Isolation and structural elucidation of the 11-residue peptaibol antibiotic, harzianin HK VI

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Harzianin HK VI, one of the major peptides of the natural mixture of peptaibol antibiotics biosynthesized by the fungus *Trichoderma pseudokoningii*, has been isolated by a multi-step chromatography procedure including HPLC. Its sequence was determined by positive ion FAB MS and two-dimensional ^{1}H and ^{13}C NMR experiments (COSY, HOHAHA, HSQC-HOHAHA, HMBC), allowing the location in the sequence of two isoleucines at positions 3 and 4 and of two leucines at positions 7 and 8, which could not be distinguished on the basis of their ^{1}H NMR spin systems. Harzianin HK VI is an 11-residue peptaibol with two Aib-Pro sequences at positions 5–6 and 9–10. From ROESY data and amide temperature coefficients, a β-bend ribbon stabilised by intramolecular hydrogen bonds of the 4→1 type is suggested. Despite the short sequence, harzianin HK VI increases the permeability of liposome bilayers to the same extent as longer 14-residue peptaibols.

Introduction

Of antibiotic peptides, peptaibols form an important class of linear hydrophobic peptides, known as membrane-modifiers. They interact with membrane lipids,[1–5] leading to permeabilisation of the bilayers[3,4] and upon voltage application, they form voltage-dependent ion channels.[2,5] Although still controversial as regards several points, the model of ion-channel formation implies a bundle of amphipathic transmembrane helices surrounding a central pore.[1,2] Biological activities of peptaibols, mainly related to the membrane-perturbing activity include antibiotic activity and inhibition of the multiplication of various types of cells,[4,6] haemolysis,[7] uncoupling of oxidative phosphorylation[8,9] and induction of the secretion of catecholamine in bovine adrenal chromaffin cells.[10]

Peptaibols are characterised by an acylated N-terminus, an amino alcohol as C-terminal residue and several α-dialkylated amino acids, mainly α-aminoisobutyric acid (Aib). According to their chain length and chemical characteristics, they are classified into three subclasses: the long-sequence peptaibols with 18–20 residues,[11–14] the short-sequence peptaibols with 11–16 residues[15,16] and the lipopeptaibols with 7 or 11 residues and having the N-terminal residue acylated by a linear aliphatic chain.[17,18]

In the course of our studies on the structure and function of antibiotic peptides, we characterised two groups of peptaibols from the culture broth of the soil fungus *Trichoderma pseudokoningii*. The first group was composed of 20-residue peptides related to trichobrachins,[19] whereas the second group consisted of a highly complex mixture of 11-residue peptaibols. As compared to long-sequence peptaibols, a great number of which are extensively described in the literature, few sequences of 11-residue peptaibols have been rigorously determined,[20–22] partly because they are biosynthesized by the fungi in even more complex mixtures than the long-sequence peptaibols.

We report herein on the isolation, complete sequence determination and conformational features in solution of harzianin HK VI (Fig. 1), which is shown to exhibit a membrane activity that is unexpected owing to the low number of residues.

1	2	3	4	5	6	7	8	9	10	11
Ac	Aib	Asn	Ile	Ile	Aib	Pro	Leu	Leu	Aib	Pro	Leuol

Fig. 1 Sequence of harzianin HK VI

Results and discussion

1 Isolation of harzianin HK VI

Fermentation of *Trichoderma pseudokoningii* (strain MVHC 662) on a synthetic medium led to a crude peptaibol mixture obtained by exclusion chromatography through Sephadex LH-20 of the culture broth butanolic extract and of the mycelium methanolic extract, which were treated independently. The culture filtrate and the mycelium extracts both led to the same peptide mixtures which were combined. The crude peptide fraction was further fractionated by silica gel chromatography to yield two main peptide groups of different polarity. The first eluted group was designated as PKA whilst a more polar group, designated as PKB, was a simpler mixture of 20-residue peptaibols.

When analysed by C18 reversed-phase HPLC, the PKA mixture appeared as a microheterogeneous peptide group showing 15 main peaks; this mixture was resolved into pure peptides by a multi-step semi-preparative HPLC separation. One of the major compounds was harzianin HK VI (Fig. 2). A three-step HPLC procedure involving two different C18 phases was developed, entailing first the separation of the PKA mixture into two fractions noted PKA-1 and PKA-2, on Spherisorb ODS2 reversed-phase (Fig. 2A). The more hydrophobic fraction PKA-2 which contained harzianin HK VI was, in turn, separated into three fractions, PKA-2-1, PKA-2-2 and PKA-2-3, on a Kromasil C18 phase (Fig. 2B). The last fraction, PKA-2-3 finally afforded pure harzianin HK VI which proved to be homogeneous from HPLC analyses (Fig. 2C), FAB MS molecular ion and NMR data.

