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Proteolytic stability of cyclic α-hydrazino acid containing peptides: a qualitative study

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ABSTRACT

Synthesis of cyclic α-hydrazino acid containing tripeptides Phe-X-Val and evaluation their proteolytic stability against digestive enzymes has been reported for the first time. Tripeptides were treated with the digestive juice of mice and incubated in buffer at 37 °C for 45 min and was found to be stable when X = 5,6,7-membered hydrazino acids with δ-NH/NHCbz whereas X = proline was digested. Digestion process was monitored by LC–MS analysis.

There is a general perception that small peptides, equipped with high selectivity, lower toxicity, and high chemical/biological diversity than small molecules, might be the best candidates for therapy of various diseases.[1]

Despite the selectivity and the perceived efficacy, rapid in vivo elimination of therapeutic peptides remains one of the major challenges to be addressed.[1] Since most therapeutic peptides are mere modifications of native sequences, imparting stability to them by a way of chemical modifications, so that they are less easily degraded (proteolysis) or less susceptible to renal clearance takes in a bit of extra effort which in turn has the potential to seriously alter the potency of the drug candidate. Chemical modifications, either within the sequence or by conjugation (to polymer, lipid) has been tried to some extent. A wide range of polymeric conjugates are now available,[2] including polyethylene glycol (PEG), hydroxyethyl starch (HES), human serum albumin (HSA), XTEN, PAS (recombinant polypeptides), polyglutamic acid, and monoclonal antibodies. Covalent modifications to create new chemical entity (NCE) and conjugating them to drug candidates[3] are also known to alter the pharmacokinetics as well as pharmacodynamics of the original drug. NCE conjugated pharmaceutical ingredients are either expected to have pro drug characteristics (if the conjugation is reversible) or, are expected to interact directly with the target. However, covalent conjugation usually lowers potency but compensates by increasing the half life of the peptide. Another relevant approach is to modify the peptide from within using unnatural or non-proteinogenic amino acids.[4] The approach exploits the substrate specificity of enzymes. A slight modification in the amino acid backbone can render it immune towards proteolytic degradation.

Since the discovery of piperazic acid (Fig. 1) (hexahydropyridazine-3-carboxylic acid) a non-proteinogenic amino acid (from a group of cyclodepsipeptide natural product[5]) by Hassall and coworkers as a component of monamycins, it or similar such motifs has been in focus for its presence in other naturally occurring cyclodepsipeptide.

Peptides containing repeating linear alpha hydrazino carboxylic acids or azapeptide linkage incorporated into various enzyme inhibitors are known to be stable under proteolytic conditions.[4c,6] Thinking alike we hypothesized that their cyclic counterparts should exhibit similar properties. The δ-nitrogen atom of cyclic α-hydrazino acid is expected to play a pivotal role in imparting stability. The similarity of the cyclic α-hydrazino acids with proline encouraged us to substitute proline with cyclic α-hydrazino acid (Fig. 1) in a model tri-peptide and probe their effect on the pharmacokinetics. Herein we wish to report a few relevant observations.

Literature study of previously reported enzymatic studies on short peptide sequences indicated that tripeptides with the general

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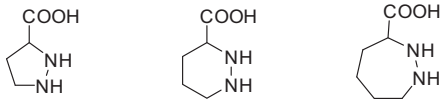


Figure 1. Structure of cyclic α-hydrazino acids.

structure Phe-ΨPro-Val are recognized by the enzymes.[7a,d] Accordingly tripeptide **1** was chosen as our model substrate (Fig. 2). The cyclic α-hydrazino acid in the central position was varied (analogues and enantiomers). The proteolytic stability was compared to the stability of proline (when X = CH_2, n = 1) containing peptide under similar conditions.

Methyl ester of mono Cbz protected δ-azaproline (**Aza-Pro**) **2**[7b–d] (racemic) was used as the starting material for the synthesis of the δ-azaproline containing protected tripeptide **3** according to our previously reported procedures[7c] (Fig. 3).

