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Prolinoamino Acids as Tools to Build Bifunctionalized, Stable β-Turns in Water

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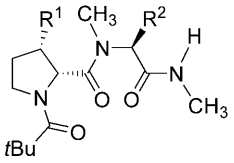
The design of peptidomimetics able to mimic the structural and binding motifs of proteins is a major challenge in the development of new pharmaceutical tools. The β-turn motif is one of the major secondary structure elements that plays an important role in the folding of globular proteins and is often implicated as a recognition element in receptor–ligand interactions.[1] Considerable effort has been devoted to the development of synthetic templates as reverse turn mimics.[2] One strategy consists in restricting the conformational space of the central positions by means of bi- or tricyclic rings. A second approach is based on stabilizing the N- and C-terminal residues by covalent bridging or folding through noncovalent cation–π interactions.[3]

Another strategy to stabilize β-turn conformations is the incorporation of proline residues that are known to have a high β-turn propensity.[4] Heterochiral D-Pro-L-Pro or L-Pro-D-Pro sequences adopt types II′ or II β-turn conformations, respectively.[5] The replacement of one proline residue by an *N*-methyl amino acid (D-Pro-L-NMeXaa or L-Pro-D-NMeXaa sequences) further stabilizes the β-turn conformation and enables the introduction of a side chain functionality in the *i*+2 position of the β-turn.[6] We have explored the possibility of recovering the side chain functionality in the *i*+1 position using *cis*-3-substituted prolinoamino acids (P^c_3Xaa).[7] These prolinoamino acids, which are easily accessible by chemical synthesis[8] when not commercially available, can be considered as chimeras between proline and a proteinogenic amino acid. The conformational constraint of the pyrrolidine template limits the conformational space around the ϕ and χ^1 torsion angles. Prolinoamino acids can have applications as conformational tools for probing the bioactive conformations of peptides and for pharmaceutical use.[9,10] We have previously introduced a *cis*-3-prolinoleucine (P^c_3Leu) in the Piv-D-P^c_3Leu-L-Pro-NHMe sequence and shown that this short peptide bearing the side chain of Leu in the *i*+1 position adopts a stable β-turn structure in organic solvents and in water.[7]

In this study, we have validated the use of prolinoamino acids in Piv-D-P^c_3Xaa-L-NMeYaa-NHMe sequences (Scheme 1) to stabilize short β-turns, in water, that possess the two side chain functionalities in the *i*+1 and *i*+2 positions. We have examined different types of side chains able to mediate van der Waals (Leu/Phe, peptide **4a**) or cation–π interactions (HTrp/Arg and HTrp/Lys, peptides **4b** and **4c**) to investigate the influence of side chains on β-turn folding and characterize the conformational space explored by side chains. The sequences of peptides **4b** and **4c** correspond to turn sequences found in tendamistat[11] and somatostatin,[12] respectively.



4a: R^1 = *i*Pr, R^2 = CH_2-Ph
4b: R^1 = CH_2-Ind, R^2 = $(CH_2)_3$-Gua
4c: R^1 = CH_2-Ind, R^2 = $(CH_2)_4$-NH_2

Scheme 1. Chemical structures of pseudotetrapeptides Piv-D-P^c_3Xaa-L-NMeYaa-NHMe (**4a**–**c**): Piv-D-P^c_3Leu-L-NMePhe-NHMe (**4a**), Piv-D-P^c_3HTrp-L-NMeArg-NHMe (**4b**) and Piv-D-P^c_3HTrp-L-NMeLys-NHMe (**4c**).

The Piv-D-P^c_3Xaa-L-NMeYaa-NHMe compounds **4a**–**c** were obtained by solution phase peptide synthesis (Scheme 2). After conversion of *N*-methylamino acids into *N*-methylcarboxamide derivatives, couplings of *N*-protected prolinoamino acids[8] were realized by using PyBrop or PyAOP/HOAt as coupling agents with satisfactory yields. After removal of the protecting group P with TFA (P = Boc) or piperidine (P = Fmoc), the pivaloyl group was introduced on the nitrogen of the pyrrolidine ring. Finally, removal of P^1 and P^2 side chain protecting groups when required led to pseudotetrapeptides **4a**–**c**.

