Structural Elucidation of Trikoningins KA and KB, Peptaibols from *Trichoderma koningii*

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A *Trichoderma koningii* strain collected in Uruguay produced two antibiotic membrane-active peptidic groups, the trikoningins KA and KB, from which the main components KA V, KB I and KB II were purified. The peptide sequences were elucidated by FAB mass spectrometry and high-field NMR experiments. KA V was a nonadecapeptaibol, whereas KB I and KB II were two 11-residue lipopeptaibols. They all displayed antibiotic activity against *Staphylococcus aureus*. Their membrane-modifying properties were examined by following the leakage of liposome-entrapped carboxyfluorescein. KA V induced similar permeability modifications to those exhibited by known 19-residue peptaibols, while the KB I and KB II activities were weaker.

The widespread soil fungi *Trichoderma* proved to be a source of metabolites that exhibit antibiotic properties. Among them a special class of hydrophobic peptides, termed as peptaibols[1–6] is notable. Such peptides which interact with biological membranes,[7,8] contain a high proportion of α-aminoisobutyric acid (Aib) and a *C*-terminal amino alcohol.[9–12]

An original strain of *Trichoderma koningii* Oudem. collected in Uruguay and selected for its antagonistic properties against other fungi, was shown to produce original peptaibols. The three main peptides of the natural mixture were isolated. Examination of their sequence revealed the presence of a 19-residue peptaibol, trikoningin KA V, and two 11-residue lipopeptaibols, the trikoningins KB I and KB II. Their antibiotic activity against Gram-positive and Gram-negative bacteria and their membrane-modifying properties were compared to those exhibited by other peptaibols.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
KA V:	Ac	Aib	Gly	Ala	Aib	Ile	Gln	Aib	Aib	Aib	Ser	Leu	Aib	Pro	Val	Aib	Ile	Gln	Gln	Leuol
KB I:	Oc	Aib	Gly	Val	Aib	Gly	Gly	Val	Aib	Gly	Ile	Leuol								
KB II:	Oc	Iva	Gly	Val	Aib	Gly	Gly	Val	Aib	Gly	Ile	Leuol								

Results and Discussion

Isolation of Trikoningins KA V, KB I and KB II.—The crude trikoningins were separated by silica gel chromatography into trikoningins KA (38%) and trikoningins KB (62%). Investigated by TLC, KA and KB groups appeared homogeneous, but the C_{18} reversed-phase HPLC analyses revealed the presence of several compounds. Nevertheless, the peptidic composition of the mixtures was not so complex as usually observed for peptaibols.[12–15] HPLC analysis of the KA group showed a major component KA V (Fig. 1a), while KB mixture revealed mainly two peptides KB I and KB II (Fig. 1b). Each peptide was isolated by repetitive semi-preparative HPLC and proved to be free of sequence analogues by further FAB-MS and NMR analyses.

Characterisation of Trikoningins KA V, KB I and KB II.—The amino acid compositions were established from derivatised residue GC analyses of the complete acid hydrolysates. They indicated the presence of 1 Ala, 7 Aib, 3 Glx, 1 Gly, 2 Ile, 1 Leu, 1 Leuol, 1 Pro, 1 Ser and 1 Val for KA V, and 4 Gly, 1 Ile, 1 Leuol and 2 Val for KB peptides. The distinction between KB I and KB II arose from the presence of 3 Aib in KB I and 2 Aib and 1 Iva in KB II. The absolute configuration of the constituent amino acids, examined on a chiral capillary column, indicated that Iva had the *R*-configuration; the other chiral residues had the *S*-configuration. The nature of the Glx residues were accurately determined by ^1H and ^{13}C NMR experiments and by FAB mass spectrometry analyses as Gln residues.

The circular dichroism spectra of the peptides exhibited negative Cotton effects at 206–208 and 222 nm characteristic of right-handed helical forms.[16,17]

Sequence Determination of KA V.—The positive ion FAB mass spectrum of KA V (Fig. 2a) exhibited a sodium adduct ion $[M + Na]^+$ at *m/z* 1912 from which a molecular weight of 1889 (H = 1.008) could be deduced, leading to the molecular formula $C_{87}H_{152}N_{22}O_{24}$. Careful inspection of the spectrum revealed the presence of two superimposed acylium ion series, suggesting the preferential cleavage of the Aib-Pro bond, as previously described for 18–20 residue peptaibols.[18,19] One series initiated at *m/z* 1122 led to successive acylium ions at *m/z* 924, 837, 752, 667, 454, 341, 256, 185 and 128. Taking into account the amino acid composition, this series of ions indicated the losses of [Leu (Ile) + Aib], Ser, Aib, Aib, [Gln + Aib], Leu (Ile), Aib, Ala, Gly, Ac-Aib and resulted from the *N*-terminal part. The *C*-terminal ion series started at *m/z* 768 and gave fragment ion peaks at *m/z* 651, 523, 395, 282 and 197, involving the successive losses of $[Lol + H]^+$, Gln, Gln, Ile (Leu), Aib and [Pro + Val].

