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ORIGINAL ARTICLE

Structure of the cyclic peptide [W8S]contryphan Vn: effect of the tryptophan/serine substitution on *trans–cis* proline isomerization

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Abstract The structural characterization of [W8S] contryphan Vn, an analogue of Contryphan Vn with tryptophan 8 substituted with a serine residue (W8S), was performed by NMR spectroscopy, molecular dynamics simulations and fluorescence spectroscopy. Contryphan Vn, a bioactive cyclic peptide from the venom of the cone snail *Conus ventricosus*, contains an S–S bridge between two cysteines and a D-tryptophan. Like other Contryphans, [W8S]contryphan Vn has proline 7 isomerized *trans*, while the proline 4 has nearly equivalent populations of *cis* and *trans* configurations. The thermodynamic and kinetic parameters of the *trans–cis* isomerization of proline 4 were measured. The isomers of [W8S]contryphan Vn with proline 4 in *cis* and *trans* show structural differences. The absence of the salt bridge between the same Asp2 and Lys6, present in Contryphan Vn, may be attributed to the lack of the hydrophobic side chain of Trp8 where it likely protects the electrostatic interactions. These results may contribute to identifying, in these cyclic peptides, the structural determinants of the mechanism of proline *trans–cis* isomerization, this being also an important step in protein folding.

Keywords Contryphan · NMR · Cyclic peptides · Three-dimensional structure · Fluorescence · Proline isomerization

Abbreviations

TFA	Trifluoroacetic acid
HMQC	Heteronuclear multiple quantum coherence
HSQC	Heteronuclear single quantum coherence
ROESY	Rotating-frame Overhauser spectroscopy
TOCSY	Total correlation spectroscopy
DOSY	Diffusion-ordered spectroscopy
TFE	Trifluoroethanol
PME	Particle-mesh Ewald method
TSP	Trimethylsilyl propionic acid

Introduction

Contryphans are natural bioactive peptides, first isolated from piscivorous cone snail venom (Olivera et al. 1985; Jimenez et al. 1996; Jacobsen et al. 1998); all of them have S–S cystine bridges and are rich in a variety of unusual post-translational modifications, including proline hydroxylation, C-terminus amidation, proline and leucine isomerization (Jacobsen et al. 1999; Pallaghy et al. 1999) and tryptophan bromination (Jimenez et al. 1997). The characterized Contryphans are reported schematically in Table 1 (Olivera et al. 1985; Jimenez et al. 1996, 1997, 2001, 2002; Jacobsen et al. 1998, 1999; Pallaghy et al. 1999, 2000; Pallaghy and Norton 2000; Massilia et al. 2001, 2003; Eliseo et al. 2004; Grant et al. 2004; Hansson et al. 2004; Sabareesh et al. 2006). The biological activity of Contryphans has been assayed (Jimenez et al. 1996, 2001; Massilia et al. 2001); specifically in the case Contryphan Vn, it was found to modulate the activity of Ca^{2+}-dependent K^+ channels

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Table 1 The Contryphans recently isolated and structurally characterized

Peptide	Sequence	Species	Prey
Contryphan R	G–CO<u>W</u>EPWC*	C. *radiatus*	Fish
Contryphan R/Tx	G–CO<u>W</u>QPYC*	C. *textile*	Mollusc
Contryphan P	G–CO<u>W</u>DPWC*	C. *purpurascens*	Fish
*Bromo*contryphan	G–CO<u>W</u>EPXC*	C. *radiatus*	Fish
Contryphan Sm	G–CO<u>W</u>QPWC*	C. *stercusmuscarum*	Fish
Leu-contryphan Tx	– –CV<u>L</u>YPWC	C. *textile*	Mollusc
Leu-contryphan P	G–CV<u>L</u>LPWC	C. *purpurascens*	Fish
Glacontryphan M	NγSγ CP<u>W</u>HPWC	C. *marmoreus*	Mollusc
Am975	GCP<u>W</u>DPWC*	C. *loroisii*	Mollusc
Lo959	GCO<u>W</u>DPWC*	C. *amadis*	Fish
Contryphan Vn	GDCP<u>W</u>KPWC*	C. *ventricosus*	Worm
[W8S]contryphan Vn	GDCP<u>W</u>KPSC*	This work	

D-Amino acids are underlined; O indicates hydroxyPro; X indicates 6-BrTrp. The asterisk indicates C-terminus amidation. Disulfide connectivity is not indicated above the cysteines of the amino acid sequences

in insect neurosecretory cells and rat fetal chromaffin cells (Massilia et al. 2003). Moreover Gla-Contryphan (Massilia et al. 2003; Eliseo et al. 2004; Grant et al. 2004; Hansson et al. 2004; Sabareesh et al. 2006) has a role of calcium-binding peptide, adding a new functionality to this scaffold (Hansson et al. 2004) that can be useful for modeling neuroactive peptides (Pallaghy and Norton 2000; Pallaghy et al. 2000) with conformational constraints as in other cyclic peptides (Juvvadi et al. 1992).

Contryphans contain two proline residues which exist as a mixture of *cis–trans* isomers. Specifically, the proline residue closest to the N-terminal is a mixture of *cis* and *trans* isomers, while the second proline is constantly the *trans* isomer (Table 1). This behavior has been attributed to a localized *trans–cis* isomerization mechanism (Jacobsen et al. 1998; Pallaghy et al. 2000; Eliseo et al. 2004) due to the constraints of the ring cyclization by the cystine S–S bridge. By contrast, Gla-Contryphan (Hansson et al. 2004) has *cis* and *trans* prolines with a unique combination. It has been hypothesized that electrostatic interaction between the charged *N*-terminal amino group and an anionic side chain conserved in position i, $i + 4$ stabilizes the *cis* isomer with respect to the *trans* one of the first proline (Pallaghy et al. 2000).

In fact, Contryphan R, P, and Sm (see Table 1) show the charged N-terminal amino group and an anionic side chain conserved in position i, $i + 4$. On the other hand in Contryphan Vn, it was found that the Asp2 and Lys 6 residues, in positions i, $i + 4$, form a salt bridge within the cycle (Eliseo et al. 2004). The salt bridge is protected by the nearby hydrophobic region of the indole rings of D-Trp5 and Trp8 and can be considered as the structural determinant of the dominant *cis* conformation of proline 4 in that cyclic peptide. The salt bridge was identified in Contryphan R (Pallaghy et al. 1999 as a result of a titration, but was first observed experimentally with a series of specific NOEs in Contryphan Vn (Eliseo et al. 2004). This paper reports the characterization of the [W8S]contryphan Vn (see Fig. 1).

