4
B14			H	65.2 ± 2.3	8611.4 ± 8.9

Table 1 (*continued*)



Compound	R_6	R_7	IC_{50}[a] (μM)	
			APN	MMP-2
C1			16.3 ± 1.2	8967.1 ± 6.9
C2			31.4 ± 1.6	1755.2 ± 5.4
C3			6.7 ± 0.9	1776.2 ± 4.7
C4			4.8 ± 0.6	630.3 ± 3.7
C5			7.2 ± 1.1	1538.5 ± 5.6
C6			4.2 ± 0.7	1649.5 ± 4.6
C7			4.3 ± 0.7	8135.5 ± 7.1
Bestatin			3.8 ± 0.5	253.2 ± 3.9

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

Finally, although the computed information assay totally supported our assumption, the exact binding mode of the L-arginine derivatives with APN should be verified from further X-ray crystal studies.

4. Conclusions

In all, we have synthesized a new series of L-arginine derivatives as APN inhibitors. Most of the compounds showed potent activity and selectivity against APN, in which compound **C6**, **C7** and **C4** were comparable to bestatin and could be used as lead compounds for the development of future low molecular-weight peptidomimetic APN inhibitors as anti-cancer agents.

5. Experimental section

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 (600 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. HRMS spectra were conducted by Shandong Analysis and Test Center. Melting points were determined on an electrothermal melting point apparatus (uncorrected).

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

The title compound was synthesized as described in our previous paper.[11]

5.1.2. Boc protected amino acid (5)

The title compound was synthesized as described by Haaina.[12]

5.1.3. (*S*)-2-(2-(*tert*-Butoxycarbonyl)-4-methylpentanamido) acetic acid (11-B1)

Compound **9** was synthesized as described by Jordis.[13]

To a stirred solution of compound **5** ((*S*)-2-(*tert*-butoxycarbonyl)-4-methylpentanoic acid, 2.31 g, 10 mmol) and *N*-methylmorpholine (1.34 mL, 12 mmol) in THF (30 mL) was added isobutyl chloroformate (1.60 mL, 12 mmol) at −15 °C. The mixture was stirred for 30 min. A solution of compound **9** (methyl 2-aminoacetate hydro-

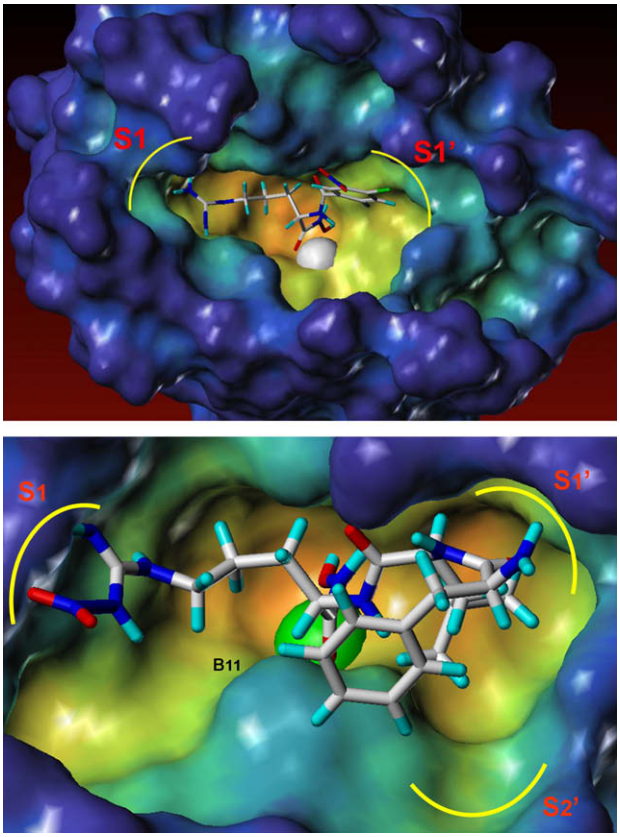


Figure 3. Comparison of the FlexX docking results of compounds **B4** and **B11**.

