*Original Article*

# 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 $S_1'$ and $S_2'$ pockets. The $S_1'$ pocket, a deep and narrow channel, is the key domain, and the $S_2'$ 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 $S_1'$ and $S_2'$ 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 $R_1$ group would be hydroxamate, carboxylate, or ester functioning as an effective ZBG to chelate the active site of catalytic zinc ions. The $R_2$ group would be introduced with various amino acids as side chains that might occupy the $S_1'$ or $S_2'$ 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

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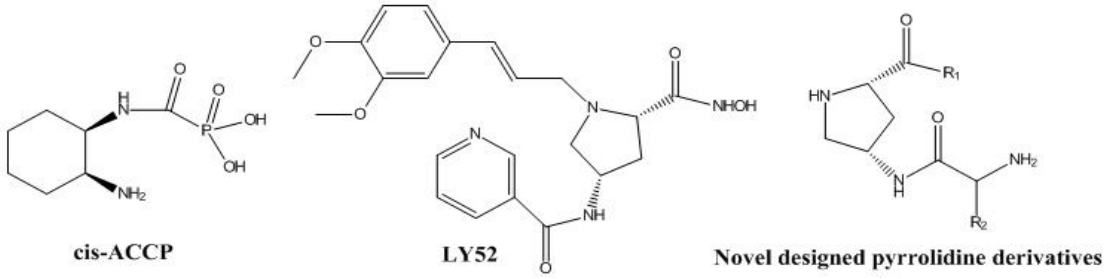
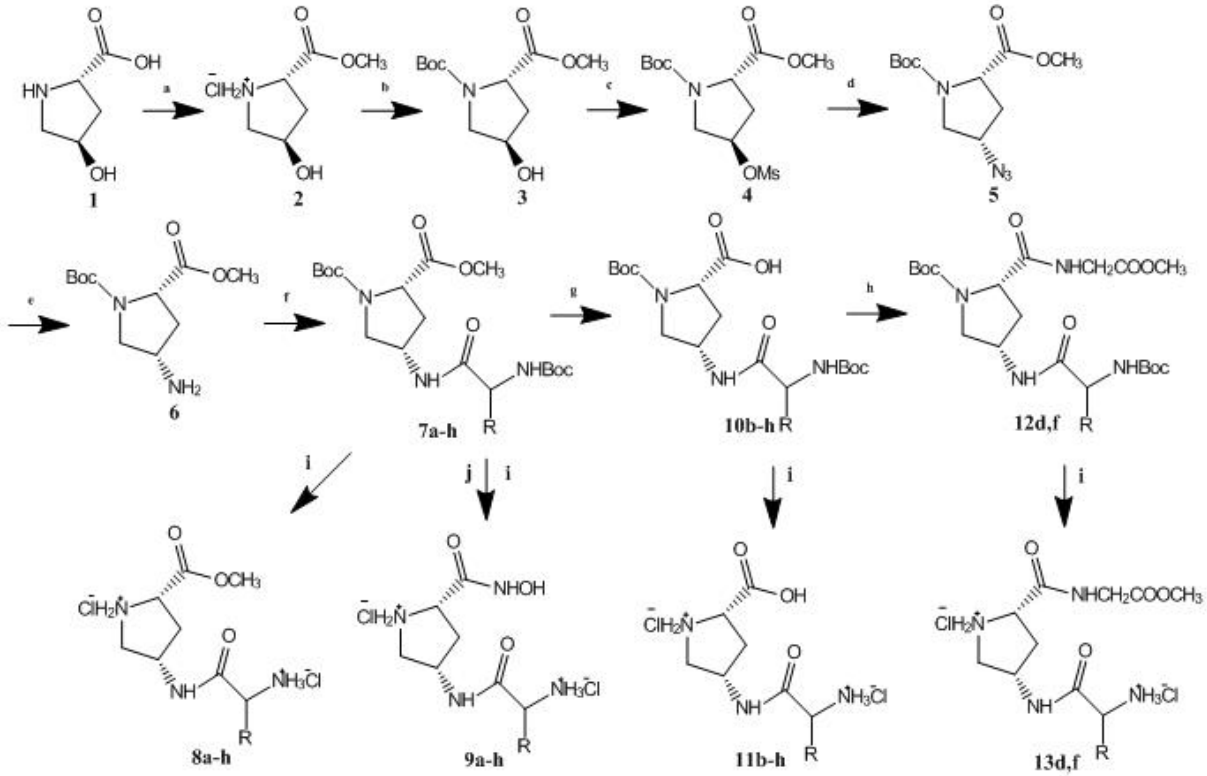



