# Comparison of proline and *N*-methylnorleucine induced conformational equilibria in cyclic pentapeptides

Hardy Weißhoff[a], Torsten Wieprecht[b], Peter Henklein[c], Cornelius Frömmel[c,*], Christof Antz[d], Clemens Mügge[a]

[a] *Humboldt Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I, Institut für Chemie, Hessische Str. 1–2, 10115 Berlin, Germany*
[b] *Forschungsinstitut für Molekulare Pharmakologie, A.-Kowalke Str. 4, 10315 Berlin, Germany*
[c] *Humboldt Universität zu Berlin, Charité, Institut für Biochemie, Hessische Str. 3–4, 10115 Berlin, Germany*
[d] *Max Planck Institut für Medizinische Forschung, Jahnstr. 29, 69120 Heidelberg, Germany*

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**Abstract The cyclic, imido acid containing pentapeptides cyclo(Asp-Trp-(NMe)Nle-Asp-Phe) (cpp[NMeNle$^3$]) and cyclo(Asp-Trp-Pro-Asp-Phe) (cpp[Pro$^3$]) have been investigated by $^1$H-NMR spectroscopy in DMSO and by restrained molecular dynamics methods. The spectra indicate the existence of at least four *cis/trans* isomers for cpp[NMeNle$^3$] and two *cis/trans* isomers for cpp[Pro$^3$]. In addition to the imido peptide bonds, cpp[NMeNle$^3$] shows *cis/trans* isomerization of the Asp$^4$–Phe$^5$ and Phe$^5$–Asp$^1$ peptide bonds whereas only the Phe$^5$–Asp$^1$ peptide bond isomerizes in the Pro-containing peptide. In cpp[Pro$^3$] all *cis* bonds are centred in βVIb turns. Also, cpp[NMeNle$^3$] prefers backbone angles around the *cis* bonds which are rather similar to the angles of a βVIb turn. The higher number of *cis/trans* isomers and slight deviations in the backbone angles of comparable isomers of both peptides are caused by an enhanced flexibility of cpp[NMeNle$^3$] due to the possibility of the ϕ-(NMe)Nle rotation.**

*Key words:* Cyclic peptide; *cis/trans* isomerization; β turn; NMR; Restrained molecular dynamics

## 1 Introduction

Conformational investigations of cyclic peptides are a general tool for the study of regular turn structures [1,2]. Regular turns are in addition to the α-helix and β-sheet the third type of secondary structures observed in proteins, comprising on average a quarter of the residues [3]. The study of cyclic peptides provides insight into backbone angles, intramolecular distances and side chain orientations of turn structures and, moreover, into the relationship of primary structure and resulting secondary structure. Cyclic peptides are therefore an appropriate model system for the investigation of regular turns. Proline is known as a turn-promoting amino acid [4]. In the *trans* conformation proline is found frequently in the $i+1$ position of βI and βII turns. An additional important property of proline is the ability to form a *cis* peptide bond. *cis*-Proline has been shown to prefer the $i+2$ position of βVIa and βVIb turns. In contrast to the βVIa turn the latter turn is not stabilized by a hydrogen bond [5,6].

*N*-methyl amino acids also have, because of their voluminous *N*-methyl group, the ability to induce *cis* peptide bonds. To our knowledge, there are no investigations dealing with the conformational influence of these amino acids in cyclic peptides.

In this work we synthesized and investigated the conformations of the cyclic pentapeptides cyclo(Asp-Trp-(NMe)Nle-Asp-Phe) (cpp[NMeNle$^3$]) **I** and cyclo(Asp-Trp-Pro-Asp-Phe) (cpp[Pro$^3$]) **II** by $^1$H-NMR spectroscopy in DMSO and rMD calculations. The sequences are derived from the naturally occurring peptide hormone CCK-4, Trp-Met-Asp-Phe-$NH_2$. Our aim was to compare the influence of proline and *N*-methylnorleucine on the *cis/trans* equilibrium and on the preferred conformations of the isomers.

## 2. Materials and methods

### 2.1. Peptide synthesis

For the preparation of the peptides we synthesized the fully protected pentapeptides using conventional Fmoc chemistry on *o*-chlorotrityl resin (Bachem). The peptides were cleaved from resin using 80% HFIP in $CH_2Cl_2$. The cyclization of the protected peptides was carried out in $10^{-3}$ M solution of DMF with HAPyU [7] and DIEA. For deprotection of the side chains the crude protected peptide was dissolved in 95% TFA. The purities of cyclic peptides were analysed by HPLC and mass spectrometry.

### 2.2. NMR measurements

Peptides were first dissolved in $H_2O$, the pH adjusted to 2.0, and then lyophilized. The samples were redissolved in 0.5 ml DMSO-$d_6$ to give a final concentration of 5 mM.

The 1D and 2D NMR spectra were recorded at 298 K on a Bruker AMX 600 NMR spectrometer interfaced to an ASPECT computer using UXNMR software package [8]. All $^1$H chemical shifts are referred to internal DMSO (2.49 ppm). $^3J_{NH\alpha}$ coupling constants were estimated from 1D $^1$H spectra, if not mentioned otherwise. All 2D experiments have been acquired in the phase-sensitive mode with quadrature detection in both dimensions, by use of TPPI. Typically 512 experiments of 32–80 scans each were performed: relaxation delay 1.5 s, complex size 2 K, spectra width in $F2$ and $F1$ 7250 Hz, zero filling in $F1$ to 1 K, apodization in both dimensions with squared sinebell shifted by π/2. TOCSY spectra were acquired with a 9 kHz MLEV-17 spinlock and 54 ms mixing time. ROESY spectra were recorded with a spinlock of 2.5 kHz and a mixing time of 120, 150 and 180 ms. The mixing times of NOESY experiments were varied in the range of 50 to 400 ms. NOESY crosspeaks were integrated by the AURELIA software [9].

### 2.3. Molecular dynamics calculations

The presented peptide structures were generated via in vacuo rMD simulations on a CONVEX 210 computer and on an IRIS 4D work-

* Corresponding author. Fax: (49) (30) 28468600.

