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Can *N*-methylated amino acids serve as substitutes for prolines in conformational design of cyclic pentapeptides?‡

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The incorporation of proline into cyclic peptides seems to be the most promising way to induce β-turn structures. Recently, however, it was shown that *N*-methylated amino acids might be even better suited than proline for introducing turn structures. Another property of proline, the ability to effect *cis*-peptide bonds, has also been reported for *N*-methylated amino acids. These findings raise the question if it might be possible to replace a proline by an *N*-methylated amino acid without altering the desired conformational features. The most important benefit of replacing proline by an *N*-methylated residue is that one recovers the side-chain functionalities, which could be used for enhancing binding selectivity, or to tune a cyclic peptide concerning its pharmacological properties.

Here, we compare cyclic peptides containing one or two prolines or *N*-methylated alanines and a combination of both with respect to preferred conformations and *cis*-peptide bonds. In addition, the positions have been investigated where an *N*-alkylated amino acid has to be incorporated to mimic structural aspects usually introduced by proline residues. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: *N*-methylated peptides; *N*-methylated amino acids; proline-containing peptides; cyclic peptides; peptide conformation; tertiary amide bond

Introduction

The presence of a tertiary amide bond both in *N*-methylated amino acids and proline-constituting peptides tempts one to consider that their corresponding stereo impact on the peptide backbone is similar. Although vivid explanations are available on the factors influencing the conformation of proline [1–11] and *N*-methylated amino acids containing peptides [12–15], there is a lack of a direct experimental comparison of peptides with *N*-methylated amino acids or prolines in peptides, with otherwise identical constitution and configuration. It is known that proline markedly influences protein architecture due to its constrained five-membered pyrrolidine ring [16] and acts as a reverse turn inducer in designed cyclic protein–epitope mimetics [17–21]. On the other hand, *N*-methylated amino acids are also able to introduce turn structures [21] into cyclic peptides [15,19,22]. It was shown that both proline [23–26] and *N*-methylated amino acids [13,15,27] have the potential to introduce *cis*-peptide bonds into peptide sequences. However, in contrast to L-proline in which the φ angle is constrained to *ca* -60° due to the pyrrolidine ring, *N*-methylated L-amino acids have much more flexibility, as the φ angle can vary between -100 and $+60^\circ$ [13]. Hence, it is not *a priori* predictable if mutual substitution of these structural units has an identical influence on the conformation.

Although *N*-methylation of amide bonds has been used in peptide chemistry for nearly 100 years [28], introduction of proline or ring-derivatized prolines in peptidic sequences was the most popular method to induce turns or *cis*-peptide bonds in peptides since preparation of *N*-methylated amino acids on solid support came along with huge problems in their synthesis. However, ring-derivatized prolines often need extensive synthesis, or do not adequately force a molecule into a single preferred conformation [29,30,31]. Nowadays, the synthetic difficulties concerning *N*-methylated amino acids have been overcome and peptides including them are easily accessible [32,33]. Recently, *N*-alkylated peptides found considerable interest in connection to the so-called peptoids [34,35]. Therefore, the question arose if it might be possible to replace a proline with an *N*-methylated amino acid without perturbing a favored conformation. In this case, one regains the side-chain functionality which is lost in proline-type amino acids because of the five-membered pyrrolidine ring. This side-chain could subsequently be used to improve the activity, selectivity or can act as a pharmacophore in the peptide. Furthermore, it has been recently shown that *N*-methylation of peptide bonds could even increase the biological activity, receptor selectivity, enzymatic stability, and could turn orally unavailable peptides into bioavailable potential drug candidates [14,22,36].