**Figure 1. The structures of *cis*-ACCP, LY52, and novel designed pyrrolidine derivatives.**




**Scheme 1. Reagents and conditions:** (**a**) MeOH, HCl; (**b**) $(Boc)_2O$, THF; (**c**) $CH_3SO_2Cl$; (**d**) NaN3,DMF; (**e**) 10% Pd-C/$H_2$; (**f**) BocNH-CH(R)-COOH; (***g***) NaOH, $CH_3OH$/HCl; (**h**) $NH_2CH_2COOCH_3$; (**i**) HCl/EtOAC; (**j**) $NH_2OK$, MeOH.

with *trans*-4-hydroxy-L-proline (**1**), the important intermediate (2*S*,4*S*)-1-*tert*-butyl-2-methyl-4-aminopyrrolidine-1,2-dicarboxylate (**6**) was prepared by esterification, acylation, sulfonation, $SN_2$ 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 $NH_2OK$ 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 $OD_{450}$ values.

2.3. *APN inhibition assay*

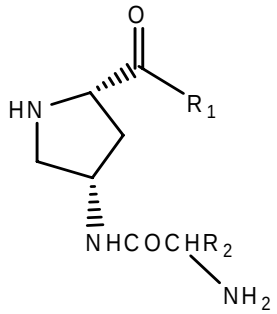
$IC_{50}$ values against APN were determined using L-Leu-*p*-nitroanilide as the substrate and microsomal aminopeptidase (Sigma) as the enzyme in 50 mM PBS, pH 7.2, at 37°C. The hydrolysis of the substrate was monitored by following the changes in absorbance measured at 405 nm. All solutions of the inhibitors 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.

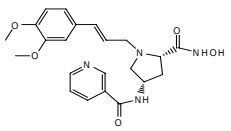
## 3. Results and Discussion

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 $IC_{50}$ (APN)/$IC_{50}$ (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

**Table 1. Compound structure and inhibitory activity against MMP-2 and APN**




| Compounds | $R_1$ | $R_2$ | $IC_{50}$/μM | | $IC_{50}$(APN)/$IC_{50}$(MMP-2) |
|---|---|---|---|---|---|
| | | | MMP-2 | APN | |
| **8a** | $OCH_3$ | H | 54.32 | 2920.6 | 53.77 |
| **8b** | $OCH_3$ | $CH_3$ | 236.3 | 132.2 | 0.56 |
| **8c** | $OCH_3$ | $CH(CH_3)_2$ | 402.7 | 1615 | 4.01 |
| **8d** | $OCH_3$ | $CH_2CH(CH_3)_2$ | 94.1 | 317.4 | 3.37 |
| **8e** | $OCH_3$ | $CH(CH_3)CH_2CH_3$ | 262.9 | 94.4 | 0.36 |
| **8f** | $OCH_3$ | $CH_2C_6H_5$ | 190.3 | 343 | 1.8 |
| **8g** | $OCH_3$ | $CH_2CH_2SCH_3$ | 214.7 | 1297.4 | 6.04 |
| **8h** | $OCH_3$ | $CH_2SH$ | 160.5 | 295.2 | 1.84 |
| **9a** | NHOH | H | 26 | 758.1 | 29.16 |
| **9b** | NHOH | $CH_3$ | 407.6 | 92.6 | 0.23 |
| **9c** | NHOH | $CH(CH_3)_2$ | 9.25 | 377 | 40.76 |
| **9d** | NHOH | $CH_2CH(CH_3)_2$ | 4.8 | 1341 | 279.38 |
| **9e** | NHOH | $CH(CH_3)CH_2CH_3$ | 1.83 | 668.8 | 365.46 |
| **9f** | NHOH | $CH_2C_6H_5$ | 110.6 | 1495.7 | 13.52 |
| **9g** | NHOH | $CH_2CH_2SCH_3$ | 3.8 | 317.1 | 83.45 |
| **9h** | NHOH | $CH_2SH$ | 320 | / | / |
| **11b** | COOH | $CH_3$ | 33 | 404.4 | 12.25 |
| **11c** | COOH | $CH(CH_3)_2$ | 86.1 | 1720 | 19.98 |
| **11d** | COOH | $CH_2CH(CH_3)_2$ | 45.8 | 1651.6 | 36.06 |
| **11e** | COOH | $CH(CH_3)CH_2CH_3$ | 22.8 | 1379.2 | 60.49 |
| **11f** | COOH | $CH_2C_6H_5$ | 149 | 90 | 0.60 |
| **11g** | COOH | $CH_2CH_2SCH_3$ | 332.9 | 83 | 0.25 |
| **11h** | COOH | $CH_2SH$ | 1515 | 20.4 | 0.01 |
| **13d** | $NHCH_2COOCH_3$ | $CH_2CH(CH_3)x_2$ | 76.2 | 502 | 6.59 |
| **13f** | $NHCH_2COOCH_3$ | $CH_2C_6H_5$ | 16.8 | 2492.8 | 148.38 |
| LY52 |  | | 5.6 | 578.9 | 103.75 |

