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Investigation of *cis/trans* ratios of peptide bonds in AVP analogues containing *N*-methylphenylalanine enantiomers

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Abstract: The solution conformation of vasopressin analogues, modified at positions 2 and 3 with *N*-methylphenylalanine or its enantiomer, [D-MePhe^2,MePhe^3]AVP and [MePhe^2,D-MePhe^3]AVP, were studied by 2D NMR spectroscopy in H_2O/D_2O and theoretical calculations (EDMC/ANALYZE). In the case of [MePhe^2,D-MePhe^3]AVP, the synthesis afforded two products, **A** and **B**, which had identical molecular ions and similar retention times on HPLC. This finding was explained by racemization of Cys^1, which gave an additional analogue, [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**). The possibility is not excluded of racemization of Cys^1 in the remaining analogues of this series. However, only in the case of [MePhe^2,D-MePhe^3]AVP was this process so distinct that two strong peaks in the HPLC chromatogram were observed. The NMR spectra of all the analogues showed several distinct sets of residual proton resonances. This suggests that the peptides adopt more than two groups of conformations in H_2O/D_2O. This fact is due to *cis/trans* isomerization. Two more populated isomers arise from the *cis/trans* isomerization across the 2–3 peptide bond in [D-MePhe^2,MePhe^3]AVP and [MePhe^2,D-MePhe^3]AVP (**A**) and across the 1–2 peptide bond in [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**). Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: EDMC; *N*-methylation; NMR spectroscopy; peptide conformation; vasopressin

INTRODUCTION

Vasopressin (AVP) agonists and antagonists have been used as pharmacological and therapeutic tools in animal and human physiology and pathophysiology. For this reason, in an attempt to design highly active and selective analogues of AVP, a number of studies have been undertaken to determine their structure-activity relationships.

In recent years, the synthesis of conformationally restricted analogues has become an important tool for reaching this goal. Steric restrictions can be imposed by means of either the formation of cyclic structures within the peptide backbone or the reduction of peptide flexibility by introducing amino acids with limited conformational freedom, which, in turn, results in specific orientations of the peptide backbone and its side chains [1,2]. Both the NMR and theoretical methods are often very useful for examining the three-dimensional structure of hormones and their analogues. They can thus provide good tools in an effort to better correlate the structure-activity relationships of peptides.

The peptide bond usually exists exclusively in the *trans* configuration. However, in the case of peptides containing either a proline residue or *N*-alkyl amino acids, the *cis/trans* isomerization of the peptide bond preceding these residues can take place. The differences in conformational behaviour arising from *cis/trans* isomerization are crucial for the biological profile of peptides.

The *cis/trans* ratio depends mostly on the sequence of peptide, solvent, pH, temperature and concentration. At room temperature, the equilibrium between the *cis* and *trans* isomers is recorded on NMR spectra. Usually the *cis* isomer is the minor species, although Dyson *et al.* have reported relative concentrations of the *cis* isomer greater than 50% for several proline-containing hexapeptides [3]. Similar results were obtained in our laboratory. Namely, the major species of vasopressin analogues substituted at positions 2 and 3 with L-N-methylphenylalanine or its D enantiomer contained a *cis* peptide bond between the second and third residues. The ratios of *cis/trans* isomers amounted to 8 : 2 and 7 : 3 for [MePhe^{2,3}]AVP and [D-MePhe^{2,3}]AVP, respectively [4]. These interesting findings prompted us to perform conformational analysis of two successive analogues of vasopressin modified at positions 2 and 3 with different enantiomers of *N*-methylphenylalanine, [D-MePhe^2,MePhe^3]AVP and [MePhe^2,D-MePhe^3]AVP. It should be remembered that apart from the reduction of the *trans* vs *cis* ratio in the peptide bond, this results in a steric constraint, suppression of the proton-donating ability of the NH group capable of hydrogen bonding

Abbreviations: AVP, [Arg^8]-vasopressin; 2D, two-dimensional; DSS, 2,2-dimethyl-2-silapentanesulfonic acid; ECEPP/3, empirical conformational energy program for peptides; EDMC, electrostatically driven Monte Carlo; MORASS, multiple Overhauser relaxation analysis and simulation; RMSD, the root mean square deviation; SRFOPT, solvent-accessible surface model.

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Table 1 Physicochemical properties of [MePhe^2,D-MePhe^3]AVP (**A**) and (**B**) and the freshly synthesized [D-Cys^1,MePhe^2,D-MePhe^3]AVP

Peptide		HPLC T_R		Molecular ion $[M + H^+]$
[MePhe^2,D-MePhe^3]AVP	**A**	9.7[a]	6.5[b]	1097
	B	10.6[a]	7.4[b]	1097
[D-Cys^1,MePhe^2,D-MePhe^3]AVP		10.6[a]	7.4[b]	1097

[a] Linear gradient from 20% to 80% of [**II**] for 20 min.
[b] Isocratic gradient 30% of [**II**].

and enhanced basicity of the carbonyl group [5]. It was also shown that further change of the L/D configuration is likely to alter the orientation of the side chains of the residues at positions 2 and 3 [6].

An additional reason for studying these analogues was the finding that after the synthesis of [MePhe^2,D-MePhe^3]AVP on an automatic peptide synthesizer, two products were obtained, **A** and **B**, which had identical molecular ions and different retention times on HPLC (Table 1). Because of this unexpected result, [MePhe^2,D-MePhe^3]AVP was re-synthesized manually in order to verify the correctness of the synthesis. This showed both preparations to be identical [7]. An explanation of this problem will be offered further in this paper.

NMR MEASUREMENTS

The NMR spectra were recorded on a 500 MHz Varian spectrometer. The experiments were carried out in H_2O/D_2O (9 : 1). The sample concentration was approximately 5–8 mM in 0.5 ml of H_2O/D_2O, pH $\approx$ 4.9. The 2D NMR spectra were recorded at 303 K. The temperature coefficients of the amide proton chemical shifts were measured for at least five temperatures: 275, 283, 293, 303 and 313 K.