For our comparison, we chose alanine as a template for *N*-methylation (because it is the simplest amino acid bearing stereo information) and screened the positions of *N*-methylated alanine (MeAla) or proline in cyclic pentapeptides (see Figure 1). Afterwards, conformational equilibria were investigated by NMR spectroscopy. The use of alanine is reasonable, as in contrast to

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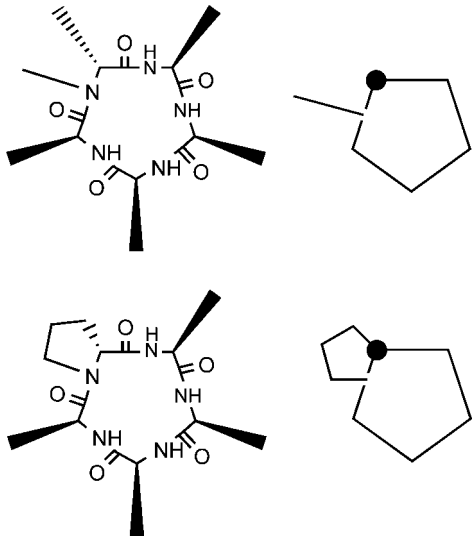

Figure 1. *c*(**Mea**AAAA) and *c*(**p**AAAA) shown as regular and schematic structures. The D-residue is marked as a black dot in the upper left corner.

aromatic residues [37,38] or β-branched amino acids [39–41], the small side-chain of alanine has no further influence on the backbone conformation.

Materials and Methods

General

Tritylchloride polystyrol (TCP) resin (0.94 mmol/g) was purchased from PepChem (Tübingen, Germany). Coupling reagents and amino acid derivatives were purchased from Merck Biosciences (Läufelfingen, Switzerland), Perseptive Biosystems GmbH (Hamburg, Germany) and Neosystem (Strasbourg, France). All other reagents and solvents were purchased from Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany), and Fluka (Neu-Ulm, Germany) and were used as received. Standard syringe techniques were applied for transferring dry solvents. Reactions on solid support were performed in filter columns (2 mL) from Abimed. RP-HPLC analyses were conducted on Amersham Pharmacia Biotech instruments using Omnicrom YMC columns (analytical: 2 mm × 250 mm, 5 µm C18, 1 mL/min), applying different 30 min linear gradients of water (0.1% TFA) and CH_3CN (0.1% TFA) and detection at 220 nm. Mass spectra (ESI) were recorded on an LCQ Finnigan instrument. Explanations for abbreviations that are not explicitly defined in the following sections can be found in Ref. 42.

Peptide Synthesis

All peptides were manually assembled on a 2-chlorotritylchloride resin applying Fmoc-based SPPS. Loading of the resin with 1 mmol amino acid was achieved using 1.1 mmol of Fmoc-Xaa-OH according to the published procedure [43]. Synthesis was carried out using 2.5 equivalents of Fmoc-Xaa-OH : TBTU/HOBt and DIPEA in *N*-methyl-2-pyrrolidone (NMP) for coupling of normal amino acids, HATU/HOAt and DIPEA in NMP for *N*-alkylated amino acids, and 20% piperidine in DMF for cleavage of the Fmoc moiety. Treatment of the resin with acetic acid (HOAc)/TFE/DCM (1/1/3) for 1 h and removal of the solvent yielded the linear peptides [44]. Addition of 3 equivalents of DPPA and 5 equivalents of solid $NaHCO_3$ and subsequent stirring in DMF for 24 h gave the crude cyclic peptides after evaporation of the solvent. Purification of the cyclopeptides was achieved by RP-HPLC on C18 columns. *N*-methylated alanine was synthesized according to the procedure of Freidinger *et al*. [45] and *N*-methylated lysine according to Biron *et al*. [33].

NMR Spectroscopy

All spectra were recorded at 300 K on a 500 MHz Bruker DMX spectrometer (Bruker, Karlsruhe, Germany) in d_6-DMSO and were processed using XWINNMR or TOPSPIN (Bruker) and analyzed with either XWINNMR, TOPSPIN, or SPARKY [46]. The assignment of proton and carbon resonances followed a strategy described in literature [47]. Sequential assignment was accomplished by through-bond connectivities from heteronuclear multiple bond correlation (HMBC) [48] spectra, whereas *N*-methyl groups served as starting point. Connectivities were proved by interresidual scalar couplings, e.g. between carbonyl carbons and adjacent amide protons. TOCSY spectra [49] were recorded with a mixing time of 60 ms, ROESY spectra [50] with a mixing time of 150 ms, thus avoiding unwanted effects caused by spin diffusion. Several compounds show more than one conformation that are in slow exchange at the NMR time scale. Conformational exchange was proven by detection of inverted signal signs in ROESY spectra [51]. Evidence of *cis*-peptide bonds in proline-containing peptides was achieved using carbon shifts in DEPT45 spectra [52]. The ratio of different conformational populations was determined via the integrals of amide and H^{α}-signals in ^1H-1D spectra.

