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Structural elucidation and conformational properties of the toxin paralysin β-Ala–Tyr

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Abstract

Larval extracts of the homotetabolous insects (i.e. Neobelleria Bullata–Insecta Diptera), cause paralysis followed by death when injected into adult flesh flies. The reason for causing these lethal effects is because the extracts contain endogenous toxins widely spread over the class of insects. Since their major effect is the paralysis they are called paralysins and are present through all the development stages. Their concentration gradually increases from larvae stage over pupation to late pharate adults indicating that paralysins have an active role in the metamorphosis. The prototype pharmacologically important dipeptide β-alanine–tyrosine was synthesized and submitted to conformational analysis studies in hydrophilic and amphoteric environments in order to reveal the stereoelectronic properties responsible for its activity.

Keywords: Paralysins; β-alanine–tyrosine; NMR; Molecular modeling; Neobelleria Bullata

1. Introduction

Acidic methanolic extracts of larvae obtained from nine different species were found to contain substances that cause a lethal effect in the adult stage of the same species and of other species. The compounds responsible for their lethal effects in adults are called paralysins. Two paralysins soluble in organic solvents and heat stable, were chromatographically purified to homogeneity by Chiou et al. [1]. These were identified by using mass spectrometry and nuclear magnetic resonance to be the β-alanine–tyrosine (β-Ala–Tyr) and 3-hydroxy-kynurenine (3-HK). The quantities of β-Ala–Tyr and 3-HK in the insect appear to increase steadily during larval development with peak values prior to the pupal stage [2].

β-Ala–Tyr was reported by Levenbook [3] (Fig. 1) to be synthesized in the fat body and to accumulate in the larval haemolymph up to the

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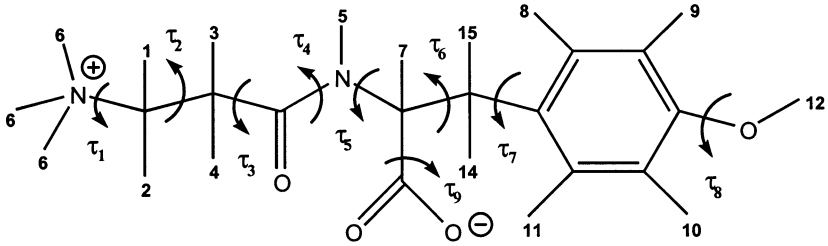

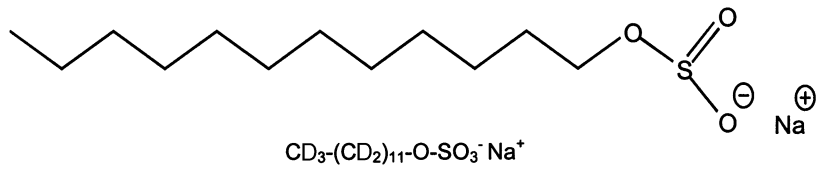

Fig. 1. Molecular structures of β-Ala–Tyr and SDS-d_{25}. The critical dihedral angles of β-Ala–Tyr that determine its conformation are also labeled on the 2D structure of the molecule.

moment of the formation of the white puparium. After that, its concentration drops to almost undetectable levels, because hydrolases from the fat body degrade the dipeptide into the resulting amino acids β-Ala and Tyr, which are subsequently incorporated in the cuticle.

The exact role of free β-Ala or of any of the β-Ala containing derivatives is unknown. It is believed that β-alanylation of the neurotransmitters octapamine, tyramine or dopamine leads to storage forms or inactivation products. Their site of action probably is on the nervous system since they cause paralysis and they may also interfere on the bioelectric activity of a yet unknown neurotransmitter receptor.

This study aims to explore the stereo electronic properties responsible for the activity of β-Ala–Tyr. Its structure was elucidated using 1D and 2D NMR techniques and the conformational properties were studied using a combination of 2D ROESY spectroscopy and molecular modeling.

