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Novel aminopeptidase N inhibitors derived from antineoplaston AS2–5 (Part I)

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ABSTRACT

Overexpression of zinc-dependent metalloproteinase, aminopeptidase N (APN/CD13), is considered to be involved in the process of tumor invasion and metastasis. Herein we describe the synthesis and in vitro enzymatic inhibition assay of antineoplaston AS2–5 scaffold peptidomimetic compounds. The results demonstrated that most of these L-*iso*-glutamine derivatives displayed selective inhibitory activity against APN as compared with MMP-2, with IC_{50} values in the micromole range. The structure–activity relationships were also briefly discussed.

1. Introduction

During the process of tumor invasion and metastasis, proteolytic degradation of the extracellular matrix (ECM) is believed to be a key step, which involves a class of zinc-dependent metalloproteinase, aminopeptidase N (APN, EC 3.4.11.2; gp150), also known as human lymphocyte surface cluster differentiation antigen CD13.[1,2] It is a 150-kDa monomeric or homodimeric type II membrane-bound glycoprotein which can release neutral or basic amino acids from the N-terminal end of peptides.[3] It is particularly noticeable that overexpression of APN/CD13 is highly correlated with various pathological disorders, such as inflammatory bowel diseases, encephalomyelitis, multiple sclerosis, ulcerative colitis, Crohn's disease, rheumatoid arthritis, and cancer.[4–8] As a consequence, it is useful to develop chemical inhibitors that could block its enzymatic activity for medical and therapeutic utilization.[9]

To date, several excellent reviews on natural or small molecule inhibitors of APN/CD13 have been published.[10–12] Amongst these inhibitors, bestatin, an antibiotic of microbial origin and known inhibitor of APN,[13] is a well-established anticancer agent which has been a useful tool as positive control in elucidating many physiological conditions.[14]

Recently, the 3D-structures of APN have been investigated by the X-ray crystallographic studies on the co-crystal of the enzyme and various inhibitors.[15,16] It has showed that beside the catalytic center zinc(II) ion of APN, there are two hydrophobic binding domains, which are called S_1 pocket and S_1' pocket, respectively (see Fig. 1). Therefore, one or more hydrophobic side chains should be introduced to insert into these pockets to generate effective interactions. Moreover, given most of the reported APN inhibitors (APNIs) are pseudodipeptides or peptidemimics bearing zinc-chelating functionality (such as hydroxamate or carboxylate), inhibitors containing zinc-binding groups (ZBGs) should be optimal selection to inhibit the enzymatic activity of APN.[17]

According to these findings, our group has previously reported a series of novel APNIs with remarkable APN inhibitory activities in the enzymatic assays.[18–21] In our ongoing work on the exploitation of potent APNIs, we herein describe the synthesis and enzymatic evaluation of analogues derived from the antineoplaston AS2-5 (N_2-phenylacetyl L-*iso*-glutamine),[22] one of active metabolites of the antineoplaston A-10 (3-phenylacetylamino-2,6-piperidinedione), which is known to be the first chemically identified antineoplaston with anti-tumor activity (see Fig. 2).[23,24]

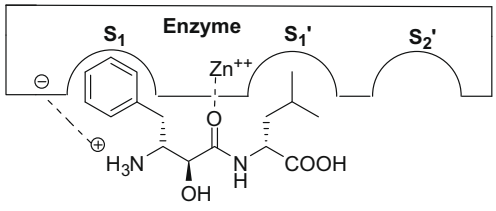

Figure 1. Schematic representation of the interaction of bestatin with APN.

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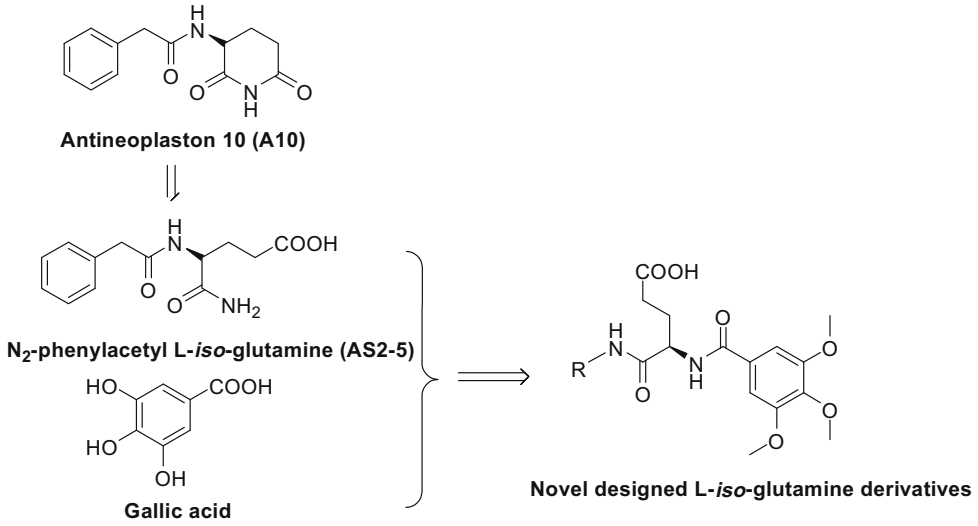


Figure 2. Chemical structures of gallic acid, antineoplaston 10 and its active metabolite N_2-phenylacetyl L-*iso*-glutamine (AS2-5).

Meanwhile, 3,4,5-trihydroxybenzoic acid (gallic acid; Fig. 2) and its derivatives are reported to possess significant anti-tumor and anti-oxidative activities, especially without any toxicities to normal cells.[25,26] According to the 'combination principle', we therefore envision that the conjunction of antineoplaston AS2–5 scaffold with gallic acid fragment might generate a more efficacious scaffold for potential enzymatic activity of APN.