The absence of reaction with ninhydrin reagent, together with the presence of a sharp singlet at 2.0 ppm in the ^{1}H NMR spectrum suggested an acetylated N-terminal amino acid. GLC analysis of the derivatised total hydrolysate showed harzianin HK VI to be composed of Aib (3), L-Asx

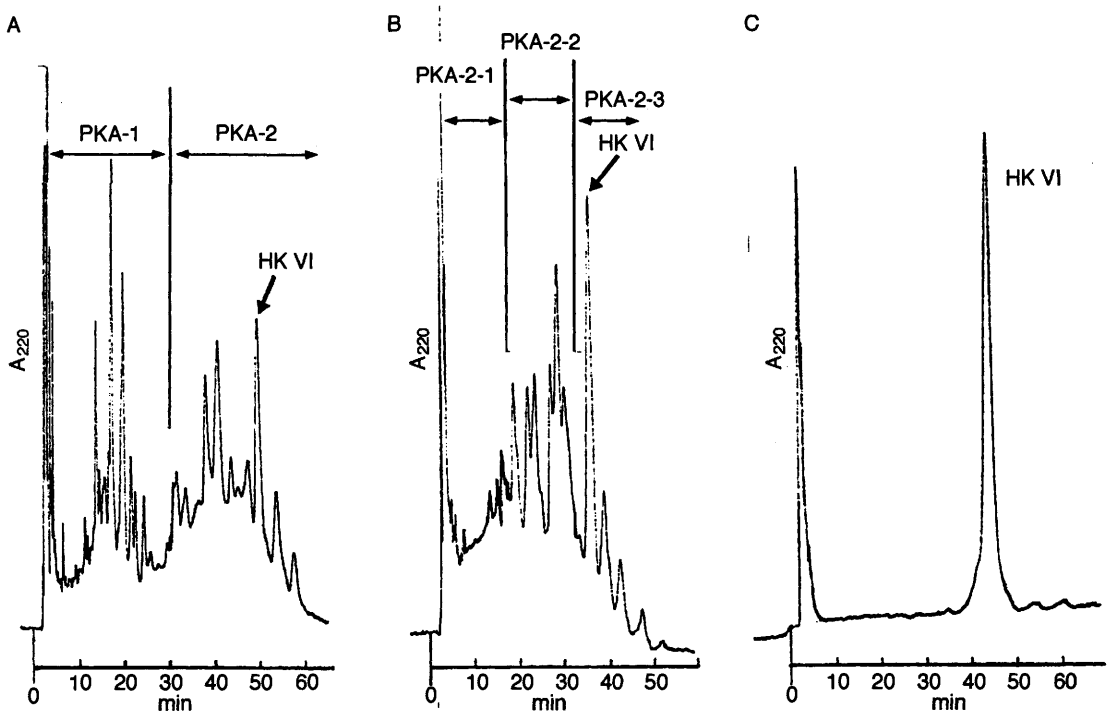

Fig. 2 HPLC procedure allowing the isolation of harzianin HK VI from *T. pseudokoningii*; A, natural PKA mixture on Spherisorb ODS2 (5μ), 7.5 × 300 mm, MeOH–H_2O (80:20), flow rate 2 cm^3 min^{-1}; B, PKA-2 fraction on Kromasil C18 (5μ), 7.5 × 300 mm, MeOH–H_2O (83:17), flow rate 2 cm^3 min^{-1}; C, purified harzianin HK VI on Kromasil C18 (5μ), 4.6 × 250 mm, MeOH–H_2O (82:18), flow rate 1 cm^3 min^{-1}; absorption monitored at 220 nm

(1), L-Ile (2), L-Leu (2), L-Leuol (1) and L-Pro (2). The L-Asx residue was assigned to L-Asn from the absence of an acid function in the peptide.

2 Structure determination of harzianin HK VI

We previously developed for the sequence determination of peptaibols the methodology employing the joint use of positive ion FAB mass spectrometry and ^1H and ^{13}C NMR spectroscopy.[12,13,16] The FAB MS technique affords the major part of the sequence, but leaves undetermined the respective location of the couples of isomeric amino acids, Leu/Ile and Val/Iva. The complete sequence results from sequential ^1H NMR assignments and stereospecific assignments are obtained from NOE effects and ^{13}C NMR data, allowing a conformational study to be undertaken. The structure determination of harzianin HK VI arose from such a methodology.

(a) FAB MS data of harzianin HK VI

Continuous series of b_n acylium ions generated from peptide fragmentation lead to sequence determination.[23] When an Aib-Pro tertiary amide link occurs in the sequence, preferential cleavage, leading to an N-terminal acylium ion N^+ and a y_n-type C-terminal ion $[HC, H]^+$[16,23–26] is observed. Such a fragmentation results in the absence of fragment ions at higher masses and in the superimposition of independent b_n type ion series at lower masses, originating from the N^+ acylium ions and the $[HC, H]^+$ ammonium ions.

The positive ion FAB mass spectra of HK VI obtained with two different matrices (α-thioglycerol and 3-nitrobenzyl alcohol) did not exhibit significant differences. As shown in Fig. 3, the pseudomolecular ion species $[M + Na]^+$ at *m/z* 1197 and $[M + K]^+$ at *m/z* 1213 (Fig. 3A) indicated a molecular weight of 1174 (nominal mass) and the molecular formula $C_{58}H_{102}O_{13}N_{12}$. Two abundant ions at *m/z* 961 and 553 suggested the formation of two N-terminal b_n ions, noted N_1^+, N_2^+, resulting from the rupture of two Aib-Pro bonds. As previously described for 14-residue peptaibols,[16] subsequent fragmentations of the N^+ ions and of their complementary

