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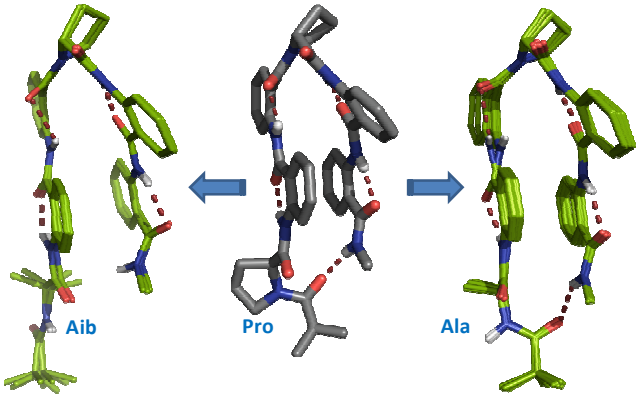
Graphical Abstract



This paper deals with the role of N-terminus proline in stabilizing the Ant-Pro zipper structure stabilized by the co-operative contribution of competing forces *viz*. hydrogen bonding, aromatic stacking and backbone chirality.

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ARTICLE TYPE

The role of N-terminus proline in stabilizing Ant-Pro zipper motif

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Hetero-chiral hybrid peptides of the general sequence $^{L}\alpha\beta_n{}^{D}\alpha\beta_n$ featuring proline (Pro, a constrained α-amino acid) and anthranilic acid (Ant, a constrained β-amino acid) as building blocks, where n = 2, 4 *etc*., form a three-dimensional zipper-like architecture. These zipper peptides attain stable conformation by balancing the co-operative contribution of two competing non-covalent forces namely hydrogen bonding, and aromatic stacking. However, the selection of the N-terminus residue also stands to be one of the key contributors in stabilising the unusually long-range intramolecular hydrogen bond, featuring 26 atoms in the H-bonded ring observed at the termini. This article deals with the substitution alterations at the N-terminus of the zipper motif and their consequent influences on its structure and stability. For this study, the N-terminus Pro residue of the zipper motif was substituted with a flexible amino acid - alanine and a constrained acylic amino acid - 2-aminoisobutyric acid to investigate the role of N-terminus proline in stabilizing Ant-Pro zipper motif, and their stabilities were assessed by employing solution-state NMR and restrained MD simulation studies.

Introduction

The field of foldamer science has helped chemists to create a large collection of structural assemblies[1] and has helped in deepening our understanding on the structural intricacy of biopolymers.[2] Last two decades have witnessed a steep ascent in the development of diverse classess of foldamers, owing to their potential applications – ranging from biomedical sciences to molecular machines.[3] Moreover, these synthetic oligomers provide deeper insights into the competition of the innate torsional prefernces of each component *vs* the non-covalent interactions, in rendering a particular stable conformation.[4] There are plenty of reports in the literature that focus largely on non-covalent interactions in designing diverse structural ensembles.[5] A few notable examples include Hunter's tailbiter,[6] knots,[7] large interwined double helices,[8] Nowick's cyclic modular β-sheets,[9] and Moore's solvophobically driven helices,[10] - to name a few. Though these rigidified systems are driven into their stable structural form through the dominant non-covalent interations, the inherent feature i.e. torsional constraints of each building block/residue selected and incorporated also are very decisive.[11] In this context, the zipper architecture assumed by heterochiral $^{L}\alpha\beta_n{}^{D}\alpha\beta_n$ possessing Pro as α-amino acid and Ant as β-amino acid serves as a good model for investigating co-operative interplay of different interactions.[12] The overplay of one interaction over the other can be determined by substitutional variation in-and-around the molecule.[13]

Different amino acids possess disitinct preferences to adopt helix or sheet structure depending on their dihedral restraints.[14] The secondary structure propensity of peptides is well accounted in the form of Ramachandran maps.[15]