2. Chemistry

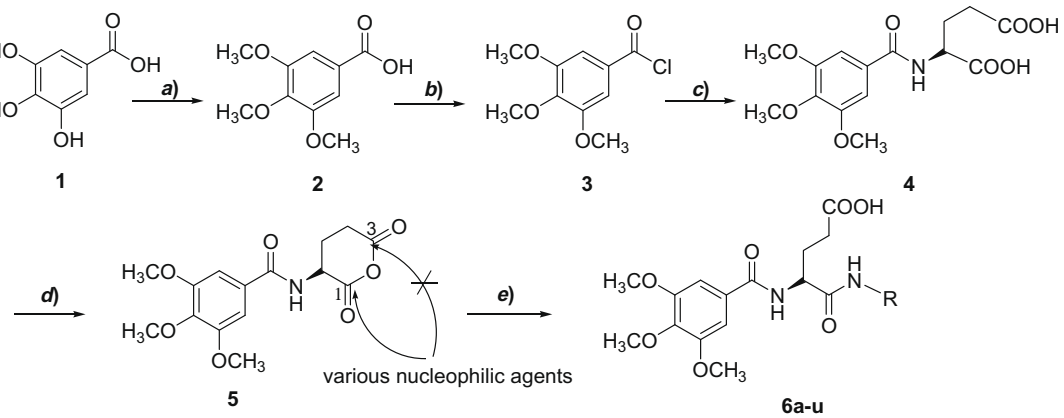
In order to study the SAR of these novel peptidomimetic compounds, different L-*iso*-glutamine derivatives were designed and synthesized via the route outlined in Scheme 1. Starting from commercially available gallic acid **1**, the key intermediate (*R*)-*N*-(2,6-dioxo-tetrahydro-2*H*-pyran-3-yl)-3,4,5-trimethoxybenzamide **5** was obtained via the sequence of methylation, acylation, nucleophilic substitution, and dehydration. This was followed by coupling with various primary amines in the presence of acetic acid to give target glutamine peptidomimetics **6a–t**. As shown in Scheme 1, when the asymmetric annular acetic anhydride (**5**) was treated with different nucleophilic agents, the six-membered anhydride ring might be opened from two sides. It revealed that only L-*iso*-glutamine derivatives were obtained, which have been confirmed by our previous work.[27] Besides, the IR, ESI-MS analytical and ^{1}H NMR data of all the synthesized compounds were in full agreement with the proposed structures (see Section 5).

3. Results and discussion

3.1. SAR studies

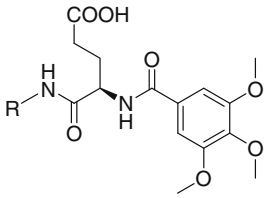
The target L-*iso*-glutamine derivatives were evaluated for their inhibitory activities toward APN/CD13 and MMP-2. Similar to APN, MMP-2 is also a zinc-dependent metalloproteinase involved in the process of tumor invasion and metastasis.[28,29] Hence the assay was performed on both of APN and MMP-2 so as to identify the selectivity of these L-iso-glutamine compounds, and all the inhibition results are summarized in Table 1. Besides, bestatin was used as the positive control.

The results showed that most of the L-*iso*-glutamine derivatives, except compound **6u**, display a better enzymatic inhibition on APN than that of MMP-2, with IC_{50} values lying in micromolar level. The resulting relatively selective inhibition against APN as compared with MMP-2, to a certain extent, confirmed our strategy for designing APNIs. 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.[1,29] As most compounds exhibited selective inhibition against APN, the following structure–activity relationships (SARs) were mainly focused on APN inhibition.



Scheme 1. Reagents and conditions: (a) Me_2SO_4, 40% NaOH, then 1 N HCl; (b) $SOCl_2$ in benzene; (c) L-Glu-Na, Na_2CO_3, then 2 N HCl; (d) Ac_2O, 55–60 °C; (e) RNH_2, AcOH.

Table 1
In vitro enzymatic assay (APN and MMP-2) results for L-*iso*-glutamine derivatives **6a–u**



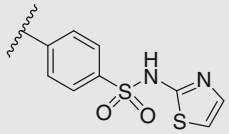
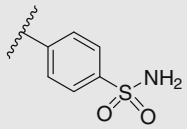
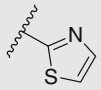
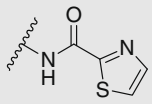
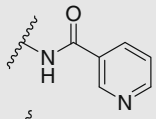
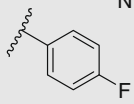
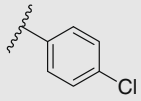
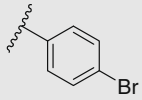
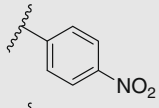
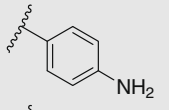
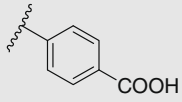
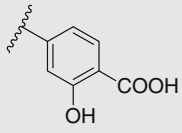
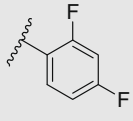
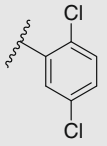
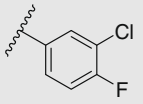
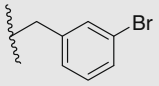
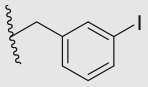
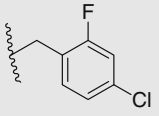
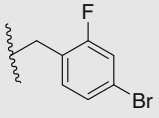
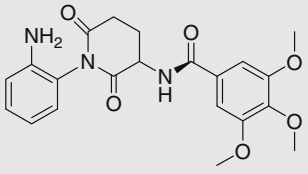
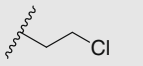
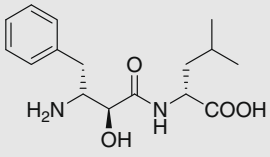
Compds	R	IC_{50} (μM)[a]	
		APN	MMP-2
6a		40.1 ± 2.1	55.3 ± 3.8
6b		102.6 ± 0.7	628.0 ± 10.1
6c		107.6 ± 2.8	369.4 ± 11.4
6d		87.7 ± 3.3	169.4 ± 5.1
6e		72.4 ± 4.4	114.9 ± 9.0
6f		24.3 ± 2.4	26.1 ± 1.8
6g		40.0 ± 1.5	52.3 ± 4.4
6h		41.9 ± 10.4	54.4 ± 9.0
6i		na[b]	na[b]
6j		23.4 ± 2.3	33.6 ± 1.9
6k		65.8 ± 7.5	71.2 ± 2.5
6l		57.8 ± 3.9	86.3 ± 3.1
6m		89.6 ± 1.6	108.7 ± 7.8

Table 1 (*continued*)

Compds	R	IC_{50} (μM)[a]	
		APN	MMP-2
6n		80.4 ± 5.5	122.4 ± 7.2
6o		78.5 ± 8.1	107.2 ± 8.0
6p		176.9 ± 1.5	287.3 ± 4.6
6q		65.9 ± 2.0	76.8 ± 1.8
6r		45.6 ± 8.4	52.8 ± 6.1
6s		51.2 ± 5.8	76.1 ± 0.9
6t		104.0 ± 10.3	659.6 ± 4.7
6u		805.1 ± 3.7	501.5 ± 7.1
Bestatin		13.1 ± 0.7	40.5 ± 1.2

[a] Values are means of three experiments, standard deviation is given.
[b] Not activity.