2. Materials and methods

2.1. Materials

DMSO (99.5%) and D_2O (99.98%) ampoules were purchased form Merck (Darmstadt, F.R. Germany) and ultra-precision NMR tubes from Peypin (France) and Wilmad 535–5 mm (SPINTEC ROTOTEC). 5-Doxyl-stearic acid free radical was obtained by Aldrich (USA) and [U-D25] dodecyl sodium sulfate (98% ATOM D) from Euriso-Top Group (France). β-Ala–Tyr was synthesized in solution by coupling Boc–Ala–OH to H–Tyr(t-Bu)–OMe with the mixed anhydride method, followed by hydrolysis of the

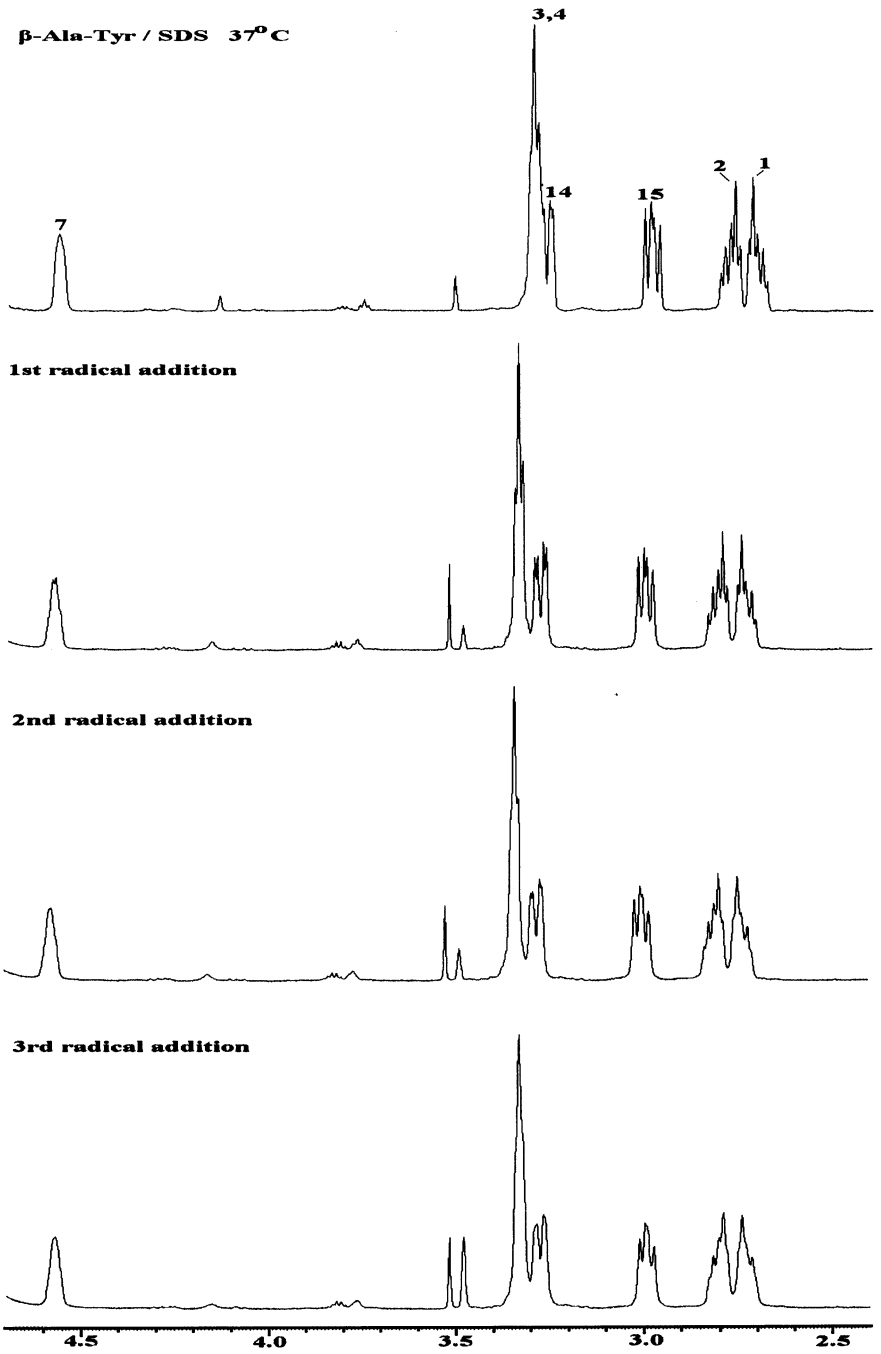

Fig. 2. ^{1}H-NMR spectra of non-aromatic region of β-Ala–Tyr in SDS micelles before (top) and after the three additions of the radicals.

