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ORIGINAL ARTICLE

Enhanced β-turn conformational stability of tripeptides containing ΔPhe in *cis* over *trans* configuration

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Abstract Conformations of three pairs of dehydropeptides with the opposite configuration of the ΔPhe residue, Boc-Gly-$\Delta^{Z/E}$Phe-Phe-*p*-NA (**Z-*p*-NA** and **E-*p*-NA**), Boc-Gly-$\Delta^{Z/E}$Phe-Phe-OMe (**Z-OMe** and **E-OMe**), and Boc-Gly-$\Delta^{Z/E}$Phe-Phe-OH (**Z-OH** and **E-OH**) were compared on the basis of CD and NMR studies in MeOH, TFE, and DMSO. The CD results were used as the additional input data for the NMR-based calculations of the detailed solution conformations of the peptides. It was found that **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, and **Z-OH** adopt the β-turn conformations and **E-OMe** and **E-OH** are unordered. There are two overlapping type III β-turns in **Z-*p*-NA**, type II' β-turn in **E-*p*-NA**, and type II β-turn in **Z-OMe** and **Z-OH**. The results obtained indicate that in the case of methyl esters and peptides with a free carboxyl group, Δ^{Z}Phe is a much stronger inducer of ordered conformations than Δ^{E}Phe. It was also found that temperature coefficients of the amide protons are not reliable indicators of intramolecular hydrogen bonds donors in small peptides.

Keywords Dehydropeptides · Dehydrophenylalanine configuration · Circular dichroism · Nuclear magnetic resonance · Dehydropeptide conformation · Temperature coefficients of amide protons

Introduction

α,β-Dehydroamino acid residues contain a double bond between the C^{α} and C^{β} atoms. When the C^{β} atom bears different substituents, they can exist in two forms—as isomers Z, with the higher ranking substituent in position *cis* to the nitrogen atom, and isomers E, with this substituent *trans* to nitrogen. Isomers Z are more thermodynamically stable than their E counterparts and hence easier to obtain (Stammer 1982; Makowski et al. 1986; Latajka et al. 2006). A great majority of papers published so far on dehydropeptides deal with dehydroamino acid residues of the Z configuration. The most studied dehydroamino acid residue has been dehydrophenylalanine. It has been found that both in the solid state and solution, a ΔPhe residue of the Z configuration induces β-turns in short sequences and a 3_{10}-helix in longer ones or peptides with more than one dehydro residue (Mathur et al. 2004; Gupta and Chauhan 2011). It may also introduce long-range interactions in peptides (Ramagopal et al. 2001, 2002) or break a β structure (Gupta et al. 2008).

The conformational preferences of a ΔPhe residue of the E configuration have been much less studied. In the solid state, this residue occupies the $i + 1$ position in type II or II' β-turn (Makowski et al. 2005, 2007, 2010). Ordered conformations have been also found for Δ^{E}Phe-containing peptides in solution (Latajka et al. 2006, 2008a, b).

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Still, in the literature there had been no direct comparison of the conformational properties of dehydropeptides containing one ΔPhe residue and differing only by its configuration. In this connection some time ago we published a paper (Lisowski et al. 2010) which presents the CD and NMR conformational studies on two pairs of tetrapeptides of a general formula Boc-Gly-$\Delta^{Z/E}$Phe-Gly-Phe-X, with X = *p*-nitroanilide or methyl ester. It was found that Boc-Gly-Δ^{E}Phe-Gly-Phe-OMe is unordered and all the other peptides adopt the β-turn conformation. There are two overlapping β-turns in Boc-Gly-Δ^{Z}Phe-Gly-Phe-OMe and Boc-Gly-Δ^{Z}Phe-Gly-Phe-*p*-NA, of type II at Gly^{1}-Δ^{Z}Phe^{2} and type III' at Δ^{Z}Phe^{2}-Gly^{3}, and two overlapping type III β-turns in Boc-Gly-Δ^{E}Phe-Gly-Phe-*p*-NA, at Δ^{E}Phe^{2}-Gly^{3} and Gly^{3}-Phe^{4}.

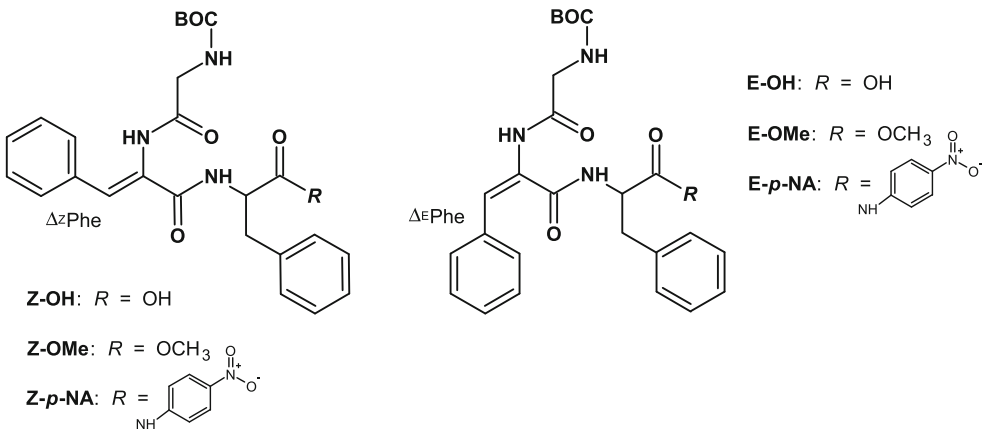
In this paper we present the results of our studies on the three following pairs of dehydropeptides:

Boc-Gly-Δ^{Z}Phe-Phe-*p*-NA	**Z-*p*-NA**
Boc-Gly-Δ^{E}Phe-Phe-*p*-NA	**E-*p*-NA**
Boc-Gly-Δ^{Z}Phe-Phe-OMe	**Z-OMe**
Boc-Gly-Δ^{E}Phe-Phe-OMe	**E-OMe**
Boc-Gly-Δ^{Z}Phe-Phe-OH	**Z-OH**
Boc-Gly-Δ^{E}Phe-Phe-OH	**E-OH**

Their structures are presented in the Scheme 1. We chose such peptides because they are shorter than the tetrapeptides described above so their conformations are determined to a larger degree by the conformational properties of a ΔPhe residue itself. At the same time their sequences are very similar to the tetrapeptides which allows the correlation of the results obtained for the two groups of peptides. Moreover, we wanted to check how the tripeptides conformation is affected by the nature of the C-terminal blocking group or the lack of it since it has been found for the tetrapeptides (Lisowski et al. 2010) that the ordered structure-inducing properties of Δ^{E}Phe depend on their C-terminal blocking group. Another important reason for this study was the observation that in one of the tetrapeptides the temperature coefficients of amide protons forming hydrogen bonds were larger than the cut-off value of 0.0046 ppm/K (Cierpicki and Otlewski 2001). We wanted to check how this criterion for detecting hydrogen bonds in proteins and peptides works for such small molecules like the peptides studied.