Piperazic (**Piz**) acid and its seven membered (**diazepane carboxylic acid**) analogue containing tripeptides were synthesized from the dipeptides **4** and **5** which in turn were synthesized using our previously reported procedure.[7b] D/L Configuration of the hydrazino acids were established previously.[7b] Compounds **4** and **5**[7b] on Boc deprotection followed by selective Cbz protection yielded **6** and **7**, respectively. The diastereomeric mixtures of mono protected dipeptides (**6a**/**6b**, **7a**/**7b**) were nicely separated by flash column chromatography using silica gel. Diastereomers were then separately converted to the corresponding protected tripeptides by treating with Fmoc-Val-Cl and AgCN in benzene to obtain **8a**, **8b** and **9a**, **9b**, respectively (Scheme 1).

The protected tripeptides were then deprotected to obtain the model peptides. Compounds **3a**, **8a,** and **9a** were deprotected partially to prepare the Cbz protected tripeptides **10a**, **11a**, **12a** (Scheme 2) and the compounds were treated with TFA to protonate the free amine in order to eliminate the chances of intramolecular cyclization[7c] during the storage or handling.

To synthesize the completely deprotected tripeptides, compounds **3**, **8,** and **9** were subjected to sequential hydrolysis, hydrogeno-lysis and Fmoc deprotection. Complete deprotection of individual isomers yielded the tripeptides **13a**, **13b**, **14a**, **14b,** and **15a**, **15b** as their respective piperidine salts (Scheme 3).

The stability of these compounds in the presence of enzymes was evaluated against the standard proline based tripeptide **18** in order to compare and test the efficacy of the enzymes. Compound **18** was prepared by sequential peptide coupling from N-Boc-L-proline. N-Boc-L-proline was subjected to EDC coupling with L-phenylalanine methyl ester to yield the protected dipeptide **16**. This dipeptide on N-Boc deprotection followed by EDC coupling with N-Boc L-valine yielded the protected tripeptide **17**. The protected tripeptide **17** on ester hydrolysis and N-Boc deprotection yielded the TFA salt of the control tripeptide **18** (Scheme 4). TFA salt was confirmed by ^{13}C NMR as peak corresponding at 161.9 (q, $J_{C,F}$ = 36 Hz) and 115.9 (q, $J_{C,F}$ = 288 Hz) are for TFA.

Proteolytic stability of cyclic α-hydrazino acid-containing peptides

Proteolytic cleavage breaks down proteins in food into smaller peptides and amino acids so that they can be absorbed and used by an organism. Similar fate is met by the therapeutic peptides when taken orally; most of the bioactive peptides undergo proteolytic degradation into many smaller peptides and amino acids.

Instead of choosing individual enzymes based on their selectivity of substrates, a broad range of them ought to be used in our study. Consequently Swiss Albino mice were chosen as model mammals for this study.

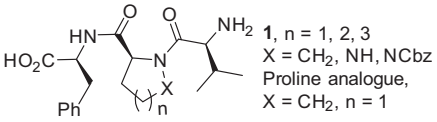


Figure 2. General structure of model tripeptide.

