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Article

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Cis - Trans Conformational Analysis of δ – Azaproline in Peptides

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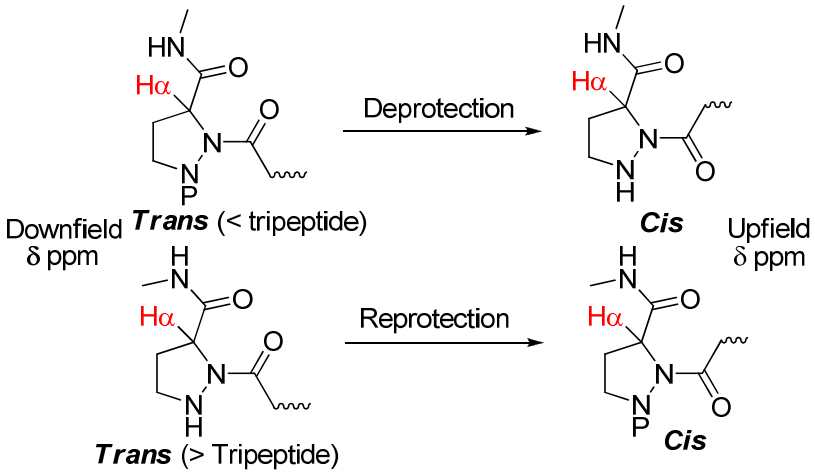


The *cis-trans* isomerisation and conformer specificity of δ-azaproline and its carbamate protected form in linear and cyclic peptides were investigated using NMR and α-chymotrypsin assay. Comparisons of chemical shift value of the α – hydrogen in each case of δ–azaproline-containing peptides with conformer specific locked diketopiperazines reveal the fact that an upfield chemical shift value corresponds to *cis* conformer and a downfield value corresponds to a *trans* conformer. δ-Azaproline adopts *cis*-conformation in simple amides, dipeptides and tripeptides whereas its carbamate protected form adopts *trans*-conformation. In the case of longer, linear or cyclic peptides, *vice-versa* results are obtained. Interestingly, in all these peptides exclusively

one conformer either *cis* or *trans* is stabilized. This *cis-trans* isomerisation is independent on both temperature and solvents, only the δ - nitrogen protecting group plays key role in the isomerization. δ-Azaproline is conformer specific in either of its protected or deprotected forms, which is a unique property of this proline. Unlike other covalently modified proline surrogates, this isomerization of δ-azaproline can be tuned easily by a protecting group. Mechanism of *cis-trans* isomerisation of δ-azaproline during deprotection and reprotection is supported by theoretical calculations.

INTRODUCTION

Cyclic α-hydrazino acids (**1**) (Figure 1) have emerged as one of the most promising non - proteinogenic amino acid candidate and they may find use as proline surrogates in order to improve peptide pharmacokinetics[1] and/or bioavailability. However before being considered as a proline substitute, their effect on the structure of peptides needs to be thoroughly investigated, since the function of peptides and proteins largely depend on their structure.

Figure 1. General structure of cyclic α - hydrazino acids

In protein structures, due to significant delocalization of the lone pair of electrons on the nitrogen atom the peptide bond attains a partial double bond character. The partial double bond renders the amide group planar, allowing it to either adopt a *cis* or *trans* conformation. These bonds are free to adopt either of the conformations in the unfolded state, but are only allowed to adopt the energetically more favorable, *trans* conformation in folded state.[2]

Proline is unique among other naturally occurring amino acid, being an imino acid proline residue restricts the conformational space of the peptide chain. However Xaa - Pro peptide

groups tend to have a roughly 3:1 *cis* and *trans* ratio, apparently because the symmetry between the C^{α} and C^{δ} atoms of proline makes both the conformers nearly equal in energy. Consequently any change in α and δ positions will be reflected in the *cis* - *trans* ratio as the symmetry will be disrupted. Because of this unique conformational property, proline plays an important role in structural and biological properties of peptides and proteins.[3a-f] Loss of symmetry owing to any substitution at the α[3g-i] or δ position[3j-o] of proline has effects on the structure of peptides as have been investigated by several groups. In most cases conformational studies of peptides or protein folding is hampered by the heterogeneity of the Xaa-Pro peptide group due to the presence of *cis*/*trans* mixtures. In order to eliminate complications arising from the *cis*/*trans* isomerisation of proline peptide groups, scientific community has constantly been on the lookout for either a *cis* or a *trans* stabilizing proline analogues[3,4] or surrogates (diminishing or enlarging the pyrrolidine ring size).[4k] In this context, the conformational behavior of pyrazolidines as proline equivalent in peptides has been extensively explored by Marshall and coworkers.[5] To some extent pyrazolidines resembled proline and could stabilize *cis* conformer in organic solvents and in solid phase, however it loses its conformer specificity in aqueous solutions. Taking a cue from these studies we contemplated the use of δ – azaproline **1** as proline surrogate in order to achieve conformer specificity.

δ - Azaproline **1**, a five membered cyclic α - hydrazino acid, closely resembles proline in structure and ring size, only difference being the nitrogen atom at the delta position instead of carbon. Herein we wish to report the effect of δ - azaproline and a carbamate protecting group at δ - nitrogen on *cis* - *trans* conformation of δ - azaproline containing linear and cyclic peptides.

RESULTS AND DISCUSSION

Model Study. The *cis* - *trans* conformation of δ – azaproline containing peptide is shown in Figure 2, which is defined by the spatial arrangement of peptide chains, either lying *syn* or *anti* to each other is termed as the *cis* or *trans* conformer, respectively.

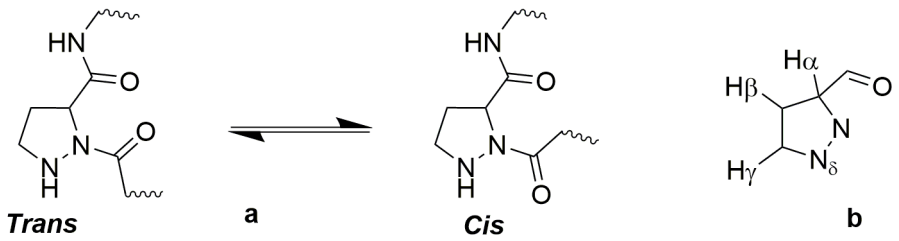
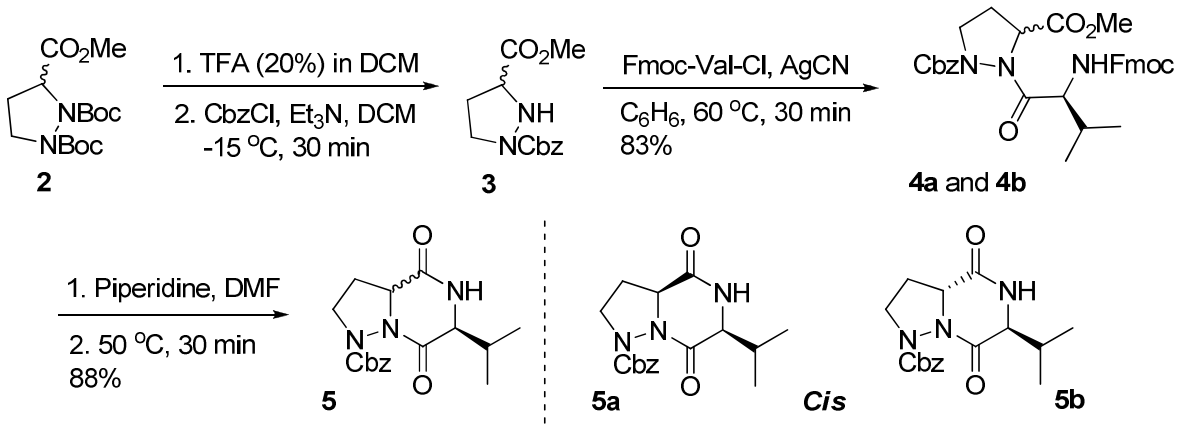


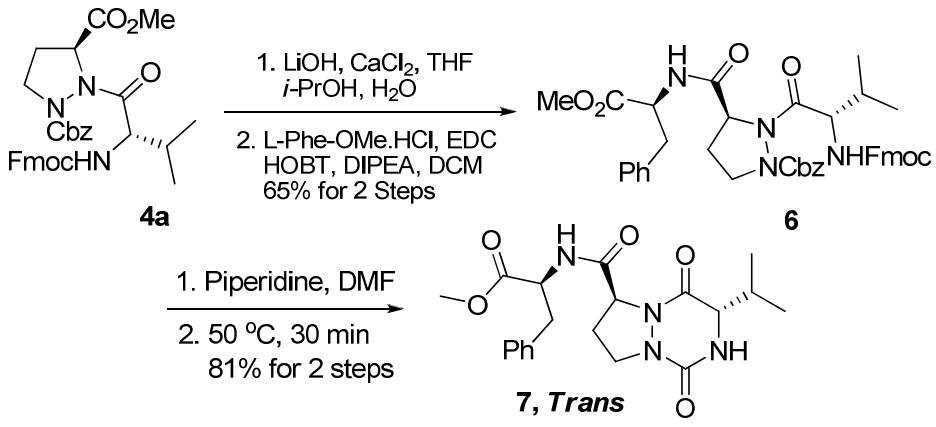
Figure 2. (**a**) *Cis* - *Trans* isomerisation in δ - azaproline, (**b**) Relative positions in δ - azaproline.

Chemical shift values of α - Hydrogen in ^{1}H - NMR was our preferred tool for the identification of *cis* and *trans* conformers of δ – azaproline and its peptides. Initially we investigated the chemical shift of α - H corresponding to the *cis* - locked and *trans* - locked diketopiperazines of δ – azaproline and accordingly, the compounds were synthesized. Compound **2** was treated with 20% TFA in DCM followed by selective benzyloxy carbonyl protection of δ-nitrogen **3**.[6] Compound **3** was heated with Fmoc-Val-Cl and AgCN in benzene[7] to yield **4**, which on Fmoc deprotection and heating in the same pot yielded the diketopiperazine **5**. The compound **5** is locked in *cis* conformation (Scheme 1).



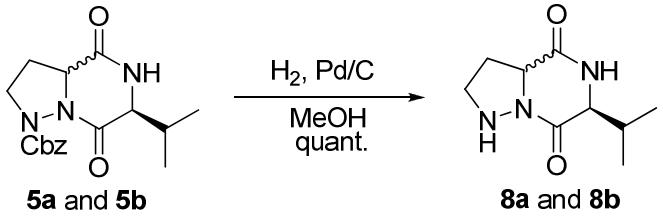
Scheme 1. Synthesis of *cis*-locked diketopiperazine **5**

Similarly, we attempted to synthesis *trans* - locked diketopiperazine from **4**, but isolated **5** as the sole product. Accordingly, scheme 2 was followed to synthesize the desired product. Compound **4a** was hydrolyzed by LiOH in $CaCl_2$ medium[8a] followed by peptide coupling with L-phenylalanine methyl ester to yield **6**.[8b] Fmoc deprotection and *in situ* heating yielded the diketopiperazine **7**. The diketopiperazine **7** is locked in *trans* conformation (Scheme 2).



Scheme 2. Synthesis of *trans*-locked diketopiperazine **7**

In order to establish the electronic effect of the carbonyl group adjoining the δ - nitrogen on the α-H ppm value, compound **5** was hydrogenated in order to remove the Cbz protection to yield **8** (Scheme 3).



Scheme 3. Synthesis of deprotected *cis*-locked diketopiperazine **8**

NMR (^{1}H, HMQC, and COSY supporting info) studies on compounds **5**, **7** and **8** were performed to establish the signals corresponding to α-hydrogen, the observation were as follows.

Compound 5. The α-hydrogen signal of diketopiperazine **5** was identified at **4.25**. Being locked in *cis* conformation, this value (ppm) was taken as the standard region for *cis* conformers of δ – azaproline-containing peptides (Figure 3).

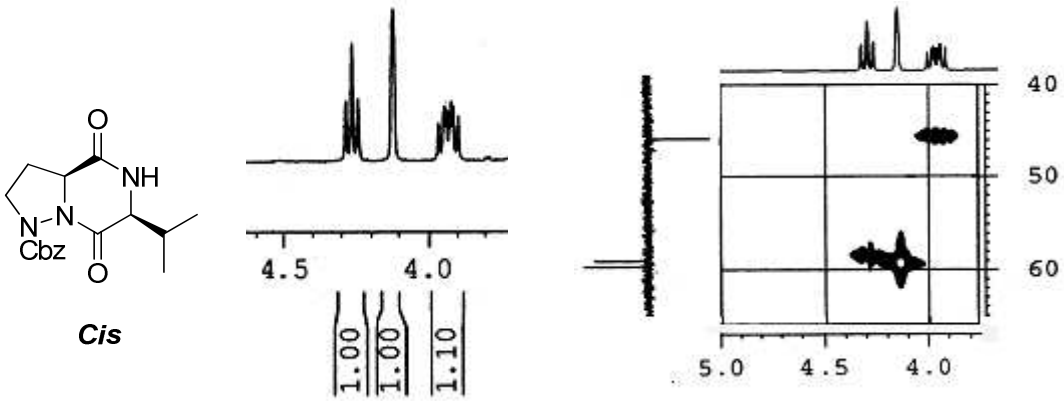


Figure 3. ^{1}**H** NMR and **HMQC** of compound **5a**

Compound 7. The α - hydrogen signal of diketopiperazine **7a** was identified at **4.86**. Being locked in *trans* conformation, this value (ppm) was taken as the standard region for *trans* conformers of δ -azaproline-containing peptides (Figure 4).

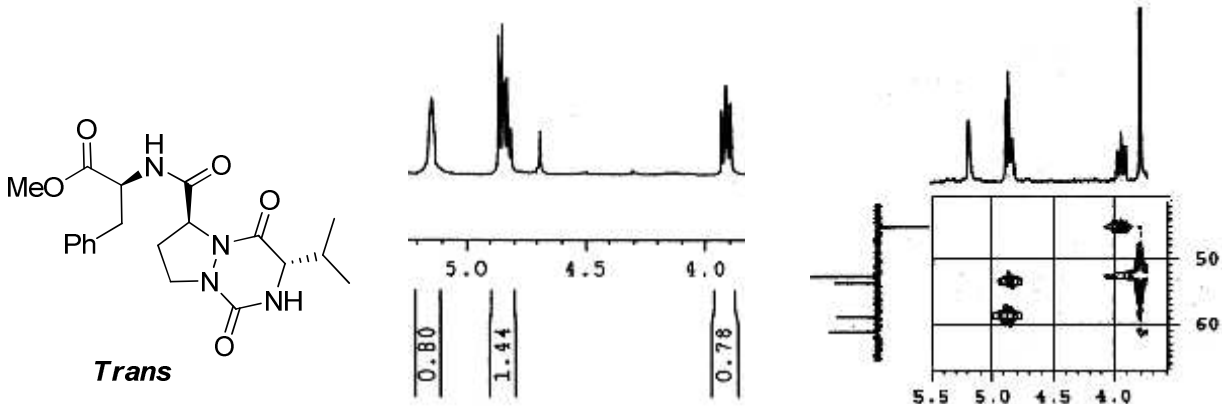


Figure 4. ^{1}**H** NMR and **HMQC** of compound **7**

Compound 8. The α - hydrogen signal of the deprotected diketopiperazine **8a** was identified at **4.27**. Being locked in *cis* conformation, this value (ppm) was taken as the standard region for deprotected *cis* conformers of δ - azaproline-containing peptides (Figure 5).

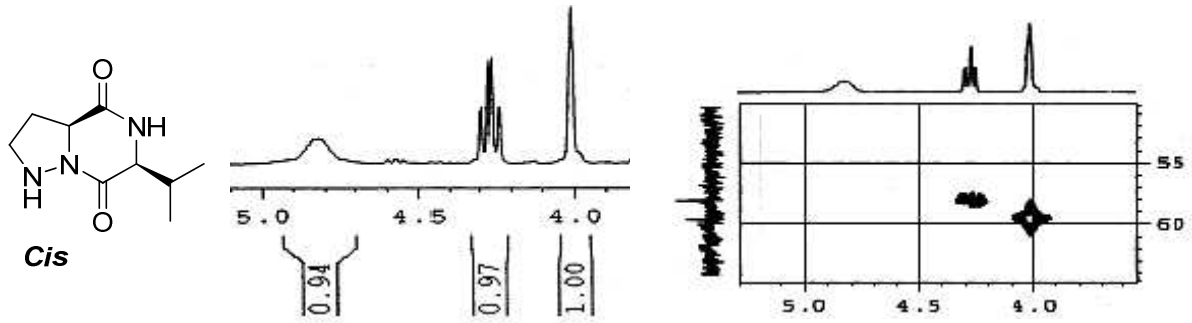


Figure 5. ^{1}**H** NMR and **HMQC** of compound **8a**

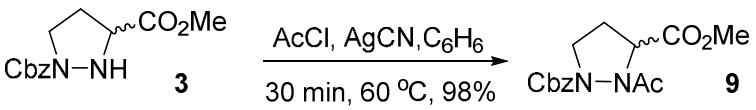
(For detailed NMR spectra see supporting information)

In conclusion the *cis* conformer generally can be identified by upfield α - hydrogen signal, the *trans* conformer by a downfield α - hydrogen signal, also the protecting groups at δ - nitrogen has no notable electronic effect on the chemical shift of α - hydrogen.

Picking up the required information from the above experiments, we proceeded to determine the actual inclination of protected and unprotected δ-azaproline towards *cis* or *trans* conformation.

To establish the *cis* - *trans* conformation in δ-azaproline, we considered a model study based on previous literature reports on *N* - acetyl proline methyl ester.[9] The ^{1}H NMR of *N* – acetyl proline methyl ester showed the presence of two isomers in roughly 3:1 ratio (78:22) *i.e.* 78% *trans* and 22% *cis* characterized by the α - H peaks at **4.33** (dd, *J* = 8.7, 3.6 Hz, 0.78H) corresponding to *trans* and at **4.27** (dd, *J* = 8.6, 2.8 Hz, 0.22H) for *cis* conformers, respectively (see Supporting Information).

Analogous azaproline based substrate **9** was prepared by acetylating **3** with acetyl chloride and AgCN in benzene (Scheme 4).