The conformation of peptides **4a**–**c** was first analyzed by CD spectroscopy in both methanol and aqueous solutions (Figure 1). The three peptides show similar CD patterns, with a broad minimum at around 210–223 nm. This signature was previously observed for Piv-D-P^c_3Leu-L-Pro-NHMe peptide and other peptide sequences and was assigned to a type II′ β-turn structure.[7] Interestingly, the CD signature is observed in both methanol and aqueous solutions; this indicates that the β-turn conformational propensity of peptides **4a**–**c** is retained in water. The variation of the minimum molar ellipticity in the 210–223 nm region (from $-33\,000$ to $-16\,000$ deg cm^2 mol^{-1}) between peptides **4a** and **4b**/**4c** might reflect different stabilization of β-turn conformers or different contributions of the phenyl or indole side chain chromophores to the CD signal.[13]

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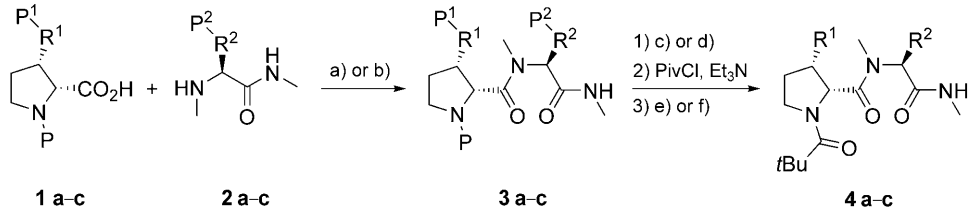
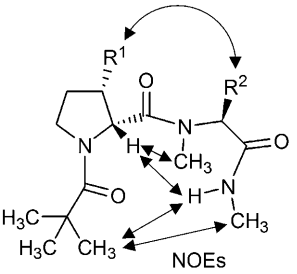


Scheme 2. Syntheses of pseudotetrapeptides **4a–c**. Reaction conditions: a) PyBroP, DIEA (**3a**); b) PyAOP, HOAt, DIEA (**3b**, **3c**); c) TFA/CH_2Cl_2 (1:1; **4a**); d) piperidine/CH_2Cl_2 (1:4; **4b**, **4c**); e) TFA/thioanisole/water (95:2.5:2.5; **4b**); f) TFA/TIS/water (95:2.5:2.5; **4c**); P = Boc (**1a**, **3a**) or Fmoc (**1b**, **1c**, **3b**, **3c**); P^1 = Boc (**1b**, **1c**, **3b**, **3c**); P^2 = Mtr (**2b**, **3b**) or Boc (**2c**, **3c**).

To address this point, the conformation of peptides **4a–c** was further investigated by NMR spectroscopy. Peptides **4b** and **4c** were examined in both methanol and aqueous solvents while peptide **4a** was studied in methanol only, owing to its low solubility at mM concentrations in water.

The NOESY or ROESY spectra of the three peptides **4a–c** in methanol revealed NOE correlations characteristic of a β-turn structure, in particular between the methyl protons of Piv N-terminal residue and the amide and methyl protons of the C-terminal NHMe group (Scheme 3). The observed NOE between the H^α proton of the prolinoamino acid residue and the amide proton of the NHMe group also supports β-turn folding. In addition, the amide proton exhibits a small temperature dependence of its chemical shift ($\Delta\delta_{NH}/\Delta T$ of ca. −3 ppb °C^{-1}); this indicates that it is involved in a hydrogen bond. The backbone sequential NOEs are compatible with (ϕ, ψ) values found in a type II′ β-turn conformation. The peptide bond between prolinoamino acid and N-methylated residues was found to be *trans*, with no sign of *cis*/*trans* isomerism. Importantly, the NMR spectroscopy data obtained in water for peptides **4b** and **4c** were very similar to those in methanol and strongly support β-turn formation in water, as already suggested by CD data.

The NMR analysis also provides information about side chain conformations and interactions. The pyrrolidine ring of the prolinoamino acid residue in the three peptides **4a–c** adopts a C^β-*exo* puckering, as shown by a strong NOE correlation between H^β and $H^{\delta S}$ protons together with the ^3J coupling constant analysis. This puckering preference restricts the χ^1 torsion angle of the Leu or HTrp side chains to the *trans* conformer. The χ^2 angle of the Leu side chain in $P^c{}_3$Leu (peptide **4a**) is also *trans*, as shown by the large value of the $^3J_{H\beta-H\gamma}$ coupling constant (9.6 Hz). In the case of $P^c{}_3$HTrp residue in peptides **4b** and **4c**, the ^3J coupling constant values and the observed NOE values suggest that the CH_2-Ind substituent does not adopt a unique conformation around its χ^2 and χ^3 torsion angles. Re-



Scheme 3. Characteristic NOE correlations observed for peptides **4a–c** in methanol (2 mM, 25 °C) or H_2O (1 mM, 20 °C). The selected NOE correlations were observed in both methanol and H_2O solvents for peptides **4b** and **4c**, except for peptide **4a** (studied in methanol only).