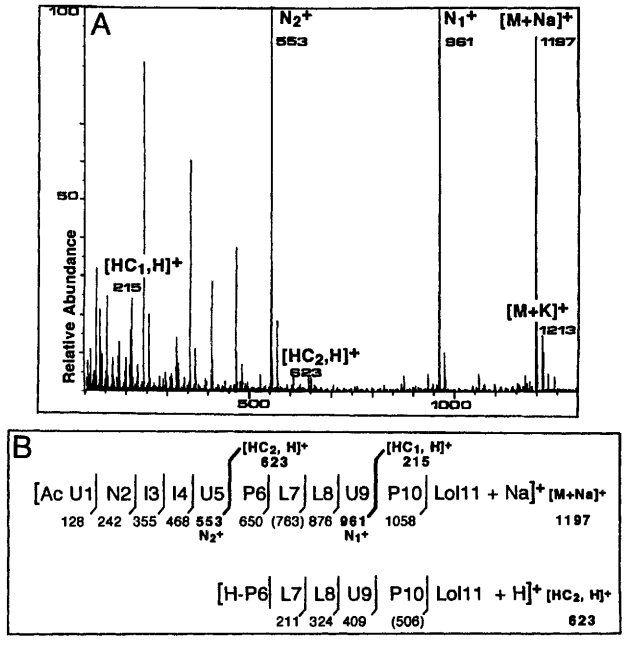

Fig. 3 A, Positive ion FAB mass spectrum of harzianin HK VI exhibiting the main fragment ions formed in the preferential cleavages at the Aib-Pro bonds. B, Mass fragmentation pattern of harzianin HK VI showing the preferential cleavages at the two Aib-Pro bonds leading to the complementary N_i^+ and $[HC_i, H]^+$.

$[HC, H]^+$ ions (Fig. 3B) gave the sequence of HK VI, but the location of the Leu and Ile residues remained unknown.

(b) Sequential and stereospecific NMR assignments of HK VI

^1H Sequential assignments. Proton sequential assignments and sequence determination of peptaibols can be obtained in two steps, according to the general procedure previously developed by Wüthrich:[27] (i) assignments of the different spin

systems to residue types result from COSY and HOHAHA experiments; (ii) sequential assignments are then afforded by exploitation of NOESY data, mainly sequential dNN (i, $i + 1$) and dαN (i, $i + 1$) connectivities. In the case of HK VI, the COSY and HOHAHA spectra assigned the spin systems of the unique Asn, of the two Pro amino acids and of the Leuol amino alcohol, but did not allow the spin systems of the two Leu and the two Ile residues to be identified unambiguously on the basis of the chemical shift values of their β and γ protons (Fig. 4). The sequential positions of the residues arose from the ROESY cross peaks (Fig. 5), but the four isomeric Leu/Ile residues could only be assigned together at positions 3, 4, 7 and 8, from observation of the appropriate dNN (i, $i + 1$) connectivities, confirming the sequence resulting from the FAB MS data. The ^{13}C NMR chemical shift values of the α carbons of Leu and Ile were thus used to differentiate between the two residue types, as the Ile α carbon (60–63 ppm) is generally more deshielded than a Leu one (53–56 ppm). From an HSQC-HOHAHA experiment, which directly relates the HOHAHA cross peak pattern for each ^1H spin system to the corresponding carbons through $^1J_{CH}$ connectivities, the Ile residues were readily assigned to positions 3 and 4 and the Leu ones to positions 7 and 8, leading to full sequence of HK VI (Fig. 1) and to the resulting ^1H sequential assignments (Table 1).

^{13}C and ^{15}N sequential assignments of HK VI. The backbone carbon atoms of HK VI were assigned from reverse-detection ^1H–^{13}C COSY (HSQC) and HSQC-HOHAHA data (Table 2; Fig. 6). The γ carbon chemical shifts of the Pro6 and Pro10 residues characterised both *trans*-Pro peptide bonds, as usually found for the peptaibols.[12,13] From a series of 2D heteronuclear reverse-detection ^1H-^{13}C COSY experiments optimised for long-range couplings of 6–8 Hz, the sequential assignment of the peptide carbonyl groups were obtained (Table 2), mostly from the $^2J_{CO_iNH_{i+1}}$ and $^3J_{CO_iNH_i}$ connectivities. The γ carbonyl group of Asn was assigned from its scalar correlation with the ββ′-protons. The two Pro carbonyl groups were assigned from the $^2J_{CO_iH\alpha_i}$ and $^3J_{CO_iH\beta_i}$ connectivities, as the ^2J and ^3J CO-NH cross-peaks were lacking. The Aib β-methyl assignments resulted from the observed $^2J_{C\alpha_iH\beta_i}$ and $^3J_{CO_iH\beta_i}$ connectivities. Finally, the nine backbone ^{15}NH assignments were accomplished from reverse-detection ^1H–^{15}N COSY (HSQC), which also gave the chemical shift of the Asn lateral chain amide (Table 2). It should be noted that the ^{15}NH signals of the three Aib were more deshielded (125–135 ppm) than those of the monoalkylated amino acids which appeared in the 100–115 ppm range.