The assignment of proton chemical shifts of the two peptides was accomplished using the proton-proton total chemical shift correlation spectroscopy (TOCSY) [8,9], the rotating-frame Overhauser enhancement spectroscopy (ROESY) [10], as well as the gradient heteronuclear single quantum coherence (^{1}H-^{13}C gHSQC, ^{1}H-^{15}N gHSQC) [11–13]. For each peptide, the mixing times of 70 ms for TOCSY and 200 and 300 ms for ROESY, were measured. The positions of the H_β and H_γ protons of Pro^7 and the N-CH_3 protons of the *N*-methylated amino acids were confirmed in the ^{1}H-^{13}C gHSQC and ^{1}H-^{13}C g HMBC [14] spectra. On the basis of the ^{1}H-^{15}N gHSQC spectra, the chemical shifts of ε-NH_2 protons of Gln^4 and those of δ-NH_2 in Asn^5 were marked for each peptide. Spectral processing was carried out using the NMRPipe/NMRDraw [15] package and analysed with XEASY [16]. The spectra were calibrated against the HDO signal, taking into account the temperature drift of the reference signal given by the equation $\delta_{1H(T)} = 5.060 - 0.0122T + (2.11 \times 10^{-5})T^2$, T[°C] [17]. External reference signals used for calibration of the correlation spectra were those of DSS (2,2-dimethyl-2-silapentanesulfonic acid) for the carbon axis in the ^{1}H-^{13}C spectra (^{13}C/^{1}H = 0.251449530) and the NH_3 signal for the nitrogen axis in the ^{1}H-^{15}N spectra (^{15}N/^{1}H = 0.101329118) [18].

The analysis of residual spin-coupling correlation systems was straightforward, being performed by a combination of the sequential-specific assignment procedure in the TOCSY and the sequential ROE network along the peptide backbone protons. The ROE cross-peaks with a mixing time of 300 ms for all peptides were picked up on the ROESY spectra. The value of temperature dependence of NH proton chemical shifts ($\Delta\delta/\Delta T$) was calculated from the 1D spectra recorded at 275, 283, 293, 303, 313 and 323 K. The coupling constants between HN and H_α ($^3J_{HNH\alpha}$) were found in the ACT-ct-COSY [19] and 1D NMR spectra.

RELATION BETWEEN PRODUCTS A AND B

To investigate the relation between two products arising from the synthesis of [MePhe^2,D-MePhe^3]AVP, **A** and **B**, a simple experiment was performed aimed at either confirmation or rejection of the existence of two stable conformers of [MePhe^2,D-MePhe^3]AVP. It was assumed that heating a solution of both peptides should eventually convert either of them to an equilibrium mixture of both. In this connection, additional 1D NMR spectra were taken at 333, 343 and 358 K for analogues **A** and **B**. In both cases, the signals of the amide protons disappeared upon increasing the temperature. The most pronounced changes were observed for the modified residues. The signals of H_α D-MePhe^3 of the **B** belonging to *trans* and *cis* isomers overlapped completely at 358 K. Aromatic protons of this peptide behaved similarly. With **A**, most of the signals of *trans* and *cis* isomers overlapped over the whole temperature range and their chemical shifts and shapes changed only slightly at higher temperatures. However, the conversion of one peptide into the other was not observed, which would unambiguously exclude the co-existence of two stable

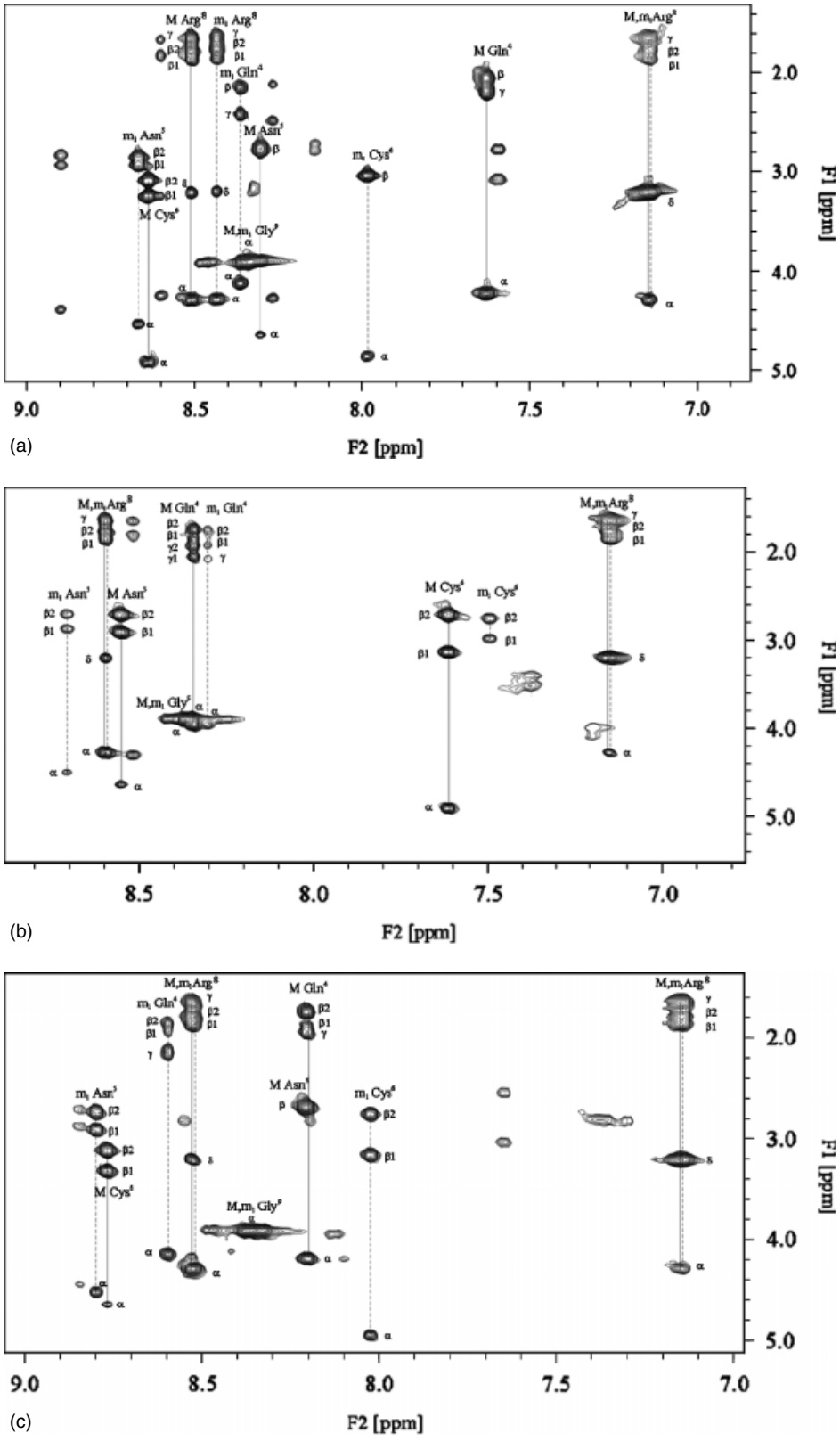

Figure 1 The fingerprint region of the TOCSY spectra of the [D-MePhe^2,MePhe^3]AVP (a), [MePhe^2,D-MePhe^3]AVP (b) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (c), showing the correlation of amide protons with the side-chain protons (α, β, γ and δ) for major (M) and minor (m_1) species.