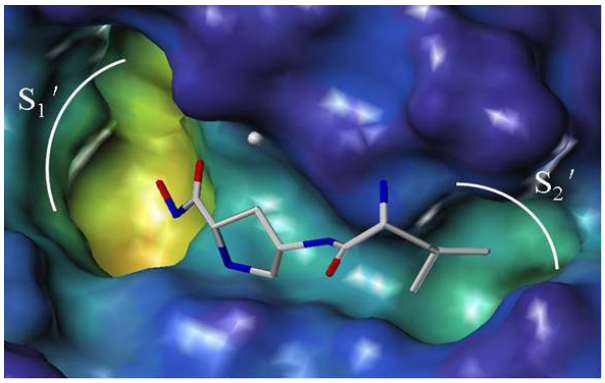



**Figure 2. The FlexX Docking of Compound 9e with MMP-2.**

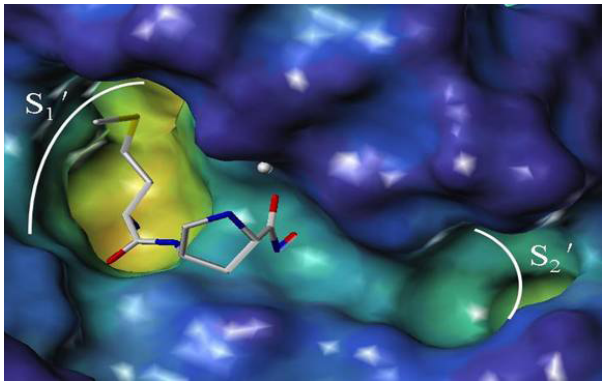



**Figure 3. The FlexX Docking of Compound 9g with MMP-2.**

endopeptidase that cleaved the peptide from its specific amino acid residue, while APN is a membrane-bound zinc exopeptidase that catalyzed the removal of $NH_2$-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 ($R_1$). 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 $IC_{50}$ (APN)/$IC_{50}$ (MMP-2) ratio of 148.38. The $IC_{50}$ (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.

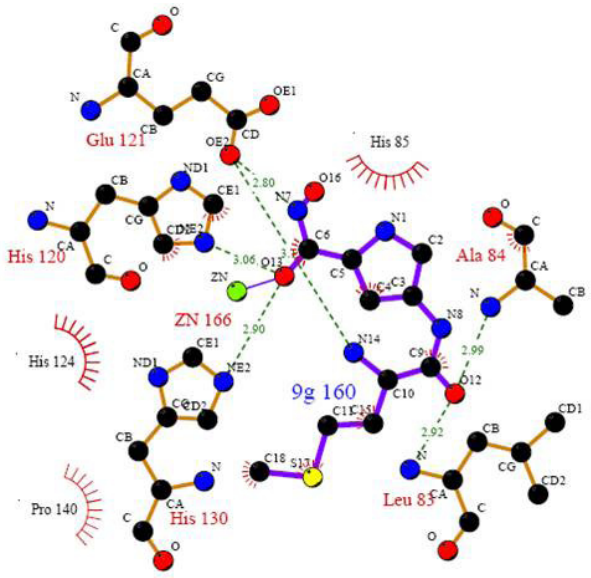



**Figure 4. The docking result of 9g with MMP-2 showed by LIGPLOT.** Compound **9g** is shown in violet.

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 $Ala^{84}$, $His^{120}$ shown in Figure 4. H-bond interactions are formed between the OE2 of $Glu^{121}$ and N7 and N14 of **9g**, the N of $Leu^{83}$ and $Ala^{84}$ and O12 of **9g**, and the NE2 of $His^{120}$, $His^{130}$, 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

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## Appedix

### 1. Chemistry: general procedures

All materials were commercial 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 $D_2O$ 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 $Et_3N$ (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 $NaHCO_3$ solution, and brine, and dried over $Na_2SO_4$. 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-(Methoxycarbonyl)-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 $Et_3N$ (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 $NaHCO_3$ solution, and brine and then dried over $Na_2SO_4$. Evaporation of DCM gave compound **4**, mp 84-86°C. ESI-MS m/z: 324.4 $(M+H)^+$.