^1H Stereospecific assignments. Exploitation of the ROESY cross-peaks allowed stereospecific assignment of a number of resonances (Table 1). The diastereotopic *pro-R* and *pro-S* ring protons of Pro6 and Pro10 were assigned unambiguously from their intra-residue NOE network: the α-proton was shown to be *cis* to the less shielded β-proton and the less-shielded δ-proton was seen as *cis* to the more-shielded γ′ proton, which in turn was *cis* to the less-shielded β proton and to the α proton. Diastereotopic Aib β-methyl groups were tentatively assigned, assuming a right-handed helix-like structure, as suggested below. Only one of the two β-methyls of each Aib residue exhibited an ROE intra-residue connectivity to the NH proton. The same β-methyl gave a stronger $^3J_{COMe}$ connectivity in the 2D inverse-detection heteronuclear ^1H-^{13}C COSY experiment optimised for a long-range coupling of 6 Hz (HMBC). It was assigned to the *pro-S*-Me, which in a right-handed helical structure, is located in the H-N-Cα plane, at a shorter distance (2–2.5 Å) from the NH proton than the *pro-R*-Me (about 3 Å).[28] It can thus give rise both to an NOE and to a $^3J_{COMe}$ coupling around 7 Hz. The *pro-R*-Me groups, orientated perpendicular to this plane, lead to zero or very low couplings.

(c) Secondary structure of HK VI. The presence of a number of α-dialkylated amino acids in peptaibols imparts a considerable conformational restriction on the peptide backbone which is constrained to adopt conformations in the 3_{10}-/α-helical region of the φ, ψ space.[29–31]

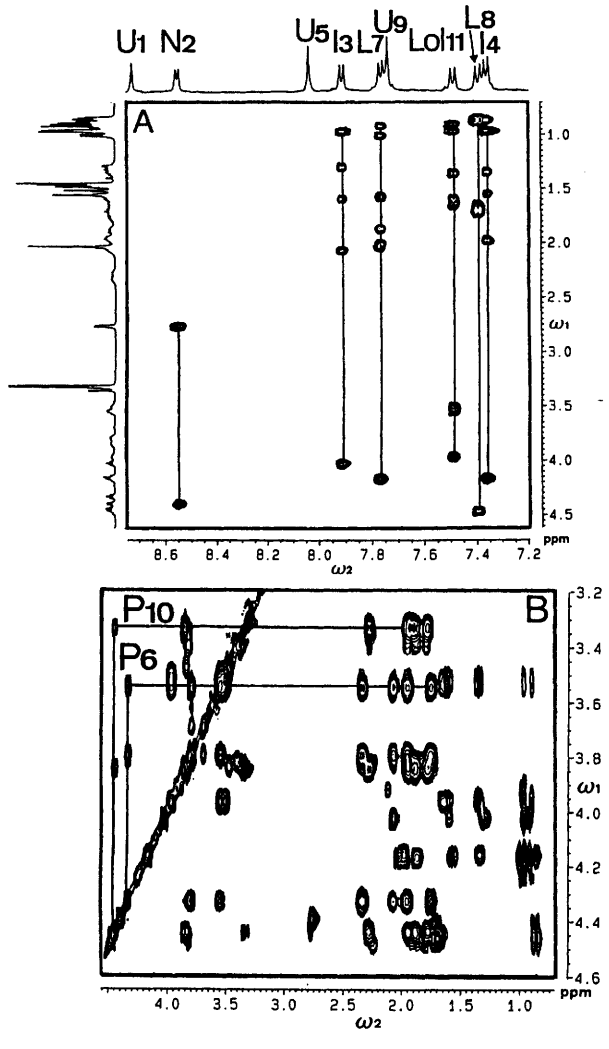

Fig. 4 Expansions of the HOHAHA spectrum of HK VI in CD_3OH with a spin-lock period of 120 ms; A, ω2 = 8.75–7.20 ppm, ω1 = 4.60–0.70 ppm; B, ω2 = 4.50–0.70 ppm, ω1 = 4.60–3.20 ppm; spin-systems are labelled with the sequential residue positions

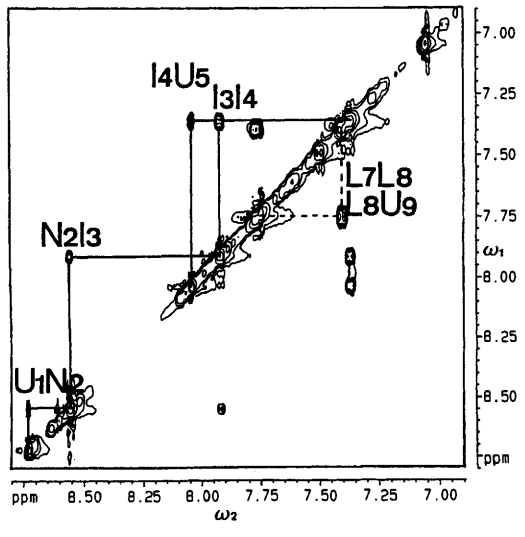

Fig. 5 Expansion of the ROESY spectrum of HK VI in CD_3OH with a mixing time of 250 ms, showing the dNN connectivities, ω2 = ω1 = 8.80–6.90 ppm

Table 1 ^{1}H sequential and stereospecific assignments of HK VI (500.13 MHz, CD_3OH, 297 K) and temperature coefficients ($-\Delta\delta/\Delta T$; ppb/K). Chemical shifts (ppm) are given to the nearest three decimals or two decimals when obtained from 1D or 2D spectra respectively; multiplicity and coupling constants (Hz) of the amino acid and amino alcohol spin systems arise, when possible, from the 1D spectra

