# Antibiotic peptides from *Trichoderma harzianum*: harzianins HC, proline-rich 14-residue peptaibols

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An original group of antibiotic-antifungal peptides of the peptaibol class, the harzianins HC, was isolated from *T. harzianum* strains selected for their antagonistic properties against phytopathogenic fungi. Eleven peptides were isolated by reversed-phase HPLC from the original complex mixtures, and sequenced.

The sequences resulted both from positive-ion FAB-MS and $^1$H NMR data. Harzianins HC are 14-residue peptaibols containing three Aib-L-Pro motives at positions 4–5, 8–9 and 12–13. ROESY data and amide temperature coefficient values agreed with a helical structure stabilised by hydrogen bonds of the 4→1 type and looking to differ from $3_{10}$- and α-helices.

Harzianins HC increased the permeability of liposome bilayers and gave rise to voltage-dependent conductance, a property generally observed for longer-sequence peptaibols.

## Introduction

Peptaibol antibiotics biosynthesized by *Trichoderma* soil fungi are linear peptides containing a high proportion of the α,α-dialkylated amino acid, α-aminoisobutyric acid (Aib, U), an N-terminal acylated residue and a C-terminal amino alcohol.[1–6] The main interest in such peptides stems from their ability to form amphipathic helices which interact with phospholipid bilayers, thus increasing the permeability of liposomes[7,8] and forming voltage-dependent transmembrane channels in planar lipid bilayer membranes.[9,10] 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.[9,11] A minimal peptide length of 18–20 residues is therefore conventionally required for an α-helical peptide to give rise to transmembrane channels. The exhibited membrane-perturbing properties are related to the various biological activities displayed by peptaibols, and particularly to their antibiotic activity.

Among this class of peptides, diversity occurs from the nature of the acyl terminus, the peptide chain length and the number of prolines in the sequences. A major group (long-sequence-peptaibols[1–5]), including the most widely described, alamethicin,[1] concerns 18- to 20-residue peptaibols containing a proline at the 2/3 of the sequence. Another group, formed by shorter peptaibols with an N-terminal amino acid acylated by octanoic acid, a great amount of glycines and without proline, has been described and termed as the lipopeptaibols.[6]

From two *Trichoderma harzianum* strains exhibiting antagonistic properties against phytopathogenic fungi, we isolated new peptaibols and pointed out their antibiotic-antifungal properties. A first group was composed of 18-residue peptides, trichorzins HA;†[10] the second one concerned short-sequence peptaibols rich in proline residues, that were named harzianins HC[12] (Fig. 1). Most of the HC peptides were original, but some of them containing a Val at position 7 (**HC I, III, VI, VIII**) were shown to be identical to trichovirins I, previously isolated by Brückner *et al.* from *T. viride* (NRLL 5234).[13]

We here report on the isolation and sequence determination of eleven harzianins HC (Fig. 1). We also discuss briefly their secondary structure which could explain how, in spite of their short sequences, harzianins HC can manage voltage-gated ion channels through lipid bilayers, in a similar manner as the 20-residue alamethicin.[14]

† Trichorzins HA were termed harzianins HA in a previous paper.[10]

## Results and discussion

### Isolation and HPLC separations of harzianins HC

Fermentation of the original M-903614 and M-903603 *T. harzianum* strains led to crude peptaibol mixtures, which were isolated from the culture broth extracts by exclusion chromatography over Sephadex LH 20. Silica gel chromatography allowed us to separate two groups of different polarity, trichorzins HA[10] and harzianins HC.[12] Each peptide group was tested for its antimicrobial activity against *Staphylococcus aureus* and *Sclerotium rolfsii* and showed similar activities. The HA and HC peptide microheterogeneous mixtures arising from strains M-903614 and M-903603 looked identical when compared by CCM, but their HPLC profiles were different (Fig. 2 A and B). Eleven pure peptides labelled on figures 2A and 2B were isolated by semi-preparative HPLC and submitted to structural elucidation.

### Sequence determination of harzianins HC

**(a) General characteristics of harzianins HC.** The purified HC peptides exhibited a sharp singlet at $\delta_H \sim 2.0$ in the proton NMR spectra, indicating the presence of an acetyl group in all the peptides. As the absence of a free $NH_2$-terminal group arose from the negative reaction obtained with ninhydrin, the N-terminal residues of harzianins HC appeared to be acetylated.

The amino acid composition and absolute configuration were determined from GLC analyses on a Chirasil-L-Val capillary column, of the total acidic hydrolysates after derivatisation of the amino acids as *N*-trifluoroacetyl isopropyl esters, and comparison with standards. The Iva residues were assigned as the D-enantiomers, whereas the chirality of other amino acids and of the amino alcohol was L. From the absence of an acidic function in harzianins HC, the L-Asx and L-Glx arose from L-Asn and L-Gln, respectively.

**(b) Positive ion FAB mass spectrometry.** Positive-ion FAB-MS proved useful in determining sequences of peptaibols by generating a continuous series of $b_n$ acylium ions,[6,15–19] but difficulties in the sequence analysis generally arise from the presence of an Aib-Pro link.[15–19] This amide bond undergoes a preferential cleavage leading to an N-terminal acylium ion $N^+$