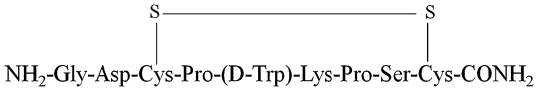

Fig. 1 Amino acid sequence of the [W8S]contryphan Vn

Materials and methods

Sample preparation and characterization

[W8S]contryphan Vn was purchased from Spectra2000, Rome, Italy. Chemical identity of the synthetic [W8S]contryphan Vn was confirmed by HPLC and LC/ESI–MS techniques. The ring closure by the S–S bridge of peptide [W8S]contryphan Vn was monitored by estimating the free thiol groups according to the method described by Ellman et al. 1959 (Riddles et al. 1979, 1983). To summarize, the sample was added to the working solution: 100 μM of Ellman's reagent 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), which was previously solubilized in 50 mM sodium acetate solution, in 1.0 M Tris HCl, pH 8.0, buffer. The reaction between DTNB and thiol groups produced the mixed disulfide and 2-nitro-5-thiobenzoic acid (NTB) which was quantified by monitoring the absorbance of NTB anion at 412 nm ($\varepsilon_{412} = 13{,}600\ M^{-1}\ cm^{-1}$). The purity of [W8S]contryphan Vn was checked by RP-HPLC using a linear gradient of 5–80 % (v/v) acetonitrile containing 0.1 % (v/v) TFA over 60 min and monitoring the absorbance at 220 nm (Gesquiere et al. 1989). The molecular mass of the cyclic peptide, confirming the intramolecular S–S ring closure, was performed by LC/ESI–MS (by Colosseum Combinatorial Chemistry Centre Tecnology, C4T, Rome, Italy) using a single-quadrupole spectrometer (Thermo Surveyor MSQ, Waltham, MA, USA) with positive modality acquisition from *m*/*z* 200 to 2,000. The instrument was calibrated with a solution standard, Ultramark 1621.

NMR spectroscopy

NMR experiments were performed using both Bruker Avance 400 MHz and Bruker Avance 700 MHz spectrometers. The sample was typically 3.0 mM of [W8S]contryphan Vn in 90 % H_2O/10 % ^2H_2O or 100 % ^2H_2O at 298 K typically at the pH measured as a meter reading.

Spectra were processed by Bruker software TOPSPIN 3.1 typically applying a resolution enhancement by multiplication of the fids by a sinebell function shifted of $\pi/2$ and a polynomial baseline correction.

All the proton resonances were assigned by 2D spectra: TOCSY (mixing time 60 and 120 ms) to identify the spin systems (Bax and Freeman 1981; Bax and Davis 1985), ^1H–^{15}N HSQC (Bodenhausen and Reuben 1980; Grzesiek and Bax 1992), ^1H-^{13}C HMQC (Bax et al. 1983) and ^1H-^{13}C HSQC (Braunschweiler and Ernst 1983) to assist with cross-peak assignment, ROESY (Bothner-By et al. 1984) (with a spinlock time of 0.050, 0.080, 0.120, and 0.150 s) using for the homo-nuclear experiments the States-TPPI phase cycle (Marion and Wuthrich 1983) following the sequential assignment method (Wüthrich 1986).

In NMR spectra, the trimethylsilyl propionic acid (TSP) was used as internal reference. At different temperatures, the same reference has been used to avoid the problem of the typical shift of water resonance on temperature.

The backbone $J_{HN C\alpha H}$ and some $JH_{C\beta C\alpha H}$ were directly measured from NMR spectrum after the complete assignment of TOCSY correlation and by dipolar correlations revealed by NOE cross-peaks in ROESY spectra (Pardi et al. 1984; Bax and Davis 1985). All the heteronuclear correlation experiments were carried out at natural abundance.

The diffusion-ordered spectroscopy (DOSY) spectra (Cohen et al. 2005; Floquet et al. 2009; Li et al. 2009) were performed using the ledbpgppr2s pulse sequence of the Bruker library to suppress the water signal at 4.7 ppm. During the DOSY experiment, 32 mono-dimensional spectra were acquired with 64 scans in a linear increasing gradient varying from 5 to 95 % with a $\Delta 1$ of 70 ms and $\Delta 2$ of 2 ms. The spectra were then analyzed using the DOSY module implemented in Bruker software TOPSPIN 3.1.

The assignments of the proline isomers was performed comparing the results of ROESY cross-peaks with the values of the ^{13}C chemical shift of the proline side chain as outlined in more detail in the results section. The population of the two isomers of proline were determined by measuring the integrals of TOCSY cross-peaks (Deber et al. 1970; Cheng and Bovey 1977).

The temperature dependence of amide proton resonances in the NMR spectra of [W8S]contryphan Vn as hydrogen bond indicators were performed (Baxter and Williamson 1997; Cierpicki and Otlewski 2001). The NMR spectra were recorded at 310, 298, 291, 285, and 281 K at pH 3.0. An accurate referencing was adopted using TSP to avoid the shift of the water resonance upon temperature increase. The values were classified as in (Baxter and Williamson 1997; Cierpicki and Otlewski 2001). By the measure of $\Delta\nu_{HN}/T$, the resonances can be classified into four classes: (a) $\Delta\nu_{HN}/T$ between -6 and -10 ppb K^{-1}, is the usual value for random coil conformations; (b) $\Delta\nu_{HN}/T$ between -2 and -4 ppb K^{-1}, is due to protons strongly shielded from the solvent with formation of intramolecular bonds; (c) $\Delta\nu_{HN}/T$ between -4 and -6 ppb K^{-1}, is due to protons weakly shielded from the solvent; and (d) $\Delta\nu_{HN}/T$ between -2 and 0 ppb K^{-1}, peptides with no hydrogen bonds but close to ring current shifted methyl groups (Baxter and Williamson 1997; Cierpicki and Otlewski 2001). From the proline isomer populations, the constant of the equilibrium was evaluated. By the inverse dependence on temperature, the thermodynamic values of the *trans–cis* isomerization were measured (Grathwohl and Wuthrich 1976; Stein 1993) by the Van't Hoff equation:

$$\ln K_{eq} = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (1)$$

Moreover, by observing resonances in the spectra, it was found that the isomerization occurs in a relatively slow exchange regime; this allowed us to measure the kinetic parameters from the rate of the interconversion. The line shape analysis was performed at different temperatures as reported (Jardetzsky and Roberts 1981), followed by evaluation of the activation energy of the proline 4 *trans–cis* interconversion under different pH conditions (3.0 and 6.0) and in the presence of a structuring solvent such as 50 % water/trifluoroethanol (TFE) mixture.

Following the approach suggested by Jardetzsky and Roberts (1981), the equation applied was:

$$\ln\left(\frac{k_1}{T}\right) = -\left(\frac{\Delta H^\#}{R}\right) \times \left(\frac{1}{T}\right) + \frac{\Delta S^\#}{R} + \ln\left(\frac{k_B}{h}\right) \quad (2)$$

The molecular dynamics simulations from NMR data (XPLOR)

After the complete assignment of the resonances of the NMR spectra, the NOEs were collected and listed for the two isomers of proline 4 to be used in restrained molecular dynamics simulation.

The structural restraints useful in determining the solution structure of [W8S]contryphan Vn were obtained from the internuclear distances evaluated by the ROESY cross-peak volumes measured from spectra obtained with 0.150 s of mixing time, and converted and grouped into distance bounds: 1.8 Å $< d <$ 2.5 Å for strong NOEs, 1.8 Å $< d <$ 3.4 Å for medium NOEs, and 1.8 Å $< d <$ 6.0 Å for weak NOEs (Wüthrich 1986). The data were used as

obtained from direct integration without using correction for the offset dependence due to the large interval of distances derived for peak intensities.