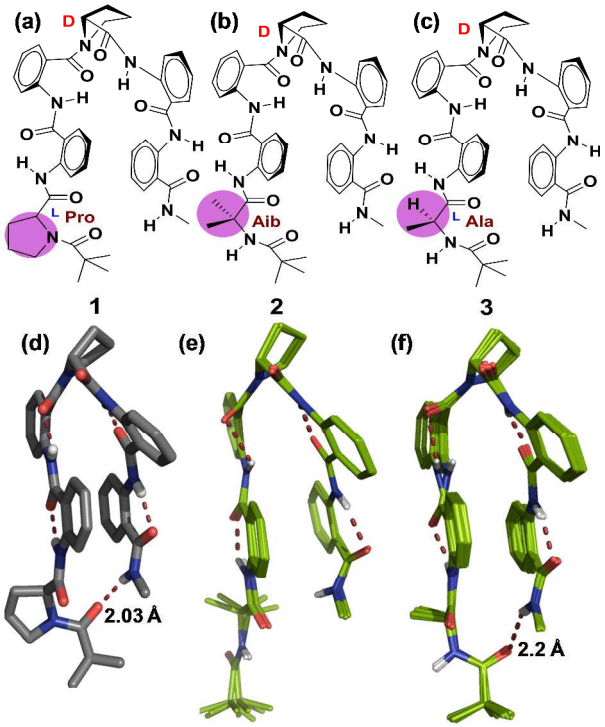


Fig. 1 Conformational investigation of zipper peptides **1**, **2** and **3**. (a, b and c) Molecular structures of the analogs **1**, **2** and **3**, respectively. (d) Crystal structure of **1**.[12] (e, f) Stereoview of 20 superimposed minimum energy structures of peptide **2** and **3** obtained from restrained MD simulations. *Note:* Hydrogens, other than the polar amide hydrogens have been removed for clarity.

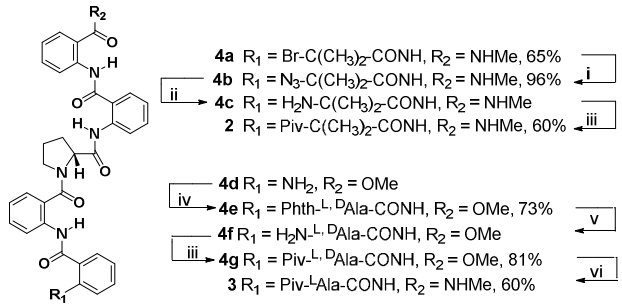
The torsional angle constraints are defined for each amino acid i.e. bond angle N- C_α-C can slightly change than its usual

tetrahedral angle 109°, in order to accommodate other strains in the structure. Aliphatic amino acid like proline with dihedral angle preferences φ = -60° and ψ = -45 to +135°, possesses strong conformational tendency to induce folding and proline oligomers are known to adopt compact PPI/PPII helical structures, totally devoid of any intramolecular H-bonding association. In comparison, Aib (α-amino isobutyric acid) is a well-known sheet breaker and its oligomers take up highly compact 3_{10} helical structure, with conformation restraints largely lying in the region φ = ±60° and ψ = ±30°. Ala is comparatively one of the most flexible amino acids with dihedral angle preferences φ = ±100° and ψ = ±120°.[16] Considering these probable influences on the backbone, we became keen on evaluating the effect on zipper architecture on substituting Pro1 with an achiral constrained residue Aib and a flexible residue Ala in the hexapeptide sequence. Therefore, two hexapeptide sequences Piv-Aib-Ant-Ant-^{D}Pro-Ant-Ant-NHMe and Piv-^{L}Ala-Ant-Ant-^{D}Pro-Ant-Ant-NHMe were prepared and their structural characteristics were evaluated *via* solution-state NMR and CD studies.