Unambiguous and complete information on the sequence of KA could not be obtained from the FAB-MS spectrum. Indeed, the lack of sequence-specific ions between the ion peaks at *m/z* 667 and 454, did not allow the asignment of the residues at position 6 and 7. In addition, the differentiation between the isomeric residues Leu/Ile at positions 5, 11 and 16 was not possible by simple focusing mass technique.

These problems were solved by NMR experiments. The strategy used for identifying the primary structures involved the unequivocal determination of stretches of $NH_i - NH_{i+1}$ connectivities in the ROESY spectra. Total assignment of resonances to the specific amino acids in the sequence arose from the HOHAHA spectrum of KA V (Fig. 3), where all side-chain resonances of the individual residues gave rise to cross peaks in the aliphatic region as well as in the amide proton

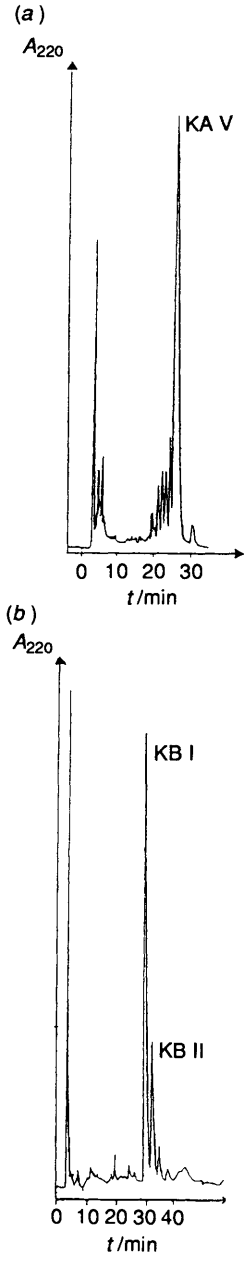

Fig. 1 HPLC chromatograms of (*a*) trikoningins KA and (*b*) trikoningins KB. Conditions: C_{18} reversed-phase Spherisorb ODS2, 5 μm (7.5 × 300 mm); flow rate 2 cm^3 min^{-1}; det. UV 220 nm; mobile phase, (*a*) MeOH–H_2O: 83/17, (*b*) MeOH–H_2O: 84/16.

region. Thus, each spin system was identified (Table 1), and the presence of two Ile and one Leu was clearly shown.

The location of isomeric and repetitive residues was immediately identified in the ROESY spectrum of KA V (Fig. 4). First, the large stretch Gly_2–Ala_3–Aib_4–Ile_5–Gln_6–Aib_7–Aib_8–Aib_9–Ser_{10} allowed the assignment of Ile, Gln and Aib residues at positions 5, 6 and 7, respectively. Then, starting from the *C*-terminal Leuol amide proton, the Ile_{16}–Gln_{17}–Gln_{18}–$Leuol_{19}$ sequence could be recognised. Finally, NN-connectivities were observed between Leu_{11}–Aib_{12} and Val_{14}–Aib_{15}.

On the basis of the above findings, the entire KA V sequence was determined unambiguously as given.

Sequence Determination of KB I and KB II.—The molecular weights of KB I and KB II were determined from their respective pseudomolecular ion species MH^+ and $(M + Na)^+$ indicative of the molecular weights 1037 and 1051, respectively. These values were in agreement with the molecular formulae $C_{50}H_{91}N_{11}O_{12}$ for KB I and $C_{51}H_{93}N_{11}O_{12}$ for KB II. Extensive series of acylium ions were exhibited, from which the whole sequence of the peptides could be deduced. In the case of KB I (Fig. 2b), the lack of isomeric residue made possible the unambiguous peptidic sequence determination by mass spectroscopy. The lower mass fragment ion at *m/z* 127 indicative of an octanoyl group,[12] agreed with ^{13}C NMR spectroscopic data which exhibited one methyl (14.32 ppm), six methylene (23.52, 26.45, 30.02, 30.12, 32.71, 36.79 ppm) and one carbonyl (176.38 ppm) groups, characterising an octanoyl (Oc) chain (Table 2).

In the mass spectrum of KB II, the fragment ions over *m/z* 127 were 14 mass units higher than those of KB I. This difference suggested the replacement of Aib at position 1 by a Val/Iva residue in KB II, taking into account the amino acid composition. The respective assignment of isomeric residues Val/Iva was solved by a ROESY experiment. First, the assignment of the ^1H NMR spectrum was accomplished from ^1H–^1H COSY and HOHAHA data (Table 3). Second, inspection of NH_i–NH_{i+1} and $C_\alpha H_i$–NH_{i+1} cross-peaks in the ROESY spectrum allowed the exhibition of the NN-connectivities between Iva_1 and Gly_2, and between Val_7 and Aib_8 and an αN-connectivity between Val_3 and Aib_4. In this manner, Iva was determined to be at position 1.

