Structural study of novel antimicrobial peptides, nigrocins, isolated from *Rana nigromaculata*

Sangho Park[a], Sang-Ho Park[a], Hee-Chul Ahn[a], Sunkyu Kim[b], Sunny S. Kim[b], Byeong Jae Lee[b,*], Bong-Jin Lee[a,1]

[a] *Research Institute of Pharmaceutical Science, College of Pharmacy, Seoul National University, Seoul 151-742, South Korea*
[b] *Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, South Korea*

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Abstract Novel cationic antimicrobial peptides, named nigrocin 1 and 2, were isolated from the skin of *Rana nigromaculata* and their amino acid sequences were determined. These peptides manifested a broad spectrum of antimicrobial activity against various microorganisms with different specificity. By primary structural analysis, it was revealed that nigrocin 1 has high sequence homology with brevinin 2 but nigrocin 2 has low sequence homology with any other known antimicrobial peptides. To investigate the structure–activity relationship of nigrocin 2, which has a unique primary structure, circular dichroism (CD) and homonuclear nuclear magnetic resonance spectroscopy (NMR) studies were performed. CD investigation revealed that nigrocin 2 adopts mainly an α-helical structure in trifluoroethanol (TFE)/H_2O solution, sodium dodecyl sulfate (SDS) micelles, and dodecylphosphocholine micelles. The solution structures of nigrocin 2 in TFE/H_2O (1:1, v/v) solution and in SDS micelles were determined by homonuclear NMR. Nigrocin 2 consists of a typical amphipathic α-helix spanning residues 3–18 in both 50% TFE solution and SDS micelles. From the structural comparison of nigrocin 2 with other known antimicrobial peptides, nigrocin 2 could be classified into the family of antimicrobial peptides containing a single linear amphipathic α-helix that potentially disrupts membrane integrity, which would result in cell death. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Antimicrobial peptide; Nuclear magnetic resonance; Circular dichroism; Solution structure

1. Introduction

It is becoming clear through recent studies that the antimicrobial peptides are an important component of the innate defenses of all species of life [1]. In the case of vertebrates, the peptides are stored in the intracellular vesicles within phagocytic cells or in the secretory glands of epithelial tissues and released upon microbial infection. On the other hand, cecropins, the hemolymph peptides, are synthesized in pupae of the *Cecropia* moth as a response to bacterial infection [2]. In mammals, defensins are stored primarily in the intracellular granules of phagocytic cells and play a role in non-oxidative killing of engulfed microorganisms [3]. A number of peptides from the skins of various amphibia have been found to have antimicrobial activity [4–11]. Many peptides have good activities against a broad range of pathogenic microbes, including Gram-positive and Gram-negative bacteria, protozoa and fungi, as well as against cancer cells [12].

Recently, solution structures of various antimicrobial peptides from amphibian skins have been studied to see the structure–activity relationship [13–18]. Most of them are lysine-rich and belong to a large group of linear amphipathic helical peptides. These unique structural features are thought to endow these peptides to act on membranes. Magainin has been extensively studied and known to interact directly on bacterial cell membrane and to destroy the ionic gradient across the cell membrane by forming ion channels [19–24]. Many antimicrobial peptides such as brevinins [5,6], ranalexin [7], and gaegurins [11] are isolated from the family of Ranidae. They have high sequence homology and are categorized into several groups by their primary structures [25]. The solution structures of these peptides were studied to characterize the structural features and to understand the action mechanism [13,14]. They are composed of α-helix and C-terminal loop region delineated by an intra-disulfide bridge (*i*, *i*+6) named rana box and are known to be active against microbial membranes like other peptides from frogs.

In the present study, novel antimicrobial peptides, named nigrocin 1 and 2, were isolated from Korean frog, *Rana nigromaculata*, and their amino acid sequences were determined. The antimicrobial activities of these peptides were assayed against various microorganisms. Because nigrocin 2 showed little sequence homology with any other known antimicrobial peptides, the solution structure of this peptide was determined and compared with other antimicrobial peptides from Ranidae in order to understand the structure–activity relationship of nigrocin 2.

*Corresponding author. Fax: (82)-2-872 3632.
E-mail addresses: lbj@nmr.snu.ac.kr (B.J. Lee), mbglmg@plaza.snu.ac.kr (B.-J. Lee).