Results and Discussion

Synthesis

Syntheses of all the hexapeptide analogues **1**–**3** were carried out using conventional solution phase peptide synthesis under standard coupling conditions (Scheme 1). Synthesis of the pentapeptide sequences **4a**, **4d** and hexapeptide **1** were undertaken, as reported earlier.[12] Hexapeptides **2** and **3** were synthesized from the pentamer derivatives, in a multi-step synthetic strategy as depicted in scheme 1 (ESI, page S3). Hexapeptide **2** was synthesized from **10d** following a few steps *via* conversion of bromo **4a** to azido **4b**, followed by reduction to yield the amine **4c** and then finally pivaloyl protection. For the compound **3**, pentamer derivative **4d** was coupled with phthaloyl protected alanine to give hexamer **4e**. The phthaloyl group on deprotection, followed by reaction with pivaloyl chloride and C-terminal amidation resulted in the hexamer **3.**



Scheme 1: Synthesis of **2** and **3. Reagents and Conditions:** (i) NaN_3, cat. LiCl, DMF, rt, 12h; (ii) $Pd(OH)_2$, MeOH, 4h; (iii) Piv-Cl, TEA, DCM, 1h; (iv) (a) Phth-^{L}Ala-OH, $(COCl)_2$, cat. DMF, DCM, 0°C-rt, 30 min., (b) TEA, DCM, 0°C-rt, 1 hr; (v) NH_2-$NH_2.H_2O$, 1:1 DCM:EtOH, 6h; (vi) methanolic methylamine, rt, 1h. *Note*: **4e**, **4f** and **4g** existed as diastereomers, presumably formed during coupling with phthaloyl alanine, although **3** could be finally purified as a single diastereomer (see, ESI page S7, S10, S13 and S19). The other diastereomer 3' was isolated in 40% yield.

Solution-state structural studies

The structural elucidation of **2** and **3** were carried out *via* solution-state NMR studies, since they were highly resistant to yield to crystal formation, despite several efforts. It has already been reported that oligomer **1** in the solution-state shows characteristic long-range inter-residual *nOe*s observed between the groups positioned at the termini ($C_{42}H/C_{40}H$ and $C_{42}H/NH_7$). Also, the *edge-to-face* stacking effect was evidenced from the *nOe* contours of $C_{10}H/C_{31}H$ (fig. 2a). These diagnostic dipolar coupling interactions unambiguously suggested that the fully folded conformation observed in the solid-state is clearly prevalant in the solution-state as well. In order to investigate the essentiality of proline at the N-terminus, the analogues **2** and **3** were evaluated comparing the similar interaction pattern and *nOe* signals observed in **1**. The hexapeptide **2** with N-terminus Aib showed similar characteristic *nOe*s ($C_{11}H/C_{39}H$, $C_{30}H/C_9H$ and $C_{11}H/NH_7$; fig. 2b). Comparison of all the three analogues **1-3** revealed that the overall zipper architecture remains preserved in all the cases.