KBI and KBII belong to the 11-residue lipopeptaibol class among which trichogin GA IV was the first mentioned.[12]

Membrane-modifying properties of Trikoningins.—Peptaibols bind to phospholipid bilayers and are able to modify their permeability.[6,20–24] Permeability properties of the isolated peptides were thus examined, by following the induced carboxyfluorescein (CF) leakage from small unilamellar vesicles (egg phosphatidylcholine–cholesterol, 70:30) for different R_i^{-1} = [peptide]/[lipid] ratios, as previously described.[6]

An $R_i^{-1} = 1.3 \times 10^{-3}$ value allowing the leakage of 50% of the entrapped CF in 20 min, was observed for KA V. As compared to known 19-residue peptaibols, KA V exhibited a similar effect, although it was slightly weaker than that of trichorzianine TA IIIc (Fig. 5a).

Both KB I and KB II exhibited the leakage of 50% of the entrapped CF for a $R_i^{-1} = 32 \times 10^{-3}$ value. Thus, the substitution of Aib_1 with a Iva residue did not appear to modify the peptide bilayer interaction. It is noticeable that this activity was much lower than that of the 19-residue peptaibol KA V (Fig. 5b).

More striking was the comparison of the results obtained for KB I and KB II with those of the 11-residue lipopeptaibol GA IV.[12] This lipopeptide, which differed from KB I only by the replacement of two Leu residues in positions 3 and 7 by two Val residues, was 25 times more efficient. As valine can be considered to be less hydrophobic than leucine,[25,26] the difference noticed between the activity of GA IV and KB peptides, could be related to the decrease of hyrophobicity in KB peptidic sequences, which would result in a lesser interaction with membranes.

Antimicrobial Activity of Trikoningins.—The antibacterial properties of trikoningins KA V, KB I and KB II were examined against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria by the agar diffusion test. No inhibition was observed with *E. coli*. The observed inhibition diameters for *S. aureus* were as given in Table 4. The results show that the 19-residue peptaibol induced similar antibacterial properties than the 11-residue lipopeptaibols, suggesting that if the membrane-perturbing activity and the antimicrobial activity are somewhat related,[27] they do not follow rigorously a parallel trend.

