Design, synthesis, and primary activity evaluation of pyrrolidine derivatives as matrix metalloproteinase inhibitors

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ABSTRACT: A series of novel pyrrolidine derivatives was designed, synthesized, and assayed to determine the derivatives' activity against matrix metalloproteinase-2 (MMP-2) and aminopeptidase N (APN)/CD13. Preliminary biological tests showed that most compounds inhibit MMP-2 in a highly selective manner compared to APN. Compounds 9d, 9e, and 9g had better inhibitory activity than LY52 and could be used as lead compounds in the future.

Keywords: Matrix metalloproteinase-2, aminopeptidase N, inhibitors, pyrrolidine derivatives, synthesis

1. Introduction

The matrix metalloproteinases (MMPs) are a family of zinc-dependent calcium-containing hydrolytic enzymes that play a crucial role in tissue remodeling and degradation of the extracellular matrix (ECM). More than 20 subfamily members have previously been identified, such as collagenases, stromelysins, gelatinases, and membrane-type MMPs (1,2). Under normal physiological conditions, MMPs are minimally expressed, while their over-expression can lead to a variety of pathological disorders. Among MMPs, MMP-2 has been considered as a potential therapeutic target for cancer due to its high correlation with tumor growth, invasion, and metastasis (2,3).

Recently, the high-resolution X-ray crystal structures of MMP-inhibitor complexes have increasingly been revealed (4). This structural information indicates that besides the conserved catalytic site of Zn$^{2+}$ of the MMP-2 enzyme there are two hydrophobic domains, named the S1$'$ and S2$'$ pockets. The S1$'$ pocket, a deep and narrow channel, is the key domain, and the S2$'$ pocket is a solvent-exposed cleft (5,6). In general, a typical inhibitor of MMPs consists of a "zinc-binding group (ZBG)" and a "backbone". In addition, at least one side chain has been reported to effectively interact with enzyme subsites such as the S1$'$ and S2$'$ pockets (7).

cis-2-Aminocyclohexylcarbamoylphosphonic acid (cis-ACCP; See Figure 1) was designed and synthesized as an efficient MMP inhibitor and can selectively block the proteolytic activity of MMP-2. cis-ACCP also significantly inhibited metastasis formation in a murine melanoma model and reduced both local tumor growth and metastasis formation in an orthotopic human prostate tumor model. Moreover, the introduction of amino groups into the molecule enhanced their zinc-binding effect and also improved their inhibitory potency by forming effective hydrogen bonds (8).

The current authors have been developing a pyrrolidine scaffold as an effective MMP inhibitor for many years. Most compounds such as LY52 (See Figure 1) substantially inhibit MMP-2 and display significant anti-cancer activity both in vitro and in vivo (9,10). Based on this finding, together with the fact that Hyp and Gly residues account for more than 60% of all amino acids in the primary structure of collagen (11), Hyp and Hyp-Gly residues were thus presumed to be the specific recognition sites for effective interaction with MMPs. Additionally, trans-s-hydroxy-L-proline is the main constituent of Hyp and Hyp-Gly residues, so amino acids fragments were introduced into the trans-s-hydroxy-L-proline scaffold to form a new integrated structural pattern (See Figure 1). The R1 group would be hydroxamate, carboxylate, or ester functioning as an effective ZBG to chelate the active site of catalytic zinc ions. The R2 group would be introduced with various amino acids as side chains that might occupy the S1$'$ or S2$'$ pocket, resulting in effective enzyme interaction.

2. Materials and Methods

2.1. Chemicals

All of the target compounds were designed and synthesized via the route shown in Scheme 1. Starting
with *trans*-4-hydroxy-L-proline (1), the important intermediate (2S,4S)-1-tert-buty1-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (6) was prepared by esterification, acylation, sulfonation, SN₂ nucleophilic substitution, and catalytic hydrogenation (12). The condensation of compound 6 with various amino acid residues led to compounds 7a-h, which were directly de-protected or treated with NH₂OK in anhydrous methanol. Subsequent de-protection yielded the target compounds 8a-h and 9a-h. Some of the compounds that were selected from 7a-h were hydrolyzed to obtain compounds 10b-h, which were also de-protected to obtain the target compounds 11b-h. Further coupling of compounds 11d and f with glycine methyl ester yielded compounds 12d and f, which were de-protected to provide the target compounds 13d and f.