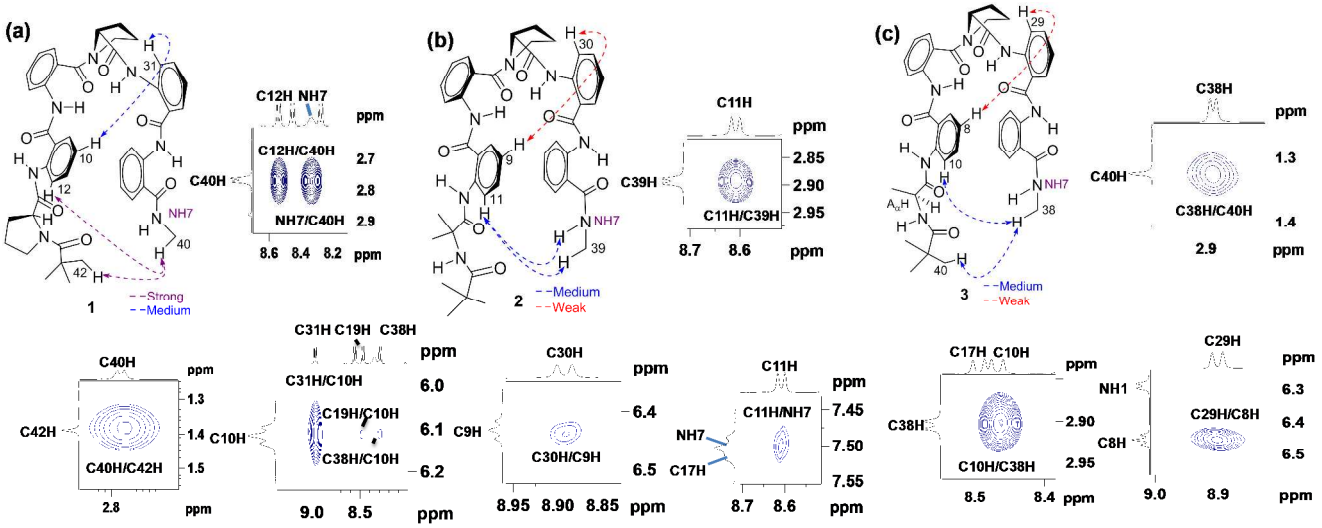


Fig. 2 2D-NMR comparison of hexapeptide sequences **1** (a), **2** (b) and **3** (c), illustrating the terminal and stacking interactions.

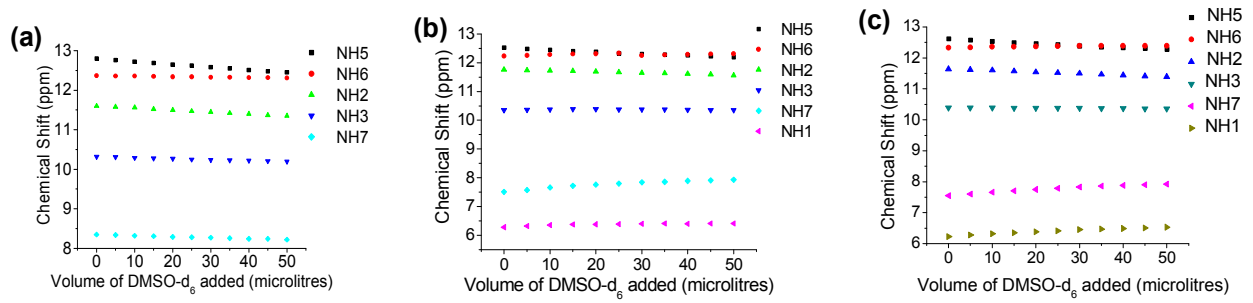


Fig. 3 NMR DMSO-d_6 titration studies of hexamers **1** (a), **2** (b) and **3** (c).

However, in **2**, the terminal interactions between N-terminus pivaloyl group (Piv) and C-terminus methylamide (CH3) were not observed, presumably due to the fraying of the end groups Similar to compound **1**, aromatic stacking effect in **2** was clearly evidenced from C_9H proton of Ant2 as it appeared at 6.4 ppm in the ^{1}H-spectrum, which showed diagnostic dipolar coupling between $C_9H/C_{30}H$ (Fig. 2b). On the other hand, the hexamer **3** showed characteristic long-range inter-residual *nOe*s observed between the groups positioned at the termini $C_{38}H/C_{40}H$ alongwith $C_{10}H/C_{38}H$ as a proof of folding in the molecule. Stacking effect was also evidenced from the dipolar coupling between $C_8H/C_{29}H$ (Fig. 2c).

Evaluation of the strength of hydrogen-bonds through solution-state NMR studies

The peptides **2** and **3** showed excellent solubility in non-polar organic solvents, despite having several amide groups, suggesting the fact that the polar H-bonding groups are not solvent exposed, thereby preventing aggregation. Investigation of intramolecular nature of H-bonding in **2** and **3** were obtained from H/D exchange studies and DMSO-d_6 titration studies. The negligible ^{1}H NMR chemical shifts difference (Δδ NH: <0.15ppm) observed for the oligomers **2** and **3** upon on DMSO-d_6 titration studies (up to 10% of DMSO-d_6 in $CDCl_3$) (Fig. 3) supported the intramolecular nature of the H-bonds in the solution-state. Also, the presence of terminal intramolecular H-bonding interaction was evidenced from the DMSO-d_6 titration experiment that revealed chemical shift variation for NH7 about ≈ 0.42 ppm and ≈ 0.37 for hexapeptide **2** and **3**, respectively. The slow H/D exchange rate in MeOD (>21h) (see ESI, page S23-S24, Fig. S1-S2) also confirms intramolecular long-range hydrogen-bondings in **2** and **3**.