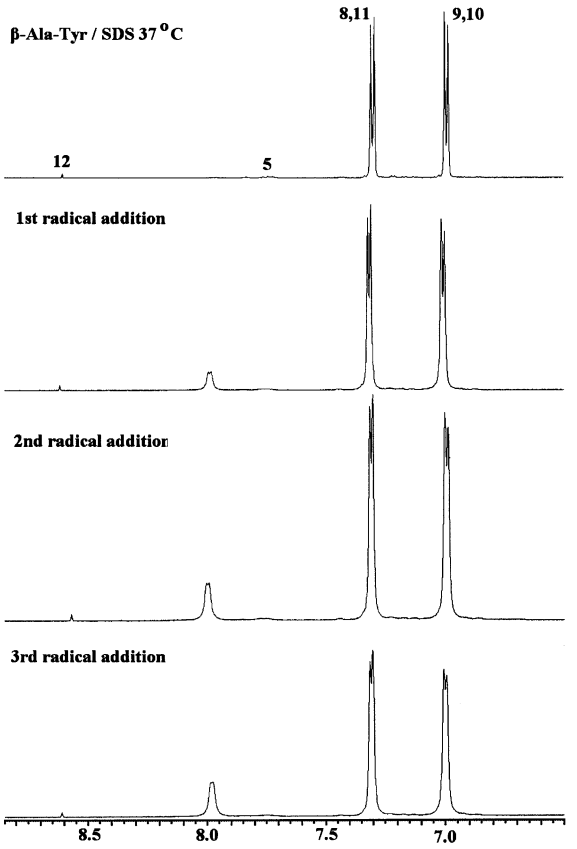

Fig. 3. ^{1}H-NMR spectra of aromatic region of β-Ala–Tyr in SDS micelles before (top) and after the three additions of the radicals.

methyl ester and deprotection of Boc- and t-Bu groups. The final product was obtained after purification by semi-preparative RP-HPLC and its purity was checked with HPLC and ES-MS.

2.2. Liquid NMR spectroscopy

The high-resolution spectra in DMSO were obtained using Bruker 300 AC instrument. The high-resolution spectra in D_2O and micelles were obtained using Varian INOVA 600 MHz instrument. All data were collected using pulse sequences and phase cycling routines provided in the Bruker and Varian libraries of pulse programs. Data processing, including sine-bell apodization, Fourier transformation, phasing, symmetrization, and plotting were performed using Bruker and Varian software packages. ^{1}H-NMR spectra in Bruker instrument were recorded using the following acquisition parameters: pulse width (PW) 3.0 μs, spectral width (SW) 2513 Hz, data size (TD) 32K, recycling delay (RD) 1.0 s, number of transients (NS) 16, and digital resolution 0.076 Hz pt^{-1}. ^{13}C-NMR spectra were performed with PW 2.1 μs, SW 20 000 Hz, TD 8K, RD 2.5 μs, NS 24 576 and digital resolution 2.441 Hz pt^{-1}. The ^{1}H–^{1}H correlation spectroscopy (COSY) was recorded using the following acquisition parameters: RD (D1)1 s, D0 increment 3 μs, SW in F_2 2487.56 Hz and in F_1 1243.78 Hz. The data sizes were 1K and 2K in F_1 and F_2, respectively, and the

Table 1
^{1}H-NMR chemical shifts in DMSO, D_2O and SDS micelles for β-Ala–Tyr

Assignment	Chemical shifts				
	D_2O	DMSO	Micelles (10 °C)	Micelles (27 °C)	Micelles (37 °C)
H-12		8.50	8.40	8.50	8.61
H-5	8.16	7.96	7.93	7.93	7.97
H-8, H-11	7.02	7.20	7.21	7.21	7.30
H-9, H-10	6.63	6.90	6.90	6.90	7.01
H-7	4.06	4.49	4.46	4.46	4.57
H-14	3.36	3.17	3.00	3.16	3.25
H-3	3.05	3.20	3.04	3.21	3.32
H-15	3.01	2.88	2.72	2.73	2.97
H-4	2.64	3.20	3.04	3.16	3.32
H-1	2.23	2.67	2.52	2.67	2.80
H-2	2.23	2.62	2.46	2.63	2.69