[1] Also corresponding author.

Abbreviations: CD, circular dichroism; CFU, colony forming units; DPC, dodecylphosphocholine; DQF-COSY, double quantum filtered correlation spectroscopy; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; r.m.s.d., root mean square deviation; SDS, sodium dodecyl sulfate; TFE, 2,2,2-trifluoroethanol; TOCSY, total correlation spectroscopy

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2. Materials and methods

2.1. Materials

2,2,2-Trifluoroethanol-d_3 99.5% (TFE-d_3) and sodium dodecyl sulfate-d_{25} (SDS-d_{25}) were obtained from Aldrich. D_2O 99.95% was obtained from Sigma and carboxymethyl-Sepharose CL-6B and Sephadex G-50 were purchased from Pharmacia. All other chemicals were of analytical grade obtained from various manufacturers.

2.2. Peptide preparation

Crude extracts were prepared by electric shock as described previously [26]. The crude extracts were applied to carboxymethyl-Sepharose CL-6B equilibrated with 0.2 M sodium acetate (pH 4.0) and eluted with 0.2 M ammonium acetate (pH 5.2). The active fractions were pooled, lyophilized, and redissolved in water, the sample then loaded onto Sephadex G-50 and eluted with 0.2 M ammonium formate (pH 4.0). The fractions containing antimicrobial activity were lyophilized and dissolved in 20% acetonitrile/0.1% trifluoroacetic acid and applied to a high performance liquid chromatography (HPLC) C_4 column (0.46 cm×25 cm, Vydac). The peptides were eluted at 0.6 ml/min in a linear gradient (20–70% acetonitrile) for 35 min. The eluted fractions containing antibiotic activities were used for sequencing. Amino acid sequence was determined by the Edman degradation method using an automated peptide sequencer (Applied Biosystems, Model 473A). The synthetic peptide with a disulfide bridge between C15 and C21, corresponding to the amino acid sequence of nigrocin 2 (Fig. 2), was purchased from AnyGen, Co., Ltd., South Korea, and used for further analysis. The sequence and purity of the synthetic peptide were confirmed by mass spectroscopy and HPLC.

2.3. Antimicrobial and hemolytic assay

Bacterial cells were grown overnight in Luria–Bertani (LB) media and inoculated into 5 ml of molten 0.6% LB agar with final 10^7 colony forming units (CFU)/ml, which was overlaid on a 150 mm Petri dish containing solidified 2% LB agar. After the top agar hardened, 3–10 μl of peptide samples were dropped into the 3 mm wells on the surface of the top agar and completely dried before incubating overnight at 37°C. Antimicrobial activity was determined by observing the zone of suppression of bacterial growth. Minimal inhibitory concentrations (MICs) against various bacteria were determined by incubating 10^6 CFU/ml of cells in LB media including a variable amount of peptides. Cell growth was measured by optical density of the culture suspension at 600 nm.

Hemolysis induced by nigrocin 1 and 2 was determined by incubating a 10% (v/v) suspension of human red blood cells (RBC) in phosphate-buffered saline with the appropriate amount of nigrocins and melittin at 37°C for 10 min. After centrifugation at 10 000×g for 10 min, the optical density at 350 nm of the supernatant was measured. The relative optical density compared to that of the suspension treated with 0.1% Triton X-100 defined % hemolysis.

2.4. Circular dichroism (CD) spectroscopy

CD experiments were performed using a Jasco J-715 spectropolarimeter to investigate the secondary structure of nigrocin 2. Samples were prepared by dissolving the peptide to the concentration of 50 μM in various solvents: aqueous solution, TFE/5 mM potassium phosphate buffer (1:1, v/v, pH 6.5), TFE/H_2O (1:9, 3:7, and 1:1, v/v, pH 4.0) solutions, 5 mM dodecylphosphocholine (DPC) (pH 4.0), and 10 mM SDS (pH 4.0). The spectra were measured between 190 and 260 nm. Three consecutive scans per sample were performed in a 2 mm cell at 20°C. Three scans were added and averaged, followed by subtraction of the CD signal of the solvent. The helicity of the peptides was estimated from the mean residue ellipticity at 222 nm [27].

