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International Journal of Biological Macromolecules
29 (2001) 251–258

INTERNATIONAL JOURNAL OF
Biological
Macromolecules
STRUCTURE, FUNCTION AND INTERACTIONS

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Molecular scaffold of a new pokeweed antifungal peptide deduced by ^{1}H nuclear magnetic resonance

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Received 24 April 2001; accepted 24 July 2001

Abstract

The antifungal peptide from seeds of *Phytolacca Americana* (Pokeweed), designated PAFP-S hereinafter, is a recently found cationic peptide which consists of 38 amino acid residues and exihibites a broad spectrum of antifungal activity, including inhibition of certain saprophytic fungi and some plant pathogens. The secondary structure and three cysteine pairings have been investigated by ^{1}H NMR analysis. The results show that the molecular scaffold of PAFP-S features a triple-stranded antiparallel β-sheet knotted by a typical disulfide bridge motif, which characterizes the knottin fold. CD spectroscopy indicates a high stability of the molecule in solution. Therefore, PAFP-S should be a new member of the knottin structural family and the first antifungal peptide that adopts the knottin-like fold.

Keywords: Antifungal peptide; Secondary structure; Cysteine pairing; Knottin-like peptide

1. Introduction

Plants have evolved highly effective defense mechanisms to restrict the growth of microorganism inside their tissues. A wide array of antimicrobial peptides, either produced in a constitutive or in an inducible manner are believed to be involved in such mechanism [1]. To date, a series of plant peptides or proteins with antimicrobial activity in vitro have been identified. They have been classified into several groups according to their characteristic sequence, such as thionins, plant defensins, lipid transfer proteins, and hevein- and knottin-type antimicrobial peptides [2]. The mode of action for these peptides is still much debated. Providing the structural and functional characterization of these plant antimicrobial peptides is essential to understand their biological activity. Recently, we found a potent antifungal peptide from the dry seeds of *Phytolacca americana* (pokeweed) and designated as PAFP-S [3]. PAFP-S is highly basic and consists of 38 amino acid residues with six cysteine residues. The peptide exihibited a broad spectrum of antifungal activity, including a visible inhibition of the growth of *T. viride* and the growth of a mushroom *Morchelia conica*, *Fusarium oxysporum* (a pathogen of cotton) and *Pyricularia oryzae* (a pathogen of rice), but PAFP-S displays no inhibitory activity towards *Escherichia coli* [3]. Here we report the secondary structure of PAFP-S uncovered by using NMR and CD analyses, which shows the presence of a knottin-like structural motif. Up to date no three-dimensional structure of an antifungal peptide adopting the knottin-like fold has been determined, although a refer-

Abbreviations: CD, circular dichroism; COSY, correlated spectroscopy; DQF-COSY, double-quantum-filtered correlated spectroscopy; PAFP-S, antifungal peptide from seeds of *Phytolacca americana* (Pokeweed antifungal peptide from seeds); NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

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PII: S0141-8130(01)00171-4

ence model from amaranth α-amylase inhibitor could indirectly provide some information [4,5].

2. Materials and Method

2.1. Sample preparation

PAFP-S was isolated from the seeds of *P. americana* and purified through four steps of chromatography on columns of CM-Sephadex C-50, Sephadex G-50, CM-cellulose 52 and S-Hyper D10, successively. Details of the procedure were described in the previous report [3]. The purity of the resulted sample was higher than 95% as showed by the SMART Mini-S chromatography and mass spectrometry. The determined molecular mass is 3929.0.

2.2. Circular dichroism spectroscopy

CD experiments were performed using a Jasco J-720 spectropolarimeter (Japan Spectroscopy Co. Ltd, Japan) to determine the secondary structure information and test the stability of the molecule in solution. The samples were prepared in 0.02 mol/l Tris–HCl pH 7.5 with 15 mg/ml. The spectra were acquired in the 260–190 nm UV region using 10 nm/min scan speed and 0.1 mm optical path length.

2.3. NMR spectroscopy

The NMR samples were prepared by dissolving 8 mg of PAFP-S in 0.5 ml of water containing 0.2 mM DSS, pH (or p^{2}H) 3.5. For the experiments in ^2H_2O, the sample was repeatedly dissolved in 99.8% ^2H_2O and