A series of structures were calculated using the NMR data as restraints for the molecular simulations in a conventional XPLOR (Brunger 1993) simulated annealing protocol in vacuo (Omichinski et al. 1997). Starting from linear template structure and different randomized initial velocity distributions, structures were subjected to an all-hydrogen force field (including covalent geometry, planarity, hard-sphere van der Waals, empirical NOE, and J-coupling energy terms, but not Lennard-Jones, electrostatic, hydrogen bonding and empirical dihedral angle terms). Simulated annealing was performed using a first phase consisting of 10,000 steps (2 fs each step) at 4,000 K, setting the initial van der Waals weight at a very low value (0.003) to allow atoms to pass each other in the early stage of simulations. The successive 7,000 steps were performed to gradually cool the system to 300 K. A set of 50 structures with the least number of violations (zero or a single violation lower than 0.5 Å) were selected. Among these, PROCHECK-NMR software (Laskowski et al. 1993) identified the best 20 structures with the greatest number of backbone torsion angles located in most favorable regions of the Ramachandran plot with none in unpermitted regions (Gly1 and D-Trp5 were not included in the analysis).

The unrestrained molecular dynamics (GROMACS)

The molecular dynamics simulation was performed with Gromacs 4.5 (Hess et al. 2008) using the AMBER03 protein, nucleic AMBER94 (Duan et al. 2003) force field and the TIP3P model for water molecules. The simulations were performed on a total time of 5 ns. The cutoff radii was set at 0.8 nm for electrostatic interactions and 0.8 nm for Lennard-Jones interactions. Long-range electrostatic interactions were treated using the particle-mesh Ewald (PME) method (Duan et al. 2003). Temperature coupling was performed with a Nose–Hoover thermostat (Essmann et al. 1995) and pressure coupling was carried out with the Parrinello–Rahman barostat (Cheng and Merz 1996). The calculations were performed on a Linux PC presenting an AMD Athlon(tm) II X2 260 Processor × 2 at a rate of about 19.6 ns day^{-1}.

The fluorescence spectroscopy

Fluorescence spectral measurements were carried out using an RF-5301PC spectrophotometer (Shimadzu) equipped with a water bath to regulate the sample temperature. Fluorescence emission spectra were recorded between 300 and 500 nm with an excitation wavelength of 280 nm. The peptide concentrations were 10 μM in water and tryptophan steady state fluorescence was measured in two different acidity conditions, pH 3.0 and pH 6.0, at different temperatures: 10, 15, 20, 25, 30, and 37 °C. Experiments were also performed in 50 % trifluoroethanol (TFE)/water mixture/at the same peptide concentration.

Results

The existence of two stable isomer populations attributed to the *trans–cis* isomerization of proline 4 previously described for Contryphan Vn (Eliseo et al. 2004) and other Contryphans (Table 1) was observed also in [W8S]contryphan Vn. In fact, the RP-HPLC chromatogram profile showed two peaks indicating the presence of two different peptide populations in solution. The Ellman's test of [W8S]contryphan Vn further indicated that free thiol groups are absent and that the open form is almost negligible or absent completely. LC/ESI–MS confirmed the presence of two isomer populations with the same mass of 988 Da and excluded the presence of polymeric forms due to intermolecular S–S bridges. Finally, the DOSY gave nearly a unique diffusive front with the hydrodynamic radius almost equivalent (data not shown).

NMR spectroscopy

The ^{1}H NMR spectra of [W8S]contryphan Vn was assigned completely. The individual and sequential assignments were obtained by TOCSY (see Fig. 2a–c) and ROESY spectrum both with different mixing times (Fig. 3a, b).

Heteronuclear 2D NMR spectra at natural abundance of ^{15}N and ^{13}C (^{1}H–^{13}C HSQC spectrum is shown in Supplementary Fig. 1) were used to overcome ambiguities and confirm the assignments of the ^{1}H NMR spectra. The spectrum of ^{15}N is reported in Fig. 4.

In the natural abundance correlation HSQC spectrum between ^{15}N and amidic protons (see Fig. 4), the two isomers of [W8S]contryphan Vn are clearly distinguishable and assigned. Particularly useful was the 2D natural abundance ^{13}C HSQC-TOCSY spectrum (not shown), leading to the complete assignment of side chain carbon-linked protons reported in Supplementary Table 1.

The isomers *cis* and *trans* of proline 4 were identified by the expected characteristic sequential NOEs. Strong and weak NOEs due to the different interatomic distances in the two isomers (Wüthrich 1986; Hinck et al. 1993) are shown in the Fig. 3a. Moreover, the ^{13}C chemical shift values of Cβ and Cγ resonances (Supplementary Table 1) (Wüthrich 1986; Hinck et al. 1993) are in line with those reported in the literature for *cis* and *trans* proline isomers, respectively (*cis*, Cβ: 33 ppm, Cγ: 23 ppm; *trans*, Cβ: 30 ppm, Cγ: 26 ppm).

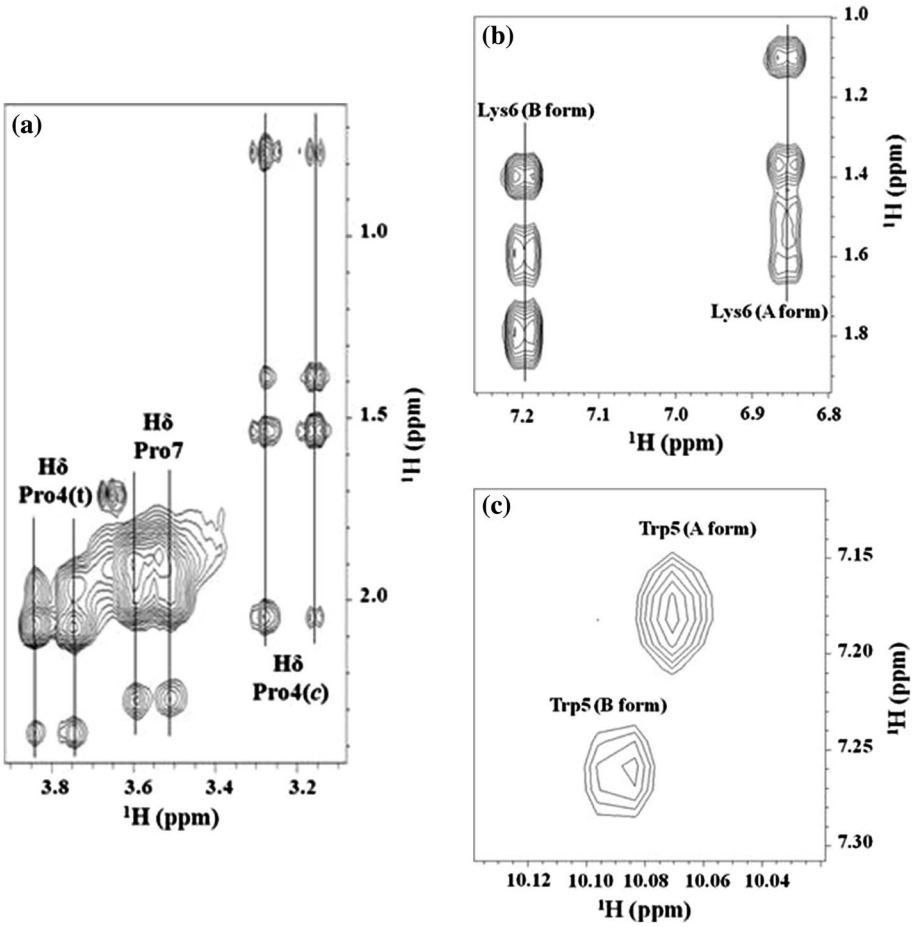

Fig. 2 Enlarged regions (**a**–**c**) of the TOCSY spectrum of [W8S]contryphan Vn obtained as reported in "Materials and methods"

While the proline 7 in [W8S]contryphan Vn is in *trans* configuration, such as observed in many other Contryphans structures (Eliseo et al. 2004), the proline 4 is present in both the isomers, *cis* and *trans*, the latter being the most common isomer found in structured proteins (Bothner-By et al. 1984). The analysis of the chemical shift values found in [W8S]contryphan Vn by the method of chemical shift index of Wishart et al. (1991) of the CHα resonances differ slightly from the mean values reported for random coil conformation. Excluding Gly 1 and Asp 2 located outside the cycle, for the other residues a low level of continuity of values indicates the absence of a regular secondary structure.