2.5. Nuclear magnetic resonance (NMR) spectroscopy

Samples for NMR measurements were prepared at concentration of 3 mM in TFE-d_3/H_2O (1:1, v/v) and in 500 mM SDS-d_{25}. There was no significant difference between the CD spectra of nigrocin 2 at pH 4.0 and at pH 6.5 (data not shown), so the NMR samples were prepared at pH 4.0 to detect amide proton more easily. Data acquisitions for the homonuclear double quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY) spectra of nigrocin 2 were carried out at 30 and 40°C on Bruker DRX-500 and DRX-600 spectrometers equipped with a gradient unit. All NMR spectra were acquired using the TPPI method. Data matrices contained 4000×512 points and 64 scans were accumulated. Two-dimensional (2D) TOCSY spectra were acquired with a mixing time of 60 ms. 2D NOESY spectra were acquired with mixing times of 200, 250, and 300 ms, respectively. For detecting the αH protons resonating at the same frequency as water, the WATERGATE sequence [28] was used to suppress the solvent signals in the NOESY experiments. Assignment of spin-systems to individual amino acids was achieved using DQF-COSY and TOCSY spectra, while complete resonance assignment was obtained by the combined use of TOCSY and NOESY spectra, following the sequential assignment strategy [29]. The amide proton exchange experiments were carried out in TFE-d_3/D_2O (1:1, v/v) at 30°C and in 500 mM SDS-d_{25}/D_2O at 40°C. Slowly exchanging amide protons were monitored with a series of 2D NOESY spectra acquired at 50, 90, 130, and 170 min immediately after dissolving the lyophilized peptide in TFE-d_3/D_2O (1:1, v/v) and in 500 mM SDS-d_{25}/D_2O, respectively.

All NMR spectra were processed by using NMRPipe/NMRDraw software and were analyzed with the NMRView program. The NOESY spectra of nigrocin 2 in TFE/water solution obtained at 30°C and in SDS micelles at 40°C were used for volume measurement of the NOE cross-peaks. Chemical shifts were referenced to methyl signals of sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

2.6. Structure calculation

Distance restraints were obtained from the NOESY spectra. NOE data from the NOESY spectra were classified into three classes: strong, medium, and weak, corresponding to upper bound inter-proton distance restraints of 3.0, 4.0, and 5.0 Å, respectively. Lower distance bounds were taken as the sum of the van der Waals radii of 1.8 Å. As no stereospecific assignment could be made for the methyl and methylene protons, appropriate pseudoatom corrections were applied [29]. φ angles were constrained to $-65\pm35°$ for backbone amides with $^3J_{HNH\alpha} < 6$ Hz from DQF-COSY experiments. Hydrogen bond restraints were determined on the basis of slowly exchanging amide protons and the pattern of the NOE characteristic of an α-helix. Three restraints were added to define the disulfide bridge between C15 and C21. The target values of S(15)–S(21), S(15)–C_β(21), and S(21)–C_β(15) were set to 2.02 (±0.2) Å, 2.99 (±0.5) Å, and 2.99 (±0.5) Å, respectively [30]. The three-dimensional structures were calculated using the simulated annealing and energy minimization protocol in the program XPLOR 3.851 [31].

3. Results and discussion

3.1. Primary structures of nigrocins

Two peaks showing antimicrobial activity were separated by HPLC and their amino acid sequences were determined. They were named nigrocin 1 and nigrocin 2, respectively (Fig.

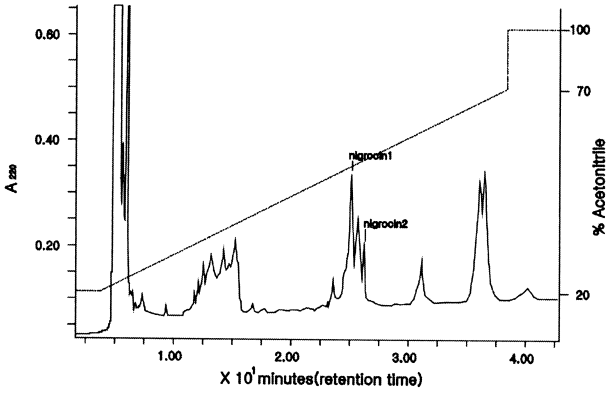

Fig. 1. Separation of nigrocins by C_4 HPLC. The fractions showing antimicrobial activity from Sephadex G-50 were loaded onto a C_4 column and eluted at 0.6 ml/min in a linear gradient of 20–70% acetonitrile (v/v)/0.1% trifluoroacetic acid (v/v) for 35 min.