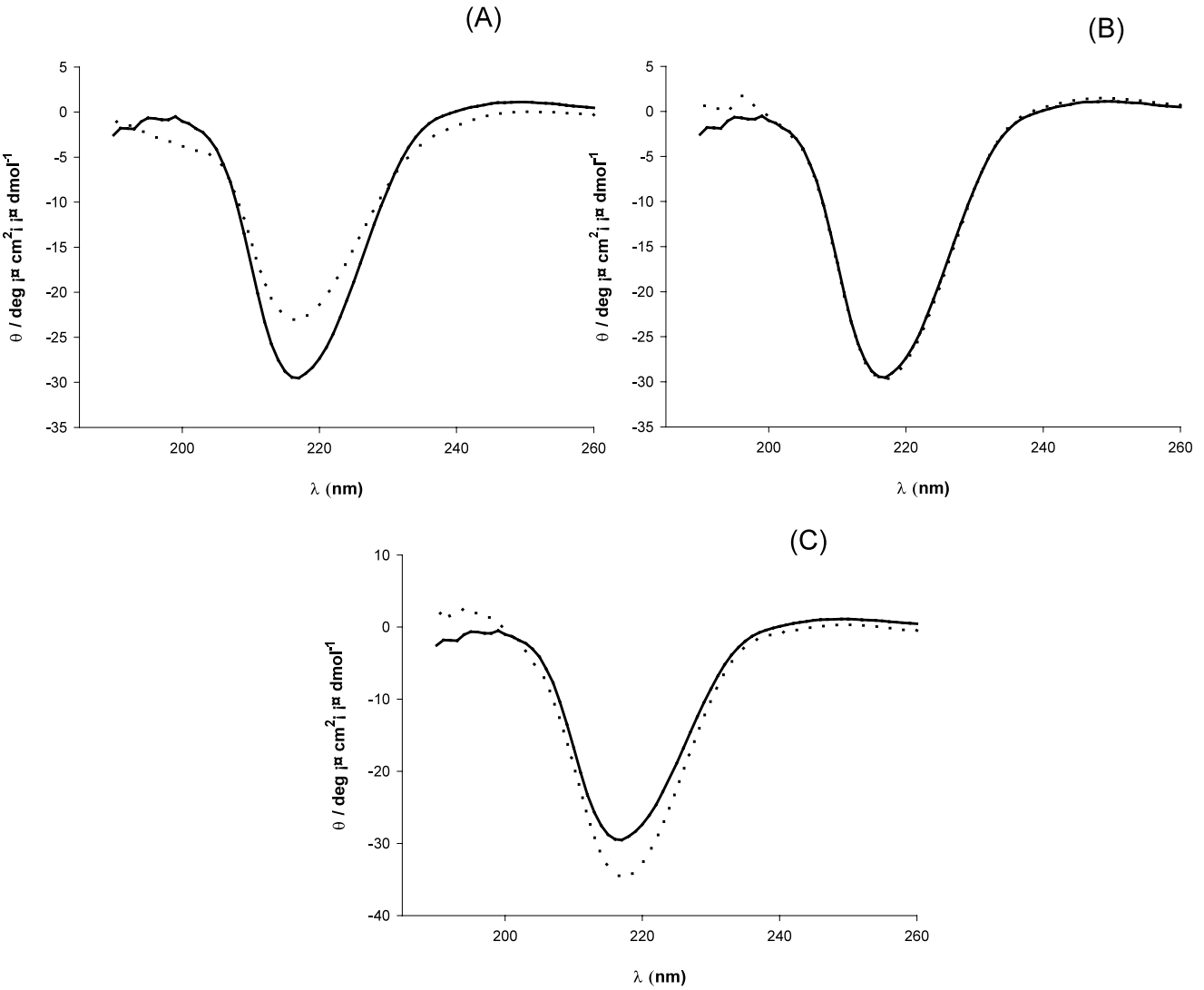

Fig. 1. Circular dicroism spectra of PAFP-S in various conditions: (A) after incubation at 100 °C for 30 min; (B) in the presence of 10 mM $CaCl_2$; (c) in the presence of 50% ethanol. In all panels spectra obtained in the conditions tested (dotted line) are superposed to those obtained in buffer without treatment (solid line). The results provide evidence for β-strand conformation and extreme stability of PAFP-S in solution.