Table 2 Position of *cis*/*trans* isomerization and the ratios of the dominant populations for each peptide

Peptide	Position of *cis*/*trans* isomerization	*cis*/*trans* ratio
[D-MePhe^2,MePhe^3]AVP	D-MePhe^2 – MePhe^3	3 : 7
[MePhe^2,D-MePhe^3]AVP (**A**)	MePhe^2 – D-MePhe^3	1 : 9
[D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**)	D-Cys^1 – MePhe^2	4 : 6

Table 3 Proton chemical shifts [ppm] and the amide proton temperature coefficients [ppb/K] of [D-MePhe^2,MePhe^3]AVP (*trans*-major and *cis*- minor isomers) in water, at 30 °C

Residue		Chemical shifts [ppm]						$\Delta\delta/\Delta T$ [ppb/K]
		NH	H_α	H_β	H_γ	H_δ	others	
Cys^1	*trans*	n.o.	4.64	2.75; 3.13				n.o.
	cis	n.o.	4.56	2.01; 2.43				n.o.
D-MePhe^2	*trans*	—	5.54	2.99; 3.33			N-CH_3 2.99 $H_{2,6}$ 7.13	—
	cis	—	4.28	2.99			N-CH_3 2.84 $H_{2,6}$ 7.14	—
MePhe^3	*trans*	—	4.98	2.18; 2.26			N-CH_3 2.89 $H_{2,6}$ 7.24; $H_{3,5}$ 7.32; H_4 7.29	—
	cis	—	5.74	3.17; 3.54			N-CH_3 3.13 $H_{2,6}$ 7.38; $H_{3,5}$ 7.31	—
Gln^4	*trans*	7.64	4.21	2.05	2.19		ε-NH_2 6.82; 7.39	–8.2
	cis	8.37	4.12	2.14	2.42		ε-NH_2 6.82; 7.55	–7.6
Asn^5	*trans*	8.30	4.63	2.76			δ-NH_2 6.87; 7.53	–2.5
	cis	8.67	4.53	2.86; 2.92			δ-NH_2 6.88; 7.59	–6.1
Cys^6	*trans*	8.64	4.91	3.09; 3.23				–7.4
	cis	7.99	4.85	3.04				–4.7
Pro^7	*trans*	—	4.41	1.91; 2.27	2.00	3.68; 3.85		—
	cis	—	4.42	1.91; 2.28	2.00	3.68; 3.83		—
Arg^8	*trans*	8.51	4.28	1.78; 1.88	1.66	3.19	ε-NH 7.16	–9.4
	cis	8.44	4.28	1.75; 1.86	4.64	3.18	ε-NH 7.15	–9.2
Gly^9	*trans*	8.35	3.90					–8.2
	cis	8.32	3.44					–6.6
C-NH_2	*trans*	7.02; 7.42						
	cis	7.00; 7.39						

conformers of the same peptide. These results prompted us to look for another explanation. In particular, it was found that **B** differs from other peptides of this series with regard to the position of *cis*/*trans* isomerization. Namely, the appropriate cross peaks, which will be discussed further in this paper, showed that of the several isomers identified in the NMR spectra, two more populated ones arise from the *cis*/*trans* isomerization across the 1–2 peptide bond. In the case of the remaining analogues with *N*-methylphenylalanine enantiomers at positions 2 and 3, two more populated isomers originate from the *cis*/*trans* isomerization between *N*-methylated amino acids. Since the study obtained all possible diastereoisomers of the AVP analogues, substituted at positions 2 and 3 with the *N*-methylphenylalanine residues, [MePhe^{2,3}]AVP, [D-MePhe^{2,3}]AVP [4], [D-MePhe^2,MePhe^3]AVP and [MePhe^2,D-MePhe^3]AVP (**A** and **B**), which have different retention times on HPLC [7] and different NMR spectra, but have the same molecular ion, a hypothesis was rejected that the reason for obtaining the mixture of **A** and **B** might be the loss of stereochemical integrity in the carboxyl component on the formation of the 2–3 peptide bond in [MePhe^2,D-MePhe^3]AVP. However, it was assumed that the appearance of two products, **A** and **B**, might be due to Cys^1-racemization. In order to check the putative racemization, [D-Cys^1,MePhe^2,D-MePhe^3]AVP was synthesized. In the next step, fraction **B** and

the freshly synthesized [D-Cys^1,MePhe^2,D-MePhe^3]AVP analogue were mixed and the retention time of the mixture was measured using HPLC. HPLC was carried out on a Waters chromatograph equipped with a UV detector. The purity of the peptides was determined in a Vydac C_{18} column (5 μm, 250 × 4.6 mm). The following solvent systems were used: [**I**] 0.1% aqueous trifluoroacetic acid (TFA), [**II**] acetonitrile: 0.1% aqueous TFA (80 : 20 v/v). A linear gradient from 20% to 80% of [**II**] for 20 min and an isocratic gradient 30% of [**II**] were applied for peptides, at a flow rate of 1 ml/min ($\lambda = 226$ nm). Preparative HPLC was carried out using a Kromasil C_8 column (5 μm, 25 × 250 mm) in a gradient running from 10% to 50% of [**II**] for 120 min at a flow rate of 10 ml/min ($\lambda = 226$ nm). Fab/MS of the peptides were recorded on a MALDI TOF mass spectrometer. The physicochemical properties of fractions **A** and **B** and the synthesized [D-Cys^1,MePhe^2,D-MePhe^3]AVP are summarized in Table 1.