2.2. MMP-2 inhibition assay

Gelatinase A (MMP-2) and trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma, St Louis, MO, USA, and the substance was synthesized as described by Vijaykumar et al. (13). The pyrrolidine derivatives were assayed for inhibitory activity against MMP-2 in 96-well microtiter plates using succinylated gelatin as the substrate. The gelatinase, substance, and inhibitor were dissolved in sodium borate (pH 8.5, 50 mM) and incubated for 30 min at 37°C, and then 0.03% TNBS was added and the solution was incubated for another 20 min. The resulting solution was detected at a
wavelength of 450 nm to yield OD values.

2.3. APN inhibition assay

IC₅₀ values against APN were determined using L-Leu-p-nitroanilide as the substrate and microsomal aminopeptidase (Sigma) as the enzyme in 50 mM PBS, pH 7.2, at 37°C. The hydrolysis of the substrate was monitored by following the changes in absorbance measured at 405 nm. All 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 inhibitors were preincubated with APN for 30 min at 37°C. The assay mixture, which contained the inhibitor solution (with its concentration depending on the inhibitor), the enzyme solution (4 μg/mL final concentration), and the assay buffer, was adjusted to 200 μL.

3. Results and Discussion

All inhibition results are listed in Table 1. Similar to MMP-2, APN is also a zinc-dependant metalloproteinase involved in tumor invasion and metastasis. Thus, the assay was performed on both of MMP-2 and APN so as to identify the compounds’ selectivity (13,14). LY52 was used as the positive control.

The pyrrolidine derivatives exhibited better activity against MMP-2 than APN. For example, 9e had an IC₅₀ (APN)/IC₅₀ (MMP-2) ratio of 365.46, while 9d had one of 279.38. To a certain extent, the results confirmed the current strategy of designing effective MMP-2 inhibitors. MMP-2 is a zinc-dependent

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LY52

5.6  578.9  103.75
endopeptidase that cleaved the peptide from its specific amino acid residue, while APN is a membrane-bound zinc exopeptidase that catalyzed the removal of NH₂-terminal amino acids from the peptide. The differences between the structures of the two enzymes lead to different requirements for their respective inhibitors. Therefore, these compounds are more suitable as MMP-2 inhibitors. Compounds 9d, 9e, and 9g were more potent MMP-2 inhibitors than the positive control LY52. The FlexX docking of compounds 9e and 9g with MMP-2 was done using Sybyl 7.0 from Tripos Inc. (St Louis, MO, USA), and the results are shown in Figures 2 and 3, respectively. Compounds 9a-h and 11b-h were more potent than 8a-h, which might be attributed to the ZBG (R₁). Hydroxamate, carboxylic acid, and carboxylate are the ZBGs for 9a-h, 11b-h, and 8a-h, respectively, all of which chelate zinc ions in the center of the enzyme's catalytic activity. However, the hydroxamate and carboxylic acid groups were more potent ZBGs than carboxylate group, as shown in Table 1. Compound 13f, a tripeptide containing Hyp-Gly residues, displayed selective inhibitory activity against MMP-2 with an IC₅₀ (APN)/IC₅₀ (MMP-2) ratio of 148.38. The IC₅₀ (MMP-2) of 13f was 16.8 μM, which is slightly higher than that of the positive control LY52 (5.6 μM). The authors are presently working on tripeptides with Hyp-Gly residues in order to obtain more potent compounds. For a further and detailed understanding of the binding mode of 9g with MMP-2, a 2D picture was also created with the program Ligplot. Hydrophobic and H-bond interactions were visualized between the inhibitor and the residues lining the active site of the protein. Significant hydrophobic interactions can be found between molecule 9g and amino acid Ala³⁰, His¹²⁰ shown in Figure 4. H-bond interactions are formed between the OE2 of Glu¹²¹ and N7 and N14 of 9g, the N of Leu⁸³ and Ala³⁰ and O12 of 9g, and the NE2 of His¹²⁰, His¹³⁰, and O13 of 9g.

4. Conclusion

In conclusion, a new series of pyrrolidine derivatives was synthesized as MMP-2 inhibitors. Most of the compounds showed potent activity and selectivity against MMP-2, and 9d, 9e, and 9g in particular were more potent than the positive control LY52. Further assays of these compounds in cell cultures and animal models are underway and will be reported in the near future.