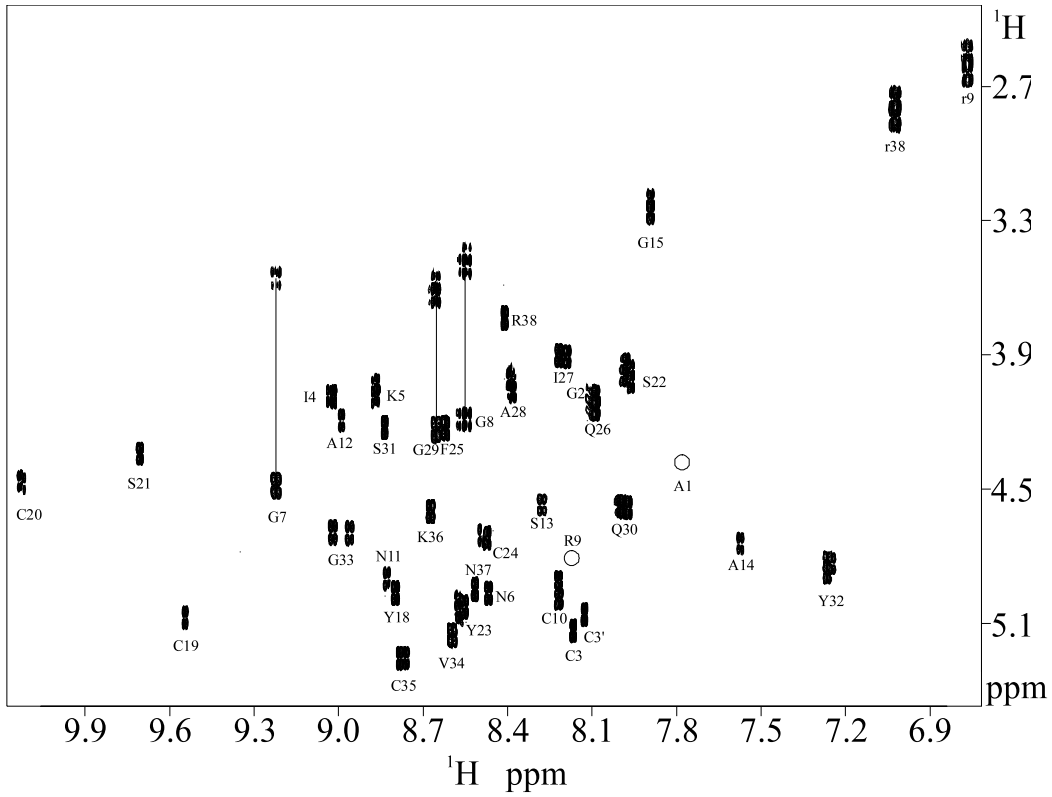

Fig. 2. H_N–H_α fingerprint region of the COSY spectrum in H_2O (300 K). The open circle indicates the position of correlation peak that appears at lower contour levels.

lyophilized with the final sample dissolved in 99.996% ^2H_2O. For the experiments in H_2O, the sample was dissolved in 90% H_2O/10% ^2H_2O.

Proton NMR spectroscopy were collected on a Bruker DMX 600 spectrometer at 300 or 310 K. The monomeric state of the peptide was checked by recording a 1D spectrum at a PAFP-S concentration of either 1 or 4 mM. No significant frequency shift was observed between the two samples. COSY, DQF-COSY, E.COSY, TOCSY and NOESY experiments were performed in phase-sensitive mode with different spin-lock and mixing times. Typically, the 2D ^{1}H NMR experiments were recorded over a period of 20–40 h, acquiring 384 or 512 $t1$ increments, with 128 scans per increment and 4096 data points per scan. The spectra were acquired with a spectral width of 7183.908 Hz in $F1$ and $F2$ dimensions. Data processing was performed on a Silicon Graphic Onyx 2 workstation using FELIX software version 97 (Biosym Technologies, San Diego, 1997). The original data were zero-filled to 1024 complex points in the $F1$ dimension, and a sinebell-squared window function with a $\pi/2$ (0 for COSY) phase shift was applied prior to Fourier transformation. Baseline correction was performed in the $F2$ dimension using the standard FLATT macro of FELIX.

3. Results

3.1. CD analysis

There is almost no change in the CD spectrum even after incubation at 100 °C for 30 min, as well as in the presence of 10 mM $CaCl_2$ or 50% ethanol (Fig. 1). The profile of these spectra shows that β-strand conformation is virtually the only secondary structure in PAFP-S. The content of β-strand conformation estimated by the program set in the instrument is 34.2%. The absence of spectral changes in various conditions indicates that the secondary structure of PAFP-S remains highly stable in solution.

3.2. Spin-system identification and sequential assignment

The fingerprint region of the COSY spectrum exihibited all 38 NH–αH cross-peaks expected for PAFP-S (Fig. 2). The complete sequence-specific assignment of the ^{1}H NMR resonance of PAFP-S was obtained using the standard methods established by Wüthrich [6]. Initially, spin systems were identified from the DQF/COSY and TOCSY spectra (Fig. 3) and then classified

by virtue of their coupling patterns and chemical shifts into amino acid types or groups. Sequential NOESY cross-peaks of types $d_{\alpha N}$, d_{NN} and $d_{\beta N}$ were used to assign systems to specific residues in the PAFP-S sequence. The good dispersion of the backbone ^1H resonances allowed us to identify at least two characteristic NOEs between neighboring residues, leading to a continuous stretch of sequential NOEs over the whole sequence. A NOESY spectrum recorded with a 250-ms mixing times in H_2O is shown in Fig. 4. Table 1 summarizes the chemical shifts of the assigned proton resonaces of PAFP-S.