Analysis of molecular species beyond isomers

An analysis of the molecular species by the DOSY (Cohen et al. 2005; Floquet et al. 2009; Li et al. 2009) may provide information about the hydrodynamic radius. All the resonances present in the DOSY spectrum clearly shows a diffusion coefficient, $D \cong 1.4 \times 10^{-9}\ m^2\ s^{-1}$, thus excluding the presence of [W8S]contryphan Vn aggregates (Supplementary Fig. 2).

The solution structure of [W8S]contryphan Vn by NMR spectroscopy and molecular dynamics simulation

The distribution of the experimentally detected NOEs used as restraints in the MD simulations, expressed as number of NOEs per residue along the sequence of [W8S]contryphan Vn in Fig. 5 shows a high number of interresidue NOEs.

The major differences from Contryphan Vn (Eliseo et al. 2004) are observed between D-Trp5 and Pro7 (D-Trp5(17)–Lys6(13)–Proline7(13)–Trp8 (32)). Differently from Contryphan Vn where NOEs were found between β-CH_2 of Asp2 and γ- and δ-CH_2 of Lys6, in [W8S]contryphan Vn were not observed. This finding was confirmed by monitoring the interatomic distances in the molecular dynamics simulation (see below).

The molecular dynamics simulation was performed separately for the two isomers of W8S Contryphan. The collected NOEs were translated into ranges of internuclear distances for the application of restrained molecular dynamics protocols in vacuo and used separately for the two isomers. Through the inclusion of backbone dihedral angle restraints from the coupling constants, a final set of 20 structures has been generated (Fig. 6a, b), giving two

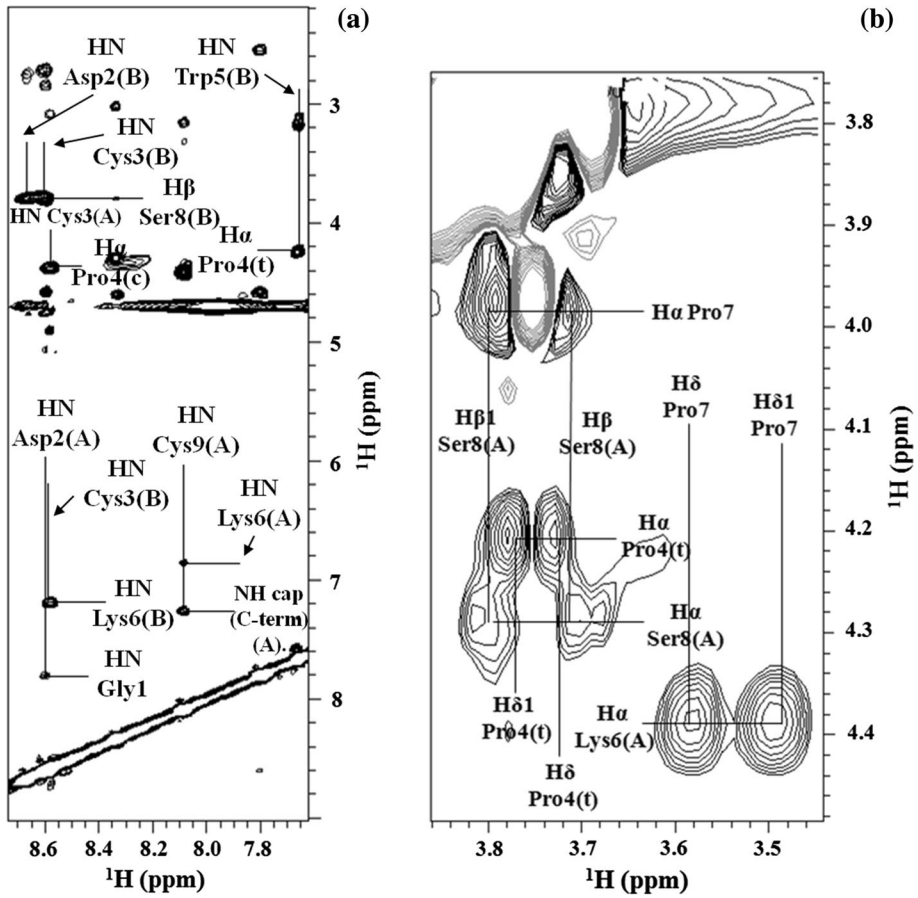

Fig. 3 Enlarged regions of ROESY spectrum of [W8S]contryphan Vn (**a**), enlarged region of ROESY spectrum of W8S Contryphan (**b**)

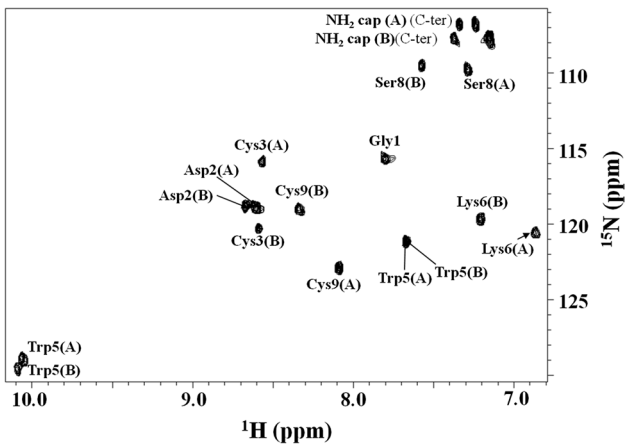

Fig. 4 Heteronuclear ^{1}H–^{15}N HSQC NMR spectrum of [W8S]contryphan Vn

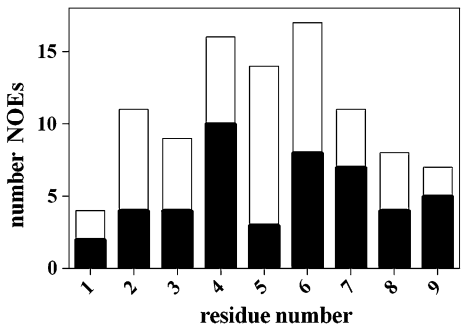

Fig. 5 Distribution of the NOEs per residue in [W8S]contryphan Vn, the interresidue and intraresidue NOEs are indicated in *white* and *black*, respectively. The NOEs of the two proline 4 isomers are reported together and used separately for the molecular dynamics simulations

unique and well-defined families with the Pro4 in *cis* or in *trans* configuration. The statistic results of the structures are reported in Supplementary Tables 2a, 2b.