Acknowledgements

This work was supported by the National High-Tech Research and Development Program of China (863 project, Grant No. 2007AA02Z314), the National Natural Science Foundation of China (Grant Nos. 30772654 and 90713041), and the Doctoral Foundation of the Ministry of Education of the People's Republic of China (Grant No. 20060422029).

References


(Appendix)

1. Chemistry: general procedures

All materials were commercially available. All the reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visualized with UV light or ferric chloride. 200-300 mesh silica gel was used in column chromatography. Proton NMR spectra were determined on a Brucker DRX spectrometer (600 MHz) with δ in parts per million and J in Hertz; TMS was used as an internal standard. Measurements were made in D2O solutions. ESI-MS were determined on an API 4000 spectrometer. Melting points were determined on an electrothermal melting point apparatus (uncorrected).

1.1. trans-4-Hydroxy-L-proline methylester hydrochloride (2)

26.2 g (200 mmol) trans-4-hydroxy-L-proline (1) in methanol (300 mL) was treated with dry hydrogen chloride until homogeneous. The solution was heated to the reflux temperature for 2 h and concentrated in vacuo. Upon cooling, the product was crystallized from the solvent, collected by filtration, washed with acetone and ether, and dried to yield trans-4-hydroxy-L-proline methylester hydrochloride (2) as white crystals (32.7 g, 90%), mp 159-162°C.

1.2. (4R)-1-(tert-Butoxycarbonyl)-4-hydroxy-L-proline methyl ester (3)

10.89 g (60 mmol) trans-4-hydroxy-L-proline methylester hydrochloride (2) was dissolved in DCM with Et3N (18 mL, 126 mmol) and treated with (Boc)2O (14.4 g, 66 mmol) in 20 mL DCM. The mixture was stirred at room temperature for 12 h, washed with 1 M citric acid, saturated NaHCO3 solution, and brine, and dried over Na2SO4. Evaporation of DCM gave a white solid (3), mp 96-98°C. ESI-MS m/z: 246.3 (M+H)+.

1.3. (3R,5S)-5-(Methoxy carbonyl)-1-(tert-butoxycarbonyl) pyrrolidin-3-ylsulfonates (4)

9.8 g (40 mmol) (4R)-1-(tert-butoxycarbonyl)-4-hydroxy-L-proline methyl ester (3) was dissolved in anhydrous DCM with Et3N (7 mL, 44 mmol) at 0°C and treated with MsCl (3.5 mL, 44 mmol) in 10 mL anhydrous DCM. After 12 h at 0°C, the mixture was washed with 1 M citric acid, saturated NaHCO3 solution, and brine, and then dried over Na2SO4. Evaporation of DCM gave compound 4, mp 84-86°C. ESI-MS m/z: 324.2 (M+H)+.

1.4. (2S,4S)-1-tert-Butyl-2-methyl-4-azidopyrrolidine-1,2-dicarboxylate (5)

(3R,5S)-5-(Methoxy carbonyl)-1-(tert-butoxycarbonyl) pyrrolidin-3-ylsulfonates (4) (12.92 g, 40 mmol) and 6.5 g NaN3 (100 mmol) were stirred overnight in DMF (40 mL) at 55-65°C. The mixture was mixed with 20 mL ice water and extracted with ethyl acetate (30 mL × 5).
The organic phase was washed with 0.1 M HCl and brine and dried over Na2SO4. Evaporation of EtOAc provided a colorless viscous oil (5). ESI-MS m/z: 271.6 (M+H)+.

1.5. (2S,4S)-1-tert-Butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (6)

(2S,4S)-1-tert-Butyl-2-methyl-4-azidopyrrolidine-1,2-dicarboxylate (5) (20 mmol) in MeOH (100 mL) was hydrogenated in the presence of a catalytic amount of 10% Pd-C at room temperature and 1 atm of pressure. After 36 h, the catalytic was filtered with a bed of kieselguhr on a funnel and the solvent was removed under a vacuum to give (2S,4S)-1-tert-butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (6). ESI-MS m/z: 245.5 (M+H)+.