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HC I : | Ac | Aib | **Asn** | Leu | Aib | Pro | **Ser** | **Val** | Aib | Pro | **Aib** | Leu | Aib | Pro | Leuol |
| HC III : | Ac | Aib | **Asn** | Leu | Aib | Pro | **Ser** | **Val** | Aib | Pro | **Iva** | Leu | Aib | Pro | Leuol |
| HC VI : | Ac | Aib | **Asn** | Leu | Aib | Pro | **Ala** | **Val** | Aib | Pro | **Aib** | Leu | Aib | Pro | Leuol |
| HC VIII : | Ac | Aib | **Asn** | Leu | Aib | Pro | **Ala** | **Val** | Aib | Pro | **Iva** | Leu | Aib | Pro | Leuol |
| HC IX : | Ac | Aib | **Asn** | Leu | Aib | Pro | **Ala** | **Ile** | Aib | Pro | **Iva** | Leu | Aib | Pro | Leuol |
| HC X : | Ac | Aib | **Gln** | Leu | Aib | Pro | **Ala** | **Val** | Aib | Pro | **Iva** | Leu | Aib | Pro | Leuol |
| HC XI : | Ac | Aib | **Asn** | Leu | Aib | Pro | **Ser** | **Ile** | Aib | Pro | **Aib** | Leu | Aib | Pro | Leuol |
| HC XII : | Ac | Aib | **Asn** | Leu | Aib | Pro | **Ser** | **Ile** | Aib | Pro | **Iva** | Leu | Aib | Pro | Leuol |
| HC XIII : | Ac | Aib | **Gln** | Leu | Aib | Pro | **Ser** | **Ile** | Aib | Pro | **Iva** | Leu | Aib | Pro | Leuol |
| HC XIV : | Ac | Aib | **Asn** | Leu | Aib | Pro | **Ala** | **Ile** | Aib | Pro | **Aib** | Leu | Aib | Pro | Leuol |
| HC XV : | Ac | Aib | **Gln** | Leu | Aib | Pro | **Ala** | **Ile** | Aib | Pro | **Iva** | Leu | Aib | Pro | Leuol |

**Fig. 1** Sequences of harzianins HC isolated from *T. harzianum* (strains M-903614 and M-903603); bold-faced letters indicate those amino acids which differ in the sequences

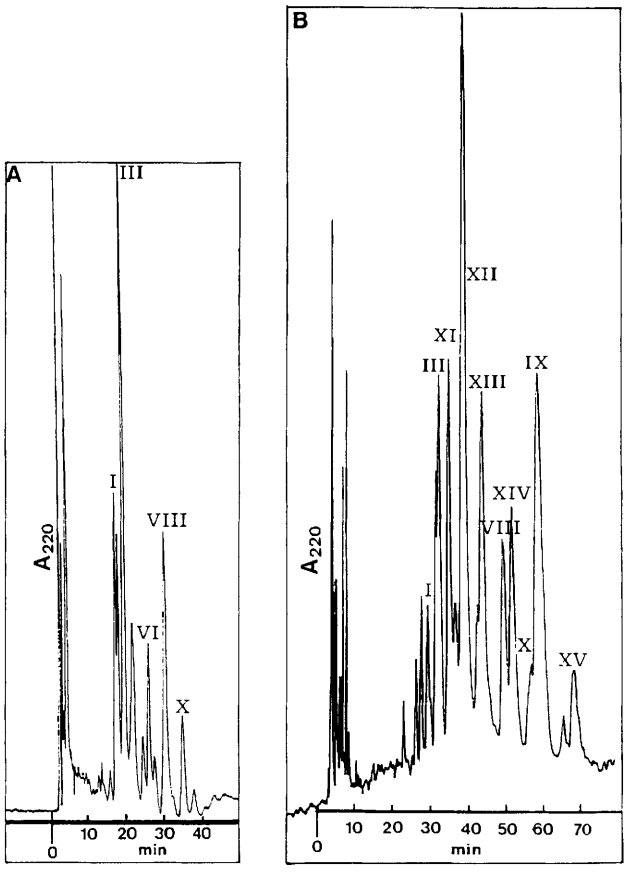


**Fig. 2** (A) HPLC chromatogram of harzianins HC from *T. harzianum* strain M-903603. Spherisorb ODS2 (5 μ), 2.5 mm × 30 cm; MeOH–water (81 : 19), flow rate 1 $cm^3$ $min^{-1}$; absorption monitored at 220 nm; (B) HPLC chromatogram of harzianins HC from *T. harzianum* strain M-903614, Spherisorb ODS2 (5 μ), 7.5 mm × 30 cm; MeOH–water (82.5 : 17.5), flow rate 2 $cm^3$ $min^{-1}$; absorption monitored at 220 nm.

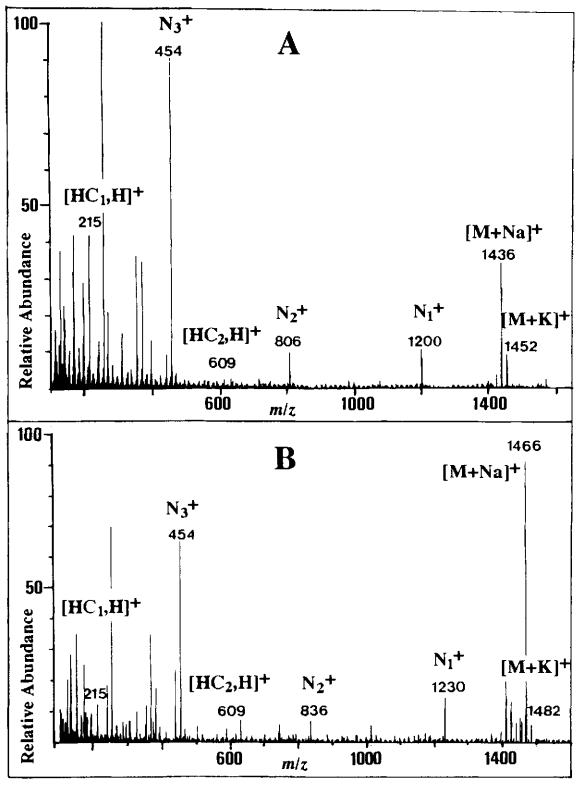


**Fig. 3** Positive-ion FAB mass spectra of **HC X** (A) and **HC XIII** (B) exhibiting the main fragment ions formed in the multiple preferential cleavages of the Aib–Pro bonds

and a diprotonated C-terminal ion $[HC, H]^+$. Such a major cleavage at the tertiary amide link Aib-Pro results in the absence of fragment ions at higher masses and in the superimposition of two independent $b_n$-type ion series at lower masses, starting from the $N^+$ and $[HC, H]^+$ ions. According to the Roepstorff nomenclature modified by Biemann,[20,21] the $[HC, H]^+$ ions are $y_n$ diprotonated ammonium ions.[22,23]

Assignments of the relative molecular masses of harzianins HC could be derived from their molecular-ion species $[M + Na]^+$ and $[M + K]^+$ which appeared at higher masses in the positive-ion FAB mass spectra (Fig. 3; Table 1). The spectra did not exhibit additional ions in this region, but showed anomalous complexity at lower masses. Taking into account the presence of three prolines in the HC sequences, this complexity suggested the superimposition of fragment ions resulting from the cleavage of Xaa-Pro bonds.