3.3. Secondary structure from NMR analysis

The delineation of regular secondary structure of PAFP-S from NMR data is based on characteristic set of the inter-strand αHi–NHj, NHi–αHj and αHi–αHj NOE connectivity, $^3J_{N\alpha}$ coupling constants, slowly exchanging amide protons and low-field-shifted Hα chemical shifts [7]. The relevant data for PAFP-S are presented in Fig. 5. This is particularly evident from the inter-strand αH_i–NH_j (Gln26–Val34), NH_i–NH_j (Gly8–Cys35, Cys10–Gly33, Ile27–Tyr32) and NH_i–αH_j (Cys10–Val34, Phe25–Cys35, Ile27–Gly33) NOE contacts as well as the well-characterized low-field-shifted Hα chemical shifts (relative to the random coil values) and large $^3J_{N\alpha}$ coupling constants, which strongly suggests the presence of a β-sheet comparing residues 8–10, 23–27 and 32–36. The antiparallel nature of the sheet is revealed by the typical inter-strand αH_i–NH_j (Arg9–Val34, Asn11–Tyr32, Cys24–Cys35, Gln26–Gly33) NOEs (Fig. 6). As expected, most of the backbone amide protons within these strands exchange very slowly in D_2O while those lying outside individual β-strands exchange rapidly. The fast exchanging NH protons and the lack of NOEs suggest that several NH_2-terminal and COOH-terminal residues, and residues from Asn11 to Tyr18 are mobile and disordered in solution. So, we conclude that PAFP-S adopt a compact three-stranded antiparallel β-sheet consisting of residues 8–10, 23–27 and 32–36 with a long loop

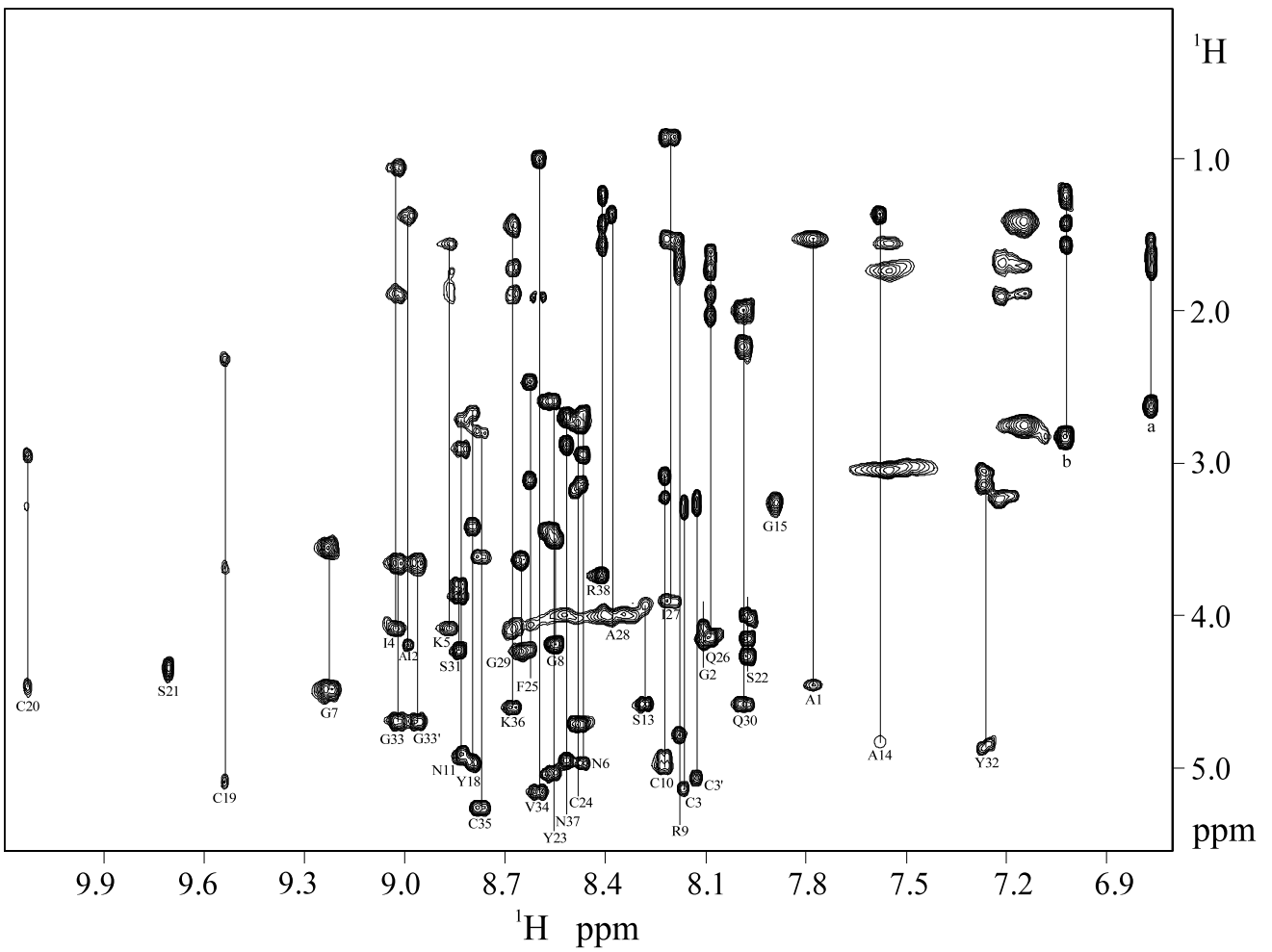

Fig. 3. A part of TOCSY spectrum of PAFP-S in H_2O at pH 3.5 and 300 K, showing intraresidue scalar connectivities. *a* and *b* marked on the right represent the connectivities between side chain nitrogen-bound protons of Arg9, Arg38 and protons of their sidechains, respectively. The open circle indicates the position of a cross peak that appears at lower contour levels. Cys3 and Gly33 exhibit unusual large coupling constants.