The comparative observations obtained from the analyses of the NMR data (DMSO titration and 2D NOESY studies) of the oligomers **2** and **3** with the zipper peptide **1** having Pro at the N-terminus are summarized as: (1) comparison of the C-terminus NH chemical shifts reveals that the zipper peptide **1** having N-terminal Pro show negligible NH7 shift difference (Δδ= 0.13 ppm), suggestive of strong intramolecular remote H-bonding. However, the Aib and Ala analogues **2** and **3** show higher NH7 Δδ value (0.42 ppm and 0.37, respectively) suggestive of weaker remote intramolecular hydrogen-bonding involving 26 atoms in the network. Thus, these observations suggest that proline is the most favoured amino acid at the N-terminus which can stabilize the crucial remote hydrogen bonded network and (2) aromatic stacking effects are observed in all cases, suggesting that they contribute significantly in stabilizing the zipper architecture.

NMR-based MD-simulated structure evaluation

The MD-simulated structures were generated for compounds **2** and **3**, using the quantitative restraints obtained from the NOESY spectra calculating relativity of cross-peak intensities of the volume integrals. The 20 superimposed minimum energy structures derived from MD calculations revealed the perspicuous folded zipper conformation for both hexapeptides **2** and **3** (Fig. 1b and c). The terminal amide NH signal appearing relatively downfield at δ ~7.5 ppm for both the molecules **2** and **3**, indicated an intramolecular association but weaker in comparison with **1** (δ 8.35 ppm). Even so, the 2D NMR and DMSO-d_6 titration solution-state studies of hexapeptide **2** projected a disparity in predictions about the terminal H-bonding pattern. Nevertheless, MD-simulated structure of **2** revealed the 5-membered intra-residual H-bonding pattern supporting the downfield shift. However, hexapeptide **3** with N-terminal ^{L}Ala forms long-range inter-residual hydrogen-bonding pattern, akin to that of **1**.

CD Studies

CD spectra of peptides **1-3** reveal similar conformational ordering pattern and strong absorption is seen between 300-350 nm owing to the backbone aromatic residues. Although, a clear conclusion cannot be drawn regarding the stability dependence on different residues, it can be summarised that all the analogs reveal similar absorption patterns.

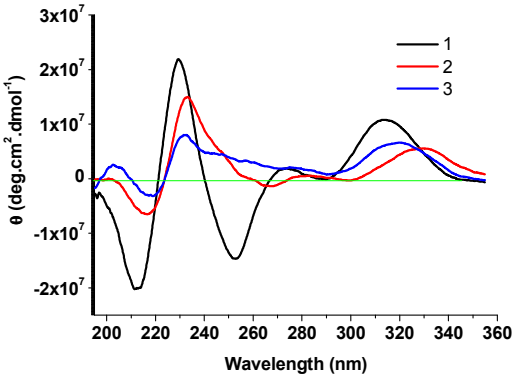


Fig. 4 Representative CD spectra of the hexapeptide **1**, **2** and **3**. All spectra were recorded at 293 K with a concentration of 0.05 mM in 2,2,2-trifluoroethanol (TFE).

DFT calculation studies

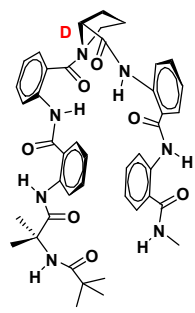
Density functional calculations were carried out by using B3LYP functional and 6-31+g (d,p) basis set as implemented in the Gaussian 09 package. Our theoretical studies are very much in accordance with the experimental studies. DFT calculations reveal that the relative strength of terminal hydrogen bond in pro analogue **1** is more than that of the Ala analogue **3** (see ESI, page S44-S45, Fig. S19-S20). The calculated terminal hydrogen bond distances were found to be 1.95 Å and 1.98 Å, respectively, for the analogues **1** and **3**. In case of Aib analogue **2**, no such termini interactions were observed. It is noteworthy that aromatic–aromatic stacking interaction is observed in all the three cases as evident from the frontier molecular orbital diagram (see ESI, page S45, Fig. S20).

