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Two Unprecedented Natural Aib-Peptides with the (Xaa-Yaa-Aib-Pro) Motif and an Unusual *C*-Terminus: Structures, Membrane-modifying and Antibacterial Properties of Pseudokonins KL III and KL VI from the Fungus *Trichoderma pseudokoningii*

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Abstract: Pseudokonins KL III and KL VI are two natural ten-residue peptides, which both contain the (Xaa-Yaa-Aib-Pro) motif and exhibit an unusual *C*-terminus. They have been isolated from the fungus *Trichoderma pseudokoningii* by intensive reversed-phase HPLC, beside peptaibols classically C-ended by a β-amino alcohol. The amino acid sequences and the chemical structures of the C-ends have been determined by the combined use of positive ion LSI-MS and two-dimensional homo- and heteronuclear NMR, including COSY, TOCSY, ROESY, ^{13}C heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC). Instead of one of the amino alcohols usually found as *C*-terminal residue in peptaibols, pseudokonins KL III and KL VI are characterized by -Pro-NH_2 and *cyclo*-(Aib-L-Proal) (Proal, prolinal), respectively. Such backbone modifications are described here for the first time for peptaibol antibiotics. The unusual *cyclo*-(Aib-L-Proal) *C*-terminus is probably the result of an intramolecular cyclization of the two last Aib and Pro residues of a ten-amino acid precursor, via a Proal intermediate. A secondary structure stabilized by –C=O···H–N-hydrogen bonds of the $1 \leftarrow 4$ type has been deduced for both peptides from ROESY data, $^3J_{NHC^{\alpha}H}$ couplings and amide proton temperature coefficient values. The (Xaa-Yaa-Aib-Pro) β-bend ribbon spiral, which has been described for the first time in the case of a 14-residue peptaibol containing three repetitive (Xaa-Yaa-Aib-Pro) motifs (Ségalas G *et al. Biopolymers* 1999; **50**: 71–85) appears to be maintained in the two shortened modified peptides. The β-bend ribbon structure thus appears to be initiated by a single (Xaa-Yaa-Aib-Pro) motif and unaffected by the *C*-terminal modifications. However, the membrane and antibiotic properties of pseudokonins KL III and KL VI, point to the unfavourable effect of both shortening and cyclization of the peptide chain. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Aib peptide; helix; mass spectrometry; membrane activity; NMR spectroscopy; structure–activity relationship

INTRODUCTION

Peptaibol antibiotics, including the well-known alamethicin [1], form an important class of linear peptides of fungal origin which are characterized by a high content in $C^{\alpha,\alpha}$-disubstituted glycines (α-aminoisobutyric acid, Aib, and isovaline, Iva), an *N*-terminal acyl (most often acetyl) group and a *C*-terminal β-amino alcohol [1–10]. According to the number and nature of residues, they form three sub-classes, the long-sequence peptaibols with 18–20 residues [1–5], the short-sequence peptaibols

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with 11–16 residues [6–8] and the lipopeptaibols with seven or 11 residues and an *N*-terminal lipid chain [9,10]. Peptaibols exhibit antibiotic properties which are mainly directed against Gram positive bacteria and mycoplasmas, the smallest self-replicating prokaryotes [11]. This characteristic is of particular interest, as several species of mycoplasmas are pathogens and resist to most of the common antibiotics.

Due to the conformational constraints imposed on the peptide backbone by the presence of $C^{\alpha,\alpha}$-dialkylated glycyl residues, Aib-containing peptides generally form helical structures, either α, 3_{10} or mixed $\alpha/3_{10}$ helices, depending on the length of the peptide and on the number and location of Aib residues [12–14]. Helix-like structures have been described in the case of $(Aib\text{-}Pro)_n$ and (Xaa-Yaa-Aib-Pro) segments which give rise to the β-bend ribbon spiral [15,16] and the (Xaa-Yaa-Aib-Pro) β-bend ribbon spiral, respectively [17]. These helical structures are involved in the antimicrobial properties of peptaibols which are related to their ability to interact with biological membranes, modify their permeability and form voltage-dependent transmembrane ion channels [18–21]. This latter property has been suggested to result from the assembly of amphiphilic helical peptide monomers, forming helix bundles that allow the passage of ions through membrane bilayers [20,21].

Peptaibols result from a nonribosomal biosynthesis process involving peptide synthetases as multienzymic templates; the peptide chain termination step usually occurs via the amino alcohol linkage [22]. Of those peptides, a few exceptions to the amino alcohol *C*-terminus concern the 20-residue aibellin, which has a modified *C*-terminal amino alcohol [23], some of the 20-residue trichobrachins [24], which exhibit deletion of the amino alcohol and are thus ended by Gln^{19}, and the 15-residue clonostachin [25], which has a sugar molecule linked to the carboxyl terminus through an ester bond. In addition, several Aib-containing peptides related to peptaibols, such as efrapeptins [26], the aminolipopeptides leucinostatins [27], trichopolyns [28] and helioferins [29], are ended by various unusual cyclic or linear moieties.

We now report on the complete structure determination of two *C*-terminally modified Aib-containing peptides which were isolated from a *Trichoderma pseudokoningii* strain beside a classical short-sequence peptaibol, harzianin HK VI [8]. Pseudokonin KL III has nine amino acids and a prolinamide (-Pro-NH_2) *C*-terminal residue, while pseudokonin

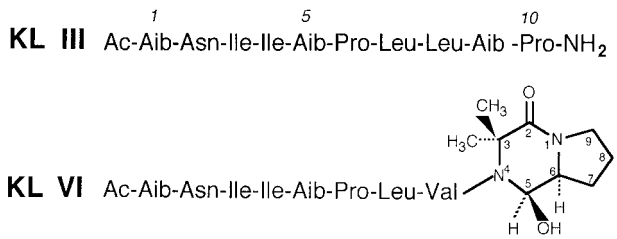

Figure 1 Chemical structures of pseudokonins KL III and KL VI showing *inter alia* the hydroxyketopiperazine ring numbering used here.

KL VI is an eight-residue peptide ended by a hydroxyketopiperazine ring system (Figure 1) [30]. This last backbone modification results from intramolecular cyclization of the two *C*-terminal residues of a ten-amino acid precursor. This original termination of the peptide elongation, instead of the usual amino alcohol linkage, is described here for the first time. In addition, the roles of main-chain shortening and *C*-terminal modification on peptide conformation and the membrane-modifying and antibiotic properties are analysed.

MATERIALS AND METHODS

Production and Isolation of Pseudokonins KL III and KL VI

The *T. pseudokoningii* strain (MVHC 662) obtained from the Institute of Biochemical Technology and Microbiology (Vienna, Austria), was maintained and further cultivated on liquid synthetic medium as previously described [7]. Briefly, a typical 20 l culture in Roux flasks (1 l), incubated for 11 days at 27°C, was separated into mycelium and fermentation broth, which were extracted separately by methanol and 1-butanol, respectively. The extracts (1.0 g from mycelium and 1.7 g from culture broth) were submitted to gel filtration over Sephadex-LH20 with methanol as eluent, leading to 700 mg of crude peptide mixture. This mixture was chromatographed over silica gel (Kieselgel 60 H Merck, Darmstadt) with CH_2Cl_2–MeOH (90:10–50:50), yielding the pseudokonins A (PKA) group (178 mg), which eluted first with CH_2Cl_2–MeOH (80:20), followed by pseudokonins B (PKB) [212 mg, CH_2Cl_2–MeOH (70:30)].