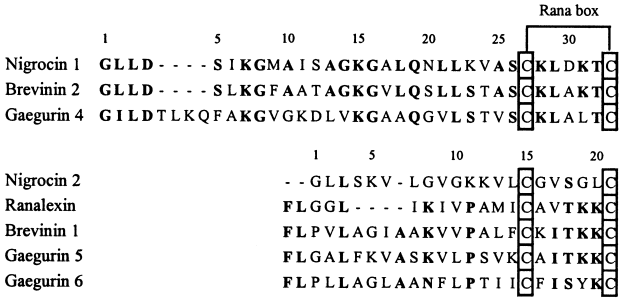

Fig. 2. Sequences of nigrocins and sequence alignment with other antimicrobial peptides from Ranidae. The sequences of brevinins [5], ranalexin [7], gaegurins [11] are reported for comparison. Bold letters indicate amino acid residues that are conserved among these peptides. Rana box is marked as boxes.

1). Both nigrocin 1 and nigrocin 2 are lysine-rich carrying a net charge of +5 and +3 at pH 7, respectively, like other antimicrobial peptides from Ranidae. These peptides have two conserved cysteines delineated by a disulfide bridge in the C-terminal region (rana box), which is characteristic of the antimicrobial peptides from Ranidae. The nigrocin 1 shows about 73% and 50% of sequence identity with brevinin 2 and gaegurin 4, respectively (Fig. 2). However, the sequence homology between nigrocin 2 and other peptides from Ranidae is low (Fig. 2). Nigrocin 2 has a very simple amino acid composition of five leucines, five glycines, four valines, three lysines, two serines, and two cysteines, which is a unique characteristic among the antimicrobial peptides from Ranidae. The N-terminus of nigrocin 2 starting with G1, L2, and L3 is different from those of relatively short (20–24 residue long) antimicrobial peptides from Ranidae containing highly conserved two N-terminal residues (F1 and L2) known to be involved in the peptide–micelle interaction due to their strong hydrophobicity [14]. Nigrocin 2 has no proline residues, while the short peptides from Ranidae have highly conserved proline residues located in the middle of the helix known to be essential for antimicrobial activity [15]. From these views, nigrocin 2 has a distinct primary structural characteristic compared with other antimicrobial peptides from Ranidae.

3.2. Antimicrobial and hemolytic assay

The antimicrobial spectra of nigrocins were determined by measuring the MIC. Table 1 shows the MIC values of nigrocins against various microorganisms. Nigrocin 1 and 2 show similar activities against the microorganisms tested in the present study despite their different primary structures. Nigrocins showed good activities in the MIC value of 10 μg/ml against the Gram-negative bacteria, *Klebsiella pneumoniae* and *Shigella dysenteriae*, and they were moderately active against *Salmonella typhimurium* whereas they were poorly active against *Pseudomonas aeruginosa* and had no effect on *Proteus mirabilis* and *Serratia marcescens*. *Micrococcus luteus* was significantly sensitive to nigrocins in the MIC value of 2.5 μg/ml among the Gram-positive bacteria that were tested. Nigrocins were also found to be active against a fungus, *Candida albicans*. In a conventional assay on human RBC, nigrocins did not exhibit hemolytic activity. Addition of nigrocins to human RBC up to 100 μg/ml did not cause significant hemolysis (Table 2). It was known that the appearance of numerous contiguous apolar residues in a helix is necessary for a significant hemolysis to occur [12]. Like other antimicrobial peptides isolated from the family of Ranidae, the polar residues in nigrocin 1 and nigrocin 2 are well interspersed among the hydrophobic residues, interrupting the contiguity of hydrophobicity, which gives the potential to form an amphipathic helix. For this reason, nigrocins probably exhibit little hemolytic activity like many other antimicrobial peptides from Ranidae. Interestingly, brevinin 1E is strongly hemolytic among the antimicrobial peptides from Ranidae [6,33]. In the case of the derivative of brevinin 1E, the elimination of the intra-disulfide bridge did not decrease the antimicrobial activity but did decrease the hemolytic activity. Kwon et al., there-

Table 1
Antimicrobial activities of nigrocins against various microorganisms

Microorganism	MIC (μg/ml)[a]	
	Nigrocin 1	Nigrocin 2
Gram-positive bacteria		
M. luteus	2.5	2.5
Gram-negative bacteria		
S. dysenteriae	10	10
K. pneumoniae	10	10
P. aeruginosa	75	100
S. typhimurium	22.5	22.5
P. mirabilis	>200	>200
S. marcescens	>200	>200
Fungus		
C. albicans	100	150

[a]MICs were the average values obtained in triplicates on three independent measurements.