1.6. (2S,4S)-1-tert-Butyl-2-methyl-4-(2-((tert-butoxycarbonyl)amino)acetamido)pyrrolidine-1,2-dicarboxylate (7b)

2-((tert-Butoxycarbonyl)amino)-propanoic acid (3.78 g, 20 mmol) and Et3N (2 equiv.) were dissolved in 100 mL anhydrous DCM. To this stirring solution was added 1,2-dimethyl 4-(2-(pyrrolidine-1,2-dicarboxylate (6)). ESI-MS m/z: 245.5 (M+H)+.

Compounds 7a and 7c-h were synthesized following the procedure described above. (2S,4S)-1-tert-Butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (7a), 2.2 g, 54.6%; (2S,4S)-1-tert-butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (7c), 2.9 g, 65.4%; (2S,4S)-1-tert-butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (7d), 3.12 g, 68.3%; (2S,4S)-1-tert-butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (7e), 3.09 g, 67.6%; (2S,4S)-1-tert-butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (7f), 3.42 g, 69.5%; (2S,4S)-1-tert-butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (7g), 2.92 g, 61.3%; (2S,4S)-1-tert-butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (7h), 2.36 g, 52.7%. 0.2 g compound 7a in 20 mL EtOAc saturated with HCl gas was stirred at room temperature for 6 h. The mixture was filtered to obtain the target compound 8a (0.12 g). Yield 87.4%, mp 75-77°C. 1H-NMR (DMSO-d6): 2.04 (m, 1H), 2.59 (m, 1H), 3.12 (m, 1H), 3.45 (m, 1H), 3.53 (s, 2H, CH2), 3.78 (s, 3H, CH3), 4.40 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, NH2).

Compounds 8b-h were synthesized following the procedure described above. (2S,4S)-Methyl 4-(2-aminopropanamido)pyrrolidine-2-carboxylate (8b)

White solid, yield 82.6%, mp 137-139°C. 1H-NMR (DMSO-d6): 1.36 (d, J = 6.6 Hz, 3H, CH3), 2.04 (m, 1H), 2.61 (m, 1H), 3.14 (m, 1H), 3.44 (m, 1H), 3.78 (s, 3H, CH3), 3.80 (d, J = 5.4 Hz, 1H), 4.39 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, NH2).

(2S,4S)-Methyl 4-(2-amino-3-methylbutanamido)pyrrolidine-2-carboxylate (8c)

White solid, yield 86.3%, mp 148-150°C. 1H-NMR (DMSO-d6): 0.93 (d, J = 6.6 Hz, 6H, CH3), 2.06 (m, 1H), 2.09 (m, 1H), 2.62 (m, 1H), 3.10 (m, 1H), 3.49 (m, 1H), 3.57 (s, 1H), 3.78 (s, 3H, CH3), 4.41 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, NH2).

(2S,4S)-Methyl 4-(2-amino-4-methylpentanamido)pyrrolidine-2-carboxylate (8d)

White solid, yield 80.2%, mp 146-148°C. 1H-NMR (DMSO-d6): 0.90 (t, 6H, CH3), 1.59 (m, 2H), 1.64 (m, 1H), 2.09 (m, 1H), 2.61 (m, 1H), 3.12 (m, 1H), 3.45 (m, 1H), 3.72 (t, 1H), 3.78 (s, 3H, CH3), 4.39 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, NH2).

(2S,4S)-Methyl 4-(2-amino-3-methylpentanamido)pyrrolidine-2-carboxylate (8e)

White solid, yield 85.7%, mp 154-156°C. 1H-NMR (DMSO-d6): 0.88 (m, 6H, CH3), 1.09 (m, 1H), 1.50 (m, 1H), 1.84 (m, 1H), 2.09 (m, 1H), 2.61 (m, 1H), 3.09 (m, 1H), 3.48 (m, 1H), 3.60 (s, 1H), 3.78 (s, 3H, CH3), 4.41 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, NH2).

(2S,4S)-Methyl 4-(2-amino-3-phenylpropanamido)pyrrolidine-2-carboxylate (8f)

White solid, yield 83.5%, mp 159-161°C. 1H-NMR (DMSO-d6): 1.96 (m, 1H), 2.61 (m, 1H), 2.85 (m, 1H), 3.04 (m, 1H), 3.09 (m, 1H), 3.34 (m, 1H), 3.89 (s, 3H, CH3), 3.98 (m, 1H), 4.34 (m, 1H), 4.45 (m, 1H), 7.27, 7.28, 7.32, 7.35 (5H, C6H5), 8.24 (s, 2H, NH2).