HPLC Purification

This operation was carried out with a Waters liquid chromatograph (model 600E Multisolvent Delivery System with gradient controller, model 717 plus

Autosampler and model 486 UV-Vis Tunable Absorbance Detector). The following three-step procedure was applied: (i) The PKA group was separated 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 ODS-2 column (5 g; 7.5 × 300 mm) with MeOH–H_2O (80:20) as eluent. (ii) The PKA-1 fraction was separated into two sub-fractions PKA-1-1 (9 mg; $t_R = 0–30$ min) and PKA-1-2 (39 mg; $t_R = 17–34$ min) on a C18 Kromasil-5 μm column (7.5 × 300 mm) with MeOH–H_2O (77:23) as eluent. (iii) The PKA-1-2 fraction was separated on a C18 Kromasil-5 μm column (7.5 × 300 mm) with MeOH–H_2O (76:24) as eluent, leading to pseudokonins KL III (3 mg; $t_R = 33$ min) and KL VI (2 mg; $t_R = 45$ min).

The purity of KL III and KL VI was checked by analytical HPLC: Kromasil C18–5 μm, 4.6 × 250 mm, MeOH–H_2O (75:25) 1 ml/min.

Amino Acid Analysis

Total acid hydrolysis of KL III and KL VI in sealed tube (6 *M* HCl, 110°C, Ar) was followed by derivatization of the obtained amino acids, as previously described [7]. The GLC analyses of the *N*-trifluoroacetylated isopropyl ester derivatives were performed with a Hewlett–Packard chromatograph on a Chirasil-L-Val quartz capillary column (25 m length, 0.2 mm i.d.; Chrompack) with He as carrier gas (0.7 bar) and the temperature programme: 50–130°C, 3°C/min; 130–190°C, 10°C/min. Separation of the proline D,L-enantiomers was performed with: 50–100°C, 3°C/min; plateau at 100°C for 10 min; 100–190°C, 10°C/min.

LSI Mass Spectrometry

Positive-ion liquid secondary ion (LSI) MS was performed on a ZAB-2SEQ (VG Analytical) mass spectrometer equipped with a standard FAB source and a caesium ion gun operating at 35 kV. The peptide methanolic solutions were mixed either with 3-nitrobenzyl alcohol or 3-nitrobenzyl alcohol saturated with LiCl as matrix. The resolution was 2000.

NMR Spectroscopy

A total of 0.5 ml of a CD_3OH solution of KL III (3.7 m*M*) or KL VI (5.7 m*M*) in a 5 mm Wilmad tube was used for the NMR experiments, which were conducted at 298 K, either on a Bruker AVANCE 400 spectrometer equipped with an ^1H-^{13}C Dual probehead and a Silicon Graphics O2 computer using XWIN-NMR 2.5 version software, or on a Bruker AVANCE 500 DMX spectrometer equipped with a quadruple resonance ^1H-^{31}P-^{13}C-^{15}N gradient probehead and an ASPECT Station 1 computer using UXNMR and AURELIA softwares. ^1H and ^{13}C chemical shifts were referenced to the central lines of methanol at δ_H 3.313 ppm and δ_C 49.0 ppm, downfield to TMS, respectively. The COSY spectra were obtained with solvent presaturation. The TOCSY and ROESY experiments were recorded with solvent signal suppression by the WATERGATE scheme included in the pulse sequences. For both experiments 512 free-induction decays (FID) of four scans were recorded, each FID consisting of 2048 time-domain points, with spin lock periods and mixing times of 120 and 250 ms, respectively. They were processed using sine bell window functions in the F1 and F2 dimensions, shifted by $\pi/2–\pi/2$. The ^1H-^{13}C HSQC (512 experiments with 2048 data points and 24 scans each) and HMBC (512 experiments with 1024 data points and 40 scans each) spectra were optimized for ^1H-^{13}C coupling constants of 135 and 6, 7, 8 Hz, respectively. They were processed using either sine bell squared functions in the F1 and F2 dimensions shifted by $\pi/4–\pi/2$ (HSQC) or sine bell functions shifted by $\pi/2–\pi/2$ (HMBC). Amide temperature coefficients ($\Delta\delta/\Delta T_{NH}$) were determined from 1D-spectra in the range 296–316 K.

Liposome Permeabilization

Egg phosphatidylcholine type V E (egg-PC) from Sigma was used without further purification; cholesterol (Chol) from Sigma and carboxyfluorescein (CF) from Eastman Kodak were purified as described [18]. CF-entrapped small unilamellar vesicles (SUV) were prepared by sonication of an egg-PC-cholesterol (7:3) mixture, followed by gel filtration of the obtained preparation on Sephadex G75 to remove the unencapsulated CF [7,8,18]. Leakage kinetics were obtained for different peptide/lipid molar ratios ([lip] = 0.6 m*M*) obtained by adding aliquots of methanolic solutions of peptides (methanol concentration kept below 0.5% by volume). Fluorescence spectra ($\lambda_{exc} = 488$ nm; $\lambda_{em} = 520$ nm) were measured at 20°C on an Aminco SPF 500 spectrofluorometer.

Antibiotic Assays

The antibiotic activity was measured against *Staphylococcus aureus* (209P) and *Escherichia coli* (RL65) by the agar diffusion test, using 6 mm diameter pits. A total of 50 μl of solutions of peptide

samples in dimethylsulfoxide (DMSO) were deposited into the pits, giving 500–50 μg/pit. Inhibition zones were measured after 24 h of incubation at 37°C.

Antibiotic activity against *Acholeplasma laidlawii* and *Spiroplasma melliferum* was measured for peptide concentrations in the range 10–100 mmol/l, as described previously [19]. Minimum inhibitory concentrations (MICs) were determined in 96-well microtitration plates by following the colour change of phenol red, resulting from acidification of the culture medium during the cell growth.

RESULTS

Isolation and HPLC Purification of Pseudokonins KL III and KL VI

Cultivation of a *T. pseudokoningii* strain (MVHC 662) on liquid synthetic medium [8], followed by separation through Sephadex LH20 of the crude extracts obtained from the culture broth and mycelium, provided a complex peptide mixture. It was separated by adsorption chromatography into short-sequence peptaibols designated as PKA and 20-residue long-sequence peptaibols, analogues of alamethicin. Pseudokonins KL III and KL VI were isolated from the PKA mixture, via a three-step reversed-phase HPLC procedure involving Spherisorb ODS2 and Kromasil C18 phases (Figure 2), in addition to the previously isolated harzianin HK VI (Ac-Aib[1]-Asn-Ile-Ile-Aib[5]-Pro-Leu-Leu-Aib-Pro[10]-Leuol), an 11-residue peptaibol classically C-ended by leucinol (Leuol) [8]. Pseudokonins KL III and KL VI were shown to be homogeneous by further HPLC analyses, LSI-MS and NMR data.