Table 2
Hemolytic activity of nigrocins against human RBC

Concentration (μg/ml)	Hemolysis[a] of human RBC (%)		
	Melittin	Nigrocin 1	Nigrocin 2
0	0	0	0
10	27.4	0	0
100	100	1.1	0.9

[a]The relative optical density compared to that of the suspension treated with 0.1% Triton X-100 defined % hemolysis. Melittin was used for positive control.

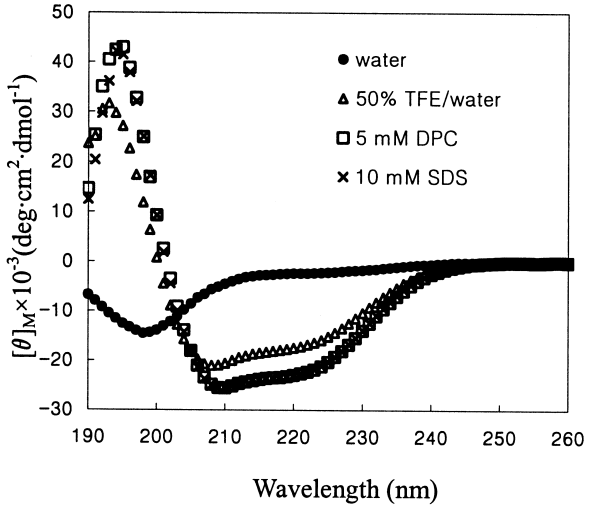

Fig. 3. CD spectra of nigrocin 2 in aqueous solution (●), in 50% TFE/water solution (△), in 5 mM DPC (□), and in 10 mM SDS (×).

fore, suggested that the hydrophobic nature of the intra-disulfide bridge is important for the strong hemolytic activity [33]. However, nigrocins as well as many other antimicrobial peptides from Ranidae [6,8,11] are not significantly hemolytic in spite of their preserved intra-disulfide bridge. These results indicate that nigrocins could be a good candidate for a new antibiotic agent.

3.3. Secondary structure

From the CD spectra of nigrocin 2, it was found that nigrocin 2 has no regular secondary structure in aqueous solution. However, in the presence of TFE, CD spectra showed two minima at 208 and 222 nm, which is characteristic of the presence of an α-helical conformation (Fig. 3). The helical content increased with increasing the concentration of TFE up to 50% (v/v), and addition of TFE above 50% caused no more change in CD spectrum (data not shown). Nigrocin 2 also revealed an α-helical conformation in membrane-mimetic environments such as DPC and SDS micelles (Fig. 3). The helix contents in 50% TFE, 5 mM DPC micelles, and 10 mM SDS micelles were estimated to be about 63, 82, and 82%, respectively. This result indicates that the helicity of the nigrocin 2 increased in micellar environments. This conformational transition of nigrocin 2 from random-coil in aqueous solution to α-helix in membrane-mimetic environments reflects its potentiality of interaction with membrane. The helical structures of nigrocin 2 in TFE/water solution and in SDS micelles were precisely investigated on the basis of the NOE data reported in Fig. 4. Several NOE connections were observed between the NH protons of residue *i*+3 and the αH protons of residue *i*. This type of NOE connectivity is indicative of an α-helical structure [32]. The medium $d_{\alpha\beta}$ (*i*, *i*+3) connections were observed in the same region except the glycine residues that have no β proton, and the $^3J_{HNH\alpha}$ coupling constants of the residues in this region were smaller than 6.0 Hz except glycine residues. These features are also the characteristics of an α-helical conformation. These results revealed

