Harzianin HB I, an 11-residue peptaibol from *Trichoderma harzianum*: isolation, sequence, solution synthesis and membrane activity

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Harzianin HB I is a minor component of the peptaibol mixture biosynthesized by a *Trichoderma harzianum* strain. It is isolated by a multi-step chromatography procedure, including HPLC; its sequence has been elucidated by LSIMS and two-dimensional ^{1}H NMR experiments. This 11-residue peptaibol is an analogue of the 14-residue peptaibol harzianin HC IX, resulting from the deletion of the tripeptide Aib-Pro-Ala. The CD and NMR data (NOE data and amide proton temperature coefficients) are similar to those of HC IX, suggesting a right-handed helix-type conformation for the two peptides. Harzianin HB I was synthesized by the solution-phase method using BOP as coupling reagent. The membrane properties examined on liposomes are compared with those of other known peptaibols.

Introduction

Peptaibols biosynthesized by *Trichoderma* soil fungi are amphipathic linear peptides with a high proportion of α,α-dialkylated amino acids such as α-aminoisobutyric acid (Aib, U), an N-terminal acetylated residue and a C-terminal amino alcohol. They can be classified into the long-sequence group containing 18 to 20 residues,[1–5] the short-sequence group with 11 to 16 residues[6,7] and the lipopeptaibols with 7 to 11 residues and an N-terminal amino acid acylated by a lipid chain.[8,9] The main interest in such peptides stems from their ability to form voltage-gated ion channels in planar lipid bilayer membranes,[10,11] and therefore they can be viewed as a prototypic pore. In the absence of a voltage, peptaibols induce permeabilization of liposomes;[12,13] long-sequence ones being more efficient than short-sequence ones. Their membrane activity is modulated by both the length and hydrophobicity of the sequence. The known biological properties of these peptides are their antibiotic[14] and catecholamine secretion-inducing abilities[15,16] which have been suggested to be related to their membrane activity. Recently, we isolated peptaibols organised in helical structures which still have significant membrane activity in spite of their short sequences, such as the 11-residue lipopeptaibol GA IV and the proline-rich 14 residue peptaibols, harzianins HC.[7]

From a Uruguayan strain of *Trichoderma harzianum* we previously isolated two groups of peptaibols, trichorzins HA and harzianins HC, containing 18 and 14 residues respectively. From the particularly complex mixture of the 14-residue peptides, a minor component of the same polarity, harzianin HB I, was isolated. Its structure was determined from liquid secondary ion mass spectrometry (LSIMS) and NMR spectroscopy. It was synthesized in order to test its antibiotic and membrane properties. Harzianin HB I was shown to modify slightly the permeability of liposomes and induce voltage-gated macroscopic conductance in planar bilayers.

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

Sequence of harzianin HB I

Results and discussion

Isolation and characterization of HB I[17]

The culture broth extract of the Uruguayan M-903603 *T. harzianum* strain was fractionated by exclusion chromatography; the peptide fraction was further chromatographed over silica gel to yield two peptaibol groups of different polarity. The first group was composed of 18-residue peptaibols, the trichorzins HA,[5,14] and the second one mostly of the 14-residue harzianins HC.[7] The HPLC chromatogram of the crude HC mixture was very complex, showing more than 16 peaks, one of them being assigned to a minor component, HB I. Its isolation was undertaken by repetitive semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and its purity was checked by analytical HPLC (Fig. 1). Further LSIMS and ^{1}H NMR experiments confirmed HB I to be homogeneous. It reacted neither with diazomethane nor with ninhydrin, indicating the presence of neither free carboxy nor amino groups. The presence of a sharp singlet at δ_H 2.02 in the 1D NMR spectrum suggested an acetylated N-terminal residue, as usually found in peptaibols.

The amino acid composition and absolute configuration of the residues resulted from GLC analysis of the total acid hydrolysate derivatives on a Chirasil L-Val capillary column: Aib (3), L-Asn (1), L-Ile (1), D-Iva (1), L-Leu (2), L-Pro (2), L-Leuol (1).

Sequence determination of HB I

Positive liquid secondary-ion mass spectrometry [(+)-LSIMS]. The amino acid sequence of HB I was examined by positive LSIMS. A relative molecular mass of 1160 was assigned to HB I from the sodium ion adduct $[M + Na]^+$ observed at *m/z* 1183, in agreement with the postulated molecular mass arising from the amino acid and amino alcohol composition.

The LSIMS spectrum exhibited several abundant b-type acylium ions[18] at *m/z* 947, 553, 468, 355, 242 and 128 and two lower-abundance ion regions between *m/z* 553 and 947, and between *m/z* 947 and 1183. This fragmentation pattern suggested the presence of two labile Aib–Pro bonds in HB I. The series of b-type ions ranging from *m/z* 128 to 553 gave the N-terminal pentapeptide sequence. The mass difference of 394 amu between *m/z* 947 and 553 was in agreement with the formation of the tetrapeptide ion observed at *m/z* 395 and