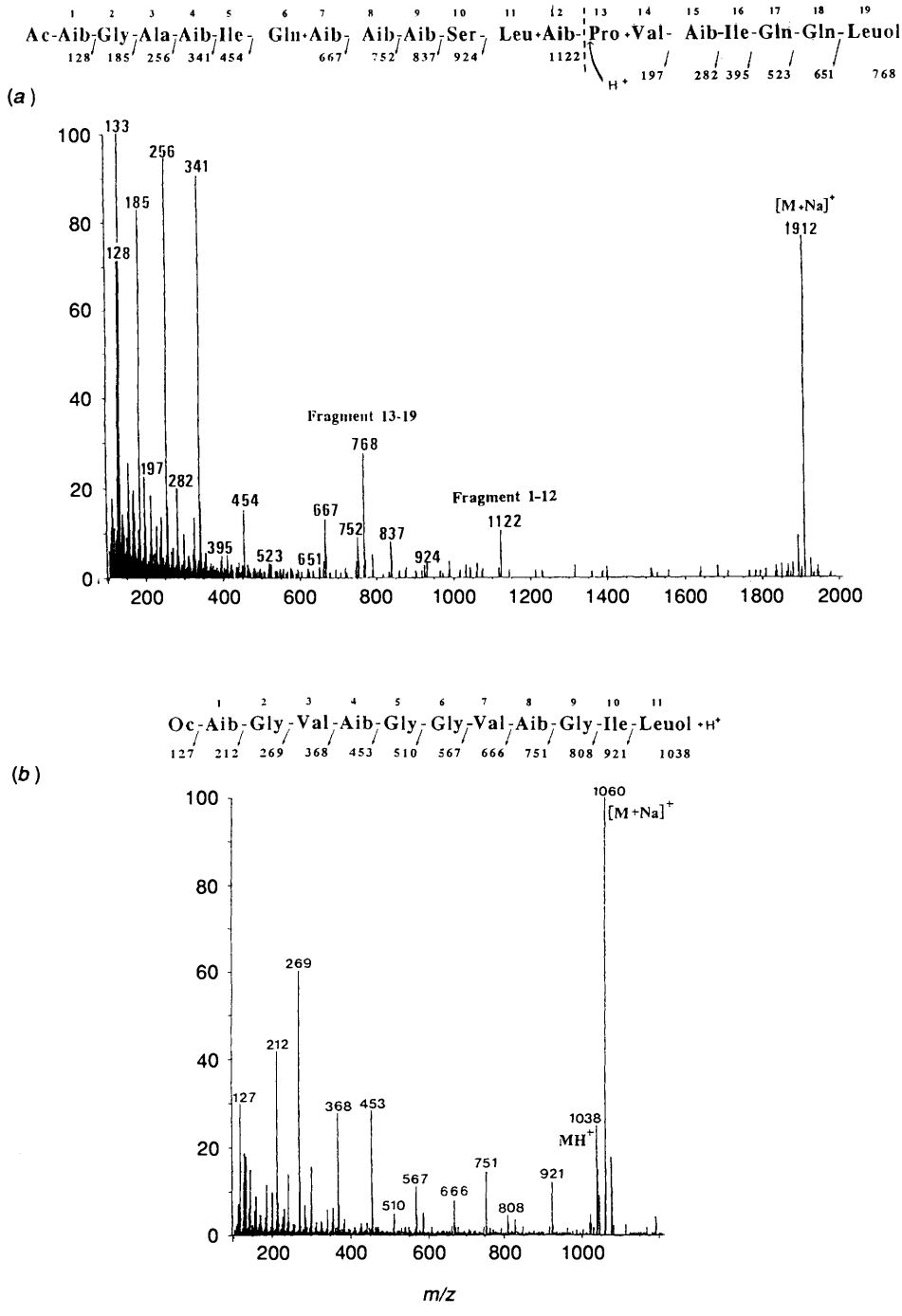

Fig. 2 Positive ion FAB mass spectrum of (*a*) KA V and (*b*) KB I

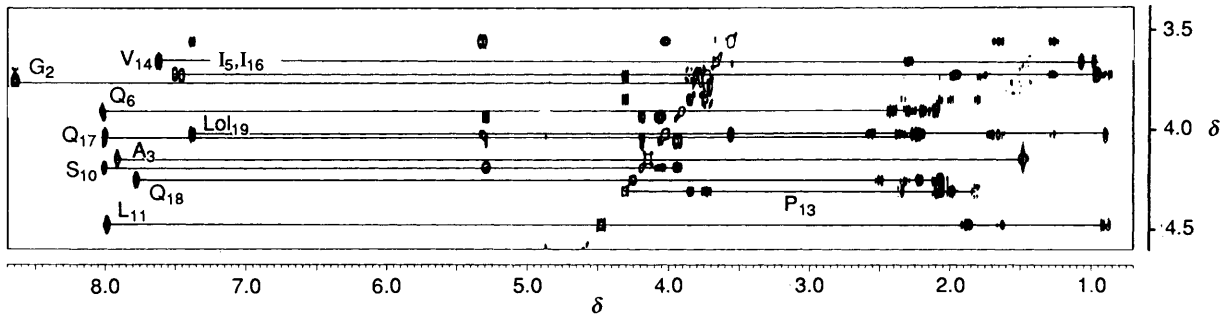

Fig. 3 Fingerprint region (ω_2 = 0.7–8.7 ppm, ω_1 = 3.0–4.6 ppm) of the HOHAHA spectrum of KA V (400.13 MHz; 296 K; CD_3OH). The spin system of each residue is connected.

Table 1 ^{1}H NMR Specific assignments for resonances of KA V

Residue	δ_H[a] (J/Hz) NH	Hα	Other
Ac			2.044 s
Aib_1	8.649 s		
Gly_2	8.658 dd (5.4, 5.4)	3.802 dd (16.4) 3.745 dd (16.4)	
Ala_3	7.914 d (5.7)	4.157 q (7.3)	β = 1.470 d (7.3)
Aib_4	7.666 s		
Ile_5	7.466 d (5.7)	3.743 d (10.0)	β = 1.96; γ_1 = 1.27; γ_1' = 1.76; γ_2 = 0.97; δ = 0.89
Gln_6	8.030 d (4.5)	3.920 t (7.8)	β = 2.12; β′ = 2.20; γ = 2.30; γ′ = 2.44
Aib_7	8.133 s		
Aib_8	7.762 s		
Aib_9	8.428 s		
Ser_{10}	8.014 d (4.9)	4.202 dd (7.0, 3.2)	β = 3.942 dd (3.2, 12.0); β′ = 4.069 dd (7.0, 12.0)
Leu_{11}	7.993 d (7.5)	4.486 dd (11.2, 3.6)	β = 1.86; β′ = 1.62; γ = 1.89; δ_1 = 0.87; δ_2 = 0.92
Aib_{12}	8.259 s		
Pro_{13}		4.324 dd (7.5)	β = 2.34; β′ = 1.80; γ = 2.08; γ′ = 1.95; δ = 3.86; δ′ = 3.72
Val_{14}	7.618 d (7.2)	3.675 d (10.0)	β = 2.31; γ_1 = 1.084 d (6.5); γ_2 = 0.982 d (6.5)
Aib_{15}	7.502 s		
Ile_{16}	7.512 d (6.6)	3.735 d (8.8)	β = 1.94; γ = 1.27; γ_1' = 1.76; γ_2 = 0.95; δ = 0.87
Gln_{17}	8.014 d (4.9)	4.036 dd (6.1, 8.5)	β = 2.26; γ = 2.38; γ′ = 2.58
Gln_{18}	7.790 d (7.2)	4.263 dd (10.9, 4.1)	β = 2.08; β′ = 2.20; γ = 2.34; γ′ = 2.50
Lol_{19}	7.395 (9.0)	4.04 m	β_1 = 1.26; β_1' = 1.66; β_2 = 3.566 m; γ = 1.66; δ = 0.88
CONH Gln_6[b]			
ε_{syn}	6.782 br s		
ε_{anti}	7.447 br s		
CONH Gln_{17}[b]			
ε_{syn}	6.672 br s		
ε_{anti}	7.432 br s		
CONH Gln_{18}[b]			
ε_{syn}	6.782 br s		
ε_{anti}	7.454 br s		
Aib 1, 4, 7, 8 9, 12, 15			Meβ = 1.611; 1.591 (×2); 1.581; 1.549; 1.529; 1.518; 1.500 (×2); 1.490; 1.476; 1.469; 1.467; 1.451