Backbones are different in the two Pro4 isomers of [W8S]contryphan Vn. In fact, the structures containing the *cis* isomer of proline 4 have a two β-turn of type IV: one from Asp2 to Trp5 and another from Lys6 to Cys9. By contrast, the structures with the *trans* proline 4 have no β-turns.

The unrestrained molecular dynamics simulations in water of both the two isomers of proline 4 of [W8S]contryphan Vn for about 5 ns showed the existence of stable intramolecular hydrogen bonds in the *cis* isomer of [W8S]contryphan Vn: one between (N–H) of Cys9 and

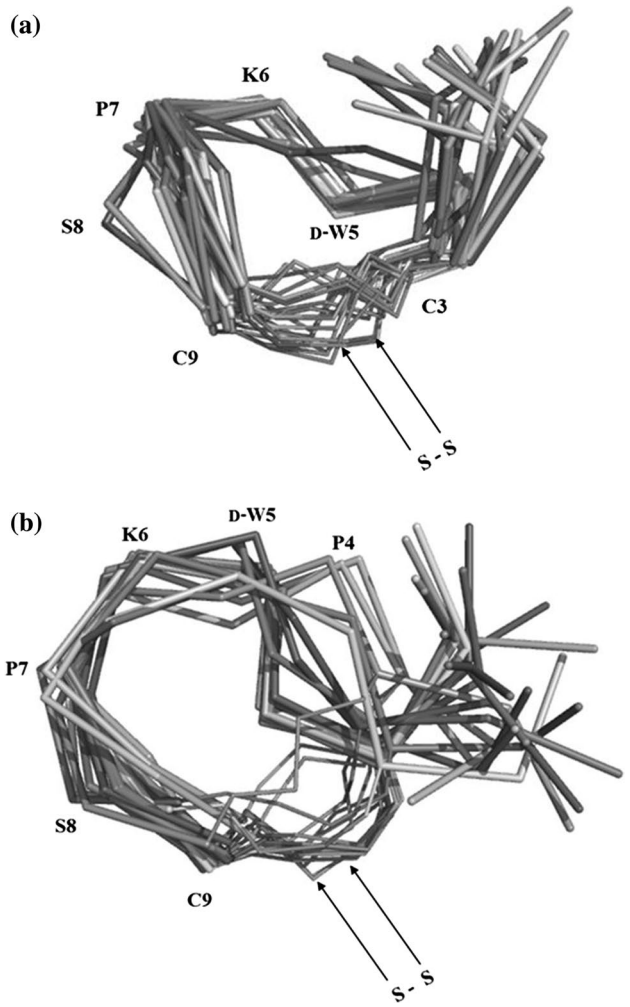

Fig. 6 Solution structure of the [W8S]contryphan Vn: **a** containing Pro 4 *cis* isomer; **b** structure Contryphan Pro 4 *trans* isomer

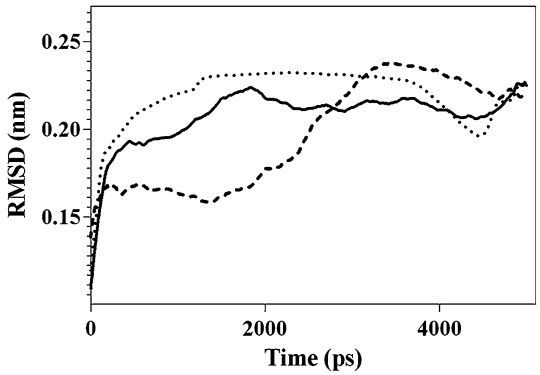

Fig. 7 RMSD of trajectories during MD simulations: Contryphan Vn (*dots*); [W8S]contryphan Vn Pro4-*trans* (*dashed*); [W8S]contryphan Vn Pro4-*cis* (*continuous*)

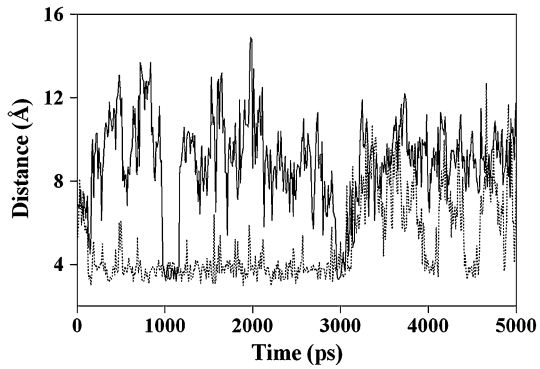

Fig. 8 Interatomic distance during the MD simulation between Asp2 COO^- and Lys6 NH^{3+}. The *gray trace* corresponds to the MD simulation of Contryphan Vn (predominant the *cis* isomer), the *black* one refers to [W8S]contryphan Vn with Pro4 *cis*

the CO group of Lys6 and another between (N–H) of D-Trp5 and the carboxyl of Asp2 side chain. Both hydrogen bonds are involved in the two observed β-turn, their existence being confirmed by the dependence on temperature of the amide NMR resonances.

The trajectories of the simulations of isomers of [W8S]contryphan Vn with proline 4 in *cis* and *trans* are reported in Fig. 7 together with the simulation of Contryphan Vn.

The *cis* isomer of [W8S]contryphan Vn reaches the conformational equilibrium similarly to Contryphan Vn. By contrast, the [W8S]contryphan Vn with the Pro4 in *trans* needs more time to equilibrate. As reported in the simulations carried out by Amadei (D'Alessandro et al. 2004), in Contryphan Vn a large mobility of Trp8 side chain (here substituted by serine in W8S Contryphan) is observed. Particularly in correspondence to the minimum in RMSD (Fig. 7) and maximum in total energy (not shown), the indole ring of Trp8 is in proximity to the peptide backbone, so playing a role of protection in the formation–disruption dynamics of salt bridge in Contryphan Vn (see Fig. 7).

Monitoring the distance between the charged side chains of Asp2 and Lys6, the two partners of the stable salt bridge of Contryphan Vn (where proline 4 is in majority in *cis* conformation) confirmed that in [W8S]contryphan Vn the occurrence of this salt bridge is unlikely (see Fig. 8). In fact, only for two short intervals are the values of distance favorable to the formation of salt bridge observed: This is in contrast with Contryphan Vn where such a favorable distance is maintained for long periods (Fig. 8).

The conformation of the *cis* and *trans* isomers of Pro4 in [W8S]contryphan Vn

The ϕ and ψ values of the two isomers of [W8S]contryphan Vn were obtained and compared with the values found in Contryphan Vn (Supplementary Fig. 3a,3b,3c,3d). The population of *cis* isomer of proline 4 of [W8S]contryphan

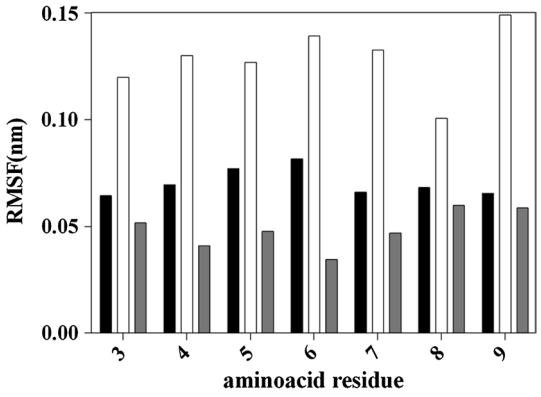

Fig. 9 Molecular flexibility of residues during the molecular dynamics simulation for [W8S]contryphan Vn (Pro4 *cis* and *trans*, *black* and *white*, respectively, and for Contryphan Vn, *gray*)