The conformations of the tripeptides were studied by CD in MeOH, TFE, and DMSO, and NMR in DMSO-d_6. Combination of the two methods is a very effective way of conformational studies on small peptides. In the case of such flexible and dynamic compounds NMR only is very often not capable of providing sufficient solution structure data due to the small number of ROE contacts and often ambiguous $^{3}J_{HH}$ couplings constants. In such a case, it is very advantageous to have additional information on the peptide conformation from CD, concerning the type of structure which dominates in its conformational equilibrium. We used then the CD results as the additional input data for the NMR-based calculations of the detailed solution conformations of the peptides studied. Such an approach has been successfully applied in our conformational studies on the decapeptide fragment of ubiquitin (Jaremko et al. 2009) and the tetrapeptides mentioned above (Lisowski et al. 2010).

Conformational properties of **Z-*p*-NA** has been studied earlier by X-ray, CD, and NMR (Lisowski et al. 2008). The only conclusion reached from solution studies was that the peptide adopts the type III β-turn conformation but without specifying its position. Likewise, little has been deduced from the CD and NMR studies on **E-*p*-NA** (Latajka et al. 2006). The authors found only that its conformation is ordered. The NMR and CD studies and ab initio calculations have been also performed recently on **Z-OMe** (Jewgiński et al. 2013). In solution, it was found merely that the



Scheme 1 Structures of **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, **E-OMe**, **Z-OH**, and **E-OH**

peptide adopts the ordered conformation and no specific information were given. In this paper, we present the detailed solution conformations of **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, and three other related dehydropeptides on the basis of our CD and NMR studies. The results presented here may be helpful in the *de novo* design of peptides with specific conformations in solution.

Materials and methods

Synthesis of peptides

The syntheses of **Z-*p*-NA**, **E-*p*-NA**, and **Z-OMe** have been described by Makowski et al. (2001); Latajka et al. (2006); and Jewgiński et al. (2013), respectively.

Boc-Gly-Δ^{E}Phe-Phe-OMe (E-OMe)

E-OMe was synthesized according to the method of Makowski et al. (1986). Boc-Gly-Δ^{E}Phe-OH (1.602 g, 5 mmol) was coupled with H-Phe-OMe (0.896 g, 5 mmol) in DMF (7.5 ml) by mixed anhydride method with *i*-BuOCOCl (0.65 ml, 5 mmol). During the reaction extensive configurational inversion took place and both **E-OMe** and **Z-OMe** were obtained with a ratio of 1:1.3. The isomers were separated on a silica gel H60 column with ethyl acetate–benzene (2–60 % v/v of ethyl acetate) as an eluent. The product was crystallized from chloroform–benzene (2:1, v/v)/hexane. Yield 24 %; mp 67.5–70 °C; elemental analysis calcd (%) for $C_{26}H_{31}N_4O_6$ (481.55): C 64.85, H 6.49, N 8.73; found: C 64.80, H 6.53, N 8.80. ^{1}H-NMR (800 MHz, DMSO-d_6), δ (ppm): 1.41 (9H, s, 3 × CH_3 Boc), 2.88–2.92 (2H, m, $C^{\beta}H_2$ Phe^3), 3.53 (3H, s, CH_3 Me), 3.68 (2H, broad s, $C^{\alpha}H_2$ Gly^1), 4.52 (1H, m, $C^{\alpha}H$ Phe^3), 6.83 (1H, s, $C^{\beta}H$ Δ^{E}Phe^2), 7.01 (1H, t, NH Gly^1), 7.14–7.27 (5H, m, aromatic protons of Phe^3), 7.19–7.22 (5H, m, aromatic protons of Δ^{E}Phe^2), 8.61 (1H, d, NH Phe^3), 9.53 (1H, broad s, NH Δ^{E}Phe^2); ^{13}C-NMR (201 MHz, DMSO-d_6), δ (ppm): 31.36 (CH_3 Boc), 39.80 (C^{β} Phe^3), 46.64 (C^{α} Gly^1), 54.85 (CH_3 Me), 57.13 (C^{α} Phe^3), 120.26 (C^{β} Δ^{E}Phe^2), 129.68 (C^{δ} Δ^{E}Phe^2), 129.98 (C^{ε} Δ^{E}Phe^2), 132.13 (C^{δ} Phe^3), 131.38 (C^{ζ} Phe^3), 131.49 (C^{ε} Phe^3).

Boc-Gly-Δ^{Z}Phe-Phe-OH (Z-OH)

Z-OH was obtained from **Z-OMe** (0.964 g, 2 mmol) by a standard basic hydrolysis with a 10 %-fold excess of 1 M NaOH in MeOH, for 2 h at room temperature. It was crystallized from ethyl acetate–MeOH (5:1, v/v)/hexane. Yield 96 %; mp 181–183 °C; elemental analysis calcd (%) for $C_{25}H_{29}N_4O_6$ (467.52): C 64.23, H 6.25, N 8.99; found: C 64.12, H 6.30, N 9.13.

^{1}H-NMR (800 MHz, DMSO-d_6), δ (ppm): 1.42 (9H, s, 3 × CH_3 Boc), 3.03–3.11 (2H, m, $C^{\beta}H_2$ Phe^3), 3.71 (2H, broad s, $C^{\alpha}H^2$ Gly^1), 4.49 (1H, m, $C^{\alpha}H$ Phe^3), 7.09 (1H, s, $C^{\beta}H$ of Δ^{Z}Phe^2), 7.12 (1H, t, NH Gly^1), 7.21–7.29 (5H, m, aromatic protons of Phe^3), 7.37–7.56 (5H, m, aromatic protons of Δ^{Z}Phe^2), 8.07 (1H, d, NH Phe^3), 9.44 (1H, broad s, NH Δ^{Z}Phe^2); ^{13}C-NMR (201 MHz, DMSO-d_6), δ (ppm): 31.36 (CH_3 Boc), 39.71 (C^{β} Phe^3), 46.81 (C^{α} Gly^1), 57.35 (C^{α} Phe^3), 132.31 (C^{β} Δ^{Z}Phe^2), 132.79 (C^{δ} Δ^{Z}Phe^2), 131.65 (C^{ε} Δ^{Z}Phe^2), 131.45 (C^{δ} Phe^3), 129.52 (C^{ζ} Phe^3), 132.33 (C^{ε} Phe^3).

Boc-Gly-Δ^{E}Phe-Phe-OH (E-OH)

E-OH was obtained from **E-OMe(0.964 g, 2 mmol)** by a standard basic hydrolysis with a 10 %-fold excess of 1 M NaOH in MeOH, for 2 h at room temperature. It was crystallized from ethyl acetate–MeOH (2:1, v/v)/hexane. Yield 96 %; mp 145–147.5 °C; elemental analysis calcd (%) for $C_{25}H_{29}N_4O_6$ (467.52): C 64.23, H 6.25, N 8.99; found: C 64.33, H 6.12, N 9.18.