Of these inhibitors, compound **6j**, with phenylamine group as hydrophobic side chain, showed the best inhibitory activity (IC_{50} = 23.4 ± 2.3 μM). Compounds, **6a**, **6f**, **6g**, **6h** and **6r** exhibited the subsequent activities toward APN, with IC_{50} value from 24.3 ± 2.4 to 45.6 ± 8.4 μM. However, all of these compounds demonstrated a little less activity than that of bestatin. Additionally, compounds with aromatic side chains (**6a–t**) gave better activities compared to aliphatic one (**6u**). The possible reason might be due to the π system of the aromatic ring enhancing the interaction with the hydrophobic region of the enzyme.

Substitution on aromatic ring (**6a–t**) also has impact on bioactivity. For the activities of sulfonylamino-containing compounds, **6a** and **6b**, the improved activity was observed via introduction of a five-membered thiazole ring (**6a**). Comparing thiazole-con-

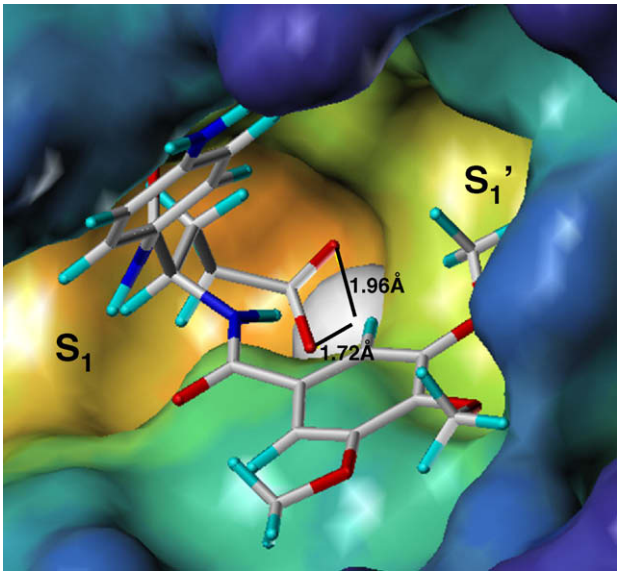


Figure 3. FlexX docking result of compound **6j** with the active site of *Escherichia coli* APN (PDB: 2DQM).

taining compounds **6a**, **6c** and **6d**, we can conclude that phenyl sulfonamide and amide groups were positively relative with the APN inhibition. This might be due to the formation of hydrogen bond with the enzyme backbone, thus stabilizing the binding between the compound and the enzyme. However, replacing five-membered thiazole ring (**6d**) with six-membered pyridinyl ring (**6e**) produced slightly improved potency.

When it comes to compounds with mono-substitution at the *para*-position in the aromatic ring (**6f–k**), to some extent, the electron-withdrawing property of *para*-substituent was negatively relative with the inhibitory activities. For example, the introduction of amino group (**6j**) displayed the highest activity, the halogen group (**6f–h**) was in the next place, following the carboxyl group (**6k**), and the nitro group (**6i)** presented the least activity, even has no APN and MMP-2 inhibition. Moreover, as compared with fluoro, chloro and bromo substitutions, it seems that the increased volume might lead to impair activity, suggesting there is a space requirement in the binding pockets to accommodate the suitable substituents. The same rule can also be applied to compound **6t**.

As compared with the halogen-substituted compounds, generally speaking, di-substitution at the phenyl moiety (**6m–o**) moderately impaired the activity in comparison with the mono-substitution counterparts (**6f–h**). However, when we changed the phenyl ring into benzyl moiety, the reverse rule can be observed. That is to say, di-substitution at the benzyl moiety (**6r** and **6s**) showed better inhibitory activity compared with the mono-substitution compounds, **6p** and **6q**. Interestingly, compound **6l**, with a hydroxyl group *ortho*- to the phenyl carboxyl core (at the 3- and 4-positions, respectively), was slightly more potent than **6k**, suggesting that hydroxyl group was positively related with the inhibitory activity by forming effective hydrogen bond with the residue of the enzyme.

3.2. Binding mode

In order to investigate the interaction of our target compound with APN, the most active compound **6j** was selected for molecular docking study by utilizing SYBYL 7.0. The binding mode of **6j** in the active site of *Escherichia coli* APN (PDB code: 2DQM) is proposed based on computational-docking results. The binding studies (Fig. 3) demonstrated that carbonyl group of **6j** can coordinate with the catalytic zinc ion with the distance of 1.96 Å and 1.72 Å, respectively. In addition, one of the methoxy group and partial carbonyl portion insert into S_1' and S_1 pocket.

According to the structure shown as Figure 4, the carbonyl group of **6j** can form hydrogen bonds with His^{297} (<2.85 Å) and His^{301} (<3.03 Å), which are the essential amino acids of the conserved sequence ($HEXXHX_{18}E$) in the catalytic domain that is well conserved in peptidase M1 family.[30] As far as the Tyr^{381} residue is concerned, the phenolic hydroxyl group of it can interact with the carbonyl group of **6j** by hydrogen interaction (<2.66 Å) which might be benefit to stabilize of interaction intermediate with the zinc ion.[15] In addition, the binding interactions were further enhanced by hydrogen bonds with Tyr275, Ala262, Gly261, and Arg293 as well. Finally, the hydrophobic parts of phenyl rings are in contact with nonpolar surface areas of APN.

Although the computed information partially supported our assumption, the exact binding model of the target compounds with APN should be verified from further X-ray crystal studies.