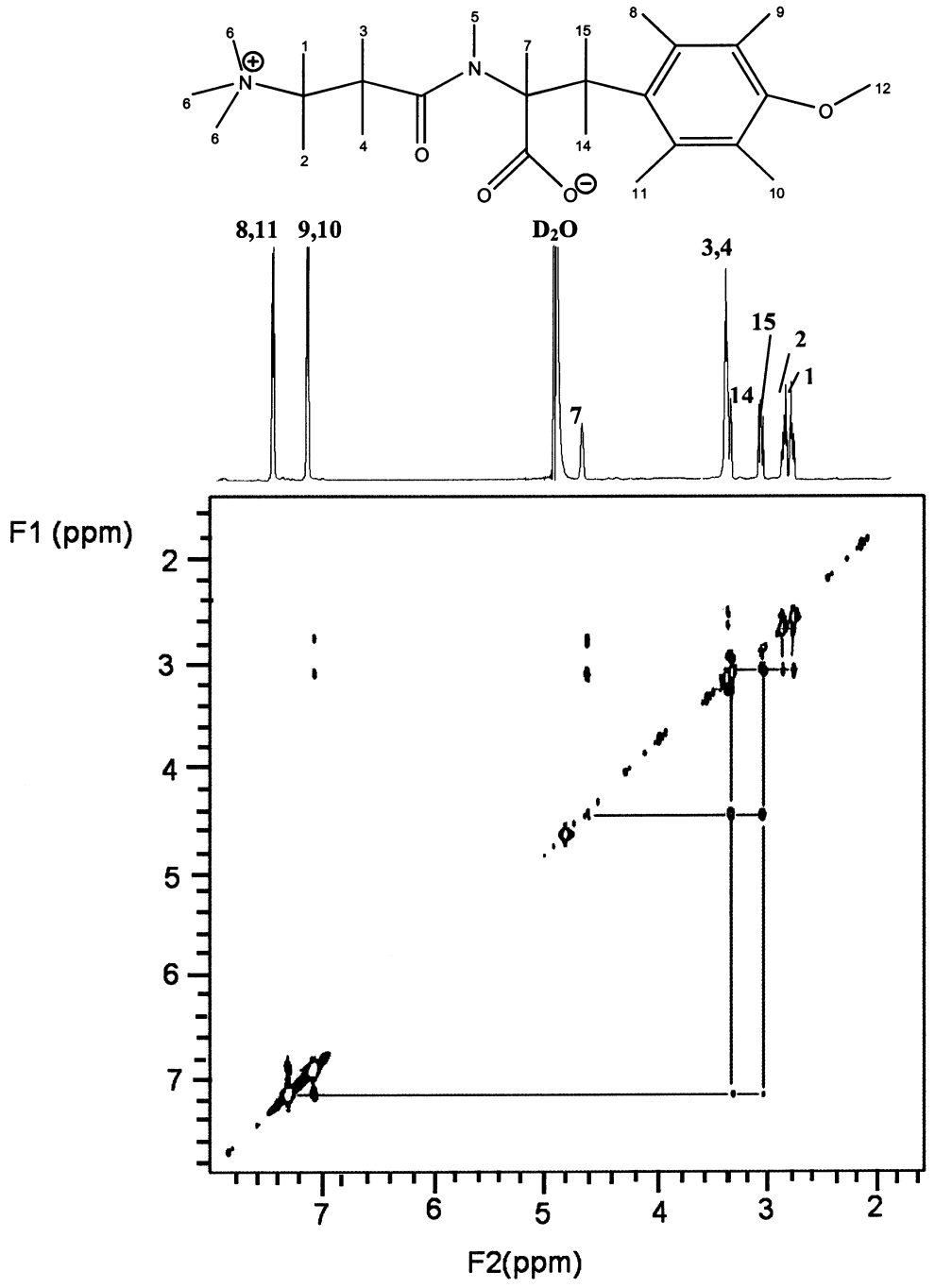

Fig. 4. ROESY experiment of β-Ala–Tyr in SDS micelles at 37 °C.