^{1}H-NMR (800 MHz, DMSO-d_6), δ (ppm): 1.40 (9H, s, 3 × CH_3 Boc), 2.91–3.06 (2H, m, $C^{\beta}H_2$ Phe^3), 3.69 (2H, d–d, $C^{\alpha}H^2$ Gly^1), 4.44 (1H, m, $C^{\alpha}H$ Phe^3), 6.84 (1H, s, $C^{\beta}H$ Δ^{E}Phe^2), 6.99 (1H, t, NH Gly^1), 7.14–7.27 (5H, m, aromatic protons of Phe^3), 7.16–7.24 (5H, m, aromatic protons of Δ^{E}Phe^2), 8.26 (1H, d, NH Phe^3), 9.51 (1H, broad s, NH Δ^{E}Phe^2); ^{13}C-NMR (201 MHz, DMSO-d_6), δ (ppm): 30.62 (CH_3 Boc), 39.07 (C^{β} Phe^3), 46.03 (C^{α} Gly^1), 56.40 (C^{α} Phe^3), 119.80 (C^{β} Δ^{E}Phe^2), 130.64 (C^{δ} Δ^{E}Phe^2), 128.63 (C^{ε} Δ^{E}Phe^2), 129.28 (C^{ζ} Δ^{E}Phe^2), 131.55 (C^{δ} Phe^3), 130.65 (C^{ζ} Phe^3), 130.53 (C^{ε} Phe^3).

Circular dichroism spectroscopy

CD spectra were recorded on a Jasco J-600 spectropolarimeter, at room temperature. Spectra were measured in MeOH, TFE, and DMSO. Pathlength of 1 mm was used. Concentrations of the solutions were in the range of 0.17–0.21 mg/ml (2.6–3.9 × 10^{-4} M). Each spectrum represents the average of at least four scans. The data are presented as molar ellipticity [θ].

NMR spectroscopy

NMR spectra were recorded at 11.7 T on a Varian Unity + 500 spectrometer at 25 °C, in DMSO-d_6. In all cases 5–10 mM peptide solutions were used. The 1D ^{1}H-NMR spectra were processed and analyzed with a VnmrJ software (Varian Inc. Palo Alto, USA). The acquired two dimensional homonuclear NMR spectra were processed by NMRPipe (Delaglio et al. 1995) and analyzed with Sparky (Goddard and Kneller 2003) programs. Complete

assignments of the ^{1}H and ^{13}C resonances for all peptides were done by application of a standard procedure (Wüthrich 1986) based on inspection of the 2D homonuclear TOCSY (with mixing times 10 and 90 ms) and ROESY (with mixing times 200 and 500 ms) experiments. The temperature coefficients of amide protons were measured in DMSO-d_6 at the temperature range from 291 to 318 K, with a temperature interval of 3 K. Temperature dependencies of all investigated amide protons were found to be linear with $R^2 > 0.995$. Through space distances between protons were determined by analysis of 2D ^{1}H-^{1}H ROESY spectra inter proton cross peaks. Only non-trivial inter-proton distance constraints were used in the calculations and all were kept in the range of 1.8 Å (sum of Van der Waals radii) up to 5.5 Å (ROE upper limit). Calculations of lowest-energy structures, carried out for peptides which exhibited ordered conformations in solutions according to CD, were performed with an X-PLOR NIH 2.21 program package (Schwieters et al. 2003). Additional dihedral angles restraints defining the β-turn type (Lewis et al. 1973) indicated by CD with the deviation of ±90° were applied. The values of φ and ψ angles typical for the β-turn type indicated by CD were used in the calculations for certain amino acids along the sequence only if their presence was justified by the inter-proton ROE contacts. Figures presenting ensembles of 50 lowest-energy structures from the 500 calculated peptide conformers were prepared with a MolMol software (Koradi et al. 1996). Supplementary data are available from the authors upon request.

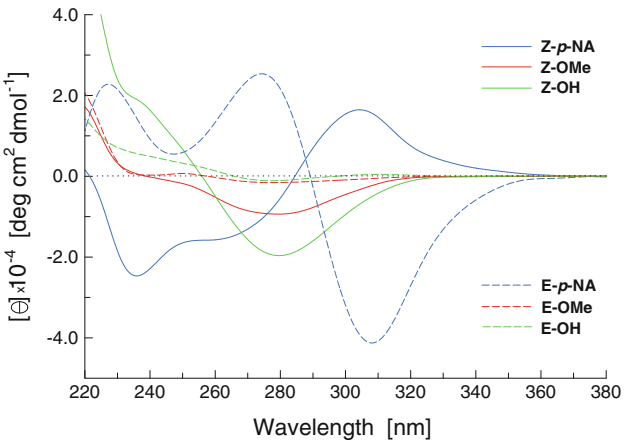


Fig. 1 CD spectra of **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, **E-OMe**, **Z-OH**, and **E-OH** in MeOH

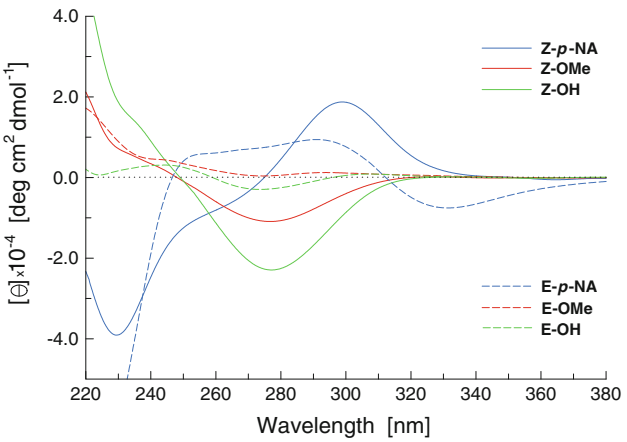


Fig. 2 CD spectra of **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, **E-OMe**, **Z-OH**, and **E-OH** in TFE

Results

CD studies

The CD spectra of **Z-*p*-NA** in MeOH, TFE, and MeCN has been measured before (Lisowski et al. 2008). We measured a new spectra of that peptide in MeOH and TFE, and for the first time in DMSO, to compare them with the spectra of its configurational counterpart, **E-*p*-NA**.

The CD spectra of all the peptides studied in MeOH are presented in Fig. 1. The spectrum of **Z-*p*-NA** consists of a large positive band of *p*-NA at 304 nm, a very distinct negative shoulder at about 264 nm of nearly equal intensity, due to the charge-transfer transition in the ΔPhe residue (usually this band appears at 280 nm), and a strong negative band at 236 nm, corresponding to the π–π* transition of the dehydro chromophore (Pieroni et al. 1996). In the case of **Z-OMe**, there is a negative band at 279 nm and positive ellipticities below 230 nm. The spectrum of **Z-OH** shows a large negative band at 280 nm, twice bigger than that of **Z-OMe**, a positive shoulder at 237 nm, and positive ellipticities below 230 nm. Very large bands, negative at 308 nm, and two positive at 274 and 227 nm, are observed for **E-*p*-NA**. The bands at 308 and 274 nm are distinctly stronger than the corresponding ones of **Z-*p*-NA**. The CD spectra of **E-OMe** and **E-OH** differ very much from those of other peptides. There are practically no bands in the near-UV region, only positive ellipticities at shorter wavelengths.