4. Conclusions

In summary, we developed a new series of L-*iso*-glutamine derivatives originated from the scaffold antineoplaston AS2-5. Most of the compounds possess selective inhibitory activity against APN as compared with MMP-2. This feature may offer a critical point in designing selective metalloproteinase inhibitors so as to optimize the recognition of APN. Besides, further in vitro and in vivo evaluation of these compounds on anti-tumor activity is underway.

5. Experimental

5.1. General procedures

Silica gel for column chromatography (CC) and thin-layer chromatography (TLC) plates precoated with Silica Gel GF_{254} were commercial available from Qingdao Haiyang Chemical Company, Qingdao, China. Reaction courses and product mixtures were routinely monitored by TLC on 0.25 mm Silica Gel 60 F_{254} plates and visualized under ultraviolet (UV) light (254 nm), or iodine (I_2) vapor. Flash-chromatography (FC) was performed using 200–300 mesh silica gel and the solvent system was indicated in the procedure. All solvents were of reagent grade and, when necessary, were purified and dried by standard methods. Melting points (Mp) were determined on an *X-6* micro-melting-point apparatus (*Beijing Tech Co., Ltd*) with no correction. Infrared spectra (IR) were recorded in the range of 4000–600 cm^{-1} using a *Nicolet Nexus 470FT-IR* spectrometer, and KBr disks were used as indicated. ^1H NMR spectra were determined on a *Brucker Avance DRX-600* spectrometer, with chemical shift (δ) given in ppm upfield from Me_4Si (TMS) as an internal standard, and coupling constants *J* were recorded in hertz (Hz). Electrospray ionization mass spectrometry (ESI-MS) was performed on an *API-4000* triple-stage quadrupole instrument. Anhydrous reactions were carried out in oven-dried glassware under a nitrogen atmosphere, and all anhydrous solvents were distilled over CaH_2 or Na/benzophenone prior to use. Yields refer to purified products and are not optimized.

5.2. Syntheses

5.2.1. 3,4,5-Trimethoxybenzoic acid (2)

To a stirred solution of gallic acid **1** (5 g, 29.4 mmol) in 50 ml of 4 N NaOH was added $(CH_3)_2SO_4$ (8.9 g, 71 mmol) dropwise, keeping the inside temperature under 20 °C. The resulting solution was allowed to stir at 30–40 °C for 20 min, another portion of gallic

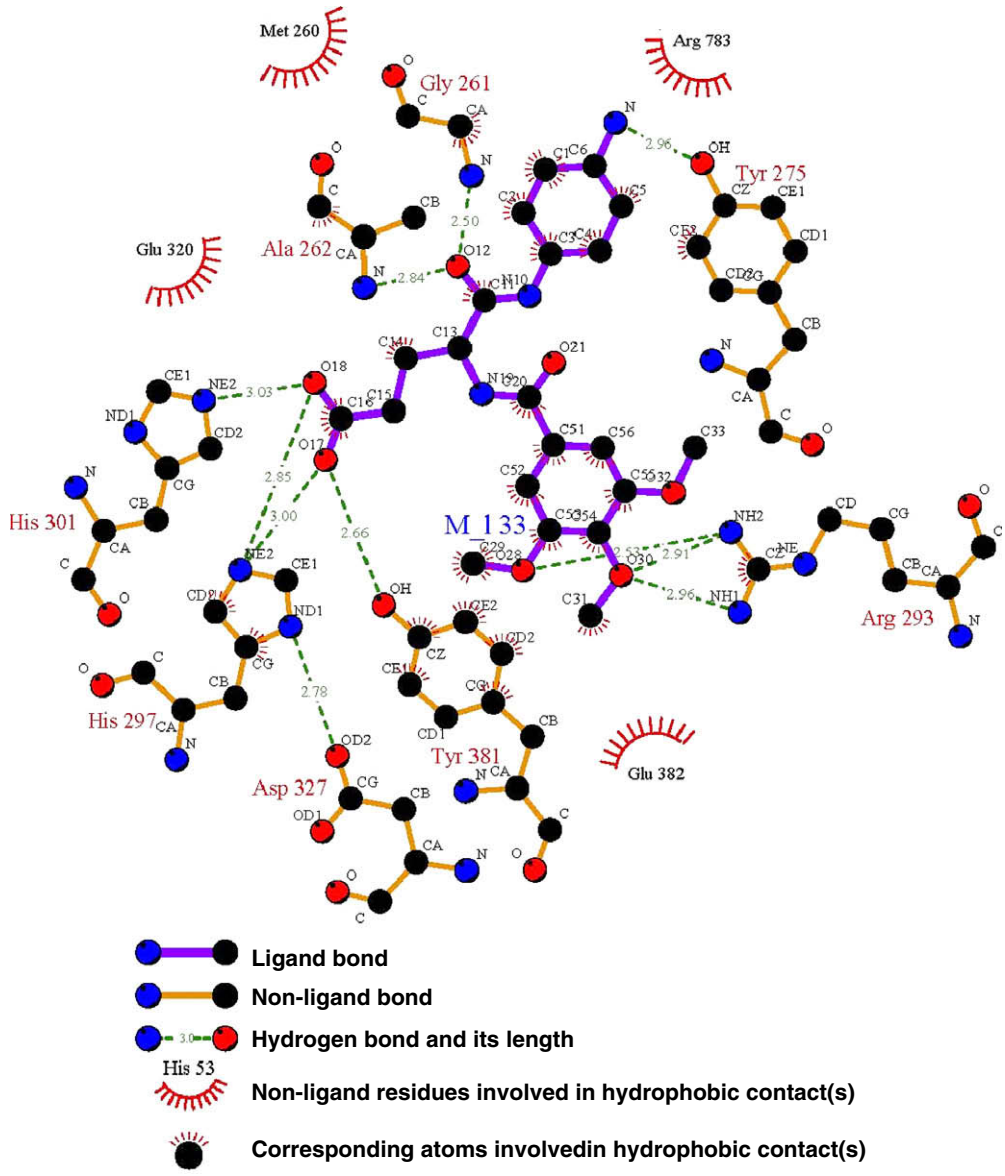


Figure 4. Diagram (LIGPLOT) of the hydrogen bonds and hydrophobic interactions of **6j** with APN.

acid (5 g) and 50 ml of 4 N NaOH were successively added. The mixture was slowly heated to 90 °C and maintained at this temperature for 1 h. After refluxing for another 2 h, the reaction mixture was allowed to cool to rt. The solution was then acidified with 2 N HCl to pH 2, filtered, and the solid was washed twice with distilled water. The crude product was recrystallized with 50% EtOH to give compound **2** (40.8 g, 65%) as a white needle crystals: mp 168–171 °C. ^{1}H NMR δ 3.97 (s, 9H), 7.23 (s, 2H), 12.11 (s, 1H). ESI-MS: m/z $[M+H]^+$ 165.8.