Residue	NH	$-\Delta\delta/\Delta T$	αH	βH/βMe	γH/γMe	δMe and other groups
Ac						2.032s
U1	8.724s	8.1		*pro-S* 1.449s/*pro-R* 1.463s*		
N2	8.552d (5.5)	5.2	4.40	2.76		ε *syn* 7.045bs;[a] ε *anti* 7.739bs[b]
I3	7.913d (7.5)	1.9	4.02	2.05	1.58/1.29 γMe 0.97	0.93
I4	7.364d (8.1)	2.4	4.15	1.98	1.55/1.35 γMe 0.95	0.86
U5	8.036s	4.9		*pro-S* 1.555s/*pro-R* 1.514s*		
P6			4.33	*pro-S* 2.35/*pro-R* 1.75	*pro-S* 2.07/*pro-R* 1.95	*pro-R* 3.80/*pro-S* 3.55
L7	7.765d (7.2)	1.1	4.17	2.02/1.58	1.85	0.999d (6.6)/0.91
L8	7.397d (9.0)	1.2	4.46	1.72/1.72	1.72	0.88/0.84
U9	7.739s	2.3		*pro-S* 1.481s/*pro-R* 1.449s*		
P10			4.44	*pro-S* 2.27/*pro-R* 1.80	*pro-S* 1.95/*pro-R* 1.90	*pro-R* 3.85/*pro-S* 3.35
Lol11	7.492d (8.9)	2.3	3.95	1.60/1.35; 3.52	1.60	0.880d (7.7)/0.96

* Assignments may be reversed. [a] $-\Delta\delta/\Delta T$ = 5.9. [b] $-\Delta\delta/\Delta T$ = 7.1

Table 2 ^{13}C (75.47 MHz) and ^{15}N (50.68 MHz) NMR chemical shifts (ppm) for HK VI (CD_3OH, 297 K)

Residue	CO	αC	βC	γC	δC	^{15}NH
Ac	173.78	23.00				
U1	177.91	57.50	26.80 *pro-S* 24.05* *pro-R*			132.5
N2	174.15	54.06	35.96	174.92		103.9; 104.0 (δ)
I3	174.63	61.34	36.74	26.54; 15.84	10.96	111.9
I4	174.52	60.44	37.28	26.71; 16.14	10.58	108.9
U5	175.10	57.98	23.54 *pro-S* 26.63* *pro-R*			127.1
P6	176.04	65.10	29.94	26.98	50.50	—
L7	176.14	55.12	40.31	25.90	23.69; 20.97	107.8
L8	174.37	53.11	41.20	25.69	23.47; 20.81	108.5
U9	174.68	57.87	24.22 *pro-S* 25.89* *pro-R*			126.0
P10	174.37	64.21	30.10	26.87	50.30	—
Lol11	—	51.13	40.31; 65.81	25.98	21.93; 24.05	113.7

* Assignments may be reversed.

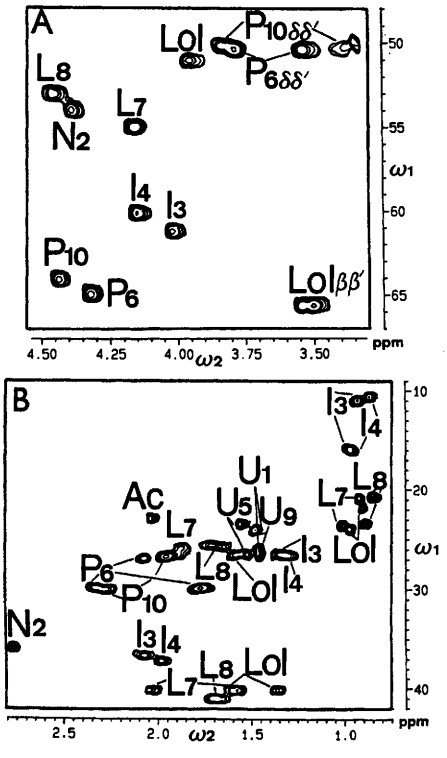

Fig. 6 Expansions of the heteronuclear ^{1}H–^{13}C HSQC spectrum: A, ω2 = 4.55–3.30 ppm, ω1 = 67.0–48.0 ppm; B, ω2 = 2.80–0.80 ppm, ω1 = 42.0–9.0 ppm

As the state of aggregation is an important consideration for the conformation of small peptides, the absence of evolution of the ^{1}H NMR chemical shifts of the NH (upfield shift of 0.003 to 0.01 ppm), and the α- and β-protons (upfield shifts of 0.001 to 0.003 ppm) upon increasing the concentration (from 0.02 to 15 mM) was verified. Conformational NMR parameters including nuclear Overhauser effects (NOEs), $^3J_{NHC\alpha H}$ coupling constant values and amide proton temperature coefficients were used to compare the secondary structure of HK VI in methanol solution to that of the longer analogues harzianins HC.[16] The NOEs were observed using two-dimensional rotating frame nuclear Overhauser effect spectroscopy (ROESY), which was used mainly for obtaining qualitative proximity information (<4.5 Å).[32] The pattern of through-space connectivities exhibited by HK VI (Fig. 7), a succession of strong dNN(i,i + 1) and low-range dαN(i,i + 1) all over the sequence, together with dβN(i,i + 1), dαβ(i,i + 3) and dαN(i,i + 3) in the Asn2–Aib9 region, characterised a helical structure as generally found for Aib-peptides. The absence of dαN(i,i + 4) in any part of the sequence accompanied by the presence of two dαδδ′ (i,i + 2) NOE cross-peaks between the α protons of Ile4 and Leu8 and the δ protons of Pro6 and Pro10 respectively, argued for a helix stabilised by 4→1 hydrogen bonds, which was in agreement with amide proton temperature coefficients measured over the range 296–316 K (Table 1). The above conformational parameters indicated thus that harzianin HK VI adopted the same structure made of a succession of consecutive β-turns as harzianins HC.[16] This conformation resulted in an amphipathic character, all the Leu and Ile lateral chains lying on the same face of the ribbon. The $^3J_{NHC\alpha H}$ coupling constants (Table 1)