In TFE (Fig. 2), the CD spectrum of **Z-*p*-NA** shows a large positive band at 299 nm, a negative shoulder at 264 nm, and a very large negative band at 229 nm. The spectra of **Z-OMe** and **Z-OH** are very similar to those in MeOH. They show a negative band at 277 nm, twice larger for **Z-OH**, a positive shoulder at about 237 nm, and positive ellipticities at shorter wavelengths. The spectrum of **E-*p*-NA** differs very much in its shape and decreased intensities from that in MeOH. It consists of a negative band at 331 nm, a positive band at 291 nm, a positive shoulder at 253 nm, and a very large, negative band below 230 nm. In the CD spectrum of **E-OMe** there are only residual

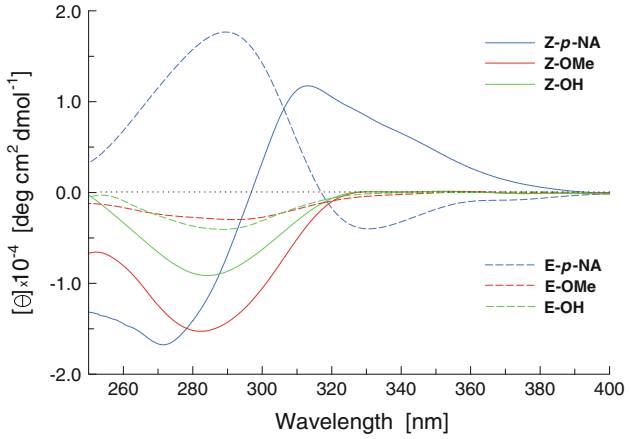


Fig. 3 CD spectra of **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, **E-OMe**, **Z-OH**, and **E-OH** in DMSO

ellipticities in the near-UV region and a weak, positive shoulder at 244 nm. In the case of **E-OH** small bands, negative at 274 nm and positive at 244 nm, are observed.

The spectra in DMSO (Fig. 3) were measured for a direct comparison of the CD and NMR results. They are characterized by smaller band intensities than in other two solvents. In the case of **Z-*p*-NA** a broad positive band at 313 nm and a negative one at 272 nm are observed. For **Z-OMe** and **Z-OH**, a negative band is present at 282 and 284 nm, respectively. In DMSO, contrary to MeOH and TFE, the intensity of that band is larger for **Z-OMe** than for **Z-OH**. The spectrum of **E-*p*-NA** shows a very weak, negative band at 331 nm and a large, positive band at 289 nm. Very small, negative bands are observed for **E-OMe** and **E-OH**, at 292 and 286 nm, respectively.

NMR studies

To check for the presence of intramolecular hydrogen bonds in the peptides investigated the temperature dependence of their amide protons was measured. Amide proton chemical shifts are sensitive to temperature changes. If a proton forms a hydrogen bond, then its chemical shift shows a very small temperature dependence. The presence or absence of hydrogen bonds is indicated by a $d\delta/dT$ (ppm K^{-1}) coefficient. It has been established that amide protons in protein systems, which are characterized by the temperature coefficient value lower than 0.0046 ppm K^{-1}, are involved in hydrogen bonds (Cierpicki and Otlewski 2001). This value is often used for hydrogen bond assignments in conformational studies on peptides and proteins. The temperature coefficients of amide protons in the peptides studied are presented in Table 1. They show the presence of three hydrogen bonds, formed by amide protons of Gly^1 and *p*-NA in **Z-*p*-NA** and the amide proton of *p*-NA in **E-*p*-NA**. Values slightly

Table 1 Temperature coefficients for amide protons of **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, **E-OMe**, **Z-OH**, and **E-OH** in DMSO-d_6

Residue	$d\delta/dT$ (ppm K^{-1} × 1,000)					
	Z-*p*-NA	**E-*p*-NA**	**Z-OMe**	**E-OMe**	**Z-OH**	**E-OH**
Gly^1	4.4	6.5	5.9	5.3	5.9	5.1
ΔPhe^2	5.2	6.0	5.0	6.5	4.7	6.3
Phe^3	4.8[a]	7.3[a]	6.0	9.6	5.7[a]	9.3
p-NA	2.8[a]	2.0	–	–	–	–

Temperature coefficients were measured at the temperature range from 291 to 318 K, with a temperature interval of 3 K. For each peptide amide, ten data points were collected and data were fitted using standard least-squares fit procedures in SigmaPlot to the y = ax + b function, where a is the amide temperature coefficient

[a] Protons forming hydrogen bonds according to calculations

bigger than the cut-off value were found for Phe^3 NH in **Z-*p*-NA** and ΔPhe^2 NH in **Z-OH**, which suggests that those protons maybe also involved in hydrogen bonds. However, the results obtained from CD and conformational calculations (see "Discussion") indicate that hydrogen bonds are formed by Phe^3 and *p*-NA of **Z-*p*-NA**, and NH's of Phe^3 in **E-*p*-NA**, **Z-OMe**, and **Z-OH**. It shows that in the case of small peptides the temperature coefficients of amide protons alone are not good indicators of hydrogen bonds.

Important conformational information can be obtained from the ROESY spectra. They allow to detect interatomic contacts in the peptides studied. It was found that trivial, intraresidue contacts are present in each investigated peptide, whereas non-trivial contacts were observed for **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, and **Z-OH** (Table 2; Fig. 5). The number of such contacts determined for **Z-*p*-NA** (11) is comparable with that found for its tetrapeptide counterpart (12) (Lisowski et al. 2010). For **E-*p*-NA**, the number of non-trivial ROE contacts is six and for its tetrapetide counterpart it is eight, whereas for **Z-OMe** it is eight and for its tetrapetide counterpart it is five. Non-trivial contacts are absent in **E-OMe** and **E-OH** which confirms the lack of intramolecular hydrogen bonds in the two peptides. Such contacts were used as the input data for calculations of the lowest-energy conformers of **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, and **Z-OH**. Besides interatomic contacts, the calculations were based on the coherent results obtained at the same time by the NMR and CD, i.e., which amide protons form hydrogen bonds and what type of a β-turn is present in a given peptide. In such a case it is important to have the results from the two methods which can be directly compared. We achieved that by measurement of the CD spectra also in DMSO, which was used for the NMR experiments. In all the cases there is a large similarity between the CD spectra measured in DMSO and other solvents (excluding **E-*p*-NA** in TFE). The only exception is largely diminished intensity of the *p*-NA band of **E-*p*-NA** at 331 nm in

Table 2 Interatomic non-trivial contacts in **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, and **Z-OH** detected from the ROESY spectra in DMSO-d_6