Scheme 4. Synthesis of model substrate **9**

^{1}H NMR and HMQC studies on **9** indicated the presence of only one conformer (Figure 6). Comparing those with the chemical shift values from previous section it can be said that Cbz protected δ-azaproline exists exclusively in the *trans* conformation.

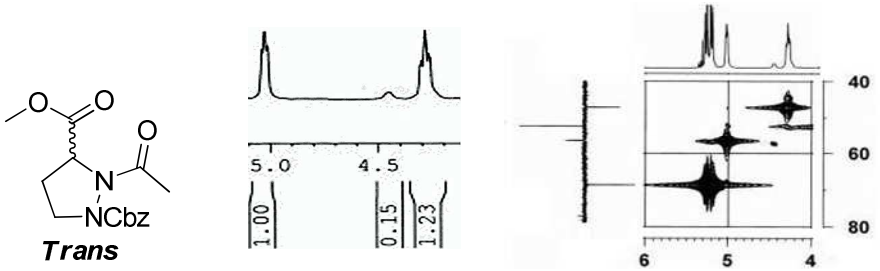
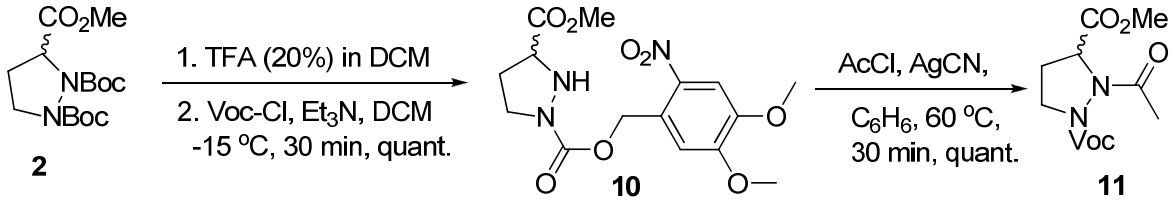


Figure 6. ^{1}**H** NMR and **HMQC** of **9**

We have synthesized another azaproline derivative **10** with a photo labile protecting group. Such protecting groups are known to cleave in the presence of light and this strategy could be useful in biological research. Compound **2** was converted to **11** by selectively protecting with veteroxy chloroformate followed by acetylation (Scheme 5).



Scheme 5. Synthesis of *N* - Voc protected model substrate **11**

1H and 2D NMR studies of **11** again illustrated the presence of *trans* conformer exclusively,

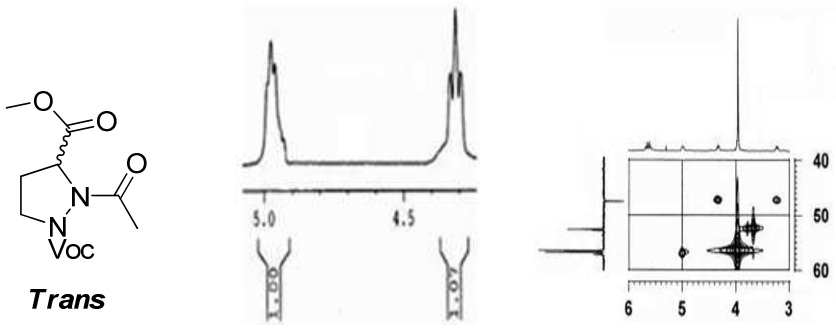
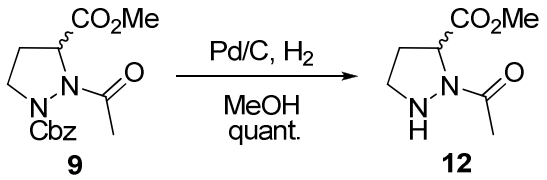


Figure 7. ^{1}**H** NMR and **HMQC** of **11**

similar to that of compound **9** (Figure 7). The role of electronic parameters (presence of NO_2) on the chemical shift of α - hydrogen was ruled out by our previous study of compound **8** (Scheme 3, Figure 5).

To spot the effect of deprotection, compound **9** was subjected to hydrogenation condition to yield **12** (Scheme 6). NMR analysis of **12** showed the presence of only *cis* conformer, indicating a crossover from *trans* conformation during the deprotection (Figure 8).



Scheme 6. Synthesis of deprotected model substrate **12**

The effect of electronic factor on the chemical shift value was again ruled out by comparing with compound **8**.

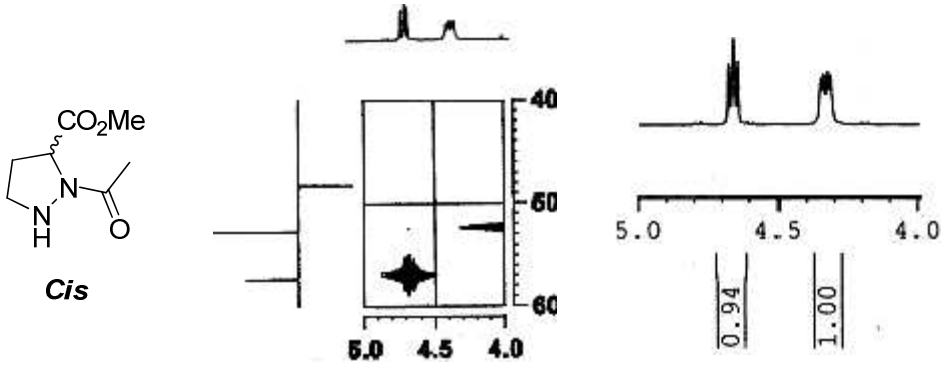


Figure 8. ^{1}**H** NMR and **HMQC** of **12**

The possible preference for *cis* conformation of the deprotected form (**12**) was initially thought to be due to the formation of an intramolecular H-bonding between the hydrogen attached to δ-nitrogen and the adjacent oxygen of *N* - acetyl (highlighted in red) but rapid deuterium exchange with D_2O suggested otherwise (Figure 9). Since, a proton which is part of an intramolecular hydrogen bond is expected to exchange slowly if at all.[10]

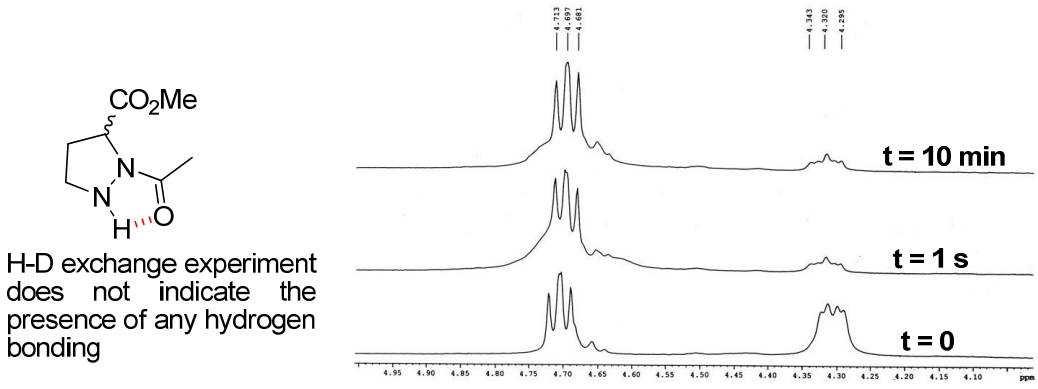
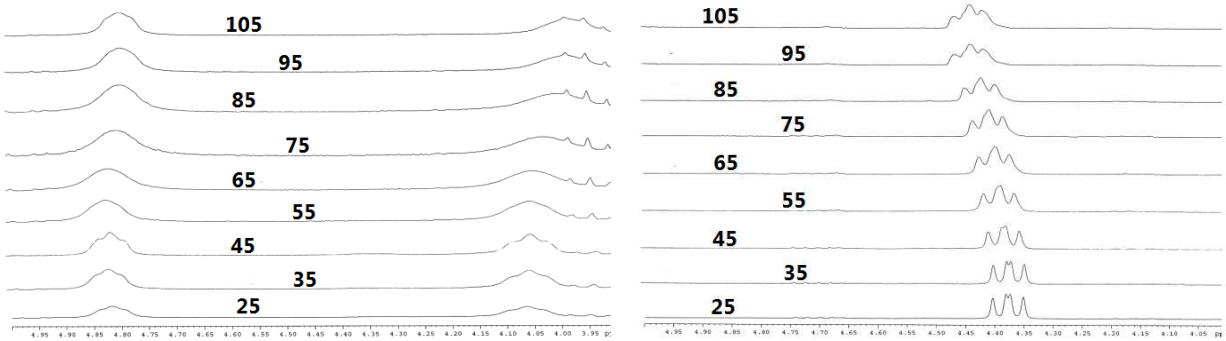


Figure 9. **H - D** exchange experiment on **12**

After this investigation, we tried to establish the temperature dependence of *cis* - *trans* isomerisation between the two conformers. Accordingly high temperature NMR studies on both the compounds **9** and **12** were performed. The compounds were dissolved in DMSO-*d6* and proton NMR was recorded at an interval of 10 ^{o}C (25 to 105 ^{o}C).

Both the Cbz protected model substrate **9** and deprotected substrate **12** did not undergo any *cis* - *trans* isomerisation in the temperature range between 25 to 105 ^{o}C (Figure 10).

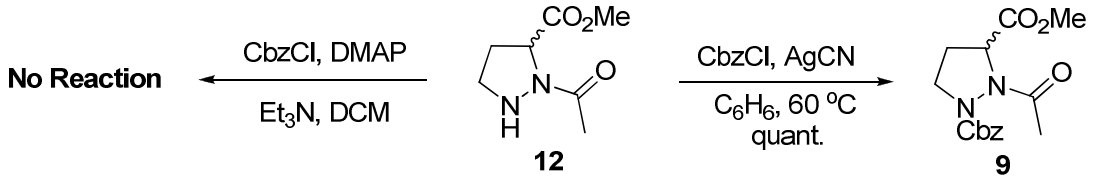


a **b**

Figure 10. (a) Variable temperature NMR of **9**, (b) Variable temperature NMR of **12**, (Values in the spectra denotes the temperature)

Apart from expected shift the change of solvent ($CDCl_3$ to DMSO-*d6*) also had no effect on the isomerisation as evident from the chemical shift values of the α-H of **9** and **12** under investigation.

These investigations also begs the question about the fate of **12** on reinstallation of a protecting group, consequently it was subjected to standard Cbz protection condition (DMAP, Et_3N and CbzCl) only to recover the starting material. Harsher conditions (AgCN, CbzCl and 60 ^oC) yielded protected δ - azaproline which was identical to previously synthesized **9** in all aspects (Scheme 7).



Scheme 7. Reprotection of model substrate **12**

From the above model studies it was concluded that the δ–azaproline, in simple amides exclusively adopts a *trans* conformation in its protected form and *cis* conformation in its native form. These conformations resist interconversion even at elevated temperatures, only protection or deprotection seems to bring about the conformational changes.

At this point to gain an insight into the mechanism of isomerization during deprotection or reprotection we turned towards theoretical calculations.

I. Computational Details:

We have optimized each intermediates and transition states using ωB97X-D[11] functional with Pople's 6-31++g(d,p) basis set on each atom except Ag atom. For Ag atom we have used LANL2 pseudopotential for core electrons and LANL2DZ basis set for valance electrons. All optimization were done in presence of continuum with the help of SMD solvent model[12] and appropriate dielectric constant of solvent. For deprotection of **9t** (**t** stands for *trans*) using Pd/H_2 we have used DMSO as solvent and for protection of **12c** (**c** stands for *cis*) using AgCN and CbzCl we have used benzene as solvent. Determination of entropy of a species in solution phase

is difficult to compute, so an approximation proposed by Wertz[13] to compute solution phase entropies from gas phase entropies based on experimental results was used. This approximation is frequently employed for evaluating free energies by static quantum chemical calculations.[14] We have calculated and incorporated the decrease of entropy in solution phase by reducing gas phase entropy to 0.5 times its actual value. All theoretical calculations are done using standard Gaussian09 quantum chemical package.[15]

II. Mechanism of deprotection of Cbz protected amide using Pd/H_2:

Optimized structure of **9t** (shown in Figure 11) shows a strong CH-π interaction between methyl CH of amide bond present nearest to the chiral center and the benzene ring present in the benzyl group which is used for protection of the amine. In the optimized structure of **9t**, amide

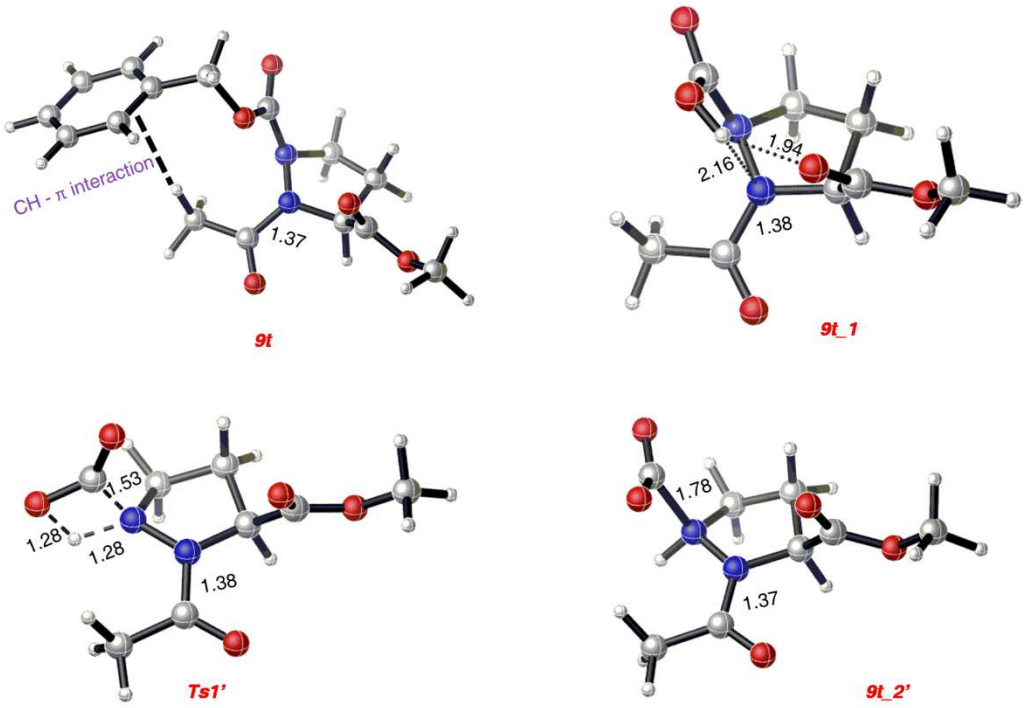


Figure 11. Optimized structures of **9t**, **9t_1**, **Ts1'**, **9t_2'**. Bond distances are shown in Å. C, N and O are shown in grey, blue, red color. H atoms are shown in grey also but they have smaller van der wall radii compared to C atom. This color coding is maintained throughout this section.

C-N bond distance is 1.37 Å. It is well known that during deprotection of Cbz protected amine by Pd/H_2, a carbamic acid intermediate is generated which undergoes decarboxylation to produce the deprotected amine.[16] We have optimized the *in situ* generated carbamic acid, **9t_1** (shown in Figure 11) from **9t**. Optimized structure of **9t_1** shows hydrogen bonding between the proton of carbamic acid with the amide nitrogen and the carbonyl group of the substituent present in the chiral carbon. Proton of carboxylic acid in **9t_1** can be transferred through a four member transition state **Ts1'** (shown in Figure 11) to the nitrogen atom to produce **9t_2'** (Shown in Figure 11) with free energy activation barrier of 37.3 kcal/mol. This barrier height is

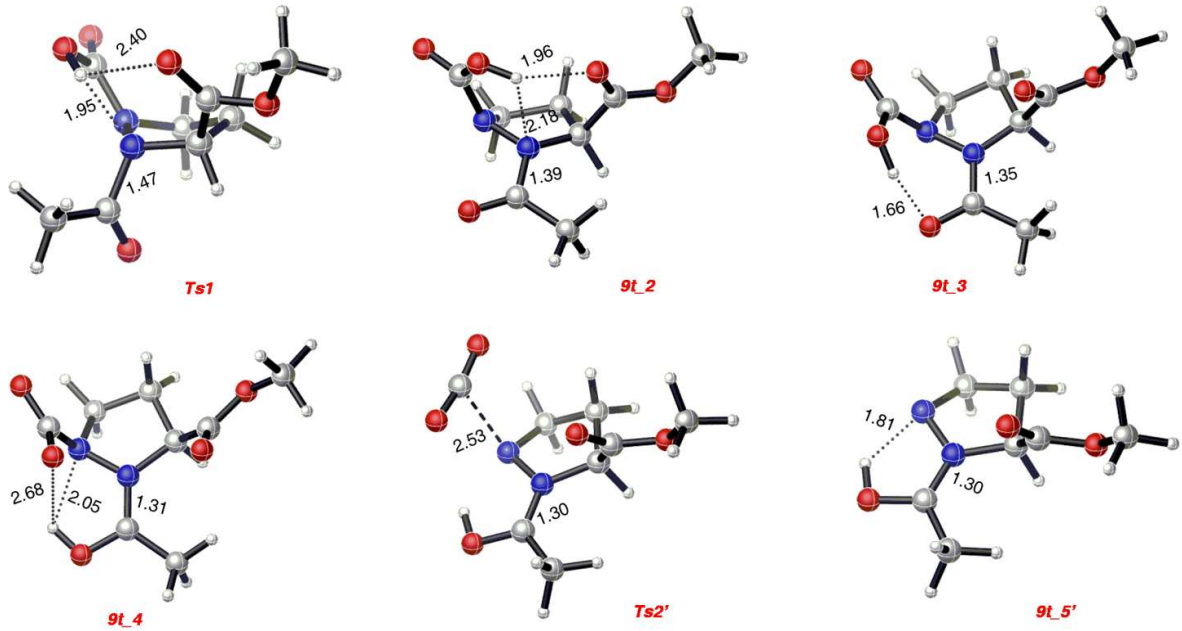


Figure 12. Optimized structure of **Ts1**, **9t_2**, **9t_3**, **9t_4**, **Ts2'** and **9t_5'**. Bond distances are shown in Å.

insurmountable in the reaction conditions. Also if this decarboxylation happens we would get a *trans* isomer of **12**. But our experimental results show that *cis* isomer of **12** is obtained selectively. Optimized structure of **9t_1** (shown in Figure 11) shows that C-N amide bond length is 1.38 Å, which is longer compared to **9t** (shown in Figure 11). So we looked into the possibility

of the rotation of the amide bond to produce *cis* isomer of carbamic acid, **9t_2** (shown in Figure 12). Our theoretical computation predicts very low free energy activation barrier ($\Delta G^{\#}$ = 15.3 kcal/mol) for the amide bond rotation via **Ts1** and the reason behind this low activation barrier is the stabilization provided by the proton of carboxylic acid to the lone pair of nitrogen during rotation. As a result of amide bond rotation **9t_3** is obtained and it consists of a strong hydrogen bonding interaction between carbonyl oxygen of the amide group and the proton of the carboxylic acid. Starting with **9t_3**, an intramolecular proton transfer from carboxylic acid to the carbonyl oxygen atom of the adjacent amide group yielded **9t_4** (shown in Figure 12).