*Abbreviations:* 1D/2D, one- and two-dimensional; DIEA, *N*-ethyldiisopropylamine; DQF-COSY, double quantum filtered correlated spectroscopy; Fmoc, 9-fluorenylmethyloxycarbonyl; HAPyU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethylenuroniumhexafluorophosphate; HFIP, hexafluoroisopropanol; (NMe)Nle, *N*-methylnorleucine; NOESY, nuclear Overhauser enhancement spectroscopy; PEM, Powell energy minimization; rMD, restrained molecular dynamics; ROESY, rotating frame Overhauser spectroscopy; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; TPPI, time proportional phase increment


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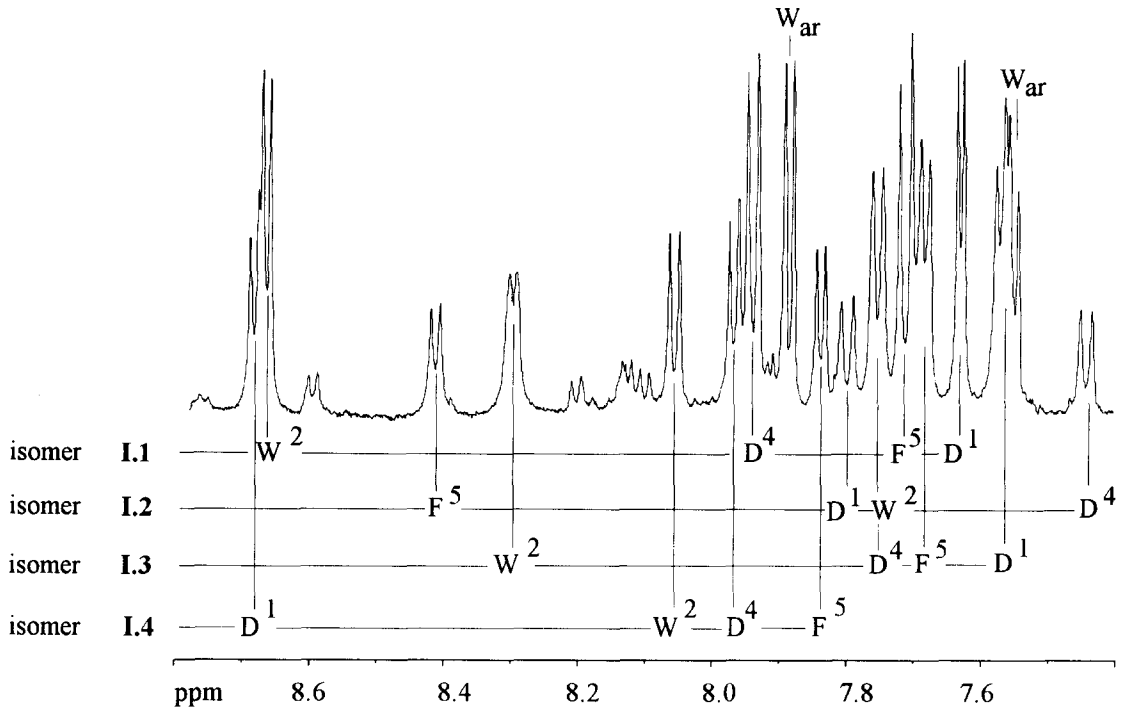


Fig. 1. The NH region of the 600 MHz $^1$H spectra of cpp[NMeNle$^3$] in DMSO-$d_6$ measured at 298 K. $W_{ar}$ denotes peaks due to aromatic Trp protons.

station using the programs XPLOR 3.1 [10] and QUANTA [11]. The calculations were carried out by considering 22, 17, 18 and 18 NMR derived interproton distances for isomer **I.1**, **I.2**, **I.3** and **I.4**, respectively, of cpp[NMeNle$^3$] and 23 and 19 NOE based restraints for isomer **II.1** and **II.2** of cpp[Pro$^3$]. Furthermore vicinal coupling constants $^3J_{NH\alpha}$ which show only two results of the modified Karplus equation by Bystrov [12] were also used as restraints.

We generated for each isomer 100 structures from complete random arrays of atoms followed by a PEM and rMD simulation at 300 K. The nonbonded energy cutoff was set to 8 Å.

In order to find starting conformations reflecting as much as possible the restraint geometries, we applied another 1000 steps of PEM with the weighting factor $k_{NOE} = 50$ for the NOE and $k_{CDIH} = 5$ for the dihedral energy term. The factors of all the other energy terms were set to 1 except $k_{VDW} = 0.002$.

All structures were submitted to a subsequent rMD protocol in conjunction with a simulated annealing procedure for a more efficient mapping of the energy surface. We first carried out 8000 steps (size 1 fs) of rMD simulation at a constant temperature of 1000 K. The force constants were the same as mentioned above except for the energy terms that reflect the angle and improper values, which were set to $k_{ANG} = 0.4$ kcal/(mol rad$^2$) and $k_{IMP} = 0.1$ kcal/(mol rad$^2$), respectively.

Table 1
Comparison of the experimental (NOESY) and calculated (rMD) backbone interproton distances of the isomers **I.1** and **I.2** of cpp[NMeNle$^3$] and the main isomer **II.1** of cpp[Pro$^3$]