When examining accurately the mass spectrum of **HC X** taken as an example, three intense ions occurring in the spectrum at *m/z* 1200, 806 and 454 suggested the formation of three N-terminal $b_n$ ions, noted $N_1^+$, $N_2^+$, $N_3^+$, which confirmed the cleavage of three Aib-Pro bonds. They underwent subsequent fragmentation as shown in Fig. 3A. The $N_1^+$ and $N_2^+$ ions were accompanied by their complementary C-terminal ions $[HC_1, H]^+$ and $[HC_2, H]^+$ at *m/z* 215 and 609, respectively. The $[HC_2, H]^+$ ion underwent subsequent fragmentation which allowed us to assign the (Val/Iva)[10]-Leu[11]-Aib[12]-Pro[13] sequence. Unless the $[HC_3, H]^+$ $y_5$ ion complementary to $N_3^+$ was not detected at *m/z* 961 in the spectrum, it gave rise to an acylium-ion series starting from *m/z* 549, which confirmed a Val/Iva residue at position 10 and assigned an Ala at position 6. The fragmentation pattern, unless complex, finally permitted full determination of the **HC X** sequence, excepting the localisation of the isomeric residues Val/Iva (Fig. 4).

The (+)-ion FAB mass spectra of the remaining HC also showed the above ion types $Ni^+$ and $[HC_i, H]^+$ (Table 1), together with sequence-specific fragment ion series, which afforded the sequence determination. Nevertheless, the unsolved problem was the identification and localisation, in the sequences, of the pairs of isomeric residues, Leu/Ile and

**Table 1** Pseudomolecular ion species and sequence-specific fragment ions arising in the (+)-ion FAB mass spectra of harzianins HC; the matrix used was either nitrobenzyl alcohol (a) or α-thioglycerol (b): the former favoured the pseudomolecular ions and the latter gave more intense fragment ions

| Peptide | **HC I** (a) | **HC III** (a) | **HC VI** (a) | **HC VIII** (a) | **HC IX** (b) | **HC X** (b) | **HC XI** (a) | **HC XII** (a) | **HC XIII** (a) | **HC XIV** (a) | **HC XV** (a) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1401 | 1415 | 1385 | 1399 | 1413 | 1413 | 1415 | 1429 | 1443 | 1399 | 1427 |
| Ion types | $C_{67}H_{115}N_{15}O_{17}$ | $C_{68}H_{117}N_{15}O_{17}$ | $C_{67}H_{115}N_{15}O_{16}$ | $C_{68}H_{117}N_{15}O_{16}$ | $C_{69}H_{119}N_{15}O_{16}$ | $C_{69}H_{119}N_{15}O_{16}$ | $C_{68}H_{117}N_{15}O_{17}$ | $C_{69}H_{119}N_{15}O_{17}$ | $C_{70}H_{121}N_{15}O_{17}$ | $C_{68}H_{117}N_{15}O_{16}$ | $C_{70}H_{121}N_{15}O_{16}$ |
| | *m/z* (%) | *m/z* (%) | *m/z* (%) | *m/z* (%) | *m/z* (%) | *m/z* (%) | *m/z* (%) | *m/z* (%) | *m/z* (%) | *m/z* (%) | *m/z* (%) |
| $[M + K]^+$ | 1440 (11) | 1454 (10) | 1424 (11) | 1438 (10) | 1452 (1) | 1452 (9) | 1454 (4) | 1468 (8) | 1482 (5) | 1438 (12) | 1466 (5) |
| $[M + Na]^+$ | 1424 (100) | 1438 (100) | 1408 (100) | 1422 (100) | 1436 (17) | 1436 (34) | 1438 (100) | 1452 (100) | 1466 (100) | 1422 (100) | 1450 (100) |
| $N1^+$ | 1188 (13) | 1202 (14) | 1172 (25) | 1186 (11) | 1200 (37) | 1200 (10) | 1202 (12) | 1216 (5) | 1230 (14) | 1186 (18) | 1214 (12) |
| | | | | | 1115 (1) | | | | | | |
| | | 905 (1) | 889 (1) | | 903 (1) | 903 (1) | 919 (1) | | 933 (1) | | |
| $N2^+$ | 808 (7) | 808 (6) | 792 (12) | 792 (8) | 806 (28) | 806 (9) | 822 (6) | 822 (5) | 836 (7) | 806 (11) | 820 (9) |
| | | | 707 (2) | 707 (1) | 721 (3) | 721 (1) | | | 751 (1) | 721 (2) | 735 (1) |
| | 537 (1) | | 537 (2) | | 537 (2) | 551 (2) | | | 551 (1) | 537 (2) | 551 (1) |
| $N3^+$ | 440 (61) | 440 (28) | 440 (55) | 440 (42) | 440 (100) | 454 (90) | 440 (44) | 440 (38) | 454 (65) | 440 (56) | 454 (48) |
| | 355 (35) | 355 (18) | 355 (32) | 355 (23) | 355 (46) | 369 (34) | 355 (26) | 355 (24) | 369 (34) | 355 (32) | 369 (23) |
| | 242 (78) | 242 (31) | 242 (55) | 242 (52) | 242 (62) | 256 (100) | 242 (49) | 242 (44) | 256 (70) | 242 (67) | 256 (56) |
| | 128 (31) | 128 (12) | 128 (26) | 128 (21) | 128 (21) | 128 (37) | 128 (19) | 128 (17) | 128 (20) | 128 (36) | 128 (15) |
| $[HC3, H]^+$ | | | | | | | | | | | |
| | 749 (1) | | 733 (1) | | 761 (1) | | | | 777 (1) | 747 (1) | |
| | 664 (1) | | 648 (1) | | | | | | | | |
| | | | | | 563 (2) | 549 (1) | | | 579 (2) | | |
| | 466 (1) | 466 (1) | 450 (2) | 450 (1) | 464 (4) | 450 (2) | 480 (1) | | 480 (2) | | 464 (1) |
| | 369 (19) | 369 (13) | 353 (17) | 353 (16) | 367 (39) | 353 (36) | 383 (14) | 383 (13) | 383 (17) | 367 (18) | 367 (12) |
| | 284 (7) | 284 (3) | 268 (12) | 268 (9) | 282 (16) | 268 (21) | 298 (5) | 298 (5) | 298 (6) | 282 (9) | 282 (8) |
| | 185 (9) | 185 (3) | 169 (10) | 169 (10) | 169 (24) | 169 (42) | 185 (6) | 185 (7) | 185 (8) | 169 (14) | 169 (12) |
| $[HC2, H]^+$ | | | | | 609 (2) | 609 (2) | | | 609 (1) | 595 (1) | |
| | | | | | 492 (2) | 492 (2) | | | | | |
| | 381 (5) | 395 (3) | 381 (4) | 395 (3) | 395 (11) | 395 (13) | 381 (4) | 395 (3) | 395 (5) | 381 (4) | 395 (4) |
| | 296 (6) | 310 (4) | 296 (6) | 310 (5) | 310 (11) | 310 (15) | 296 (5) | 310 (5) | 310 (7) | 296 (6) | 310 (5) |
| | 183 (12) | 197 (5) | 183 (11) | 197 (7) | 197 (13) | 197 (29) | 183 (8) | 197 (7) | 197 (9) | 183 (11) | 197 (8) |
| $[HC1, H]^+$ | 215 (15) | 215 (6) | 215 (12) | 215 (9) | 215 (20) | 215 (41) | 215 (9) | 215 (8) | 215 (12) | 215 (13) | 215 (7) |