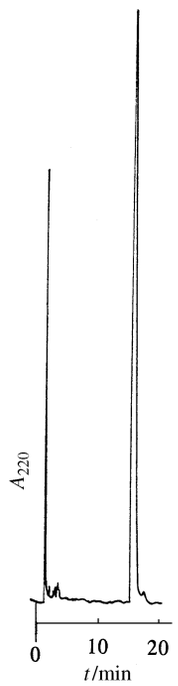
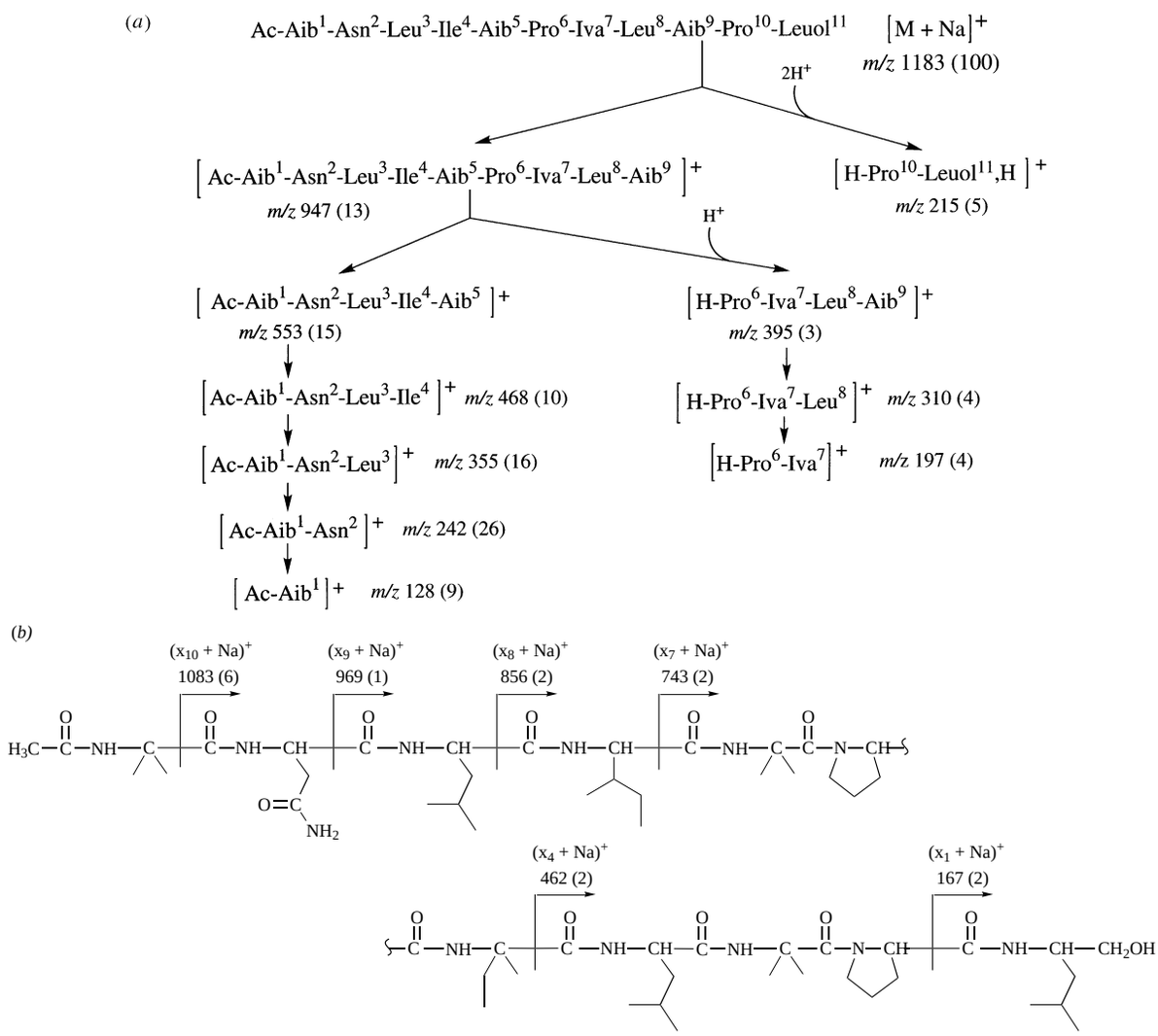


Fig. 1 HPLC chromatogram of purified natural harzianin HB I (Spherisorb ODS2, 5μm), 4.6 × 250 mm, MeOH–water (83:17), flow rate 1 cm^3 min^{-1}; absorption monitored at 220 nm

obtained *via* cleavage of the Aib[5]–Pro[6] amide bond.[6,19] The C-terminal dipeptide, resulting from cleavage of the Aib[9]–Pro[10] amide bond was observed as a y_2 ion at *m/z* 215. Furthermore, a series of weak and unusual but significant $[x_n + Na]^+$ ions at *m/z* 1083, 969, 856, 743, 462 and 167 was observed and allowed the confirmation of the HB I sequence.[18] Nevertheless, the respective locations of the isomeric residues Leu/Ile remained to be determined.

^{1}H NMR spectroscopy. The complete amino acid sequence of HB I was determined by two-dimensional ^{1}H NMR spectroscopy. Assignments of ^{1}H chemical shifts to specific protons of individual residues (Table 1) were obtained by 2D homonuclear (COSY) and phase-sensitive 2D total (TOCSY) chemical-shift correlation experiments, showing complete spin systems of one Asn, two Pro and one Leuol (Fig. 2), as well as those of one Ile and two Leu residues in agreement with ^{13}C resonances of one Ile C^{α} at δ_C 64.1 and two Leu C^{α} at δ_C 53.2 and 54.1 (data not shown). The sequence-specific assignments of the backbone NH proton signals arose from the rotating-frame nuclear Overhauser effect (ROESY) spectrum, and were completely carried out by using inter-residue connectivities $d_{NN}(i,i+1)$ and $d_{\alpha N}(i,i+1)$ [Fig. 3(*a*)]. The lowest-field singlet NH proton showing a cross-peak with the acetyl $C^{\beta}H_3$ protons was assigned to Aib[1]. Then, $d_{NN}(i,i+1)$ connectivities extending from Asn[2] to Aib[5] and from Iva[7] to Aib[9] confirmed the location of Ile at position 4. Finally, sequential assignment of Pro[6] and Pro[10] arose from $d_{N\delta}(i,i+1)$ connectivities between their δ protons and the amide protons of Aib[5] and Aib[9] [Fig. 3(*b*)].



Mass fragmentation pattern of harzianin HB I (positive-ion LSIMS), exhibiting the b_n ions (*a*) and the x_n + Na ions (*b*) (relative intensities in parentheses)

Table 1 ^{1}H sequential and stereospecific assignments of harzianin HB I (500.13 MHz; CD_3OH; 296 K). Chemical shifts (ppm) are given to the nearest three or two decimal places when obtained from 1D or 2D spectra, respectively; $^3J_{NH\text{-}C\alpha H}$ coupling constants (Hz) arise from the 1D spectrum, and are given in parentheses

	Residue	NH	α-H	β-H/β-Me	Other groups
	Ac				Me 2.027 s
	Aib[1]	8.679 s		*pro-R* 1.46*/*pro-S* 1.450	
	Asn[2]	8.610 d (5.6)	4.38	2.76	ε *syn* 7.04/ε *anti* 7.77
	Leu[3]	8.111 d (7.1)	4.25	1.93/1.56	γ 1.74/δ^1 0.89/δ^2 0.96
	Ile[4]	7.356 d (9.1)	4.22	1.95	γ 1.55/γ′ 1.32/γ-Me 0.95/δ-Me 0.86
	Aib[5]	7.806 s		*pro-R* 1.498*/*pro-S* 1.501	
	Pro[6]		4.21	*pro-R* 1.78/*pro-S* 2.35	*pro-R* γ 1.94/*pro-S* γ 2.10/*pro-R* δ 3.85/*pro-S* δ 3.41
	Iva[7]	7.484 s		2.45/1.77/β-Me 1.46	γ 0.82
	Leu[8]	7.627 d (8.5)	4.39	1.75/1.75	γ 1.75/δ^1 0.85/δ^2 0.96
	Aib[9]	7.836 s		*pro-R* 1.450*/*pro-S* 1.501	
	Pro[10]		4.42	*pro-R* 1.78/*pro-S* 2.29	*pro-R* γ 1.87/*pro-S* γ 1.93/*pro-R* δ 3.88/*pro-S* δ 3.44
	Leuol[11]	7.534 d (8.9)	3.96	1.60/1.35/3.53	γ 1.64/δ^1 0.90/δ^2 0.95

* May be exchanged.