5.2.2. 3,4,5-Trimethoxybenzoic chloride (3)

To a stirred solution of **2** (21.2 g, 100 mmol) in benzene (320 ml) was added dropwise $SOCl_2$ (40 ml, 548 mmol). The reaction mixture was refluxed for 3 h, and the solvent was removed to obtain compound **3** as pale yellow oil. Successively another 50 ml of benzene was added and evaporated until the excess $SOCl_2$ was completely removed. The crude product was used immediately in the next reaction without further purification.

5.2.3. (*R*)-2-(3,4,5-Trimethoxybenzamido)pentanedioic acid (4)

To a suspension of anhydrous Na_2CO_3 (19.6 g, 185 mmol) and L-glutamic acid (16.9 g, 115 mmol) in distilled water (150 ml) at −5 °C was added a solution of compound **3** in 200 ml benzene dropwise over 1 h. Introduce a mechanical stirrer and stir vigorously until the solid has almost completely dissolved (about 5 h). The mixture was acidified with 2 N HCl to pH 5–6, the layers were separated, and the aqueous phase was acidified again with 2 N HCl to pH 3–4, and extracted twice with chloroform. Remove the combined organics, and the obtained aqueous phase was acidified with 2 N HCl to pH 2. Cool and leave in a refrigerator overnight, the white precipitate was filtered with glass filter, washed with water, and dried in vacuo to give compound **4** (28.6 g, 89.3%). Mp 120–121 °C, IR (KBr, cm^{-1}): 3299.7 and 3255.4 (NH), 2942.5 (CH), 1744.7 and 1699.5 (C=O), 1500.7 (δ NH), 1235.5 and 1127.7 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.41 (s, 2H, 2-COOH), 8.53 (d, 1H, J = 7.2 Hz, NH), 7.22 (s, 2H, Ar-H), 4.41 (m, 1H, CH), 3.83 (s, 6H, 2-OCH_3), 3.71 (s, 3H, OCH_3), 2.35 (m, 2H, CH_2), 2.21 (m, 1H, CH), 1.96 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 341.8.

5.2.4. (*R*)-*N*-(2,6-Dioxo-tetrahydro-2*H*-pyran-3-yl)-3,4,5-trimethoxybenzamide (5)

Place 10 g (2.9 mmol) of compound **4** and 80 ml of redistilled acetic anhydride in a 250 ml round-bottomed flask and boil the mixture steadily under 55–60° for 5 h. Remove a small quantity of insoluble substance by rapidly filtering the hot reaction mixture through a small plug of cotton wool loosely inserted into the stem of a preheated glass filter funnel. After 10 ml of anhydrous diethyl ether was added, the filtrate was allowed to cool to room temperature. The collected white precipitate was dried in vacuo to give **5** as white solid (5.2 g, 55%). Mp 150–152 °C, IR (KBr, cm^{-1}): 3310.0 (NH), 2945.1 (CH), 1777.0 and 1640.7 (O=C–O–C=O), 1504.2 (δ NH), 1239.6 and 1129.6 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 6.99 (s, 2H, Ar-H), 8.13 (d, 1H, J = 7.2 Hz, NH), 4.45 (m, 1H, CH), 3.73 (s, 9H, 3-OCH_3), 1.96–2.25 (m, 2H, CH_2), 2.18–2.28 (m, 2H, CH_2). ESI-MS: m/z $[M+H]^+$ 323.8.

5.2.5. (*R*)-5-Oxo-5-4-[(1,3-thiazole-2-amino)sulfonyl] phenylamino-4-[(3,4,5-trimethoxybenzamido)amino] pentanoic acid (6a)

A suspension of compound **5** (3.23 g, 10 mmol) and 4-amino-*N*-(thiazol-2-yl)benzenesulfonamide (2.8 g, 11 mmol) in acetic acid (30 ml) was stirred at room temperature until the disappearance of the starting material, checking via TLC. Collect the white flaky crystals on a Buchner funnel, wash with ice-cold water and dry to give **6a** (5.75 g, 78%). Mp 143–145 °C, IR (KBr, cm^{-1}): 3324.3, 3117.5 (NH), 2939.5 (CH), 1706.3 (O=C–NH), 1590.3 (C=C), 1127.0 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.6 (s, 1H, COOH), 12.21 (s, 1H, NH), 10.44 (s, 1H, NH), 8.58 (d, 1H, NH, J = 7.2 Hz), 7.75 (d, 2H, Ar-H, J = 9.3 Hz), 7.71 (d, 1H, =CH, J = 4.3 Hz), 7.25 (s, 2H, Ar-H), 7.23 (d, 2H, Ar-H, J = 9.1 Hz), 6.80 (d, 1H, =CH, J = 4.4 Hz), 4.56 (d, 1H, CH, J = 5.5 Hz), 3.83 (s, 6H, 2-OCH_3), 3.71 (s, 3H, -OCH_3), 2.41 (m, 1H, CH), 2.34 (m, 1H, CH), 2.09 (m, 1H, CH), 2.03 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 581.4.

The other L-*iso*-glutamine derivatives were synthesized following the general procedure as the above mentioned.

5.2.6. (*R*)-5-[4-(Aminosulfonyl)phenylamino]-5-oxo-4-[(3,4,5-trimethoxybenzamido)amino]pentanoic acid (6b)

Yield: 67%, mp 206–208 °C, IR (KBr, cm^{-1}): 3334.7, 3299.9, 3245.2 (NH), 1733.9 (O=C–NH), 1668.3 (O=C–O), 1529.1 (C=C), 1340.9 and 1134.0 (S–O, C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.04 (s, 1H, COOH), 10.37 (s, 1H, NH), 8.54 (d, 1H, NH, J = 7.08 Hz), 7.77 (s, 4H, Ar-H), 7.26 (s, 2H, Ar-H), 7.18 (s, 2H, NH_2), 4.59 (d, 1H, CH, J = 6.5 Hz), 3.84 (s, 6H, 2-OCH_3), 3.73 (s, 3H, –OCH_3), 2.42 (m, 1H, CH), 2.35 (m, 1H, CH), 2.12 (m, 1H, CH), 2.06 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 497.9.