ANALYSIS OF NMR SPECTRA

The NMR spectra of each analogue displayed several distinct sets of residual proton resonances (Figure 1). This fact indicates that the peptides adopt more than two groups of conformations in H_2O/D_2O (9 : 1). Their appearance is due to the *cis/trans* isomerization. In this paper, only two more populated isomers of each peptide will be considered, one major (M) and one minor (m_1). Table 2 shows the position of *cis/trans* isomerization and the ratios of dominant populations for each peptide. The proton chemical shifts for the major (M) and minor (m_1) species together with the H_N temperature coefficients for each peptide are given in Tables 3–5.

In the ROESY map, the following number of intraresidual, sequential, medium-range and long-range interactions for the major species of [D-MePhe^2,MePhe^3]AVP, [MePhe^2,D-MePhe^3]AVP (**A**) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**) peptides, respectively, were identified: 63, 23, 4 and 0; 78, 35, 4 and 1; and 59, 26, 3 and 8. The smaller number of ROE connectivities in [D-Cys^1,MePhe^2,D-MePhe^3]AVP may be due to a lower sample concentration.

Figure 2 presents the ROE pattern, the coupling constants, as well as $^3J_{HNH\alpha}$ and temperature coefficients for the peptides. The presence of the cross-peaks of $H_\alpha Cys^6 - H_\delta Pro^7$ indicates the *trans* geometry of this peptide bond for the major species. It should be also emphasized that the signals of C_β and C_γ of Pro appearing, respectively, at 30.5 ± 0.6 and 25.1 ± 0.5 ppm for the *trans* and at 32.2 ± 0.4 and 23.4 ± 0.3 ppm for the *cis* isomer, provide additional evidence for the geometry of the X-Pro peptide bond [20]. The carbon chemical shifts of C_β and C_γ of Pro for the major species were found at 29.07 and 24.50; 29.14 and 24.52, and 29.22 and 24.61 for [D-MePhe^2,MePhe^3]AVP, [MePhe^2,D-MePhe^3]AVP (**A**) and [D-Cys^1, MePhe^2,D-MePhe^3]AVP (**B**), respectively. On this basis, it was found that the Cys^6-Pro^7 peptide bond exists in the *trans* geometry in the major species of each peptide studied here. The $H_\alpha(1) - H_{N-CH3}(2)$ contact reveals the *trans* peptide bond between the first and second residues.

In the ROESY spectra of [D-MePhe^2,MePhe^3]AVP, the presence of strong exchange cross-peaks between M H_αMePhe^3 and m_1 H_αMePhe^3 suggests the *cis/trans* isomerization of the D-MePhe^2 – MePhe^3 peptide bond. For more populated isomer, the ROE H_α(D-MePhe^2) – H_{N-CH3}(MePhe^3) cross-peak indicates the *trans* configuration on that peptide bond. In the case of [MePhe^2,D-MePhe^3]AVP (**A**), the exchange cross-peak of M H_αD-MePhe^3 – m_1 H_αD-MePhe^3 is missing in the ROESY spectra. This is because the signals of the second and third residues have comparable proton chemical shifts for the *trans* and *cis* isomers. However, the presence of the cross-peaks between the H_α proton of MePhe^2 and H_{N-CH3} protons of D-MePhe^3, as well as between the H_α proton of MePhe^2 and H_α proton of D-MePhe^3, suggest that the MePhe^2-D-MePhe^3 peptide bond is involved in the *cis/trans* isomerization. These connectivities recognize the *trans* and *cis* MePhe^2 – D-MePhe^3 peptide bond for the major and minor species, respectively. As regards the missing the signals of Cys^1 in the TOCSY spectrum of [MePhe^2,D-MePhe^3]AVP (**A**), the proton chemical shift of H_β in Cys^1 was identified in the heteronuclear, ^{1}H-^{13}C gHSQC and ^{1}H-^{13}C gHMBC spectra. Furthermore, the $H_\beta(1) - H_\alpha(6)$ connectivity in the ROESY spectrum points to the position of H_β protons of Cys^1. The cross-peak between Cys^1 and Cys^6 reveals the preference for conformation in which the S-S dihedral angle is confined to a positive value of approximately 90° [21].

The exchange cross-peak between M H_αMePhe^2 – m_1 H_αMePhe^2 in the ROESY spectra of [D-Cys^1, MePhe^2,D-MePhe^3]AVP (**B**), suggests that the D-Cys^1 – MePhe^2 peptide bond is involved in the *cis/trans* isomerization. In addition, the presence of H_αD-Cys^1 – H_{N-CH3}MePhe^2 contact for the major (M) and of H_αD-Cys^1 – H_αMePhe^2 for the minor species (m_1) indicates that the H_αD-Cys^1 – MePhe^2 peptide bond can adopt *trans* geometry for the major (M) species and *cis* geometry for the minor (m_1) one.

The analysis of ROE patterns and the vicinal coupling constants, $^3J_{HNH\alpha}$, shows that the main structural elements of the peptides are β-turns (Figure 2). The strong $H_\alpha(i) - H_N(i+1)$ and $H_\alpha(6) - H_\delta(7)$ cross peaks in the *C*-terminal part of each molecule and the medium $H_N(8) - H_N(9)$ one indicate the β-turn at position 7,8. Moreover, the $H_\alpha(i) - H_{N-CH3}(i+1)$ and strong or medium $H_\alpha(3) - H_N(4)$ connectivities suggest that β-turn at position 2,3 are populated. In the case of [D-MePhe^2,MePhe^3]AVP, the presence of $H_{N-CH3}(2) - H_N(5)$, $H_{N-CH3}(2) - H_N(6)$ and $H_{N-CH3}(3) -$