Table 2
^{1}H-NMR Chemical shifts in SDS micelles and after the three additions of radicals for β-Ala–Tyr

Assignment	Chemical shifts					
	Micelles (37 °C)	1st radical addition	2nd radical addition	3rd radical addition	$\bar{x}$	$\bar{s}$
H-12	8.60	8.62	8.61	8.61	8.62	0.010
H-5	7.97	7.98	7.99	7.98	7.98	0.008
H-8, H-11	7.30	7.32	7.32	7.30	7.31	0.012
H-9, H-10	7.01	7.02	7.01	7.01	7.01	0.005
H-7	4.57	4.57	4.57	4.57	4.57	0.000
H-14	3.25	3.26	3.27	3.26	3.26	0.008
H-3	3.32	3.30	3.29	3.33	3.31	0.018
H-15	2.97	3.01	3.00	3.00	3.00	0.017
H-4	3.32	3.30	3.29	3.33	3.31	0.018
H-1	2.80	2.80	2.80	2.79	2.80	0.005
H-2	2.69	2.75	2.75	2.74	2.73	0.029

data were zero-filled in F_1 prior to 2D Fourier transformation to yield a 2×2K data matrix. The spectrum was processed using sine-bell window function both in F_1 and F_2 (WDW = S) and the data were symmetrized about the diagonal (SYM). The DQF-COSY experiment was performed with gradients [4–6] at 600 MHz. The ROESY experiment was measured at 150 ms mixing time. The ^{1}H sweep width was 9820 at 600 MHz [7]. Typically homonuclear proton spectra were acquired with 4096 data points in t_2, 16–64 scans, 256–512 complex points in t_1, and relaxation delay of 1–1.5 s.

Data were processed and analyzed with V-NMR software package from VARIAN. Spectra were zero-filled two times and apodized with a square sine-bell function shifted by π/2 in both dimensions. Distances were calculated from cross-peak volumes in ROESY spectra using the V-NMR program.

The micelle experiments were carried out on a 5 mM sample in aqueous solution (90% H_2O–10% D_2O) containing 4 M SDS-d_{25} using Varian INOVA 600 MHz. The micelles were formed prior to the addition of β-Ala–Tyr using sonification. From ROESY spectra the distances were calcu-

Table 3
Observed ROEs, their intensities and calculated distances in low energy conformers

No	Protons	Intensity[a]			Distance (Å)	
		DMSO	D_2O	Micelles	D_2O	Micelles
1	5–11	M	M	–	3.22	–
2	5–15	L	M	–	2.47	–
3	7–3	M	S	M	1.89	2.77
4	7–15	S	S	M	2.10	3.31
5	3–15	–	S	M	2.06	2.41
6	2–4	S	S	M	1.86	2.59
7	1–3	S	S	M	2.08	2.46
8	8/11–9/10	S	S	S	2.46	2.46
9	9/10–3/4	L	M	M	2.45	3.17
10	8/11–3/4	M	S	M	2.21	3.09
11	11–15	L	S	M	1.95	2.47
12	7–11	–	M	–	2.32	–

[a] L, light; M, medium; S, strong.

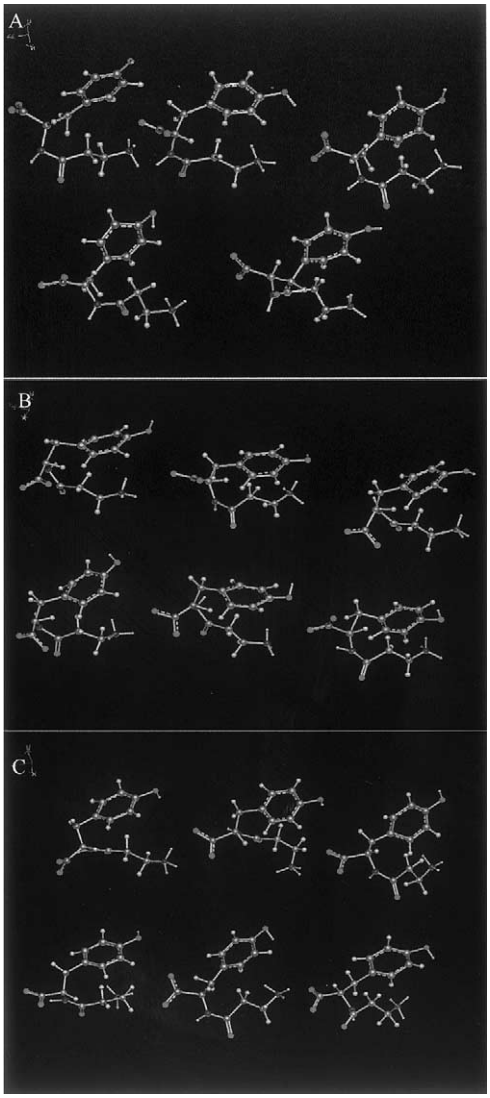

Fig. 5. The lowest energy family representative conformers obtained using distance geometry of the ROE data in (a) DMSO; (b) D_2O and (c) micelle environments.

lated as described above. In the solution, a 5-doxylsterate spin label was added in a ratio of 1:4 with respect to the dipeptide using three cumulative additions.