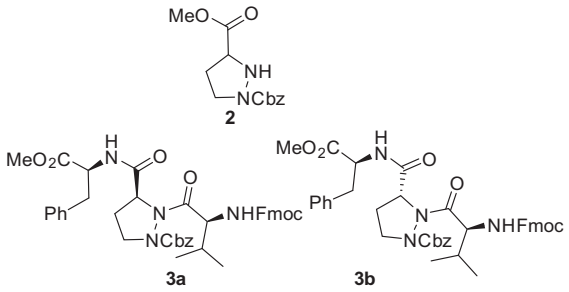
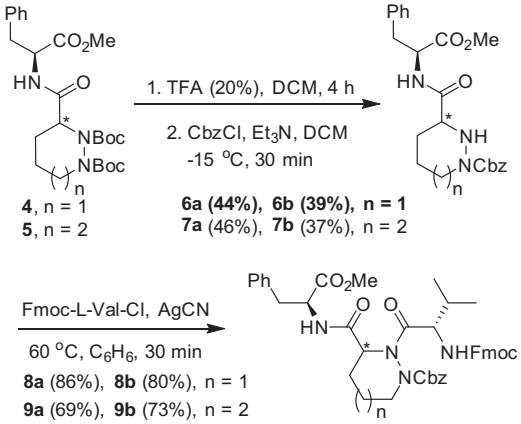
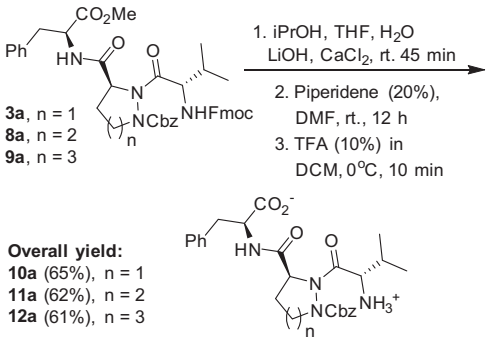


Figure 3. δ-Azaproline containing tripeptides.

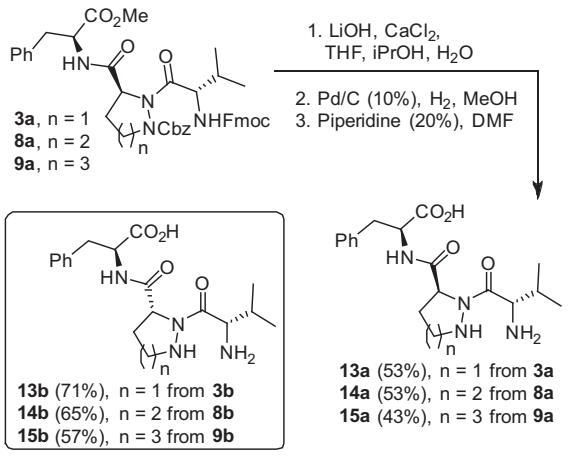


Scheme 1. Synthesis of protected tripeptides **8** and **9**.



Scheme 2. Synthesis of Cbz-protected tripeptides **10a**, **11a**, **12a**.

Inbred mice were purchased and maintained in the laboratory at 24 °C, 12 h day night cycle and fed on chick peas twice daily. The mice were anaesthetized before being sacrificed. Extraction of the digestive enzymes from mouse was done following the reported procedure of Rao et al.[8] The abdomen was opened and the duodenum and a part of the proximal ileum was removed under sterile condition and kept in 0.05 M Tris–HCl buffer (pH 9) at 4 °C for immediate processing. The alimentary canal was slit opened and washed with 0.05 M Tris–HCl buffer (pH 9) to recover



Scheme 3. Synthesis of unprotected tripeptides **13**, **14**, **15**.

the pancreatic enzyme. The extracts were centrifuged, the supernatant (enzyme solutions) were separately stored at 4 °C until the experiment.

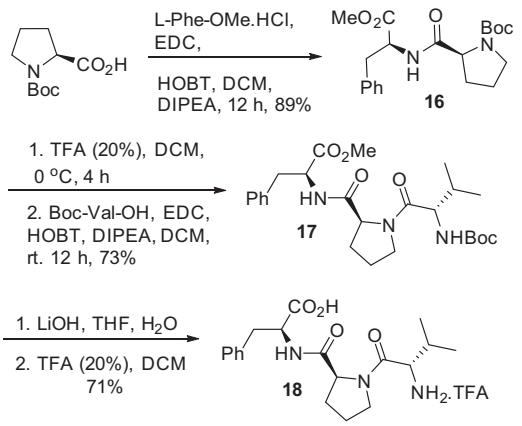
Solutions of peptides were treated with the enzyme solution[9] and the mixtures were analyzed with LCMS (Table 1).