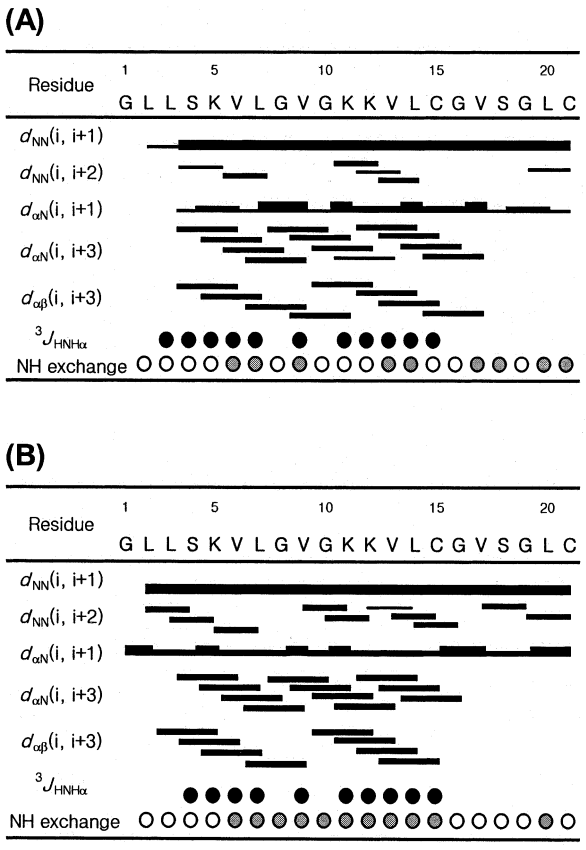

Fig. 4. Overview of the NOE connectivities, amide proton exchange rates, and coupling constants of nigrocin 2 in TFE/water solution (A) and in SDS micelles (B). The thickness of bar is indicative of NOE intensities classified into three groups. Slowly exchanging amide protons are represented as gray circles. The small (<6 Hz) coupling constants are represented as filled circles.

Table 3
^{1}H chemical shifts of nigrocin 2 in 50% TFE (303 K) and in 500 mM SDS micelles (313 K) at pH 4.0

	50% TFE					500 mM SDS				
	H^N	H^α	H^β	H^γ	Others	H^N	H^α	H^β	H^γ	Others
G1	n.d.[a]	n.d.[a]				n.d.[a]	3.96			
L2	8.54	4.24	1.70	n.d.[a]	n.d.[a]	8.60	4.07	1.75/1.66	1.66	H^δ0.9/0.92
L3	8.10	4.21	1.70	1.70	H^δ0.98/1.64	8.23	3.99	1.72	1.72	H^δ0.9/0.91
S4	7.89	4.16	4.00			7.99	4.08	3.97		
K5	7.78	4.26	2.03	1.59/1.50	$H^{\delta/\varepsilon}$1.71/3.01	7.74	4.23	2.07/1.89	1.57/1.49	$H^{\delta/\varepsilon}$1.68/2.97
V6	7.94	3.82	2.23	1.08/0.99		8.06	3.64	2.26	1.05/0.91	
L7	8.28	4.25	1.84	1.54	H^δ1.00/0.91	8.30	4.00	1.83/1.57	1.83	H^δ0.90/0.87
G8	8.24	3.96				7.97	4.00			
V9	7.95	3.86	2.24	1.10/1.00		7.95	3.81	2.2	1.12/0.97	
G10	8.28	3.83/3.89				8.59	3.61			
K11	7.89	3.95	1.96	1.61/1.54	$H^{\delta/\varepsilon}$1.76/3.03	8.47	3.87	1.92	1.52/1.45	$H^{\delta/\varepsilon}$1.72/2.95
K12	8.03	4.08	2.04	1.61/1.51	$H^{\delta/\varepsilon}$1.73/3.03	7.52	3.97	2.01	1.61/1.44	$H^{\delta/\varepsilon}$1.70/2.99
V13	8.19	3.78	2.21	1.06/0.97		8.25	3.75	2.18	1.07/0.94	
L14	8.23	4.2	1.84	1.75	H^δ0.96/0.93	8.50	4.04	1.78/1.67	1.74	H^δ0.87/0.86
C15	8.43	4.47	3.35/3.44			8.74	4.35	3.13/3.03		
G16	8.30	3.96				8.07	3.94			
V17	8.45	4.09	2.21	1.10/1.01		8.36	4.08	2.16	1.08/1.00	
S18	8.03	4.49	4.02			7.85	4.43	3.98/3.90		
G19	8.06	4.24/4.05				8.02	4.12			
L20	7.96	4.47	1.70	1.60	H^δ0.94/0.90	7.85	4.29	1.64	1.55	H^δ0.91/0.86
C21	7.69	4.47	3.38/3.31			7.64	4.37	3.38		

[a]n.d., not detected.