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

(3*R*,5*S*)-5-(Methoxycarbonyl)-1-(*tert*-butoxycarbonyl) pyrrolidin-3-ylsulfonates (**4**) (12.92 g, 40 mmol) and 6.5 g $NaN_3$ (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 $Na_2SO_4$. 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)

(2*S*,4*S*)-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 $Et_3N$ (2 equiv.) were dissolved in 100 mL anhydrous DCM. To this stirring solution was added TBTU (1.3 equiv.) followed by compound **6**. The mixture was stirred for 10 h and washed with saturated $NaHCO_3$ solution, 1 M citric acid, and brine. 2.53 g **7b** was obtained by flash column chromatography, yield 60.8%. ESI-MS m/z: 416.7 $(M+H)^+$. $^1$H-NMR (DMSO-$d_6$): 1.12 (d, $J$ = 6.6 Hz, 3H, $CH_3$), 1.32 (m, 18H, $CH_3$), 1.80 (m, 1H), 2.49 (m, 1H), 3.06 (m, 1H), 3.61 (m, 1H), 3.68 (s, 3H, $CH_3$), 3.86 (m, 1H), 4.21 (m, 2H).

Compounds **7a** and **7c-h** were synthesized following the procedure described above. (2S,4S)-1-*tert*-Butyl 2-methyl 4-(2-((*tert*-butoxycarbonyl)amino)acetamido)-pyrrolidine-1,2-dicarboxylate (**7a**), 2.2 g, 54.6%; (2S,4S)-1-*tert*-butyl 2-methyl 4-(2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)pyrrolidine-1,2-dicarboxylate (**7c**), 2.9 g, 65.4%; (2S,4S)-1-*tert*-butyl 2-methyl 4-(2-((*tert*-butoxycarbonyl)amino)-4-methylpentanamido)pyrrolidine-1,2-dicarboxylate (**7d**), 3.12 g, 68.3%; (2S,4S)-1-*tert*-butyl 2-methyl 4-(2-((*tert*-butoxycarbonyl)amino)-3-methylpentanamido)pyrrolidine-1,2-dicarboxylate (**7e**), 3.09 g, 67.6%; (2S,4S)-1-*tert*-butyl 2-methyl 4-(2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanamido)pyrrolidine-1,2-dicarboxylate (**7f**), 3.42 g, 69.5%; (2S,4S)-1-*tert*-butyl 2-methyl 4-(2-((*tert*-butoxycarbonyl)amino)-4-(methylthio)butanamido)pyrrolidine-1,2-dicarboxylate (**7g**), 2.92 g, 61.3%; (2S,4S)-1-*tert*-butyl 2-methyl 4-(2-((*tert*-butoxycarbonyl)amino)-3-mercaptopropanamido)pyrrolidine-1,2-dicarboxylate (**7h**), 2.36 g, 52.7%.

### 1.7. (2S,4S)-Methyl 4-(2-aminoacetamido)pyrrolidine-2-carboxylate (8a)

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. $^1$H-NMR (DMSO-$d_6$): 2.04 (m, 1H), 2.59 (m, 1H), 3.12 (m, 1H), 3.45 (m, 1H), 3.53 (s, 2H, $CH_2$), 3.78 (s, 3H, $CH_3$), 4.40 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, $NH_2$).

Compounds 8b-h were synthesized following the procedure described above.

### (2*S*,4*S*)-Methyl 4-(2-aminopropanamido)pyrrolidine-2-carboxylate (8b)

White solid, yield 82.6%, mp 137-139°C. $^1$H-NMR (DMSO-$d_6$): 1.36 (d, $J$ = 6.6 Hz, 3H, $CH_3$), 2.04 (m, 1H), 2.61 (m, 1H), 3.14 (m, 1H), 3.44 (m, 1H), 3.78 (s, 3H, $CH_3$), 3.80 (d, $J$ = 5.4 Hz, 1H), 4.39 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, $NH_2$).

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

White solid, yield 80.2%, mp 146-148°C. $^1$H-NMR (DMSO-$d_6$): 0.93 (d, $J$ = 6.6 Hz, 6H, $CH_3$), 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, $CH_3$), 4.41 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, $NH_2$).

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

White solid, yield 86.3%, mp 148-150°C. $^1$H-NMR (DMSO-$d_6$): 0.90 (t, 6H, $CH_3$), 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, $CH_3$), 4.39 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, $NH_2$).