[a] CD_3OH; 296 K; 400.13 MHz for amide protons; CD_3OD; 296 K; 300.13 MHz for non-exchangeable protons; Δδ are with 3 decimals when obtained from 1D spectra and with 2 decimals when obtained from 2D spectra. [b] Assignments determined from the connectivities between the γ protons of the Gln residues and the syn and anti protons in the ROESY.

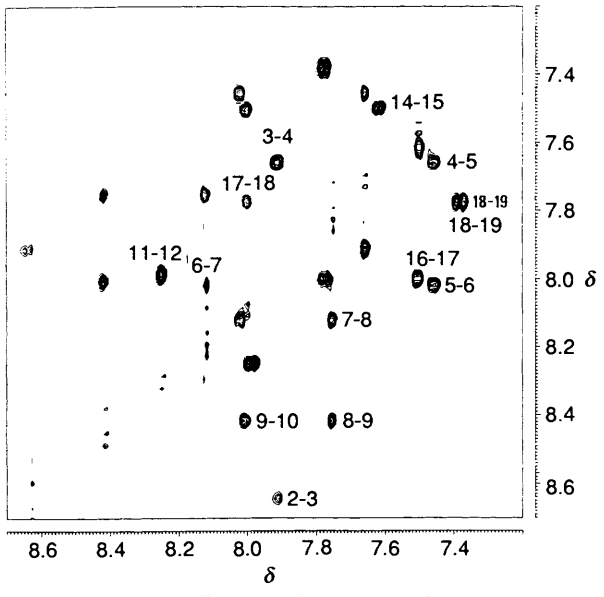

Fig. 4 NH–NH region of the ROESY spectrum of KA V (400.13 MHz; 296 K; CD_3OH). Inter-residue connectivities are indicated by the residue numbers.

Experimental

General Data.—The CD spectra were obtained on a Jobin Yvon CD6 dichrograph. Fluorescence spectra were recorded at 20 °C on an Aminco SPF 500 spectrofluorimeter.

Fermentation.—*Trichoderma koningii* (No. 90 3589) was obtained from the 'Collection de souches fongiques du Muséum National d'Histoire Naturelle', Paris (France). Lyophilised preparation was transferred to agar slants that contained a seeding medium (2% w/v malt-agar). To maintain the ability of subsequent fermentations to produce peptide antibiotics, fresh malt-agar cultures were prepared every two months from lyophilised samples of the original spore isolates. Fermentations were performed in Roux flasks (1 dm^3) on a synthetic medium previously described.[21] After autoclaving, the Roux flasks were inoculated with a suspension of *T. koningii* spores, and the stationary culture was incubated for 20 days at 27 °C.

Isolation of Trichoningins.—A typical culture (6 dm^3) of *T. koningii* was filtered to separate the mycelium from the culture broth. The filtrate was extracted three times with butanol, and the wet mycelium three times with methanol. The concentrated and combined extracts (1.63 g) were submitted to gel chromatography on Sephadex LH 20 with methanol as eluent. A crude peptide mixture (238 mg) of KA and KB was obtained and chromatographed on silica gel; it was eluted first with methylene chloride–methanol (9:1) affording KB (90 mg) and then with methylene chloride–methanol (7:3) to give KA (56 mg); TLC R_f value (SiO_2, Merck 60 F_{254}, 6:2:2 butanol–acetic acid–water) of KA: 0.35 and KB: 0.50. The spots gave no reaction with ninhydrin but were detected by spraying anisaldehyde reagent (1:1:50 anisaldehyde–sulfuric acid–acetic acid) and heating.