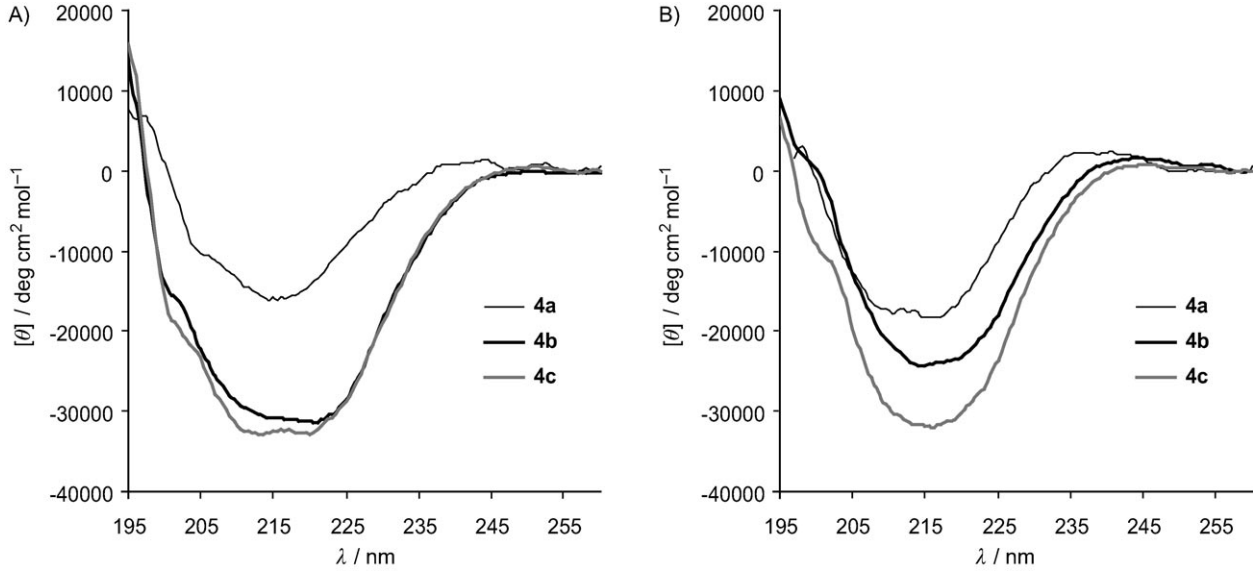


Figure 1. CD spectra of peptides **4a–c** (20–50 μM, 20 °C) in: A) methanol, and B) H_2O.

garding the *i*+2 position, the side chain of NMePhe residue in peptide **4a** adopts the *gauche*− rotamer around its χ^1 angle, as shown by $^3J_{H\alpha-H\beta}$ coupling constant analysis. In the case of peptides **4b** and **4c**, the same analysis reveals a conformational equilibrium around the χ^1 angle of NMeArg and NMeLys residues between *gauche*− and *trans* rotamers with respective populations of ~3:1.

The 3D structures of peptides **4a–c** (Figure 2) were calculated by restrained molecular dynamics and energy minimization with the DISCOVER program by using interproton distance restraints derived from NOEs and dihedral angle restraints derived from homonuclear vicinal coupling constants measured in methanol solvent. A unique conformation of the backbone was found in the three peptides with (ϕ, ψ) values around (60°, −125°) and (−125°, 55°) for prolinoamino acid and *N*-methylamino acid residues, respectively. The Leu and Phe side chains in peptide **4a** adopt unique orientations and interact with each other through van der Waals interactions, in agreement with several NOE values observed between the aliphatic and aromatic resonances. In peptides **4b** and **4c**, the observed NOEs cannot be satisfied by a single conformation of HTrp and Arg or Lys side chains. Cation–π interactions are observed in different conformers of the NMR structure family and are experimentally confirmed by the up-field shifts of Arg and Lys side chain protons together with weak NOE correlations observed between indolic protons of $P^c{}_3$HTrp and side chain protons of the cationic residues.