2.3. Molecular modeling

Computer calculations were performed on a Silicon Graphics O_2 workstation using QUANTA 97 version of the Molecular Simulation Incorporated program. The conformational energy of the dipeptide was first minimized using the algorithm of Steepest Descents to approach its local minimum and then Newton Raphson method to reach the local minimum. The minimized structure was used as the starting conformer in the dynamics experiments [8]. The molecular dynamics calculations were run for the molecule using simulation time 6 ps at a temperature of 2000 K and dielectric constants $\varepsilon = 45$ and 81 that simulate the DMSO and D_2O environment respectively. The same procedures were used for exploring the low energy conformers of β-Ala–Tyr in micelles. Family structures were generated using the dihedral angle criterion. The lowest energy conformers from each family were considered as the representatives ones. The initial MD phase of the calculation involved a gradual heating starting from 0 K and then increase to 2000 K with a time step of 0.001, by direct scaling of the velocities. The NMR derived distances restraints with a force constant of 10 kcal mol^{-1} $Å^{-1}$ were applied during the complete simulation. Conformers derived during the 200 ps dynamics process were saved every 1 ps. The last 100 ps of the trajectory were used for analysis of the ROEs distance violations and dihedral angles.

3. Results and discussion

3.1. Structural identification of β-Ala–Tyr

Figs. 2 and 3 depict representative ^{1}H-NMR regions of a spectrum of β-Ala–Tyr in micelles (tops) and Table 1 shows the chemical shift values for the ^{1}H-NMR spectra of the molecule in DMSO, D_2O solvents and micelles. Observed peaks are referenced to tetramethylsilane. The peak identification was confirmed by integration of the peaks, a 2D COSY spectrum and 2D ROESY.

3.2. Conformational properties of β-Ala–Tyr

The conformational properties of β-Ala–Tyr in D_2O, DMSO and micelles were sought using a

Table 4
Values of dihedral angles of the lowest energy conformers generated form molecular dynamics

Dihedrals	DMSO conformers					D_2O conformers						Micelle conformers					
	Conf. 1	Conf. 2	Conf. 3	Conf. 4	Conf. 5	Conf. 1	Conf. 2	Conf. 3	Conf. 4	Conf. 5	Conf. 6	Conf. 1	Conf. 2	Conf. 3	Conf. 4	Conf. 5	Conf. 6
τ_1	−163.3	123.9	31.4	60.9	66.4	65.5	−163.7	−50.9	110.9	63.1	75.4	65.5	−86.9	−74.1	87.4	−9.5	179.1
τ_2	−179.7	−175.3	−147.9	173.0	−175.0	−175.5	141.7	−162.9	145.3	137.0	154.3	−175.5	150.8	−143.4	177.8	177.7	3.5
τ_3	−5.4	83.0	77.5	82.1	26.2	21.9	−15.3	20.4	12.8	34.9	−13.7	21.9	−9.1	31.9	−18.9	−30.2	32.4
τ_4	33.9	−8.0	−54.9	−61.8	25.8	−31.9	−36.0	−44.8	−37.6	−48.7	−36.0	−31.9	8.4	−85.3	4.7	−17.2	−9.7
τ_5	45.9	52.1	89.7	90.8	50.0	108.4	117.2	102.0	122.0	116.3	116.0	108.4	62.7	137.7	104.8	100.7	86.3
τ_6	−110.8	−120.8	−104.7	−134.6	−116.0	−59.6	−59.2	−70.3	−99.4	−50.0	−82.4	−59.6	−123.0	−84.2	−79.5	−81.1	−57.3
τ_7	−83.1	−106.1	−111.0	−101.9	−78.4	62.1	64.9	46.5	55.7	123.6	124.4	62.1	0.8	37.6	175.5	−123.3	122.1
τ_8	−3.7	−23.7	−37.4	26.1	−28.5	87.3	71.2	104.9	96.3	78.9	101.1	87.3	138.8	135.7	121.5	125.9	109.6
τ_9	46.6	48.1	82.8	−156.0	21.5	63.6	−41.2	82.8	44.9	87.9	110.2	63.6	−61.7	−69.3	−66.5	156.3	−9.2