Analysis of the LCMS of control tripeptide, 18

The control tripeptide **18** (Phe-Pro-Val-OH) is expected to undergo proteolytic degradation since it contains only naturally occurring amino acids (L-Phe, L-Pro, L-Val). The degradation will also indicate the activity of extracted digestive enzymes. The fragment at 263.8 corresponds to the Phe-Pro dipeptide whereas the peak at 118.0 corresponds to the released Val (Fig. 4a). It has shown the efficacy of the extracted digestive enzymes, as well as demonstrated the fate of the peptide (or protein) based drugs under digestive conditions.

Analysis of the LCMS of Cbz protected δ-L-azaproline containing tripeptide 10a

Compound **10a**, being protected is not expected to undergo hydrolysis. The Cbz group at the δ-nitrogen is expected to inhibit the formation of enzyme substrate complex. The molecular ion peak of **10a** at 495.2 and the presence of very little related metabolites at 396.3 indicated its resistance towards proteolytic enzymes (Fig. 4b).



Scheme 4. Synthesis of control tripeptide **18**.

Table 1
Relative qualitative analysis of proteolysis

Entry		Peptides	Stability[i]
(1)	**18**	Phe-Pro-Val-OH	–
(2)	**10a**	Phe-(L)-AzaPro(Cbz)-Val-OH	+
(3)	**11a**	Phe-(L)-Piz(Cbz)-Val-OH	+
(4)	**12a**	Phe-(L)-Diazepane(Cbz)-Val-OH	+
(5)	**13a**	Phe-(L)-AzaPro-Val-OH	++
(6)	**13b**	Phe-(D)-AzaPro-Val-OH	++
(7)	**14a**	Phe-(L)-Piz-Val-OH	+
(8)	**14b**	Phe-(D)-Piz-Val-OH	+
(9)	**15a**	Phe-(L)-Diazepane-Val-OH	++
(10)	**15b**	Phe-(D)-Diazepane-Val-OH	++

[i] (++) = Stable with negligible proteolysis; (+) = stable with slight proteolysis; (–) = complete proteolysis, **a**/**b** indicates the diastereomers.

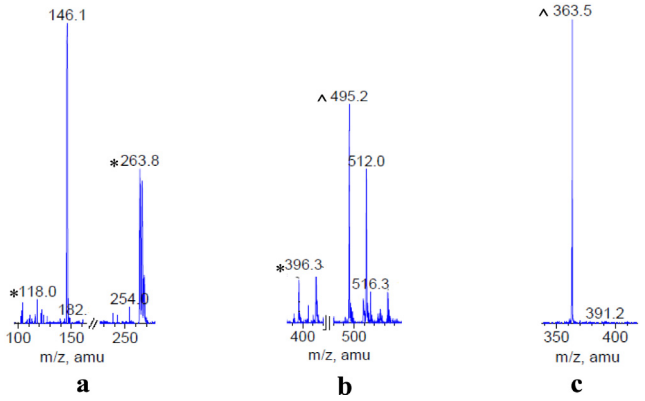


Figure 4. Digestion of (a) Control peptide **18** (b) Phe-(L)-AzaPro(Cbz)-Val-OH, **10a** (c) Phe-(L)-AzaPro-Val-OH, **13a**. (*) Denotes metabolites and (^) denotes peptides.

Analysis of the LCMS of L-δ-azaproline containing tripeptide Phe-(L)-Aza Pro-Val-OH, 13a

L-Configuration of δ-azaproline in **13a** is the closest to that of L-proline and consequently, stability of this tripeptide is of utmost interest. Without any protection as that of **10a**, peptide **13a** is expected to be prone to proteolysis.