The acetylated *N*-terminus was identified in both pseudokonins from the absence of reactivity for ninhydrin and the presence of a sharp singlet at ~2 ppm in the ^{1}H NMR spectrum. Composition and chirality of the constitutive amino acids were determined by GC of the total hydrolysates on a Chirasil-L-Val capillary column. None of the amino alcohols usually found in peptaibols was detected in the hydrolysate. Five peaks and six peaks were observed on the chromatograms of KL III and KL VI, respectively, indicating: Aib (3), L-Asx (1), L-Ile (2), L-Leu (2), L-Pro (2) in KL III and Aib (2), L-Asx (1), L-Ile (2), L-Leu (1), L-Pro (1), L-Val (1) in KL VI. No additional peak was noticed in the chromatograms. As the peptides did not give any reactivity to diazomethane, and as the *syn* and *anti* protons of a carboxamide group were observed in the ^{1}H NMR spectra of both pseudokonins, the L-Asx residues were assigned to asparagine in both peptides.

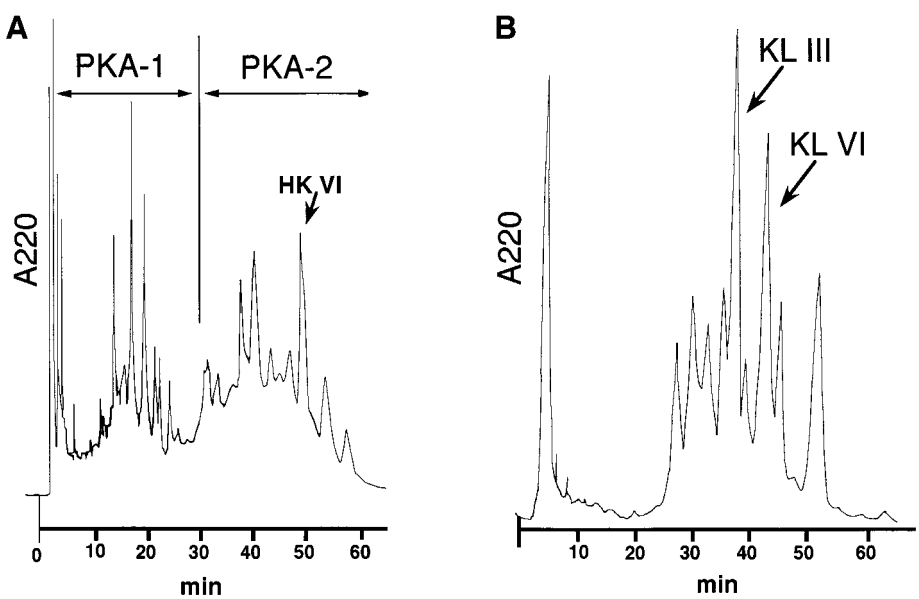

Figure 2 HPLC procedure resulting in the isolation of pseudokonins KL IIII and KL VI from *T. pseudokoningii*. (A) PKA mixture on Spherisorb ODS2–5 μm; 7.5 × 300 mm; MeOH–H_2O (80:20); flow rate 2 ml/min. (B) PKA-1 fraction on Kromasil C18–5 μm; 7.5 × 300 mm; MeOH–H_2O (76:24); flow rate 2 ml/min; absorption monitored at 220 nm.

Structure Analysis of Pseudokonins KL III and KL VI

The main part of peptaibol sequences, containing or not Aib-Pro labile bonds, generally arises from analysis of acylium ion series in the positive ion FAB or LSI mass spectra [31–33]. In addition, lithium cationization results in the formation of cationized $[a_n + Li\text{-}H]^+$ ions which allow easy sequencing together with location of Leu/Ile isomers from $[d_n + Li\text{-}H]^+$ ion formation [34]. The positive ion LSI mass spectra of pseudokonins KL III and KL VI exhibited molecular ion species $[M + K]^+$, $[M + Na]^+$ and MH^+, which allowed us to assign a molecular mass of 1074 to KL III and 1045 to KL VI (Table 1; Figure 3). Occurrence of Aib-Pro bonds in both peptides was demonstrated by the presence in the spectra of the complementary *N*-terminal b_n acylium ions (N_n^+) and diprotonated *C*-terminal y_{m-n} ammonium ions ($[HC_{m-n}, H]^+$), arising from the preferential cleavage at these bonds. This type of fragmentation generally prevails when Aib-Pro tertiary amide links occur in the peptides [31,33,35]. An Aib[5]-Pro[6] bond was thus assigned in both peptides from observation of the N_5^+ ions at *m*/*z* 553 in both spectra and of the $[HC_5, H]^+$ ammonium ions at *m*/*z* 523 and 494, for KL III and KL VI, respectively. The presence of the additional Aib[9]-Pro[10] labile bond in KL III was established from the observation of the N_9^+ and $[HC_1, H]^+$ ions at *m*/*z* 961 and 113, respectively. In the case of KL VI, the Aib[5]-Pro[6] bond cleavage and its subsequent fragmentation were accompanied by a continuous series of sequence specific b_n acylium ions originating from the molecular ion species (Table 1; Figure 3(B) and (D)). Finally, the complete fragmentation patterns (Table 1; Figure 3) allowed the 1–10 and 1–8 sequences to be assigned for KL III and KL VI, respectively. Taking into account the amino acid composition of pseudokonin KL III, which indicated the presence of two Pro residues,

Table 1 Quasimolecular Ion Species and Fragment Ions in the (+) LSI Mass Spectra of Pseudokonins KL III ($C_{52}H_{90}N_{12}O_{12}$) and KL VI ($C_{51}H_{87}N_{11}O_{12}$)

(a) Origin		KL III		KL VI		(b) Origin	KL III		KL VI	
		m/*z*	%	*m*/*z*	%		*m*/*z*	%	*m*/*z*	%
$[M+K]^+$		1113	7	1084	2	$[M+Li]^+$	1081	100	1052	100
$[M+Na]+$		1097	34	1068	14	$[M+2Li]^{++}$	544	6	529.5	8
$[MH]^+$		1075	1	1046	3					
$[M\text{-}Na\text{-}CH_3]^+$				1031	13					
				1029	9					
				948	18					
				183	18					
N_9^+	b_9	961	44			$[a_9+Li\text{-}H]^+$	939	8		
N	b_8	876	6	862	76	$[a_8+Li\text{-}H]^+$	854	10	840	9
						$[d_8+Li\text{-}H]^+$	812	4		
N	b_7	763	1	763	5	$[a_7+Li\text{-}H]^+$	741	8	741	7
						$[d_7+Li\text{-}H]^+$	699	10	699	9
N	b_6	650	3	650	5	$[a_6+Li\text{-}H]^+$	628	3	628	4
N_5^+	b_5	553	82	553	100	$[a_5+Li\text{-}H]$	531	15	531	12
N	b_4	468	33	468	36	$[a_4+Li\text{-}H]^+$	446	18	446	16
						$[d_4+Li\text{-}H]^+$	418	10	418	7
N	b_3	355	63	355	51	$[a_3+Li\text{-}H]^+$	333	12	333	12
N	b_2	242	100	242	66	$[a_2+Li\text{-}H]^+$	220	11	220	12
N	b_1	128	38	128	37	$[a_1+Li\text{-}H]^+$	106	15	106	13
$[HC_5, H]^+$	y_5	523	2	494	4	$[y_5+Li\text{-}3H]^+$	527	12	498	8
C		409	17	395	7					
C		324	11	310	16					
C		211	17	211	15					
$[HC_1, H]^+$	y_1	113	6							

[a] (a) 3-nitrobenzyl alcohol; (b) 3-nitrobenzyl alcohol saturated with lithium chloride; the origin of ions from N^+ or $[HC, H]^+$ ions is indicated by *N* or *C*, respectively.

and the 113 mass unit of the *C*-terminal residue, a -Pro-NH_2 *C*-terminus could be assigned. However, the 183 a.m.u. *C*-terminal end of KL VI, which did not correspond to any known amino alcohol among the peptaibols, remained undetermined. Also unknown was the respective location of the Leu and Ile residues in both sequences.