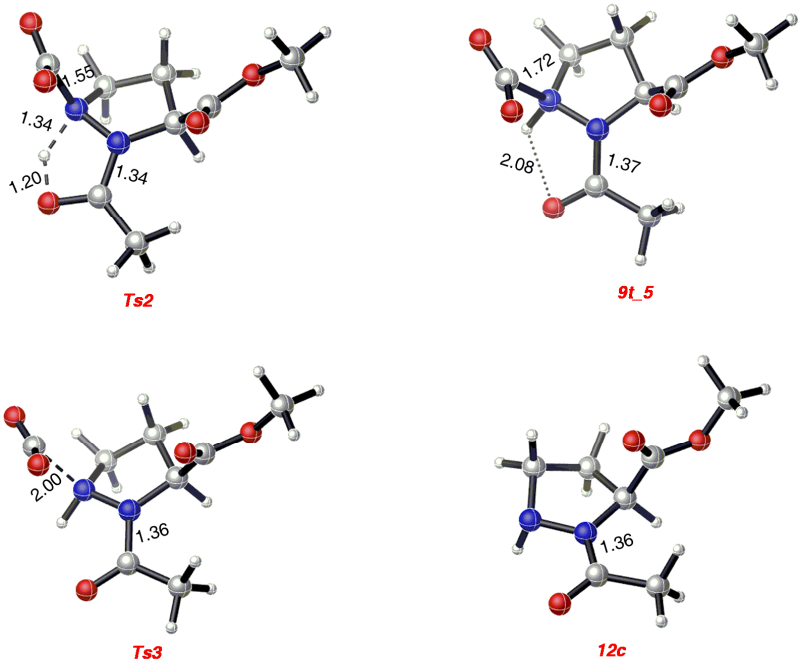


Figure 13. Optimized structure of **Ts2**, **9t_5**, **Ts3** and **12c**. Bond distances are shown in Å.

The optimized structure of **9t_4** shows that the amide C-N bond length is shortened and the adjacent carbonyl C-O bond length has increased appreciably than that of **9t_3**, suggesting the lone pair donation tothe π* orbital of the protonated carbonyl group. We did not get any transition state for the **9t_3** to **9t_4** transformation. We have performed a relaxed scan to locate

the transition state for the above mentioned process but no transition state for this proton transfer process could be identified. Hydrogen bonding interaction between proton of the imidic acid form of protonated amide and the lone pair of adjacent nitrogen atom in **9t_4** (shown in Figure 12), clearly suggests another possible intramolecular proton transfer. This proton transfer happens via **Ts2** (structure shown in Figure 13) with free energy activation barrier of 21.8 kcal/mol.

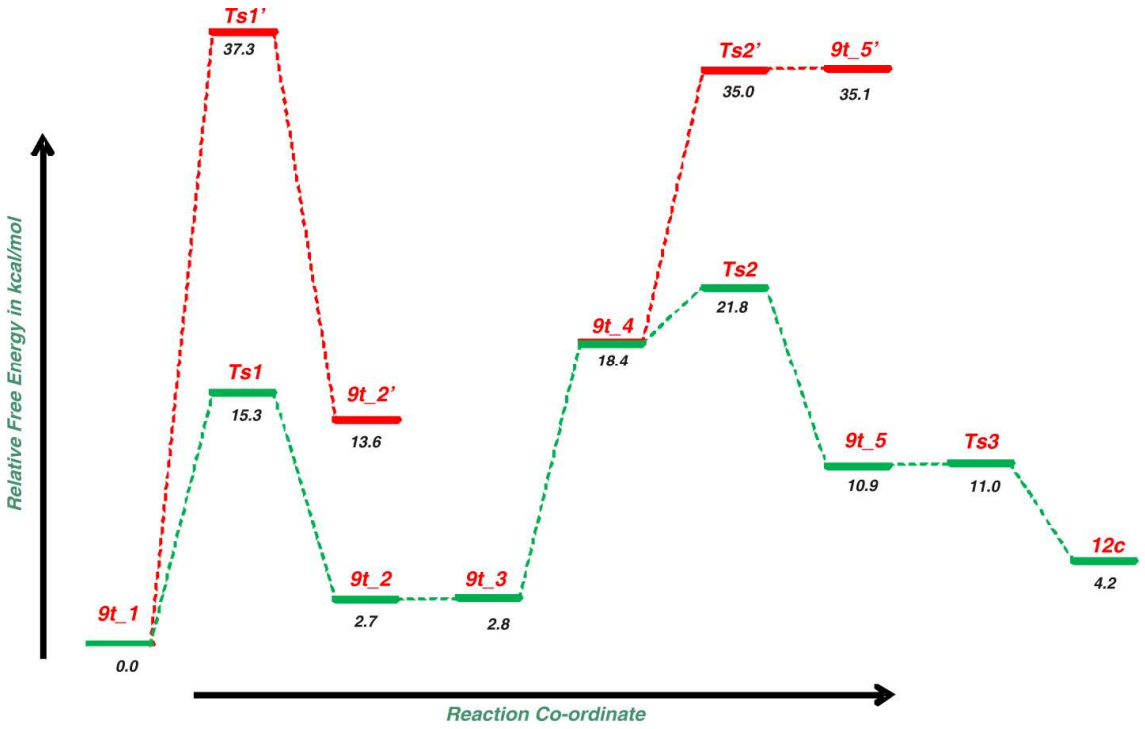


Figure 14. Relative free energy profile for decarboxylation process of carbamic acid generated from **9t** during deprotection using Pd/H_2.

As a result of this proton transfer **9t_5** is formed (an exoergic process), followed by decarboxylation of **9t_5** via **Ts3** with an activation barrier of 11.0 kcal/mol leading to the formation of **12c**. Though formation of **12c** is endoergic in nature, the deprotection process is

favored as the release of CO_2 from solution phase to gas phase creates a non equilibrium situation.

Decarboxylation can also happen before intramolecular proton transfer *i.e.* from **9t_4** via **Ts2' (**Figures 12 and 14). Since this route requires an activation energy of 35.0 kcal/mol. it is very unlikely for the reaction to follow this path.

Our detail theoretical investigation shows that the minimum energy pathway for deprotection of **9t** involves rotation of the amide bond which plays a crucial role in the deprotonation of carbamic acid and subsequently facilitates the decarboxylation process. So our theoretical calculations offer an explanation for the crossover from *trans* to *cis* isomer during deprotection of amide (**9t**).

III. Mechanism for protection of amine using Cbz and AgCN:

In this section we have investigated the mechanistic details of conformational change in the amide group during protection of **12c** with AgCN and Cbz. We found that carbonyl oxygen of the Cbz can coordinate with AgCN to produce **int1** (see Figure 15). Optimized structure of **int1** shows the increase in C-O (present in Cbz) bond length (the bond length of C=O group of **int1** is 1.21 Å whereas the C=O bond length of Cbz is 1.19 Å) (see Figure 15). So AgCN acts as a Lewis acid to polarize the carbonyl group facilitating subsequent nucleophilic addition by amine **12c**. Formation of **int1** is exothermic by 7.2 kcal/mol in terms of free energy. Nucleophilic addition of **12c** to the carbonyl group of **int1** produces **int2** via **Ts4** with free energy activation barrier of 3.7 kcal/mol (see Figure 15). Formation of **int2** is endothermic compared to the separated reactants (**int1** and **12c)** by 2.34 kcal/mol. Optimized structure of **int2** shows that amide C-N bond length is 1.39 Å (Figure 15), which is longer compared to **9t_1**. Starting with **int2**, the amide bond can rotate through **Ts5** (with free energy activation barrier of 17.6 kcal) to

attain *trans* form of the nucleophilic addition product, **int3** (Figure 15). So this low free energy activation barrier allows the conversion of *cis* to *trans* form of the amide during protection of **12c** by AgCN and Cbz which is observed experimentally.

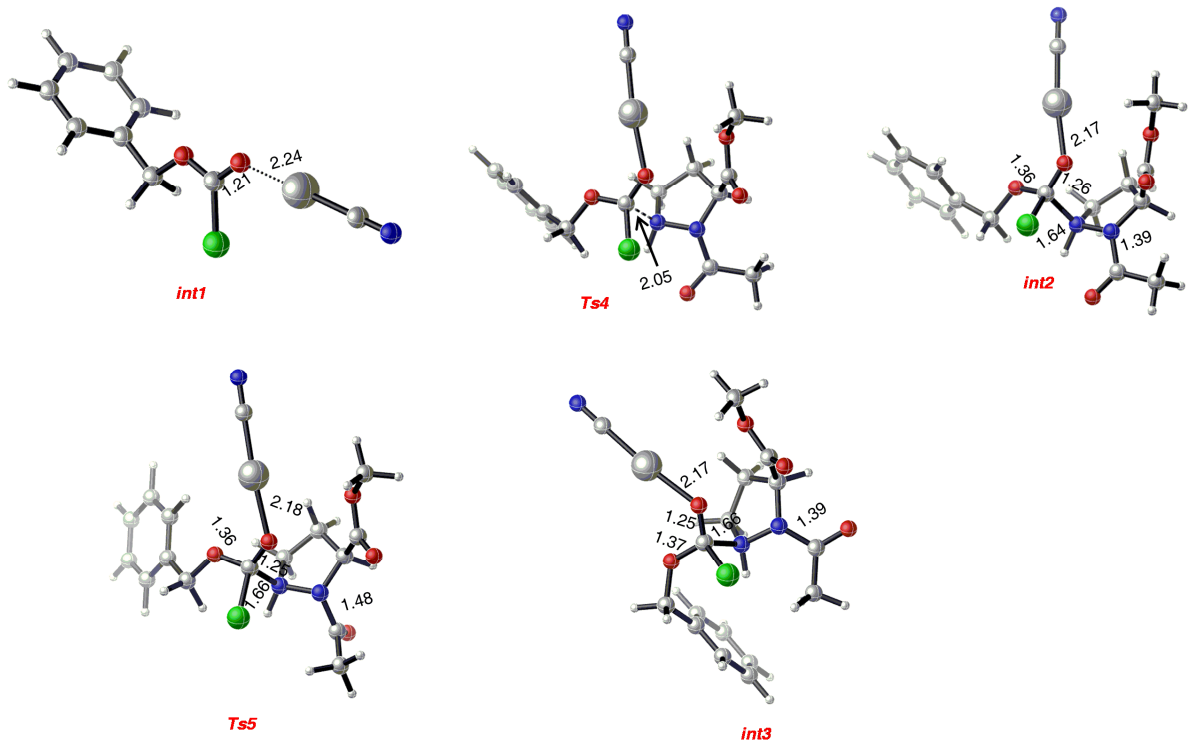
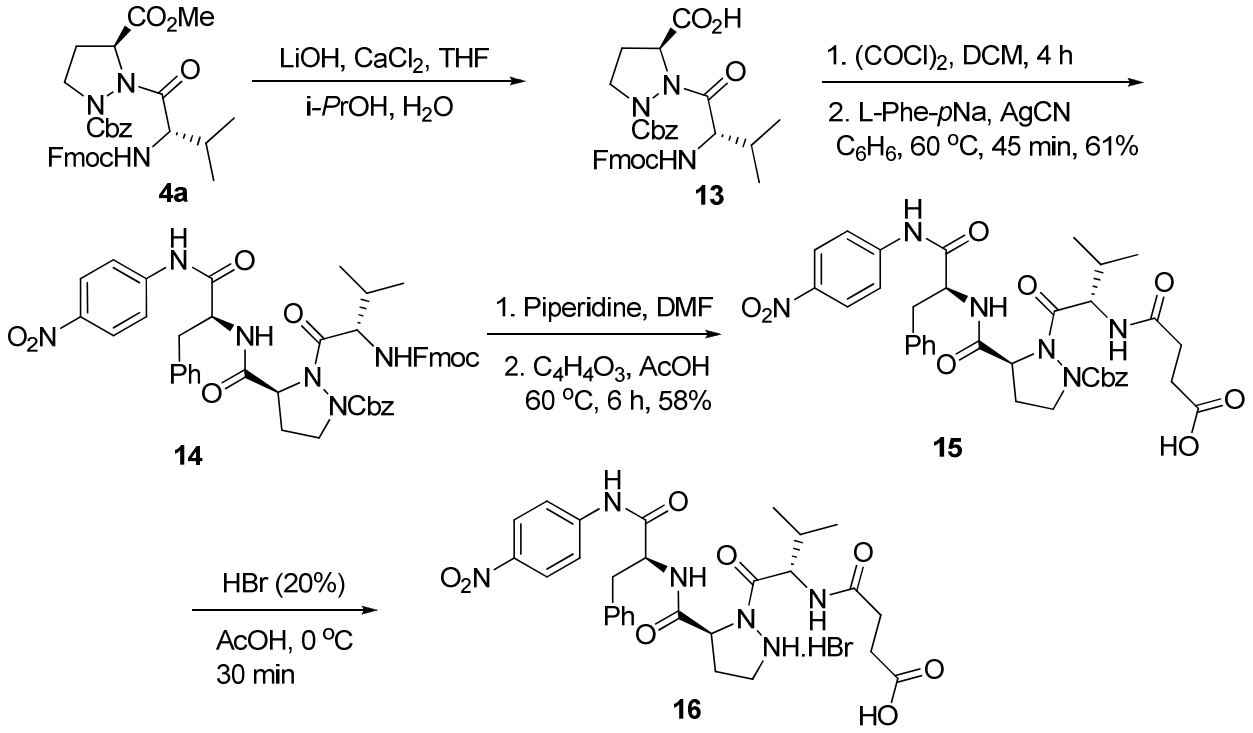


Figure 15. Optimized structures of important intermediates and transition states related to the conformational change of the amide bond of **12c** during protection using AgCN and Cbz. Bond distances are shown in Å.

To experimentally establish and generalize this observation in tripeptides, the chymotrypsin coupled assay developed by *Fischer et al.*[17a] was used. Short model peptides of type **Suc-Val-ΨPro-Phe-*p*NA** are hydrolyzed by chymotrypsin between Phe and *p*NA only if the Val-ΨPro peptide bond is in the *trans* conformation (the degree of tolerance of enzymes towards proline surrogates was demonstrated by M. Mutter *et.al.*).[18,3a] High rate of *Phe - pNA* hydrolysis by enzyme is expected of the protected δ-azaproline-containing model peptide

(known as burst phase kinetics), on the other hand the deprotected δ-azaproline containing model peptide is not expected to undergo hydrolysis.

The azaproline-containing model peptides for chymotrypsin studies were prepared from **4a**. The free acid **13** on treatment with oxalyl chloride followed by AgCN-mediated coupling with L-Phe-*p*NA provided the tripeptide **14**. The compound **14** on Fmoc deprotection and subsequent *N* – succinylation[19] provided the protected model peptide **15**. The tripeptide **15** was then treated with 20% HBr in acetic acid[20] to yield the deprotected tripeptide **16** (Scheme 8).



Scheme 8. Synthesis of chymotrypsin substrates **15** and **16**.

NMR analysis of the tripeptides **15** (protected) and **16** (deprotected) showed anticipated results, *i.e.* **15** adopted a *trans* conformation (Figure 16a) and compound **16** adopted a *cis* conformation (Figure 16b).