HPLC Separation of Trikoningins.—High-performance liquid chromatography (HPLC) was carried out on a Merck (L 6200 pump, AS 200 autosampler, L 4000 UV–VIS detector)

Table 2 ^{13}C NMR shifts for KB I and KB II ($^{12}CD_3OH$; 296 K; 75.47 MHz)

		KB I		KB II	
Residue	Carbon	δ	δ CO	δ	δ CO
Oc	C-8′	14.32		14.29	
	C-7′	23.53		23.54	
	C-6′	32.71		32.74	
	C-5′	30.12[a]		30.21	
	C-4′	30.02[a]		30.04	
	C-3′	26.45		26.23	
	C-2′	36.79		36.79	
	C-1′		176.38		176.21
Gly 2	α	44.70	173.25	44.51	173.20
Val 3	α	62.27	174.57	62.20	174.57
	β	30.71		30.91[a]	
	γ_1	19.45[b]		19.45[b]	
	γ_2	19.53[b]		19.45[b]	
Gly 5	α	44.79	173.23	44.72	173.17
Gly 6	α	44.29	172.93	44.23	172.87
Val 7	α	62.71	174.23	62.50	174.15
	β	30.79		30.76[a]	
	γ	19.45[b]		19.70[b]	
	γ′	19.78[b]		19.52[b]	
Gly 9	α	44.70	172.47	44.67	172.44
Ile 10	α	60.58	173.68	60.50	173.65
	β	37.29		37.30	
	γ	26.20		26.58	
	γ	15.92		15.91	
	δ	11.46		11.42	
Leuol 11	α	50.78		50.82	
	β_1	65.90		65.89	
	β_2	40.73		40.78	
	γ	25.61		26.30	
	δ_1	22.03		22.03	
	δ_2	23.82		23.80	
Aib 1, 4, 8	α	57.86	178.28 (1)	60.57 (Iva 1)	178.06
	α	57.82	174.04 (4)	57.89	177.99
	α	57.39	178.03 (8)	57.84	177.97
	β_1	24.78		24.91	
		24.70		22.52 (Iva 1, βCH_3)	
		24.65		29.56 (Iva 1, βCH_2)	
	β_2	25.89		25.69 (×2)	
		26.30		25.63	
		25.78		7.97 (Iva 1, γCH_3)	

[a,b] May be reversed within the same column.

liquid chromatograph equipped with a Gilson 201 fraction collector using a semi-preparative C_{18}-column (Spherisorb ODS 2, 5 μm, 7.5 × 300 mm) (SFCC, France). **KA**: eluent methanol–water (87:13) flow rate 2 cm^3 min^{-1}; R_t (KA V) = 26 min; **KB**: eluent methanol–water: 84:16; flow rate 2 cm^3 min^{-1}; R_t (KB I) = 30 min and R_t (KB II) = 32 min.

Amino Acid Analysis.—Total hydrolyses of KA V, KB I and KB II were performed according to the usual procedure for peptides (6 mol dm^{-3} HCl at 110 °C in sealed tubes for 24 h). Identification of the amino acids was accomplished by gas chromatography after derivatisation.[5,15] Retention times of the *N*-trifluoroacetyl isopropyl ester derivatives were compared with those of standards. The GC analyses were performed on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxan) quartz capillary column (Chrompack, 25 m, 0.2 mm i.d.) with He (1.1 bar) as carrier gas and a temperature program 50 °C for 10 min, then 50–190 °C: 2 °C min^{-1}. Retention times (min): Aib (8.7), D-Iva (10.6), L-Ala (18.7), Gly (22.9), L-Val (23.2), L-Pro (27.5), L-Ile (28.0), L-Lol (29.0), L-Ser (30.7), L-Leu (32.7) and L-Glu (47.2).

CD Measurements.—0.1 mm path cells; T = 22 °C; λ (nm) $[\theta]_M$ (deg cm^2 $dmol^{-1}$); **KA V** 208 (−549 000), 222 (−380 000); **KB I** 207 (−121 000), 222 (−70 900); **KB II** 206 (−180 000) and 222 (−122 000).

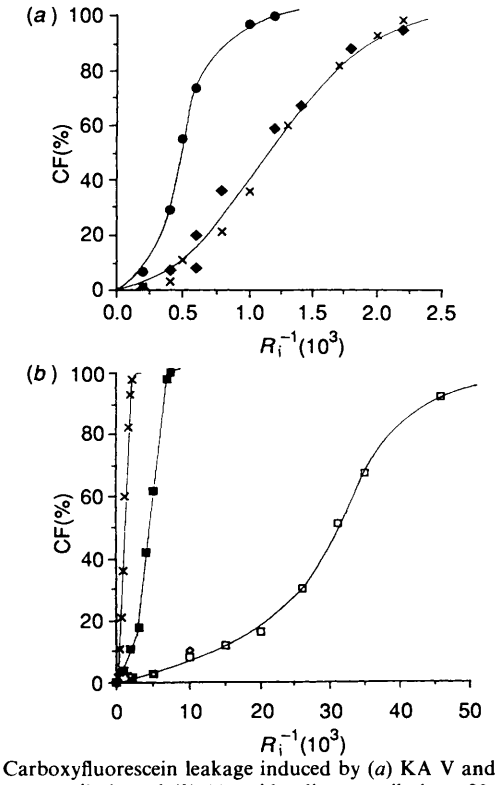

Fig. 5 Carboxyfluorescein leakage induced by (*a*) KA V and known 19-residue peptaibols and (*b*) 11-residue lipopeptaibols at 20 min for different ratios R_i^{-1} = [peptide]/[lipid], from egg PC/cholesterol (70/30) vesicles. (*a*) (**X**) KA V, (◆) LB I, (●) TA IIIc; (*b*) (■) KB I, (◇) KB II, (■) GA IV, (**X**) KA V.