**Table 2** Chemical shifts ($\delta$/ppm) and $^3J_{NH,C\alpha H}$ coupling constants ($J$/Hz) of the amide protons for the isolated harzianins HC (300.13 MHz; 296 K; $CD_3OH$)

| Peptide | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Position | **HC I** | **HC III** | **HC VI** | **HC VIII** | **HC IX** | **HC X** | **HC XI** | **HC XII** | **HC XIII** | **HC XIV** | **HC XV** |
| $U^1$ | 8.686 | 8.678 | 8.679 | 8.669 | 8.671 | 8.634 | 8.677 | 8.671 | 8.638 | 8.670 | 8.636 |
| $N^2$ ($Q^2$)[a] | 8.588 (6.1) | 8.594 (6.1) | 8.591 (6.1) | 8.592 (6.1) | 8.594 (6.1) | 9.189[a] (4.8) | 8.593 (6.0) | 8.589 (6.2) | 9.146[a] (4.5) | 8.594 (6.1) | 9.183[a] (4.7) |
| $L^3$ | 8.157 (8.1) | 8.164 (8.1) | 8.140 (8.2) | 8.143 (8.3) | 8.151 (8.3) | 8.173 (8.3) | 8.169 (8.3) | 8.168 (8.3) | 8.194 (8.6) | 8.152 (8.1) | 8.176 (8.2) |
| $U^4$ | 7.847 | 7.843 | 7.837 | 7.834 | 7.843 | 7.873 | 7.860 | 7.859 | 7.874 | 7.845 | 7.877 |
| $A^6$ ($S^6$)[b] | 7.884[b] (7.0) | 7.889[b] (6.7) | 7.809 (7.1) | 7.815 (7.0) | 7.780 (7.0) | 7.828 (6.5) | 7.860[b] (5.9) | 7.853[b] (6.0) | 7.884[b] (6.9) | 7.771 (6.5) | 7.795 (7.1) |
| $I^7$ ($V^7$)[c] | 7.463[c] (9.1) | 7.469[c] (8.9) | 7.475[c] (9.1) | 7.465[c] (9.0) | 7.427 (9.2) | 7.465 (9.1) | 7.421 (9.0) | 7.427 (9.2) | 7.439 (8.4) | 7.418 (9.2) | 7.426 (9.2) |
| $U^8$ | 7.983 | 7.982 | 7.860 | 7.856 | 7.918 | 7.859 | 7.987 | 7.984 | 8.018 | 7.921 | 7.921 |
| $J^{10}$ ($U^{10}$)[d] | 7.760[d] | 7.522[d] | 7.782[d] | 7.540 | 7.537 | 7.540 | 7.758[d] | 7.517 | 7.520 | 7.782[d] | 7.540 |
| $L^{11}$ | 7.611 (8.1) | 7.651 (8.5) | 7.619 (8.5) | 7.654 (8.6) | 7.640 (8.6) | 7.654 (8.6) | 7.601 (8.3) | 7.628 (8.7) | 7.631 (8.4) | 7.611 (8.5) | 7.637 (8.6) |
| $U^{12}$ | 7.814 | 7.814 | 7.850 | 7.842 | 7.843 | 7.840 | 7.860 | 7.874 | 7.840 | 7.854 | 7.833 |
| $Lol^{14}$ | 7.535 (8.4) | 7.534 (8.1) | 7.537 (8.8) | 7.528 (8.3) | 7.540 (8.8) | 7.525 (8.2) | 7.559 (9.0) | 7.628 (8.7) | 7.535 (9.0) | 7.539 (9.0) | 7.527 (7.9) |
| $\delta_a$ $N^2/\varepsilon_a$ $Q^2$ | 7.624 | 7.734 | 7.740 | 7.736 | 7.733 | 7.691 | 7.733 | 7.701 | 7.727 | 7.734 | 7.700 |
| $\delta_s$ $N^2/\varepsilon_s$ $Q^2$ | 6.977 | 6.993 | 6.979 | 6.992 | 6.989 | 6.990 | 6.992 | 6.983 | 6.987 | 6.991 | 6.989 |

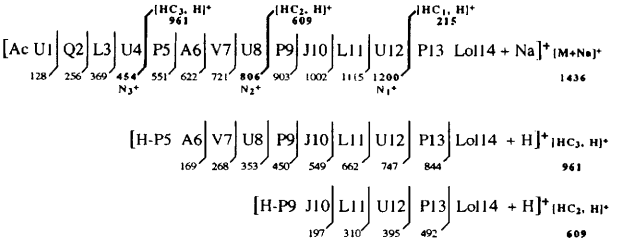


**Fig. 4** Mass-fragmentation pattern of harzianin **HC X** exhibiting the preferential cleavages at the three Aib–Pro bonds leading to the complementary $N_i^+$ and $[HC_i, H]^+$ ions and the expected fragment ions

Val/Iva. The differentiation of Ile and Leu by FAB MS/MS using a double-focusing mass spectrometer has been previously reported,[24,25] but such a tentative determination experienced on the peptaibol trichosporin TS-B-VIb[16] did not succeed.