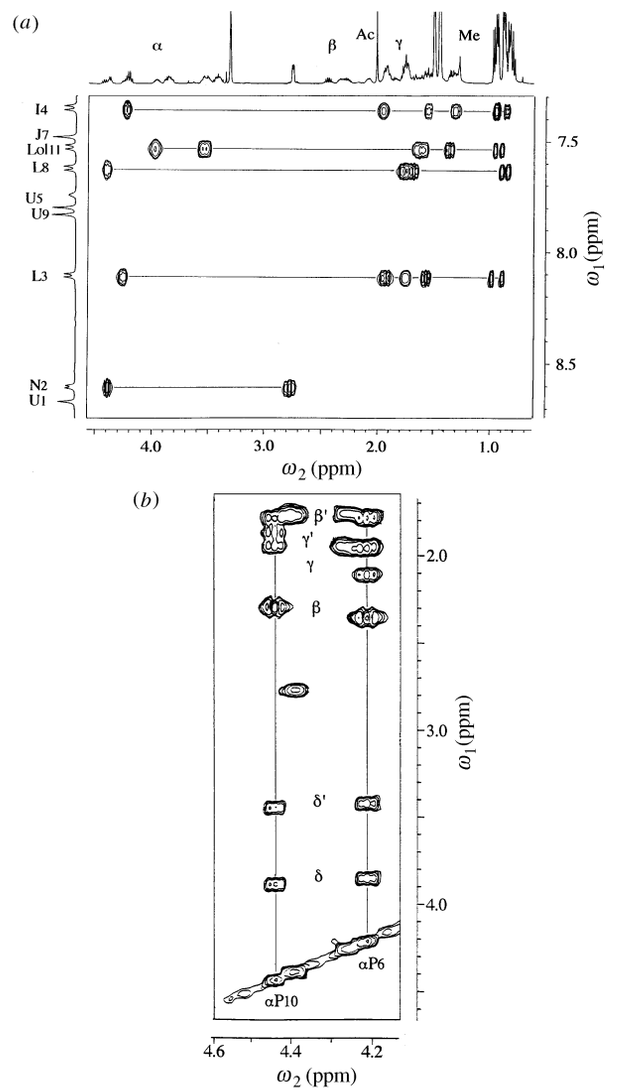


Fig. 2 Expansion of the TOCSY spectrum of HB I in CD_3OH (spin lock period 120 ms): (*a*) ω_2 = 0.6–4.5 ppm, ω_1 = 7.3–8.7 ppm; (*b*) ω_2 = 4.1–4.6 ppm, ω_1 = 1.7–4.6 ppm; spin-systems are labelled with the sequential residue positions

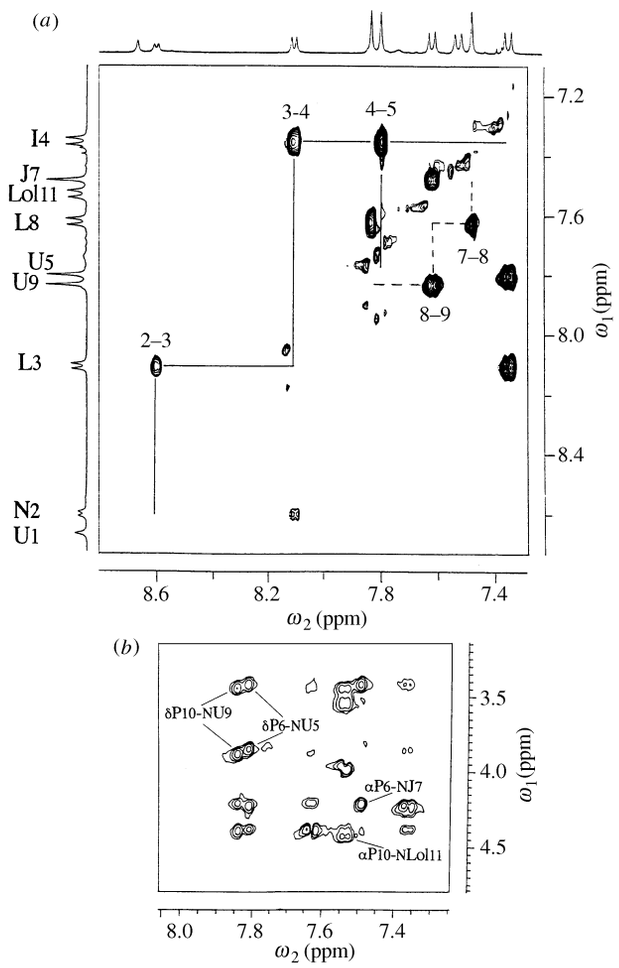


Fig. 3 Parts of the ROESY spectrum of HB I in CD_3OH (mixing time 250 ms): (*a*) ω_2 = 7.3–8.8 ppm, ω_1 = 7.1–8.7 ppm; (*b*) ω_2 = 7.3–8.0, ω_1 = 3.2–4.8 ppm

Conformation of HB I

The conformation of HB I in methanol solution was examined by CD and NMR spectroscopy, using inter-residue NOE connectivities, $^3J_{NH\text{-}C\alpha H}$ coupling constants and amide temperature coefficients. The CD spectrum of HB I showed two transitions, at 192 (+) and 205 (−) nm characteristic of a right-handed helix.