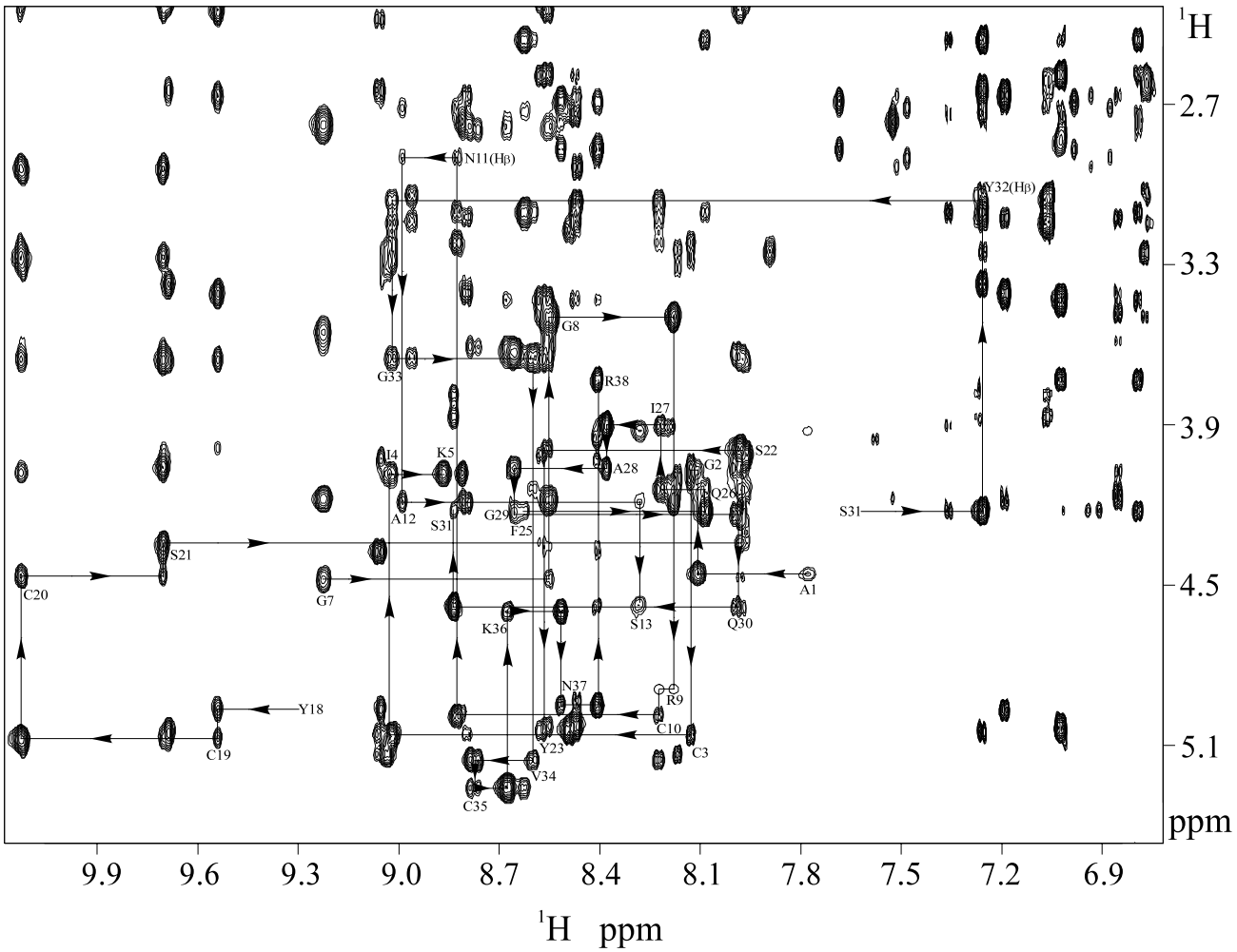

Fig. 4. The low-field region of the NOESY spectrum of PAFP-S recorded with 250 ms mixing time at pH 3.5 and 300 K. The sequential $d_{\alpha N}$ or $d_{\beta N}$ connectivities for residues 1–5, 7–13, 18–23 and 25–38 are illustrated by lines. The open circles indicate the position of an NOE cross peak that appears at lower contour levels.

connecting the first to the second β-strand as the core of the molecule (Fig. 7).