**(c) $^1$H NMR spectroscopy.** Complete and unambiguous sequence determination of harzianins HC arose from the standard $^1$H NMR strategy, including 2D homonuclear chemical-shift correlation (COSY) and 2D homonuclear Hartmann–Hahn magnetisation transfer (HOHAHA) experiments for assignment of the spin-system resonances to residue types and rotating-frame nuclear Overhausser enhancement spectroscopy (ROESY) for sequential assignments and sequence determination.[26] Cross-peaks between $NH_i$ and $C\alpha H_i$ protons were easily identified by examination of the COSY spectra. HOHAHA experiments with a mixing time of 120 ms were used to correlate the side-chain spin systems with the NH–C$\alpha$H cross peaks (Fig. 5). This was particularly useful for assignment of the side-chain protons of leucines and isoleucines and for the $\beta$ and $\gamma$ protons of prolines.

ROESY spectra providing sequential inter-residue dipolar couplings were performed for **HC III**, **HC VIII** (Fig. 6), **HC IX** and **HC X**. The dNN($i$, $i$ + 1) connectivities (Fig. 6A) afforded three partial sequences interrupted by the prolines at positions 5, 9 and 13; the complete sequences (Fig. 1) arose from observation of the d$\alpha$N($i$, $i$ + 1) connectivities which confirmed the results of the FAB-MS study and afforded the location of Ile (for **HC IX**) and Val (for **HC III**, **HC VIII**, **HC X**) at position 7 in the sequences. Sequential assignments of their amide protons, together with $^3J_{NH,C\alpha H}$ coupling-constant values are given in Table 2. Unequivocal sequences of the other isolated HC were proposed from comparison of 1D and 2D NMR data, particularly of their amide (Table 2) and $\alpha$ proton chemical shifts.

## Conformation of harzianins HC

The conformation of harzianins HC was tentatively examined in methanol solution, based on several experimental criteria including circular dichroism (CD) data and NMR parameters such as inter-residue NOE connectivities, $^3J_{NH,C\alpha H}$ coupling constants and NH temperature coefficients. The inter-residue NOE patterns observed for **HC III**, **HC VIII**, **HC IX** and **HC X** in the ROESY spectra were completely similar. A helical structure was obvious from the series of strong dNN($i$, $i$ + 1) and d$\beta$N($i$, $i$ + 1) and low-range d$\alpha$N($i$, $i$ + 1) all along the sequence, accompanied by d$\alpha$N($i$, $i$ + 3) mainly in the $Pro^5$–$Aib^{12}$ region (Fig. 6). The prevalence of a helix stabilised by 4→1 intramolecular hydrogen bonds ($3_{10}$-helix type) over an $\alpha$-helix (which is stabilised by 5→1 hydrogen bonds) arose from the presence of d$\alpha$N($i$, $i$ + 2) NOEs in the $Leu^3$–$Aib^{12}$ region and the absence of d$\alpha$N($i$, $i$ + 4) all along the sequence. The amide proton thermal coefficients ($\Delta\delta/\Delta T_{NH}$) which were very similar for all the peptides (Table 3) were consistent with such a hydrogen-bonding pattern. Also similar were the $^3J_{NHC\alpha H}$ coupling constants for all the analysed HC (Table 2): values of 6–7 Hz were observed for residues at positions 2 and 6, located three residues before a proline, whereas higher values (8–9 Hz) characterised the other residues, $Leu^3$, $Val/Ile^7$, $Leu^{11}$ (two residues before a Pro) and $Leuol^{14}$. Such unusual values for a helical structure and the observed periodicity suggested a regular helical structure made of a succession of $\beta$-turns, as proposed in the $\beta$-bend ribbon spiral, a structure observed in the case of alternation of Aib and Pro residues.[27,28] In addition, the results indicated that the substitutions Asn → Gln, Ala→Ser, Val→Ile and Aib→Iva at positions 2, 6, 7, 10 respectively, which are responsible for the microheterogeneity of harzianins HC, were without significant influence on the secondary structure.

## Conclusions

Although a close identity of the two examined *T. harzianum* strains appeared from morphological and biological criteria, their harzianin HC contents were quantitatively and qualitatively different, as exemplified by HPLC analyses. This observation points to the usefulness of peptaibols as fingerprints for the characterisation of *Trichoderma* fungi.

The 14-residue pore-forming peptaibols, harzianins HC, the sequences of which include three Aib-Pro motives at positions 4–5, 8–9 and 12–13 separated by dipeptides, appear to exhibit a regular helical structure stabilised by intramolecular hydrogen bonds of the 4→1 type. The detailed study of the solution structure of such peptides by NMR and molecular dynamics

**Table 3** Temperature coefficients ($-\Delta\delta/\Delta T$, ppb/K) of amide protons of harzianins **HC III, HC VIII, HC IX, HC X, HC XI, HC XIV** (n.d., not determined because of saturation transfer) (300.13 MHz; $CD_3OH$).