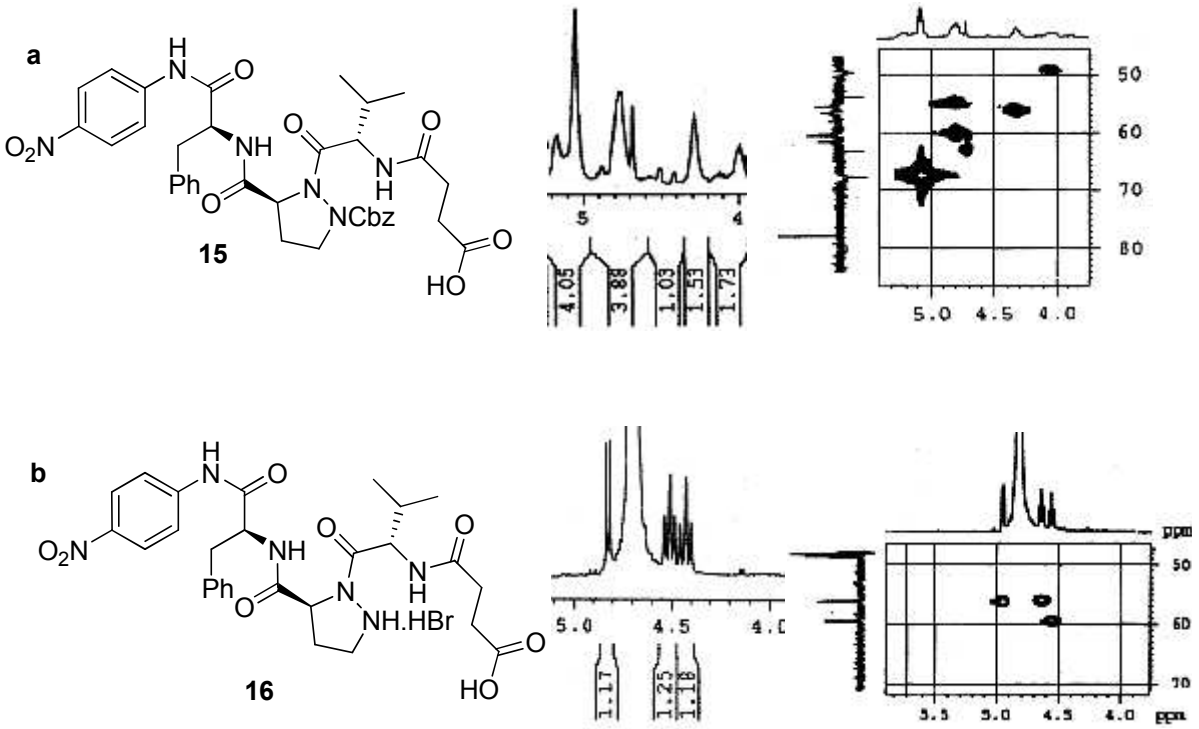
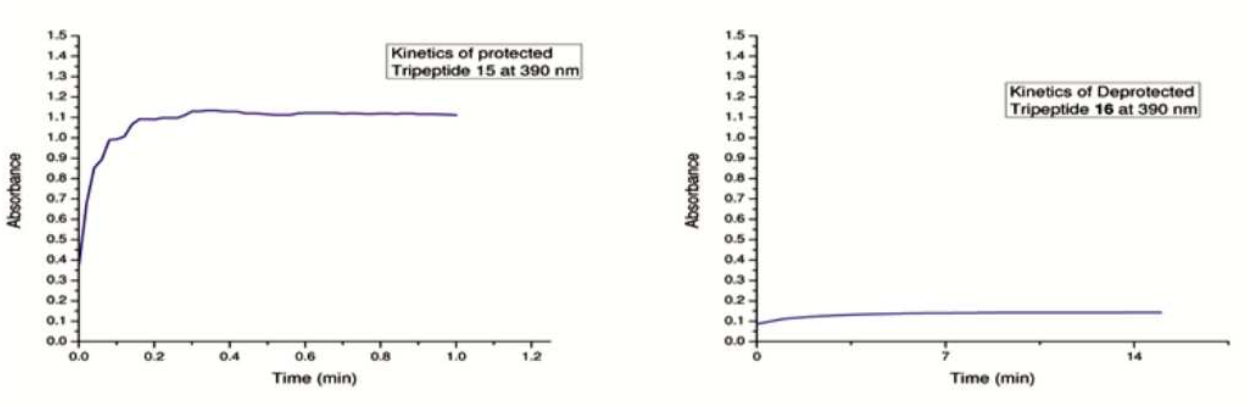


Figure 16. (**a**) **^{1}H** NMR and **HMQC** of **15**, (**b**) **^{1}H** NMR and **HMQC** of **16**

Chymotrypsin assay. The compound **15** (protected) has a *trans* δ-azaproline and hence is expected to undergo hydrolysis releasing *p* - nitroaniline. This was confirmed by the assay as the reaction (release of *p* -nitroaniline) completed within 12 seconds (Figure 17a).

Compound 16 (deprotected). Since the compound **16** has a *cis* δ-azaproline, consequently it is not expected to undergo hydrolysis in presence of chymotrypsin, which was also corroborated with the experiment (Figure 17b).

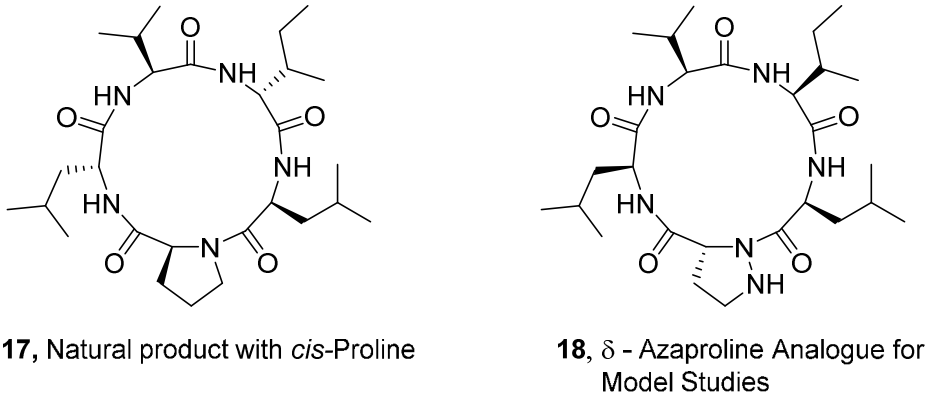
Steep rise in case of **15** (Figure 17a) is the characteristic of a burst phase kinetics denoting the exclusive presence of *trans* conformer in solution. On the other hand a flat line (Figure 17b) in case of **16** indicates no reaction at all due to the exclusive presence of *cis* conformer.



a **b**

Figure 17. (**a**) Chymotrypsin assay of **15**, (**b**) Chymotrypsin assay of **16**.

After a thorough investigation of the *cis-trans* profile of δ-azaproline **1** in amide and tripeptides we decided to investigate the same for a longer linear and a cyclic peptide. Accordingly a proline containing naturally occurring cyclic pentapeptide **17**, isolated from endolichenic *Xylaria* sp.[21] was chosen as a target, the linear precursor will serve the purpose of a linear model and the compound **18** will serve as the cyclic model. The D-amino acids were replaced by their corresponding L isomers and the D isomer of δ-azaproline instead of proline, was used for this study (Figure 18).

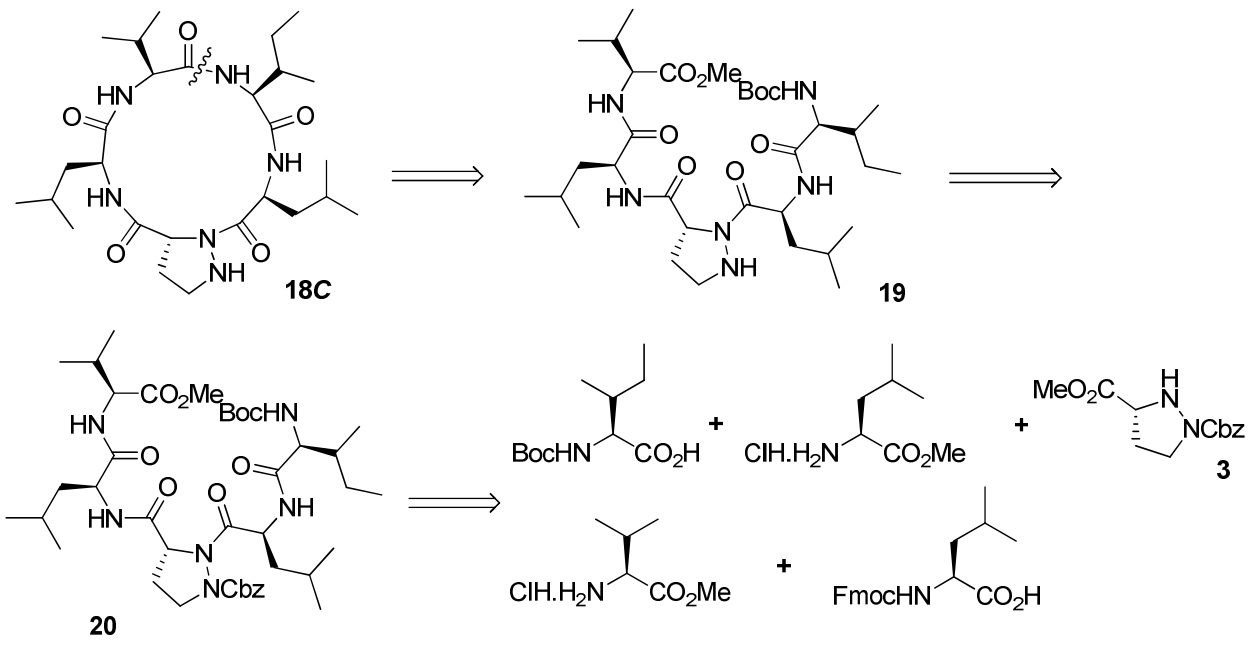


17, Natural product with *cis*-Proline

18, δ - Azaproline Analogue for Model Studies

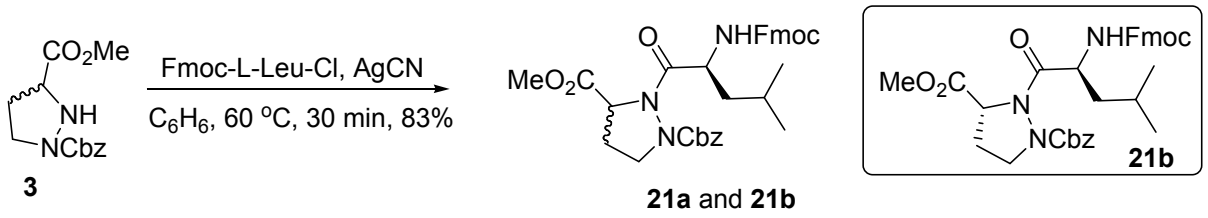
Figure 18. Naturally occurring cyclic peptide **17** and our target **18**.

The retrosynthetic analysis of our target **18** identified Fmoc-L-Leu, Boc-L-Ile, L-Leu-OMe, L-Val-OMe and δ-*N*-Cbz-D-Azaproline-OMe **3** as the required starting materials (Scheme 9).



Scheme 9. Retrosynthetic analysis of target molecule **18*C***.

The forward synthesis commenced from the synthesis of δ-azaproline-containing dipeptide **21** using the previously employed silver cyanide method. The diastereomers hence obtained were separated and the dipeptide δ-*N*-Cbz-D-azaproline-OMe-Fmoc-L-Leu **21b** was used for further reactions (Scheme 10).

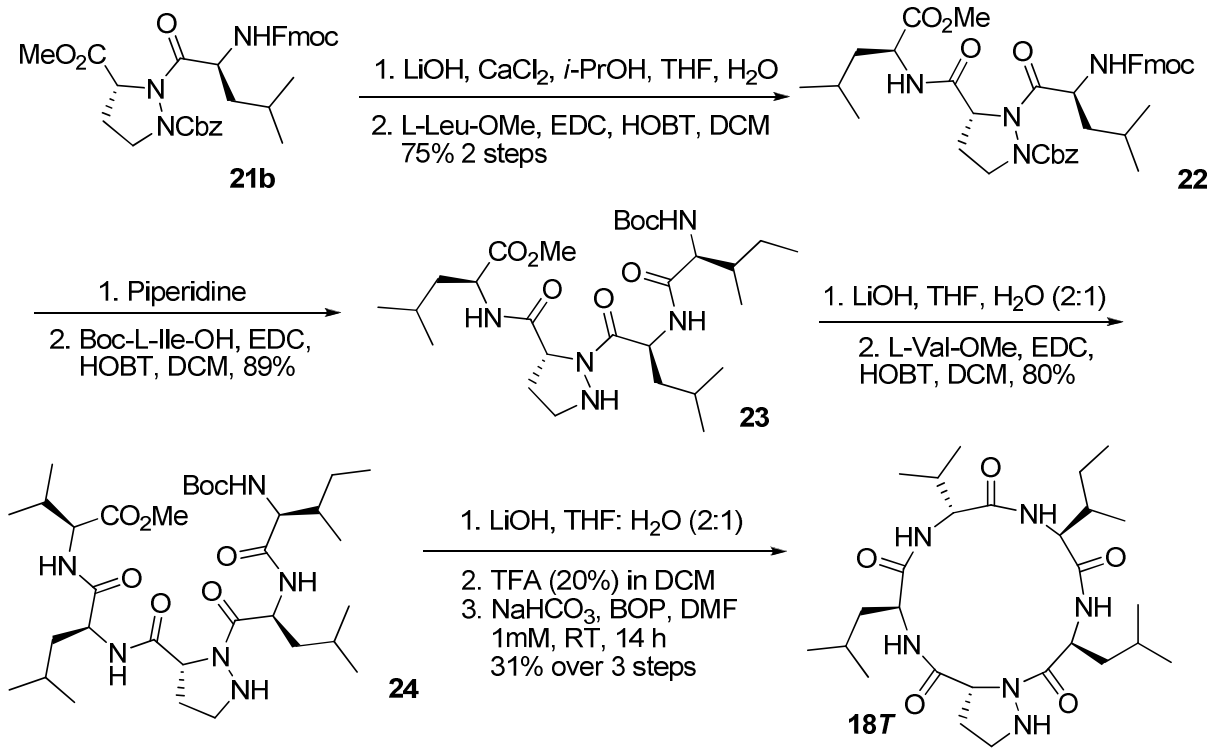


Scheme 10. Synthesis of dipeptide **21**.

Methyl ester deprotection followed by coupling with L-Leu-OMe yielded the tripeptide **22**. The compound **22** on Fmoc deprotection in piperidine followed by another coupling with Boc-L-Ile yielded the *N*-Cbz deprotected tetrapeptide **23**.

At this point we envisaged that due to steric factors the secondary δ-nitrogen would be unable to take part in further peptide coupling and hence proceeded with **23**. The tetrapeptide **23** on ester

hydrolysis followed by coupling with L-Val-OMe yielded the linear pentapeptide **24**. Ester hydrolysis and N-Boc deprotection of **24** yielded the linear precursor which was cyclized using $NaHCO_3$ and BOP in DMF[22] to yield the cyclic peptide **18*T*** (***T*** stands for the *trans* conformer, Scheme 11).



Scheme 11. Synthesis of cyclic peptide **18*T***.

NMR studies of the compound **18*T*** showed epimerization at the valine center which is indicative of slower rate of cyclization which in turn designates the *trans* conformation at the δ-azaproline amide bond. It was confirmed by X-ray studies of **18*T*** (Figure 19).

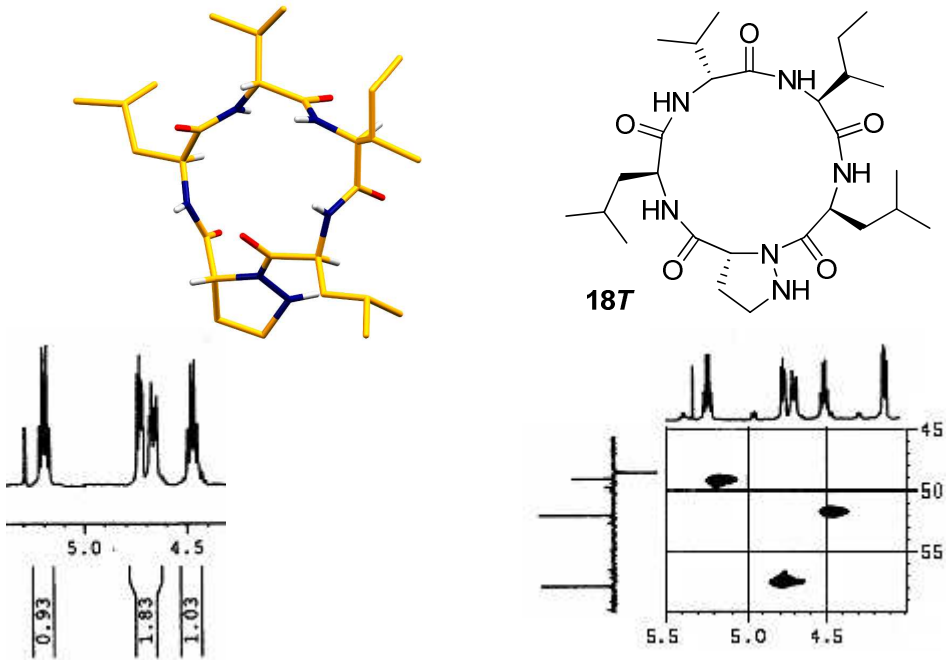
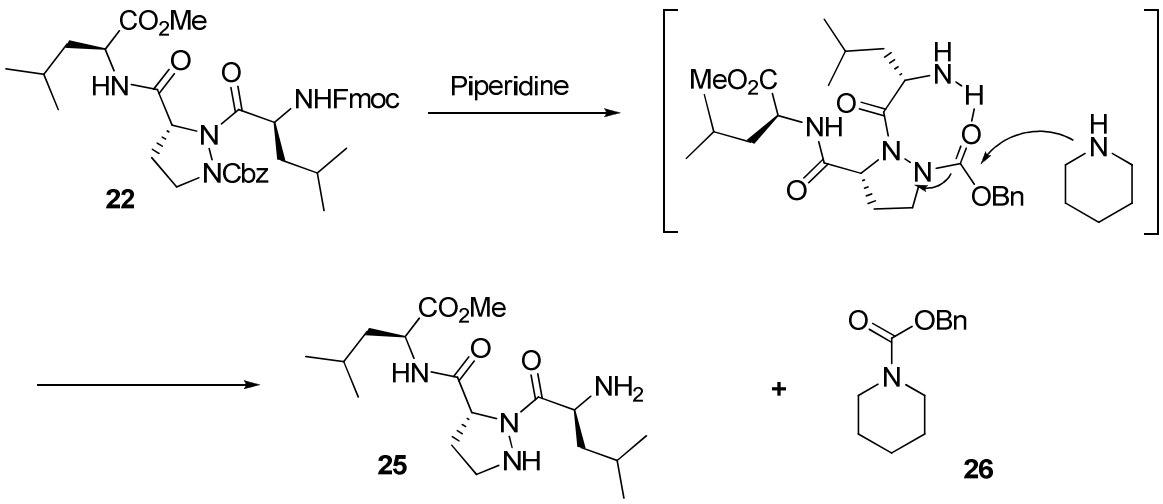


Figure 19. X-Ray structure, ^{1}H NMR and HMQC of epimerized cyclic peptide **18*T***.