Conclusions

This study points out that a sterically constrained residue such as proline is unequivocally vital in orienting the H-bonding cores appropriately to result in strong zipper conformation. Chirality alteration has already been established to be another key contributor in the formation of this unique zipper structure *i.e.* heterochirality of both the Pro units as aliphatic components is necessary.[12] However, as an extension to those investigations, variation of residues at the N-terminus reveals yet another requirement for the stability of the long range H-bonding network formation. It has become conclusive that the conformationally arrested residue like Pro fulfills all the requirements for the stable zipper architecture. Comparatively, substitutions at the N-terminus with Aib or ^{L}Ala support zipper architecture, but considerably weaker terminal H-bonding interaction indicates the interplay of dihedral constraints in stability of these unique structures. In conclusion, the comparitive evaluation of the three residues (Pro, Aib and Ala) reveal that the dihedral restraints by different units are necessary to bring in an appropriate orientation of H-bonding sites and to form stable zipper architecture. In a broad sense, the study reveals that torsion angles also co-operatively affects the 3D-structure formation, modulation and stability.

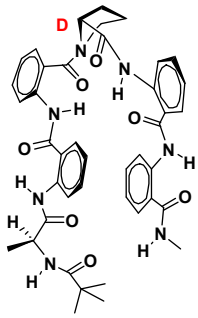
Experimental Section

(R)-1-(2-(2-(2-methyl-2-pivalamidopropanamido)benzamido)benzoyl)-N-(2-((2-(methylcarbamoyl)phenyl)carbamoyl)phenyl)pyrrolidine-2-carboxamide 2:



To a solution of **4c** (0.1, 0.145 mmol, 1 equiv.) in dry DCM (2 mL), Et_3N (0.030 mL, 0.217 mmol, 1.5 equiv.) was added followed by the addition of Piv-Cl (0.019 mL, 0.159 mmol, 1.1 equiv.) at 0°C. After completion of reaction, DCM (5 mL) was added to the reaction mixture and the combined DCM layer was washed sequentially with saturated $NaHCO_3$ solution, water and brine solution. DCM layer was then dried over Na_2SO_4, filtered and the solvent was stripped off under reduced pressure. Purification by column chromatography (eluent: 60% AcOEt/ pet. Ether, R_f: 0.3) afforded **2** (60%) as a fluffy white solid. mp:119-121°C; $[\alpha]^{25.36}_{D}$: 111.160° (c 0.5, $CHCl_3$); IR ($CHCl_3$) ν (cm^{-1}): 3339, 3020, 2927, 2856, 1655, 1585, 1518, 1436, 1408, 1299, 1162; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.49 (s, 1H, amide), 12.23 (s, 1H, amide), 11.74 (s, 1H, amide), 10.36 (s, 1H, amide), 8.88-8.87 (d, J = 8.24 Hz, 1H), 8.60-8.58 (d, J = 8.24 Hz, 1H), 8.42-8.40 (d, J = 8.24 Hz, 1H), 8.34-8.32 (d, J = 8.24 Hz, 1H), 7.75-7.73 (d, J = 7.93 Hz, 2H), 7.68-7.66 (d, J = 7.63 Hz, 1H), 7.58-7.54 (d, J = 7.93 Hz, 1H),7.58-7.54 (d, J = 7.93 Hz, 1H), 7.50-7.47 (m, 2H), 7.40-7.38 (d, J = 7.63 Hz, 1H), 7.30-7.27 (m, 2H), 7.23-7.19 (d, J = 7.93 Hz, 1H), 7.19-7.16 (d, J = 7.32 Hz, 1H), 7.10-7.07 (d, J = 7.32 Hz, 1H), 6.43-6.40 (d, J = 7.32 Hz, 1H), 6.30 (s, 1H, amide), 4.86-4.83 (d, J = 3.66 Hz, J = 8.54 Hz, 1H), 4.01-3.98 (m, 1H), 3.88-3.85 (m, 1H), 2.88-2.87 (d, J = 4.58, 3H), 2.45-2.39 (m, 1H), 2.36-2.32 (m, 1H), 2.15-2.09 (m, 1H), 2.04-2.01 (m, 1H), 1.66 (s, 3H), 1.57 (s, 1H), 1.27 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 178.5, 173.2, 170.5, 169.9, 169.4, 167.7, 166.5, 140.4, 138.6, 137.3, 133.2, 132.7, 131.9, 131.2, 128.3, 127.5, 127.3, 127.1, 123.8, 123.5, 123.3, 123.0, 122.9, 121.8, 121.0, 120.9, 120.5, 119.5, 118.9, 63.3, 57.6, 50.8, 38.9, 30.1, 27.5, 26.9, 26.0, 25.2, 24.5; MALDI-TOF/TOF: 797.1769 $(M+Na)^+$, 813.2659 $(M+K)^+$; Elemental analysis calculated for $C_{43}H_{47}N_7O_7$: C, 66.74; H, 6.12; N, 12.67. Found: C, 66.61; H, 6.20; N, 12.87.