| Isomer | | **I.1** | | | | **I.2** | | **II.1** | |
|---|---|---|---|---|---|---|---|---|---|
| Proton pair | | $r^{NOE}$ (Å) | $r^{MD}$ (Å) | Proton pair | | $r^{NOE}$ (Å) | $r^{MD}$ (Å) | $r^{NOE}$ (Å) | $r^{MD}$ (Å) |
| Asp$^1$NH | Phe$^5$NH | 2.26 | 2.17 | Asp$^4$NH | Xaa$^3$N* [a] | 2.95 | 2.84 | 2.61 | 2.57 |
| Asp$^1$NH | Asp$^4$NH | 3.51 | 3.65 | Asp$^4$NH | Trp$^2$NH | 3.69 | 3.95 | 3.98 | 4.02 |
| Asp$^1$NH | Trp$^2$NH | 2.73 | 2.99 | Asp$^4$NH | Phe$^5$NH | 2.56 | 2.34 | 2.53 | 2.44 |
| Trp$^2$NH | Asp$^4$NH | 3.01 | 3.12 | Phe$^5$NH | Trp$^2$NH | 3.83 | 4.21 | 3.78 | 4.02 |
| Nle$^3$NMe | Asp$^4$NH | 2.51 | 2.85 | Asp$^1$NH | Trp$^2$NH | overlay | 2.34 | 2.31 | 2.45 |
| Asp$^1$NH | Asp$^1$Hα | 3.05 | 2.85 | Asp$^4$NH | Asp$^4$Hα | 2.68 | 2.99 | 2.76 | 2.99 |
| Asp$^1$NH | Phe$^5$Hα | 3.07 | 3.43 | Asp$^4$NH | Xaa$^3$Hα | 3.25 | 3.22 | n.d. | 3.39 |
| Trp$^2$NH | Trp$^2$Hα | 2.93 | 2.97 | Phe$^5$NH | Phe$^5$Hα | 2.77 | 2.99 | 2.68 | 2.97 |
| Trp$^2$NH | Asp$^1$Hα | 3.77 | 3.61 | Phe$^5$NH | Asp$^4$Hα | 3.51 | 3.60 | 3.43 | 3.60 |
| Trp$^2$NH | Nle$^3$Hα | 3.16 | 3.12 | Phe$^5$NH | Asp$^1$Hα | 3.62 | 3.75 | 3.64 | 3.92 |
| Nle$^3$NMe | Nle$^3$Hα | n.d.[b] | 3.44 | Asp$^1$NH | Asp$^1$Hα | 2.90 | 2.97 | 3.10 | 2.93 |
| Asp$^4$NH | Asp$^4$Hα | 2.93 | 2.99 | Trp$^2$NH | Trp$^2$Hα | 2.71 | 2.99 | 3.03 | 2.98 |
| Asp$^4$NH | Nle$^3$Hα | 2.85 | 3.03 | Trp$^2$NH | Asp$^1$Hα | 3.35 | 3.58 | 3.22 | 3.56 |
| Phe$^5$NH | Phe$^5$Hα | 2.72 | 2.99 | Xaa$^3$N* | Xaa$^3$Hα | n.d. | 3.45 | n.d. | 3.63 |
| Phe$^5$NH | Asp$^4$Hα | 3.77 | 3.60 | Xaa$^3$N* | Trp$^2$Hα | n.d. | 3.89 | 3.89 | 4.20 |
| Trp$^2$Hα | Nle$^3$Hα | 2.08 | 1.81 | Phe$^5$Hα | Asp$^1$Hα | 1.95 | 1.72 | 1.88 | 1.70 |
| Asp$^4$Hα | Phe$^5$Hα | 2.08 | 1.77 | Trp$^2$Hα | Xaa$^3$Hα | 1.82 | 1.65 | 1.85 | 2.02 |

Amino acids overlaid in Fig. 3 are arranged in the same row.
[a]Xaa$^3$ represents amino acids (NMe)Nle$^3$ in isomer **I.2** or Pro$^3$ in isomer **II.1**; N* denotes NMe for (NMe)Nle or Hδ* for Pro.
[b] n.d., not determined.