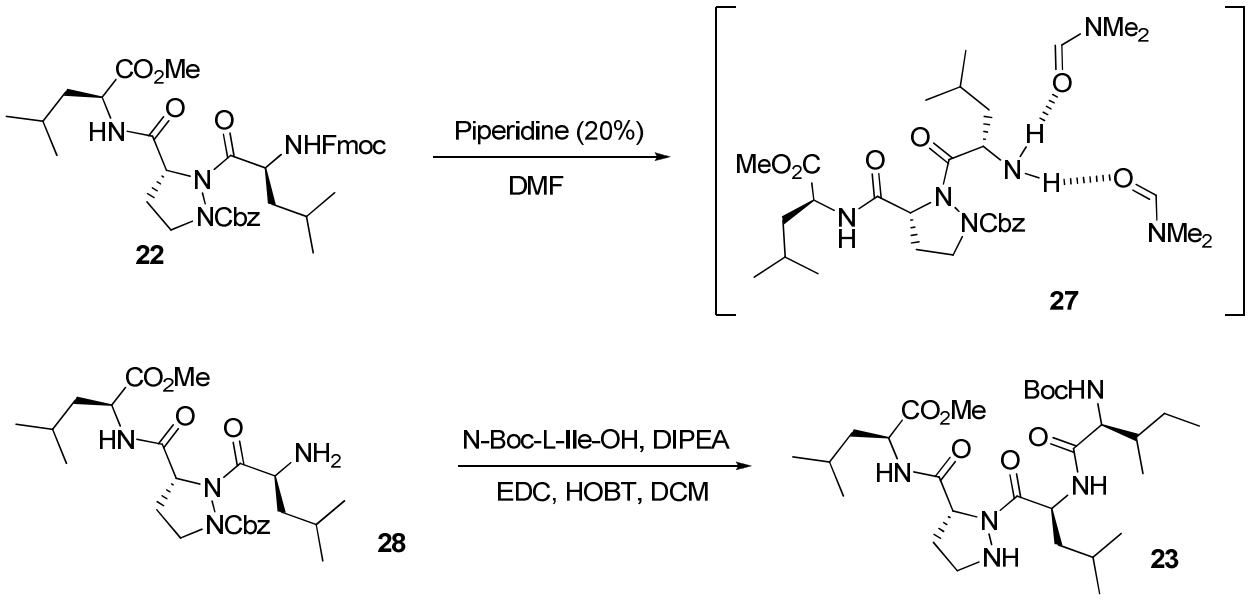
This might appear as a contradiction to our claim and theoretical studies in the previous section but an analysis of the Cbz deprotection (Scheme 12) provided the answer *i.e.* for base mediated deprotection of an otherwise acid labile protecting group, the *trans* conformation is essential and the conversion of *trans* to *cis* becomes restricted once the deprotection takes place. This *trans* conformation is carried forward till the final step and is evident from the NMR and X-Ray studies (Figure 19).



Scheme 12. Mechanism of Cbz deprotection of **22**.

The product **25** and the byproduct **26** were identified using HRMS (Supporting info) substantiating the above proposed mechanism. The H-bonding between the NH and the carbonyl of the Cbz increases the electrophilicity at the carbonyl carbon allowing nucleophilic piperidine to carry out the deprotection.

From the above analysis it was hypothesized that the in situ Cbz deprotection could be arrested by solvating the liable NH_2 group with a polar aprotic solvent like DMF (**27**). Accordingly, Fmoc deprotection was done in DMF using piperidine which yielded the desired Fmoc deprotected product **28**. The product **28** under EDC coupling condition in DCM yielded the Cbz deprotected tetrapeptide **23** with a *trans* conformation (Scheme 13).

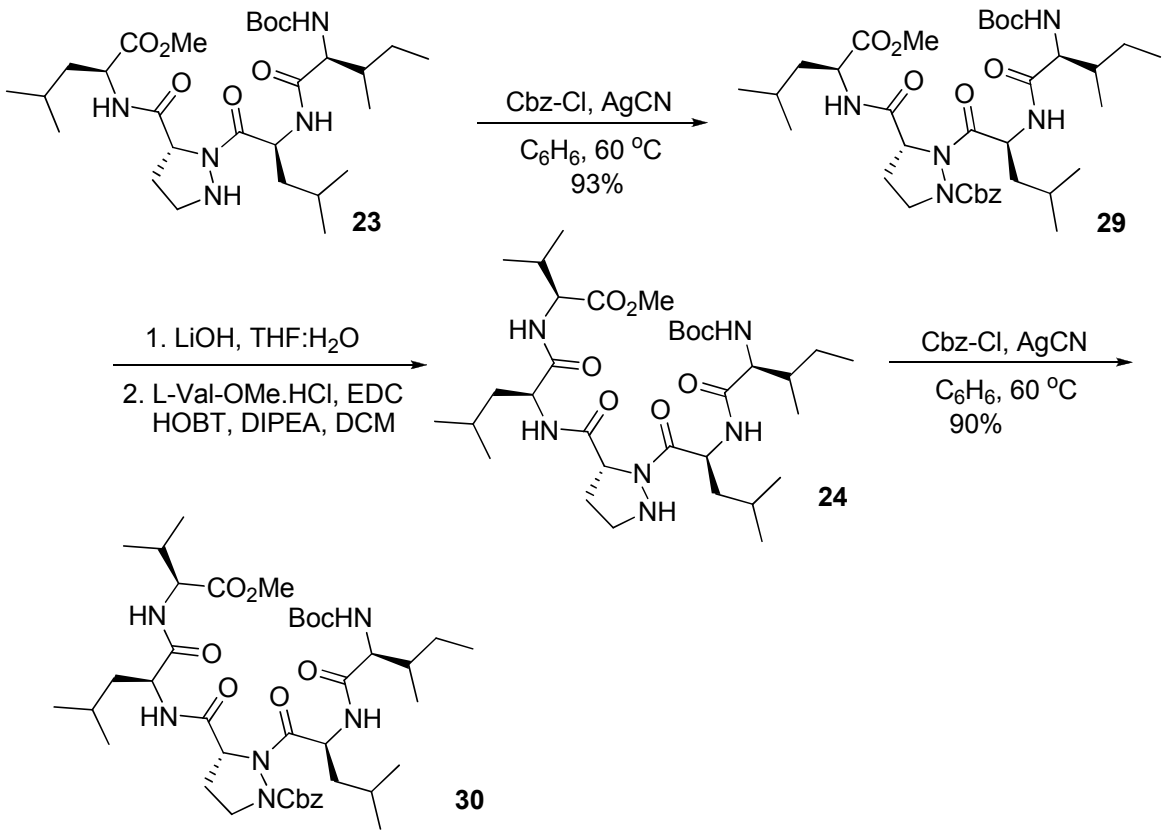


Scheme 13. Addressing *in situ* Cbz deprotection.

The effect of reprotection at the δ-*N* will be noteworthy at this point (since in the previous section and theoretical calculations we observed that reprotection induces isomerization); as a result the deprotected linear tetrapeptide **23** was treated with Cbz-Cl in presence of AgCN. The reaction proceeded with a change in the conformation to yield **29** (Scheme 14). The isolated product was in *cis* conformation (confirmed from 2D NMR studies, SI).

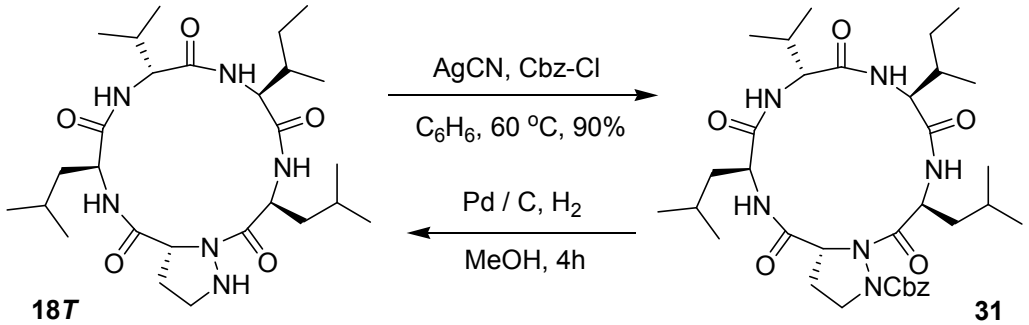
The compound **29** on hydrolysis followed by EDC mediated peptide coupling with L-Val-OMe.HCl proceeded with Cbz deprotection and conformation change to yield the pentapeptide **24** which was indistinguishable to the previously synthesized **24** (Scheme 11) in all aspects.

The pentapeptide **24** on reprotection exhibits similar trend as that of the tetrapeptide **23** on Cbz protection **24** yield **30** (Scheme 14).



Scheme 14. Effect of isomerization after Cbz reprotection of linear peptides.

In order to investigate the effect of reprotection of δ – nitrogen on cyclic peptide **18*T*** with a *trans* conformation was subjected to Cbz protection to yield **31** (Scheme 15).



Scheme 15. Cbz reprotection of **18*T*** and deprotection of **31**

NMR analysis shows an upfield shift of the α-hydrogen to 4.02 ppm from 4.72 indicating a crossover to *cis* conformation (Figure 20).

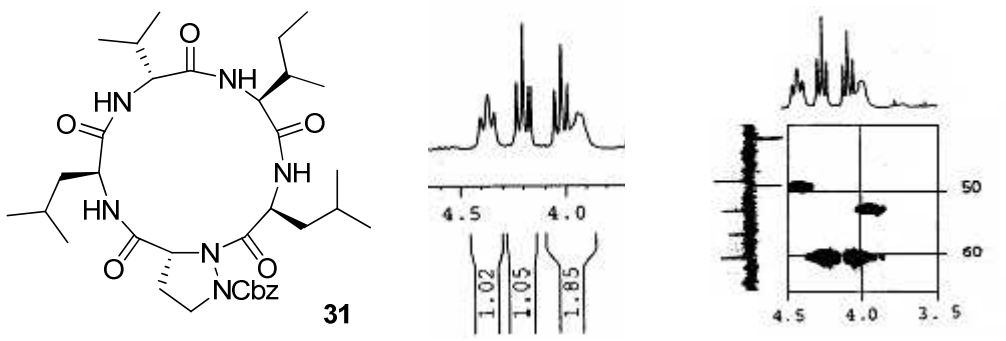


Figure 20. ^{1}H NMR and HMQC of Cbz-protected *cis*-cyclic peptide **31**.

The compound **31** had a *cis* conformation, interestingly removal of the Cbz group from the δ-*N* under hydrogenolysis condition yielded the deprotected cyclic peptide **18*T*** with a *trans* conformation (Scheme 15).

NMR analysis of **18*T*** and tallying it with that of previously synthesized **18*T*** indicated that both the compounds are indeed identical.

CONCLUSION

δ-Azaproline, exclusively adopts a *trans* conformation in simple amides to tripeptides when its δ-*N* is protected with a carbamate group and changes over to *cis* conformation during deprotection which was confirmed by NMR spectroscopy and chymotrypsin-mediated digestion studies. In longer linear and cyclic peptides due to an *in situ* deprotection the behavior reverses but the reluctance of isomerization holds true in all the cases. This behavior of δ-azaproline will be beneficial in case of solid phase peptide synthesis where its deprotected from in longer peptides will stabilize the *trans* conformation thereby minimizing aggregate formation. Theoretical studies give a clear insight into the mechanism of isomerization during protection or deprotection. The behavior of unprotected δ-azaproline, in case of tripeptides remains the same even if the δ-*N* is protonated, which was evident from the NMR studies of **16** which despite being an hydrobromide salt, adopted a *cis* conformation. Interesting observation is the chemical

shift values of α - H of δ-azaproline, that is upfield for *cis* - conformer than the *trans* - conformer, *cis – trans* conformers of proline also exhibit similar trend.[9] During the course of our work, a related work has been published by Jamart - Grégoire *et. al.* where they tried to prove similar preference of δ-azaproline in simple tripeptides with the help of IR and NMR studies.[23] For the first time, δ-azaproline has also been used for the synthesis of medicinally important diketopiperazines, which are employing both the "*N*"s. Among several substituted prolines, δ-azaproline is unique because of its ability to stabilize one conformer exclusively and the δ - *N* provides a much required site for the spatio-temporal control of bioactive peptides *e.g.* prolyloligopeptidase inhibitors with a suitable protecting group or a photo labile group. Such applications of δ-azaproline with a photo labile protecting group in biological systems are ongoing and will be reported shortly. Effects of non carbamate protecting groups on the *cis-trans* profile of δ – azaproline are also being investigated.

EXPERIMENTAL SECTION

General Methods.

All the reactions were done in argon atmosphere with dry solvents and reagents unless otherwise mentioned. Reagents purchased from commercial sources were used directly except DCM, DMF (dried from CaH_2), THF, benzene, toluene (dried over sodium). Column chromatography was done on a 230 – 400 mesh silica gel. Thin-layer chromatography (TLC) was carried out on aluminum sheets, Silica Gel 60 F254 (layer thickness 0.25 mm). Visualization of the developed chromatogram was performed by ceric ammonium molybdate (CAM) or $KMnO_4$ stains. ^1H and 2-D spectra were recorded at 300, 400 or 500 MHz and ^{13}C NMR on 75, 100 or 125 MHz, using $CDCl_3$, CD_3OD or D_2O as solvent. Chemical shifts (δ) are given in parts per million. The following abbreviations are used for multiplicity of NMR signals: s = singlet, d = doublet, t =

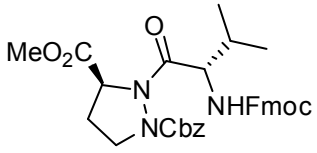
triplet, m = multiplet, br = broad. High resolution mass spectra (HRMS) were measured in a QTOF I (quadrupole-hexapole-TOF) mass spectrometer. Kinetics was measured by a diode array spectrophotometer with a thermostated cuvette holder.

1-Benzyl 3-methyl 2-(2-(((9H-fluoren-9-yl) methoxy) carbonylamino)-3-methylbutanoyl) pyrazolidine-1, 3-dicarboxylate, (4).

Fmoc protected L-Valine (0.689 g, 2.03 mmol) was stirred with oxalyl chloride (0.72 ml, 8.4 mmol) in DCM at rt. for 4 h. The deep yellow (to red) solution was then freed from DCM and excess oxalyl chloride *in vacuo*.

The acid chloride generated above was redissolved in dry benzene and added to a benzene solution of **3** (0.447 g, 1.7 mmol). To the above mixture AgCN (0.453 g, 3.38 mmol) was added and refluxed at 60 ^{o}C for 30 min. On completion the reaction (TLC) benzene was removed, the mixture was extracted with EtOAc, filtered through a sintered funnel. The organic layer was then washed with satd. $NaHCO_3$ followed by water and brine. Removal of the organic layer yielded the crude diasteriomeric mixture which was separated by a flash column chromatography (25% ethyl acetate in petroleum ether). Overall yield 83% (0.826 g, 1.41 mmol, mixture 0.099 g).

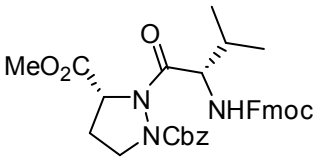
(*S*)-1-Benzyl-3-methyl-2-((*R*)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-methylbutanoyl)pyrazolidine-1,3-dicarboxylate, (4a).



Yield, 35%, 0.35 g, 0.6 mmol. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (d, 2H, J = 7.5 Hz), 7.57 (d, 2H, J = 7.5 Hz), 7.24 – 7.39 (m, 9H), 5.59 (d, 0.23H, J = 9 Hz), 5.05 – 5.30 (m, 4H), 4.62 – 4.73 (m, 0.77H), 4.17 – 4.41 (m, 3H), 3.74 (s, 0.86H), 3.61 (s, 2.13H), 3.35 – 3.41 (m, 0.35H), 3.04 – 3.16 (m, 0.65H), 2.12 – 2.38 (m, 4H), 0.82 – 1.02 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.3, 170.8, 170.5,

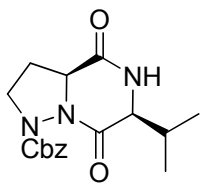
158.7, 158.1, 156.3, 156.0, 143.8, 141.3, 135.2, 135.1, 128.6, 128.5, 127.9, 127.7, 127.3, 127.0, 125.1, 120.0, 69.5, 68.9, 66.9, 57.6, 57.4, 57.2, 56.8, 55.5, 52.5, 48.5, 48.1, 47.1, 31.4, 31.1, 29.7, 29.0, 19.9, 19.5, 16.7, 16.3. HRMS (ESI) $(M + Na)^+$ calculated for $C_{33}H_{35}N_3O_7Na^+$ = 608.2373, found 608.2371.

(*R*)-1-Benzyl-3-methyl-2-((*R*)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-methylbutanoyl)pyrazolidine-1,3-dicarboxylate, (4b). Yield, 37%, 0.38 g, 0.64 mmol. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74 (d, 2H, J = 7.2 Hz), 7.54 (d, 2H, J = 7.5 Hz), 7.14 – 7.40 (m, 9H), 5.42 (br d, 1H, J = 8.1 Hz), 5.29 (d, 1H, J = 12 Hz), 5.09 (d, 1H, J = 12.3 Hz), 4.25 – 4.31 (m, 2H), 4.76 – 4.86 (m, 2H), 3.51 (s, 3H), 3.08 – 3.16 (br m, 1H), 2.26 – 2.41 (m, 2H), 1.84 – 1.95 (m, 1H), 0.87 (dd, 6H, J = 13.8, 6.9 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.7, 170.3, 157.9, 155.8, 143.9, 143.8, 141.2, 141.1, 135.3, 128.3, 128.0, 127.6, 127.0, 125.1, 125.0, 119.9, 68.9, 66.8, 57.2, 56.4, 52.4, 47.4, 47.0, 31.5, 29.7, 19.2, 17.3. HRMS (ESI) $(M + Na)^+$ calculated for $C_{33}H_{35}N_3O_7Na^+$ = 608.2373, found 608.2371.



(6)-Benzyl-6-isopropyl-4,7-dioxohexahydropyrazolo[1,5-*a*]pyrazine-1(2*H*)-carboxylate(5).

To a solution of **4** (mixture of diasterioisomer, 0.099 g, 0.17 mmol) in DMF (2.4 ml), piperidine (0.6 ml) was added, the mixture was stirred for overnight. The reaction mixture was then heated at 50 ^{o}C for 30 min following which the DMF was removed *in vacuo*. The crude reaction mixture was then purified in a flash column chromatography (50% ethyl acetate in petroleum ether) to yield the two diasterioisomers. Overall yield 88% (0.049 g, 0.15 mmol).