The LSIM spectra of KL III and KL VI, performed under cationization by lithium ions, both exhibited abundant $[M + Li]^+$ adduct ions, accompanied by doubly charged $[M + 2Li]^{++}$ and by series of $[a_n + Li\text{-}H]^+$ fragment ions (Table 1) which confirmed the sequences previously proposed. The location of one Ile at position 4 in both KL III and KL VI

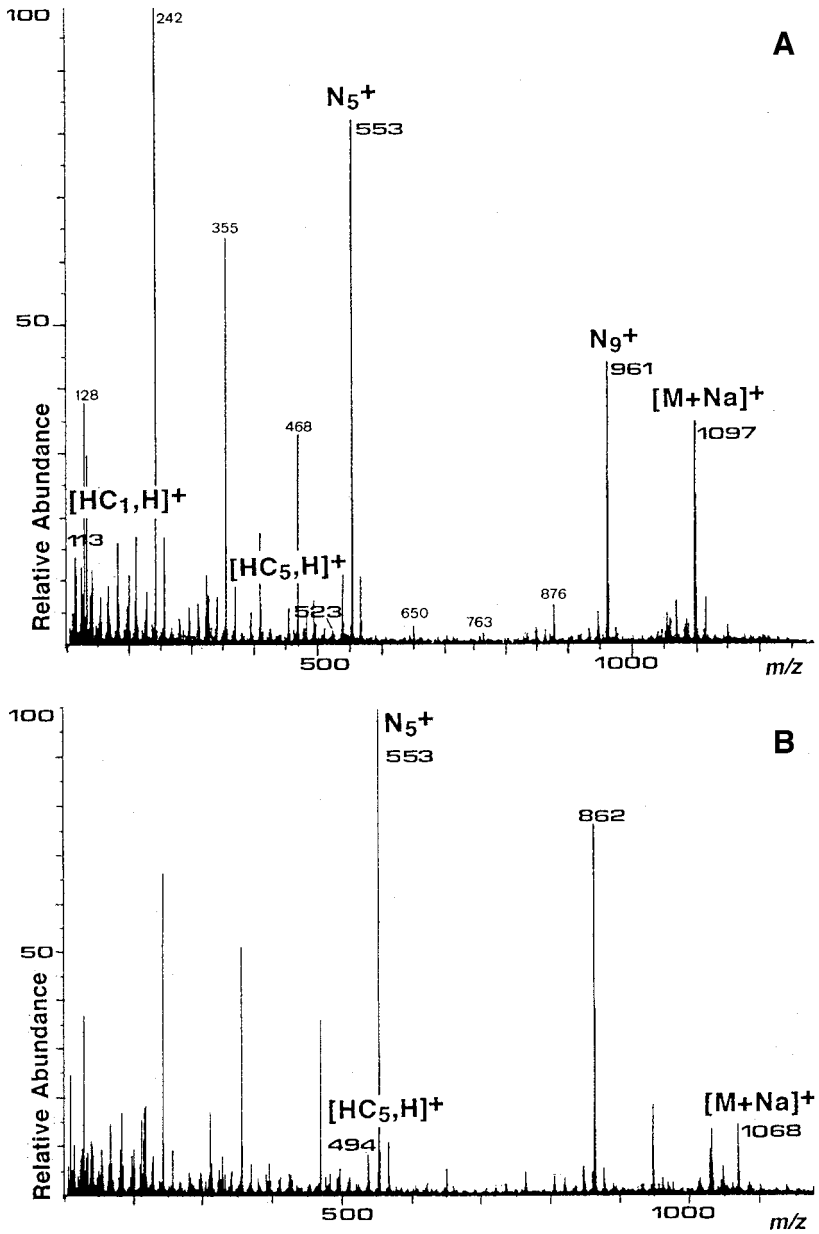

Figure 3 Positive ion LSI mass spectra of pseudokonins KL III (A) and KL VI (B) (3-nitrobenzyl alcohol as matrix) and mass fragmentation patterns of KL III (C) and KL VI (D).

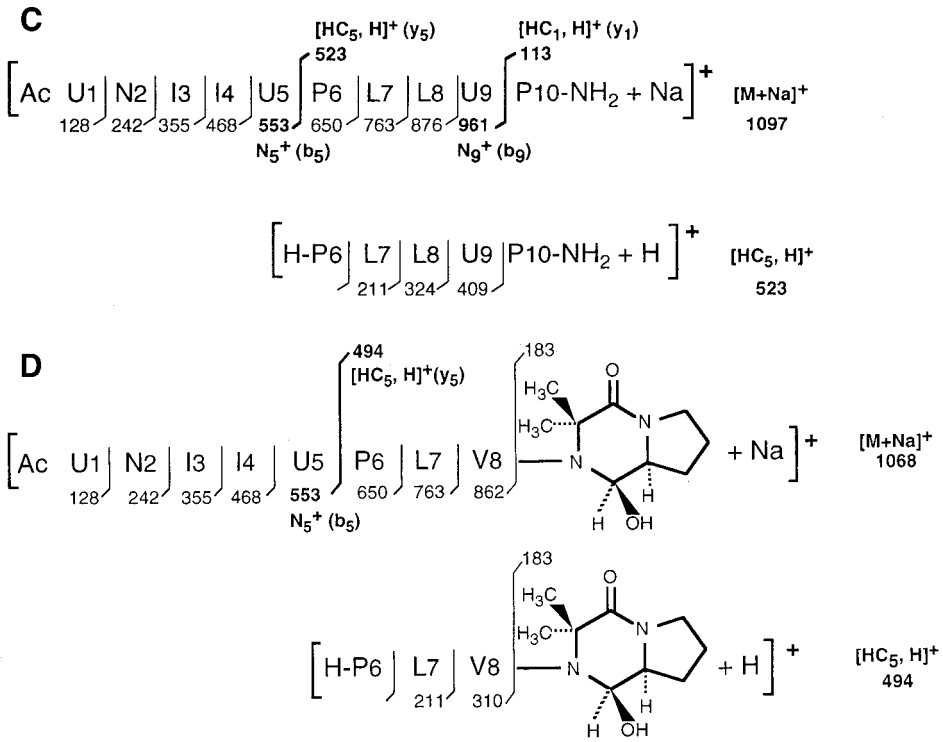

Figure 3 (*Continued*)

resulted from the characteristic mass difference of 28 a.m.u. between the $[a_4 + Li\text{-}H]^+$ ions and the corresponding $[d_4 + Li\text{-}H]^+$ ions, which arose from the loss of a radical from the lateral chain (Table 1) [34]. Similarly, the Leu residues were located at positions 7 and 8 in KL III and at position 7 in KL VI from the 42 a.m.u. mass differences observed between the corresponding $[a_n + Li\text{-}H]^+$ and $[d_n + Li\text{-}H]^+$ ions (Table 1). The amino acid sequences of both pseudokonins were thus completely determined (Figure 1). However, the structure of the KL VI *C*-terminus (*m*/*z* 183) remained unsolved.