A stretch of strong sequential $d_{NN}(i,i+1)$ accompanied by $d_{\alpha N}(i,i+1)$ and by a series of medium and strong $d_{\alpha N}(i,i+3)$ all along the sequence was observed in the ROESY spectrum (Fig. 3), in agreement with a helical structure. The presence of $d_{\alpha N}(i,i+2)$ NOEs all along the sequence and the complete absence of $d_{\alpha N}(i,i+4)$ connectivities suggested a succession of turns stabilized by 4→1 intramolecular hydrogen bonds, as observed for the 3_{10}-helix. This was in agreement with the amide protons' thermal coefficients ($\Delta\delta/\Delta T_{NH}$) (Fig. 4). Little information was obtained from the $^3J_{NH\text{-}C\alpha H}$ coupling constants, as only half of the residues could give such data (Fig. 4). The Asn[2] and Leu[3] residues showed values ⩽7 Hz, whereas Ile[4], Leu[8] and Leuol[11] had higher values, around 9 Hz, apparently inconsistent

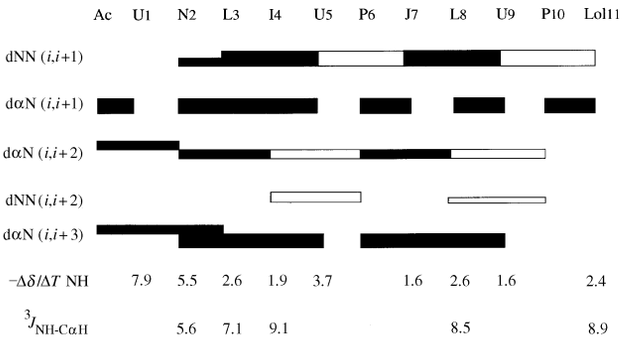


Fig. 4 Amino acid sequence of harzianin HB I (the one-letter code of amino acid residues is used with U = Aib, J = Iva, Lol = Leuol) and a survey of the NOE connectivities involving NH and CαH (dαδ connectivities observed for prolines are indicated by white boxes), of the $^3J_{NH\text{-}C\alpha H}$ coupling constants, and of the temperature coefficients of the amide protons. The observed NOEs are classified as strong, medium and weak (based on counting the cross-peak contour levels) and shown by thick, medium and thin lines, respectively.

with a helical structure. However, such values have frequently been observed for the two amino acids flanking Aib-Pro segments in α-helical peptaibols.[4,5,20–22] The studied residues in HB I have such a location in the sequence. Comparison of the thermal coefficients and coupling constants of HB I with those of the longer analogue HC IX, which contains an additional Aib-Pro-Ala tripeptide after Leu[3], showed extensive similarity of the data.[17] The results thus suggested a structure stabilized by 4→1-type intramolecular hydrogen bonds, forming a ribbon of β-turns.

Synthesis of HB I

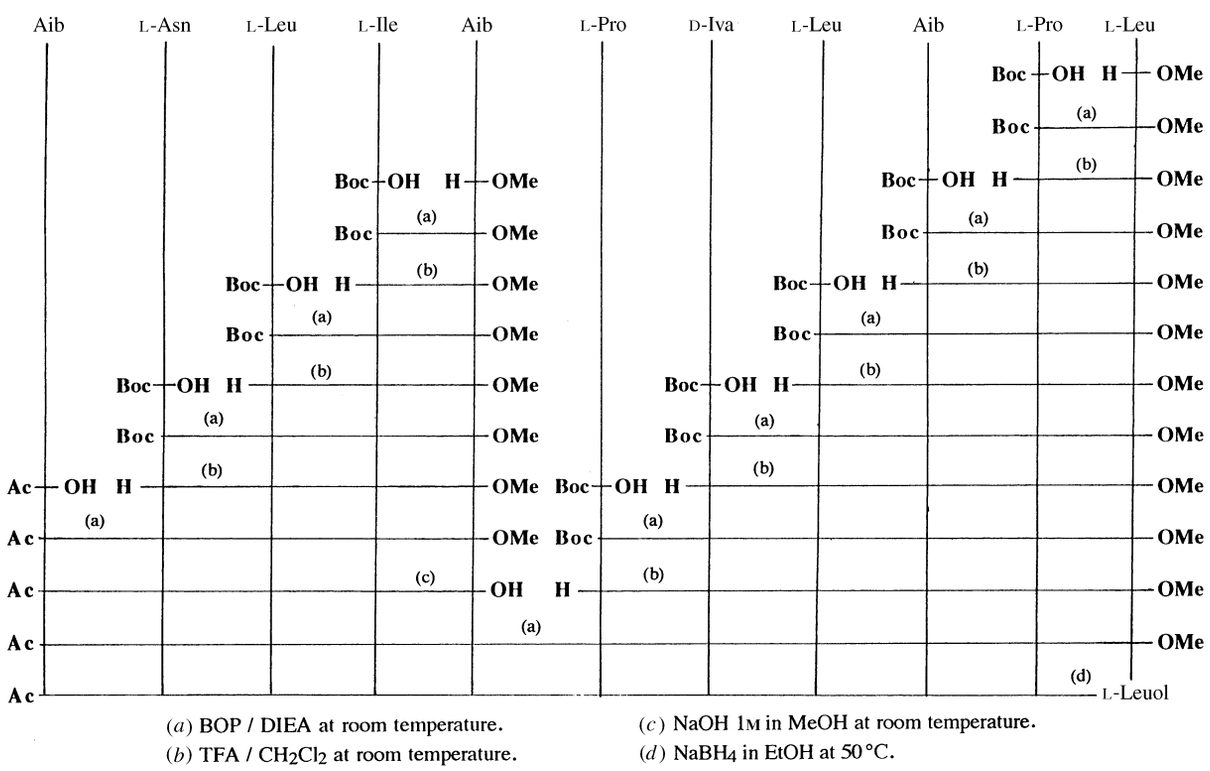
In order to make available a sufficient amount of harzianin HB I for bioassays, it was synthesized by a solution-phase method in the presence of the (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) coupling reagent in CH_2Cl_2 at room temperature, according to the synthetic route shown in Scheme 1. The penta- and hexa-peptide fragments were built up in a stepwise manner using Boc/OMe strategy. They were designed so that Aib was placed at the C-terminal position in order to avoid racemization during the deprotection and activation steps. The synthesis of HB I was achieved by reduction of the C-terminal methyl ester group into an alcohol function by $NaBH_4$–EtOH. Synthetic HB I was finally purified by semi-preparative HPLC. The analytical HPLC retention time (t_R) and the ^{1}H NMR spectrum of synthetic HB I were identical to those of natural HB I.

Antibacterial activity

The antibacterial activity of HB I examined against *S. aureus* and *E. coli* showed it to be inactive against *E. coli*, in agreement with previous observations on other peptaibols.[4,5,7] However, no antibacterial activity against *S. aureus* was detected even at 200 μg pit^{-1}, whereas growth inhibition induced by short-sequence peptaibols harzianins HC and trichogin GA IV could be detected up to 50 and 1.5 μg pit^{-1}, respectively.[7,8] This result was in agreement with the absence of antibacterial activity noticed for C_2-GA IV,[23] the analogue of GA IV with an acetyl group instead of the lipid chain. The absence of antibacterial activity for this 11-residue peptaibol confirms the role of the N-terminal lipid chain in the lipopeptaibol activity.