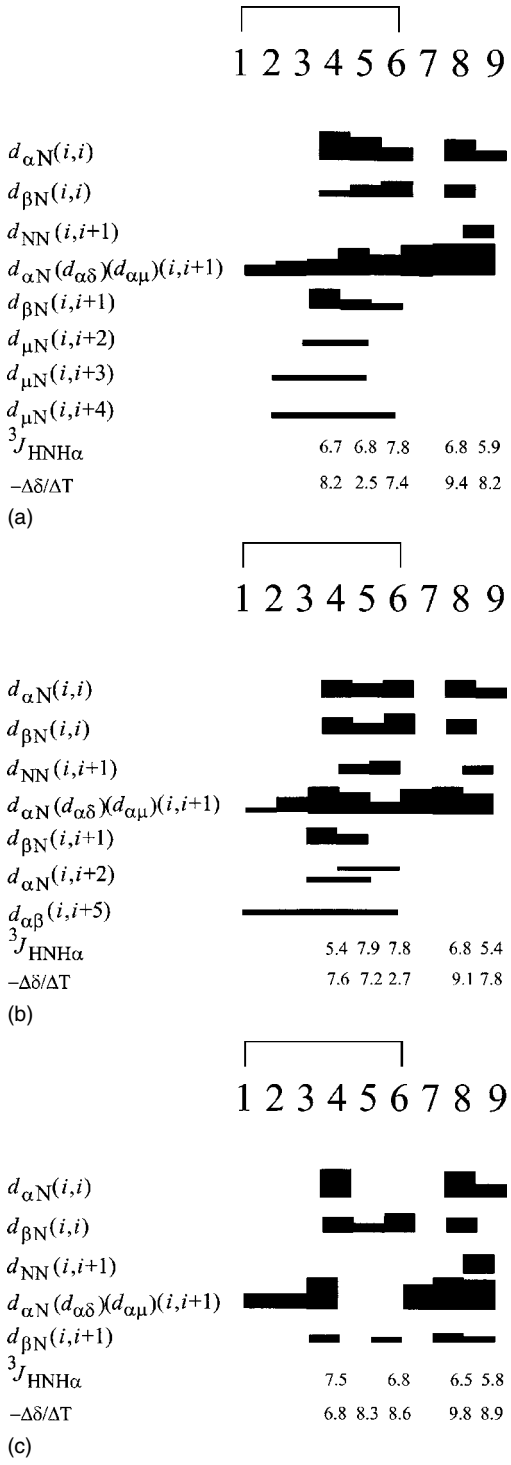

Figure 2 The ROE effects corresponding to the inter-proton distances and $^3J_{HNH\alpha}$ measured for the [D-MePhe^2, MePhe^3]AVP (a), [MePhe^2,D-MePhe^3]AVP (**A**) (b) and [D-Cys^1, MePhe^2,D-MePhe^3]AVP (**B**) (c).

$H_N(6)$ are likely to indicate β-turns in the middle part of the analogue. The low temperature coefficient of the amide proton of Asn^5 (−2.5 ppb/K) suggests that it is probably engaged in the hydrogen bond with the oxygen atom of the carbonyl group of D-MePhe^2. This hydrogen bond stabilizes the β-turn at position 3,4.

The ROE effects $H_N(4) - H_N(5)$, $H_N(5) - H_N(6)$ and strong $H_\alpha(6) - H_\delta(7)$ cross-peak in [MePhe^2, D-MePhe^3]AVP (**A**) assume β-turn at position 5,6. Furthermore, the $H_\alpha(4) - H_N(6)$ contact seems to confirm that suggestion. The $H_N(4) - H_N(5)$ connectivity together with a weaker $H_\alpha(4) - H_N(6)$ one suggest either a type I or II β-turn at position 4,5. Moreover, the temperature coefficient of the amide proton of Cys^6 (−2.7 ppb/K) shows this proton to be involved in the hydrogen bond, which may stabilize either a β-turn at position 4,5 or γ-turns. On the other hand, the coupling constants, $^3J_{HNH\alpha}$, 5.4 and 7.9 Hz for Gln^4 and Asn^5, respectively, depart a little from those characteristic of type I and II β-turns which is likely to disqualify this type of β-turns.

In the case of [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**), the temperature coefficients (Figure 2c), found in the range −6 to −10 ppb/K do not identify the hydrogen bonds along the backbone, whilst the ROE pattern reveals the tendency of the peptide to form a β-turn at the MePhe^2-D-MePhe^3 residues.

THE EDMC AND ANALYZE CALCULATIONS

The global conformational search of the peptides studied here was carried out using the electrostatically driven Monte Carlo (EDMC) method [22] with the ECEPP/3 force field [23] which assumes rigid valence geometry. The force field included a hydration contribution, which was evaluated in the SRFOPT solvent-accessible surface model [24] whose parameters pertain to solvation by water. A total of 3000 energy-minimized conformations were generated for each peptide. The working temperature was 1000 K. The conformations were subsequently clustered into families using the minimum-variance algorithm [25]. The root mean square deviation (RMSD) between heavy atoms at optimal superposition was taken as a measure of the distance between conformations, and a cut-off value of 1.2 Å was used to separate the families to afford 726, 931 and 875 families of conformations for [D-MePhe^2,MePhe^3]AVP, [MePhe^2,D-MePhe^3]AVP (**A**) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**), respectively. In the next step, for the lowest energy conformation of each family, a NOESY spectrum and vicinal coupling constants, $^3J_{HNH\alpha}$, were calculated by using a MORASS [26,27] program. This program solves the system of Bloch differential equations [28] for the cross-relaxation of a system of interacting proton spins. The vicinal couplings, $^3J_{HNH\alpha}$, were calculated from the empirical Bystrov-Karplus relationship [29]. The NOE effects were generated using a correlation time of 0.45 ms [30], mixing time of 300 ms and cut-off value of 6 Å. The weight of the coupling-constant term was 0.1 in the minimized sum and the Marquardt convergence criterion [31] equal to 10^{-5} was used. The entropy factor, $\alpha = 0.2$, was used for all peptides. The populations of

Table 4 Proton chemical shifts [ppm] and the amide proton temperature coefficients [ppb/K] of [MePhe^2,D-MePhe^3]AVP (**A**) (*trans*- major and *cis*- minor isomers) in water at 3 °C