A NMR study of pseudokonins KL III and KL VI was thus undertaken to complete the KL VI structure and to obtain conformational parameters for the two peptides. By the use of homo- and heteronuclear 2D NMR techniques including COSY, TOCSY, ROESY, ^{13}C HSQC and HMBC, full sequential and stereospecific assignments of the ^1H and ^{13}C chemical shifts of KL III were achieved (Tables 2–4). In particular, the 1–9 sequence of KL III and its unusual -Pro-NH_2 *C*-terminus, previously deduced from the MS data, were confirmed by this technique. Actually, the -Pro-NH_2 residue was clearly identified from the ROE correlations between the $\delta\delta'$-protons belonging to the Pro[10] spin system and the *syn* and *anti* carboxamide protons characterized by broad singlets at 6.9 and 7.5 ppm, respectively, in addition to those belonging to the Asn[2] residue at 7.0 and 7.7 ppm. Two *trans* Aib[5]-Pro[6] and Aib[9]-Pro[10] peptide bonds were characterized in KL III from the chemical shifts of the Pro[7]-carbons observed at about 27 ppm [36].

Detailed analysis of the two-dimensional ^1H-^1H COSY, TOCSY, ROESY, and ^1H-^{13}C HSQC and HMBC spectra enabled us to perform a complete assignment of the ^1H and ^{13}C spin systems of the eight amino acids involved in the KL VI sequence, including the stereospecific assignments of the β-methyl groups of the Aib residues. Sequential dNN(*i*, $i+1$) and dαN(*i*, $i+1$) inter-residue dipolar couplings in the ROESY spectrum led to the sequential assignments of KL VI from Aib[1] to Val[8].

Complete ^1H assignments of the 1–8 residues being thus achieved (Table 3), a remaining signal was observed at 5.8 ppm, which gave rise to a set of connectivities in the TOCSY spectrum (Figure 4), defining a modified proline, in agreement with the ^{13}C chemical shifts (Table 4). The ten carbonyl signals of the ^{13}C 1D spectrum were assigned from the ^2J and ^3J connectivities they exhibited to the NH and Hα protons in the HMBC experiment optimized

Table 2 ^{1}H Sequential and Stereospecific Assignments of Pseudokonin KL III (500.13 MHz, CD_3OH, 298 K)[a]

Residue	NH	αH	βH/βMe	γH/γMe	δH/δMe and other groups
Ac					2.02
U[1]	8.717		*pro-S* 1.45 s/*pro-R* 1.52		
N[2]	8.544	4.40	2.76		δ *syn* 7.031; δ *anti* 7.739
I[3]	7.914	4.03	2.08	1.58/1.30 γMe 0.97	δMe 0.93
I[4]	7.362	4.15	1.98	1.55/1.35 γMe 0.95	δMe 0.86
U[5]	8.031		*pro-S* 1.55 s/*pro-R* 1.46		
P[6]		4.32	*pro-S* 2.34/*pro-R* 1.76	*pro-S* 2.06/*pro-R* 1.95	*pro-R* 3.80/*pro-S* 3.54
L[7]	7.771	4.17	2.02/1.57	1.86	δMe 0.999/0.91
L[8]	7.386	4.45	1.70/1.70	1.71	δMe 0.87/0.84
U[9]	7.721		*pro-S* 1.48/*pro-R* 1.46		
P[10]-NH_2		4.39	*pro-S* 2.26/*pro-R* 1.79	*pro-S* 1.98/*pro-R* 1.85	*pro-R* 3.85/*pro-S* 3.35 *syn* 6.932; *anti* 7.465

[a] Chemical shifts (δ, ppm) are given to the nearest three decimals or two decimals when obtained from 1D or 2D spectra, respectively; U, Aib; P-NH_2, Pro-NH_2.

Table 3 ^{1}H Sequential and Stereospecific Assignments of Pseudokonin KL VI (500.13 MHz, CD_3OH/CD_3OD, 298 K)[a]

Residue	NH	αH	βH/βMe	γH/γMe	δH/δMe and other groups
Ac					2.032
U[1]	8.685		*pro-S* 1.466/*pro-R* 1.454		
N[2]	8.523	4.386 t (5.8)[b]	2.764 d (5.8)		δ *syn* 7.027; δ *anti* 7.743 s
I[3]	7.906	4.010 d (8.4)[b]	2.06	1.59/1.30 γMe 0.960 d (6.9)	δMe 0.919 t (7.4)
I[4]	7.300	4.168 d (8.1)[b]	1.97	1.54/1.32 γMe 0.931 d (7.1)	δMe 0.845 t (7.5)
U[5]	7.886		*pro-S* 1.512/*pro-R* 1.512		
P[6]		4.367[b]	*pro-S* 2.30/*pro-R* 1.71	*pro-S* 1.98/*pro-R* 1.90	*pro-R* 3.79/*pro-S* 3.40
L[7]	8.203	4.228[b]	2.03/1.65	1.80	δMe 1.002 d (6.5)/0.893 d (6.5)
V[8]	7.904	4.572 d (9.5)[b]	2.31	γMe 0.938 d (6.6)/ γMe 0.950 d (6.9)	
Ring (U[9])			*pro-S* 1.674/*pro-R* 1.705		
Ring (Proal[10])		3.80 (H6)	*pro-S* 2.15/*pro-R* 2.05 (H_7, $H_{7'}$) 5.797 d (1.6) (H_5)	*pro-S* 1.99/*pro-R* 1.85 (H_8, H'_8)	*pro-R* 3.46/*pro-S* 3.52 (H_9, H'_9)

[a] Chemical shifts (δ, 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 spin systems arise, when given, from the 1D spectra.
[b] $^3J_{\alpha,\beta}$ couplings determined for CD_3OD solution (U, Aib; Proal, prolinal).

for a 6 Hz coupling. The carbonyl group at 174.7 ppm appeared to be involved in the *C*-terminal ring system from its ^3J HMBC connectivities to both the Aib[9] β-methyl groups and the Proal[10] β-protons. Assignments of the six Aib β-methyl groups resulted from the ^2J and ^3J HMBC connectivities to their respective Cα and CO carbons. The *cis* configuration of the Aib[9]-Proal[10] peptide bond in the keto-piperazine ring was in agreement with its γ-carbon chemical shift (C_8 at 23.6 ppm), while a *trans* Aib[5]-Pro[6] peptide bond was assigned (Pro[6] γC at 27.0 ppm). An octapeptide (Ac-Aib[1]-Asn-Ile-Ile-Aib-

Table 4 ^{13}C NMR Chemical Shifts (ppm) for Pseudokonins KL III and KL VI (CD_3OH, 298 K)