Peptide	Interatomic contacts
Z-*p*-NA	CH_3 Boc ↔ $C^{\alpha}H^{\alpha 1}$ Gly^1
	CH_3 Boc ↔ $C^{\alpha}H^{\alpha 2}$ Gly^1
	CH_3 Boc ↔ aromatic H's $\Delta^{Z}Phe^2$
	CH_3 Boc ↔ aromatic H's Phe^3
	NH $\Delta^{Z}Phe^2$ ↔ $C^{\alpha}H^{\alpha 1}$ Gly^1
	NH $\Delta^{Z}Phe^2$ ↔ $C^{\alpha}H^{\alpha 2}$ Gly^1
	NH $\Delta^{Z}Phe^2$ ↔ NH Gly^1
	NH Phe^3 ↔ NH $\Delta^{Z}Phe^2$
	NH Phe^3 ↔ $C^{\beta}H$ $\Delta^{Z}Phe^2$
	NH *p*-NA ↔ NH Phe^3
	NH *p*-NA ↔ $C^{\alpha}H$ Phe^3
E-*p*-NA	NH $\Delta^{E}Phe^2$ ↔ $C^{\alpha}H^{\alpha 1}$ Gly^1
	NH $\Delta^{E}Phe^2$ ↔ $C^{\alpha}H^{\alpha 2}$ Gly^1
	NH $\Delta^{E}Phe^2$ ↔ aromatic H's *p*-NA
	$C^{\beta}H$ $\Delta^{E}Phe^2$ ↔ aromatic H's *p*-NA
	NH *p*-NA ↔ NH Phe^3
	NH *p*-NA ↔ $C^{\alpha}H$ Phe^3
Z-OMe	$C^{\alpha}H_2$ Gly^1 ↔ CH_3 Boc
	NH $\Delta^{Z}Phe^2$ ↔ $C^{\alpha}H_2$ Gly^1
	NH $\Delta^{Z}Phe^2$ ↔ NH Phe^3
	aromatic H's $\Delta^{Z}Phe^2$ ↔ $C^{\alpha}H_2$ Gly^1
	$C^{\alpha}H$ Phe^3 ↔ CH_3 Boc
	$C^{\beta}H^{\beta 1}Phe^3$ ↔ CH_3 Boc
	$C^{\beta}H^{\beta 2}Phe^3$ ↔ CH_3 Boc
	NH Phe^3 ↔ $C^{\beta}H$ $\Delta^{Z}Phe^2$
Z-OH	CH_3 Boc ↔ $C^{\alpha}H_2$ Gly^1
	CH_3 Boc ↔ aromatic H's $\Delta^{Z}Phe^2$
	CH_3 Boc ↔ $C^{\beta}H^{\beta 2}$ Phe^3
	CH_3 Boc ↔ aromatic H's Phe^3
	NH Gly^1 ↔ NH $\Delta^{Z}Phe^2$
	$C^{\alpha}H_2$ Gly^1 ↔ NH $\Delta^{Z}Phe^2$
	$C^{\alpha}H_2$ Gly^1 ↔ aromatic H's $\Delta^{Z}Phe^2$
	$C^{\alpha}H_2$ Gly^1 ↔ NH Phe^3
	$C^{\alpha}H_2$ Gly^1 ↔ aromatic H's Phe^3
	NH $\Delta^{Z}Phe^2$ ↔ NH Phe^3
	$C^{\beta}H$ $\Delta^{Z}Phe^2$ ↔ NH Phe^3

DMSO. The calculations were performed with the assumption that there are two overlapping type III β-turns in **Z-*p*-NA**, stabilized by two hydrogen bonds Phe^3 NH···Boc CO and *p*-NA NH···$\Delta^{Z}Phe^2$ CO, and type II β-turns in **Z-OMe** and **Z-OH**, at the Gly^1-$\Delta^{Z}Phe^2$ residues, with no hydrogen bonds. In the case of **E-*p*-NA**, the calculations were performed with a type II' β-turn at either Gly^1-$\Delta^{E}Phe^2$ or $\Delta^{E}Phe^2$-Phe^3 as the starting conformation. In each case very similar results were obtained. The lowest-energy conformers calculated for **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, and **Z-OH** are presented in Fig. 4a–d and the values of their dihedral angles are given in Table 3. Figure 4e–h also shows the ensembles of 50 lowest-energy structures of those peptides, chosen from 500 calculated.

Discussion

The CD spectra were analyzed in the near-UV region only since there are overlapping contributions of the peptide, aromatic, and unsaturated chromophores in the far-UV which makes their analysis in that region quite difficult. The dehydropeptides studied contain a ΔPhe residue which gives a large CD band in the near-UV region, at about 280 nm, which is very sensitive to a dehydropeptide conformation (Pieroni et al. 1993, 1996; Ramagopal et al. 2002). In this connection this residue is a very good conformational probe in the studies on dehydropeptides. In **Z-*p*-NA** and **E-*p*-NA**, there is also the *p*-NA group which has been found to be a useful tool in the conformational studies on peptides (Sato et al. 1981; Baldwin et al. 1994; Halab et al. 2000; Lisowski et al. 2008, 2010). This group gives Cotton effects in the near-UV as well which can be either positive or negative depending on the peptide conformation (Latajka et al. 2006; Lisowski et al. 2008, 2010).

Large intensities of the CD bands of **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, and **Z-OH** in the near-UV region (Figs. 1, 2, 3) show that the peptides adopt chiral, rigid conformations in solution. Taking into account the conformational properties of a ΔPhe residue, one can expect these are folded structures of the β-turn type stabilized by intramolecular hydrogen bonds. The analysis of the CD spectra of **Z-*p*-NA** in MeOH, TFE, and MeCN has been presented before (Lisowski et al. 2008). It was found that the peptide adopts different, solvent-dependent forms of the type III β-turn conformation, with one or two overlapping turns. The CD spectrum of **Z-*p*-NA** in DMSO (Fig. 3) shows that its conformation is quite rigid. DMSO is known to lower the amount of ordered conformations of dehydropeptides (Pieroni et al. 1993, 1996). A smaller intensity of the *p*-NA band of **Z-*p*-NA** in DMSO at 313 nm as compared with MeOH and TFE shows that the conformational equilibrium of **Z-*p*-NA** in that solvent shifts toward unordered structures but this change is not substantial.