Computational Methods

Proton distances were calculated according to the isolated two-spin approximation from volume integrals of ROESY spectra [53]. No ROE offset correction was performed since biasing offset effects at the field strength used in this study are rather small. The integrated volumes of ROE crosspeaks were converted to proton–proton distances with the help of calibration to an averaged alanine Hα–Hβ* distance as reference (2.45 Å; including pseudoatom correction). Upper and lower distance restraints were obtained by adding and subtracting 10% to the calculated experimental values, thus accounting for experimental errors and simulation uncertainties. Metric matrix DG calculations were carried out with a (slightly modified version) distance geometry program utilizing random metrization [54]. Experimental distance restraints which are more restrictive than the geometric distance bounds (holonomic restraints) were used to create the final distance matrix. All structure templates were first embedded in four dimensions and then partially minimized using conjugate gradient minimization followed by distance-driven dynamics (DDD) [55] wherein only distance constraints were used. The DDD simulation was carried out at 1000 K for 50 ps with a gradual reduction in temperature over the next 30 ps. The DDD procedure utilized holonomic and experimental distance constraints plus a chiral penalty function for the generation of violation energies and forces. A distance matrix was calculated from each structure, and the EMBED procedure was used to compute Cartesian coordinates in three dimensions [54]. One hundred structures were calculated for each peptide, and >90% of the structure bundle of each peptide did not show any significant violations (>0.2 Å). MD calculations were carried out with the program DISCOVER, using the CVFF force field [56]. Structures resulting from DG calculations were placed in a cubic box with a vector length of 3.0 nm and soaked with DMSO. Intramolecular distances

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of the peptides were kept constant according to the experimental values. After energy minimization using steepest descent and conjugate gradient algorithms, the system was gradually heated in 50 K steps (equilibration time at each temperature was 2 ps) starting from 10 K, each by direct scaling of velocities. The system was equilibrated for 50 ps with temperature bath coupling (300 K). Configurations in the subsequent production runs (150 ps) were saved every 100 fs. Finally, 150 ps free MD simulations at 300 K were carried out in order to prove that no significant structural changes occur when no distance restraints are present during the simulation.

Results and Discussion

The first comparison where only one *N*-methylation or one proline was varied led to the promising result that, in nearly every case, *N*-methylated alanine and proline containing peptides show similar results with respect to the highest populated conformation. In addition, both reveal a good agreement concerning the *cis/trans*-ratios of amide bonds (see Figure 2). Especially when introduced at the *i*-position (the position of the D-residue in the cyclic peptide; please note that '*i*' is also often used as starting residue in turn structures) Me-D-alanine and D-proline both revealed only one highly populated conformation and both peptides are in the all-*trans*-conformation. Both structures possess a β-turn in the upper part which is introduced by the D-residue and a γ-turn in the lower part of the molecule, whereas the peptide bond between Ala2 and Ala3 is known to flip around its adjacent φ and ψ dihedral angles (flip of $\sim 180^{\circ}$). As both structures have been extensively discussed in literature there is no need to go into further detail [5,8,10,11,13,15].

Also when being introduced in the $i+1$ position, the all-*trans*-conformation is preferred in both cases with a population of 85% for the alanine and 92% for the proline peptide. It is worth mentioning that both peptides show a nearly identical structure that enables the replacement of one with another without changing the backbone conformation (see superposition in Figure 3).