| | Peptide | | | | | |
|---|---|---|---|---|---|---|
| Position | **HC III** | **HC VIII** | **HC IX** | **HC X** | **HC XI** | **HC XIV** |
| $U^1$ | 8.2 | 6.6 | 7.5 | 7.0 | 7.0 | 8.4 |
| $N^2$ ($Q^2$)[a] | 6.3 | 5.3 | 5.5 | 5.8[a] | 5.1 | 6.2 |
| $L^3$ | 3.1 | 2.7 | 2.9 | 2.8 | 2.7 | 3.0 |
| $U^4$ | 2.8 | 2.2 | 2.4 | 2.3 | 2.8 | 2.6 |
| $A^6$ ($S^6$)[b] | 1.4[b] | 1.7 | 1.3 | 1.3 | 1.0[b] | 1.3 |
| $I^7$ ($V^7$)[c] | 2.0[c] | 1.7[c] | 1.8 | 1.5 | 1.6 | 1.8 |
| $U^8$ | 3.1 | 2.0 | 2.9 | 2.3 | 2.7 | 3.0 |
| $J^{10}$ ($U^{10}$)[d] | 2.0 | 2.0 | 1.8 | 1.8 | 2.1[d] | 2.8[d] |
| $L^{11}$ | 2.8 | 2.6 | 2.5 | 2.3 | 2.2 | 2.6 |
| $U^{12}$ | 1.6 | 1.3 | 1.5 | 1.2 | 1.5 | 1.8 |
| $Lol^{14}$ | 2.6 | 2.2 | 2.3 | 2.0 | 2.2 | 2.5 |
| $\delta_a$ $N^2/\varepsilon_a$ $Q^2$ | n.d. | 5.7 | 6.4 | 5.6 | 5.6 | n.d. |
| $\delta_s$ $N^2/\varepsilon_s$ $Q^2$ | 8.4 | 7.0 | 7.3 | 6.5 | 6.8 | 8.3 |

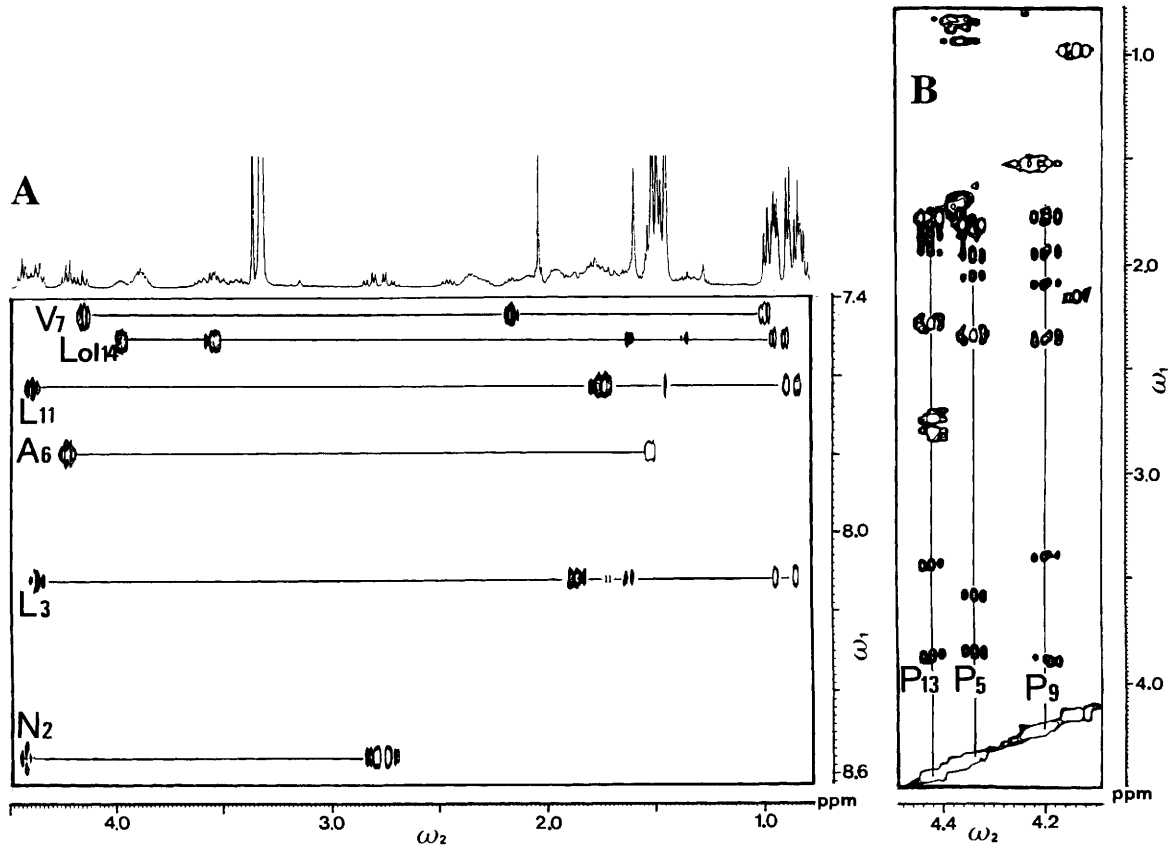


**Fig. 5** Parts of the HOHAHA spectrum of **HC VIII** ($CD_3OH$; 296 K; mixing time 120 ms): **A** $\omega 2$ = 4.5–0.8 ppm, $\omega 1$ = 8.6–7.4 ppm; **B** $\omega 2$ = 4.4–4.1 ppm, $\omega 1$ = 4.5–0.8 ppm

calculations is now under investigation in order to correlate the exhibited channel-forming properties to the secondary structure.

## Experimental

### Isolation of harzianins HC

The *T. harzianum* strains (M-903614; M-903603), collected in Uruguay, were obtained from the 'Collection de Souches fongiques du Muséum National d'Histoire Naturelle' (Paris); they were maintained and cultivated as previously described.[5,6] Typical 20 $dm^3$ cultures disposed into 120 Roux flasks (1 $dm^3$), each containing 160–170 $cm^3$ of the sterilised usual synthetic medium[6] were incubated for 11 days at 27 °C. The filtered fermentation broths of the two *T. harzianum* strains were independently extracted three times with butan-1-ol, to give, after removing the solvent under reduced pressure, crude extracts (1.6 g and 1.2 g, respectively) for strains M-903614 and M-903603. The residues were submitted to gel filtration on Sephadex LH 20 with methanol as eluent. The crude peptide mixtures (M-903614: 215 mg; M-903603: 468 mg) were then chromatographed over silica gel (Kieselgel 60 H Merck, Darmstadt) with $CH_2Cl_2$–MeOH (90:10 to 50:50) as eluent. Harzianins HC (M-903614: 130 mg; M-903603: 274 mg) eluted