Residue	CO	αC	βC	γC	δC
KL III					
Ac	173.8	22.9			
U[1]	177.9	57.5	26.6 *pro-S* 23.8 *pro-R*		
N[2]	174.1	54.0	35.9	174.8	
I[3]	174.6	61.4	36.5	26.5; 15.6	10.9
I[4]	174.5	60.2	37.1	26.4; 15.9	10.4
U[5]	175.0	58.0	23.2 *pro-S* 26.4 *pro-R*		
P[6]	176.0	65.0	29.8	26.9	50.4
L[7]	176.1	55.1	40.1	25.7	23.5; 20.8
L[8]	174.4	53.1	41.0	25.4	23.2; 20.4
U[9]	174.7	58.0	24.2 *pro-S* 25.7 *pro-R*		
P[10]-NH_2	176.7	63.8	30.0	26.6	50.1
KL VI					
Ac	173.7	23.0			
U[1]	177.8	57.5	27.2 *pro-S* 24.2 *pro-R*		
N[2]	174.1	54.0	36.0	175.0	
I[3]	174.5	61.3	36.8	26.8; 16.0	11.1
I[4]	173.7	59.9	37.4	27.6; 16.2	10.7
U[5]	174.8	58.1	23.8 *pro-S* 26.8 *pro-R*		
P[6]	175.7	64.4	30.8	27.0	50.4
L[7]	175.1	54.0	40.5	26.1	24.0; 20.8
V[8]	171.1	57.8	31.2	20.0; 18.9	
Ring (U[9])	174.7 (C_2)	62.4 (C_3)	23.2 *pro-S* 26.0 *pro-R*		
Ring (Proal[10])		61.8 (C_6)	29.4 (C_7) 76.4 (C_5)	23.6 (C_8)	48.1 (C_9)

U, Aib; P-NH_2, Pro-NH_2; Proal, prolinal.

Pro-Leu-Val[8]) ended by a *cyclo*-(Aib[9]-L-Proal[10]) *C*-terminus was thus defined.

Finally, the stereospecific assignments of the Aib β-methyl groups and of the proline β-, γ- and δ-protons (Table 3) were achieved from ROESY networks together with the relative configuration of the piperazine ring. The H_5 proton at 5.8 ppm exhibited strong ROEs with both the α-proton of Val[8] and H_6 (Proal[10] α-proton), medium-size ROEs with H_7 and $H_{7'}$ (Proal[10] β,β'-protons) and a weak ROE with the β-proton of Val[8] (Figure 5). Thus, H_5 and H_6 were on the same side of the piperazine ring, as confirmed by the very small $^3J_{H5,H6}$ coupling constant (1.6 Hz). Assuming the highly probable *S*-configuration of the Proal[10] α-carbon, as usually found for the prolines, the other C^{α}-monoalkylated amino acids and the amino alcohols in peptaibols [1–10], the absolute configuration shown in Figure 1, was proposed for pseudokonin KL VI.

Solution Conformational Analysis of Pseudokonins KL III and KL VI

A β-bend ribbon structure stabilized by intramolecular hydrogen bonds of the $1 \leftarrow 4$ type and exhibiting specific ϕ and ψ angles related to the presence of the repetitive (Xaa-Yaa-Aib-Pro) motif has been previously described for the 14-residue peptaibol harzianin HC IX [16]. The other harzianins HC have been shown to adopt a similar structure, very close to that of the related 11-residue harzianin HK VI [8]. In order to examine the possible conformational modifications induced by both shortening the

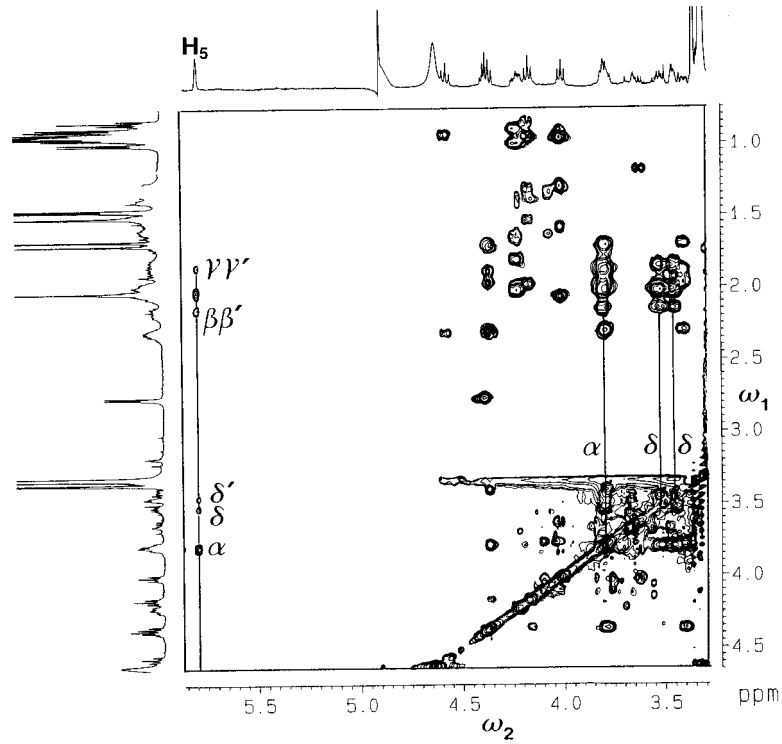

Figure 4 Part of the TOCSY spectrum of KL VI (500.13 MHz, 298 K, CD_3OH, spin lock period 120 ms; $\omega_2 = 5.8–3.3$ ppm; $\omega_1 = 4.6–0.5$ ppm) showing the characteristic coupling pattern of the *C*-terminal hydroxyketopiperazine ring.

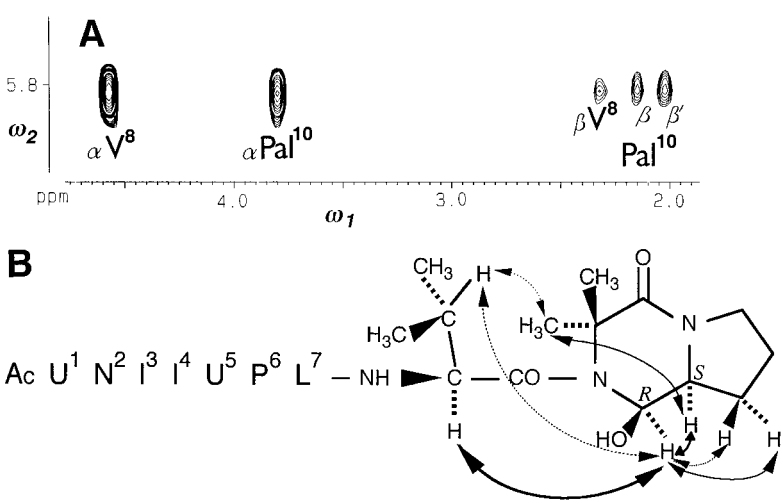

Figure 5 (A) Part of the ROESY spectrum of KL VI (500.13 MHz, 298 K, CD_3OH, mixing time 300 ms; $\omega_2 = 5.78–5.82$ ppm; $\omega_1 = 4.75–2.00$ ppm) and (B) summary of the ROEs characterizing the *C*-terminal hydroxyketopiperazine ring of KL VI; ◄—►, strong; ◄ – ►, medium; ◄ · · · · ►, weak.