When the *N*-alkylated residue is introduced in the $i+2$ or in the $i+3$ position, the all-*trans*-conformation is disfavored. Especially for proline, a *cis*-peptide bond is preferred at the position of the *N*-alkylation finally resulting in a conformation with a population of about 90% for *c*(aA**P**AA) (in general, a small letter indicates a D-residue) with a *cis*-peptide bond at the position of proline. Being introduced at the $i+4$ position, the all-*trans*-conformation is again favored. However, for *c*(aAAA***MeA***), three conformations with a population of 84/13/3 are present where the two main conformations possess an all-*trans*-conformation. *c*(aAAA**P**) also shows three conformations with a ratio of 75/19/6 where the main conformation also exhibits an all-*trans* arrangement.

To summarize, a MeAla has the ability to replace a single proline in cyclic pentapeptides without having an undesired impact on the backbone conformation. Furthermore, the *N*-methylated amino acid might be preferred because one regains the side-chain functionality which is lost when using proline. To prove this hypothesis, we replaced the Me-D-alanine in *c*(***Mea***AAAA) with an *N*-methylated D-lysine and succeeded in getting *c*(***Mek***AAAA) with a population of 100% (see Figure 4).

Another possibility for introducing functionalities into peptides is *N*-alkylation. For example, transitional sulfonamidic protection of the amine to be alkylated and subsequent alkylation under Mitsunobu conditions or halide displacement has been recently described by Demmer *et al*. [57].

As in the case of *c*(aAA***MeA***A) and *c*(aAA**P**A), the differences between the all-*trans*-conformation and the conformation con-

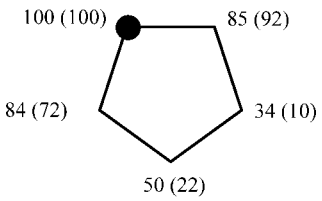

Figure 2. Populations of the all-*trans*-conformations for the Ala-peptides with one Me-alanine (proline) at the different positions in percentage (%). The remaining percentages are the populations containing a *cis*-peptide bond between the *N*-alkylated amino acid and the preceding one. The D-residue is marked as a black dot in the upper left corner.

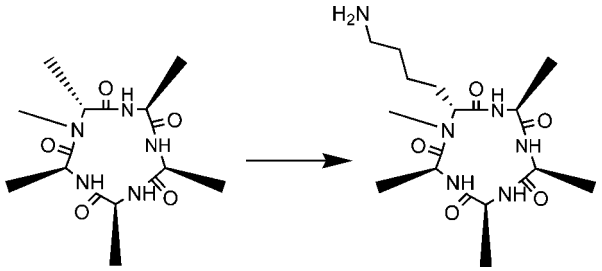

Figure 4. *c*(***Mea***AAAA) that shows an all-*trans* population of 100% was used as template for *c*(***Mek***AAAA). The generated peptide also revealed a population of 100% which serves as a proof that alanine can be used as a template for amino acids with a longer side-chain.

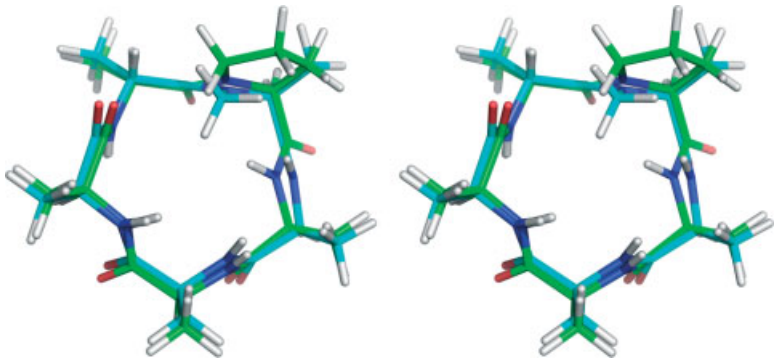

Figure 3. Stereo view of the superposition of *c*(a**MeA**AAA) and *c*(a**P**AAA). Small characters refer to a D-residue.

taining one *cis*-peptide bond were the lowest, we decided to fix the *N*-alkylated amino acid at the $i + 3$ position and to rotate another proline or MeAla through the different positions of the cyclic pentapeptide. Thus, we investigated if the presence of a second *N*-alkylated amino acid alters the *cis*/*trans* equilibrium, and finally, tested the potential occurrence of new preferred conformations.