5.2.7. (*R*)-5-Oxo-5-(thiazol-2-ylamino)-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6c)

Yield: 80%, mp 204–205 °C, IR (KBr, cm^{-1}): 3411.7 and 3328.3 (NH), 2940.7 (CH), 1696.1 (O=C-NH), 1583.0 and 1497.8 (C=C), 1128.3 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.27 (s, 1H, COOH), 12.20 (s, 1H, NH), 8.62 (d, 1H, NH, J = 7.0 Hz), 7.48 (d, 1H, =CH, J = 3.5 Hz), 7.25 (s, 2H, Ar-H), 7.22 (d, 1H, =CH, J = 3.6 Hz), 4.63 (m, 1H, CH), 3.83 (s, 6H, 2-OCH_3), 3.71 (s, 3H, -OCH_3), 2.41 (m, 1H, CH), 2.34 (m, 1H, CH), 2.09 (m, 2H, CH_2). ESI-MS: m/z $[M+H]^+$ 423.8.

5.2.8. (*R*)-5-Oxo-5-(2-(thiazole-2-carbonyl)hydrazinyl)-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6d)

Yield: 52%, mp 213–214 °C, IR (KBr, cm^{-1}): 3414.2 and 3326.9 (NH), 2944.7 (CH), 1706.4 (O=C–NH), 1671.4 and 1656.0 (O=C–O), 1497.7 (C=C), 1126.9 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.20 (s, 1H, COOH), 12.08 (s, 1H, NH), 8.61 (d, 1H, NH, J = 3.8 Hz), 8.06 (d, 1H, NH, J = 5.5 Hz), 7.39 (d, 1H, =CH, J = 7.5 Hz), 7.25 (s, 2H, Ar-H), 7.17 (d, 1H, =CH, J = 3.6 Hz), 4.58 (m, 1H, CH), 3.81 (s, 6H, 2-OCH_3), 3.73 (s, 3H, –OCH_3), 2.44 (m, 1H, CH), 2.29 (m, 1H, CH), 2.11 (m, 2H, CH_2). ESI-MS: m/z $[M+H]^+$ 466.4.

5.2.9. (*R*)-5-(2-Nicotinoylhydrazinyl)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6e)

Yield: 67%, mp 237–238 °C, IR (KBr, cm^{-1}): 3297.3 (NH), 2942.3 (CH), 1715.2 (O=C–NH), 1673.0 and 1654.9 (O=C–O), 1498.3 (C=C), 1124.5 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 10.65 (s, 1H, COOH), 10.07 (s, 1H, NH), 8.76 (d, 2H, Ar-H, J = 4.5 Hz), 8.68 (d, 1H, NH, J = 7.5 Hz), 7.75 (d, 2H, Ar-H, J = 4.5 Hz), 7.23 (s, 2H, Ar-H), 4.42 (m, 1H, CH), 3.83 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.37 (m, 2H, CH_2), 2.17 (m, 1H, CH), 2.03 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 460.9.

5.2.10. (*R*)-5-(4-Fluorophenylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6f)

Yield: 60%, mp 205–207 °C, IR (KBr, cm^{-1}): 3424.5 and 3276.9 (NH), 2939.2 (CH), 1715.7 (C=O), 1640.0 and 1508.2 (C=C), 1125.1 (C-O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.06 (s, 1H, COOH), 10.07 (s, 1H, NH), 8.49 (d, 1H, NH, J = 7.5 Hz), 7.63 (m, 2H, Ar-H), 7.25 (s, 2H, Ar-H), 7.13 (m, 2H, Ar-H), 4.57 (m, 1H, CH), 3.84 (s, 6H, 2-OCH_3), 3.72 (s, 3H, –OCH_3), 2.39 (m, 2H, CH_2), 2.12 (m, 1H, CH), 2.05 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 434.8.

5.2.11. (*R*)-5-(4-Chlorophenylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6g)

Yield: 68%, mp 217–218 °C, IR (KBr, cm^{-1}): 3420.1 and 3265.5 (NH), 2937.8 (CH), 1712.0 (C=O), 1639.5 and 1491.8 (C=C), 1128.4 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.14 (s, 1H, COOH), 10.24 (s, 1H, NH), 8.57 (d, 1H, NH, J = 7.4 Hz), 7.65 (m, 2H, Ar-H), 7.36 (m, 2H, Ar-H), 7.25 (s, 2H, Ar-H), 4.56 (m, 1H, CH), 3.84 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.39 (m, 1H, CH), 2.34 (m, 1H, CH), 2.09 (m, 1H, CH), 2.03 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 450.8.

5.2.12. (*R*)-5-(4-Bromophenylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6h)

Yield: 79%, mp 221–222 °C, IR (KBr, cm^{-1}): 3420.6 (OH), 3291.8 and 3264.5 (NH), 2938.2 (CH), 1712.3 (O=C–NH), 1639.8 (O=C–O), 1489.9 (C=C), 1128.3 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.06 (s, 1H, COOH), 10.16 (s, 1H, NH), 8.51 (d, 1H, NH, J = 6.4 Hz), 7.60 (d, 2H, Ar-H, J = 7.8 Hz), 7.60 (d, 2H, Ar-H, J = 7.8 Hz), 7.25 (s, 2H, Ar-H), 4.57 (m, 1H, CH), 3.84 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.39 (m, 1H, CH), 2.35 (m, 1H, CH), 2.12 (m, 1H, CH), 2.04 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 497.1.