Positive Ion FAB Mass Spectrometry.—Positive ion FAB mass spectra were recorded on a VG analytical MM ZAB-HF mass spectrometer. Peptide solutions were mixed with α-monothioglycerol as matrix on the FAB probe tip. Ions were formed by fast atom bombardment with a 8 keV Xe atom beam generated in a saddle field neutral gun (Ion Tech. Ltd, Teddington, UK). Resolving was about 2500. For KA, the *N*- or *C*-terminal origin of ions is indicated as N or C.

KA V: *m/z* (relative intensity): 128 N (71), 133 (100), 185 N (82), 197 C (22), 256 N (94), 282 C (20), 341 N (90), 395 C (3), 454 N (15), 523 C (3), 651 C (2), 667 N (13), 752 N (9), 768 C (27), 837 N (8), 924 N (2), 1122 N (10) and 1912 $[M + Na]^+$ (76).

KB I: 127 (12), 212 (41), 269 (60), 368 (27), 453 (28), 510 (5), 567 (11), 666 (8), 751 (14), 808 (4), 921 (12), 1038 MH^+ (25) and 1060 $[M + Na]^+$ (100).

KB II: 127 (6), 226 (14), 283 (24), 382 (10), 467 (10), 524 (3), 581 (4), 680 (2), 765 (4), 822 (2), 935 (3) and 1074 $[M + Na]^+$ (100).

NMR Spectroscopy.—^1H NMR spectroscopy experiments were conducted on Bruker AC 300 or AM 400 spectrometers, equipped with Aspect 3000 and X 32 computers, respectively. They were referenced to the central component of the quintet due to CHD_2 resonance of methanol at 3.313 ppm downfield from $(CH_3)_4Si$. Spectra in CD_3OH were obtained by solvent pre-saturation. ^{13}C NMR spectra were collected at 75.47 MHz on a Bruker AC 300 spectrometer. ^{13}C NMR chemical shifts were referenced to internal CD_3OH taken at 49.00 ppm relative to $(CH_3)_4Si$. The one-dimensional spectra were obtained with 300–700 scans.

For KA V, the COSY spectrum (300.13 MHz; CD_3OH; 296

Table 3 ^{1}H NMR Specific assignments for resonances of KB I[a] and KB II[b]

	KB I δ_H(J/Hz)			KB II δ_H(J/Hz)		
Residue	NH	Hα	Other groups	NH	Hα	Other groups
Oc CH_3 (C_8')			0.895 t (7.3)			0.90 t
CH_2 (C_7' to C_4')			1.30 m; 1.35 m; 1.37 m; 1.42 m			1.313 m
CH_2 (C_3')			1.62 m			1.60 m
CH_2 (C_2')			2.266 t (7.5)			2.275 t (6.8)
Aib 1	8.430 s		1.493 s; 1.439 s			
Iva 1				8.318 s		β_1 2.02 m; β'_1 1.81 m β_2 1.386 s; γ 0.874 t (7.4)
Gly 2	8.463 dd (5.1; 5.1)	3.885 d (16.2) 3.750 d (16.2)		8.442 dd (5.8; 5.8)	3.78 m 3.87 m	
Val 3	7.903 d (7.5)	4.043 d (8.0)	β 2.26 m γ_1 0.970 d (6.1) γ_2 1.012 d (6.6)	8.001 d (7.3)	4.015 dd (7.5; 7.3)	β 2.26 m γ_1 0.972 d (6.7) γ_2 1.010 d (6.7)
Aib 4	7.985 s		1.517 s; 1.504 s	8.064 s		β 1.500 s; β′ 1.493 s
Gly 5	8.197 dd (5.5; 5.5)	3.849 d (16.1) 3.810 d (16.1)		8.202 dd (5.5; 5.5)	3.84 m	
Gly 6	8.075 dd (5.6; 5.6)	3.946 d (16.2) 3.917 d (16.2)		8.101 dd (5.9; 5.9)	3.94m	
Val 7	7.960 d (6.5)	3.932 d (5.4)	β 2.186 m γ_1 0.983 d (6.3) γ_2 1.034 d (6.8)	7.965 d (6.6)	3.93 m	β 2.16 m γ_1 0.972 d (6.7) γ_2 1.021 d (6.6)
Aib 8	8.162 s		1.479 s; 1.455 s	8.243 s		1.474 s; 1.468s
Gly 9	7.987 dd (5.9; 5.9)	3.830 d (16.9) 3.801 d (16.9)		8.028 dd (5.9; 5.9)	3.82 m	
Ile 10	7.738 d (8.1)	4.169 d (7.8)	β 2.036 m γ_1 1.27 m; γ_1 1.672 m γ_2 0.977 d (6.3) δ 0.908 t (6.9)	7.763 d (8.1)	4.158 dd (8.0; 8.2)	β 2.02 m γ_1 1.30 m; γ'_1 1.63 m γ_2 0.960 d (6.7) δ 0.881 t (6.4)
Leuol 11	7.383 d (8.9)	4.02 m	β_1 1.35 m; β_1' 1.46 m β_2 3.485 dd (11.2; 5.6) β'_2 3.517 dd (11.2; 5.6) γ 1.692 m δ_1 0.882 d (6.0) δ_2 0.901 d (6.9)	7.453 d (9.0)	4.03 m	β_1 1.36 m; β'_1 1.50 m β_2 3.498 m γ 1.66 m δ_1 0.90 m; δ_2 0.90 m