Residue		Chemical shifts [ppm]						$\Delta\delta/\Delta T$ [ppb/K]
		NH	H_α	H_β	H_γ	H_δ	others	
Cys^1	*trans*	n.o.	n.o.	3.21				n.o.
	cis	n.o.	n.o.	n.o.				n.o.
MePhe^2	*trans*	—	5.87	2.56; 2.79			N-CH_3 3.29 $H_{2,6}$ 7.24; $H_{3,5}$ 7.38	—
	cis	—	5.86	2.55; 2.78			N-CH_3 3.29 $H_{2,6}$ 7.24; $H_{3,5}$ 7.38	—
D-MePhe^3	*trans*	—	5.14	2.68; 3.12			N-CH_3 3.09 $H_{2,6}$ 7.22; $H_{3,5}$ 7.35	—
	cis	—	5.15	2.68; 3.10			N-CH_3 3.09 $H_{2,6}$ 7.22; $H_{3,5}$ 7.35	—
Gln^4	*trans*	8.35	3.96	1.76; 1.87	1.95; 2.06		ε-NH_2 6.80; 7.34	−7.6
	cis	8.30	3.95	1.75; 1.84	1.93; 2.07		n.o.	−7.0
Asn^5	*trans*	8.56	4.64	2.71; 2.91			δ-NH_2 6.88; 7.55	−7.2
	cis	8.71	4.48	2.70; 2.86			n.o.	−7.6
Cys^6	*trans*	7.62	4.90	2.72; 3.14				−2.7
	cis	7.49	n.o.	2.75; 2.98				−1.8
Pro^7	*trans*	—	4.52	1.89; 2.29	2.03	3.65; 3.83		—
	cis	—	4.59	1.78; 2.33	1.93; 2.15	3.45; 3.60		—
Arg^8	*trans*	8.59	4.27	1.78; 1.88	1.66	3.20	ε-NH_2 7.16	−9.1
	cis	8.51	4.29	1.82	1.65	3.20	ε-NH_2 7.14	−8.0
Gly^9	*trans*	8.36	3.89					−7.8
	cis	8.41	3.89					−8.0
C-NH_2	*trans*	7.02; 7.41						
	cis	7.02; 7.41						

the families were determined by fitting a linear combination of the generated spectra and coupling values to the experimental data.

ANALYSIS OF THE CALCULATED STRUCTURES

To describe the structural preference of both peptides, the structures constituting about 75% of the ensemble obtained from calculations were used. As a result, 43, 166 and 63 conformations of [D-MePhe^2,MePhe^3]AVP,[MePhe^2,D-MePhe^3]AVP (**A**) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**), respectively, were considered. Superposition of each peptide is shown in Figure 3 aligned to their first coordinates using N, C_α and C atoms in the backbone of the cyclic part of the molecule. RMSD values for the ensemble of structures are 0.750; 0.523 and 0.458 Å for [D-MePhe^2,MePhe^3]AVP, [MePhe^2,D-MePhe^3]AVP (**A**) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**), respectively, from the first structure.

Table 6 summarizes both measured and computed vicinal couplings, $^3J_{HNH\alpha}$. The β-turn types and positions detected in the conformations were defined according to Lewis *et al.* [32]. The hydrogen bonds were calculated using the HBPLUS program [33]. For displaying and analysing the three-dimensional structure, a molecular graphics program MOLMOL [34] was used.

The main structural element of each peptide are the β-turn at positions 2,3 (Table 7). This is mainly a type IV β-turn, which is not stabilized by the hydrogen bond. Moreover, the [D-MePhe^2,MePhe^3]AVP and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**) show the tendency to form a β-turn at position 3,4. Few of the conformations of [MePhe^2,D-MePhe^3]AVP (**A**) involve the D-MePhe^3-Cys^6 residues in βI-turn, whilst the [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**) may adopt structures with a reverse turn at position 5,6. Of the presented peptides, [MePhe^2,D-MePhe^3]AVP (**A**) possesses the greatest population of conformations with a β-turn at the Pro^7-Arg^8 residues. This analogue, in contrast to [D-MePhe^2,MePhe^3]AVP, and similar to [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**), shows the strongest propensity for the formation of a type III or IV β-turn at position 7,8, whereas [D-MePhe^2,MePhe^3]AVP forms only a βIV-turn at this position. Most of the conformations of [MePhe^2,D-MePhe^3]AVP (**A**) with the βIII-turn at Pro^7-Arg^8 residues take the hydrogen bond between the amide proton of Gly^9 and the oxygen atom of the carbonyl group of Cys^6. The common feature of all peptides is a γ-turn over Gly^9, which is stabilized by an

Table 5 Proton chemical shifts [ppm] and the amide proton temperature coefficients [ppb/K] of [D-Cys^1,MPhe^2,D-MePhe^3]AVP (**B**) (*trans*- major and *cis*- minor isomers) in water at 30 °C

Residue		Chemical shifts [ppm]						$\Delta\delta/\Delta T$ [ppb/K]
		NH	H_α	H_β	H_γ	H_δ	others	
Cys^1	*trans*	n.o.	4.62	2.38; 2.77				n.o.
	cis	n.o.	4.38	2.85; 2.98				n.o.
MePhe^2	*trans*	—	5.37	3.06			N-CH_3 2.72 $H_{2,6}$ 7.22; $H_{3,5}$ 7.30	—
	cis	—	4.91	2.48			N-CH_3 2.98 $H_{2,6}$ 7.20; $H_{3,5}$ 7.38	—
D-MePhe^3	*trans*	—	5.49	2.99; 3.20			N-CH_3 2.97 $H_{2,6}$ 7.28; $H_{3,5}$ 7.35	—
	cis	—	5.52	3.16; 3.28			N-CH_3 3.33 $H_{2,6}$ 7.35; $H_{3,5}$ 7.38	—
Gln^4	*trans*	8.21	4.18	1.74; 1.89	1.95		ε-NH_2 6.77; 7.27	−6.8
	cis	8.59	4.13	1.86; 1.93	2.13		ε-NH_2 6.84; 7.40	−8.2
Asn^5	*trans*	8.21	n.o	2.69			δ-NH_2 6.91; 7.55	−8.3
	cis	8.79	4.51	2.72; 2.89			δ-NH_2 6.87; 7.57	−8.9
Cys^6	*trans*	8.77	4.64	3.11; 3.31				−8.6
	cis	8.02	4.92	2.80; 3.15				−7.3
Pro^7	*trans*	—	4.45	1.96; 2.34	2.15	3.83; 3.88		—
	cis	—	4.41	1.88; 2.29	1.99	3.62; 3.83		—
Arg^8	*trans*	8.53	4.28	1.78; 1.87	1.65	3.20	ε-NH_2 7.16	−9.8
	cis	8.51	4.27	1.77; 1.85	1.65	3.19	ε-NH_2 7.14	−9.6
Gly^9	*trans*	8.37	3.90					−8.9
	cis	8.33	3.89					−8.3
C-NH_2	*trans*	7.03; 7.42						
	cis	7.06; 7.45						

appropriate hydrogen bond. An inverse γ-turn at position 5 is characteristic of [D-MePhe^2,MePhe^3]AVP and [MePhe^2,D-MePhe^3]AVP (**A**), whilst in the case of [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**), this element appears very seldom. In [MePhe^2,D-MePhe^3]AVP (**A**), the ψ torsion angle of Asn^5 strays a little from that considered for an inverse γ-turn. Thus, it is confirmed that it is non-ideal. However, this structure is supported by the HN^6-CO^4 hydrogen bond. With the above described conformational preferences, it is assumed that the L – D interconversion of Cys^1 affects only slightly the conformation and induces major structural changes over the Asn^5 residue only.