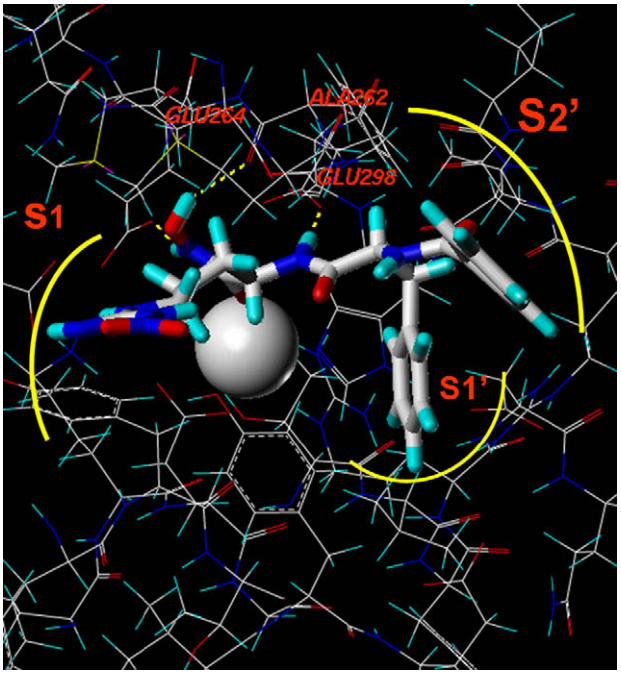


Figure 4. The docking mode of compound **C6** with APN.

chloride, 1.26 g, 10 mmol) and *N*-methylmorpholine (1.34 mL, 12 mmol) in THF (30 mL) was added dropwise to the reaction mixture. The stirring was continued for 24 h at −5 °C, and then the solvent was evaporated in vacuum. The residue was dissolved in EtOAC and washed with saturated $NaHCO_3$, 10% citric acid and brine in turn. Then the organic phage was dried with $MgSO_4$ and concentrated with a rotary evaporator to give the crude product compound **10** ((*S*)-methyl 2-(2-(*tert*-butoxycarbonyl)-4-methyl pentanamido) acetate).

Compound **10** (3.02 g, 10 mmol) was dissolved in 25 mL MeOH and then 25 mL 2 mol/L NaOH was added in 5 min. The reaction mixture was stirred for 5 h at room temperature. The organic phase was evaporated off. The resulting solution was adjusted to pH 3 with 2 mol/L citric acid and compound **11-B1** precipitated as a white solid, which was filtrated and dried in vacuum oven.

5.1.4. (*S*)-2-(4-Methoxybenzamido) propanoic acid (17)

The title compound was synthesized following the procedure described for compound **11-B1**.

5.1.5. (*S*)-Methyl 2-((*S*)-2-(*tert*-butoxycarbonyl)-3-methylbutanamido)-5-(3-nitroguanidino)pentanoate (6-A1)

A solution of DCC (4.50 g, 22 mmol) in 30 mL anhydrous THF was added dropwise to a solution of compound **5** ((*S*)-2-(*tert*-butoxycarbonyl)-3-methylbutanoic acid, 2.17 g, 20 mmol) and HOBt (2.94 g, 22 mmol) in 60 mL of anhydrous THF at −5 °C for 20 h. Then the HOBt active ester was filtrated and the filtrate would be used the next step.

Compound **3** (2.70 g, 20 mmol) was suspended in 30 mL anhydrous THF, and TEA (2.78 mL, 20 mmol) was added. After 0.5 h, the former filtrate was added in. The mixture was stirred at room temperature overnight. The dicyclohexylurea (DCU) was filtrated off and THF was evaporated in vacuum. The residues obtained were dissolved in EtOAC and the DCU appeared again was filtrated off. The filtrate was washed with saturated $NaHCO_3$, 10% citric acid and brine, and then dried with $MgSO_4$. The solvent was evaporated to give compound **6**.

5.1.6. *tert*-Butyl (*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-3-methyl-1-oxobutan-2-ylcarbamate (7-A1)

To a solution of compound **6-A1** (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, compound **A1** was obtained.

5.1.7. *tert*-Butyl (*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-3-methyl-1-oxobutan-2-ylcarbamate (A1)

Yield 72%, mp 121–124 °C. ^{1}H NMR (D_2O): 0.79–0.87 (m, 6H), 1.38 (s, 9H), 1.48–1.59 (m, 4H), 1.90–1.93 (m, 1H), 3.34–3.40 (m, 2H), 3.79–3.82 (t, J = 7.2 Hz, 1H), 4.14–4.18 (dd, J_1 = 6.6 Hz, J_2 = 7.8 Hz, 1H). HRMS calcd for $C_{16}H_{31}N_7O_7$ $(M+H)^+$ 434.2363, found 434.2356.