While sequences containing two prolines are not well suited for application in medicinal chemistry as two of the possible side-chain functionalities are lost, the incorporation of two *N*-methylated amino acids is preferable because all side-chains can be used to gain activity or selectivity, respectively.

As can bee seen in Figure 5, there is a high population difference between *c*(**p**AA**P**A) and *c*(***Mea***AA***MeA***A) since the latter shows an all-*trans* arrangement with a population of about 70%, while in *c*(**p**AA**P**A) the conformation with one *cis*- and all other peptide bonds in *trans*-configuration is the highest populated conformation (~65%). For the two cyclic pentapeptides *c*(a***MeA***A***MeA***A) and *c*(a**P**A**P**A), the peptide with *N*-methylations prefers a highly populated conformation where one amide bond is in *cis* and the other *N*-alkylated amide bond is in *trans*-conformation. In contrast, the cyclic peptide with two prolines shows a main population with two *cis*-amide bonds at the positions of the prolines. In the case of *c*(aA***MeA******MeA***A) and *c*(aA**PP**A), the all-*trans*-conformation is preferred for the peptide with the *N*-methylations while for the proline–peptide, the conformation with one *cis*-amide bond is favored. For *c*(aAA***MeA******MeA***) and *c*(aAA**PP**), both compounds show a preferred conformation which is highly populated bearing one *cis*-peptide bond between $i + 3$ and $i + 4$ (see superposition in Figure 6).

In conclusion, structural features of a cyclic peptide are no longer predictable when two *N*-methylations or two prolines are present since peptides bearing more than one *N*-methylated amino acid often differ in their conformation compared to the proline-containing analogs. One benefit of this finding is the fact that replacement of proline by an *N*-methylated amino acid most often results in a different structure, which could be useful for the search for better candidates to fit into a binding pocket.

Finally, we were interested in the conformation of cyclic peptides when both, a proline and an *N*-methylated amino acid, are incorporated. In this study, we fixed proline at the $i + 3$ position and varied the position of the *N*-alkylation (and vice versa). The obtained set of compounds showed preferred conformations,

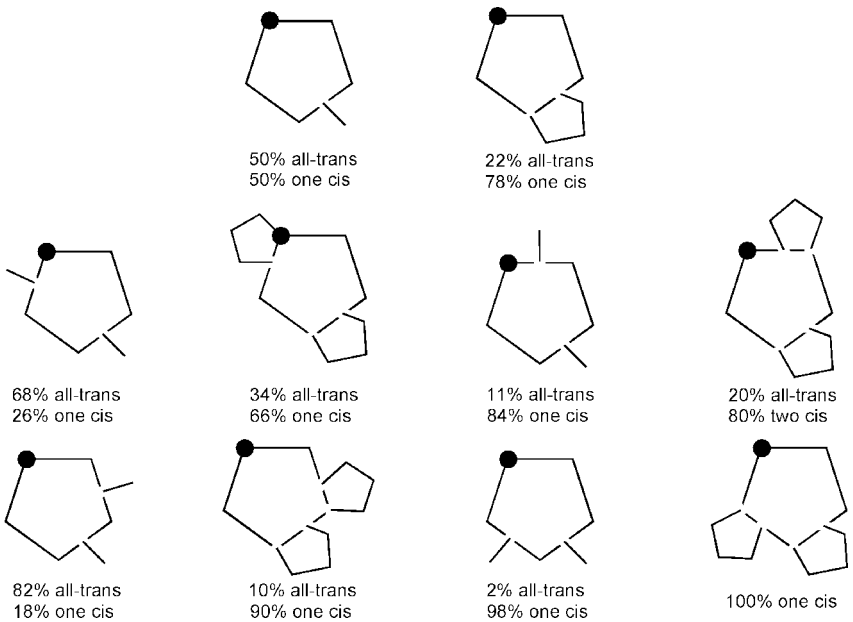

Figure 5. Populations of the different conformations. The *cis*-amide bond occurs between an *N*-alkylated amino acid and the previous one. Two peptides revealed more than two conformations at the NMR time scale. In that case, only the two major conformations are given, leading to percentage statements that do not sum up to 100%.