peptide chain and the presence of *C*-terminal extremity differing from the usual amino alcohols, NMR conformational parameters were collected in methanol solution for pseudokonins KL III and KL VI (Table 5; Figure 6). As generally observed for peptaibols in methanol solution, no aggregation occurred with pseudokonins, as shown from the absence of significant evolution of the ^1H chemical shifts of the NH

Table 5 $^3J_{NHC^{\alpha}H}$ Coupling Constants (Hz, 298 K) and Temperature Coefficients of Amide Protons ($-\Delta\delta/\Delta T$, ppb/K) for Pseudokonins KL III and KL VI (500.13 MHz, CD_3OH)

Residue	$^3J_{NHC^{\alpha}H}$		$-\Delta\delta/\Delta T$	
	KL III	KL VI	KL III	KL VI
U^1			8.1	8.3
N^2	5.4	5.7	5.2	5.2
δ *syn*			5.9	6.8
δ *anti*			7.1	7.1
I^3	7.5	7.6[a]	1.9	2.6
I^4	8.2	9.0	2.4	2.2
U^5			4.9	4.9
P^6				
L^7	7.4	7.6	1.1	3.1
V^8/L^8	9.7	9.1[a]	1.2	2.1
U^9			2.3	
P^{10}-NH_2				
γ *syn*			7.1	
γ *anti*			7.1	

[a] Measured at 316 K; U, Aib; P-NH_2, Pro-NH_2.

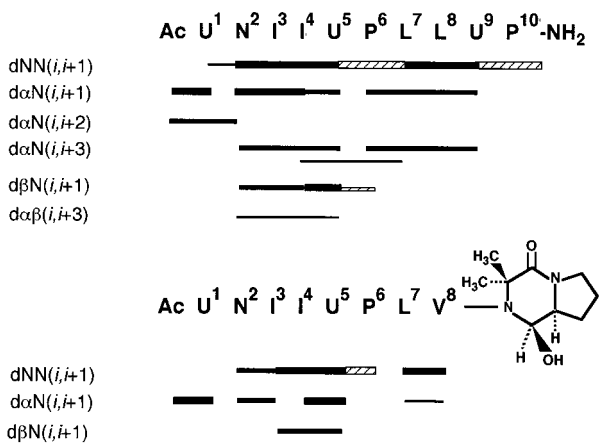

Figure 6 Amino acid sequences (the one-letter code for amino acids is used with U = Aib) and summary of the conformational parameters of KL III and KL VI: survey of interresidue ROE connectivities involving, NH, $C^{\alpha}H$ and $C^{\beta}H$ protons ($d\alpha\delta$ connectivities observed for prolines are indicated by hatched lines). The observed ROEs are classified as strong, medium and weak (based on counting the cross-peak contour levels) and shown by thick, medium and thin lines, respectively.

protons (upfield shift of 0.003–0.01 ppm), and α and β-protons (upfield shift of 0.001–0.003 ppm) in the 0.02–5 m*M* concentration range. The chemical shift values, temperature coefficients of the NH resonances, $^3J_{NHC^{\alpha}H}$ values and ROE scheme were thus analysed and compared with those of harzianins HC IX [17] and HK VI [8].

The pattern of through-space connectivities exhibited by pseudokonin KL III (Figure 6), a succession of strong dNN(i, $i+1$) and lower-range dαN(i, $i+$l) throughout the sequence, accompanied by dβN(i, $i+1$), d$\alpha\beta$(i, $i+3$) and dαN(i, $i+3$), consistent with a helical structure, was very close to those obtained for harzianins HC IX and HK VI [7,8,17]. Such a ROE pattern is usually found for Aib-peptides, as the presence of a number of Aib residues constrains the peptide backbone to adopt conformations in the 3_{10}-/α-helical region of the ϕ, ψ space [12–14]. The absence of dαN(i, $i+4$) in any part of the sequence, accompanied by the presence of dαN(i, $i+2$) and d$\alpha\delta$(i, $i+2$) nOe cross-peaks, argued for a helix stabilized by $1\leftarrow4$ hydrogen bonds. Amide proton temperature coefficients also agreed with this hypothesis. The $^3J_{NHC^{\alpha}H}$ coupling constants (Table 5) exhibited lower values (≤ 7.5 Hz), consistent with a helix, for Asn^2, Ile^3 and Leu^7, whereas Ile^4 and Leu^8 showed higher values (around 9 Hz). Such high couplings, which are apparently inconsistent with helical structures, are frequently observed for the amino acids flanking Aib-Pro segments in α-helical peptaibols [4,8,37]. The same periodicity of lower and higher values, as regard to the location of the Pro residues has been observed previously for related peptides [7,8,17].

Fewer ROEs were detected in the case of pseudokonin KL VI. However, the pattern was very similar to those of pseudokonin KL III and harzianin HK VI, as well as the thermal coefficients of amide protons and $^3J_{NHC^{\alpha}H}$ coupling constants. Long-range ROE cross-peaks were not observed between the ketopiperazine ring and the peptide moiety, in addition to those noticed to the α- and β-protons of Val^8. The whole data indicated that pseudokonins KL III, KL VI and harzianins HK VI [8] adopted a very similar structure, which appears very close to that described for harzianin HC IX [17]. This structure consists in a ribbon of successive overlapping β-turns twisted into a continuous spiral. This short helix-like structure should result in a slight amphipathic character, with the hydrophobic bulky lateral chains of Leu and Ile lying on the same face of the spiral.

Membrane and Antibiotic Properties of Pseudokonins KL III and KL VI

The membrane-modifying, antibacterial and antimycoplasmic properties of pseudokonins KL III

Table 6 Liposome Permeabilization and Antimycoplasmic Properties of Pseudokonins KL III and KL VI, as Compared to Peptides Taken as References [37]

Peptide	Number of residues	R_{i50} [a]	Peptide concentration	MIC (μ*M*)	
		($\times 10^{-2}$)	(μ*M*)	*Acholeplasma laidlawii*	*Spiroplasma melliferum*
KL VI	8	0.03	162.0	R	R
KL III	10	0.25	24.0	R	R
HK VI [8]	11	1.42	4.2	12.5	25.0
HC IX [7]	14	0.25	0.8	12.5	50.0
PA VI [35]	18	12.20	0.5	3.1	12.5
TA VII [33]	19	11.80	0.3	1.5	25.0
SA IV [42]	20	10.0	0.4	1.5	6.2

[a] [Lipid]/[peptide] ratios allowing 50% leakage in 20 min of the entrapped CF from ePC-Chol (7:3) liposomes ([lipid] = 0.6 m*M*) for pseudokonins KL III and KL VI as compared to: short-sequence peptaibols: harzianins HK VI (Ac-Aib^{1}-Asn-Ile-Ile-Aib-Pro-Leu-Leu-Aib-Pro^{10}-Leuol); harzianin HC IX (Ac-Aib^{1}-Asn-Leu-Aib-Pro-Ala-Ile-Aib-Pro-Iva^{10}-Leu-Aib-Pro-Leuol); and-long-sequence peptaibols: trichorzin PA VI (Ac-Aib^{1}-Ser-Ala-Aib-Iva-Gln-Aib-Val-Aib-Gly^{10}-Leu-Aib-Pro-Leu-Aib-Aib-Gln-Pheol); trichorzianin TA VII (Ac-Aib^{1}-Ala-Ala-Aib-Iva-Gln-Aib-Aib-Aib-Ser^{10}-Leu-Aib-Pro-Val-Aib-Ile-Gln-Gln-Pheol) and saturnisporin SA IV (Ac-Aib^{1}-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib^{10}-Gly-Aib-Aib-Pro-Val-Aib-Iva-Gln-Gln-Pheol^{20}). R, resistant to 100 μ*M* peptide concentration.

and KL VI were compared to those previously described for longer, related Aib-containing peptides (Table 6).