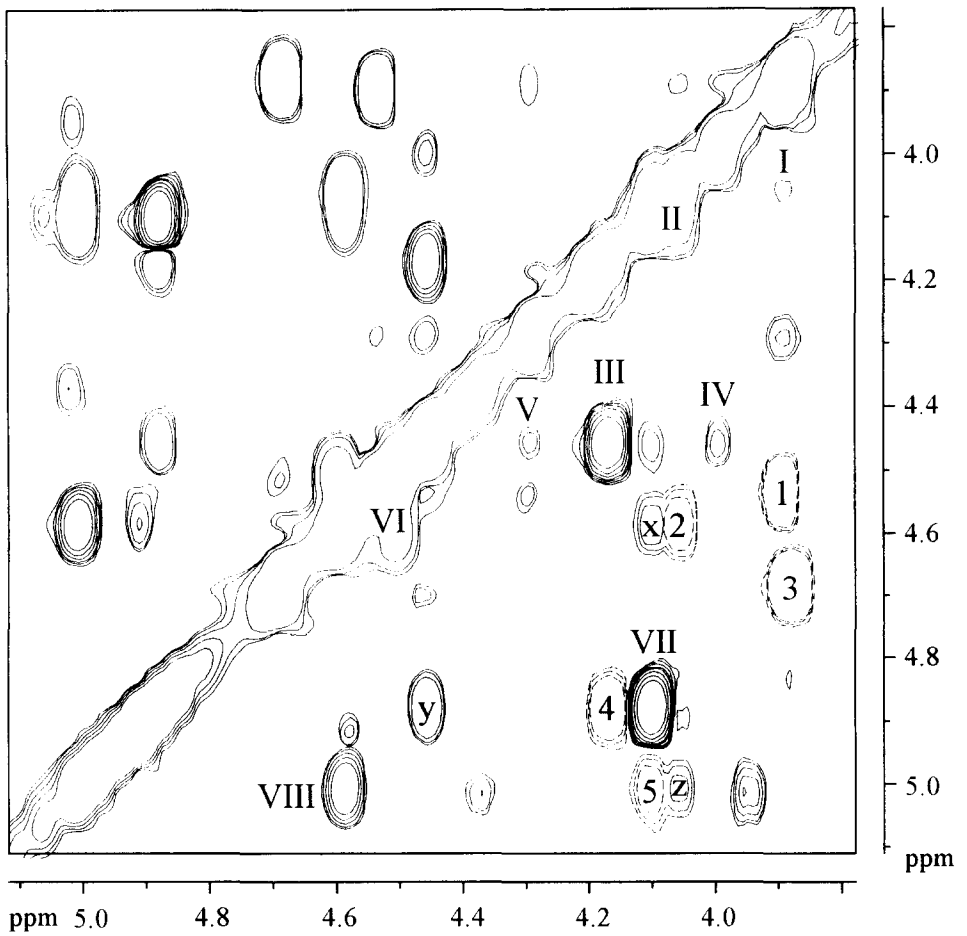


Fig. 2. Hα/Hα region of a 600 MHz ROESY spectrum of cpp[NMeNle$^3$] in DMSO-$d_6$ measured at 298 K. The spectrum was recorded with a mixing time of 180 ms and a spin lock power of 2.5 kHz. Pure NOEs are lettered with Arabic numerals (dashed lines), chemical exchange peaks (EP) with Roman numerals and 'relayed' peaks (RP) are lettered with characters. Unlettered peaks are due to a fifth minor populated isomer which could not be analysed. **I** EP Nle$^3$**(I.3)**/Nle$^3$**(I.1)**, **II** EP Nle$^3$**(I.2)**/Nle$^3$**(I.3)**, **III** EP Asp$^1$**(I.3)**/Asp$^1$**(I.2)**, **IV** EP Asp$^1$**(I.3)**/Asp$^1$**(I.4)**, **V** EP Asp$^1$**(I.3)**/Asp$^1$**(I.1)**, **VI** EP Asp$^4$**(I.2)**/Asp$^4$**(I.3)**, **VII** EP Phe$^5$**(I.2)**/Phe$^5$**(I.3)**, **VIII** EP Trp$^2$**(I.2)**/Trp$^2$**(I.3)**, **1** NOE Trp$^2$**(I.1)**/ Nle$^3$**(I.1)**, **2** NOE Trp$^2$**(I.3)**/Nle$^3$**(I.3)**, **3** NOE Asp$^4$**(I.1)**/Phe$^5$**(I.1)**, **4** NOE Phe$^5$**(I.2)**/Asp$^1$**(I.2)**, **5** NOE Trp$^2$**(I.2)**/ Nle$^3$**(I.2)**, **x** RP Trp$^2$**(I.3)**/ Nle$^3$**(I.2)**, **y** RP Phe$^5$**(I.2)**/Asp$^1$**(I.3)**, **z** RP Trp$^2$**(I.2)**/ Nle$^3$**(I.3)**.

The second step consists of a 10 ps rMD simulation under the same conditions as in step 1, except that the asymptote of the NOE energy term was linearly increased from 0.1 to 1.0. We used this scaling in order to find more conformations that reflect better the NOE restraints. In the third step the system was hyperbolically cooled in 50 steps from 1000 K to 100 K. During cooling $k_{VDW}$ was linearly increased from 0.002 to 1.0. This step yields conformations which agree well with all experimental restraints. In the last step, the 10 structures with the lowest energies were used for an additional rMD simulation which consists of 90000 integration steps (size 1 fs) at 300 K. All weighting factors of force constants were set to 1.0 and the Coulomb energy term with a dielectrical constant of ε=48 for DMSO was employed. The first 30 ps were used to equilibrate the system and the following 60 ps served for the analysis of the trajectory. All structures were finally relaxed with a 20000 step PEM (including Coulomb interaction).

## 3 Results

### *3.1. Resonance assignment and isomer analysis*

The occurrence of more than 20 NH resonances in the 1D proton spectrum of cpp[NMeNle$^3$] is very unusual for a pentapeptide with only four NH-containing amino acids (Fig. 1). Employing the exchange peaks in the ROESY spectra the first suspicion of an impurity could be ruled out by identifying five different isomers in slow conformational exchange on the NMR time scale. The strong Hα-Hα ROEs (Fig. 2) proved that the multitude of resonance signals is caused by a complex *cis/trans* equilibrium. Only four of the five isomers are in a population sufficient for conformational analysis. The $^1$H-NMR spectrum of cpp[Pro$^3$] shows only two different sets of resonance lines, reflecting a less complex equilibrium of isomers.

Chemical shift assignments for both peptides were performed by the combined use of DQF-COSY and ROESY experiments [13]. A great difficulty in assignment of cpp[NMeNle$^3$] arises from the existence of 'relayed' peaks (Fig. 2), which suggest ROE connectivities between protons of different isomers. These peaks (negative in ROESY spectra) arise from ROE transfer combined with conformational exchange between two isomers within the spinlock time of the experiment. Therefore, the best way to assign the spin systems to the different isomers was the consequent utilization of the positive exchange peaks.

Employing the Hα-Hα NOEs the *cis/trans* isomerism of

Table 2
$^3J_{NH\alpha}$ coupling constants, dihedral angles calculated from coupling constants and backbone dihedral angles obtained from the lowest energy rMD structures of isomers of cpp[NMeNle$^3$] and cpp[Pro$^3$]