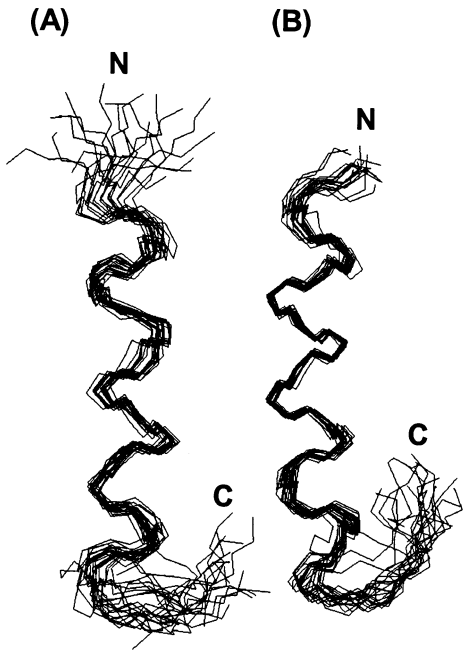

Fig. 5. Superimposition of the backbone atoms (N, C_α, and C′) of 20 structures from residues 1 to 21 in TFE/water solution (A) and in SDS micelles (B). Residues 4–17 of nigrocin 2 could be overlapped with the average r.m.s.d. values of 0.53 and 0.46 Å for the backbone, respectively.

that nigrocin 2 is composed of an α-helix like other antimicrobial peptides from Ranidae such as brevinin 1E [33] and ranalexin [14] in spite of low sequence homology.

3.4. Three-dimensional structure

Many of the sequential connections were completed mainly on the basis of the strong d_{NN} and $d_{\alpha N}$ (i, i+1) connections. These connections were compared with the $d_{\alpha N}$ (i, i+3) and $d_{\alpha\beta}$ (i, i+3) connections. The NH–NH and the αH–NH connectivities from L2 to C21 were observed. The complete assignment of the ^{1}H resonances is given in Table 3. 171 distance restraints, 11 ϕ angle restraints, and five hydrogen bond restraints were used for calculating the structure of nigrocin 2 in TFE/water solution and 191 distance restraints, 10 ϕ angle restraints, and 10 hydrogen bond restraints were used for calculating the structure of nigrocin 2 in SDS micelles.

A set of 50 structures of nigrocin 2 was calculated and the 20 structures with the lowest energy were chosen to represent the solution structure of nigocin 2. As shown, the structure of the peptide is a well defined linear α-helix spanning residues 3–18 (Fig. 5). These 20 structures exhibited a root mean square deviation (r.m.s.d.) about the mean coordinate position for helical region of 0.53 and 0.46 Å for the backbone atoms in TFE/water solution and in SDS micelles, respectively (Table 4). The backbones of the N- and C-terminal are poorly defined in TFE/water solution, being probably due to some flexibility of this region. However, the backbone of the N-terminal region is ordered better in SDS micelles than in TFE/water solution (Fig. 5), which results in a smaller backbone r.m.s.d. than in TFE/water solution. The α proton of G1 in SDS micelles was observed in NOESY spectra though the α proton of G1 in TFE/water solution was not observed (Table 3). These results indicate that the N-terminal region may be involved in the interaction with micelles through the hydrophobic residues (L2, L3), which may play the similar role like F1 and L2 of ranalexin [14]. The amide protons of G8, G10, K11, K12, and C15 showed a large chemical shift difference between in TFE/water solution and in SDS micelles (Table 3). These amide protons were exchanged slowly in SDS micelles, whereas they were exchanged fast in TFE/water solution (Fig. 4). These results are probably due to the stabilization of α-helical conformation in a micellar environment.