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

White solid, yield 85.7%, mp 154-156°C. $^1$H-NMR (DMSO-$d_6$): 0.88 (m, 6H, $CH_3$), 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, $CH_3$), 4.41 (m, 1H), 4.48 (m, 1H), 8.24 (s, 2H, $NH_2$).

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

White solid, yield 83.5%, mp 159-161°C. $^1$H-NMR (DMSO-$d_6$): 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, $CH_3$), 3.98(m, 1H), 4.34 (m, 1H), 4.45 (m, 1H), 7.27, 7.28, 7.29, 7.32, 7,35 (5H, $C_6H_6$), 8.24 (s, 2H, $NH_2$).

### (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, $NH_2$).

**(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, $NH_2$).

**1.8. (2S,4S)-4-(2-Aminoacetamido)-*N*-hydroxypyrrolidine-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. $^1$H-NMR (DMSO-$d_6$): 1.80 (m, 1H), 2.58 (m, 1H), 3.07 (m, 1H), 3.43 (m, 1H), 3.53 (d, $J$ = 3.6 Hz, 2H, $CH_2$), 4.11 (m, 1H), 4.40 (m, 1H), 8.21 (s, 2H, $NH_2$).

Compounds **9b-h** were synthesized following the procedure described above.

**(2S,4S)-4-(2-Aminopropanamido)-*N*-hydroxypyrrolidine-2-carboxamide (9b)**

White solid, yield 43.2%, mp 167-169°C. $^1$H-NMR (DMSO-$d_6$): 1.84 (m, 1H), 2.60 (m, 1H), 3.08 (m, 1H), 3.44 (m, 1H), 3.82 (d, $J$ = 5.4 Hz, 1H), 4.11 (m, 1H), 4.37 (m, 1H), 8.31 (s, 2H, $NH_2$).

**(2S,4S)-4-(2-Amino-3-methylbutanamido)-*N*-hydroxypyrrolidine-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, $NH_2$).

**(2S,4S)-4-(2-Amino-4-methylpentanamido)-*N*-hydroxypyrrolidine-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, $NH_2$).

**(2S,4S)-4-(2-Amino-3-methylpentanamido)-*N*-hydroxypyrrolidine-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, $NH_2$).

**(2S,4S)-4-(2-Amino-3-phenylpropanamido)-*N*-hydroxypyrrolidine-2-carbox-amide (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, $C_6H_6$), 8.37 (s, 2H, $NH_2$).

**(2S,4S)-4-(2-Amino-4-(methylthio)butanamido)-*N*-hydroxypyrrolidine-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, $NH_2$).

**(2S,4S)-4-(2-Amino-3-mercaptopropanamido)-*N*-hydroxypyrrolidine-2-carboxamide (9h)**

White solid, yield 33.6%, mp 142-145°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), 3.84 (m, 1H), 4.12 (m, 1H), 4.40 (m, 1H), 8.58 (s, 2H, $NH_2$).

**(2S,4S)-4-(2-Aminopropanamido)pyrrolidine-2-carboxylic acid (11b)**

Compound **7a** (0.42 g, 1mmol) in 10 mL methanol was treated with 1 M NaOH (3 mL), and stirred for 3 h at room temperature. The solvent was evaporated in a *vacuum* and the residue was adjusted to pH 2-3 with 1 M HCl. The mixture was extracted 3 times by EtOAc. The organic phase was dried over $Na_2SO_4$. Evaporation of EtOAc provided compound **10b**. De-protection of Boc group as the synthesis of **8a** provided the target compound **11b** (0.11g). Yield 54.8%, mp 195-198°C. $^1$H-NMR (DMSO-$d_6$): 1.37 (d, $J$ = 7.2 Hz, 3H, $CH_3$), 2.07 (m, 1H), 2.60 (m, 1H), 3.13 (m, 1H), 3.43 (m, 1H), 3.81 (d, $J$ = 6.6 Hz, 1H), 4.36 (m, 1H), 4.48 (m, 1H), 8.33 (s, 2H, $NH_2$).

Compounds **11c-h** were synthesized following the procedure described above.

**(2S,4S)-4-(2-Amino-3-methylbutanamido) pyrrolidine-2-carboxylic acid (11c)**

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, $J$ = 4.2Hz, 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, $C_6H_6$), 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-$d_6$): 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 $Et_3N$ (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-(2-((*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 $(M+H)^+$.

**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 (m, 2H, $CH_2$), 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), 2.72 (m, 1H), 2.94 (m, 1H), 3.58 (m, 2H, $CH_2$), 3.62 (s, 3H, $CH_3$), 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, $C_6H_6$), 8.24 (s, 2H, $NH_2$).