| Isomer | Amino acid | NMR $^3J_{NH\alpha}$ | Calculated[a] $\phi$ | $\phi$ | rMD $\psi$ | *cis/trans* | Turn type |
|---|---|---|---|---|---|---|---|
| | cpp[NMeNle$^3$] | | | | | | |
| **I.1** | Asp$^1$ | 5.3 | −72 | −72 | −67 | *trans* | |
| | Trp$^2$ | 7.0 | −158 | −146 | 81 | *trans* | βVIb; *i*+1 |
| | (NMe)Nle$^3$ | – | – | −128 | 19 | *cis* | βVIb; *i*+2 |
| | Asp$^4$ | 9.5 | −142 | −129 | 85 | *trans* | βVIb; *i*+1 |
| | Phe$^5$ | 10.6 | −110 | −104 | −11 | *cis* | βVIb; *i*+2 |
| **I.2** | Asp$^1$ | 10.8 | −114 | −94 | −33 | *cis* | βVIb; *i*+2 |
| | Trp$^2$ | 10.6 [b] | −110 | −114 | 121 | *trans* | βVIb; *i*+1 |
| | (NMe)Nle$^3$ | – | – | −94 | 9 | *cis* | βVIb; *i*+2 |
| | Asp$^4$ | 10.0 | −102 | −115 | −61 | *trans* | |
| | Phe$^5$ | 8.0 | −153 | −139 | 85 | *trans* | βVIb; *i*+1 |
| **I.3** | Asp$^1$ | 7.6 | −85 | −89 | −70 | *trans* | |
| | Trp$^2$ | 5.9 | −165 | −155 | 105 | *trans* | βVIb; *i*+1 |
| | (NMe)Nle$^3$ | – | – | −92 | −19 | *cis* | βVIb; *i*+2 |
| | Asp$^4$ | 7.9 [b] | −87 | −77 | 118 | *trans* | |
| | Phe$^5$ | 7.8 | 79 | 67 | −65 | *trans* | γ |
| **I.4** | Asp$^1$ | 7.2 | −83 | −86 | −42 | *trans* | |
| | Trp$^2$ | 8.8 | −147 | −142 | −61 | *trans* | |
| | (NMe)Nle$^3$ | – | – | −72 | −37 | *trans* | |
| | Asp$^4$ | 8.4 | −150 | −133 | −65 | *trans* | |
| | Phe$^5$ | 7.5 | −84 | −98 | −53 | *trans* | |
| | cpp[Pro$^3$] | | | | | | |
| **II.1** | Asp$^1$ | 10.5 | −108 | −83 | −33 | *cis* | βVIb; *i*+2 |
| | Trp$^2$ | 10.6 | −130 | −129 | 135 | *trans* | βVIb; *i*+1 |
| | Pro$^3$ | – | – | −80 | −5 | *cis* | βVIb; *i*+2 |
| | Asp$^4$ | 10.2 | −105 | −114 | −62 | *trans* | |
| | Phe$^5$ | 9.1 | −145 | −144 | 97 | *trans* | βVIb; *i*+1 |
| **II.2** | Asp$^1$ | 8.9 | −93 | −111 | −53 | *trans* | |
| | Trp$^2$ | 6.4 | −162 | −159 | 118 | *trans* | βVIb; *i*+1 |
| | Pro$^3$ | – | – | −87 | −18 | *cis* | βVIb; *i*+2 |
| | Asp$^4$ | 8.9 | −93 | −85 | −99 | *trans* | |
| | Phe$^5$ | 7.6 | −85 | −50 | −59 | *trans* | |

[a]The Karplus equation modified by Bystrov [12] was used for the calculation. We noted only the solutions which are next to the results of the rMD in the table.
[b]$^3J_{NH\alpha}$ coupling constants were determined from peak separation in DQF-COSY maps for Trp$^2$ **I.2** and Asp$^4$ **I.3**.

both peptides was investigated. The most populated isomer **I.1** of cpp[NMeNle$^3$] (40%) shows two *cis* peptide bonds between Trp$^2$–(NMe)Nle$^3$ and Asp$^4$–Phe$^5$. Surprisingly, one *cis* bond exists between the two non-imido acids Asp and Phe, which usually do not show *cis/trans* isomerization. A similar situation has been found in isomer **I.2** (13%) where two *cis* bonds exist between Trp$^2$–(NMe)Nle$^3$ and between Phe$^5$–Asp$^1$. The third isomer **I.3** of cpp[NMeNle$^3$] (18%) contains only one *cis* peptide bond between Trp$^2$ and (NMe)Nle$^3$, whereas the fourth isomer **I.4** (25%) has all the amide bonds in the *trans* form.

A less complex situation has been observed in cpp[Pro$^3$], characterized by a major isomer (**II.1**, 80%) with two *cis* peptide bonds between Trp$^2$–Pro$^3$ and Phe$^5$–Asp$^1$ and a minor isomer (**II.2**, 20%) with a *cis* bond between Trp$^2$–Pro$^3$.

### 3.2. Conformational analysis

*3.2.1. Isomers containing two cis peptide bonds.* The medium range NOEs and dihedrals $\phi$ derived from the $^3J_{NH\alpha}$ coupling constants of the isomers (Tables 1 and 2) were used as inputs for the rMD calculations. Table 1 shows a comparison of the NOE derived backbone interproton distances for the isomers with two *cis* amide bonds (isomer **I.1**, **I.2** of cpp[NMeNle$^3$] and **II.1** of cpp[Pro$^3$]) and the calculated (rMD) interproton distances for the lowest energy conformations.

For isomer **I.1** of cpp[NMeNle$^3$] the 10 lowest energy structures belong to the same conformational family and the trajectories did not reveal any significant fluctuations during the dynamic, indicating a stable conformation with all the distances within the NOE derived distance ranges. The residues Trp$^2$–(NMe)Nle$^3$ and Asp$^4$–Phe$^5$ show a very similar spatial arrangement around the two *cis* peptide bonds (Table 2) with the main chain torsion angles close to these of a βVIb turn. Because of slight deviation from the ideal turn ($\phi_{i+1} = -120°$, $\psi_{i+1} = 120°$, $\phi_{i+2} = -90°$, $\psi_{i+2} = 0°/150°$ [5,14]) we use the designation βVIb-like turn. Hence, the amino acids around the *cis* peptide bonds occupy the *i*+1 (Trp$^2$, Asp$^4$) and *i*+2 ((NMe)Nle$^3$, Phe$^5$) positions of two βVIb-like turns. Neither turn is stabilized by a hydrogen bond, which agrees with the βVIb turns found in proteins and cyclic peptides [1,5,6]. Also, for isomer **I.2** of cpp[NMeNle$^3$] both *cis* bonds are located in the centre of turns with dihedrals similar to βVIb turns. A comparison such that the (NMe)Nle$^3$ of isomer **I.1** and Asp$^1$