5.1.8. *tert*-Butyl (*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2- ylamino)-4-methyl-1-oxopentan-2-ylcarbamate (A2)

Yield 73%, mp 156–159 °C. ^{1}H NMR (D_2O): 0.83–0.87 (m, 6H), 1.37 (s, 9H), 1.40–1.44 (m, 2H), 1.47–1.55 (m, 2H) 1.58–1.62 (m, 3H), 3.13–3.14 (m, 2H), 3.94–3.98 (m, 1H), 4.13–4.15 (m, 1H). HRMS calcd for $C_{17}H_{33}N_7O_7$ $(M+H)^+$ 448.2520, found 448.2506.

5.1.9. *tert*-Butyl (*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-4-(methylthio)-1-oxobutan-2-ylcarbamate(A3)

Yield 64%, mp 135–137 °C. ^{1}H NMR (D_2O): 1.38 (s, 9H), 1.48–1.58 (m, 2H), 1.69–1.84 (m, 2H), 2.02 (s, 3H), 2.40–2.48 (m, 2H), 2.67–2.70 (m, 2H), 3.18–3.19 (m, 2H), 3.97–4.00 (m, 1H), 4.06–

4.07 (m, 1H). HRMS calcd for $C_{16}H_{31}N_7O_7S$ $(M+H)^+$ 466.2084, found 466.2074.

5.1.10. (*S*)-2-((*S*)-2-Amino-3-methylbutanamido)-*N*-hydroxy-5-(3-nitroguanidino) pentanamide (8-A4)

Compound **A1** (1.73 g, 4 mmol) was dissolved in 20 ml 2 mol/L HCl/EtOAC. After 30 min, the solvent was filtrated and the precipitate was washed with EtOAC to get compound **A4**.

5.1.11. (*S*)-2-((*S*)-2-Amino-3-methylbutanamido)-*N*-hydroxy-5-(3-nitroguanidino)pentanamide (A4)

Yield 88%, mp 76–78 °C. ^{1}H NMR (D_2O): 0.87–0.96 (m, 6H), 1.47–1.67 (m, 4H), 2.03–2.13 (m, 1H), 3.14–3.17 (m, 2H), 3.64–3.69 (m, 1H), 4.20–4.24 (m, 1H).

HRMS calcd for $C_{11}H_{23}N_7O_5$ $(M+H)^+$ 334.1833, found 334.1852.

5.1.12. (*S*)-2-((*S*)-2-Amino-4-methylpentamido)-*N*-hydroxy-5-(3-nitroguanidino)pentanamide (A5)

Yield 86 %, mp 95–97 °C. ^{1}H NMR (D_2O): 0.87–0.93 (m, 6H), 1.50–1.60 (m, 4H), 1.62–1.66 (m, 2H), 1.68–1.73 (m, 1H), 3.17–3.18 (m, 2H), 3.82–3.84 (m, 1H), 4.21–4.27 (m, 1H).

HRMS calcd for $C_{12}H_{25}N_7O_5$ $(M+H)^+$ 348.1990, found 348.1985.

5.1.13. (*S*)-2-((*S*)-2-Amino-4-(methylthio)butanamido)-*N*-hydroxy-5-(3-nitroguanidino)pentanamide (A6)

Yield 83%, mp 92–94 °C. ^{1}H NMR (D_2O): 1.54–1.71 (m, 4H), 1.94–2.04 (m, 2H), 2.06 (s, 3H), 2.50–2.52 (m, 2H), 3.16–3.18 (m, 2H), 3.89–3.91 (m, 1H), 4.19–4.22 (m, 1H).

HRMS calcd for $C_{11}H_{23}N_7O_5S$ $(M+H)^+$ 366.1554, found 366.1558.

5.1.14. (*S*)-Methyl 2-(2-((*S*)-2-(*tert*-butoxycarbonyl)-4-methylpentanamido) acetamido)-5-(3-nitroguanidino)pentanoate (12-B1)

The title compound was synthesized as described for compound **6**.

5.1.15. *tert*-Butyl (*S*)-1-(2-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-2-oxoethylamino)-4-methyl-1-oxopentan-2-ylcarbamate (13-B1)

The title compound was synthesized as described for compound **7**.