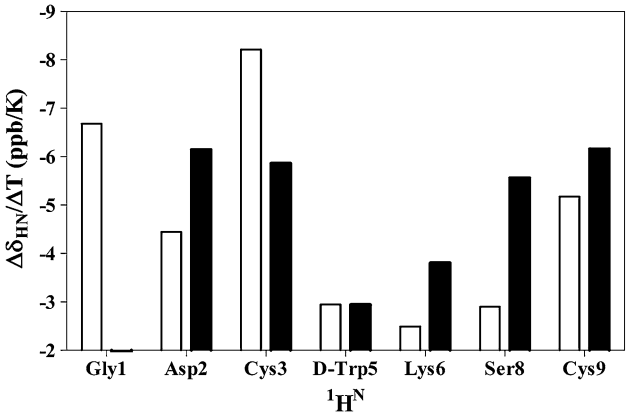

Fig. 10 Chemical shift dependence on temperature of the NMR resonances of the amidic protons of W8S Contryphan. As reported in Table 2, the resonance assignments of forms A are those in the *cis* Pro4 isomer; on the other hand, forms B are in the *trans* Pro4 isomer. In figure, *white bars* refer to forms A residues, while *black* ones refer to forms B residues

Vn shows a similarity with Contryphan Vn (predominantly *cis*) which, in turn, is similar to Contryphan R and Contryphan Sm (Pallaghy et al. 1999, 2000). The observation that in [W8S]contryphan Vn with the same charged side chains of Contryphan Vn the stable salt bridge between Asp2 and Lys6 side chains does not occur seems to conflict with the hypothesis concerning the role played by the electrostatic interaction i, $i + 4$, in the local conformation. It must be said that the only difference between Contryphan Vn and [W8S]contryphan Vn is the absence of the Trp indole ring in position 8, possibly indicating its important protecting role in the formation of the salt bridge. On the other hand, this residue is a common feature of all the Contryphans (only the Contryphan R/Tx, see Table 1, has a Tyr in place of a Trp in position 8).

The comparison of the fluctuations (r.m.s.f.) values between averaged backbone coordinates of [W8S]contryphan Vn in the molecular dynamics simulation in water (both Proline 4 *cis* and *trans* isomers) and compared with that of Contryphan Vn are reported in Fig. 9. The values indicate a marked flexibility in [W8S]contryphan Vn (the Pro4 *cis* isomer) with respect to the ring of Contryphan Vn. In addition, in [W8S]contryphan Vn the *cis* isomer is less flexible than the Pro4 *trans* isomer. Thus the role of Trp 8 appears important in the stability of the molecular conformation and, hence, to the high population of the *cis* form in the other Contryphans.

The temperature dependence of amidic protons in peptides and proteins ($\Delta\nu_{HN}/T$) are considered good indicators of intramolecular hydrogen bonds (Baxter and Williamson 1997; Cierpicki and Otlewski 2001).

In the TOCSY spectra, the $\Delta\nu_{HN}/T$ of the amidic protons at different temperatures of [W8S]contryphan Vn has been measured at pH 3.0 (Fig. 10).

The values obtained indicate the presence of intramolecular hydrogen bonds involving amidic protons of D-Trp5A, and to some degree D-Trp5B and Lys6 (namely D-Trp5A and Lys6A in the Pro4 *cis* isomer, D-Trp5B and Lys 6B in the Pro4 trans isomer). In particular, the result of the low dependence on temperature of the resonance of the amidic resonance of D-Trp5 in the *cis* isomer matches the simulation results. In fact, a relatively more flexible structure was found in the [W8S]contryphan Vn containing the trans isomer of proline 4 than that found in the *cis* containing one.

The *trans–cis* isomerization of the proline 4, the thermodynamic and the kinetic parameters

The effect of temperature on the *trans–cis* equilibrium in Contryphans has been studied previously (Gesquiere et al. 1989). The isomerization of Pro4 in [W8S]contryphan Vn was studied by measuring the ratio of the volumes of the cross-peaks of the resonances in the TOCSY spectra between the proline 4 *cis* and *trans* isomer (Hβ–Hδ) in TOCSY NMR spectrum (Troganis et al. 2000) under different conditions (i.e., pH 3.0 and 6.0).

It was found that the amount of *cis* and *trans* isomers of Pro4 are dependent on temperature and pH. These measures were also performed in trifluoroethanol/water mixture (50 % TFE/H_2O at pH 5.0). The values found are reported in Table 2. From the values found, the equilibrium constant of the *trans* to *cis* isomerization of the amidic bond Cys3–Pro4 has been determined so as to evaluate the thermodynamic parameters of the proline isomerization.

Summarizing the ΔH and ΔS values obtained reported in Table 2, the *trans* form is thermodynamically more stable at pH 6.0 than at pH 3.0. In TFE/H_2O solution, pH 5.0, [W8S]contryphan Vn shows an increase of the *cis* isomer

Table 2 Equilibrium constants and thermodynamic parameters for the Pro4 *cis/trans* isomerization

Condition	T (K)	K_{eq} cis/trans	ΔH and ΔS
[W8S]contryphan Vn in H_2O, pH 3.0	281.1	0.613	$\Delta H^\circ = 28.76$ KJ mol^{-1}
	285.1	0.666	
	291.1	0.818	$\Delta S^\circ = 97.86$ J K^{-1} mol^{-1}
	298.1	1.222	
[W8S]contryphan Vn in H_2O, pH 6.0	281.1	0.523	$\Delta H^\circ = 9.947$ KJ mol^{-1}
	291.1	0.628	$\Delta S^\circ = 30.13$ J K^{-1} mol^{-1}
	298.1	0.665	
[W8S]contryphan Vn in TFE/H_2O, pH 5.0	281.1	1.702	$\Delta H^\circ = 7.398$ KJ mol^{-1}
	291.1	1.941	$\Delta S^\circ = 3.706$ J K^{-1} mol^{-1}
	298.1	2.030	

Table 3 Activation parameters for the Pro4 *cis/trans* isomerization in [W8S]contryphan Vn

Condition	T (K)	k_1 (Hz)	Activation parameters
[W8S]contryphan Vn in H_2O, pH 3.0	281.1	742.1	$\Delta H^\# = 151.41$ J mol^{-1}
	285.1	735.9	
	291.1	732.8	$\Delta S^\# = -90.01$ J K^{-1} mol^{-1}
	298.1	725.2	
[W8S]contryphan Vn in H_2O, pH 6.0	281.1	765.9	$\Delta H^\# = 1.534$ KJ mol^{-1}
	291.1	755.0	$\Delta S^\# = -90.00$ J K^{-1} mol^{-1}
	298.1	750.1	
[W8S]contryphan Vn in TFE/H_2O, pH 5.0	281.1	744.0	$\Delta H^\# = -4.626$ KJ mol^{-1}
	291.1	716.7	$\Delta S^\# = -24.77$ J K^{-1} mol^{-1}
	298.1	705.2	

of proline 4 with respect to the aqueous environment, so indicating that a structuring medium favors the *cis* isomer.