Membrane-modifying properties of HB I

Long-sequence peptaibols have been previously shown to exhibit membrane-modifying properties by increasing the permeability of liposomes.[5,11] Optimal membrane activity was observed for a hydrophobic neutral α-helix of 18–19 residues, while the liposome permeabilization decreased for shorter-sequence peptaibols.[5] The membrane-modifying activity of HB I was studied by fluorescence spectroscopy, following the leakage of a carboxyfluorescein (CF) fluorescent probe, previously entrapped at self-quenched concentration in small unilamellar vesicles. The results, presented as a percentage of escaped CF at 20 min as a function of R_i^{-1} = [peptide]/[lipid], were compared with those of other short peptides such as the 14-residue HC IX and the 11-residue lipopeptaibol GA IV (Fig. 5). Comparison of R_i^{-1} values characteristic of 50% release of the entrapped material showed HB I ($R_i^{-1} = 83 \times 10^{-3}$) to be less efficient than HC IX ($R_i^{-1} = 12 \times 10^{-3}$) and GA IV



Scheme 1 Scheme for the total synthesis of HB I

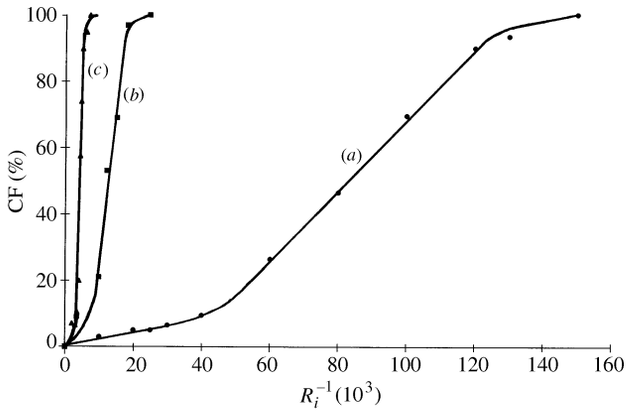


Fig. 5 Peptide-induced CF at $t = 20$ min for different [peptide]/[lipid] ratios (R_i^{-1}) from egg PC/cholesterol 70/30 vesicles: (*a*) HB I, (*b*) HC IX (Ac-Aib-Asn-Leu-Aib-Pro-Ala-Ile-Aib-Pro-Iva-Leu-Aib-Pro-Leuol) and (*c*) GA IV (Oc-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leuol)

($R_i^{-1} = 4 \times 10^{-3}$). This result points to the major role of the sequence length, the presence of the N-terminal lipidic chain also favouring the liposome permeabilization.

The voltage-dependent channel-forming properties of harzianin HB I were also examined by macroscopic current–voltage experiments (G. Molle, H. Duclohier, unpublished results). In such conditions, HB I exhibited channel-forming activity for concentrations ranging between 10^{-6} and 10^{-5} M, in the same way as the 11-residue peptaibol trichorozin TZ-IV,[6] or the 14-residue harzianins HC.[7]

Experimental

Isolation of harzianin HB I

The *T. harzianum* strain (M-903603), collected in Uruguay, was obtained from the 'Collection de souches fongiques du Muséum National d'Histoire Naturelle' (Paris); the strain was maintained and cultivated as previously described.[7] The culture was incubated for 11 days at 27 °C. The filtered fermentation broth was extracted three times with butan-1-ol to give, after removal of the solvent under reduced pressure, 1.2 g of crude extract. The residue was submitted to gel filtration on Pharmacia Sephadex LH 20 with methanol as eluent. The crude peptide mixture (468 mg) was then chromatographed over silica gel (Kieselgel 60 H Merck, Darmstadt) with CH_2Cl_2–MeOH (9:1 to 5:5) as eluent. The HC/HB mixture (130 mg) was eluted with CH_2Cl_2–MeOH (80:20).

HPLC separation

This was carried out with a Waters liquid chromatograph (6000 A and M45 pumps, a 680 automated solvent programmer, a WISP 712 automatic injector and a 481 UV–VIS detector) on a semi-preparative C18 column (Spherisorb ODS2; 5 μm; 7.5 × 300 mm; AIT France); eluent methanol–water (83:17); flow rate 2 cm^3 min^{-1}. The purity of HB I (3 mg) was confirmed on an analytical column (3.5 × 250 mm); eluent methanol–water (83:17); flow rate 1 cm^3 min^{-1}; t_R 16 min.

Amino acid analysis

Total hydrolysis of HB I was carried out according to the usual procedure for peptides (6 M HCl at 110 °C in sealed tubes for 24 h). Identification of the amino acids was accomplished by gas chromatography after derivatization.[4] Retention times of the *N*-trifluoroacetyl isopropyl ester derivatives were compared with those of standard samples. The GLC analyses 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 (0.7×10^5 Pa) as carrier gas and a temperature programme: 50–130 °C, 3 °C min^{-1}; 130–190 °C, 10 °C min^{-1}; t_R (min): Aib (10.4), L-Asp (29.5), L-Ile (20.4), D-Iva (11.2), L-Leu (24.2), L-Leuol (22.2). A special temperature programme was used for the separation of proline enantiomers: 50–110 °C, 3 °C min^{-1}; plateau at 110 °C for 10 min; 100–190 °C, 10 °C min^{-1}; t_R: L-Pro (25.1).

Secondary ion mass spectroscopy

Positive LSIMS was 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 solution was mixed with α-monothioglycerol as matrix. The resolution was 1000. Positive HR-LSIMS were recorded on a ZAB-HF spectrometer. The MS spectra were registered with either Li^+ or Na^+ added to the matrix.

NMR Spectroscopy

A 0.4 cm^3 aliquot of 7 mM methanolic (CD_3OH) solution of HB I peptide in a 5 mm tube (Wilmad) was used for all the NMR experiments. Proton NMR spectroscopy experiments were conducted at 296 K on a Bruker AC 300 equipped with an Aspect 3000 computer or on a Bruker Avance DMX 500 spectrometer equipped with a Bruker Station 1 computer and an indirect quadruple-resonance ^1H–^{31}P–^{13}C–^{15}N gradient probehead. Spectra were processed using UXNMR and AURELIA software (Bruker, Inc). Chemical shifts were referenced to the central component of the quintet due to the CD_2H resonance of methanol at δ_H 3.313, downfield from $SiMe_4$. *J* Values are given in Hz. TOCSY[24] experiments were run with the MLEV 17 sequence for spin locking and a mixing time of 120 ms (9 kHz). The ROESY[25] experiment was carried out with a mixing time of 250 ms and a spin lock field of 2 kHz to reduce Hartmann–Hahn transfers. Two-dimensional spectra were obtained with quadrature detection in both dimensions using the hypercomplex method in the F1 dimension.[26] The solvent signal was suppressed using the WATERGATE scheme[27] included in the standard and ROESY pulse sequences. A total of 2048 data points were acquired in the F2 dimension, and 512 complex points in the F1 dimension. For each complex data point in the F2, 4 free induction decays were accumulated with a relaxation delay of 2 s. All spectra were apodized with π/2-shifted sine-bell functions in both dimensions.