(3a*S*,6*S*)-Benzyl-6-isopropyl-4,7-dioxohexahydropyrazolo[1,5-a]pyrazine-1(2H)-carboxylate, (5a). Yield, 53%, 0.03 g, 0.09 mmol, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.37 (m, 5H), 5.95 (br s, 1H), 5.23 (d, 2H, J = 12.4

Hz), 4.26 (td, 1H, *J* = 8.4, 0.8 Hz), 4.12 (t, 1H, *J* = 1.2 Hz), 3.93 (qt, 1H, *J* = 8.4, 2.4 Hz), 3.44 – 3.51 (m, 1H), 2.60 – 2.67 (m, 2H), 2.26 – 2.34 (m, 1H), 1.07 (d, 3H, *J* = 7.2 Hz), 0.91 (d, 3H, *J* = 6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.9, 162.9, 156.1, 135.7, 128.8, 128.5, 128.2, 68.8, 59.5, 58.9, 45.8, 30.9, 29.4, 19.1, 16.2. HRMS (ESI) $(M + Na)^+$ calculated for $C_{17}H_{21}N_3O_4Na^+$ = 354.1430, found 354.1433.

(3a*R*,6*S*)-Benzyl-6-isopropyl-4,7-dioxohexahydropyrazolo[1,5-a]pyrazine-1(2H)-carboxylate, (5b). Yield, 33%, 0.02 g, 0.057 mmol, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.36 (m, 5H), 6.54 (d, 1H, *J* = 2.4 Hz), 5.21 (d, 1H, *J* = 12Hz), 5.16 (d, 1H, *J* = 12 Hz), 4.23 (t, 1H, *J* = 8.8 Hz), 3.94 (qt, 1H, *J* = 8.8, 2 Hz), 3.79 (t, 1H, *J* = 4.8 Hz), 3.47 (td, 1H, *J* = 10, 5.2 Hz), 2.61 – 2.70 (m, 1H), 2.29 – 2.38 (m, 1H), 2.15 – 2.19 (m, 1H), 0.98 (d, 3H, *J* = 7.2 Hz), 0.94 (d, 3H, *J* = 6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.0, 162.5, 156.2, 135.4, 128.7, 128.6, 128.5, 69.0, 62.55, 58.3, 45.2, 34.0, 31.0, 18.6, 17.1. HRMS (ESI) $(M + Na)^+$ calculated for $C_{17}H_{21}N_3O_4Na^+$ = 354.1430, found 354.1428.

(*S*)-Benzyl-2-((*S*)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-methylbutanoyl)-3-((*S*)-1-methoxy-1-oxo-3-phenylpropan-2-ylcarbamoyl)pyrazolidine-1-carboxylate, (6). The methyl ester **4a** (0.152 g, 0.26 mmol) was dissolved in isopropanol (4.5 ml) and tetrahydrofuran (2 ml). $CaCl_2$ was added (0.461 g, 4.15 mmol). Separately, $LiOH.H_2O$ (0.042 g, 1.03 mmol) was dissolved in H_2O (2 ml). The aqueous solution was then added to the reaction mixture and stirred the cloudy white solution for 45 min. The organic solvents were removed under reduced pressure, and the resulting residue was taken up in 10% potassium carbonate (K_2CO_3) (15 ml) as a cloudy white suspension. The aqueous layer was partitioned in diethyl ether (Et_2O) (4 × 3 ml) to remove the Fmoc deprotection side products (if any), after which it

was acidified to pH 2 with concentrated HCl. It was then extracted with EtOAc (30 ml). The organic layers were dried over Na_2SO_4, and concentrated to a white foamy solid **13**.

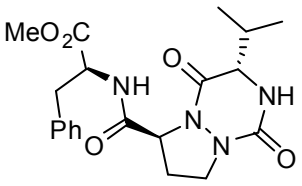
Diisopropylethylamine (45.28 µl, 0.26 mmol) was added dropwise to a stirred suspension of L-phenylalanine methyl ester hydrochloride (0.084 g, 0.39 mmol) in dichloromethane (20 ml) at room temperature under an atmosphere of nitrogen. On dissolution, the solution was cooled to 0 ^oC then **13** (crude from above reaction) and 1-hydroxybenzotriazole (0.53 g, 0.39 mmol) were added successively, each in one portion. The suspension was stirred at 0 ^oC for further 15 min and then EDC.HCl (0.075 g, 0.39 mmol) was added in one portion. The mixture was allowed to warm to room temperature over the course of 12 hr, the mixture was evaporated *in vacuo*.

The residue was taken up in ethyl acetate (40 ml), and washed with saturated aqueous sodium bicarbonate solution and brine. The combined organic extracts were dried and evaporated *in vacuo* to leave the crude product which was purified by chromatography (50% EtOAc in petroleum ether) to give the tripeptide **6** in 65% yield (0.127 g, 0.17 mmol) as a light yellow solid.

^1H NMR (500 MHz, $CDCl_3$) δ 7.45 (d, 2H, J = 7.5 Hz), 7.56 – 7.60 (m, 3H), 7.08 – 7.40 (m, 13H), 7.06 (d, 2H, J = 10 Hz), 5.20 (d, 1H, J = 9 Hz), 5.13 (d, 1H, J = 12 Hz), 4.98 (br s, 1H), 4.78 – 4.81 (m, 2H), 4.59 (dd, 1H, J = 9, 4 Hz), 4.37 (dd, 1H, J = 10.5, 7 Hz), 4.29 – 4.32 (m, 1H), 4.15 – 4.20 (m, 2H), 3.17 (s, 3H), 3.07 – 3.17 (m, 2H), 2.93 (dd, 1H, J = 14.5, 7 Hz), 2.38 – 2.44 (m, 1H), 2.15 – 2.17 (m, 1H), 1.88 – 1.96 (m, 1H), 0.84 – 0.89 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.3, 171.5, 169.4, 158.8, 156.4, 143.9, 141.5, 136.3, 135.0, 129.4, 129.0, 128.9, 128.7, 128.5, 127.9, 127.2, 127.1, 125.2, 120.1, 69.3, 67.1, 60.6, 55.9, 53.3, 52.4, 49.7, 47.3, 38.1, 30.2, 30.0, 19.9, 16.5. HRMS (ESI) $(M + Na)^+$ calculated for $C_{42}H_{44}N_4O_8Na^+$ = 755.3057 found 755.3054.

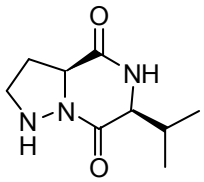
(*S*)-Methyl 2-((3*S*,6*S*)-3-isopropyl-1,4-dioxohexahydro-1H-pyrazolo[1,2-a][1,2,4]triazine-6-carboxamido)-3-phenylpropanoate, (7).

Compound **6** (0.019 g, 0.026 mmol) was dissolved in 1.6 ml DMF and 0.4 ml piperidine. The mixture was stirred overnight followed by heating at 50 ^{o}C for 30 min. After the completion of the reaction (TLC, 5% MeOH in DCM) excess DMF and piperidine were removed in *vacuo*. The compound was purified by column chromatography to yield **7** in 81% yield (8.4 mg, 0.021 mmol).



^{1}H NMR (500 MHz, $CDCl_3$) δ 7.50 (d, 1H, J = 7.5 Hz), 7.20 – 7.30 (m, 2H), 7.08 (d, 2H, J = 7 Hz), 5.14 (br s, 1H), 4.81 – 4.86 (m, 2H), 3.91 (t, 1H, J = 8.5 Hz), 3.76 (s, 3H), 3.61 (dd, 1H, J = 5, 2.5 Hz), 3.38 – 3.44 (m, 1H), 3.23 (dd, 1H, J = 14, 5 Hz), 2.97 (dd, 1H, J = 14, 7.5 Hz), 2.71 (dd, 1H, J = 13, 5.5 Hz), 2.22 – 2.27 (m, 1H), 2.09 – 2.17 (m, 1H), 1.21 (t, 1H, J = 7 Hz), 1.03 (d, 3H, J = 7 Hz), 0.97 (d, 3H, J = 11.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.6, 167.4, 161.9, 154.5, 136.0, 129.3, 128.7, 127.2, 61.4, 58.9, 53.7, 52.6, 45.2, 38.0, 30.5, 25.4, 19.0, 17.6. HRMS (ESI) $(M + Na)^+$ calculated for $C_{20}H_{26}N_4O_5Na^+$ = 425.1801, found 425.1803.

(3a*S*,6*S*)-6-Isopropylhexahydropyrazolo[1,5-a]pyrazine-4,7-dione, (8a).

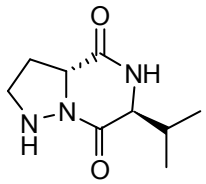


To a solution of **5a** (5 mg, 0.015 mmol) in MeOH (1 ml), 10% Pd/C (3 mg) was added, the mixture was stirred for 4 h in hydrogen atmosphere. The reaction mixture was filtered through celite followed by MeOH removal *in vacuo*. The crude reaction mixture was then purified by flash column chromatography (5% MeOH in DCM) to yield **8a** in quantitative yield (3 mg, 0.015 mmol).

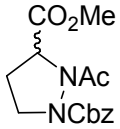
^{1}H NMR (300 MHz, $CDCl_3$) δ 5.81 (br s, 1H), 4.83 (br s, 1H), 4.27 (td, 1H, J = 8.4, 1.2 Hz), 4.01 (t, 1H, J = 2.1 Hz), 3.25 – 3.33 (m, 1H), 3.10 – 3.18 (m, 1H), 2.61 – 2.73 (m, 1H), 2.48 – 2.58

(m, 2H), 1.09 (d, 3H, J = 7.2 Hz), 0.94 (d, 3H, J = 6.9 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.4, 161.2, 150.1, 129.9, 59.8, 58.1, 45.3, 30.4, 28.6, 19.4, 16.1. HRMS (ESI) $(M + Na)^+$ calculated for $C_9H_{15}N_3O_2Na^+$ = 220.1062, found 220.1059.

(3a*R*,6*S*)-6-Isopropylhexahydropyrazolo[1,5-a]pyrazine-4,7-dione, (8b). Yield, 83%, 5 mg, 0.025 mmol from 9 mg, 0.03 mmol of **5b**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.42 – 6.44 (m, 1H), 5.30 (br s, 1H), 4.28 (t, 1H, J = 8.4 Hz), 3.77 (t, 1H, J = 4.8), 3.21 – 3.30 (m, 1H), 3.07 – 3.16 (m, 1H), 2.54 – 2.63 (m, 1H), 2.38 – 2.53 (m, 1H), 2.18 – 2.27 (m, 1H), 1.08 (d, 3H, J = 6 Hz), 1.03 (d, 3H, 5.7). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.1, 161.4, 64.5, 63.0, 62.9, 57.6, 44.7, 33.2, 31.1, 29.7, 19.2, 19.1, 17.9, 17.6. HRMS (ESI) $(M + Na)^+$ calculated for $C_9H_{15}N_3O_2Na^+$ = 220.1062, found 220.1060.



1-Benzyl 3-methyl 2-acetylpyrazolidine-1, 3-dicarboxylate (9).

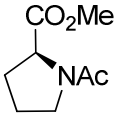


To a solution of **3** (0.057 g, 0.21 mmol) in benzene (3 ml), acetyl chloride (33 µl, 0.46 mmol) and AgCN (43 mg, 0.34 mmol) were added. The mixture was then heated at 60 ^{o}C for 30 min followed by removal of benzene *in vacuo*. The residue was taken up in EtOAc (10 ml) and filtered through celite (to remove AgCl ppt.). The filtrate was then washed with satd. $NaHCO_3$ and brine. Organic extract was dried and evaporated *in vacuo* to leave the crude product which was purified by chromatography (30% EtOAc in petroleum ether) to give **9** as colorless liquid in quantitative yield (0.064 g, 0.21 mmol) which slowly formed white solid on cooling.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 – 7.38 (m, 5H), 5.21 (dd, 2H, J = 21.6, 12 Hz), 5.00 (dd, 1H, J = 8.7, 5.7 Hz), 4.22 – 4.30 (m, 1H), 3.61 (s, 3H), 3.12 – 3.24 (m, 1H), 2.22 – 2.44 (m, 2H), 2.12 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3, 171.0, 157.9, 135.4, 128.6, 128.4, 128.1, 68.7,

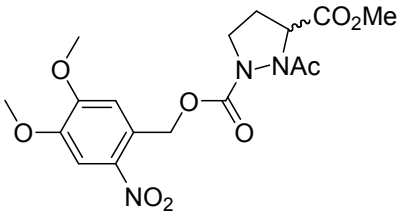
56.7, 52.4, 47.2, 29.8, 20.7. HRMS (ESI) $(M + Na)^+$ calculated for $C_{15}H_{18}N_2O_5Na^+$ = 329.1113, found 329.1114.

_N_-Acetyl-L-proline methyl ester[9]



^{1}H NMR (300 MHz, $CDCl_3$) δ 4.33 (dd, J = 8.7, 3.6 Hz, 0.78H), 4.27 (dd, J = 8.6, 2.8 Hz, 0.22H), 3.63 (s, 0.6H), 3.58 (s, 2.4H), 3.48 – 3.56 (m, 1H), 3.33 – 3.42 (m, 1H), 1.78 – 2.23 (m, 7H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 172.4, 169.4, 169.2, 59.9, 58.3, 52.4, 51.9, 47.6, 46.1, 31.2, 29.2, 24.6, 22.6, 22.0.

1-(4, 5-Dimethoxy-2-nitrobenzyl) 3-methyl 2-acetylpyrazolidine-1, 3-dicarboxylate (11).



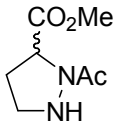
A solution of **2** (0.035 g, 0.1 mmol) in DCM (1.6 ml) was cooled to 0 ^{o}C and to it TFA (0.4 ml) was added. The reaction mixture was allowed to stir at the same temperature for 4 h. Removal of the organic solvent yielded the *N*-Boc deprotected compound.

DCM (1 ml) solution of 4,5-dimethoxy-2-nitrobenzyl alcohol (0.022 g, 0.1 mmol) was added to a solution of triphosgene (16 mg, 0.053 mmol) and aliquot 336 (catalytic) in DCM at 0 ^{o}C and stirred for 12 hr at r.t. Removal of the organic solvent yielded the crude chloroformate. The residue was taken up in DCM (5 ml) and added a solution of *N*-Boc deprotected compound in DCM (2 ml). The reaction mixture was then cooled to -20 ^{o}C (ice salt mixture) followed by addition of Et_3N (60 µl, 0.4 mmol). It was then allowed to stir at the same temperature for 30 min. Upon completion (TLC, 15% EtOAc in DCM) the reaction was quenched with satd. NH_4Cl solution (0.1 ml). DCM was removed *in vacuo* from the reaction mixture followed by extraction with EtOAc (10 ml). The EtOAc layer was washed with satd. $NaHCO_3$ solution and brine. Organic extract was dried and evaporated *in vacuo* to leave the crude product **10** (0.046 g).

The crude compound **10** was dissolved in dry benzene (5 ml), to it acetyl chloride (15 µl, 0.21 mmol) and AgCN (0.021 g, 0.16 mmol) were added. The mixture was heated at 60 ^{o}C for 30 min followed by the removal of benzene. The residue was taken up in EtOAc (10 ml) and filtered through celite (to remove AgCl ppt.). The filtrate was then washed with satd. $NaHCO_3$ and brine. Organic extract was dried and evaporated *in vacuo* to leave the crude product which was purified by chromatography (50% EtOAc in petroleum ether) to give **11** as yellow liquid in quantitative yield (0.043 g, 0.1 mmol).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69 (s, 1H), 7.25 (s, 1H), 5.54 – 5.65 (m, 2H), 4.95 (t, 1H, J = 7 Hz), 4.28 (t, 1H, J = 9 Hz), 3.92 (s, 6H), 3.62 (s, 3H), 3.19 (q, 1H, J = 11 Hz), 2.44 – 2.46 (m, 1H), 2.21 – 2.26 (m, 1H), 2.15 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.1, 171.0, 157.5, 154.1, 148.2, 139.3, 127.0, 109.9, 108.1, 65.9, 57.1, 56.7, 56.4, 52.5, 47.3, 30.2, 20.7, 12.2. HRMS (ESI) $(M + Na)^+$ calculated for $C_{17}H_{21}N_3O_9Na^+$ = 434.1175, found 434.1176.

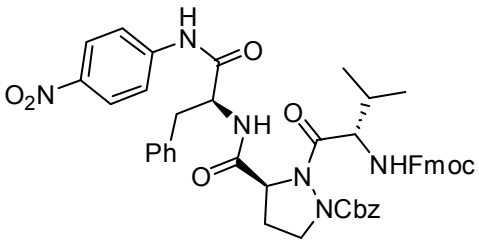
Methyl 2-acetylpyrazolidine-3-carboxylate (12).



To a solution of **9** (0.052 g, 0.17 mmol) in MeOH (3 ml) 10 % Pd/C (6 mg) was added. The reaction mixture was stirred in hydrogen atmosphere at rt. for 4h. Upon completion (TLC, 50% EtOAc in petroleum ether) the mixture was filtered through celite. Organic extract was dried and evaporated *in vacuo* to yield the pure product **12** as colorless liquid in quantitative yield (0.029 g, 0.17 mmol).

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.66 (t, 1H, J = 8.5 Hz), 4.33 (dd, 1H, J = 12, 5.5 Hz), 3.71 (s, 3H), 3.20 – 3.25 (m, 1H), 2.80 – 2.88 (m, 1H), 2.47 – 2.51 (m, 1H), 2.17 (s, 3H), 1.98 – 2.06 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.9, 171.3, 57.1, 52.5, 48.0, 33.5, 21.4. HRMS (ESI) $(M + Na)^+$ calculated for $C_7H_{12}N_2O_3Na^+$ = 195.0746, found 195.0740.

(*S*)-Benzyl-2-((*S*)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-methylbutanoyl)-3-((*S*)-1-(4-nitrophenylamino)-1-oxo-3-phenylpropan-2-ylcarbamoyl)pyrazolidine-1-carboxylate, (14).