(2S,4S)-Methyl 4-(2-amino-4-(methylthio)butanamido)pyrrolidine-2-carboxylate (8g)
White solid, yield 85.8%, mp 151-153°C. \(^1\)H-NMR (DMSO-\(d_6\)): 1.99 (m, 1H), 2.07 (s, 3H, CH\(_3\)), 2.10 (m, 2H, CH\(_2\)), 2.12 (m, 1H), 2.51 (m, 1H), 2.61 (m, 1H), 3.17 (m, 1H), 3.47 (m, 1H), 3.78 (s, 3H, CH\(_3\)), 3.84 (m, 1H), 4.40 (m, 1H), 4.49 (m, 1H), 8.24 (s, 2H, NH\(_3\)).

\((2S,4S)\)-Methyl 4-(2-amino-3-mercaptopropanamido) pyrrolidine-2-carboxylate (8h)

White solid, yield 63.2%, mp 174-176°C. \(^1\)H-NMR (DMSO-\(d_6\)): 1.87 (m, 1H), 2.60 (m, 1H), 2.65, 2.76 (q, 2H, CH\(_2\)), 3.07 (m, 1H), 3.41 (m, 1H), 3.43 (d, \(J = 6\) Hz, 1H), 3.78 (s, 3H, CH\(_3\)), 3.84 (m, 1H), 4.12 (m, 1H), 4.40(m, 1H), 8.53 (s, 2H, NH\(_3\)).

1.8. \((2S,4S)\)-4-(2-Aminoacetamido)-N-hydroxy-pyrrolidine-2-carboxamide (9a)

To a solution of compound 7a (0.4 g, 1 mmol) in 10 mL anhydrous methanol at room temperature was added dropwise a solution of NHOK (3 mmol) in methanol (1.7 mL). The mixture was stirred for 12 h and the solvent was evaporated in vacuum. De-protection of Boc group as the synthesis of 8a provided the target compound 9a (0.07 g). Yield 39%, mp 124-126°C.

\((2S,4S)\)-Methyl 4-(2-amino-4-(methylthio)butanamido) pyrrolidine-2-carboxylic acid (11c)

White solid, yield 33.6%, mp 176-178°C. \(^1\)H-NMR (DMSO-\(d_6\)): 1.85 (m, 1H), 2.09 (m, 1H), 2.59 (m, 1H), 3.03 (m, 1H), 3.48 (m, 1H), 3.54 (d, \(J = 5.4\) Hz, 1H), 4.10 (m, 1H), 4.41 (m, 1H), 8.37 (s, 2H, NH\(_3\)).

\((2S,4S)\)-4-(2-Amino-3-methylbutanamido)-N-hydroxy-pyrrolidine-2-carboxamide (9c)

White solid, yield 47.8%, mp 170-172°C. \(^1\)H-NMR (DMSO-\(d_6\)): 0.93 (d, \(J = 7.2\) Hz, 3H, CH\(_3\)), 1.85 (m, 1H), 2.09 (m, 1H), 2.59 (m, 1H), 3.03 (m, 1H), 3.48 (m, 1H), 3.54 (d, \(J = 5.4\) Hz, 1H), 4.10 (m, 1H), 4.41 (m, 1H), 8.24 (s, 2H, NH\(_3\)).

\((2S,4S)\)-4-(2-Amino-4-methylpentanamido)-N-hydroxy-pyrrolidine-2-carboxamide (9d)

White solid, yield 41.8%, mp 176-178°C. \(^1\)H-NMR (DMSO-\(d_6\)): 0.90 (t, 3H, CH\(_3\)), 1.57 (m, 2H, CH\(_2\)), 1.63 (m, 1H), 1.83 (m, 1H), 2.59 (m, 1H), 3.05 (m, 1H), 3.46 (m, 1H), 3.73 (t, 1H), 4.10 (m, 1H), 4.41 (m, 1H), 8.31 (s, 2H, NH\(_3\)).

\((2S,4S)\)-4-(2-Amino-3-methylpentanamido)-N-hydroxy-pyrrolidine-2-carboxamide (9e)

White solid, yield 46.5%, mp 171-173°C. \(^1\)H-NMR (DMSO-\(d_6\)): 0.90 (t, 3H, CH\(_3\)), 1.12 (m, 2H, CH\(_2\)), 1.51 (m, 1H), 1.87 (m, 1H), 2.61 (m, 1H), 3.05 (m, 1H), 3.40 (m, 1H), 3.63 (t, 1H), 4.11 (m, 1H), 4.41 (m, 1H), 8.23 (s, 2H, NH\(_3\)).