5.1.16. *tert*-Butyl (*S*)-1-(2-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-2-oxoethylamino)-4-methyl-1-oxopentan-2-ylcarbamate (B1)

Yield 63%, mp 132–134 °C. ^{1}H NMR (D_2O): 0.84–0.87 (m, 6H), 1.38 (s, 9H), 1.41–1.43 (m, 3H), 1.48–1.51 (m, 2H), 1.57–1.64 (m, 2H), 3.12–3.13 (m, 2H), 3.68–3.75 (m, 2H), 3.94–3.98 (q, J = 7.8 Hz, 1H), 4.14–4.17 (q, J = 7.8 Hz, 1H). HRMS calcd for $C_{19}H_{36}N_8O_8$ $(M+H)^+$ 505.2729, found 505.2723.

5.1.17. *tert*-Butyl (*S*)-1-((*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-1-oxopropan-2-ylamino)-4-methyl-1-oxopentan-2-ylcarbamate (B2)

Yield 72%, mp 132–134 °C. ^{1}H NMR (D_2O): 0.84–0.86 (m, 6H), 1.16–1.17 (d, J = 7.2 Hz, 3H), 1.37 (s, 9H), 1.40–1.42 (m, 3H), 1.48–1.50 (m, 2H), 1.58–1.60 (m, 2H), 3.12–3.13 (m, 2H), 3.92–3.96 (m, 1H), 4.10–4.14 (q, J = 7.2 Hz, 1H), 4.27–4.29 (m, 1H). HRMS calcd for $C_{20}H_{38}N_8O_8$ $(M+H)^+$ 519.2891, found 519.2879.

5.1.18. *tert*-Butyl (*S*)-1-((*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-3-methyl-1-oxobutan-2-ylamino)-1-oxo-3-phenylpropan-2-ylcarbamate(B3)

Yield 75%, mp 116–119 °C. ^{1}H NMR (D_2O): 0.76–0.78 (m, 3H), 0.83–0.88 (m, 3H), 1.29 (s, 9H), 1.40–1.43 (m, 2H), 1.58–1.62 (m, 2H), 1.91–1.96 (m, 1H), 2.70–2.75 (m, 1H), 2.95–2.98 (m, 1H), 3.14–3.15 (m, 2H), 4.12–4.19 (m, 2H), 4.25–4.27 (m, 1H), 7.16–7.19 (m, 2H), 7.25–7.29 (m, 3H). HRMS calcd for $C_{25}H_{40}N_8O_8$ $(M+H)^+$ 581.3047, found 581.3042.

5.1.19. *tert*-Butyl (*S*)-1-((*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-4-methyl-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-ylcarbamate (B4)

Yield 70%, mp 133–135 °C. ^{1}H NMR (D_2O): 0.77–0.89 (m, 6H), 1.30 (s, 9H), 1.36–1.47 (m, 4H), 1.59–1.69 (m, 3H), 2.69–2.75 (m, 1H), 2.89–2.96 (m, 1H), 3.14–3.15 (m, 2H), 4.09–4.20 (m, 2H), 4.31–4.38 (m, 1H), 7.17–7.19 (m, 2H), 7.25–7.26 (m, 3H). HRMS calcd for $C_{26}H_{42}N_8O_8$ $(M+H)^+$ 595.3204, found 595.3220.

5.1.20. (*S*)-*tert*-Butyl 2-(((*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)carbamoyl)pyrrolidine-1-carboxylate (B5)

Yield 68%, mp 139–142 °C. ^{1}H NMR (D_2O): 1.37 (s, 9H), 1.48–1.57 (m, 4H), 1.62–1.64 (m, 3H), 1.99–2.00 (m, 1H), 2.73–2.82 (m, 1H), 2.96–3.01 (m, 1H), 3.13–3.15 (m, 2H), 3.18–3.40 (m, 2H), 4.15–4.16 (m, 2H), 4.59–4.60 (m, 1H), 7.15–7.28 (m, 5H). HRMS calcd for $C_{25}H_{38}N_8O_8$ $(M+H)^+$ 579.2891, found 579.2879.