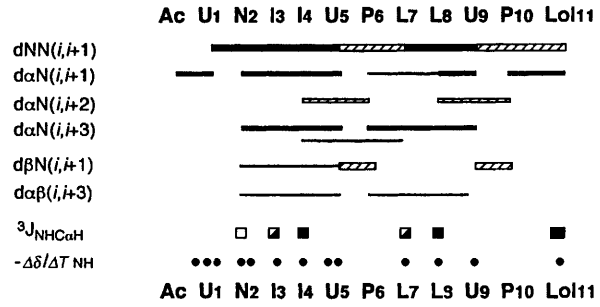

Fig. 7 Amino acid sequence (the one-letter code of amino acids is used with U = Aib, Lol = Leuol) and summary of the conformational parameters of HK VI: survey of interresidue NOE connectivities involving, NH, CαH and CβH protons (dαδ connectivities observed for prolines are indicated by hatched lines), of the temperature coefficients of the NH (● low, ●● high, ●●● very high) and of the $^3J_{NHC\alpha H}$ couplings (□ < 6 Hz, ◪ 7 Hz, ■ ⩾ 8 Hz). The observed NOEs are classified as strong, medium and weak (based on counting the cross-peak contour levels) and shown by thick, medium and thin lines, respectively.

showed a regular periodicity as regard to the location of the two proline residues in the sequence, with values around 7 Hz for the residues three before a Pro (Ile3, Leu7) and around 8–9 Hz for the residues two before a Pro (Ile4, Leu8). These values, anomalously high for residues involved in an α- or a 3_{10}-helix, could be interpreted by conformational averaging. However, values around 8 Hz are systematically observed for the two residues flanking an Aib-Pro segment in the case of well characterised α-helical Aib-containing peptides, such as alamethicin[28] and analogues;[12,13,33,34] when replacing Pro for Aib in the sequences, these couplings decrease to 5 Hz.[33] Such a location as regards to Aib-Pro segments is found for Ile4, Leu7 and Leu8 in the HK VI sequence. Restrained molecular dynamics calculations are in progress on one of the longer harzianins HC in order to characterise such a ribbon of β-turns and particularly to visualise the accommodation in the structure of dihedral angles ranging in the φ space around −90° which arise from the $^3J_{NHC\alpha H}$ couplings. Such angles were previously found in the crystallographic analysis of zervamicins,[15,35] a 15-residue peptaibol containing a subtype of the 3_{10}-helix, the β-bend ribbon spiral.[15,35,36]

3 Permeabilisation of liposomes by harzianin HK VI

Long-sequence peptaibols, among which is included the well-known alamethicin,[11] have been shown to promote voltage-gated ion channels (10^{-8} M) and to modify in the absence of voltage the membrane permeability of liposomes to ions or small molecules (10^{-7}–10^{-6} M). Recently, the 14-residue harzianins HC have also been shown to exhibit such membrane properties for concentrations in the range 10^{-6}–10^{-5} M.[16] The barrel-stave model of ion channels generally accepted for peptaibols needs self-assembly of parallel bundles of helices.[1,2] A minimal chain length of 18–20 residues for an α-helix, or 14–16 residues for a 3_{10}-helix is thus required to span the 30 Å thick hydrophobic region of a bilayer. However, the formation of voltage-dependent ion channels at a 10^{-6} M concentration of trichorovin TV-XIIa and trichorozin TZ-III,[21,22] 11-residue peptaibols which only differ from HK VI, either by the location of the isomeric Leu/Ile at positions 3, 4, 7, 8, or by a Leuol/Ileol substitution at the C-terminal part, was recently described, suggesting a different mode of action of these short peptides.

Another approach to study the peptaibol/membrane interaction consists in following the induced leakage of a fluorescent probe previously entrapped in small unilamellar vesicles (SUV) composed of zwitterionic lipids (egg phosphatidylcholine–cholesterol 7:3).[3–5,12,13,16,17,37] This method allows a comparison between the permeabilisation properties of peptaibols with different length and hydrophobicity. The [lipid]/[peptide] ratios allowing 50% leakage in 20 min of the entrapped probe (R_{i50}) are taken as a measure of the peptide efficiency; the values for selected peptaibols are given in Table 3. The long-sequence peptaibols organised in α-helices and exemplified here by the 19-residue trichorzianin TA VII and the 18-residue trichorzin HA V, exhibit the most efficient permeabilisation of liposomes, whereas the *O*-methylated 1–12 N-terminal α-helical fragment of TA VII (NA VII-OMe) is completely devoid of activity, even for a [lipid]/[peptide] ratio of 9:1 showing the requirement of a minimal peptide chain length for the activity.[37] In the case of harzianins HC, the 14-residue peptaibols organised in a multiple β-turn structure, an increase in the bilayer permeability is observed but in a lesser extent than for long-sequence peptaibols;[37] the activity is increased upon increasing the residue hydrophobicity.