3.4. Disulfide pairing and molecular scaffold

The complete amino acid sequence of PAFP-S showed 6 cysteine residues [3], which should be paired into three disulfide bonds as indicated by mass analysis. The structure of PAFP-S is stabilized by these covalent bonds, as indicated by the CD study. In the absence of chemical characterization, these covalent links were determined from NMR data. The distance between the Hβ protons of one Cys residue and the Hα proton of the second one engaged in the same disulfide bridge is in a range in which NOEs can be theoretically detected. Nevertheless, local dynamics or the mutual overlap between the Hβ resonances of cystinyl and other residues can strongly hamper the detection of these NOEs. Several inter-cystinyl NOE connectivities (HA Cys10—HB1 Cys24, HB1 Cys19—HA Cys35, HB2 Cys19—HA Cys35; HA Cys19—HB1 Cys35, HB1 Cys19—HB2 Cys35, HB2 Cys19—HB1 Cys35) allowed us to identify unambiguously two disulfide bridges: Cys10–Cys24 and Cys19–Cys35. The third bridge (Cys3–Cys20) was deduced indirectly via long range connectivities between residues Cys20 and Ile4 (HN Ile4—HN Cys20). By the proposed disulfide pairings, Cys3–Cys20, Cys10–Cys24 and Cys19–Cys35, the three-stranded antiparellel β-sheet described above is firmly knotted to form the scafford of PAFP-S molecule as shown in Fig. 7.

4. Discussion

Ten years ago Le Nguyen et al. [8] first recognized a unique three-dimensional fold which featured a cystein-knotted triple-stranded β-sheet. Based on this 'knotlike' feature they introduced the term 'knottins' for this molecular scaffold. We now know that the knottins are

a group of structurally related small proteins, typically less than 40 residues in length. Knottins bind to a diverse range of molecular targets to perform distinct functional activities but share a common scaffold comprising a small triple-stranded antiparallel β-sheet and disulphide bond framework with a consensus sequence (C…C…CC…C…C) and pairing pattern (1–4, 2–5, 3–6). To date a series of knottins showing binding activity towards specific molecular targets, such as cellulose [9,10], K^+ channel [11], N- and P-type Ca^{2+} channel [12], trypsin and α-amylase [5], sweet test receptor [13], have been reported. However, so far no knottin structure with antifungal activity having been yet reported.

The alignment of PAFP-S sequence with that of some knottin peptides (Fig. 8) indicates that PAFP-S possesses the same cysteine motif and pairing of knottins. The secondary structure deduced from CD analysis (39.7% β-strand conformation) is in good agreement with that from NMR determination (34.2% β-strand conformation). All data reported in this paper, including CD spectra and NMR analysis, show that the molecular scaffold of PAFP-S features a triple-stranded antiparallel β-sheet knotted by three disulfide bridges with a very high stability in solution. This is coincident with the characteristics of the knottin fold. Therefore, PAFP-S should be a new member of the knottin peptide family. In fact, this is the first antifungal peptide that has been identified to adopt the knottin-like fold by structural analysis. Up-to-date only two antifungal peptide, Mj-AMP1 and Mj-AMP2, were reported to have the knottin like cysteine motif. They were purified from the seeds of *Mirabilis jalapa* L., comprise 36 and 37 residues, respectively, and exhibited high sequence

Table 1
Chemical shifts obtained for PAFP-S in H_2O solution at 300 K and pH 3.5

Residue	NH	Hα	Hβ	Others
Ala 01	7.78	4.46	1.52	
Gly 02	8.11	4.07		
Cys 03	8.13	5.07	3.28, 3.23	
Ile 04	9.03	4.08	1.90	γCH_2 1.40, 1.00; γCH_3 1.06; δCH_3 0.80
Lys 05	8.87	4.08	1.84	γCH_2 1.56; δCH_2 1.74; εCH_2 3.23; εNH_3^+ 7.22
Asn 06	8.47	4.97	2.94, 2.67	γNH_2 7.51,6.93
Gly 07	9.23	4.49, 3.56		
Gly 08	8.55	4.19, 3.49		
Arg 09	8.18	4.78	1.73, 1.63	γCH_2 1.53; δCH_2 2.62; ε-NH 6.77
Cys 10	8.22	4.96	3.22, 3.08	
Asn 11	8.83	4.91	2.90, 2.71	γNH_2 7.48,6.88
Ala 12	8.99	4.19	1.38	
Ser 13	8.28	4.57	3.93	
Ala 14	7.58	4.74	1.36	
Gly 15	7.89	3.26		
Pro 16	–	4.38	2.39, 1.99	γCH_2 2.22; δCH_2 3.02
Pro 17	–	4.20	2.25, 1.91	γCH_2 1.83,1.68; δCH_2 3.13
Tyr 18	8.80	4.96	3.41, 2.67	2,6H 7.19; 3,5H 6.85
Cys 19	9.54	5.08	3.67, 2.35	
Cys 20	10.13	4.47	3.28, 2.95	
Ser 21	9.71	4.35	4.35	
Ser 22	7.97	4.01	4.27, 4.15	
Tyr 23	8.57	5.03	3.44, 2.59	2,6H 7.03; 3,5H 6.79
Cys 24	8.48	4.71	3.15, 2.74	
Phe 25	8.63	4.23	3.10, 2.46	2,6H 7.36; 3,5H 7.26; 4H 6.78
Gln 26	8.08	4.15	1.71, 1.60	γCH_2 2.02,1.88; δNH_2 6.94,6.32
Ile 27	8.22	3.90	1.52	γCH_2 1.30,1.02; γCH_3 0.86; δCH_3 0.86
Ala 28	8.38	4.06	1.36	
Gly 29	8.65	4.23, 3.63		
Gln 30	7.99	4.59	1.99	γCH_2 2.23; δNH_2 7.53,6.69
Ser 31	8.84	4.23	3.87, 3.79	
Tyr 32	7.26	4.88	3.14, 3.04	2,6H 7.07; 3,5H 6.76
Gly 33	9.02	4.70, 3.66		
Val 34	8.60	5.16	1.92	γCH_3 1.00
Cys 35	8.77	5.26	3.61, 2.79	
Lys 36	8.67	4.60	1.88, 1.70	γCH_2 1.43; δCH_2 1.43; εCH_2 2.75; εNH_3^+ 7.15
Asn 37	8.52	4.95	2.87, 2.69	γNH_2 7.68, 6.98
Arg 38	8.41	3.74	1.57, 1.43	γCH_2 1.24; δCH_2 2.83; ε-NH 7.02