5.2.13. (*R*)-5-(4-Nitrophenylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6i)

Yield: 71%, mp 228–230 °C, IR (KBr, cm^{-1}): 3392.3 and 3289.7 (NH), 2940.5 (CH), 1708.9 (O=C–NH), 1583.9 and 1499.9 (C=C), 1326.1 and 1130.3 (N–O, C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.18 (s, 1H, COOH), 10.74 (s, 1H, NH), 8.65 (d, 1H, NH, J = 7.2 Hz), 8.24 (d, 1H, Ar-H, J = 9.2 Hz), 7.89 (d, 1H, Ar-H, J = 9.3 Hz), 7.25 (s, 2H, Ar-H), 4.59 (m, 1H, CH), 3.84 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 3.31 (s, 2H, CH_2), 2.43 (m, 1H, CH), 2.37 (m, 1H, CH), 2.12 (m, 1H, CH), 2.06 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 461.4.

5.2.14. (*R*)-5-(4-Aminophenylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6j)

Yield: 75%, mp >255 °C, IR (KBr, cm^{-1}): 3399.8 and 3279.9 (NH), 2939.6 (CH), 1714.0 (O=C–NH), 1637.8 (O=C–O), 1584.1 and 1500.4 (C=C), 1129.0 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.13 (s, 1H, COOH), 10.04 (s, 1H, NH), 8.54 (d, 1H, NH, J = 7.5 Hz), 7.55 (s, 2H, Ar-H), 7.25 (s, 2H, Ar-H), 4.56 (m, 1H, CH), 3.84 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.41 (m, 1H,

CH), 2.34 (m, 1H, CH), 2.10 (m, 1H, CH), 2.04 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 431.8.

5.2.15. (*R*)-4-(4-Carboxy-2-(3,4,5-trimethoxybenzamido) butanamido)benzoic acid (6k)

Yield: 64%, mp 248–249 °C, IR (KBr, cm^{-1}): 3512.0 (OH), 3361.4 (NH), 2944.6 (CH), 1710.9 and 1640.1 (O=C-NH), 1589.8 and 1498.4 (C=C), 1120.8 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 12.35 (br, 2H, 2-COOH), 10.34 (s, 1H, NH), 8.53 (d, 1H, NH, J = 7.2 Hz), 7.90 (d, 2H, Ar-H, J = 8.4 Hz), 7.74 (d, 2H, Ar-H, J = 8.4 Hz), 7.25 (s, 2H, Ar-H), 4.61 (m, 1H, CH), 3.84 (s, 6H, 2-OCH_3), 3.72 (s, 3H, –OCH_3), 2.41 (m, 1H, CH), 2.35 (m, 1H, CH), 2.12 (m, 1H, CH), 2.06 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 462.9.

5.2.16. (*R*)-4-(4-Carboxy-2-(3,4,5-trimethoxybenzamido) butanamido)-2-hydroxybenzoic acid (6l)

Yield: 88%, mp 222–224 °C, IR (KBr, cm^{-1}): 3335.2 (NH), 2992.2 (CH), 1718.2 (O=C-NH), 1668.9 and 1604.9 (O=C–O), 1500.4 (C=C), 1130.0 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 13.69 (s, 1H, COOH), 12.20 (s, 1H, COOH), 11.35 (s, 1H, OH), 10.42 (s, 1H, NH), 8.63 (d, 1H, NH, J = 7.2 Hz), 7.72 (d, 1H, Ar-H, J = 8.7 Hz), 7.39 (s, 1H, Ar-H), 7.21 (s, 2H, Ar-H), 7.10 (d, 1H, Ar-H, J = 7.7 Hz), 4.56 (m, 1H, CH), 3.84 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.43 (m, 1H, CH), 2.34 (m, 1H, CH), 2.09 (m, 1H, CH), 2.04 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 476.8.

5.2.17. (*R*)-5-(2,4-Difluorophenylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6m)

Yield: 56%, mp 174–176 °C, IR (KBr, cm^{-1}): 3328.3 (NH), 2940.0 (CH), 1719.1 and 1639.5 (O=C–NH), 1501.2 (C=C), 1131.0 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 12.16 (s, 1H, COOH), 9.84 (s, 1H, NH), 8.57 (d, 1H, NH, J = 7.5 Hz), 7.76 (m, 1H, Ar-H), 7.32 (m, 1H, Ar-H), 7.29 (s, 2H, Ar-H), 7.06 (m, 1H, Ar-H), 4.67 (m, 1H, CH), 3.84 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.37 (m, 2H, CH_2), 2.12 (m, 1H, CH), 2.03 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 452.8.

5.2.18. (*R*)-5-(2,5-Dichlorophenylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6n)

Yield: 89%, mp 219–220 °C, IR (KBr, cm^{-1}): 3367.8 and 3281.4 (NH), 2942.1 (CH), 1704.9 (O=C), 1587.2 (C=C), 1132.8 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 12.11 (s, 1H, COOH), 9.60 (s, 1H, NH), 8.62 (d, 1H, NH, J = 7.2 Hz), 7.97 (d, 1H, Ar-H, J = 2.16 Hz), 7.54 (d, 1H, Ar-H, J = 8.6 Hz), 7.26 (s, 3H, Ar-H), 4.71 (d, 1H, CH, J = 5.5 Hz), 3.85 (s, 6H, 2-OCH_3), 3.73 (s, 3H, –OCH_3), 2.42 (m, 2H, CH_2), 2.20 (d, 1H, CH, J = 6.1 Hz), 2.07 (d, 1H, CH, J = 6.1 Hz). ESI-MS: m/z $[M+H]^+$ 407.1.

5.2.19. (*R*)-5-(3-Chloro-4-fluorophenylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6o)

Yield: 58%, mp 204–205 °C, IR (KBr, cm^{-1}): 3333.4 (NH), 2940.5 (CH), 1715.7 (O=C), 1641.4 and 1500.4 (C=C), 1130.1 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 12.10 (s, 1H, COOH), 10.32 (s, 1H, NH), 8.59 (d, 1H, NH, J = 7.3 Hz), 7.94 (m, 1H, Ar-H), 7.52 (m, 1H, Ar-H), 7.37 (m, 1H, Ar-H), 7.25 (s, 2H, Ar-H), 4.53 (m, 1H, CH), 3.84 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.39 (m, 1H, CH), 2.35 (m, 1H, CH), 2.10 (m, 1H, CH), 2.03 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 468.8.