5.1.21. (*S*)-*tert*-Butyl 2-(((*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)carbamoyl)pyrrolidine-1-carboxylate (B6)

Yield 60%, mp 166–168 °C. ^{1}H NMR (D_2O): 1.37 (s, 9H), 1.50–1.53 (m, 2H), 1.59–1.63 (m, 2H), 1.64–1.66 (m, 3H), 2.01–2.03 (m, 1H), 2.62–2.67 (m, 1H), 2.84–2.89 (m, 1H), 3.13–3.16 (m, 2H), 3.19–3.40 (m, 2H), 4.13–4.17 (m, 2H), 4.46–4.50 (m, 1H), 6.99–7.04 (m, 4H). HRMS calcd for $C_{25}H_{38}N_8O_9$ $(M+H)^+$ 595.2840, found 595.2832.

5.1.22. (*S*)-*tert*-Butyl 2-((*S*)-2-(((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)carbamoyl)pyrrolidine-1-carbonyl)pyrrolidine-1-carboxylate (B7)

Yield 62%, mp 101–103 °C. ^{1}H NMR (D_2O): 1.37 (s, 9H), 1.44–1.66 (m, 4H), 1.72–1.83 (m, 8H), 3.13–3.14 (m, 2H), 3.44–3.58 (m, 2H), 3.62–3.75 (m, 2H), 4.04–4.18 (m, 1H), 4.19–4.26 (m, 1H), 4.36–4.42 (m, 1H). HRMS calcd for $C_{21}H_{36}N_8O_8$ $(M+H)^+$ 529.2734, found 529.2728.

5.1.23. (*S*)-2-(2-((S)-2-Amino-4-methylpentanamido) acetamido)-*N*-hydroxy-5-(3-nitroguanidino)pentanamide (14-B8)

The title compound was synthesized as described for compound **8**.

5.1.24. (*S*)-2-(2-((*S*)-2-Amino-4-methylpentanamido) acetamido)-*N*-hydroxy-5-(3-nitroguanidino)pentanamide (B8)

Yield 82%, mp 88–90 °C. ^{1}H NMR (D_2O): 0.88–0.91 (m, 6H), 1.53–1.60 (m, 4H), 1.62–1.75 (m, 3H), 3.16–3.17 (m, 2H), 3.56–3.58 (m, 1H), 3.78–3.88 (m, 2H), 4.15–4.22 (m, 1H). HRMS calcd for $C_{14}H_{28}N_8O_6$ $(M+H)^+$ 405.2205, found 405.2216.

5.1.25. (*S*)-2-((*S*)-2-((*S*)-2-Amino-4-methylpentanamido)propanamido)-*N*-hydroxy-5-(3-nitroguanidino)pentanamide (B9)

Yield 83%, mp 96–98 °C. ^{1}H NMR (D_2O): 0.87–0.91 (m, 6H), 1.19–1.27 (m, 3H), 1.50–1.58 (m, 4H), 1.60–1.63 (m, 2H), 1.66–1.70 (m, 1H), 3.15–3.16 (m, 2H), 3.78–3.79 (m, 1H), 4.11–4.17 (m, 1H), 4.36–4.39 (q, J = 7.2 Hz, 1H). HRMS calcd for $C_{15}H_{30}N_8O_6$ $(M+H)^+$ 419.2361, found 419.2348.

5.1.26. (*S*)-2-((*S*)-2-((*S*)-2-Amino-3-phenylpropanamido)-3-methylbutanamido)-*N*-hydroxy-5-(3-nitroguanidino)pentanamide (B10)

Yield 86%, mp 123–125 °C. ^{1}H NMR (D_2O): 0.88–0.94 (m, 3H), 1.07–1.10 (m, 3H), 1.56–1.64 (m, 4H), 1.91–2.01 (m, 1H), 2.97–

3.08 (m, 1H), 3.13–3.17 (m, 2H), 3.37–3.40 (m, 1H), 4.13–4.18 (m, 2H), 4.22–4.26 (m, 1H), 7.22–7.36 (m, 5H). HRMS calcd for $C_{20}H_{32}N_8O_6$ $(M+H)^+$ 481.2518, found 481.2526.

5.1.27. (*S*)-2-((*S*)-2-((*S*)-2-Amino-3-phenylpropanamido)-4-methylpentanamido)-*N*-hydroxy-5-(3-nitroguanidino)pentanamide (B11)

Yield 85%, mp 121–123 °C. ^{1}H NMR (D_2O): 0.83–0.85 (m, 6H), 1.47–1.54 (m, 4H), 1.58–1.71 (m, 2H), 1.73–1.77 (m, 1H), 2.94–2.98 (m, 1H), 3.02–3.04 (m, 1H), 3.14–3.16 (m, 2H), 4.11–4.13 (m, 2H), 4.38–4.41 (m, 1H), 7.26–7.28 (m, 5H). HRMS calcd for $C_{21}H_{34}N_8O_6$ $(M+H)^+$ 495.2674, found 495.2713.