The kinetic parameters of the isomerization were determined by application of NMR line shape analysis in the relatively slow exchange condition of the NMR resonances of the Pro4 isomers (Jardetzsky and Roberts 1981) for the application of the Eyring equation. The value of the resonance separation ($k_1 = \pi\Delta\nu$) in the slow exchange regime and the plotting $k_1(T)$ versus ($1/T$) for the resonances (Hβ–Hδ) of the Pro4 *cis* and *trans* was used to determine the activation energy. The results are reported in Table 3.

The investigation in water at pH 6.0 showed that the transition is thermodynamically unfavored (ΔG° ~974 J mol^{-1}), having a kinetic energy barrier of $\Delta G^\#$ ~28.4 kJ mol^{-1} while at pH 3.0 ΔG° ~ −402 J mol^{-1} and $\Delta G^\#$ ~26.9 kJ mol^{-1}. The isomerization is thermodynamically favored and the activation barrier is decreased to some extent. The values found are in line with those reported for short peptides (Troganis et al. 2000).

As expected in the equilibrium the entropy change is the equilibrium's driving force. Increasing the pH the entropy change becomes important, the equilibrium being shifted toward the *trans* form. In the less polar environment such as in the presence of 50 % TFE/water the *cis* form of [W8S]contryphan Vn appears dominant. At room temperature, the *trans–cis* isomerization is thermodynamically favored with a ΔG° ~ −9.17 kJ mol^{-1}.

These results are in line with several findings that reported that TFE/water solution mimics the biological environment, namely the membrane-like environment, and is known to induce marked structuring effects in peptides and proteins. The comparison of the values of activation energy parameters for the Pro4 *trans–cis* isomerization in TFE/water solution shows a difference in the activation enthalpy (see Table 3), particularly low when the pH is at 3.0 and higher at pH 6.0.

Fluorescence studies

Tryptophan fluorescence has been used extensively to study peptide and protein conformation changes, due to the sensitivity of both emission wavelength and intensity to the indole chromophore's local environment (Adams et al. 2002). The interest in this spectroscopy was the high sensitivity in the change of polar environment around the fluorophore and the possibility of monitoring very small conformation changes. Here, the conformational differences induced by the *trans–cis* isomerization of Proline 4 were of interest. Such fluorescence studies were facilitated by the presence of a single D-Trp5. The presence of two Trp

residues (as in the case of native contryphans) prevents fluorescence studies from being conducted since the two fluorophores within the same molecule cannot be distinguished.

The fluorescence spectroscopy of [W8S]contryphan Vn was carried out at different temperatures and under different conditions. The study was performed in water solution at pH 3.0 and 6.0 and in 50 % TFE at pH 5.0. Figures 11a, b and 12 report the fluorescence emission curves of W8S Contryphan.

The spectra indicated that at different pH the fluorescence is dependent on the temperature. More particularly, an important decrease of the emission is observed at increasing temperature values (Fig. 11a, b). This may be due to the change in populations of proline 4 isomers in [W8S]contryphan Vn as determined by NMR spectroscopy. In fact, by increasing the temperature, the proportion of *cis* isomer increases, while the proportion of *trans* isomer decreased as previously observed. Moreover, the intrinsic fluorescence changes, Δ (difference between the value at temperature *T* and that at temperature 37°) behaves in a pH dependent manner. In fact, the Δ fluorescence decrease being higher at pH 3.0 than at 6.0 as shown in Fig. 12. The fluorescence change likely corresponds to a conformational change induced by temperature with a different exposure to the solvent. It is known that the different polarity of the solvent changes the maximum of emission of the Trp fluorescence. In fact, an apolar solvent produces a blue-shifted emission, while a polar solvent produces a red one. Also, an increase of temperature produces, in general, a decrease of the emission quantum yield due to the increase in non-radiative processes (Valeur 2001).

The differences in water exposure of Trp side chain is relevant in fluorescence quantum yield for small peptides (Adams et al. 2002). Thus, the fluorescence spectra of [W8S]contryphan Vn acquired in water at different pH and temperatures together with NMR data both suggest that the tryptophan residue has two different conformations in *cis* and *trans* isomers of proline 4.

The molecular dynamics simulation of [W8S]contryphan Vn provided an explanation. The distance of the indole of D-Trp5 from Asp2 side chain (Fig. 13a) and from disulfide bridge (Fig. 13b) in two isomers was monitored. These two groups are considered efficient fluorescence quenchers (Adams et al. 2002). Thus it is possible to hypothesize the existence of an intramolecular quenching mechanism of the indole D-Trp5 fluorescence due to these quenching groups.

During the molecular dynamics simulation, a major proximity of the –COOH group of Asp2 to the D-Trp5 indole group in the Pro4 *cis* isomer with respect to the *trans* isomer was detected and reported in Fig. 13a.

In Fig. 13b, the proximity of indole of D-Trp5 to the S–S bridge is evident for a large part of the trajectory for the Pro4 *cis* isomer while the distance for the Pro4 isomer in *trans* oscillates predominantly around large values. These observations may well explain the lower fluorescence quantum yield of *cis* isomer compared with the *trans* one.

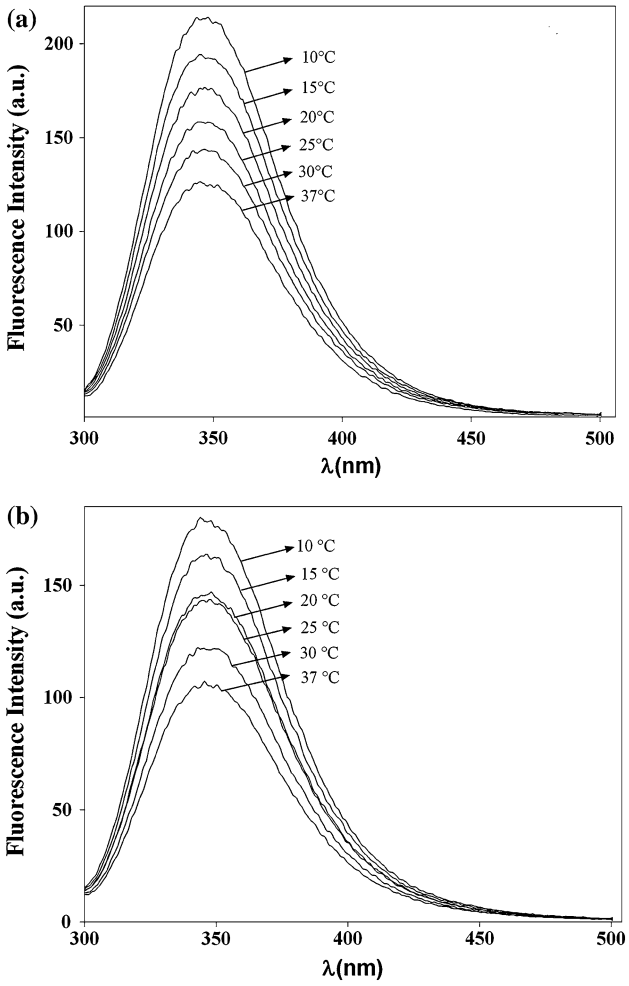

Fig. 11 Fluorescence spectra of [W8S]contryphan Vn at different temperatures in H_2O: **a** at pH 6.0 and **b** at pH 3.0