combination of computational analysis and NMR spectroscopy. The observed ROEs were quantitatively analyzed to produce interproton distances characterized by spatial proximity. The calculated distances were used as distance constraints to produce low energy conformers that do not violate ROE distances. In Fig. 4 is shown a ROESY experiment of β-Ala–Tyr in micelles at 37 °C and in Table 2 are shown the major ROEs which govern the conformational properties of β-Ala–Tyr. The proton–proton distances were calculated from experimental ROESY spectra and measured using a mixing time of 150 ms and the two-spin approximation. The integrated intensity of an aromatic pair of protons is assumed to have distance of 2.46 Å. From the observed ROEs the H10–H3, H9–H4, are critical for the conformational properties of the molecule. They establish a spatial proximity between the alkyl chain and the phenyl ring. Representative low energy conformers in the hydrophilic and amphoteric environments are depicted in Fig. 5. These conformers were derived after clustering the processed simulated ones from molecular dynamics, using the dihedral angle criterion. Thus, the conformers shown in Fig. 5 are the lowest energy representatives from each cluster.

As it can be observed from Table 3 there are some differences in ROEs observed in the three environments. ROEs between protons 5–11 and 5–15 are observed only in DMSO and D_2O environments. ROE between protons 7–11 are observed only in D_2O. However, these differences appear not significant for the overall conformation of the molecule. In all three environments β-Ala is placed above the phenyl ring of Tyr. This spatial proximity is more significant in D_2O environment. In conclusion, β-Ala–Tyr independently of the environment prefers a compact conformation. The phenolic hydroxyl group appears to be flexible adopting different orientations. It is interesting that in its lowest conformations τ_8 is almost identical in D_2O and micelle environments but differs in DMSO environment. This may show that phenolic hydroxyl groups adopt a preferable orientation in different environments. Differences were also observed with τ_7 dihedral angle which

are not however critical to determine the conformation of the molecule (Table 4).

3.3. Location of β-Ala–Tyr in micelles

To investigate if the dipeptide affects membrane properties we have performed preliminary studies using NMR relaxation probes in micelle environment. These studies will aid our experiments using membrane bilayers which will be reported elsewhere. Due to the amphotericity of the peptide, as a spin label was chosen the 5-doxylsterate. This has a nitroxide radical near the head of the sterate and affects relaxation of the NMR signals in the proximity of the micelle surface. 5-doxylsterate was added in the β-Ala–Tyr/SDS solutions three times in a ratio of 1:4 relatively to the dipeptide. The observed chemical shifts for the ^{1}H-NMR in 37 °C, before and after the addition of the radicals are shown in Figs. 2 and 3 and Table 2.

Figs. 2 and 3 and Table 2 show that not significant changes are caused by the addition of the spin label in the chemical shifts except in H-2. This may designate that the dipeptide is anchored in the vicinity of micelles with its $CH_2N^+H_3$ to be in the proximity of the spin label nitroxide.

Additional support that the molecule is located near the surface of the micelle and not in the hydrophobic core can be provided by the small broadening of the observed peaks. The broadening is spin-label concentration dependent. Not specific broadening effect is observed, indicating probably that the whole molecule is located near the surface of the bilayer and not a specific segment. Additional information is sought in our under way experiments using various biophysical techniques such as differential scanning calorimetry and solid state NMR spectroscopy.

4. Conclusions

We carried out a systematic exploring of the conformational properties of the insect toxin paralysin β-Ala–Tyr in an attempt to comprehend on its stereoelectronic properties. The conformational analysis results showed that β-Ala–Tyr is a dipeptide that adopts a compact conformation independently of its environment. Its phenolic hydroxyl group adopts specific orientation according to its environment. Other differences observed in the lowest energy conformers calculated and in accordance with ROEs by the dipeptide in the environments studied do not modify its conformational properties significantly. Experiments using spin-label radicals in a micelle environment showed that this dipeptide is probably a surface acting agent. Further experiments with its interactions in membrane bilayers will provide more information about its physical properties in membrane environment.

Acknowledgements

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