The liposome permeabilisation by harzianin HK VI was examined for different R_i^{-1} = [peptide]/[lipid] ratios: 50% leakage of the entrapped material was observed for a lipid to peptide ratio of 142:1 (Table 3). Therefore, and unexpectedly owing to the number of residues, HK VI exhibited noticeable membrane activity of the same extent as the 14-residue harzianins HC. The length and amphipathicity resulting from the proposed HK VI structure thus allowed the peptide to interact with phospholipid bilayers and perturb their organisation.

Experimental

Isolation of harzianin HK VI

The *T. pseudokoningii* strain (MVHC 662), obtained from the Institute of Biochemical Technology and Microbiology (Vienna, Austria), was maintained and cultivated as previously described.[12,36] A typical 20 dm^3 culture disposed between 90 Roux flasks (1 dm^3), each containing 160–170 cm^3 of the usual sterilised synthetic medium was incubated for 11 days at 27 °C. The fermentation broth was separated from the mycelium by filtration over a fritted glass. The mycelium and the culture broth were extracted three times each with methanol and with butan-1-ol, from which the crude extracts were obtained after removing the solvent under reduced pressure (1.0 g from mycelium and 1.7 g from culture broth). The obtained extracts were submitted independently to gel filtration over Sephadex LH 20 with methanol as eluent, yielding 100 mg (from mycelium) and 600 mg (from culture broth) of the same peptide mixture, as shown by further HPLC analyses. Crude peptide fractions were then combined and chromatographed over silica gel (Kieselgel 60 H Merck, Darmstadt) with CH_2Cl_2–MeOH (90:10 to 50:50) as eluent. The PKA group (178 mg) eluted first with CH_2Cl_2–MeOH (80:20) followed by PKB [212 mg; CH_2Cl_2–MeOH (70:30)].

HPLC separations

These were carried out with a Waters liquid chromatograph (600 E Multisolvent Delivery System with gradient controller, 717 plus Autosampler and 486 UV–VIS Tunable Absorbance Detector). A three-step procedure was used: (i) the PKA group was first fractionated into two fractions PKA-1 (56 mg; t_R = 0–30 min) and PKA-2 (90 mg; t_R = 30–60 min) on a C18 Spherisorb ODS2 column (7.5 × 300 mm; AIT France) with MeOH–H_2O (80:20) as eluent; (ii) the PKA-2 fraction was separated into three fractions PKA-2-1 (23 mg; t_R = 0–17 min), PKA-2-2 (33 mg; t_R = 17–34 min), PKA-2-3 (20 mg; t_R = 34–51 min) on a C18 Kromasil column (7.5 × 300 mm; AIT France) with MeOH–H_2O (83:17) as eluent; (iii) a last chromatography using the same conditions as in (ii) allowed the isolation of pure harzianin HK VI (4 mg) from fraction PKA-2-3. The HK VI t_R were: Spherisorb ODS2 7.5 × 300 mm, MeOH–H_2O (80:20) = 50 min; Kromasil C18 7.5 × 300 mm, MeOH–H_2O (83:17) = 36 min; Kromasil C18 4.6 × 250 mm, MeOH–H_2O (82:18) = 43 min.

Table 3 [Lipid]/[peptide] ratios allowing 50% leakage in 20 min of the entrapped CF (R_{i50}) from ePC-Chol (7:3) liposomes ([lipid] = 0.6 mM) for harzianins HK VI as compared to long-sequence peptaibols: trichorzianin TA VII (Ac Aib Ala Ala Aib Iva Gln Aib Aib Aib Ser Leu Aib Pro Val Aib Ile Gln Gln Pheol) and trichorzin HA V (Ac Aib Gly Ala Aib Iva Gln Aib Val Aib Gly Leu Aib Pro Leu Aib Iva Gln Leuol); the *O*-methylated 1-12 N-terminal fragment of TA VII, NA VII-OMe (Ac Aib Ala Ala Aib Iva Gln Aib Aib Aib Ser Leu Aib-OMe); short-sequence peptaibols: harzianins HC III (Ac Aib Asn Leu Aib Pro Ser Val Aib Pro Iva Leu Aib Pro Leuol) HC X (Ac Aib Gln Leu Aib Pro Ala Val Aib Pro Iva Leu Aib Pro Leuol) and HC XV (Ac Aib Gln Leu Aib Pro Ala Ile Aib Pro Iva Leu Aib Pro Leuol)

Number of residues	Peptide	$R_{i50}(\times 10^{-2})$
12	NA VII-OMe	< 0.1
11	HK VI	1.4
14	HC III	0.2
14	HC X	1.1
14	HC XV	2.5
18	HA V	11.1
19	TA VII	11.8