(R)-N-(2-((2-(methylcarbamoyl)phenyl)carbamoyl)phenyl)-1-(2-(2-((S)-2-pivalamidopropanamido)benzamido)benzoyl)pyrrolidine-2-carboxamide 3:



The ester **4g** (0.2g, 0.263 mmol) was taken in saturated methanolic methylamine solution (2 mL) and stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure. Column chromatographic purification (eluent: 70% AcOEt/pet. Ether, R_f: 0.3 of the residue afforded **3** (60%) as a white fluffy solid. mp: 156-158 °C; $[\alpha]^{24}_{D}$: +154.28°(c 1, $CHCl_3$); IR ($CHCl_3$) ν (cm^{-1}): 3681, 3583, 3346, 3019, 2400, 1653, 1602, 1585, 1526, 1437, 1323, 1294, 1215, 756; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 12.56 (s, 1H), 12.33 (s, 1H), 11.62 (s, 1H), 10.38 (s, 1H), 8.89-8.88 (d, J = 8.24Hz, 1H), 8.47-8.44 (m, 2H), 8.36-8.34 (d, J = 8.24Hz, 1H), 7.72-7.68 (m, 3H), 7.55-7.49 (m, 3H), 7.40-7.38 (d, J = 7.93Hz, 1H), 7.32-7.29 (t, J = 7.94Hz, 1H), 7.25-7.23 (m, 1H), 7.20-7.16 (m, 2H), 7.14-7.11 (t, J = 7.63Hz, 1H), 6.45-6.42 (t, J = 7.63Hz, 1H), 6.28-6.27 (d, J = 7.02Hz, 1H), 4.83-4.81 (m, 1H), 4.41-4.35 (pentet, J = 7.02Hz, 1H), 4.01-3.98 (m, 1H), 3.92-3.87 (m, 1H), 2.88-2.87 (d, J = 4.58Hz, 3H), 2.47-2.40 (m, 1H), 2.35-2.33 (m, 1H), 2.18-2.09 (m, 1H), 2.05-2.03 (m, 1H), 1.45-1.44 (d, J = 7.02Hz, 3H), 1.31 (s, 9H); ^{13}C NMR (125MHz, $CDCl_3$) δ: 179.0, 171.1, 170.4, 169.8, 169.4, 167.6, 166.2, 140.4, 139.9, 138.8, 137.2, 133.2, 132.6, 132.1, 131.2, 128.0, 127.2, 127.0, 123.7, 123.3, 123.2, 123.1, 123.0, 121.8, 120.9, 120.7, 120.6, 120.5, 119.0, 118.7, 63.3, 50.7, 50.3, 38.7, 30.2,

27.4, 26.8, 25.0, 18.6; MALDI-TOF/TOF: 782.1917 $(M+Na)^+$, 798.1660 $(M+K)^+$; Elemental analysis calculated for $C_{42}H_{45}N_7O_7$: C, 66.39; H, 5.97; N, 12.90; Found: C, 66.48; H, 5.87; N, 12.69.

Acknowledgement

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Notes and references

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Electronic Supplementary Information (ESI) available: ^1H, ^{13}C, DEPT-135 NMR, 2D study spectra, ESI mass spectra and theoretical study of new compounds are included. See DOI: 10.1039/b000000x

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