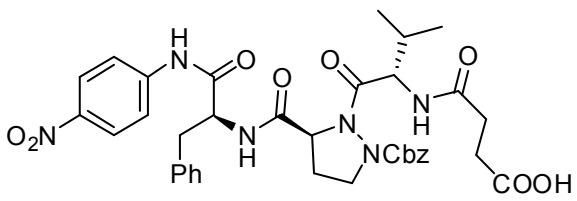


A solution of **13** (0.11 g, 0.19 mmol) and oxalyl chloride (83 µl, 0.97 mmol) in DCM (3 ml) was stirred at rt. for 4h during which it turned deep yellow (indicating completion of reaction). The excess DCM and oxalyl chloride were removed and the residue was taken up in dry benzene. To it AgCN (0.04 g, 0.29 mmol) and L-phenylalanine 4-nitroanilide (0.061 g, 0.21 mmol) were added. The mixture was heated at 60 ^{o}C for 30 min. Upon completion (TLC, 50% EtOAc in petroleum ether) the excess benzene was removed. The residue was taken up in EtOAc (15 ml) and filtered through a celite bed. The filtrate was washed with satd. $NaHCO_3$ followed by 0.2N HCl solution and brine. Organic extract was dried and evaporated *in vacuo*. Column chromatography yielded the pure product **14** in 61% yield (0.097 g, 0.116 mmol) as a yellow solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.54 – 8.56 (m, 2H), 8.10 – 8.15 (m, 1H), 7.96 (d, 1H, J = 10 Hz), 7.75 (d, 2H, J = 9.5 Hz), 7.64 (d, 1H, J = 10 Hz), 7.58 (d, 2H, J = 9.5 Hz), 7.48 (t, 1H, J = 10 Hz), 7.39 (s, 6H), 7.22 – 7.32 (m, 6H), 5.16 – 5.29 (m, 3H), 4.91 (q, 1H, J = 6 Hz), 4.64 – 4.69 (m, 2H), 4.54 (t, 1H, J = 10 Hz), 4.31 – 4.41 (m, 2H), 4.12 – 4.21 (m, 2H), 3.44 (dd, 1H, J = 18, 5 Hz), 3.15 – 3.30 (m, 1H), 2.93 – 2.99 (m, 1H), 2.45 – 2.55 (m, 1H), 2.08 – 2.13 (m, 2H), 1.60 (br s, 1H), 0.82 – 0.90 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.2, 170.0, 169.4, 160.3, 156.5, 148.7, 143.9, 143.8, 141.5, 139.1, 136.7, 134.6, 129.9, 129.8, 129.3, 129.0, 128.6, 128.5, 127.9, 127.2, 127.1, 125.7, 125.3, 125.2, 120.2, 119.0, 115.1, 70.1, 67.3, 62.9, 61.7, 56.3, 54.7, 50.8,

47.3, 37.1, 32.1, 30.5, 20.0, 16.9. HRMS (ESI) $(M + Na)^+$ calculated for $C_{47}H_{46}N_6O_9\ Na^+$ = 861.3218, found 861.3220.

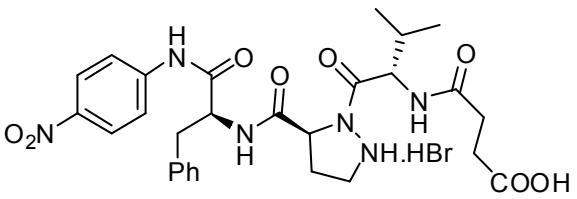
4-((*S*)-1-((*S*)-2-(Benzyloxycarbonyl)-5-((*S*)-1-(4-nitrophenylamino)-1-oxo-3-phenylpropan-2-ylcarbamoyl)pyrazolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-4-oxobutanoic acid, (15).



To a solution of **14** (0.058 g, 0.069 mmol) in DMF (1.6 ml) piperidine (0.4 ml) was added. The mixture was allowed to stir for 12 hr at rt. Upon completion (TLC, 5% MeOH in DCM) the excess DMF was removed *in vacuo*. The residue was taken up in AcOH (0.7 ml) and succinic anhydride (0.014 g, 0.14 mmol) was added. The mixture was heated at 60 ^oC for 6h. Excess AcOH was removed, the residue was washed with 10% EtOAc in petroleum ether (4 × 3 ml, to remove the Fmoc deprotection byproduct). Column chromatography purification yielded the pure product **15** in 58% (0.028 g, 0.04 mmol) yield as a foamy yellow solid.

^1H NMR (500 MHz, $CDCl_3$) δ 8.67 (br s, 1H), 8.43 – 8.45 (m, 2H), 7.84 – 8.10 (m, 5H), 7.16 – 7.45 (m, 7H), 5.05 – 5.17 (m, 2H), 4.68 – 4.89 (m, 2H), 4.29 (br s, 1H), 3.99 – 4.19 (m, 1H), 3.30 – 3.35 (m, 2H), 2.88 – 2.95 (m, 2H), 2.31 – 2.73 (m, 3H), 1.96 – 2.23 (m, 2H), 1.86 (br s, 1H), 0.68 – 1.04 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.3, 170.6, 169.7, 168.7, 163.0, 156.5, 148.6, 139.0, 136.5, 129.9, 129.7, 129.3, 129.2, 129.1, 128.9, 128.7, 128.6, 128.3, 127.1, 125.8, 119.0, 115.0, 67.4, 60.2, 56.5, 55.3, 49.4, 37.1, 30.6, 30.6, 29.7, 29.0, 28.2, 20.0, 17.2. HRMS (ESI) $(M + Na)^+$ calculated for $C_{36}H_{40}N_6O_{10}\ Na^+$ = 739.2704, found 739.2747.

4-((*S*)-3-Methyl-1-((*S*)-5-((*S*)-1-(4-nitrophenylamino)-1-oxo-3-phenylpropan-2-ylcarbamoyl)pyrazolidin-1-yl)-1-oxobutan-2-ylamino)-4-oxobutanoic acid hydrobromide salt (16).



To a cooled solution (0 °C) of **15** (0.017 g, 0.024 mmol) in AcOH (0.4 ml) 33% HBr in AcOH (0.6 ml) was added. The mixture was allowed to stir at the same temperature for 1h. Upon completion of the reaction (TLC, 10% MeOH in DCM) excess AcOH and HBr were removed *in vacuo*. The residue was washed with hexane (5 × 1 ml) to remove the benzyl bromide (byproduct of Cbz deprotection). Drying the mixture yielded the pure product in 71% (0.011 g, 0.017 mmol) yield as a foamy white solid.

^{1}H NMR (500 MHz, CD_3OD) δ 8.18 (t, 1H, J = 2.8 Hz), 7.90 (ddd, 1H, J = 11, 3.2, 1.2 Hz), 7.52 – 7.56 (m, 1H), 7.44 (t, 1H, J = 10.8 Hz), 7.11 – 7.23 (m, 5H), 4.82 (d, 1H, J = 9.2 Hz), 4.51 (t, 1H, J = 10.8 Hz), 4.43 (t, 1H, J = 10.8 Hz), 2.97 – 3.14 (m, 3H), 2.74 – 2.83 (m, 1H), 2.39 – 2.52 (m, 3H), 1.82 – 2.02 (m, 2H), 1.14 – 1.20 (m, 2H), 0.81 (t, 6H, J = 8.8 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 175.5, 173.8, 173.7, 172.3, 149.1, 139.1, 137.0, 131.0, 129.6, 128.2, 128.0, 120.6, 116.4, 60.4, 57.3, 57.1, 38.3, 33.4, 31.4, 31.2, 30.9, 19.4, 19.2, 18.3 (1Peak merged with solvent). HRMS (ESI) $(M + Na)^+$ calculated for $C_{28}H_{34}N_6O_8Na^+$ = 605.2330, found 605.2321.

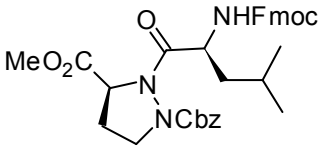
Chymotripsin coupled assay

A solution of **15** in DMSO (~10 mg/mL) was prepared, of which 3 µl was pipetted to a solution of 50 µl of α-chymotrypsin (~25 mg/mL, 1 mM HCl) and 1150 µl of buffer (HEPES 0.035 M, pH 7.8) at room 25 °C. Kinetics was measured by a diode array spectrophotometer with a thermostated cuvette holder. Total measuring time was kept at 10 min with each cycle time 10 s. The absorption of the released *p*-nitroaniline (ε = 11814 $M^{-1}cm^{-1}$) was monitored at 390 nm. Same procedure was used for **16** as a substrate.

1-Benzyl-3-methyl-2-(2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-4-methylpentanoyl)-pyrazolidine-1,3-dicarboxylate (21).

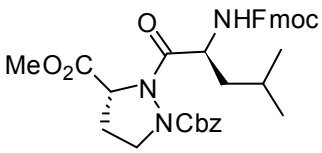
Compound **3** (0.496 g, 1.88 mmol) was dissolved in benzene (15 ml) and AgCN (0.3 g, 2.26 mmol) was added followed by the addition of Fmoc-L-Leu-Cl (0.77 gm, 2.07 mmol) in benzene (10 ml). The mixture was then heated at 60 ^{o}C for about 1 h. The completion of the reaction was adjudged by TLC. Solvent was removed *in vacuo*. The residue was taken up in ethyl acetate, filtered, and the filtrate was then washed with saturated aqueous sodium bicarbonate solution, water and brine. The combined organic extracts were dried and evaporated *in vacuo* to leave the crude product which was purified by a flash column chromatography using 7:3 petroleum ether-EtOAc as eluent to give the overall dipeptide as a yellow solid in 83 % yield (0.94 g, 1.56 mmol).

(*R*)-1-Benzyl-3-methyl-2-((*S*)-2-(((9H-fluoren-9-yl)methoxy)carb-onylamino)-4-methylpe-ntanoyl)pyrazolidine-1,3-dicarboxylate, (21a).



Yield, 40%, 0.45 g, 0.75 mmol, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (d, 2H, J = 7.5 Hz), 7.58 (d, 2H, J = 7.2 Hz), 7.41 – 7.18 (m, 9H), 5.50 (d, 1H, J = 8.4 Hz), 5.32 – 5.09 (m, 2H), 4.91 (br s, 2H), 4.12 – 4.39 (m, 4H), 3.53 (br s, 3H), 3.17 (d, 1H, J = 7.8 Hz), 2.31 – 2.40 (m, 2H), 1.66 – 1.61 (m, 1H), 1.41 – 1.44 (m, 1H), 1.26 – 1.30 (m, 1H), 0.88 – 0.90 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.9, 170.4, 157.9, 155.7, 144.1, 143.9, 141.3, 135.4, 128.6, 128.5, 128.3, 127.7, 127.1, 125.2, 125.1, 120.0, 69.2, 66.9, 60.4, 57.5, 53.5, 52.6, 50.7, 47.2, 42.7, 29.9, 24.7, 23.4, 21.7, 21.1, 14.2. HRMS (ESI) $(M + Na)^+$ calculated for $C_{34}H_{37}N_3O_7Na^+$ = 622.2529, found 622.2528.

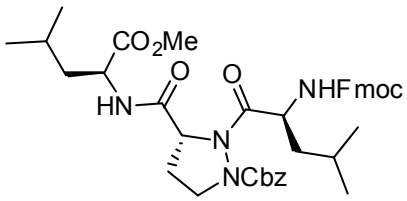
(*S*)-1-Benzyl-3-methyl-2-((*S*)-2-(((9H-fluoren-9-yl)methoxy)carbonyl-amino)-4-methylpentanoyl)pyrazolidine-1,3-dicarboxylate, (21b).



Yield, 43%, 0.48 g, 0.80 mmol), ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (d, 2H, J = 7.5 Hz), 7.58 (d, 2H, J = 7.2 Hz), 7.19 – 7.48 (m, 9H),

5.47 (br d, 1H, J = 8.1 Hz), 5.14 – 5.32 (m, 2H), 4.91 (br s, 2H), 4.15 – 4.36 (m, 4H), 3.54 (s, 3H), 3.18 (br d, 1H, J = 8.7 Hz), 2.42 – 2.32 (m, 2H), 1.55 – 1.61 (m, 1H), 1.36 – 1.44 (m, 1H), 1.26 – 1.28 (m, 1H), 0.89 (d, 6H, J = 6.3 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.3, 170.5, 157.9, 155.8, 144.1, 144.0, 141.4, 135.4, 128.5, 128.3, 127.7, 127.1, 125.3, 120.0, 69.2, 67.0, 57.5, 52.6, 50.8, 46.8, 42.7, 29.9, 24.8, 23.4, 21.8. HRMS (ESI) $(M + Na)^+$ calculated for $C_{34}H_{37}N_3O_7Na^+$ = 622.2529, found 622.2528.

(*R*)-Benzyl-2-((*S*)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-4-methylpentanoyl)-3-((*S*)-1-methoxy-4-methyl-1-oxopentan-2-ylcarbamoyl)pyrazolidine-1-carboxylate, (22).



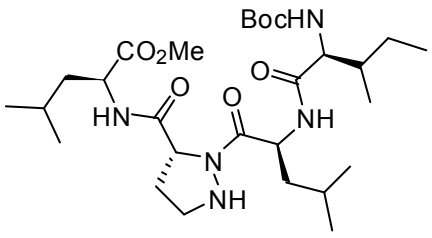
The methyl ester **21b** (0.48 g, 0.80 mmol) was dissolved in isopropanol (14 ml) and tetrahydrofuran (4.5 ml). $CaCl_2$ (1.42 g, 12.81 mmol) was added. Separately, $LiOH.H_2O$ (0.135 g, 3.2 mmol) was dissolved in H_2O (6 ml). The aqueous solution was then added to the reaction mixture and the cloudy white solution was stirred for 45 min. The organic solvents were removed under reduced pressure, and the resulting residue was taken up in 10% potassium carbonate (K_2CO_3) (40 ml) as a cloudy white suspension. The aqueous layer was partitioned in diethylether (Et_2O) (2 × 10 ml) to remove the Fmoc deprotection side products (if any), after which it was acidified to pH 2 with concentrated HCl. It was then extracted with EtOAc (100 ml). The organic layers were dried over Na_2SO_4, and concentrated to a white foamy solid in 89% yield (0.42 g, 0.71 mmol).

DIPEA (0.12 ml, 0.71 mmol) was added dropwise to a stirred suspension of L-Leu-OMe.HCl (0.155 g, 0.85 mmol) in dichloromethane (10 ml) at room temperature under an atmosphere of nitrogen. On dissolution, the solution was cooled to 0 ^{o}C and then the acid obtained above (0.42 g, 0.71 mmol) and 1-hydroxybenzotriazole (0.12 g, 0.85 mmol) were added successively, each in

one portion. The suspension was stirred at 0 ^{o}C for a further 15 min and then EDC (0.16 g, 0.85 mmol) was added in one portion. The mixture was allowed to warm to room temperature over the course of 12 hr, then filtered, and the filtrate was evaporated in vacuo. The residue was taken up in ethyl acetate, filtered, and the filtrate was then washed with 10% aqueous citric acid solution followed by saturated aqueous sodium bicarbonate solution. The combined organic extracts were dried and evaporated in vacuo to leave the crude product which was purified by chromatography on silica using 1:1 petroleum ether-EtOAc as eluent to give the tripeptide **22** (0.43 g, 0.60 mmol) as a yellow solid in 85 % yield.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74 (d, 2H, J = 7.5 Hz), 7.56 (d, 2H, J = 7.5 Hz), 7.26 – 7.41 (m, 9H), 5.27 – 5.29 (m, 1H), 5.14 – 5.18 (m, 3H), 4.87 (t, 1H, J = 7.8 Hz), 4.78 (t, 1H, J = 9.0 Hz), 4.47 – 4.55 (m, 1H), 4.32 – 4.36 (m, 2H), 4.16 – 4.21 (m, 2H), 3.72 (s, 3H), 3.26 (q, 1H, J = 9.6 Hz), 2.34 – 2.42 (m, 2H), 1.50 – 1.69 (m, 6H), 0.87 – 0.95 (m, 10H), 0.78 (d, 2H, J = 5.4 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.0, 172.8, 169.2, 158.5, 156.3, 147.1, 143.9, 141.4, 135.0, 128.9, 128.6, 128.5, 128.3, 127.8, 127.2, 125.2, 120.1, 69.3, 67.2, 59.8, 52.4, 51.1, 49.7, 49.4, 47.3, 41.2, 40.7, 29.01, 24.9, 24.8, 23.4, 23.0, 21.9, 20.9. HRMS (ESI) $(M + Na)^+$ calculated for $C_{40}H_{48}N_4O_8Na^+$ = 735.3370, found 735.3371.

(*S*)-Methyl 2-((*R*)-2-((*S*)-2-((2*S*,3*S*)-2-(*tert*-butoxycarbonylamino)-3-methylpentanamido)-4-methylpentanoyl)pyrazolidine-3-carboxamido)-4-methylpentanoate, (23).

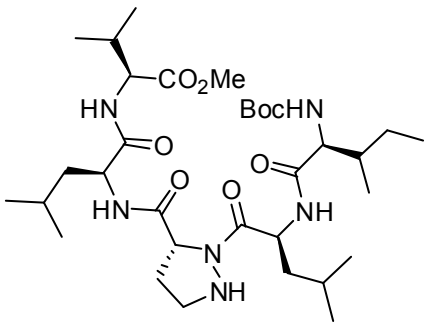


Compound **22** (0.070 g, 0.098 mmol) was treated with piperidine (1 ml) and stirred at room temperature for about 10 min. Piperidine was then removed *in vacuo* and a white solid obtained which was directly used for the next step without purification.