5.1.28. (*S*)-*N*-((*S*)-1-((*S*)-1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-yl)pyrrolidine-2-carboxamide (B12)

Yield 85%, mp 152–154 °C. ^{1}H NMR (D_2O): 1.55–1.69 (m, 4H), 1.71–1.84 (m, 4H), 2.29–2.30 (m, 1H), 2.70–2.83 (m, 1H), 3.03–3.07 (m, 1H), 3.08–3.13 (m, 1H), 3.14–3.18 (m, 2H), 4.15–4.17 (m, 1H), 4.21–4.24 (m, 1H), 4.59–4.62 (m, 1H), 7.19–7.34 (m, 5H). HRMS calcd for $C_{20}H_{30}N_8O_6$ $(M+H)^+$ 479.2361, found 479.2378.

5.1.29. (*S*)-*N*-((*S*)-1-((*S*)-1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)pyrrolidine-2-carboxamide (B13)

Yield 88%, mp 160–162 °C. ^{1}H NMR (D_2O): 1.54–1.68 (m, 4H), 1.73–1.83 (m, 4H), 2.28–2.31 (m, 1H), 2.58–2.70 (m, 1H), 2.91–2.98 (m, 1H), 3.10–3.12 (m, 1H), 3.13–3.17 (m, 2H), 4.08–4.10 (m, 1H), 4.20–4.24 (m, 1H), 4.49–4.54 (m, 1H), 6.64–6.67 (m, 2H), 7.03–7.10 (m, 2H). HRMS calcd for $C_{20}H_{30}N_8O_7$ $(M+H)^+$ 495.2310, found 495.2341.

5.1.30. (*S*)-*N*-((*S*)-1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-yl)-1-((*S*)-pyrrolidine-2-carbonyl)pyrrolidine-2-carboxamide (B14)

Yield 80%, mp 73–75 °C. ^{1}H NMR (D_2O): 1.45–1.68 (m, 4H), 1.70–1.90 (m, 3H), 2.08–2.14 (m, 4H), 2.25–2.34 (m, 1H), 3.15–3.16 (m, 2H), 3.32–3.37 (m, 2H), 3.46–3.51 (m, 2H), 4.10–4.17 (m, 1H), 4.22–4.30 (m, 1H), 4.43–4.45 (m, 1H). HRMS calcd for $C_{16}H_{28}N_8O_6$ $(M+H)^+$ 429.2205, found 429.2223.

5.1.31. (*S*)-Methyl 2-((*S*)-2-(4-methoxybenzamido)propanamido)-5-(3-nitroguanidino) pentanoate (18-C1)

The title compound was synthesized as described for compound **6**.

5.1.32. *N*-((*S*)-1-((*S*)-1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-1-oxopropan-2-yl)-4-methoxybenzamide (19-C1)

The title compound was synthesized as described for compound **7**.

5.1.33. *N*-((*S*)-1-((*S*)-1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-1-oxopropan-2-yl)-4-methoxybenzamide (C1)

Yield 68%, mp 181–183 °C. ^{1}H NMR (D_2O): 1.48–1.56 (m, 3H), 1.70–1.73 (m, 4H), 3.16–3.17 (m, 2H), 3.81 (s, 3H), 4.31–4.35 (m, 2H), 6.98–7.00 (m, 2H), 7.87–7.89 (m, 2H). HRMS calcd for $C_{17}H_{25}N_7O_7$ $(M+H)^+$ 440.1888, found 440.1876.

5.1.34. 2,4-Dichloro-*N*-((*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-1-oxopropan-2-yl) benzamide (C2)

Yield 78%, mp 125–127 °C. ^{1}H NMR (D_2O): 1.26–1.29 (m, 3H), 1.42–1.67 (m, 4H), 3.15–3.17 (m, 2H), 4.17–4.20 (m, 1H), 4.45–4.49 (m, 1H), 7.44–7.51 (m, 2H), 7.66–7.68 (m, 1H). HRMS calcd for $C_{16}H_{21}Cl_2N_7O_6$ $(M+H)^+$ 478.1003, found 478.1027.