CD spectrum

The spectrum of HB I was recorded with a Jobin-Yvon CD6 dichrograph, with a 0.1 mm path cell at 22 °C (1 mmol cm^{-3}; CH_3OH); λ (nm) and $[\theta]_M$ (deg cm^2 $dmol^{-1}$) 192 (56 000) and 205 (−110 000).

Antimicrobial activity

The antibacterial activity of HB I was examined against *Staphylococcus aureus* (strain 209P) and *Escherichia coli* (strain RL 65) by the agar diffusion test using the Mueller Hinton culture medium and 6 mm diameter pits. The peptide sample was dissolved in dimethyl sulfoxide (DMSO) such as to give a 4 mg cm^{-3} solution. Eight other concentrations were obtained by successive dilutions, and 50 mm^3 of each solution was deposited into the pits (1.5 to 200 μg). Inhibition zones were measured after 24 h of incubation at 37 °C.

Liposome permeabilization

Egg phosphatidylcholine (egg PC) type V E and cholesterol were purchased from Sigma; egg PC was used without further purification and cholesterol was recrystallized from methanol. CF from Eastman Kodak was separated from hydrophobic contaminants and recrystallized from ethanol as previously described.[11] Fluorescence spectra were measured at 20 °C on an Aminco SPF 500 spectrofluorometer. The peptide-induced release of intravesicular content was monitored by the method introduced by Weinstein,[28] 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,[11] by sonication of an egg PC–cholesterol (7:3) mixture ([lip] = 0.6 mM). The SUV obtained by sonication were separated from unencapsulated CF by gel filtration (Sephadex G 75). 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).

Synthesis of HB I

Diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), di-*tert*-butyl dicarbonate (Boc_2O) and L-leucine were purchased from Sigma-Aldrich Chimie, and D-isovaline [(*R*)-(−)-2-amino-2-methylbutanoic acid] from Acros (France). All *N*-*tert*-butoxycarbonyl-protected L-amino acids and BOP were purchased from Propeptide (France), and used without subsequent purification. H-Leu-OMe was prepared according to Boissonnas *et al.*[29] Boc-Iva-OH was prepared according to Bodanszky and Bodanszky.[30]

Column chromatography was carried out with 230–400 mesh Merck grade 60 silica gel. Analytical TLC was performed on aluminium sheets covered with Merck grade 60 silica gel. Gel filtration was carried out with Pharmacia Sephadex LH 20.

General procedure A: BOP-mediated peptide coupling. The N-protected amino acid and BOP reagent were added to a solution of the TFA salt of the C-protected amino acid or peptide in CH_2Cl_2. The stirred solution was cooled in an ice-bath and DIEA was added. The mixture was stirred at room temperature until TLC analysis indicated that consumption of the amino component was no longer proceeding. It was then concentrated *in vacuo* to leave an oil, which was dissolved in ethyl acetate and washed successively with 1 M HCl, water, 1 M NaOH and saturated aq. NaCl. The combined organics were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to leave an oil, which was purified by chromatography on a silica gel column.

General procedure B: Removal of *N*-*tert*-butoxycarbonyl protection with 50% TFA solution in CH_2Cl_2. A stirred solution of the *N*-*tert*-butoxycarbonyl-protected peptide in CH_2Cl_2 was cooled in an ice-bath and TFA was added. The mixture was stirred at room temperature until TLC analysis indicated complete consumption of the starting material, and was then evaporated *in vacuo* to leave an oil. The crude product was used without purification for the next coupling.

General procedure C: Removal of *N*-*tert*-butoxycarbonyl protection with pure TFA. The *N*-*tert*-butoxycarbonyl-protected peptide was treated with TFA (0.5 cm^3 for 1 mmol of peptide). The mixture was stirred at room temperature until TLC analysis indicated that the totality of the peptide was deprotected, and it was then evaporated *in vacuo* to give an oil. The crude product was used without purification for the next coupling.

Boc-Pro-Leu-OMe. HCl, H-Leu-OMe (0.92 g, 5.07 mmol) and Boc-Pro-OH (1.20 g, 5.58 mmol) were treated with BOP (2.47 g, 5.58 mmol) and DIEA (2.7 cm^3, 15.71 mmol) *via* procedure A, to yield the crude product which was used without purification.

Boc-Aib-Pro-Leu-OMe. Boc-Pro-Leu-OMe (1.73 g, 5.07 mmol) was deprotected according to procedure C. The crude TFA salt and Boc-Aib-OH (1.12 g, 5.50 mmol) were treated with BOP (2.43 g, 5.50 mmol) and DIEA (2.7 cm^3, 15.7 mmol) according to procedure A to yield, after purification by chromatography on silica gel (ethyl acetate), 1 g of a powder; TLC R_f (ethyl acetate) 0.40; LSIMS *m/z* 434 $[M + Li]^+$ (100), 378 (5), 334 (27), 332 (2) and 247 (9); HR-LSIMS $[M + Li]^+$ 434.2822 (Calc. for $C_{21}H_{37}LiN_3O_6$: *m/z* 434.2842); δ_H(300 MHz; CD_3OH) 0.91 (d, *J* 6.0, 3 H, δ Leu), 0.94 (d, *J* 6.0, 3 H, δ Leu), 1.36 (s, 3 H, β Aib), 1.45 (s, 12 H, β Aib, Boc), 1.81 (m, 6 H, 2 × β Leu, γ Leu, β′ Pro, 2 × γ Pro), 2.19 (m, 1 H, β Pro), 3.59 (m, 1 H, δ′ Pro), 3.68 (s, 3 H, ester), 3.81 (m, 1 H, δ Pro), 4.37 (m, 1 H, α Leu), 4.45 (dd, *J* 8.4 and 5.9, 1 H, α Pro), 7.29 (s, 1 H, NH Aib) and 8.23 (d, *J* 7.7, 1 H, NH Leu).