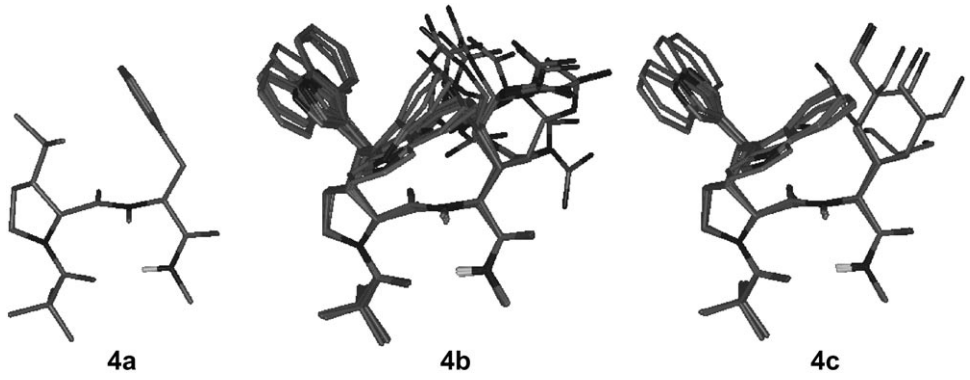


Figure 2. NMR structures of peptides **4a–c** obtained from NMR spectroscopy data collected in methanol solvent. The low energy minima structures corresponding to different side chain conformers were superimposed by best fitting of the backbone heavy atoms.

It can be concluded from CD and NMR spectroscopy data that peptides **4b** and **4c** adopt a stable type II′ β-turn conformation in both methanol and aqueous solutions. Such a β-turn structure was also observed by NMR spectroscopy of peptide **4a** in methanol and the similarity of the CD signatures in both solvents strongly supports a similar type II′ β-turn conformation of peptide **4a** in water. Therefore, we have shown that heterochiral peptide sequences incorporating a prolinoamino acid and an *N*-methylamino acid in positions *i*+1 and *i*+2 adopt stable β-turn conformations in water. The pyrrolidine scaffold and the N-methylation provide a strong conformational constraint driving type II′ β-turn formation. In addition to type II′ β-turns, type II β-turn peptides could be easily obtained by inverting the C^α configurations of both amino acids.

The incorporation of prolinoamino acid and *N*-methylamino acid enables the introduction of two side chain functionalities in positions *i*+1 and *i*+2 of the β-turn. The pyrrolidine scaffold leads to minimal deviation from ideal staggered rotamers of side chains and enables side chain contacts through van der Waals or π–cation interactions. The χ^1 torsion angle of prolinoamino acid side chain is dependent on the puckering of pyrrolidine ring. In *cis*-prolinoamino acids, the C^β-*exo* ring pucker predominates and causes a unique χ^1 *trans* orientation of the substituent ($\chi^1 \sim -155°$). However, other side-chain orientations might be accessible by using *trans*-prolinoamino acids. Indeed, we have shown previously that the energy difference was smaller between C^β-*exo* and C^β-*endo* ring puckers in the corresponding *trans*-prolinoamino acids, so that both *trans* ($\chi^1 \sim 155°$) and *gauche*+ ($\chi^1 \sim 90°$) conformations around the χ^1 angle might be populated.[9] The side-chain orientations of the *N*-methylamino acid in position *i*+2 are restricted to *gauche*− and *trans* orientations around the χ^1 angle, the *gauche*+ rotamer being destabilized by unfavorable interaction with the *N*-methyl group. The structures of peptides **4b** and **4c** were compared to bioactive conformations that are found in tendamistat and somatostatin, respectively. For the NMR structure family of peptide **4c**, several low energy conformers fit well with the conformation of a somatostatin analogue (Figure S5 in the Supporting Information). In the case of peptide **4b**, the side-chain positions deviate more significantly from those in tendamistat because the *gauche*+ conformer of the Arg residue in position *i*+2 of the tendamistat β-turn was not observed in the NMR structures of **4b**.

In conclusion, prolinoamino acids represent valuable peptidomimetic tools that can be directly incorporated in peptide sequences and can mimic most side chains of proteinogenic amino acids. They can be used to stabilize β-turns in water that retain the side-chain functionalities on both *i*+1 and *i*+2 positions of β-turns. The heterochiral prolinoamino acid/*N*-methylamino acid sequence enables the design of short β-turn mimetics and can be easily incorporated in longer β-hairpin peptides.

Experimental Section

All experimental details can be found in the Supporting Information.

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