As the conformations of the presented peptides differ only slightly from each other, it is believed that the geometry of the disulfide linkage and/or the locations of aromatic rings of the residues at positions 2 and 3, may play an important role in fostering the activity of the analogues. Thus, conformations of [D-MePhe^2,MePhe^3]AVP revealed the preference for a positive value (90°) of the Cβ-S-S-Cβ dihedral angle, whilst in the case of [MePhe^2,D-MePhe^3]AVP (**A**) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**), the Cβ-S-S-Cβ dihedral angle was constantly changed from positive to negative, although in [MePhe^2,D-MePhe^3]AVP (**A**) a slight domination was observed of the left-handed disulfide bridge, whereas in [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**), that of the right-handed one. The orientation of the aromatic rings of *N*-methylated phenylalanine residues at positions 2 and 3 seems to be very important for binding with receptors. In [D-MePhe^2,MePhe^3]AVP, the D-MePhe^2 and MePhe^3 side chains overlap but are not parallel, as in the case of vasopressin [35,36], whereas in most of the structures of [MePhe^2,D-MePhe^3]AVP (**A**) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**), the aromatic rings of MePhe^2 and D-MePhe^3 are situated opposite each other.

COMPARISON OF STRUCTURE AND ACTIVITY

The residues referred to as 'active elements' [37,38] in [Arg^8]-vasopressin (the side chains of Asn^5, Tyr^2 and Phe^3) interact with the receptor and have been suggested to be directed away from the centre of the 20-member ring. The orientation of Tyr^2 and Phe^3 side chains in [Arg^8]-vasopressin [36] results from the possibility of a ring-stacking interaction of the aromatic side chains of the neighbouring residues. This interaction causes an increase in the time that the

Table 6 Measured and EDMC/ANALYZE computed values of the coupling constants, $^3J_{HNH\alpha}$ [Hz], for each peptide

Residue	[D-MePhe^2,MePhe^3]AVP		[MePhe^2,D-MePhe^3]AVP		[D-Cys^1,MePhe^2,D-MePhe^3]AVP	
	J_{exp}	J_{calc}	J_{exp}	J_{calc}	J_{exp}	J_{calc}
Cys^1 or D-Cys^1	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
X	—	—	—	—	—	—
Y	—	—	—	—	—	—
Gln^4	6.7	7.1	5.4	7.1	7.5	7.8
Asn^5	6.8	6.3	7.9	6.4	n.o.	n.o.
Cys^6	7.8	8.3	7.8	7.3	6.8	6.8
Pro^7	—	—	—	—	—	—
Arg^8	6.8	6.7	6.8	7.0	6.5	6.2
Gly^9	5.9	5.8	5.4	5.3	5.8	5.2
S_d	0.39315		1.02777		0.59781	

n.o., not observed.
X and Y, L-*N*-methylphenylalanine or its enantiomer D.
S_d, standard deviation in vicinal coupling.

tyrosine side chain spends pointing away from the 20-member cyclic component. The aliphatic Ile^3 residue in oxytocin does not offer the possibility of π-π interaction and the aromatic ring of Tyr at position 2 is probably oriented over the pressin moiety [37,39].

Recently, the synthesis and pharmacological evaluation of four new analogues of AVP substituted at positions 2 and 3 with all possible combinations of *N*-methylphenylalanine enantiomers was described. In the present study two analogues of this series were selected and studied their conformational properties.

Among the selected peptides, only [D-MePhe^2, MePhe^3]AVP displayed low antiuterotonic and antipressor activities. The previously studied analogue, [D-MePhe^{2,3}]AVP [4], is a weak but selective blocker of OT receptors in the uterus [7]. These results showed that in this series the D conformation at position 2 is necessary for inducing antagonism. It has also been established that the pressor or oxytocic activities of vasopressin is determined by the orientation of the Tyr^2 and Asn^5 side chains [37]. Because the side chain of the Asn^5 points away from the macrocyclic ring in each peptide, the lack of activity of [MePhe^2,D-MePhe^3]AVP (**A**) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**) may be due to an inappropriate orientation of the side chain of the residue at position 2.

A comparison of the preferred structures of [D-MePhe^{2,3}]AVP [4] and [D-MePhe^2,MePhe^3]AVP shows that the former exhibits a preference for a negative value (−90°) of the Cβ-S-S-Cβ dihedral angle, whilst the latter for a positive value (90°). This may be due to the fact that the major species of [D-MePhe^{2,3}]AVP, in contrast to [D-MePhe^2,MePhe^3]AVP, possesses a *cis* peptide bond between the *N*-methylphenylalanine residues at positions 2 and 3.

The different activities of vasopressin are mediated by binding to different receptor types, and evidence