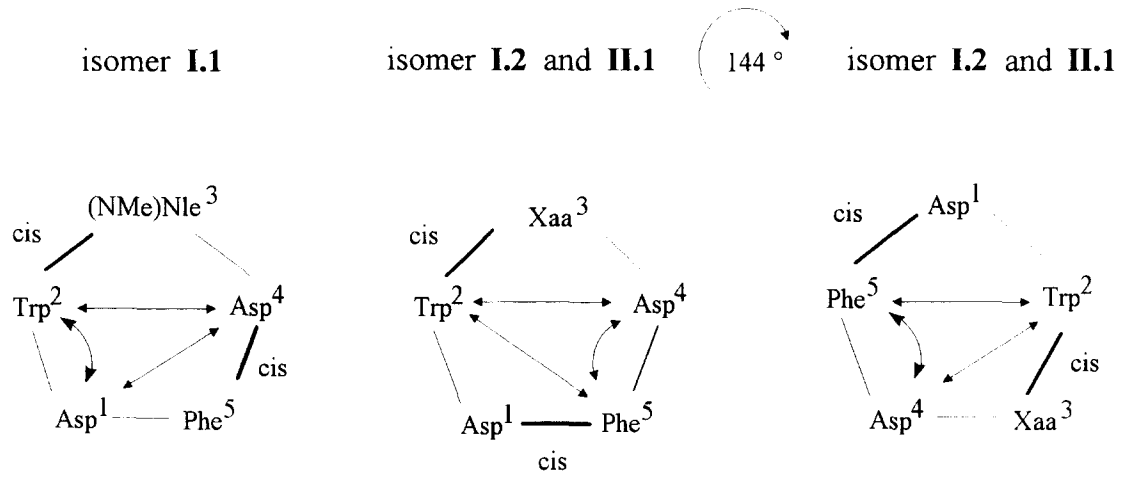


Fig. 3. Schematic presentation of the two *cis* peptide bonds containing isomers **I.1**, **I.2** and **II.1** of the both cyclic pentapeptides. A comparison of the left and right schemes reveals a close relationship between all these isomers as indicated by the same NOE patterns and the same turn structures. Selected NH-NH NOEs are drawn as arrows.

of **I.2** as well as $Phe^5$ of **I.1** and (NMe)$Nle^3$ of **I.2** are superimposed (Fig. 3) shows identical NOE patterns and backbone angles indicating the strong conformational relationship of these isomers (Table 1).

The major isomer of cpp[$Pro^3$] **II.1** shows two *cis* bonds, which are in the same positions as the *cis* bonds of isomer **I.2** of cpp[NMeNle$^3$] (Fig. 3). As recently reported for proline-containing cyclic model pentapeptides [1] the dihedrals of this isomer are identical to the values of a βVIb turn [5] with $Trp^2$–$Pro^3$ and $Phe^5$–$Asp^1$ in the positions $i$+1 and $i$+2.

*3.2.2. Isomers containing one cis peptide bond.* Isomer **I.3** of cpp[NMeNle$^3$] is characterized by a *cis* peptide bond between $Trp^2$ and (NMe)$Nle^3$. The results of rMD calculations (Table 2) reveal that the backbone angles on $Trp^2$ and (NMe)$Nle^3$ are similar to these of a βVIb turn. Isomer **II.2** has the *cis* bond in the same position as isomer **I.3**. It is not surprising that this isomer shows the βVIb turn on $Trp^2$ and $Pro^3$ (Table 2). Also for these two isomers a close conformational relationship can be concluded as additionally indicated by similar NOE patterns and backbone angles.

The other three residues $Asp^4$, $Phe^5$ and $Asp^1$ in these isomers (**I.3** and **II.2**) could theoretically describe three different structural elements, a γ turn on $Phe^5$, an inverse γ turn on $Phe^5$ or an arrangement of $Asp^1$ and $Phe^5$ with both amide protons oriented to one side of the ring plane. The latter structural feature should be characterized by a strong $Asp^1$NH–$Phe^5$NH NOE. Because of the very similar chemical shifts of the $Asp^1$NH and $Phe^5$NH protons of isomer **I.3**, a determination of such a NOE was impossible. The analysis of the rMD results revealed that γ turns with hydrogen bond stabilization between $Asp^1$NH and $Asp^4$CO as well as conformations with $Asp^1$NH and $Phe^5$NH oriented to one side of the ring are consistent with the experimental results.

However, a strong $Asp^1$NH–$Phe^5$NH NOE proved the close contact of these protons in isomer **II.2** of cpp[$Pro^3$] and it must be concluded, that the conformation with the $Asp^1$NH and $Phe^5$NH oriented to the same side of the ring is preferred. In this conformation no hydrogen bond stabilizes the structure.

*3.2.3. All-trans isomer.* Isomer **I.4** of cpp[NMeNle$^3$] was found to be in an all-*trans* conformation. This isomer shows a large number of NH-NH NOEs and only a few other medium range backbone NOEs. The rMD simulation shows the existence of only one conformational family. Interestingly, all φ and ψ angles are negative (Table 2). As a consequence, all carbonyl groups are directed to one side of the ring plane and the NH protons to the opposite side with a slight orientation to the inside of the ring. An analogous structure was recently reported for the all-*trans* peptide c(DWMDF) [15].

*3.3. Conformational equilibrium of cpp[NMeNle$^3$]*

Exchange peaks in the ROESY spectra of cpp[NMeNle$^3$] could easy be identified by their 180° phase shift compared with ROEs. Interestingly, exchange peaks have been found between the protons of isomer **I.3** and these of all other isomers, but not between **I.1** and **I.2**, **I.1** and **I.4** or **I.2** and **I.4**. The lack of exchange peaks between two isomers indicates the absence of a direct conformational transition between these isomers within the NMR time scale. In consequence, isomer **I.3** can be assumed to be in a central position in the *cis/trans* equilibrium (Fig. 4). Therefore, the isomerizations, from **I.1** to **I.2**, from **I.1** to **I.4**, from **I.2** to **I.4** and the back isomerizations pass probably in a two-step mechanism with isomer **I.3** as intermediate. For example, for the isomerization of isomer **I.1** to **I.2**, first the *cis* peptide bond between $Asp^4$ and $Phe^5$ isomerizes to *trans* (isomer **I.3**) and in the second step the *trans* peptide bond $Phe^5$–$Asp^1$ isomerizes to *cis*.

The intensity of the exchange peaks is a relative measure of the exchange rate and hence of the free activation energy of the isomerization. Particularly strong are the exchange peaks between isomer **I.2** and **I.3**, indicative of a very low energy barrier and therefore of a fast *cis/trans* isomerization of the $Phe^5$–$Asp^1$ peptide bond.