To probe the liposome permeabilization property, different peptide concentrations between 3 and 100 μ*M* were added to small unilamellar vesicles composed of phosphatidylcholine/cholesterol, 7:3, loaded with carboxyfluorescein (CF). This fluorescent probe was previously entrapped in the liposomes at a self-quenched concentration, according to the method described by Weinstein *et al.* [38]. The [lipid]/[peptide] ratios, allowing 50% leakage in 20 min of the entrapped probe (R_{i50}), were taken as a measure of the peptide efficiency and compared to those of peptaibols taken as references (Table 6). The results indicated almost complete loss of membrane-modifying capacity for the *C*-terminally modified KL VI peptide, containing the ketopiperazine ring, while the activity was only slightly decreased for KL III, as compared to that of harzianin HK VI [8,37].

The two pseudokonins were tested for their activity against *S. aureus*, *E. coli* and mycoplasma cells (*A. laidlawii* and *S. melliferum*). Pseudokonins KL III and KL VI were inactive on all the examined target cells. Under the same conditions harzianin HK VI was also inactive on the Gram positive and Gram negative bacteria, but still exhibited a moderate activity on the mycoplasmas, in the same range as that exerted by harzianin HC IX.

DISCUSSION

We have isolated two new Aib-containing peptides from the filamentous fungus *T. pseudokoningii*, which have short eight- and nine-amino acid sequences and are ended by unprecedented *C*-termini among the peptaibol antibiotics. Pseudokonin KL III exhibits a *C*-terminal -Pro-NH_2, while pseudokonin KL VI bears a hydroxyketopiperazine ring. The fungus biosynthesizes these two peptides together with long-sequence 20-residue peptaibols, analogues of alamethicin, and a complex mixture of short-sequence peptaibols from which we have previously isolated the 11-residue harzianin HK VI [8]. This is the first time that the production of *C*-terminally modified peptaibols by a fungal strain is described together with that of peptaibols classically ended by an amino alcohol. Peptaibols are assembled on non-ribosomal large protein templates, termed peptide synthetases [22,39]. The identity and sequence of amino acids in the peptides arising from this polyenzymic pathway are dictated by the organization of sets of iterated modules in the peptide synthetase, each module activating a specific amino acid. The low substrate specificity of these systems is responsible for the microheterogeneity of the resulting peptide mixtures. In the case of peptaibols, the termination of the peptide chain is generally achieved by attachment of an amino alcohol, by a mechanism which is still poorly understood. The concomitant presence of harzianin HK VI and of

pseudokonins KL III and KL VI in the natural peptide mixtures produced by *T. pseudokoningii* and the structures of these new *C*-terminally modified peptaibols show that other processes than the linkage of an amino alcohol may take place on the same peptide synthetase for the termination of peptide chain elongation. The three peptides might thus result from a ten-residue precursor peptide [Ac-Aib-Asn-Ile-Ile-Aib^{5}-Pro-Leu-Leu(Val)-Aib-Pro^{10}-OH] that could undergo different peptide chain terminations, either by amidation of Pro^{10} in KL III or by cyclization of Aib^{9} and Pro^{10} into the hydroxyketopiperazine ring in KL VI. This latter *C*-terminus might be formed presumably in two steps, starting from the precursor: (i) reduction of the carboxylic acid function of the *C*-terminal proline to an aldehyde (Proal^{10}); and (ii) intramolecular cyclization of the Proal^{10}-containing intermediate to a hemiaminal function, leading to the *cyclo*-(Aib^{9}-Proal^{10}) *C*-terminus. This cyclization thus results in the unprecedented termination of the peptaibol chain.

The (Xaa-Yaa-Aib-Pro)-β-bend ribbon spiral structure, which is characterized by four sets of specific ϕ, ψ torsion angles, has been described for the first time in the case of a 14-residue peptide containing three repetitive (Xaa-Yaa-Aib-Pro) motifs [17]. This unusual structure appears to be maintained in harzianin HK VI [8] and in pseudokonin KL III, which contain this motif only twice, and even in pseudokonin KL VI, where a single (Xaa-Yaa-Aib-Pro) motif occurs. The factors governing the α-over 3_{10}-helical preference of peptides containing multiple $C^{\alpha,\alpha}$-dialkylated amino acids have been extensively analysed [12–14]. In peptides of at least eight residues, the α-helix is preferred if the Aib content does not exceed 50%. In addition, there is no critical chain length dependence for 3_{10}-helix formation. The β-bend ribbon spiral, which occurs in the case of (Aib-Pro)$_n$ sequences and is considered a subtype of the 3_{10}-helix, has been shown to be formed even at the (Aib-Pro-Aib) tripeptide level [15,40]. The present data show that a single (Xaa-Yaa-Aib-Pro) motif is able to fold an eight-residue peptide containing two Aib residues into a β-bend ribbon spiral structure.

Long-sequence and short-sequence peptaibols have been shown previously to exert antibiotic activity against Gram positive bacteria and particularly against mycoplasma cells, as a consequence of their membrane-modifying properties [37]. Assuming a very similar conformation for harzianins HC IX, HK VI and pseudokonins KL III, KL VI, a comparison of their membrane-modifying and antibiotic properties was undertaken (Table 6). Of the membrane-active peptaibols, the long-sequence 20- to 18-residue peptides organized in α-helices are the most potent. A maximal hydrophobicity and an amphipathic character of the peptide helices are required for a high efficiency. As regards the peptides with a low number of residues, we observed that the 11-residue harzianin HK VI exhibits a rather strong membrane activity, to the same extent as several 14-residue harzianins HC [8,37]. Here, we showed that the length and hydrophobicity of the ten-residue KL III still permit the peptide to interact with phospholipid bilayers and to perturb their organization, although the resulting membrane-activity is decreased as compared to that of HK VI. By contrast, this property is suppressed when cyclization of the two last residues at positions 9 and 10 into a ketopiperazine ring occurs.

As a number of diketopiperazine compounds have proved to be potent antimicrobial agents [41], the *C*-terminal hydroxyketopiperazine ring of pseudokonin KL VI could be expected to result in an increase of the antimicrobial activity of this short peptide, as compared to that of harzianin HK VI or pseudokonin KL III. The above results show the loss of antibiotic activity against *S. aureus*, *S. melliferum* and *A. laidlawii* for both KL III and KL VI. In conclusion, a minimal 11-residue length of the structured peptide chain appears to be required for the antimycoplasmic activity, while a longer chain is required for the activity on *S. aureus*. Thus, the modifications in the usual biosynthetic pathway of peptaibol antibiotics which are here described, such as shortening and cyclization of the peptide chain, appear to result in strong decrease or loss of the membrane-perturbing and antimycoplasmic properties usually exhibited by such natural Aib peptides.

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