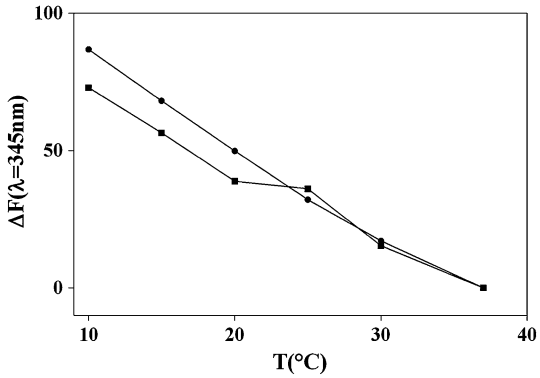

Fig. 12 Comparison between the maximum of Δ fluorescence at pH 3.0 and 6.0 at 37 °C

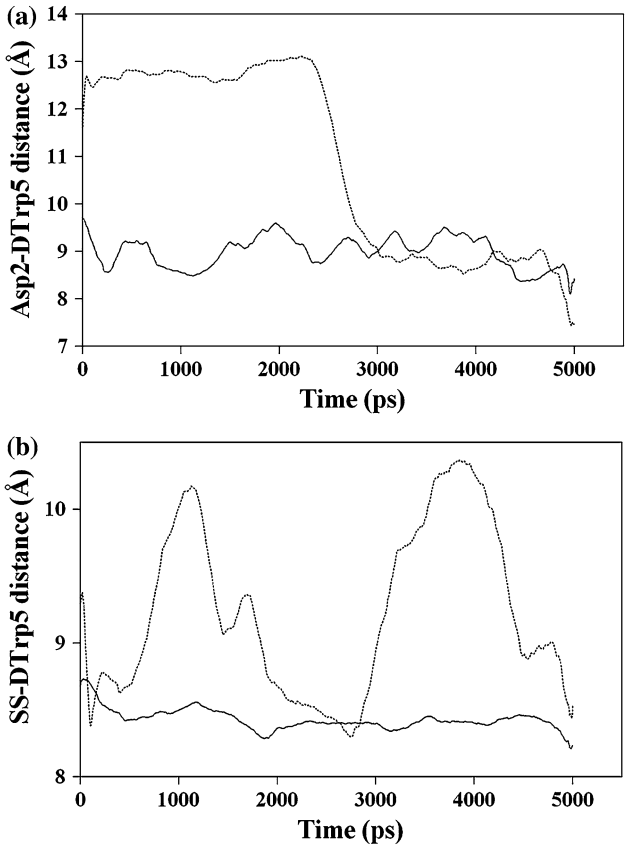

Fig. 13 Interatomic distance during molecular dynamics simulation between Asp2–COOH and Trp5 indole group (**a**); and between disulfide bridge (indicated in figure as *SS*) and Trp5 indole group (**b**). *Upper gray line* corresponds to the molecular dynamics simulation of [W8S]contryphan Vn with Pro4 in *trans* configuration, the *black lower* one refers to *cis* isomer

In the TFE/water mixture (50 % of TFE at pH 5.0) [W8S]contryphan Vn shows a fluorescence with a higher intensity with respect to water solutions. Upon temperature increase, the fluorescence emission decreases upon the same interval of temperature from 507 to 448 a.u. (not shown). This change in fluorescence is also a valid indicator of the *trans–cis* equilibrium in [W8S]contryphan Vn, confirming that at higher temperature the *cis* population decreases. However, from NMR data, the isomer *cis* in TFE/water mixture and at lower temperature is more prevalent than it is in water solution.

Discussion

The *trans–cis* isomerization of the two proline residues was characterized with values similar to those found in other peptides (Rabenstein et al. 2000; Shi et al. 2004).

In some of those cases, the interconversion mechanism was assisted by other nearby residues (Reimer et al. 1997); i.e., the protonation of imidazole of the preceding histidine was found to assist the proline interconversion. Interestingly, the *trans*-Pro4 isomer in [W8S]contryphan Vn is associated to a high internal flexibility. An entropic factor could contribute to the *trans*/*cis* interconversion, toward the *cis* form, in both highly constrained peptides and structured proteins (Reimer et al. 1997; Fischer 2000; Rabenstein et al. 2000; Adams et al. 2002; Shi et al. 2004). The fluorescence spectroscopy of the D-Trp5 demonstrated that [W8S]contryphan Vn has a higher exposure to water in the proline 4 *trans* form than in the *cis* isomer. Its quantum yield appears quenched by the nearby intramolecular quencher as the side chain carboxylate of Asp2 and of the S–S bridge.

Proline isomerization may be an important factor to consider in the design of cyclic peptides as drugs. It is known that the two isomers are thermodynamically equivalent (Grathwohl and Wuthrich 1976; Cheng and Merz 1996) with a modest free energy difference, and also that the kinetic barrier's activation energy is not particularly high (Fischer 2000). The study of the poly-L-proline revealed that the *cis* isomer of proline is conformationally compact and that, conversely, the *trans* isomer appears more stable in solvents such as water and TFE (Schimmel and Flory 1967; Deber et al. 1970; Andreotti 2003). Kinetic studies performed by NMR (Buevich et al. 2000) directly indicated that proline isomerizes by a single-step mechanism with an energy of 14–24 kcal mol^{-1} (Stein 1993; Fischer 2000) and that the interconversion is considered the rate limiting step in the folding of protein (Schmid and Baldwin 1978; Fischer 2000; Wedemeyer et al. 2002) with a defined transition state (Yonezawa et al. 2009).

In the case of [W8S]contryphan Vn, the absence of Trp8 causes the lack of a persistent salt bridge even in the presence of the same residues able to induce the $i/i + 4$ electrostatic interaction in Contryphan Vn, Contryphan R, P, and Contryphan Sm, so resulting in different effects in the proline 4 *cis–trans* isomerization. Accordingly neutralization through acetylation of the N-terminal charge in Contryphan R reduces the ratio from 8:1 to approximately 3:1 (Jacobsen et al. 1999).

In addition, the effect of temperature on the isomerization of Pro4 in Contryphan Vn indicates that at 298 K, the ratio between the *cis* and the *trans* isomer is about 1/0.15, increasing to 1/0.34 at 333 K.

[W8S]contryphan Vn shows the *cis–trans* ratio 1:1 (Contryphan Vn was 7:1, very similar to that observed in Contryphan R and Contryphan P, in similar experimental conditions). The final structures obtained indicate the fundamental role of the indole of Trp8 of Contryphan Vn (substituted by Ser8 in [W8S]contryphan Vn) near the salt bridge (D'Alessandro et al. 2004). This model of hydrophobic protection of salt bridge could provide valuable help

in designing specific peptides with a predetermined isomerization of prolines in constrained cycles (Yaron and Naider 1993), as well as facilitating the identification of structural determinants relating to the mechanisms of enzymatic proline isomerization in proteins. Finally in the the Contryphan Vn, the presence of D-Trp5 appeared to be an important structural feature for biological activity (Pallaghy et al. 1999, 2000; Massilia et al. 2003; Hansson et al. 2004; Sabareesh et al. 2006). The substitution of different residues in the cyclic Contryphan did not identify unambiguously the determinants of the activity (Jimenez et al. 1996, 1997; Pallaghy et al. 1999, 2000; Hansson et al. 2004; Sabareesh et al. 2006). In the case of [W8S]contryphan Vn, biological activity needs investigating to verify these novel compounds' true potential.

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

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