## 4. Discussion

Proline is the amino acid in globular proteins with the highest probability of adopting a *cis* amide bond [16]. Linear oligopeptides containing proline exist generally in a *cis/trans* ratio of the Xaa-Pro peptide bond. However, the *cis* isomer is usually the minor form [17,18]. In contrast, the *cis* isomer is often favoured in cyclic peptides and *cis* proline has been shown to occupy preferentially the $i$+2 position of βVI turns. The decision as to whether a βVIa or βVIb turn is formed is

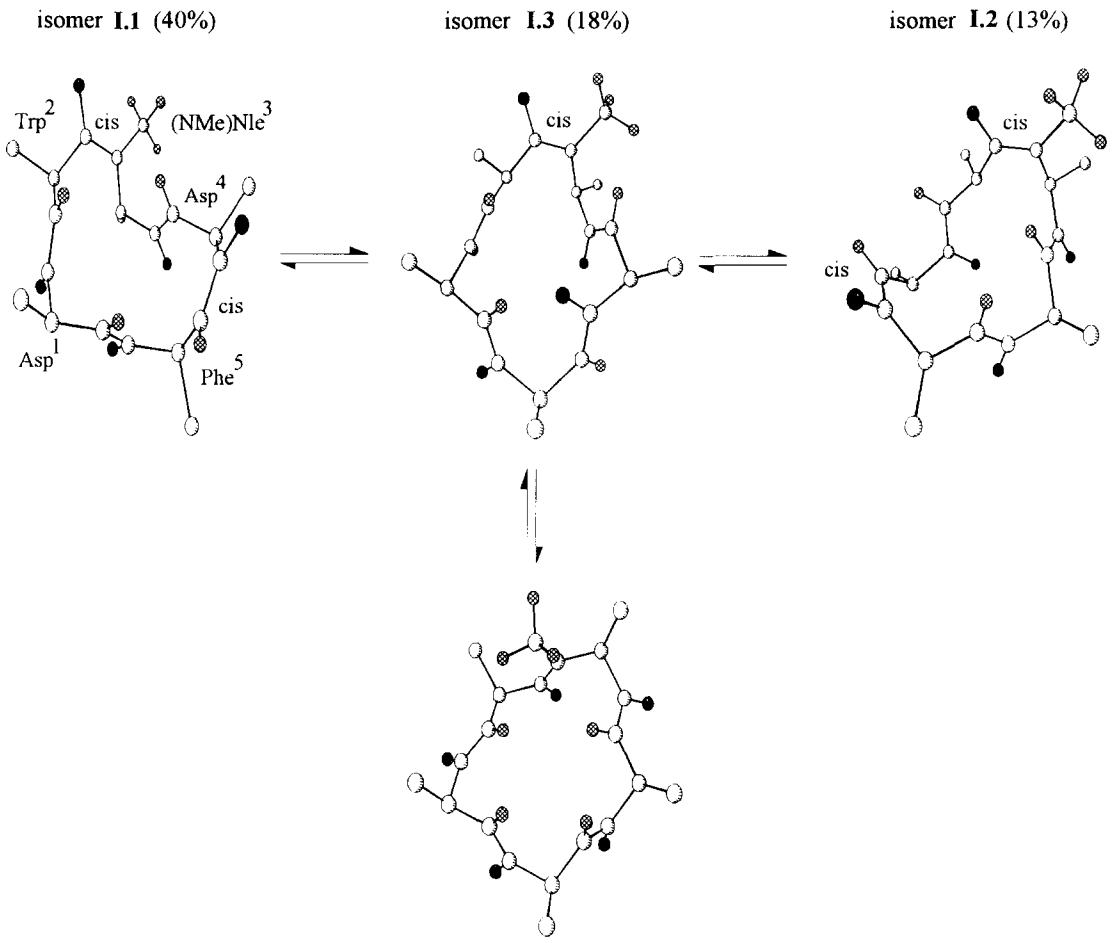


Fig. 4. Conformational equilibrium of the four isomers of cpp[NMeNle$^3$] derived from the existence and absence of exchange peaks between the isomers in the ROESY spectra. The figure shows the conformational transition pathway. All *cis* peptide bonds are indicated.

complicated by the fact that both turn types are characterized by similar backbone angle ranges. The main difference between them is the absence of a hydrogen bond between CO($i$) and NH($i$+3) in the βVIb turn. Two contradictory values (0°/150°) for the $\psi_{i+2}$ dihedral angle of the βVIb turn exist in the literature [5,6,14].

From previous investigations of only L-amino acid containing cyclic peptides a relation between preferred βVI turn type and ring size may be proposed. Hydrogen stabilization and hence βVIa turns were determined around the prolines in cyclic hexapeptides (ring size: 18 atoms) with one and two *cis*-prolines [1]. Likewise, disulfide-constrained cyclic pentapeptides (ring size: 17 atoms) with a *cis*-proline also adopt βVIa turns [2,19]. In contrast, investigations of cyclic pentapeptides (ring size: 15 atoms) containing one or two *cis*-prolines showed the existence of βVIb turns [1].

D-amino acid containing cyclic hexapeptides have been found to prefer βVIb turns in solution and in the crystal [20-22]. In these peptides proline is followed by a D-amino acid and the inverse chirality is probably the cause of the conformational preference. Cyclic pentapeptides with a D-amino acid and *cis*-proline have also been analysed, however, βVI turns have not been reported [23,24].

Besides proline, *N*-methyl amino acids are known to induce *cis/trans* isomerization in peptides. *N*-methyl amino acids are often used in synthetic peptides (i) to prevent digestion by peptidases, (ii) to induce a steric barrier to $\phi$ torsion angle rotation and (iii) to induce *cis/trans* isomerization [25,26]. As yet, little is known about the conformational influence of these amino acids in peptides. We compared in this study the influence of proline and *N*-methylnorleucine on the conformational equilibria of two cyclic pentapeptides, which differ only in the imido acid in position 3. The isomer analysis of cpp[NMeNle$^3$] revealed the existence of five *cis/trans* isomers, whereas only two were found for cpp[Pro$^3$]. Two isomers of the complex conformational equilibrium of cpp[NMeNle$^3$] (**I.2** and **I.3**) are very similar to the isomers of cpp[Pro$^3$] (**II.1** and **II.2**) as indicated by identical positions of the *cis* bonds and NOE patterns (Table 1 and Fig. 3).