\((2S,4S)\)-4-(2-Amino-3-phenylpropanamido)-N-hydroxy-pyrrolidine-2-carboxamide (9f)

White solid, yield 42.7%, mp 182-184°C. \(^1\)H-NMR (DMSO-\(d_6\)): 1.78 (m, 1H), 2.60 (m, 1H), 2.75 (m, 1H), 3.05 (d, \(J = 6.6\) Hz, 2H, CH\(_2\)), 3.30 (m, 1H), 3.98 (t, 1H), 4.09 (m, 1H), 4.37 (m, 1H), 7.26, 7.29, 7.34 (5H, C\(_6\)H\(_5\)), 8.37 (s, 2H, NH\(_3\)).

\((2S,4S)\)-4-(2-Amino-3-mercaptopropanamido)-N-hydroxy-pyrrolidine-2-carboxamide (9g)

White solid, yield 45.4%, mp 158-160°C. \(^1\)H-NMR (DMSO-\(d_6\)): 1.87 (m, 1H), 1.99 (m, 2H, CH\(_2\)), 2.07 (s, 3H, CH\(_3\)), 2.60 (m, 1H), 2.65, 2.74 (q, 2H, CH\(_2\)), 3.07 (m, 1H), 3.49 (m, 1H), 3.84 (m, 1H), 4.11 (m, 1H), 4.40(m, 1H), 8.38 (s, 2H, NH\(_3\)).

\((2S,4S)\)-4-(2-Amino-4-(methylthio)butanamido)-N-hydroxy-pyrrolidine-2-carboxamide (9h)

White solid, yield 33.6%, mp 142-145°C. \(^1\)H-NMR (DMSO-\(d_6\)): 1.87 (m, 1H), 1.99 (m, 2H, CH\(_2\)), 2.07 (s, 3H, CH\(_3\)), 2.60 (m, 1H), 2.65, 2.74 (q, 2H, CH\(_2\)), 3.07 (m, 1H), 3.49 (m, 1H), 3.84 (m, 1H), 4.12 (m, 1H), 4.40(m, 1H), 8.58 (s, 2H, NH\(_3\)).
White solid, yield 57.4%, mp 201-203°C. $^1$H-NMR (DMSO-$d_6$): 0.94 (d, 3H, CH$_3$), 1.85 (m, 1H), 2.04 (m, 1H), 2.12 (m, 1H), 2.63 (m, 1H), 3.09 (m, 1H), 3.46 (m, 1H), 3.59 (d, $J = 4.8$ Hz, 1H), 4.40 (m, 1H), 4.47 (m, 1H), 8.37 (s, 2H, NH$_2$).

(2S,4S)-4-(2-Amino-4-methylpentanamido) pyrrolidine-2-carboxylic acid (11d)

White solid, yield 61.8%, mp 207-209°C. $^1$H-NMR (DMSO-$d_6$): 0.90 (t, 3H, CH$_3$), 1.57 (m, 2H, CH$_2$), 1.63 (m, 1H), 2.01 (m, 1H), 2.62 (m, 1H), 3.08 (m, 1H), 3.46 (m, 1H), 3.73 (t, 1H), 4.32 (m, 1H), 4.41 (m, 1H), 8.38 (s, 2H, NH$_2$).

(2S,4S)-4-(2-Amino-3-methylpentanamido) pyrrolidine-2-carboxylic acid (11e)

White solid, yield 56.3%, mp 203-205°C. $^1$H-NMR (DMSO-$d_6$): 0.90 (t, 3H, CH$_3$), 1.09 (m, 1H), 1.49 (m, 1H), 1.84 (m, 1H), 2 (m, 1H), 2.61 (m, 1H), 3.06 (m, 1H), 3.47 (m, 1H), 3.60 (d, $J = 4.2$ Hz, 1H), 4.33 (m, 1H), 4.41 (m, 1H), 8.29 (s, 2H, NH$_2$).