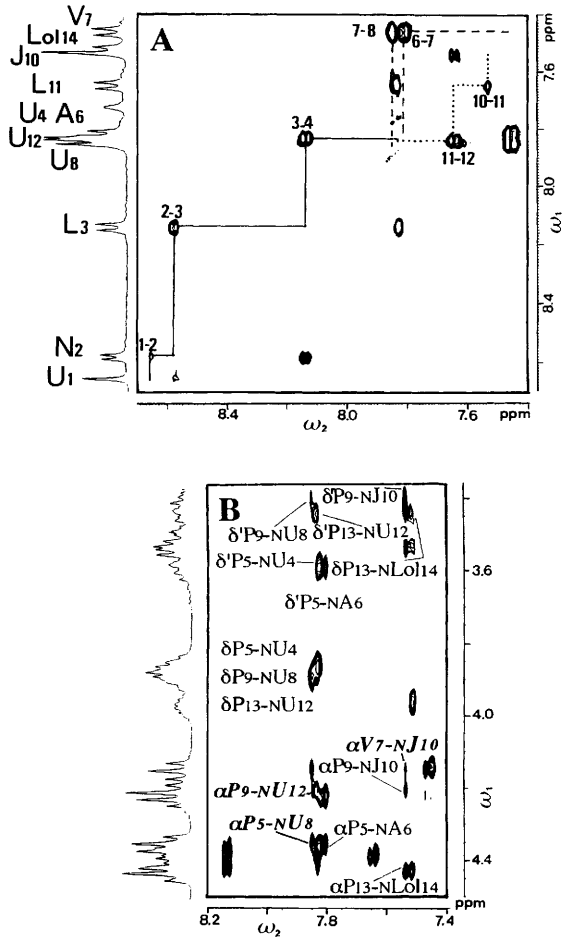


**Fig. 6** Parts of the ROESY spectrum of **HC VIII** ($CD_3OH$; 296 K; mixing time 300 ms): **A** $\omega 2 = \omega 1 = 8.7$–$7.4$ ppm; **B** $\omega 2 = 8.2$–$7.4$ ppm, $\omega 1 = 4.5$–$3.3$ ppm. Italic bold-faced characters indicate the $d\alpha N(i, i + 3)$ connectivities.

first [$CH_2Cl_2$–MeOH (80:20)] followed by trichorzins HA (M-903614 (42 mg); M-903603 (124 mg); $CH_2Cl_2$–MeOH (70:30)].

### HPLC separations

were carried out with a Waters liquid chromatograph (6000 A and M45 pumps, a 680 automated solvent programmer, a WISP 701 automatic injector and a 481 UV–Vis detector) on a semi-preparative C18 column (Spherisorb ODS2, 5 μ, 7.5 × 300 mm; AIT France); eluent methanol–water (82.5:17.5); flow rate 2 $cm^3\ min^{-1}$; $t_R$ (min): **HC I** = 29, **HC III** = 31, **HC VI** = 43, **HC VIII** = 49, **HC IX** = 58, **HC X** = 56, **HC XI** = 34, **HC XII** = 38, **HC XIII** = 42, **HC XIV** = 51, **HC XV** = 67. Purity was checked on an analytical column (3.5 × 250 mm); eluent methanol–water (81:19); flow rate 1 $cm^3\ min^{-1}$.

### Amino acid analysis

Hydrolysis of HC peptides (6 mol $dm^{-3}$ HCl; 110 °C, $N_2$) was followed by derivatisation of the given amino acids and amino alcohols, as previously described.[5,6] Classically, 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-Ala 14.8 ($\alpha = 1.16$), L-Asp 29.5 ($\alpha = 1.01$), L-Glu 33.2 ($\alpha = 1.05$), L-Ile 20.4 ($\alpha = 1.10$), D-Iva 11.2 ($\alpha = 1.02$), L-Leu 24.2 ($\alpha = 1.11$), L-Leuol 22.2 ($\alpha = 0.98$), L-Ser 23.0 ($\alpha = 1.05$), L-Val 17.7 ($\alpha = 1.08$). A special temperature programme was used for separation of the proline D,L enantiomers: 50–110 °C, 3 °C $min^{-1}$; plateau at 110 °C for 10 min; 100–190 °C, 10 °C $min^{-1}$; $t_R$ (α): L-Pro 25.1 ($\alpha = 1.02$). Amino acid compositions and absolute configurations of amino acids and Leuol:

**HC I**: Aib (5), L-Asx (1), L-Leu (2), L-Leuol (1), L-Pro (3), L-Ser (1), L-Val (1)

**HC III**: Aib (4), L-Asx (1), D-Iva (1), L-Leu (2), L-Leuol (1), L-Pro (3), L-Ser (1), L-Val (1)

**HC VI**: Aib (5), L-Ala (1), L-Asx (1), L-Leu (2), L-Leuol (1), L-Pro (3), L-Val (1)

**HC VIII**: Aib (4), L-Ala (1), L-Asx (1), D-Iva (1), L-Leu (2), L-Leuol (1), L-Pro (3), L-Val (1)

**HC IX**: Aib (4), L-Ala (1), L-Asx (1), L-Ile (1), D-Iva (1), L-Leu (2), L-Leuol (1), L-Pro (3)

**HC X**: Aib (4), L-Ala (1), D-Iva (1), L-Glx (1), L-Leu (2), L-Leuol (1), L-Pro (3), L-Val (1)

**HC XI**: Aib (5), L-Asx (1), L-Ile (1), L-Leu (2), L-Leuol (1), L-Pro (3), L-Ser (1)

**HC XII**: Aib (4), L-Asx (1), L-Ile (1), D-Iva (1), L-Leu (2), L-Leuol (1), L-Pro (3), L-Ser (1)

**HC XIII**: Aib (4), L-Ile (1), D-Iva (1), L-Glx (1), L-Leu (2), L-Leuol (1), L-Pro (3), L-Ser (1)

**HC XIV**: Aib (5), L-Ala (1), L-Asx (1), L-Ile (1), L-Leu (2), L-Leuol (1), L-Pro (3)

**HC XV**: Aib (4), L-Ala (1), L-Ile (1), D-Iva (1), L-Glx (1), L-Leu (2), L-Leuol (1), L-Pro (3).

### FAB Mass spectrometry

Positive-ion FAB mass spectra were recorded on a ZAB2-SEQ (VG Analytical, Manchester, UK) mass spectrometer equipped with a standard FAB source and a caesium ion gun operating at 35 kV. Peptide methanolic solutions were mixed with α-monothioglycerol or 3-nitrobenzyl alcohol used as matrices. The resolution was 2000.