Boc-Leu-Aib-Pro-Leu-OMe. Boc-Aib-Pro-Leu-OMe (1.00 g, 2.34 mmol) was deprotected according to procedure B. The crude TFA salt and Boc-Leu-OH (0.64 g, 2,58 mmol) were treated with BOP (1.14 g, 2.58 mmol) and DIEA (1.3 cm^3, 7.3 mmol) according to general procedure A. The mixture was purified by chromatography on silica gel eluted by CH_2Cl_2–MeOH (92:8), to yield 1.06 g (85%) of a powder; TLC R_f (CH_2Cl_2–MeOH, 92:8) 0.50; LSIMS *m/z* 547 $[M + Li]^+$ (100), 491 (8), 447 (17), 445 (3) and 247 (8); HR-LSIMS $[M + Li]^+$ 547.3657 (Calc. for $C_{27}H_{48}LiN_4O_7$ *m/z* 547.3683); $[\alpha]_D^{22}$ −77 (*c* 0.1, MeOH); δ_H(300 MHz; CD_3OH) 0.93 (m, 12 H, δ Leu^1, δ Leu^4), 1.44 (s, 12 H, β Aib, Boc), 1.46 (s, 3 H, β Aib), 1.47 (m, 2 H, 2 × β Leu^1), 1.60 (m, 1 H, β′ Leu^4), 1.69 (m, 2 H, γ Leu^1, γ Leu^4), 1.75 (m, 1 H, β Leu^4), 1.88 (m, 3 H, β′ Pro, 2 × γ Pro), 2.08 (m, 1 H, β Pro), 3.62 (m, 2 H, 2 × δ Pro), 3.68 (s, 3 H, ester), 4.09 (m, 1 H, α Leu^1), 4.34 (m, 1 H, α Leu^4), 4.47 (m, 1 H, α Pro), 6.68 (d, *J* 8.1, 1 H, NH Leu^1) and 8.21 (br s, 2 H, NH Aib, NH Leu^4).

Boc-Iva-Leu-Aib-Pro-Leu-OMe. Boc-Leu-Aib-Pro-Leu-OMe (150 mg, 0.28 mmol) was deprotected according to procedure B. The crude TFA salt and Boc-Iva-OH (73 mg, 0.34 mmol) were treated with BOP (149 mg, 0.34 mmol) and DIEA (180 mm^3, 1 mmol) according to procedure A. The mixture was purified by chromatography on silica gel (CH_2Cl_2–MeOH, 94:6) to yield 152 mg (85%) of the expected product; TLC R_f (CH_2Cl_2–MeOH, 93:7) 0.43; LSIMS *m/z* 662 $[M + Na]^+$ (15), 495 (3), 398 (44), 313 (4) and 243 (100); HR-LSIMS $[M + Li]^+$ 646.4385 (Calc. for $C_{32}H_{57}LiN_5O_8$: *m/z* 646.4367); δ_H(300 MHz; CD_3OH) 0.84 (m, 3 H, Me^{γ} Iva), 0.95 (m, 12 H, δ Leu^2, δ Leu^5), 1.34 (s, 3 H, Me^{β} Iva), 1.47 (s, 15 H, β Aib, Boc), 1.62 (m, 2 H, β′ Leu^2, β′ Leu^5), 1.70 (m, 2 H, γ Leu^2, γ Leu^5), 1.77 (m, 2 H, β Leu^2, β Leu^5), 1.78 (m, 1 H, β′ Iva), 1.85 (m, 1 H, β′ Pro), 1.88 (m, 2 H, γ Pro), 1.92 (m, 1 H, β Iva), 2.16 (m, 1 H, β Pro), 3.53 (m, 1 H, δ′ Pro), 3.3 (m, 1 H, δ Pro), 3.68 (s, 3 H, ester), 4.35 (m, 2 H, α Leu^2, α Leu^5), 4.48 (m, 1 H, α Pro), 6.93 (s, 1 H, NH Iva), 7.94 (d, *J* 7.0, 1 H, NH Leu^2), 8.14 (s, 1 H, NH Aib) and 8.16 (d, *J* 8.3, 1 H, NH Leu^5).

Boc-Pro-Iva-Leu-Aib-Pro-Leu-OMe. Boc-Iva-Leu-Aib-Pro-Leu-OMe (140 mg, 0.22 mmol) was deprotected according to procedure B. The crude TFA salt and Boc-Pro-OH (71 mg, 0.33 mmol) were treated with BOP (146 mg, 0.33 mmol) and DIEA (134 mm^3, 0.77 mmol) according to procedure A to yield, after purification by silica gel chromatography (CH_2Cl_2–MeOH, 95:5), 149 mg (92%) of a powder; TLC R_f (CH_2Cl_2–MeOH, 94:6) 0.44; LSIMS *m/z* 743 $[M + Li]^+$ (100), 643 (14), 641 (5), 473 (3), 388 (9) and 247 (5); HR-LSIMS $[M + Li]^+$ 743.4897 (Calc. for $C_{37}H_{64}LiN_6O_9$: *m/z* 743.4895); $[\alpha]_D^{22}$ −33 (*c* 0.1, MeOH); δ_H(300 MHz; CD_3OH) 0.82 (m, 3 H, Me^{γ} Iva), 0.92 (m, 12 H, δ Leu^3, δ Leu^6), 1.36 (s, 3 H, Me^{β} Iva), 1.49 (s, 3 H, β Aib), 1.50 (s, 12 H, β Aib, Boc), 1.62 (m, 2 H, β′ Leu^3, β′ Leu^6), 1.70 (m, 2 H, γ Leu^3, γ Leu^6), 1.80 (m, 2 H, β Leu^3, β Leu^6), 1.82 (m, 1 H, β′ Iva), 1.83 (m, β′ Pro^5), 1.86 (m, 2 H, γ Pro^5), 1.95 (m, 1 H, β′ Pro^1), 1.98 (m, 2 H, γ Pro^1), 2.18 (m, 1 H, β Pro^5), 2.23 (m, 1 H, β Iva), 4.31 (m, 1 H, β Pro^1), 3.51 (m, 2 H, δ′ Pro^1, δ′ Pro^5), 3.60 (m, 1 H, δ Pro^1), 3.69 (s, 3 H, ester), 3.74 (m, 1 H, δ Pro^5), 4.08 (m, 1 H, α Pro^1), 4.36 (m, 2 H, α Leu^3, α Leu^6), 4.49 (m, 1 H, α Pro^5), 7.82 (d, *J* 8.5, 1 H, NH Leu^3), 7.87 (s, 1 H, NH Iva), 8.12 (s, 1 H, NH Aib) and 8.16 (d, *J* 7.7, 1 H, NH Leu^6).