The conclusions reached for **Z-*p*-NA** from CD are supported by the NMR studies. The temperature coefficients of the amide protons of Phe^3 and *p*-NA in **Z-*p*-NA** (Table 1) indicate that they may be involved in intramolecular hydrogen bonds stabilizing two overlapping β-turns predicted by the CD. It is consistent with the results of calculations which show the presence of two intramolecular hydrogen bonds of the 4 → 1 type, i.e., Phe^3 NH···Boc CO and *p*-NA NH···Gly^1 CO. On the other hand, the calculations do not show the presence of a hydrogen bond

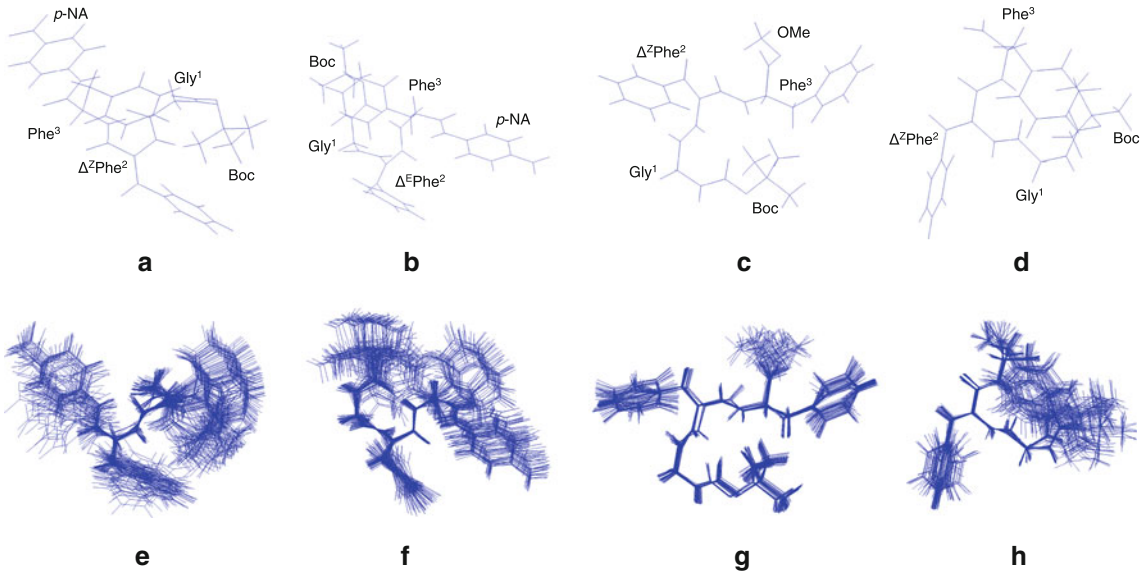
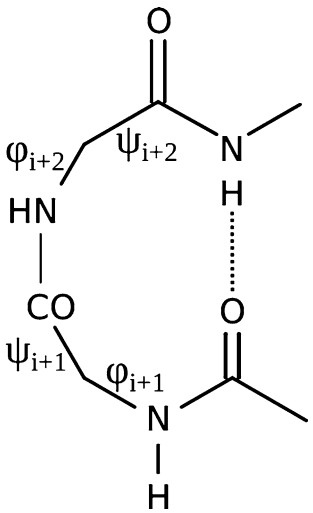


Fig. 4 The lowest-energy conformers of **Z-*p*-NA**. (**a**), **E-*p*-NA** (**b**), **Z-OMe** (**c**), and **Z-OH** (**d**), and ensembles of the 50 lowest-energy structures of **Z-*p*-NA**. (**e**), **E-*p*-NA** (**f**), **Z-OMe** (**g**), and **Z-OH** (**h**)

Table 3 Torsion angle values of **Z-*p*-NA**, **E-*p*-NA**, **Z-OMe**, and **Z-OH** calculated on the basis of NMR and CD parameters with an X-PLOR program. Standard deviations for 50 lowest-energy structures are given in brackets

Peptide	Residue					
	Gly[1]		ΔPhe[2]		Phe[3]	
	φ	ψ	φ	ψ	φ	ψ
Z-*p*-NA	−20° (±89°)	−47° (±28°)	−18° (±34°)	−60° (±33°)	−75° (±25°)	−8° (±33°)
E-*p*-NA	48° (±7°)	−110° (±16°)	−115° (±14°)	44° (±10°)	53° (±6°)	13° (±7°)
Z-OMe	−46° (±35°)	74° (±6°)	93° (±16°)	33° (±44°)	−38° (±37°)	–
Z-OH	−48° (±4°)	137° (±38°)	57° (±10°)	−17° (±24°)	−20° (±63°)	–

Standard torsion angles φ_{i+1}, ψ_{i+1}, φ_{i+2}, ψ_{i+2} for type I, II, and III β-turns (Lewis et al. 1973) are as follows, respectively: type I −60, −30, −90, 0; type II −60, 120, 80, 0; type III −60, −30, −60, −30. Types I', II', and III' have the same angles as types I, II, and III, respectively, but with the opposite sign



formed by the amide proton of Gly^1 indicated by its temperature coefficient. The dihedral angles values obtained for **Z-*p*-NA** (Table 3) show that the 4 → 1 hydrogen bonds stabilize two overlapping type III β-turns, at the Gly^1–ΔPhe^2 and ΔPhe^2–Phe^3 residues. The values presented for **Z-*p*-NA** differ quite distinctly, however, from the standard values of −60° and −30° for φ and ψ angles, respectively (Lewis et al. 1973), in a type III β-turn. Another factor indicating the presence of a β-turn is the distance between the C^{α}_{i} and C^{α}_{i+3} atoms, which should be less than 7 Å (Lewis et al. 1973). The interatomic distances derived from the ROESY spectra are very helpful in detection of β-turns. In the case of the first turn in **Z-*p*-NA** the Boc O–Phe^3 C^{α} distance (the oxygen atom of the Boc group corresponds to the C^{α}_{i} atom) is 7.38 ± 0.65 Å so it is close to the limit value. For the second turn, the Gly^1 C^{α}-*p*-NA C^{α} distance (the C^{α} atom of the aromatic ring of *p*-NA corresponds to the C^{α}_{i+3} atom) is 5.23 ± 0.44 Å (the presented values are the mean values for 50 lowest-energy structures). These results are consistent with the frequency of occurrence of the two 4 → 1 hydrogen bonds in the lowest-energy structures ensemble, which is very distinctly lower for the Phe^3 NH···Boc CO hydrogen bond. Taken together, the dihedral angles values and the values corresponding to the C^{α}_{i}–C^{α}_{i+3} distances show that the type III β-turns present in **Z-*p*-NA** are quite distorted as compared with the standard and the first one at Gly^1-$\Delta^Z Phe^2$ is less stable than the second at $\Delta^Z Phe^2$–Phe^3. The solution conformation of **Z-*p*-NA** differs from its crystal structure (Lisowski et al. 2008) where only one type II β-turn at ΔPhe^2–Phe^3, with the *p*-NA NH···Gly^1 CO hydrogen bond, is present. The lowest-energy conformation of the peptide is presented in Fig. 4a. The ensemble of 50 lowest-energy conformers (Fig. 4e) shows that **Z-*p*-NA** retains a marked conformational freedom in solution.