Where no chemical shifts are given, these resonances were not observed or assigned.

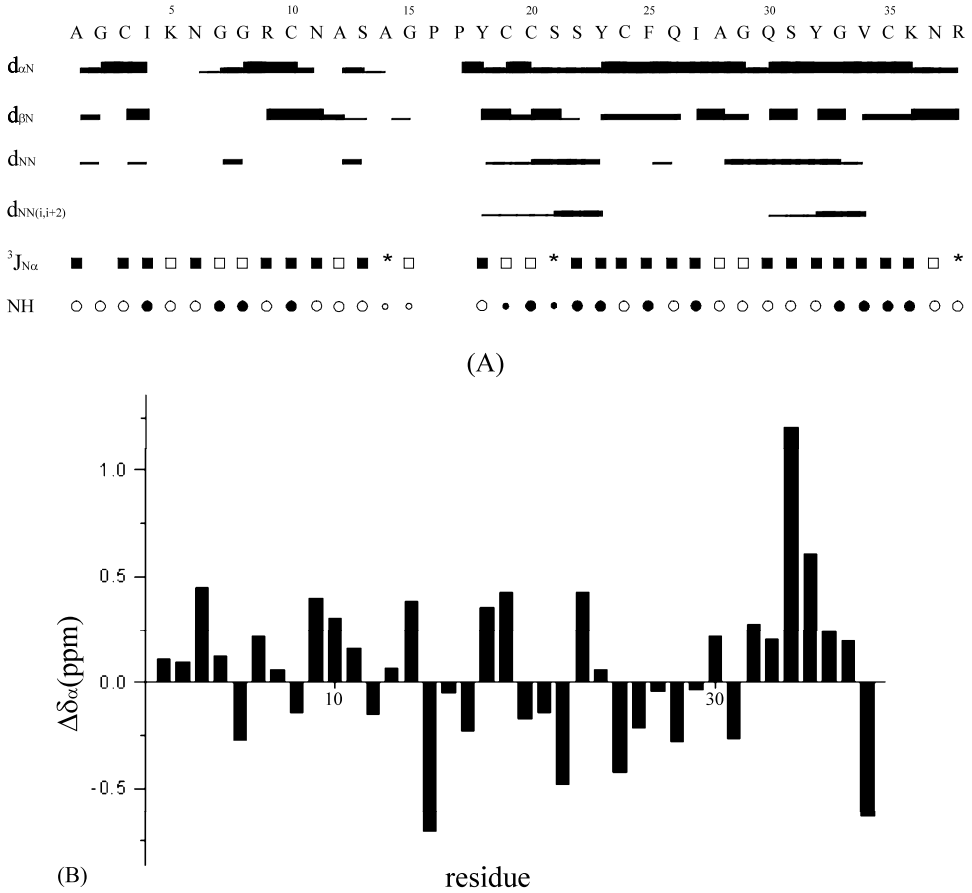

Fig. 5. (A) Overview of the structural data PAFP-S obtained from the NMR data. Sequential NOEs are categorized in distance ranges $d < 2.70$Å (■), $2.70 \leq d \leq 3.50$ Å (■) and $d > 3.50$ Å (▮). Amide H/^{2}H exchange rates (○, fast; ○, < 6 h; •, > 6 h; ●, > 48 h) and coupling constants (□, $J < 6$ Hz; ✱, $6 \leq J \leq 8$ Hz; ■, > 8 Hz). (B) The consensus chemical shift index for $^1H_\alpha$ of PAFP-S.