The *cis* bonds of all isomers of both peptides, as well as of recently investigated model peptides c(PAAAA) and c(PAPAA) [1], are centred on βVIb or βVIb-like turns. The differences in the primary structure between all these peptides led to propose that the formation of this turn in cyclic pentapeptides is essentially independent of the amino acid sequence. Consequently, the existence of a *cis* amide bond, induced by an amino acid tending to *cis/trans* isomerization (e.g. proline or NMe-amino acid), results in backbone angles similar to these

of βVIb turns. The amino acid sequence is expected to have an effect on the number and the relative amounts of the isomers. The backbone angles around *cis*-(NMe)Nle have been found to differ somewhat from the angles of an ideal βVIb turn. The higher torsional angle variability and the larger number of isomers in cpp[NMeNle$^3$] lead to the conclusion that the conformational flexibility is enhanced in this peptide by the possibility of the ϕ-(NMe)Nle rotation.

Three isomers have been analysed with *cis* peptide bonds between amino acids which usually do not tend to *cis/trans* isomerization. Isomers **I.1**, **I.2** of cpp[NMeNle$^3$] and **II.1** of cpp[Pro$^3$] are characterized by *cis* bonds between Asp$^4$–Phe$^5$ and Phe$^5$–Asp$^1$, respectively. A similar *cis* bond was described for c(PAAAA) between Ala$^3$ and Ala$^4$ [1]. However, we did not find *cis*-Asp-Phe and *cis*-Phe-Asp bonds in crystal structures of the Brookhaven Protein Data Bank. Probably, Phe-Asp and Asp-Phe *cis* amide bonds require a relatively high conformational stabilization to compensate the energetically discriminated *cis* isomerization. Such high conformational stabilization can be achieved by reduction of the ring tension of a cyclic peptide through the formation of a *cis* bond. Cyclic pentapeptides may therefore be a tool for the design of *cis* amide bonds between any two amino acids.

Isomer **I.4** of cpp[NMeNle$^3$] is the only isomer with exclusively *trans* peptide bonds. The conformation of this isomer is very similar to the structure recently reported for the cyclic all-*trans* pentapeptide c(DWMDF) which lacks N-substituted amino acids [15]. Both structures are characterized by the absence of intramolecular hydrogen bonds. Assuming that Nle and Met are isosteric amino acids, cpp[NMeNle$^3$] and c(DWMDF) differ only in the *N*-methyl group on Nle in cpp[NMeNle$^3$]. Therefore, substitution of the NH proton by methyl drastically enhanced the conformational variability of the peptide.

In summary, we have shown that the substitution of proline by *N*-methylnorleucine in a cyclic pentapeptide prevents the conformational preference of βVIb turns of the *cis* peptide bonds, but enhances the conformational variability and leads to deviations from ideal backbone angles. Consequently, the formation of βVIb turns seems to be a property of a *cis* peptide bond rather than of a proline residue.

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## References

[1] Müller, G., Gurrath, M., Kurz, M. and Kessler, H. (1993) Proteins Struct. Funct. Genet. 15, 235–251.
[2] Weißhoff, H., Frost, K., Brandt, W., Henklein, P., Mügge, C. and Frömmel, C. (1995) FEBS Lett. 372, 203–209.
[3] Kabsch, W. and Sander, C. (1983) Biopolymers 22, 2577–2637.
[4] Chou, P. and Fasman, G. (1977) J. Mol. Biol. 115, 135–175.
[5] Richardson, J.S. (1981) Adv. Protein Chem. 34, 167–339.
[6] Rose, G.D., Gierasch, L.M. and Smith, J.A. (1985) Adv. Protein Chem. 37, 1–109.
[7] Ehrlich, A., Rothemund, S., Brudel, M., Beyermann, M., Carpino, L.A. and Bienert, M. (1993) Tetrahedron Lett. 34, 4781–4784.
[8] UXNMR, Version 940501, Bruker Analytische Messtechnik GmbH, Germany.
[9] AURELIA, Version 941101.3, Bruker Analytische Messtechnik GmbH, Germany.
[10] X-PLOR, Version 3.1, Brünger, A.T., Yale University, New Haven, CT.
[11] QUANTA, Version 3.3, Molecular Simulations Inc., Waltham, MA.
[12] Bystrov, V.F. (1976) Prog. NMR Spectrosc. 10, 41–81.
[13] Wüthrich, K. (1986) in: NMR of Proteins and Nucleic Acids, pp. 117–199, Wiley, New York.
[14] Smith, J.A. and Peace, L.G. (1980) CRC Crit.Rev. Biochem. 8, 315–399.
[15] Weißhoff, H., Wieprecht, T., Henklein, P., Antz, C. and Mügge, C. (1995) Biochem. Biophys. Res. Commun. 213, 506–512.
[16] Frömmel, C. and Preissner, R. (1990) FEBS Lett. 277, 159–163.
[17] Grathwohl, C.and Wüthrich, K. (1976) Biopolymers 15, 2025–2041.
[18] Dyson, H.J., Rance, M., Houghten, R.A., Lerner, R.A. and Wright, P.E. (1988) J. Mol. Biol. 201, 161–200.
[19] Rossi, F., Saviano, M., Di Blasio, B., Zanotti, G., Maione, A.M., Tancredi, T. and Pedone, C. (1994) Biopolymers 34, 273–284.
[20] Torchia, D., Wong, S.C.K., Deber, C.M. and Blout, E.R. (1972) J. Am. Chem. Soc. 94, 616–620.
[21] Kopple, K.D., Schamper, T.J. and Go, A. (1974) J. Am. Chem. Soc. 96, 2597–2605.
[22] Kartha, G., Krishna Bhandary, K., Kopple, K.D., Go, A. and Zhu, P.P. (1984) J. Am. Chem. Soc. 106, 3844–3850.
[23] Stroup, A.N., Rockwell, A.L. and Gierasch, L.M. (1992) Biopolymers 32, 1713–1725.
[24] Bruch, M.D., Rizo, J. and Gierasch, L.M. (1992) Biopolymers 32, 1741–1754.
[25] Tonelli, A.E. (1976) Biopolymers 15, 1615–1622.
[26] Manavlan, P. and Momany, F.A. (1980) Biopolymers 19, 1943–1973.