The CD spectrum of **E-*p*-NA** in MeOH in the near-UV (Fig. 1) consists of large Δ^EPhe and *p*-NA bands which indicates the ordered conformation of the β-turn type. It has been found (Pieroni et al. 1996) that a negative band at 280 nm of the ΔPhe residue is typical of dehydropeptides in the type II β-turn conformation, with the dehydro residue at the $i + 2$ position. In the case of dehydropeptides adopting a type III β-turn conformation, a positive ΔPhe band at 280 nm is observed, accompanied by a negative band at 235 nm which may be diagnostic for distinguishing between type II and type III β-turns. The positive sign of the Δ^EPhe residue and the absence of the negative band at 235 nm in the spectrum of **E-*p*-NA** indicates that the peptide adopts the type II' β-turn conformation stabilized by the 4 → 1 hydrogen bond. This is a single β-turn because due to the φ and ψ angles values typical of various β-turn types, the only two-β-turn combination possible is II'–III and the presence of a type III β-turn in **E-*p*-NA** is very doubtful on the basis of the peptide CD spectrum. The type II' β-turn is located either at the Gly^1-$\Delta^E Phe^2$ or $\Delta^E Phe^2$–Phe^3 residues. A very large intensity of the band at 308 nm shows that the conformational freedom of the *p*-NA group is distinctly restricted which can result from its participation in the *p*-NA NH···Gly^1 CO hydrogen bond. It suggests that **E-*p*-NA** adopts the type II' β-turn conformation at the $\Delta^E Phe^2$–Phe^3 residues. This is, however, in some contradiction with the results obtained in DMSO (Fig. 3). On transition from MeOH to DMSO the intensity of the Δ^EPhe band is decreased to a small extent while the intensity of the *p*-NA band is drastically reduced. These changes suggest that the $\Delta^E Phe^2$ residue is a part of the ordered, rigid structure of the same type in both solvents whereas the *p*-NA group is much more conformationally labile and becomes unordered in DMSO. This in turn would indicate that a β-turn in **E-*p*-NA** is located at the Gly^1–$\Delta^E Phe^2$ residues and stabilized by the Phe^3 NH···Boc CO hydrogen bond.

The position of a β-turn in **E-*p*-NA** was determined by NMR studies. The temperature coefficients of the amide protons of the peptide (Table 1) indicate very distinctly that only that of *p*-NA forms a hydrogen bond. Its temperature coefficient (2.0) is the smallest found for the peptides studied. It shows that a β-turn is located at $\Delta^E Phe^2$–Phe^3. However, the calculations gave a different result. The results presented in Table 1 were obtained with the type II' β-turn at Gly^1–$\Delta^E Phe^2$. They show that the only 4 → 1 hydrogen bond present in **E-*p*-NA** is that between Phe^3 NH and Boc CO. It is interesting that the temperature coefficient of Phe^3 NH (7.3) is the largest among amide protons of **E-*p*-NA**. The Phe^3 NH···Boc CO hydrogen bond stabilizes the β-turn at the Gly^1–$\Delta^E Phe^2$ residues. It can be seen from the values of dihedral angles (Table 3) that this is a β-turn of type II', in accordance with the CD results. In this case the distance Boc O–Phe C^{α} is 5.71 ± 0.34 Å. The lowest-energy structure of **E-*p*-NA** is presented in Fig. 3b and the ensemble of its 50 lowest-energy conformations in Fig. 3f. The latter figure shows that **E-*p*-NA** is comparably flexible in solution like **Z-*p*-NA**.

A surprising result obtained for **E-*p*-NA** is its CD spectrum in TFE (Fig. 2). It is markedly different from that in MeOH. Largely decreased intensities of the Δ^EPhe and *p*-NA bands and their shift to the longer wavelengths suggest a substantial increase of the conformational freedom of the peptide and/or a change in its conformational equilibrium consisting in appearance of larger amounts of unordered or other structures. It is unexpected since TFE is well known as an ordered structures-inducing solvent (Cammers-Goodwin et al. 1996; Rajan and Balaram 1996; Luo and Baldwin 1997). This observation indicates that the conformation of **E-*p*-NA** is considerably solvent-dependent.

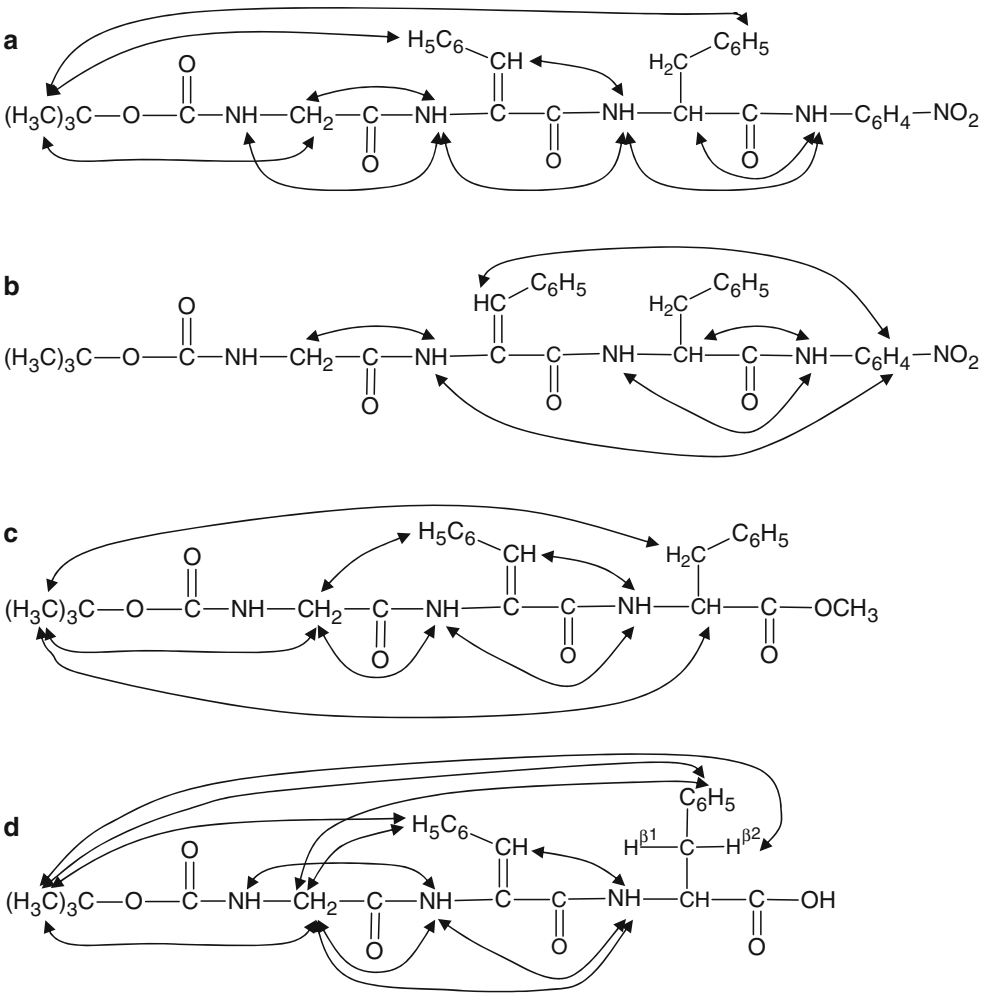


Fig. 5 Detailed chemical formulas of **Z-*p*-NA**. (**a**), **E-*p*-NA** (**b**), **Z-OMe** (**c**), and **Z-OH** (**d**) with indicated observed non-trivial ROESY contacts