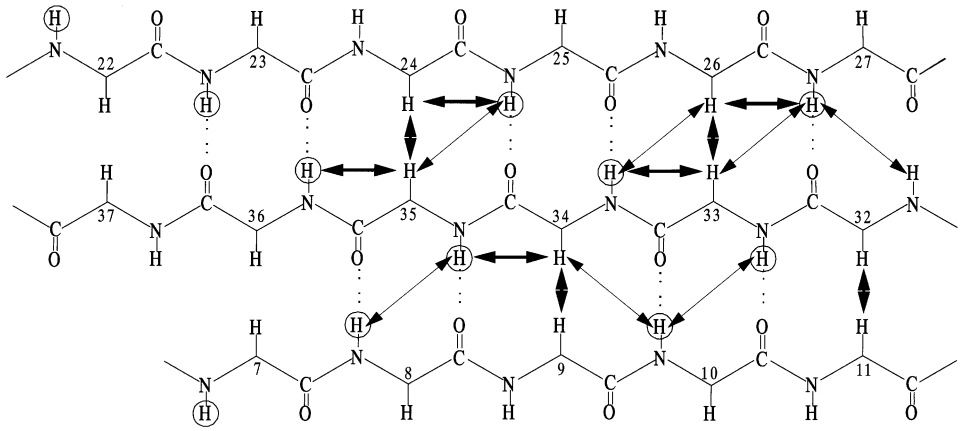

Fig. 6. Schematic representation of the antiparallel β-sheet structure of PAFP-S deduced from the analysis of the NMR data. The observed interstrand NOE connectivities between backbone NH and $C_\alpha H$ protons are indicated by arrows. The observed slowly exchanging amide protons (> 48 h) are circled. The hydrogen bonds deduced from the NOE patterns and slowly exchanging amide protons are shown as dashed lines.

homology (89%) [14]. The sequence comparison between PAFP-S and Mj-AMPs shows 57% homology and the same cysteine framework [3]. Thus, it can be predicted that Mj-AMPs should also adopt the knottin-like fold similar to that of PAFP-S.

A series of data have showed that the knottin-type peptide usually use different face of their molecular scaffold to interact with different biological targets [8–10,15–17] thus exhibiting distinct bioactivities. On this basis, the knottin scaffold appears to be a promising architecture for the design of new small proteins with diverse activities (e.g. [10,18]) of potential biotechnological and/or pharmacological interest. The fine structure of PAFP-S will provide a new model for mimicking.

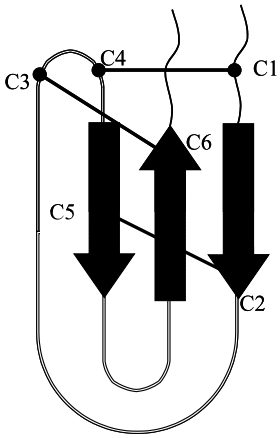

Fig. 7. Schematic view of the molecular scaffold of PAFP-S deduced from NMR analysis, which features a triple-stranded antiparallel β-sheet knotted by a disulfide bridge motif with a pairing pattern (C1–C4, C2–C5, C3–C6) (bold lines).

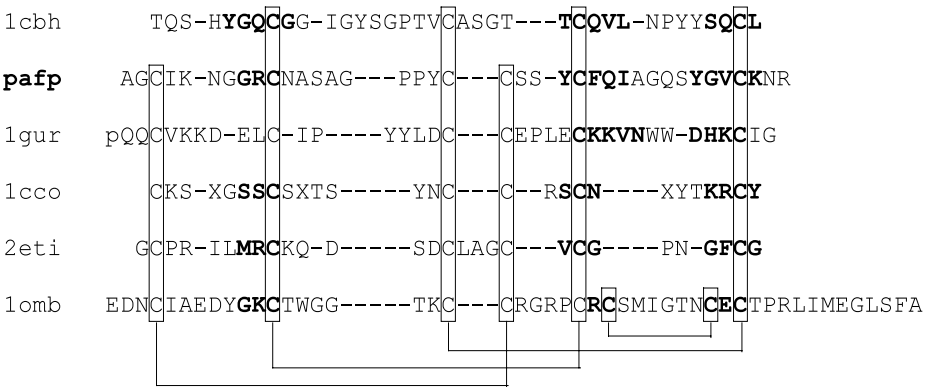

Fig. 8. Sequence comparison of some knottin peptides. All sequences have been aligned forcing the matching of cysteine residues (boxed). Except PAFP-S (in bold) [3], others are: 1cbh—the cellulose binding domain from *T. reesei* [9], 1qur—the sweet-suppressing peptide Gurmarin [13], 1cco—the ω-conotoxin GVIA from the cone snit *Conus gegraphicus* [15], 2eti—the squash trypsin inhibitor EETI-II from the seeds of *Ecballium elarium* [8], and 1omb—ω-agatoxin IVB from the American funnel-web spider *Agelonopsis aperta* [19]. All peptides listed here have their 3D structural model, in which the residues involved in β-strands are marked with bold characters.

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