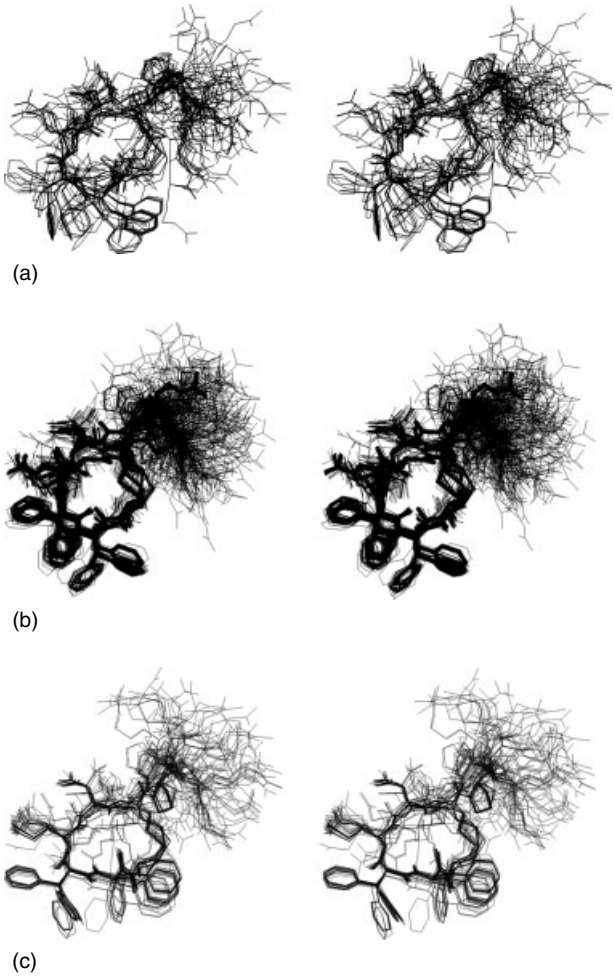

Figure 3 Stereoview showing the conformations with the highest statistical weights for three AVP analogues, obtained with EDMC/ANALYZE. [D-MePhe^2,MePhe^3]AVP (a), [MePhe^2,D-MePhe^3]AVP (**A**) (b) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**) (c) $RMSD_{1-6} = 0.750$, 0.523 and 0.458 Å for backbone atoms, respectively.

Table 7 Fractions of β-turns and the most frequently appearing γ- or an inverse γ turns within the backbone of the peptides obtained by fitting EDMC ensembles to NMR data. The set of conformations, constituting about 75% of conformation, were considered in our analysis

Peptide	Position of β-turn						γ- or γ^*-turns		
	2,3	3,4	4,5	5,6	6,7	7,8	5	7	9
[D-MePhe^2,MePhe^3]AVP	0.837	0.326	0	0	0	0.163	0.535	0.279	0.419
[MePhe^2,D-MePhe^3]AVP	0.976	0.048	0.012	0	0	0.651	0.427	0.066	0.223
[D-Cys^1,MePhe^2,D-MePhe^3]AVP	0.984	0.270	0	0.032	0	0.524	0.016	0.143	0.206

γ^*, an inverse gamma turn.

has been presented indicating that differentiation of binding to various types emerges from the conformation of the disulfide bridge [40,41]. The different geometry of the disulfide bridge could be one of the reasons why [D-MePhe^2,MePhe^3]AVP blocks both V_{1a} and oxytocin receptors, while [D-MePhe^{2,3}]AVP blocks only oxytocin receptors. The lack of activity of [MePhe^2,D-MePhe^3]AVP (**A**) and [D-Cys^1,MePhe^2,D-MePhe^3]AVP (**B**) is likely to be due to the unparalleled arrangement of the aromatic rings, which despite the existence of π-π interactions are often directed apart from each other. This orientation of aromatic rings might the reason that hydrophobic residues of the analogues were not found to be located in the binding pocket of receptors.

CONCLUSIONS

The conformational preference of neurohypophyseal peptide hormones and their analogues has been investigated by various techniques, such as laser Raman spectroscopy and CD [40], NMR spectroscopy in different solvents [21,42–49], x-ray crystallography [50–52] and other physicochemical methods [35,53].

It is generally believed that the cyclic part contains a β- or γ-turn structure, whereas the acyclic tail is very flexible. A β-turn at Tyr^2-Asn^5 in oxytocin and vasopressin, which was indicated by NMR studies [21,54–58], was also found in the crystal structures of deamino-oxytocin [51] and pressinoic acid [50]. Furthermore, a β-turn at positions 4 and 5 was detected in a trifluoroethanol solution of desmopressin [43]. An inverse γ-turn centred at Gln^4 was detected in analogues of oxytocin and vasopressin [37,47–49,51]. Both in aqueous solution and at a pH similar to physiological conditions, desmopressin was found to contain a stable inverse γ-turn centred around Gln^4 [45]. A γ-turn was also predicted for vasopressin docked into a three-dimensional computer model of V_{1a} receptor [59]. Thus, the formation of a hydrogen bond between an oxygen atom of the carbonyl group of Phe^3 and the amide proton H_N of Asn^5 leading to generation of a γ-turn is conformationally favourable in desmopressin and may be compatible with receptor binding [44]. In the peptides studied in this paper, β-turns generally occur at positions 2,3 and 7,8. The non-cyclic part of each analogue is characterized by an enhanced flexibility. Moreover, contrary to earlier findings suggesting a γ-turn centred around Gln^4, the presence of either a γ-turn or an inverse γ-turn at positions 5 and 9 stabilized by appropriate hydrogen bonds was found.

The *cis*/*trans* ratio depends mostly on the chirality of the amino acids forming the peptide bond involved in the *cis*/*trans* isomerization. Incorporation into small peptides of *N*-modified amino acids with the same chirality as the preceding amino acid implicated in the peptide bond undergoing the *cis*/*trans* isomerization, moves the equilibrium significantly towards the *cis* form. A homochiral sequence exhibits a strong preference for the β VI-folded conformation in contrast to the heterochiral sequence which retains the β II-folded conformation with a *trans* middle amide bond [5,60].

The most popular isomer of AVP analogues with the different enantiomers of *N*-methylphenylalanine at positions 2 and 3 possessed all the peptide bonds in *trans* geometry and revealed a preference to form the β-turn of type IV at position 2,3.

The synthesis of [MePhe^2,D-MePhe^3]AVP afforded two products, **A** and **B**. One-dimensional NMR spectra at an elevated temperature allowed the hypothesis to be excluded that these products are two stable conformers of the same peptide, whereas the hypothesis concerning racemization of Cys^1 in [MePhe^2,D-MePhe^3]AVP seems to be correct. In order to check the putative racemization, [D-Cys^1,MePhe^2,D-MePhe^3]AVP was synthesized. A comparison of retention times on HPLC of product **B** and that of the synthesized [D-Cys^1,MePhe^2,D-MePhe^3]AVP shows that both peptides are identical. The possibility is not excluded of racemization of Cys^1 in the remaining analogues of this series. However, only in the case of [MePhe^2,D-MePhe^3]AVP, did this process turn out to be so much advanced that two strong peaks in the HPLC chromatogram were noticed.

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