The three-dimensional fold of nigrocin 2 is totally amphipathic in the helix region, that is, leucines and valines are aligned on a portion of the helical cylinder whereas the lysines and serines occupy the remaining surface (Fig. 6). In many cases, the amphipathic nature of α-helical peptide is known to be important for membrane binding [34–37]. The amphipathic feature of nigrocin 2 is expected to mediate the interaction with microbial membranes, ultimately leading to disruption of membrane integrity. The helix region of nigrocin 2 shows some difference from that of ranalexin [14], brevinin 1E [33], and gaegurin 6 analogue [15]. The conserved proline residue induces a kink in the α-helix region in the case of ranalexin, brevinin 1E, and gaegurin 6 analogue. However nigrocin 2 has no proline and therefore adopts a stable single α-helix. Although the proline makes a kink in the middle of the helix,

Table 4
NMR restraints and statistics for the ensemble of 20 structures calculated for nigrocin 2

	50% TFE	500 mM SDS
Statistics for structure calculations		
R.m.s.d. from idealized covalent geometry		
Bonds (Å)	0.002 ± 0.0001	0.002 ± 0.0002
Bond angles (°)	0.457 ± 0.007	0.513 ± 0.014
Improper torsions (°)	0.361 ± 0.007	0.411 ± 0.015
R.m.s.d. from experimental restraints		
Distances (Å)	0.034 ± 0.002	0.030 ± 0.002
Final energies (kcal/mol)		
E_{total}	33.8 ± 1.6	42.7 ± 3.5
E_{bond}	1.3 ± 0.1	1.7 ± 0.3
E_{angles}	17.9 ± 0.6	22.6 ± 1.3
$E_{impropers}$	2.7 ± 0.1	3.4 ± 0.2
E_{vdW}	2.0 ± 0.4	4.5 ± 1.0
E_{NOE}	10.0 ± 1.0	10.5 ± 1.4
Average r.m.s.d. to the mean structure for all atoms/the backbone (Å)		
Whole (2–21)	1.74/1.09	1.56/0.97
Helix (4–17)	1.11/0.53	1.01/0.46

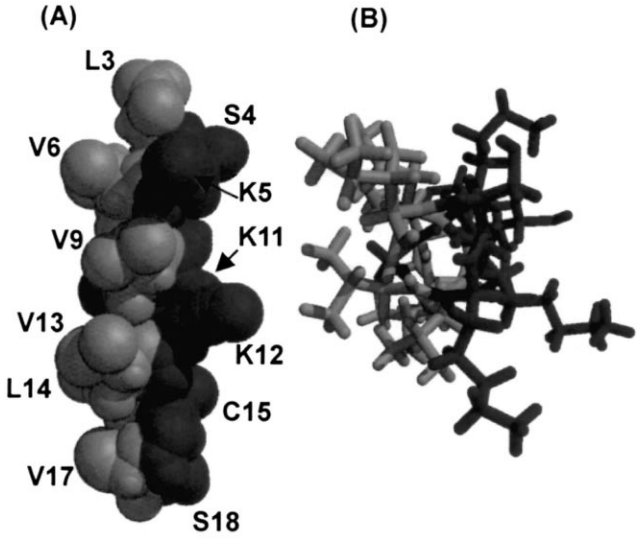

Fig. 6. Refined average structure of nigrocin 2 in SDS micelles. A: Backbone and side chains of residues 3–18 are shown as a conic presentation. B: Residues 3–18 are shown as a neon presentation. Hydrophobic and hydrophilic residues are colored light and dark gray, respectively. The direction of view is perpendicular (A) and parallel (B) to the helical axis, respectively. The program MIDAS was used for this presentation.

the overall amphipathic properties are well preserved in the region before and after the kink in ranalexin and gaegurin 6 analogue, which means that they are composed of a linear amphipathic moiety. Even though nigrocin 2 has some different α-helical conformation compared with ranalexin, brevinin 1E, and gaegurin 6 analogue, the length of the amphipathic region is similar to them. These results suggest that the overall three-dimensional fold of nigrocin 2 is similar to those of other antimicrobial peptides of about 20 residues from Ranidae, and therefore they may have a similar action mechanism at the structural level. It could be concluded that nigrocin 2 is classified into the family of antimicrobial peptides containing a single linear amphipathic α-helix that interact with target microbial membrane, even though it has low sequence homology with other peptides.

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