5.1.35. *N*-((*S*)-1-((*S*)-1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-3-methyl-1-oxobutan-2-yl)-2-iodobenzamide (C3)

Yield 77%, mp 125–127 °C. ^{1}H NMR (D_2O): 0.84–0.88 (m, 6H), 1.42–1.58 (m, 4H), 1.91–1.94 (m, 1H), 3.14–3.15 (m, 2H), 4.13–4.16 (m, 1H), 4.28–4.31 (m, 1H), 7.10–7.12 (m, 1H), 7.15–7.19 (m, 1H), 7.28–7.33 (m, 1H), 7.43–7.46 (m, 1H). HRMS calcd for $C_{18}H_{26}IN_7O_6$ $(M+H)^+$ 564.1062, found 564.1067.

5.1.36. 2-Chloro-*N*-((*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-4-methyl-1-oxopentan-2-yl) benzamide (C4)

Yield 70%, mp 116–118 °C. ^{1}H NMR (D_2O): 0.84–0.90 (m, 6H), 1.36–1.60 (m, 4H), 1.71–1.83 (m, 3H), 3.14–3.15 (m, 2H), 4.16–4.21 (m, 1H), 4.46–4.51 (m, 1H), 7.40–7.41 (m, 2H), 7.44–7.47 (m, 1H), 7.49–7.50 (m, 1H). HRMS calcd for $C_{19}H_{28}ClN_7O_6$ $(M+H)^+$ 486.1862, found 486.1886.

5.1.37. 2,4-Dichloro-*N*-((*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-4-methyl-1-oxopentan-2-yl) benzamide (C5)

Yield 79%, mp 129–131 °C. ^{1}H NMR (D_2O): 0.89–0.90 (d, J = 6.6 Hz, 6H), 1.46–1.58 (m, 4H), 1.63–1.69 (m, 3H), 3.14–3.15 (m, 2H), 4.16–4.20 (m, 1H), 4.46–4.52 (m, 1H), 7.41–7.42 (d, J = 7.8 Hz, 1H), 7.49–7.51 (m, 1H), 7.67–7.69 (d, J = 7.8 Hz, 1H). HRMS calcd for $C_{19}H_{27}Cl_2N_7O_6$ $(M+H)^+$ 520.1473, found 520.1498.

5.1.38. *N*-((*S*)-1-((*S*)-1-(Hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-yl) benzamide (C6)

Yield 64%, mp 150–152 °C. ^{1}H NMR (D_2O): 1.33–1.63 (m, 4H), 2.94–3.00 (dd, J_1 = 7.2 Hz, J_2 = 7.2 Hz, 1H), 3.16–3.17 (m, 2H), 3.36–3.40 (dd, J_1 = 7.2 Hz, J_2 = 7.2 Hz, 1H), 4.15–4.21 (m, 1H), 4.70–4.76 (m, 1H), 7.14–7.17 (m, 1H), 7.24–7.27 (dd, J_1 = 7.2 Hz, J_2 = 7.2 Hz, 2H), 7.36–7.37 (d, J = 7.2 Hz, 2H), 7.43–7.45 (m, 2H), 7.50–7.52 (m, 1H), 7.75–7.79 (dd, J_1 = 7.2 Hz, J_2 = 7.2 Hz, 2H). HRMS calcd for $C_{22}H_{27}N_7O_6$ $(M+H)^+$ 486.2096, found 486.2151.

5.1.39. 2,4-Dichloro-*N*-((*S*)-1-((*S*)-1-(hydroxyamino)-5-(3-nitroguanidino)-1-oxopentan-2-ylamino)-1-oxo-3-phenylpropan-2-yl) benzamide (C7)

Yield 76%, mp 141–143 °C. ^{1}H NMR (D_2O): 1.48–1.77 (m, 4H), 2.81–2.86 (m, 1H), 3.05–3.09 (m, 1H),3.11–3.16 (m, 2H), 4.17–4.19 (m, 1H), 4.74–4.79 (m, 1H), 7.17–7.21 (m, 2H), 7.23–7.32 (m, 2H), 7.46–7.47 (m, 1H). HRMS calcd for $C_{22}H_{25}Cl_2N_7O_6$ $(M+H)^+$ 554.1316, found 554.1327.

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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