The CD spectra of **Z-OMe** and **Z-OH** in MeOH and TFE (Figs. 1, 2) are very similar which shows that the conformational equilibria of the peptides are dominated by the same type of structures. The negative ΔPhe bands at 280 nm indicate that the dominant form of both peptides is a type II β-turn with the $\Delta^{Z}Phe^{2}$ residue at the $i + 2$ position (Pieroni et al. 1996). The intensities of those bands show that type II β-turn population is about twice larger in **Z-OH** than in **Z-OMe**. The CD spectra of **Z-OMe** and **Z-OH** in DMSO (Fig. 3) show that their β-turn populations are retained in that solvent which shows that they are quite stable. The β-turns in both peptides are located at the Gly^{1}–$\Delta^{Z}Phe^{2}$ residues and they are stabilized by a $4 \rightarrow 1$ hydrogen bond between Phe^{3} NH and Boc CO. The presence of such a conformation in **Z-OMe** and **Z-OH** is also confirmed by the distances Boc O–Phe^{3} C^{α} derived from the ROESY spectra. They are 5.37 ± 0.07 Å and 5.49 ± 0.42 Å for **Z-OMe** and **Z-OH**, respectively, i.e., they are typical of β-turns. Surprisingly, the presence of hydrogen bonds in the both peptides is decidedly contradicted by the temperature coefficients values of their Phe^{3} NH's (Table 1). The calculations performed for **Z-OMe** confirmed the absence of the Phe^{3} NH···Boc CO hydrogen bond. At the same time they indicated its presence in **Z-OH**. Besides CD spectra, interatomic distances, and a $4 \rightarrow 1$ hydrogen bond in **Z-OH**, the presence of a type II β-turn in **Z-OMe** and **Z-OH** was confirmed by their calculated dihedral angles values. These values indicate that the both peptides adopt the type II β-turn conformation at the Gly^{1}–$\Delta^{Z}Phe^{2}$ residues. The lowest-energy structures of **Z-OMe** and **Z-OH** are presented in Fig. 4c, d and the ensembles of their 50 lowest-energy conformers in Fig. 4g, h. The presented ensembles show that the conformation of **Z-OMe** is the most rigid from the peptides studied and that the flexibility of **Z-OH** is comparable with that of **Z-*p*-NA** and **E-*p*-NA**.

A very significant result obtained from the presented CD and NMR studies concerns the temperature coefficients of the amide protons. This parameter is used for detection of intramolecular hydrogen bonds in peptides and proteins (Cierpicki and Otlewski 2001). It follows from our studies that it should be used with caution when applied to small and flexible linear molecules like the tripeptides studied. The temperature coefficients presented in Table 1 show

that there are three amide protons with the $d\delta/dT$ values smaller than 0.0046 (of Gly^1 and *p*-NA in **Z-*p*-NA** and *p*-NA in **E-*p*-NA**) and two with slightly greater values (of Phe^3 in **Z-*p*-NA** and $\Delta^Z Phe^2$ in **Z-OH**). Then it may be expected that these five amide protons are involved in hydrogen bonds. Meanwhile, the calculations based on interatomic contacts and information from the CD spectra indicate that this expectation is correct only in the case of Phe^3 and *p*-NA in **Z-*p*-NA** and three other hydrogen bonds are wrongly predicted. Other hydrogen bonds present in the peptides studied are formed by the amide protons of Phe^3 in **E-*p*-NA** and **Z-OH**. CD data, interatomic distances, and dihedral angles strongly suggest that there is also another hydrogen bond formed by the Phe^3 amide proton in **Z-OMe**. In the case of analogous tetrapeptides (Lisowski et al. 2010) a similar situation with temperature coefficients of the amide protons occurred but it was not as drastic like in the present case. It shows that amide protons temperature coefficients alone are not good indicators of hydrogen bonds in small molecules and may lead to erroneous conclusions. Our results indicate that more reliable in detection of hydrogen bonds in such compounds is interatomic distances and calculations based on interatomic contacts from the ROESY spectra (Table 2; Fig. 5) in combination with the results obtained from CD or another method like IR or Raman.

Very small ellipticities observed in the CD spectra of **E-OMe** and **E-OH** in the near-UV region (Figs. 1, 2, 3) in all the solvents used indicate that the conformational equilibria of those peptides are distinctly dominated by unordered structures. The same results were obtained from the NMR studies. The ROESY spectra did not show any sign of non-trivial interatomic contacts that could suggest the presence of ordered conformations.

Conclusions

The detailed solution conformations of three pairs of dehydropeptides (*p*-nitroanilides, methyl esters, and peptides with a free C-terminal carboxyl group), differing by the configuration of a dehydroamino acid residue, were determined on the basis of CD and NMR studies, as a function of the ΔPhe residue configuration and the nature of their C-terminal ends. It was found for all the peptides studied that the ΔPhe residue configuration plays an essential role in their conformational properties. In the case of *p*-nitroanilides, both **Z-*p*-NA** and **E-*p*-NA** are ordered but there are two β-turns in the former and only one β-turn in the latter which shows a higher order in **Z-*p*-NA**. It distinguishes them from the analogous tetrapeptide *p*-nitroanilides (Lisowski et al. 2010) studied also by CD and NMR which are ordered to a similar extent. **Z-OMe** and **Z-OH** are both ordered and they adopt the same type II β-turn conformation whereas their configurational isomers, **E-OMe** and **E-OH**, are by far unordered. These results show that a ΔPhe residue of the Z configuration is a much stronger inducer of ordered conformations than its E isomer. The results obtained for **Z-OMe** and **E-OMe** are consistent with the conclusion reached for analogous tetrapeptide methyl esters (Lisowski et al. 2010). The differences in the ordered structures-inducing potential of the ΔPhe residue depending on its configuration result probably from the different steric interactions of the ΔPhe side chain with other parts of a peptide. The results presented show also that the nature of the C-terminal end of a peptide is important for the Δ^EPhe-containing peptides whereas it is of no meaning for peptides with a Δ^ZPhe residue. Of **E-*p*-NA**, **E-OMe**, and **E-OH**, only **E-*p*-NA** is ordered which shows that a Δ^EPhe residue alone is not sufficient to induce ordered conformations in a peptide unless it is present in combination with the *p*-NA group. The same result was obtained for the analogous tetrapeptides (Lisowski et al. 2010).

It was also found that the temperature coefficients values of the amide protons in small molecules like the peptides studied are not a good criterion of presence of hydrogen bond so a caution should be observed in their applications. When used alone, they may be the source of wrong conclusions. Much better way of determination of hydrogen bonds is taking into account the interatomic distances and contacts derived from the ROESY spectra, based on their calculations, and additional information obtained from other methods like CD or IR.

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Conflict of interest The authors declare that they have no conflict of interest.

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