(2S,4S)-4-(2-Amino-3-phenylpropanamido) pyrrolidine-2-carboxylic acid (11f)

White solid, yield 62.7%, mp 215-218°C. $^1$H-NMR (DMSO-$d_6$): 1.90 (m, 1H), 2.57 (m, 1H), 2.78 (m, 1H), 3.04 (d, $J = 6$ Hz, 2H, CH$_2$), 3.29 (m, 1H), 3.97 (t, 1H), 4.29 (m, 1H), 4.36 (m, 1H), 7.26, 7.29, 7.33 (5H, C$_6$H$_5$), 8.36 (s, 2H, NH$_2$).

(2S,4S)-4-(2-Amino-4-(methylthio)butanamido) pyrrolidine-2-carboxylic acid (11g)

White solid, yield 57.6%, mp 193-196°C. $^1$H-NMR (DMSO-$d_6$): 0.94 (d, 3H, CH$_3$), 1.87 (m, 1H), 2.02 (m, 2H, CH$_2$), 2.07 (s, 3H, CH$_3$), 2.60 (m, 1H), 2.65, 2.74 (q, 2H, CH$_2$), 3.07 (m, 1H), 3.44 (m, 1H), 3.84 (d, $J = 6$ Hz, 1H), 4.11 (m, 1H), 4.47 (m, 1H), 8.46 (s, 2H, NH$_2$).

(2S,4S)-4-(2-Amino-3-mercaptopropanamido) pyrrolidine-2-carboxylic acid (11h)

White solid, yield 41.2%, mp 181-185°C. $^1$H-NMR (DMSO-$d_6$): 2.05 (m, 1H), 2.61 (m, 1H), 3.08 (m, 1H), 3.16 (m, 1H), 3.43 (m, 1H), 4.11 (m, 1H), 4.35 (m, 1H), 4.42 (m, 1H), 8.58 (s, 2H, NH$_2$).

1.9. Methyl 2-((2S,4S)-4-(2-amino-4-methylpentanamido) pyrrolidine-2-carboxamido)acetate (12d)

0.94 g 10d (2 mmol) and Et$_3$N (2 equiv.) were dissolved in 30 mL anhydrous DCM. To this stirring solution was added TBTU (1.3 equiv.) followed by methyl 2-aminoacetate. The mixture was stirred for 10 h and washed with saturated NaHCO$_3$ solution, 1 M citric acid, and brine. 0.57 g of 12d was obtained by flash column chromatography. Yield 55.2%, mp 54-57°C. ESI-MS m/z: 515.6 (M+H)$^+$. Compound 12f was synthesized following the procedure described above.

(2S,4S)-tert-Butyl 4-((tert-butoxycarbonyl)amino)-3-phenylpropanamido)-2-((2-methoxy-2-oxoethyl) carbamoyl)pyrrolidine-1-carboxylate (12f)

White solid, yield 58.7%, mp 62-64°C. ESI-MS m/z: 549.6 (M+H)$^+$. Compound 13f was synthesized following the procedure described above.

1.10. Methyl 2-((2S,4S)-4-(2-amino-4-methylpentanamido) pyrrolidine-2-carboxamido)acetate (13d)

Compound 13d was obtained following the synthesis of 8a described above. White solid, yield 83.5%, mp 164-167°C. $^1$H-NMR (DMSO-$d_6$): 0.90 (s, 6H, CH$_3$), 1.58 (m, 2H), 1.64 (m, 1H), 1.87 (m, 1H), 2.76 (m, 1H), 3.07 (m, 1H), 3.46 (m, 1H), 3.66 (s, 3H, CH$_3$), 3.75 (t, 1H), 3.97 (s, 2H, CH$_3$), 4.33 (m, 1H), 4.40 (m, 1H), 8.43 (s, 2H, NH$_2$).

Compound 13f was synthesized following the procedure described above.

Methyl 2-((2S,4S)-4-(2-amino-3-phenylpropanamido) pyrrolidine-2-carboxamido)acetate (13f)

White solid, yield 83.5%, mp 172-175°C. $^1$H-NMR (DMSO-$d_6$): 1.71 (m, 1H), 2.43 (m, 1H), 2.76 (m, 1H), 3.07 (m, 1H), 3.82 (m, 1H), 3.93 (m, 1H), 4.13 (m, 1H), 4.18 (m, 1H), 7.27, 7.28, 7.29, 7.32, 7.35 (5H, C$_6$H$_5$), 8.24 (s, 2H, NH$_2$).