### NMR Spectroscopy

A 0.4 $cm^3$ amount of a 15 mmol $dm^{-3}$ methanolic ($CD_3OH$) solution of HC peptides in a 5 mm tube (Wilmad) was used for all the NMR experiments. Proton NMR spectroscopy experiments were routinely conducted at 296 K on a Bruker AC 300 or an AM 400 spectrometer equipped with Aspect 3000 and X 32 computers, respectively. Spectra were obtained by solvent presaturation and referenced to the central component of the quintet due to the $CD_2H$ resonance of methanol at 3.313 ppm, downfield from TMS. The HOHAHA (300.13 MHz) and ROESY (400.13 MHz) experiments were acquired with mixing times of 120 and 300 ms, respectively; typically, 256 experiments of 96–128 scans each were performed; relaxation delay 1.5 s; size 2K; 9.5 ppm spectral width in $\omega 2$; zero filling to 1K in $\omega 1$; sine bell ($\pi/2$) in both dimensions.

### CD spectrum

This spectrum of **HC IX** was recorded with a Jobin-Yvon CD6 dichrograph, with a 0.1 mm path cell at 22 °C (1.15 mmol $dm^{-3}$; $CH_3OH$); $\lambda$ (nm) $[\theta]_M$ (deg $cm^2\ dmol^{-1}$) 192.8 (+25 100) and 205.4 (−101 000).

### Antibacterial activity

The antibacterial activity of trichorzin HA and harzianin HC mixtures arising from strains M-903614 and M-903603 were examined against *S. aureus* (strain 209P) and *E. coli* (strain RL 65) by the agar diffusion test, using 6 mm diameter pits. The peptide samples were dissolved in dimethyl sulfoxide (DMSO) such as to give a 4 mg $cm^{-3}$ solution. Other concentrations were obtained by dilutions. Aliquots (50 $mm^3$) of each solution were deposited into the pits (500–50 μg by pit). Inhibition zones were measured after 24 h of incubation at 37 °C.

*S. aureus*: Inhibition diameters (mm) [concentration (μg pit$^{-1}$)]; HC: 17 (500); 17 (200); 15 (100); 12 (50); n.i. (25); HA: 20 (500); 20 (200); 19 (100); 18 (50); 17 (25); 13 (12); 9 (6); n.i. (3). Identical results were obtained with the HA and HC mixtures arising from the two strains. *E. coli*: no inhibition for 500 μg pit$^{-1}$.

### Antifungal activity

The antifungal properties of trichorzins HA and harzianins HC were examined against *Sclerotium cepivorum* grown on malt-agar medium in micro-plates of 4.5 cm diameter. The appropriate amounts of peptide mixtures were dissolved in MeOH in order to give a final peptide concentration of 100 μg cm$^{-3}$ of culture medium and a 0.5% amount of MeOH. The *S. cepivorum* strain was sown in the middle of the plate and incubated for 72 h at 25 °C; the control plates without peptides were then completely spread over. Four plates were prepared for each peptide mixture. Growth diameters were then measured and percentage inhibition determined as regards to the control plates. Percentage inhibition, [pept] = 100 μg cm$^{-3}$; HA: 75; HC: 40. Identical results were obtained with the HA and HC peptide samples arising from the two *T. harzianum* strains.

### Membrane-modifying properties

**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 previously described.[10] Fluorescence spectra were measured at 20 °C on an Aminco SPF 500 spectrofluorometer. The peptide-induced release of intravesicular content was monitored by the well-known method introduced first by Weinstein,[29] that uses the property of quenching relief upon dilution of an encapsulated fluorescent probe, CF. CF-entrapped small unilamellar vesicles (SUV) were prepared, as previously described,[10] by sonication of an egg PC/cholesterol (7:3) mixture ([lip] = 0.6 mmol dm$^{-3}$). The SUV obtained by sonication were separated from unencapsulated CF by gel filtration (Sephadex G75). Leakage kinetics were obtained for different peptide:lipid molar ratios obtained by adding aliquots of methanolic solutions of peptides (methanol concentration kept below 0.5% by volume). For the different isolated HC, 50% CF leakage in 20 min was induced for concentrations ranging between 2 × 10$^{-6}$ and 8 × 10$^{-5}$ mol dm$^{-3}$.

**Bilayer conductances.** Phospholipids, 1-palmitoyl-2-oleoylllecithin (POPC), dioleoylphosphatidylethanolamine (DOPE) and dimyristoylphosphatidylcholine (DMOPC) were from Avanti Polar Lipids (Birmingham, AL). Both macroscopic (room temp.) and single-channel conductance (17.5 °C) experiments were conducted as previously described.[10,30] The lipid mixture (POPC–DOPE 7:3) was dissolved in hexane either at 5–10 mg cm$^{-3}$ or 0.5–2 mg cm$^{-3}$ in macroscopic and patch-clamp configuration, respectively. The peptides in methanolic solutions were added to the *cis*- or positive side of the bilayer.

Macroscopic experiments showed concentration- and voltage-dependent conductances for 10$^{-6}$ to 6 × 10$^{-6}$ mol dm$^{-3}$ concentrations of **HC III** and **HC VIII**.

For single-channel current fluctuation recording, lipid bilayers were preformed at the tip of patch-clamp pipettes by the tip-dip method; channels with great lifetime and amplitude (≈3000 ps) were observed for **HC III** and **HC VIII** (2.5 to 5 × 10$^{-8}$ mol dm$^{-3}$, −100 to −130 mV); for **HC III**, they were accompanied by more rapid events with lower amplitude (≈700 ps).

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