The crude compound was dissolved in dry DCM, on dissolution, the solution was cooled to 0 ^{o}C and then L-Ile-NHBoc (0.025 g, 0.108 mmol) and 1-hydroxybenzotriazole (0.016 g, 0.118 mmol) were added successively, each in one portion. The suspension was stirred at 0 ^{o}C for a further 15 min and then EDC (0.023 g, 0.118 mmol) was added in one portion. The mixture was allowed to warm to room temperature over the course of 12 hr, filtered, and the filtrate was evaporated in vacuo. The residue was taken up in ethyl acetate, filtered, and the filtrate was then washed with 10% aqueous citric acid solution followed by saturated aqueous sodium bicarbonate solution. The combined organic extracts were dried and evaporated *in vacuo* to leave the crude product which was purified by chromatography on silica using 1:1 petroleum ether-EtOAc as eluent to give the tetrapeptide **23** (0.055 g, 0.096 mmol, 89 %) as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44 (d, 1H, J = 7.8 Hz), 6.65 (d, 1H, J = 6.9 Hz), 5.23 (dd, 1H, J = 13.8, 7.8 Hz), 5.15 (d, 1H, J = 9 Hz), 4.72 (dd, 1H, J = 8.7, 6.9 Hz), 4.47 – 4.55 (m, 1H), 4.42 (dd, 1H, J = 12.6, 4.5 Hz), 3.96 (t, 1H, J = 8.0 Hz), 3.69 (s, 3H), 3.27 – 3.34 (m, 1H), 2.70 – 2.85 (m, 1H), 2.33 – 2.57 (m, 2H), 1.79 (br s, 1H), 1.48 – 1.69 (m, 7H), 1.46 (s, 9H), 1.11 – 1.19 (m, 1H), 0.87 – 0.97 (m, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3, 173.1, 171.6, 170.5, 155.8, 79.7, 59.2, 58.9, 52.2, 51.1, 49.7, 48.0, 41.0, 40.8, 37.5, 30.8, 28.4, 25.0, 24.9, 23.2, 22.9, 22.0, 21.8, 15.5, 11.4. HRMS (ESI) $(M + Na)^+$ calculated for $C_{28}H_{51}N_5O_7Na^+$ = 592.3686, found 592.3685.

(*S*)-Methyl-2-((*S*)-2-((*R*)-2-((*S*)-2-((2*S*,3*S*)-2-(*tert*-butoxycarbonylamino)-3-methylpentan-amido)-4-methylpentanoyl)pyrazolidine-3-carboxamido)-4-methylpentanamido)-3-methy-lbutanoate, (24):



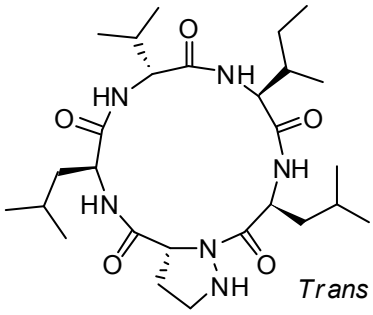
The compound **23** (76 mg, 0.13 mmol) was taken with THF (3 ml) and MeOH (0.3 ml). A solution of $LiOH.H_2O$ (56 mg, 1.3 mmol) in H_2O (0.9 ml) was added dropwise ultimately

attaining a molarity of 1.5(M). The reaction mixture was stirred for about 30 min. The solvent was removed, acidified to pH 4 at 0 ^{o}C, extracted with ethyl acetate. The organic layer was washed with water & brine to yield the acid (70 mg, 0.1 mmol) in 77% yield.

Diisopropylethylamine (73 µl, 0.42 mmol) was added dropwise over to a stirred suspension of L-Val-OMe.HCl (22 mg, 0.0.13 mmol) in dichloromethane (3 ml) at room temperature under an atmosphere of nitrogen. On dissolution, the solution was cooled to 0 ^{o}C and then the acid obtained above (70 mg, 0.1 mmol) and 1-hydroxybenzotriazole (18 mg, 0.13 mmol) were added successively, each in one portion. The suspension was stirred at 0 ^{o}C for a further 15 min and then EDC (25 mg, 0.13 mmol) was added in one portion. The mixture was allowed to warm to room temperature over the course of 12 hr, the solvent was then evaporated *in vacuo*. The residue was taken up in ethyl acetate, washed with cold 0.1 N HCl followed by saturated aqueous sodium bicarbonate solution. The combined organic extracts were dried and evaporated *in vacuo* to leave the crude product which was purified by chromatography on silica using 1:1 petroleum ether-EtOAc as eluent to give the pentapeptide **24** (70 mg, 0.10 mmol, 80 %) as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44 (d, 1H, J = 7.2 Hz), 6.96 (d, 1H, J = 9.0 Hz), 6.67 (d, 1H, J = 5.7 Hz), 5.18 – 5.11 (m, 1H), 5.01 (d, 1H, J = 9.3 Hz), 4.87 (dd, 1H, J = 12.6, 4.8 Hz), 4.72 (dd, 1H, J = 9.0, 6.9 Hz), 4.51 (dd, 1H, J = 9, 5.7 Hz), 4.35 – 4.42 (m, 1H), 3.94 (t, 1H, J = 8.4 Hz), 3.72 (s, 3H), 3.27 – 3.35 (m, 1H), 2.72 – 2.86 (m, 1H), 2.35 – 2.58 (m, 2H), 2.08 – 2.20 (m, 1H), 1.48 – 1.80 (m, 8H), 1.41 (s, 9H), 1.08 – 1.19 (m, 1H), 0.87 – 0.97 (m, 24H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.2, 172.9, 172.4, 172.1, 170.8, 155.7, 79.9, 59.0, 57.0, 53.3, 52.2, 50.1, 47.9, 40.4, 37.5, 31.2, 30.8, 28.4, 25.0, 23.1, 22.9, 22.2, 22.0, 19.1, 18.1, 17.9, 15.6, 11.3. HRMS (ESI) $(M + Na)^+$ calculated for $C_{33}H_{60}N_6O_8Na^+$ = 691.4370, found 691.4368.

(3a*R*,6*S*,9*R*,12*S*,15*S*)-12-*sec*-Butyl-6,15-diisobutyl-9-isopropyldecahydro-1H-pyrazolo[1,5-a][1,4,7,10,13]pentaazacyclopentadecine-4,7,10,13,16(2H)-pentaone, (18*T*).



Trans

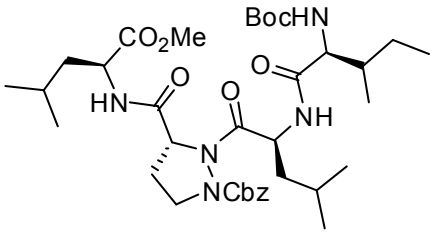
The compound **24** (55 mg, 0.082 mmol) was taken in THF : MeOH (2 ml : 0.2 ml) mixture. A solution of $LiOH.H_2O$ (42 mg, 0.82 mmol) in 0.5 ml H_2O was added dropwise ultimately attaining a molarity of 1.5 M. The reaction mixture was stirred for 30 min. The solvent was removed, acidified to pH 4 at 0 ^oC, extracted with ethyl acetate. The organic layer was washed with water and brine to yield the carboxylic acid as white foamy solid. The crude product so obtained was dissolved in dry DCM (1.6 ml) and 0.4 ml TFA was added at 0 ^oC and stirred for 2 h. Removal of DCM *in vacuo* yielded a brownish viscous liquid which was used in the next step without further purification.

The brownish solid obtained above (54 mg, 0.08 mmol) was dissolved in dry DMF (80 ml) to attain a concentration of 1 mM. To it $NaHCO_3$ (34 mg, 0.4 mmol) was added followed by the addition of BOP (39 mg, 0.088 mmol) at room temperature. The reaction mixture was then stirred for overnight at rt. Removal of DMF followed by a flash column chromatography (5 % MeOH–DCM) chromatography yielded **18*T*** (14 mg, 0.025 mmol) in 31 % yield.

^1H NMR (500 MHz, $CDCl_3$) δ 7.79 (d, 1H, J = 9.5 Hz), 7.09 (d, 1H, J = 8.0 Hz), 6.61 (d, 1H, J = 9.0 Hz), 6.44 (d, 1H, J = 7.0 Hz), 5.20 (dd, 1H, J = 16.5, 7.5 Hz), 4.73 (dd,1H, J = 9, 5.5 Hz), 4.67 (dd, 1H, J = 12, 5 Hz), 4.47 (dd, 1H, J = 17.0, 7.5 Hz), 4.09 (dd, 1H, J = 7.5, 6.0 Hz), 3.77 (t, 1H, J = 9.0 Hz), 3.32 – 3.33 (m, 1H), 2.74 – 2.83 (m, 2H), 2.09 (s, 1H), 1.94 – 1.99 (m, 1H), 1.43 – 1.70 (m, 8H), 1.10 – 1.16 (m, 1H), 0.86 – 1.00 (m, 24H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.3, 172.6, 171.9, 170.6, 169.3, 61.2, 60.1, 57.7, 51.8, 48.9, 48.4, 40.4, 39.0, 35.7, 30.0, 27.1,

25.0, 24.9, 22.9, 22.8, 22.7, 22.5, 19.4, 16.3, 11.6. HRMS (ESI) $(M + Na)^+$ calculated for $C_{27}H_{48}N_6O_5Na^+ = 559.3584$, found 559.3582.

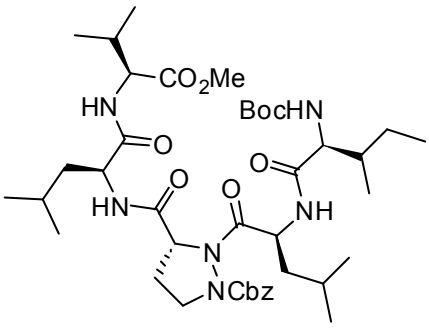
(*R*)-Benzyl-2-((*S*)-2-((2*S*,3*S*)-2-(*tert*-butoxycarbonylamino)-3-methylpentanamido)-4-methylpentanoyl)-3-((*S*)-1-methoxy-4-methyl-1-oxopentan-2-ylcarbamoyl)pyrazolidine-1-carboxylate, (29).



Cbz-Cl (0.026 ml, 0.18 mmol) was added to a benzene solution of **23** (68 mg, 0.12 mmol). To the above mixture AgCN (23 mg, 0.024 mmol) was added and refluxed at 60 ^oC for 30 min. After completion the reaction (as indicated by TLC) benzene was removed, the mixture was extracted with EtOAc, filtered through a sintered funnel. The organic layer was then washed with satd. $NaHCO_3$ followed by water and brine. Removal of the organic layer yielded the crude mixture which was purified by a flash column chromatography (3% MeOH in DCM) to yield **29** (78 mg, 0.11 mmol) in 93% yield.

^1H NMR (300 MHz, $CDCl_3$) δ 7.31 – 7.41 (m, 6H), 6.36 (d, 1H, J = 8.4 Hz), 5.26 (s, 2H), 5.00 – 5.10 (m, 2H), 4.83 (br s, 1H), 4.37 – 4.43 (m,1H), 4.16 – 4.23 (m, 1H), 3.93 (t, 1H, J = 8.4 Hz), 3.65 (s, 3H), 3.08 – 3.25 (m, 1H), 2.39 – 2.55 (m, 2H), 2.00 (br s, 1H), 1.76 – 1.87 (m, 1H), 1.48 – 1.66 (m, 5H), 1.44 (s, 9H), 1.12 – 1.33 (m, 2H), 0.85 – 1.00 (m, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 171.7, 171.2, 169.9, 155.8, 135.0, 128.8, 79.9, 70.0, 60.1, 59.3, 57.0, 52.3, 51.5, 51.0, 50.4, 49.3, 42.0, 40.8, 40.2, 37.6, 37.4, 30.7, 28.4, 25.1, 25.0, 24.9, 23.5, 23.3, 23.0, 22.0, 21.9, 21.7, 21.5, 15.6, 14.3, 11.5. HRMS (ESI) $(M + Na)^+$ calculated for $C_{36}H_{57}N_5O_9Na^+ = 726.4054$, found 726.4051.

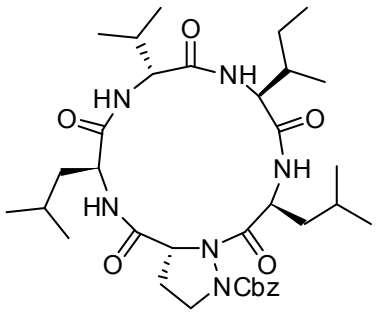
(*S*)-Benzyl-2-((*S*)-2-((2*S*,3*S*)-2-(*tert*-butoxycarbonylamino)-3-methylpentanamido)-4-methylpentanoyl)-3-((*S*)-1-((*S*)-1-methoxy-3-methyl-1-oxobutan-2-ylamino)-4-methyl-1-oxopentan-2-ylcarbamoyl)pyrazolidine-1-carboxylate, (30).



Cbz-Cl (0.013 ml, 0.087 mmol) was added to a benzene solution of **24** (39 mg, 0.058 mmol). To the above mixture AgCN (15 mg, 0.12 mmol) was added and heated at 60 ^{o}C for 30 min. After completion the reaction (as indicated by TLC) benzene was removed, the mixture was extracted with EtOAc, filtered through a sintered funnel. The organic layer was then washed with satd. $NaHCO_3$ followed by water and brine. Removal of the organic layer yielded the crude diasteriomeric mixture which was separated by a flash column chromatography (3% MeOH in DCM) to yield **30** (35 mg, 0.052 mmol) in 90% yield.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33 – 7.40 (m, 6H), 6.54 (d, 1H, J = 8.0 Hz), 6.28 (d, 1H, J = 7.5 Hz), 5.19 – 5.28 (m, 2H), 5.06 (m, 1H), 4.91 (dd, 1H, J = 11.5, 4 Hz), 4.80 (br s, 1H), 4.50 (dd, 1H, J = 9, 5.5 Hz), 4.28 – 4.32 (m, 1H), 4.24 (br s, 1H), 3.92 (t, 1H, J = 8 Hz), 3.69 (s, 3H), 3.16 – 3.19 (m, 1H), 2.55 – 2.61 (m, 1H), 2.31 (br s, 1H), 2.11 – 2.17 (m, 1H), 1.81 (br s, 1H), 1.56 – 1.63 (m, 6H), 1.44 (s, 9H), 1.24 – 1.30 (m, 1H), 1.12 – 1.21 (m, 1H), 0.77 – 0.94 (m, 24H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.7, 172.2, 171.6, 171.4, 170.1, 159.1, 155.9, 148.6, 134.9, 128.8, 79.9, 70.1, 60.0, 59.3, 57.1, 52.8, 52.2, 49.6, 41.5, 40.4, 40.3, 37.3, 32.1, 31.6, 31.3, 29.5, 28.5, 25.0, 24.9, 23.3, 23.1, 23.0, 22.8, 22.0, 21.9, 21.5, 19.1, 18.1, 18.0, 15.6, 14.3, 11.5, 11.4. HRMS (ESI) $(M + Na)^+$ calculated for $C_{41}H_{66}N_6O_{10}Na^+$ = 825.4738, found 825.4741.

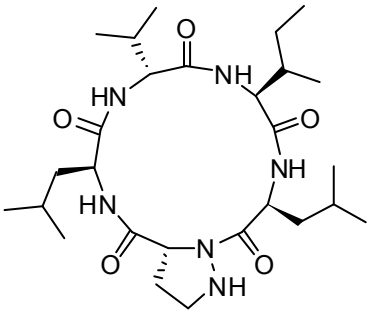
(3a*R*,6*S*,9*R*,12*S*,15*S*)-Benzyl-12-*sec*-butyl-6,15-diisobutyl-9-isopropyl-4,7,10,13,16-pentaoxohexadecahydro-1H-pyrazolo[1,5-a][1,4,7,10,13]pentaazacyclopentadecine-1-carboxylate, (31).



Cbz-Cl (5.3 µl, 0.037 mmol) was added to a benzene solution of **18*T*** (10 mg, 0.019 mmol). To the above mixture AgCN (5 mg, 0.037 mmol) was added and refluxed at 60 ^{o}C for 30 min. On completion of the reaction (as indicated by TLC) benzene was removed, the mixture was extracted with EtOAc, filtered through a sintered funnel. The organic layer was then washed with satd. $NaHCO_3$ followed by water and brine. Removal of the organic layer yielded the crude mixture which was purified by a flash column chromatography (5% MeOH in DCM) to yield **31** (9 mg, 0.013 mmol) in 71% yield.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 (d, 1H, J = 8.4 Hz), 7.49 (d, 2H, J = 7.2 Hz), 7.28 – 7.41 (m, 3H), 7.20 (br s, 1H), 6.82 (d, 1H, J = 7.8 Hz), 5.95 (brs, 1H), 5.49 (d, 1H, J = 12.6 Hz), 4.99 – 5.10 (m, 2H), 4.78 (dd, 1H, J = 8.4, 4.8 Hz), 4.37 (t, 1H, J = 9.7 Hz), 4.20 (t, 1H, J = 8.7 Hz), 4.02 (t, 1H, J = 9.3 Hz), 3.94 (br m, 1H), 3.10 (q, 1H, J = 9.0 Hz), 2.80 – 2.94 (m, 1H), 1.98 – 2.17 (m, 2H), 1.79 (br s, 2H), 1.35 – 1.63 (m, 6H), 1.02 – 1.19 (m, 1H), 0.84 – 0.98 (m, 24H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.6, 172.1, 170.8, 170.0, 167.8, 158.5, 136.3, 128.7, 128.4, 128.3, 69.6, 60.5, 60.4, 56.9, 53.6, 53.2, 49.5, 48.8, 42.3, 42.1, 36.3, 30.8, 25.6, 25.3, 25.1, 22.9, 22.7, 22.3, 19.6, 19.0, 15.7, 10.9. HRMS (ESI) $(M + Na)^+$ calculated for $C_{35}H_{54}N_6O_7Na^+$ = 693.3952, found 693.3950.

(3a*R*,6*S*,9*R*,12*S*,15*S*)-12-*sec*-Butyl-6,15-diisobutyl-9-isopropyldecahydro-1H-pyrazolo[1,5-a][1,4,7,10,13]pentaazacyclopentadecine-4,7,10,13,16(2H)-pentaone, (18*T*).



To a solution of **31** (4 mg, 0.006 mmol) in MeOH (1 ml), 10% Pd/C (3 mg) was added, the mixture was stirred for 4 h in hydrogen atmosphere. The reaction mixture was filtered through celite and MeOH was removed *in vacuo*. The crude reaction mixture was then purified by flash column chromatography (5% MeOH in DCM) to yield **18*T*** in 72% yield (2.3 mg, 0.0043 mmol).

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Supporting Information Available: Copies of NMR (^{1}H, ^{13}C) spectra of all the new compounds along with 2-D NMR (COSY and HMQC) of relevant compounds and X-ray crystallographic data for **18*T*** have been provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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