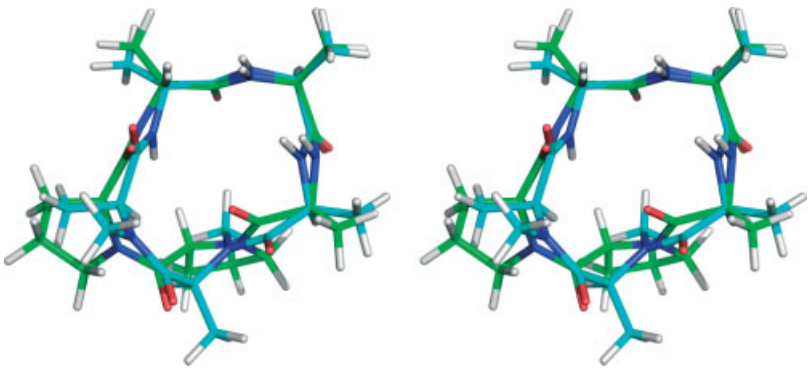

Figure 6. Stereo view of the superposition of *c*(aAA**MeAMeA**) and *c*(aAA**PP**). Small characters indicate a D-residue.

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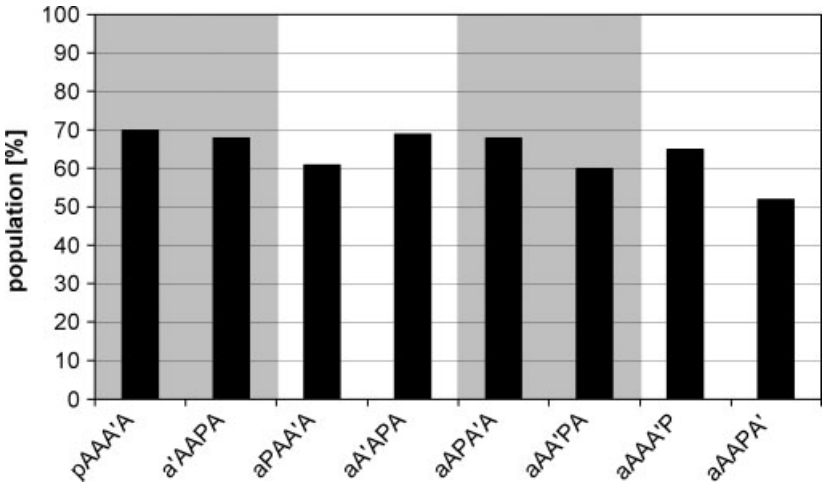

Figure 7. Comparison of peptides, which contain one proline (or D-proline) and one *N*-methylated alanine (A′ for Me-L-Ala and a′ for Me-D-Ala). They are grouped in pairs in which both units are interchanged. The populations of the dominating conformations are given.

whereas the highest populated conformation of each peptide was in the range of 65–70% (see Figure 7). Hence, we decided not to go into further detail about *cis*- or *trans*-amide bond distributions as peptides that show such poor population preferences are of limited interest in medicinal chemistry.

Summing up, we were able to show that cyclic peptides containing one proline or one *N*-methylated amino acid reveal comparable conformational features. This allows substituting a proline residue by an *N*-methylated amino acid without changing the overall conformation. Proline is often used as inducer of distinct conformations, e.g. turns or breaking helices. For conformational design it is also important to identify strongly preferred structures. Hence, the use of *N*-methylated amino acids allows for bringing additional side-chain functionality into conformationally restricted (cyclic) peptides, and therefore opens new perspectives for drug design. However, care should be taken when two prolines are to be replaced by *N*-methylated amino acids because the resulting structures might differ in their conformation. Finally, incorporation of one proline and one *N*-methylated amino acid into cyclic pentapeptides seems not to be useful since no strong preference of one single conformation was observed in the cases demonstrated here.

Supporting information

Supporting information may be found in the online version of this article.

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