Contrary to the expected degradation, compound **13a** showed remarkable stability towards proteolysis which is evident from the ion peak at 363.5 (Fig. 4c).

Analysis of the LCMS of D-δ-azaproline containing tripeptide, Phe-(D)-AzaPro-Val-OH, 13b

D-Amino acids are reported to enhance the pharmacokinetics of peptides principally by exploiting the substrate specificity of enzymes, and hence it was anticipated that the tripeptide **13b** would not undergo proteolysis under these conditions. LCMS of the mixture showed the anticipated results and the molecular ion peak at 363.1 corresponding to protonated tripeptide was observed.

Analysis of the LCMS of Cbz protected L-piperazic acid containing tripeptide, 11a

Similar to compound **10a** (Cbz protected δ-azaproline) **11a** was also expected to resist proteolytic degradation, which was confirmed from LCMS analysis with a major peak at 511.4 ($M+H^+$) and slightly hydrolysed products confirmed by corresponding peaks at 412.2 and 119.4.

Analysis of the LCMS of piperazic acid-containing tripeptides, 14

Although piperazic is a non-proteinogenic naturally occurring amino acid the tripeptides **14** containing both L and D-isomers did not undergo proteolysis in the presence of mammalian digestive enzymes. Peaks were obtained for L-isomer: 377.2 $(M+H)^+$, 278.2 for fragments and for D-isomer: 377.1 $(M+H)^+$, 278.3 and 152 (for piperazic acid).

Analysis of the LCMS of Cbz protected L-7-membered cyclic α-hydrazino acid-containing tripeptides, 12a

Similar to the previous two tripeptides (**10a** and **11a**) compound **12a** also resists proteolysis as the major molecular ion peak was obtained at 525.4 $(M+H)^+$ except two additional short peaks at 279.1 and 426.3 correspond to the fragments.

Analysis of the LCMS of L and D-7-membered cyclic α-hydrazino acid-containing tripeptides, 15a/b

The structures closely resembling proline (δ-azaproline) and naturally occurring piperazic acid containing tripeptides were stable in the presence of mammalian digestive enzymes and analogously structurally more varied 7-membered cyclic α-hydrazino acid-containing tripeptides **15a/b** was anticipated to be resistant as well. This was confirmed by LCMS analysis from a major peak at 391.3 $(M+H)^+$ (**15a** and a minor metabolite peak at 292.5) and 391.2 $(M+H)^+$ for **15b**.

In conclusion, the above experiments strongly suggest the capability of cyclic α-hydrazino acids (protected or native) in imparting proteolytic stability to peptides. After establishing the overall pharmacokinetics of these peptides, the cyclic α-hydrazino acids particularly δ azaproline can find their applicability in imparting the stability to relevant bioactive peptides or peptide drug candidates to the replacement of pseudoproline for the treatment of Alzheimer's disease.[10] In addition, synthetic methods of cyclic α-hydrazino acids-containing peptides with the selective protection at the δ-N and the peptide elongation at the α-N has been reported which could be useful in designing the peptides for biological interest.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.07.050.

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9. Experimental procedure: (i) A stock solution of the tripeptides (approx 2 mg/ml) was prepared in the same buffer (0.05 M Tris–HCl, pH 9).
(ii) The enzyme extracts were mixed and mixed well prior to the experiment.
(iii) 50 μl of peptide stock solution and 20 μl of enzyme solution (to make a final peptide concentration of 100 μg/70 μl) were added into a 0.5 ml Eppendorf tube. The mixture was kept at 37 ± 1 °C for 45 min.
(iv) 20 μl of trichloroacetic acid (TCA) solution (0.5 g TCA into 0.35 ml d H_2O) was added to the above solution and mixed well. The cloudy reaction sample was cooled (4 °C) for 15 min, then spun at 13,000 g (Eppendorf centrifuge) for 2 min to pellet the precipitated serum proteins.
(v) The supernatant solution was then analyzed using LCMS.
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