Amino acid analysis

Hydrolysis of HK VI (6 mol dm^{-3} HCl, 110 °C, N_2) was followed by derivatisation of the given amino acids and amino alcohols, as previously described.[25] Classically, the GLC analyses of the *N*-trifluoroacetyl isopropyl ester derivatives were performed with a Girdel 3000 chromatograph on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column (Chrompack, 25 m length, 0.2 mm i.d.), with He (0.7 bar) as carrier gas and a temperature programme: 50–130 °C, 3 °C min^{-1}; 130–190 °C, 10 °C min^{-1}; t_R (separation factor $\alpha_{L/D}$ for the D and L enantiomers): Aib 10.4, L-Asp 29.5 (α = 1.01), L-Ile 20.4 (α = 1.10), L-Leu 24.2 (α = 1.11), L-Leuol 22.2 (α = 0.98). The temperature programme used for separation of the proline D,L-enantiomers was: 50–100 °C, 3 °C min^{-1}; plateau at 100 °C for 10 min; 100–190 °C, 10 °C min^{-1}; t_R (α): L-Pro 25.1 (α = 1.02).

FAB mass spectrometry

Positive ion FAB mass spectra were recorded on a ZAB-2SEQ (VG Analytical, Manchester, UK) mass spectrometer equipped with a standard FAB source and a caesium ion gun operating at 35 kV. The peptide methanolic solution was mixed with 3-nitrobenzyl alcohol or α-thioglycerol as matrices. The resolution was 2000. (+) FAB mass fragmentation of HK VI, *m/z*; relative intensities (RI) are given in parentheses; the first value is obtained with 3-nitrobenzyl alcohol as matrix and the second one with α-thioglycerol: $[M + K]^+$ 1213 (15; 9), $[M + Na]^+$ 1197 (92; 44), fragment ions: 1058 (5; 3), 961 (99; 96), 876 (4; 3), 650 (3; 3), 623 (0; 2), 553 (100; 100), 468 (37; 39), 409 (29; 37), 355 (60; 67), 324 (14; 20), 242 (86; 96), 215 (24; 35), 211 (16; 22) and 128 (32; 36).

NMR spectroscopy

A 0.5 cm^3 amount of 2–15 mM methanolic (CD_3OH) solutions of HK VI in 5 mm Wilmad tubes was used for all NMR experiments (except 1D ^{13}C spectra) which were recorded at 297 K on a Bruker DMX 500 spectrometer equipped with a quadruple-resonance ^1H-^{31}P-^{13}C-^{15}N gradient probehead. Data were collected and processed on a Bruker Aspect Station 1 computer using UXNMR and AURELIA softwares. 1D ^{13}C NMR experiments were acquired at 75.47 MHz on a Bruker AC 300 spectrometer equipped with a ^1H-^{13}C Dual probehead and an Aspect 3000 computer using DISNMR software. ^1H and ^{13}C chemical shifts were referenced to the central component of the quintet due to the CD_2H resonance of methanol at 3.313 ppm, downfield from TMS and to internal CD_3OH taken at 49.00 ppm relative to TMS, respectively. ^{15}N NMR chemical shifts were referenced to external formamide taken at 112.4 ppm downfield from liquid NH_3, according to Srinivasan *et al.*[38]

The HOHAHA and ROESY experiments were performed with spin-lock times of 120 and 250 ms, respectively. For both experiments, 512 free-induction decays (FID) of four scans were recorded, each FID consisting of 2048 time-domain points. To avoid $H\alpha$ proton resonance saturation and NH proton resonance saturation transfer during relaxation and mixing delays, solvent signal was suppressed with a WATERGATE[39] scheme included in the standard HOHAHA[40] and ROESY[41] pulse sequences.

For the ^{13}C HSQC,[42] 512 experiments with 2048 data points and 24 scans each were recorded; the ^{13}C HSQC with TOCSY transfer[43] was recorded with 1024 experiments (2048 data points and 20 scans each). A series of ^{13}C HMBC[44] experiments was acquired with 512 experiments of 1024 data points and 40 scans each and delay times τ set to $1/2\ ^3J_{CH}$ or $1/2\ ^2J_{CH}$, with *J* values ranging over 5–10 Hz (*i.e.* τ values in the range 50–100 ms). For the ^{15}N HSQC experiment, 512 experiments with 2048 data points and 40 scans each were acquired. These ^{13}C and ^{15}N 2D spectra were processed using sine-bell squared functions in F1 and F2 dimensions shifted by 4–2, 10–6, 3–3 and 6–1, respectively.

Liposome permeabilisation

Egg phosphatidylcholine (egg PC) type V E and cholesterol were purchased from Sigma; egg PC was used without further purification and cholesterol was recrystallised from methanol. Carboxyfluorescein (CF) from Eastman Kodak was separated from hydrophobic contaminants and recrystallised from ethanol as described.[5] Fluorescence spectra were measured at 20 °C on an Aminco SPF 500 spectrofluorometer. The peptide-induced release of intravesicular content was monitored by the method introduced by Weinstein,[45] that uses the property of quenching relief upon dilution of an encapsulated fluorescent probe, CF. CF-entrapped small unilamellar vesicles (SUV) were prepared, as described,[3,4] by sonication of an egg PC-cholesterol (7:3) mixture. The obtained SUV were separated from unencapsulated CF by gel filtration (Sephadex G75). Leakage kinetics were obtained for different peptide/lipid molar ratios ([lip] = 0.6 mM) obtained by adding aliquots of methanolic solutions of peptides (methanol concentration kept below 0.5% by volume).

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