5.2.20. (*R*)-5-(3-Bromobenzylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6p)

Yield: 89%, mp 177–179 °C, IR (KBr, cm^{-1}): 3334.8 (NH), 2992.0 and 2940.4 (CH), 1714.4 (O=C–NH), 1635.9 (O=C–O), 1584.2 and 1500.4 (C=C), 1130.6 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 8.59 (s, 1H, NH), 8.51 (s, 1H, NH), 7.47 (s, 2H, Ar-H), 7.41 (s, 2H, Ar-H), 7.27 (s, 4H, Ar-H), 4.45 (m, 1H, CH), 4.29 (s, 2H, CH_2), 3.83 (s, 6H, 2-OCH_3), 3.70 (s, 3H, –OCH_3), 2.29 (m, 2H, CH_2), 2.06 (m, 1H, CH), 1.97 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 508.8.

5.2.21. (*R*)-5-(3-Iodobenzylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6q)

Yield: 59%, mp 165–167 °C, IR (KBr, cm^{-1}): 3275.5 (NH), 2937.8 (CH), 1708.7 and 1628.8 (O=C–NH, O=C–O), 1582.9 (C=C), 1128.5 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 12.11 (s, 1H, COOH), 8.49 (m, 1H, NH), 8.47 (d, 1H, NH, J = 7.8 Hz), 7.65 (s, 1H, Ar-H), 7.59 (d, 1H, Ar-H, J = 8.4 Hz), 7.26 (m, 3H, Ar-H), 4.45 (m, 1H, CH), 4.26 (m, 2H, CH_2), 3.83 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.31 (m, 2H, CH_2), 2.07 (m, 1H, CH), 1.95 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 556.7.

5.2.22. (*R*)-5-(4-Chloro-2-fluorobenzylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6r)

Yield: 62%, mp 145–147 °C, IR (KBr, cm^{-1}): 3292.3 (NH), 2940.8 (CH), 1713.2 (O=C), 1583.6 and 1498.9 (C=C), 1128.2 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 8.63 (d, 1H, NH, J = 6.2 Hz), 8.46 (t, 1H, NH, J = 7.2 Hz), 7.36 (m, 3H, Ar-H), 7.23 (s, 2H, Ar-H), 4.47 (m, 1H, CH), 4.30 (t, 2H, CH_2, J = 6.3 Hz), 3.83 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.32 (m, 2H, CH_2), 2.29 (m, 1H, CH), 2.84 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 482.8.

5.2.23. (*R*)-5-(4-Bromo-2-fluorobenzylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6s)

Yield: 54%, mp 146–148 °C, IR (KBr, cm^{-1}): 3292.3 (NH), 2940.7 (CH), 1712.4 and 1642.3 (O=CO=C), 1583.6 and 1499.5 (C=C), 1128.9 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 8.63 (d, 1H, NH, J = 7.2 Hz), 8.46 (t, 1H, NH, J = 7.2 Hz), 7.49 (t, 1H, Ar-H, J = 7.8 Hz), 7.38 (m, 2H, Ar-H), 7.23 (s, 2H, Ar-H), 4.28 (m, 1H, CH), 4.24 (m, 2H, CH_2), 3.83 (s, 6H, 2-OCH_3), 3.70 (s, 3H, –OCH_3), 2.32 (m, 2H, CH_2), 2.31 (m, 1H, CH), 2.28 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 528.8.

5.2.24. *N*-(1-(2-Aminophenyl)-2,6-dioxopiperidin-3-yl)-3,4,5-trimethoxybenzamide (6t)

Yield: 70%, mp >230 °C, IR (KBr, cm^{-1}): 3427.3 and 3245.8 (NH), 2939.8 (CH), 1633.8 (O=C–NH), 1584.1 and 1499.7 (C=C), 1127.9 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 12.23 (br, 2H, NH), 8.86 (d, 1H, NH, J = 4.0 Hz), 7.47 (m, 2H, Ar-H), 7.30 (s, 2H, Ar-H), 7.14 (m, 2H, Ar-H), 5.35 (m, 1H, CH), 3.83 (s, 6H, 2-OCH_3), 3.71 (s, 3H, –OCH_3), 2.46 (m, 2H, CH_2), 2.43 (m, 1H, CH), 2.40 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 411.1.

5.2.25. (*R*)-5-(2-Chloroethylamino)-5-oxo-4-(3,4,5-trimethoxybenzamido)pentanoic acid (6u)

Yield: 62%, mp 158–159 °C, IR (KBr, cm^{-1}): 3292.9 and 3260.9 (NH), 2942.7 (CH), 1727.6 and 1659.4 (O=C-NH), 1578.4 and 1499.8 (C=C), 1128.3 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 12.10 (s, 1H, COOH), 8.42 (d, 1H, NH, J = 7.8 Hz), 8.19 (d, 1H, NH, J = 5.7 Hz), 7.23 (s, 2H, Ar-H), 4.45 (m, 1H, CH), 3.83 (s, 6H, 2-OCH_3), 3.70 (s, 3H, –OCH_3), 3.61 (m, 2H, CH_2), 3.45 (m, 1H, CH), 3.35 (m, 1H, CH), 2.31 (m, 2H, CH_2), 2.04 (m, 1H, CH), 1.91 (m, 1H, CH). ESI-MS: m/z $[M+H]^+$ 402.8.

6. Enzymatic inhibiton assay (in vitro)

6.1. In vitro APN assay

The IC_{50} values against APN were determined using L-Leu-*p*-nitroanilide as substrate and microsomal aminopeptidase from Porcine Kidney Mocrosomes (Sigma) as the enzyme in 50 mM PBS, pH 7.2 at 37 °C.[31] 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 adjusted to 7.5 by the addition of 0.1 M HCl or 0.1 M NaOH. All inhibitors were preincubated with APN for 30 min at room temperature. The assay mixture, which contained the inhibitor solution (concentration dependent on the inhibitor), the enzyme solution (4 μg/ml final concentration), and the assay buffer, was adjusted to 200 μL.

6.2. In vitro MMP-2 assay

MMP-2, also called as Gelatinase A, assay was performed as described by Baragi et al.[32] The gelatinase, substrate 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% trinitrobenzenesulfonic acid (TNBS, Sigma) was added and incubated for another 20 min, the resulting solution was detected under 450 nm wavelength to gain absorption.

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