[a] CD_3OH; 296 K; 500.13 MHz for exchangeable protons; CD_3OD; 296 K; 300.13 MHz for non-exchangeable protons. [b] CD_3OH; 296 K; 300.13 MHz; Δδ are with 3 decimals when obtained from 1D spectra and with 2 decimals when obtained from 2D spectra.

Table 4 Inhibition diameters (mm) of *S. aureus* induced by different concentrations of trikoningins KA V, KB I and KB II

Concentration (μg/pit)	200.0	100.0	50.0	25.0	12.5	6.2	3.1	1.5
KA V	17	16	15	15	14	12	11	n.i.
KB I	18	17	16	15	13	11	n.i.	n.i.
KB II	14	13	13	12	11	11	n.i.	n.i.

n.i. = no inhibition.

K) was run with a total of 256 experiments of 32 scans each with a sweep width in F_2 of 2590 Hz (size 2 K) and in F_1 of 1295 Hz (size 1 K), zero filling to 2 K in F_1 and 4 K in F_2. For the HOHAHA spectrum (400.13 MHz; CD_3OH; 296 K), 512 experiments of 32 scans were performed with a sweep width in F_2 of 3597 Hz (size 2 K) and in F_1 of 1798.5 Hz (size 1 K); sine bell weighing functions shifted by π/4 in F_2 and F_1 were applied. ROESY data (400.13 MHz; CD_3OH; 296 K) were obtained with a mixing time of 350 ms. A total of 256 experiments of 84 scans each were acquired with a sweep width in F_2 of 3597 Hz (size 2 K) and in F_1 of 1798.5 Hz (size 1 K); zero filling to 2 K in F_1 and 4 K in F_2.

For KB II, the COSY (300.13 MHz; CD_3OH; 296 K) was run with 256 experiments of 96 scans each; sweep width in F_2 of 2500 Hz (size 2 K) and in F_1 of 1250 Hz (size 1 K); zero filling to 2 K in F_1 and 4 K in F_2. For the ROESY (400.13 MHz; CD_3OH; 296 K) spectrum, recorded in the phase sensitive mode, 256 experiments of 32 scans were performed with a mixing time of 250 ms; spectral width of 4001 Hz in F_1 and 2000.5 in F_2, size 1 K in F_1 and F_2; zero filling to 2 K in F_1 and F_2; sine bell functions shifted by π/2 in F_1 and F_2.

Antimicrobial Activity.—The antimicrobial properties of KA V, KB I and KB II were examined against *Staphylococcus aureus* (strain 209 P) and *Escherichia coli* (strain RL 65) by the agar diffusion test using 6 mm diameter pits. The peptide samples were dissolved in DMSO to obtain a 4 mg cm^{-3} solution. Eight concentrations were obtained by successive dilutions. Each solution (50 mm^3) was deposited into the pits (0.2–0.0015 mg). Inhibition zones were measured after 24 h of incubation at 37 °C.

Membrane Permeability Measurements.—Leakage from vesicles was measured employing the carboxyfluorescein (CF)-entrapped vesicle technique, as previously described.[6] The CF-encapsulated small unilamellar vesicles [egg phosphatidylcholine (egg PC)–cholesterol, 7:3] were prepared by sonication. The phospholipid concentration was kept constant (4.10^{-4} mol dm^{-3}), and increasing [peptide]/[lipid] molar ratios (R_i^{-1}) were obtained by adding aliquots of methanolic solutions of peptide (final methanol concentration kept below 0.5% by volume). After rapid and vigorous stirring, the time-course of fluorescence change corresponding to CF efflux was recorded at 520 nm (1 nm band pass) with λ_{exc} 488 nm (1 nm band pass). Percentage of released CF at 20 min was determined as % CF = $(F_t - F_0)/(F_T - F_0) \times 100$, where F_0 is the fluorescence intensity of the vesicle suspension in the absence of peptide, F_t the fluorescence intensity measured at 20 min in the presence of peptide, and F_T the total fluorescence determined by disrupting the vesicles by addition of a 10% Triton X-100 solution (50 mm^3).

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