Boc-Ile-Aib-OMe. According to procedure A, H-Aib-OMe (1.16 g, 7.6 mmol) and Boc-Ile-OH (2.21 g, 9.2 mmol) were treated with BOP (4.07 g, 9.2 mmol) and DIEA (2.6 cm^3, 15.2 mmol). The crude product was used without purification for the next coupling. TLC R_f (ethyl acetate–cyclohexane, 1:1) 0.61.

Boc-Leu-Ile-Aib-OMe. Boc-Ile-Aib-OMe (2.51 g, 7.6 mmol) was deprotected according to procedure B. The TFA salt and Boc-Leu-OH (2.27 g, 9.1 mmol) were treated with BOP (4.03 g, 9.1 mmol) and DIEA (4.2 cm^3, 24 mmol) according to procedure A. The mixture was purified by silica gel chromatography (ethyl acetate–cyclohexane, 5:5), to yield 1.08 g (32% for the two steps) of a solid; TLC R_f (ethyl acetate–cyclohexane,

1:1) 0.53; LSIMS *m/z* 450 $[M + Li]^+$ (100), 394 (12), 350 (24), 348 (3), 305 (2) and 192 (8); HR-LSIMS $[M + Li]^+$ 450.3159 (Calc. for $C_{22}H_{41}LiN_3O_6$: *m/z* 450.3155); δ_H(300 MHz; CD_3OH) 0.93 (m, 12 H, δ Leu, Me^{γ} Ile, Me^{δ} Ile), 1.16 (m, 1 H, γ′ Ile), 1.42 (s, 3 H, β Aib), 1.43 (s, 9 H, Boc), 1.45 (s, 3 H, β Aib), 1.53 (m, 3 H, γ Ile, 2 × β Leu), 1.66 (m, 1 H, γ Leu), 1.77 (m, 1 H, β Ile), 3.64 (s, 3 H, ester), 4.07 (m, 1 H, α Leu), 4.18 (m, 1 H, α Ile), 6.86 (d, *J* 7.7, 1 H, NH Leu), 7.71 (d, *J* 8.6, 1 H, NH Ile) and 8.35 (s, 1 H, NH Aib).

Boc-Asn-Leu-Ile-Aib-OMe. Boc-Leu-Ile-Aib-OMe (1.03 g, 2.3 mmol) was deprotected according to procedure C. The crude TFA salt and Boc-Asn-OH (0.65 g, 2.8 mmol) were treated with BOP (1.24 g, 2.8 mmol) and DIEA (1.3 cm^3, 7.5 mmol) according to procedure A. After purification by silica gel chromatography (CH_2Cl_2–MeOH, 95:5), it yielded 890 mg (69%) of a powder; TLC R_f (ethyl acetate) 0.17; LSIMS *m/z* 564 $[M + Li]^+$ (100), 508 (4), 464 (31), 462 (3), 419 (4) and 306 (3); HR-LSIMS $[M + Li]^+$ 564.3583 (Calc. for $C_{26}H_{47}LiN_5O_8$: *m/z* 564.3585); δ_H(300 MHz; CD_3OH) 0.89 (m, 3 H, Me^{δ} Ile), 0.92 (m, 6 H, δ Leu), 0.94 (m, 3 H, Me^{γ} Ile), 1.17 (m, 1 H, γ′ Ile), 1.41 (s, 3 H, β Aib), 1.42 (s, 3 H, β Aib), 1.43 (s, 9 H, Boc), 1.43 (m, 1 H, β′ Leu), 1.55 (m, 1 H, γ Ile), 1.62 (m, 1 H, β Leu), 1.73 (m, 1 H, γ Leu), 1.90 (m, 1 H, β Ile), 2.66 (m, 2 H, 2 × Asn), 3.64 (s, 3 H, ester), 4.12 (m, 1 H, α Ile), 4.37 (m, 1 H, α Leu), 4.41 (m, 1 H, α Asn), 6.86 (d, *J* 7.7, 1 H, NH Asn), 6.94 (s, 1 H, δ *syn* Asn), 7.60 (s, 1 H, δ *anti* Asn), 7.96 (d, *J* 8.7, 1 H, NH Ile), 8.03 (s, 1 H, NH Aib) and 8.17 (d, *J* 7.2, 1 H, NH Leu).

Ac-Aib-Asn-Leu-Ile-Aib-OMe. Boc-Asn-Leu-Ile-Aib-OMe (0.54 g, 0.97 mmol) was deprotected according to procedure C. The TFA salt and Ac-Aib-OH (0.27 g, 1.84 mmol) were treated with BOP (0.81 g, 1.84 mmol) and DIEA (0.5 cm^3, 3.0 mmol) according to procedure A. After purification by silica gel chromatography (CH_2Cl_2–MeOH, 90:10), it yielded 190 mg of a powder; TLC R_f (CH_2Cl_2–MeOH, 88:12) 0.30; LSIMS *m/z* 591 $[M + Li]^+$ (100), 333 (6), 291 (16), 220 (11), 209 (40), 122 (7) and 106 (15); HR-LSIMS $[M + Li]^+$ 591.3678 (Calc. for $C_{27}H_{48}LiN_6O_8$: *m/z* 591.3694); δ_H(300 MHz; CD_3OH) 0.90 (m, 12 H, δ Leu, Me^{γ} Ile, Me^{δ} Ile), 1.21 (m, 1 H, γ′ Ile), 1.43 (m, 12 H, β Aib^1, β Aib^5), 1.55 (m, 2 H, β′ Leu, γ Ile), 1.69 (m, 1 H, β Leu), 1.87 (m, 2 H, γ Leu, β Ile), 1.99 (s, 3 H, Ac), 2.71 (ABX system, *J* 7.6 and 15.4, 1 H, β′ Asn), 2.81 (ABX system, *J* 6.3 and 15.4, 1 H, β Asn), 3.65 (s, 3 H, ester), 4.14 (m, 1 H, α Ile), 4.27 (m, 1 H, α Leu), 4.41 (m, 1 H, α Asn), 6.94 (s, 1 H, δ *syn* Asn), 7.38 (d, *J* 8.9, 1 H, NH Ile), 7.69 (s, 1 H, δ *anti* Asn), 7.83 (s, 1 H, NH Aib^5), 8.11 (d, *J* 7.2, 1 H, NH Leu), 8.46 (d, *J* 6.2, 1 H, NH Asn) and 8.54 (s, 1 H, NH Aib^1).

Ac-Aib-Asn-Leu-Ile-Aib-OH. A stirred solution of Ac-Aib-Asn-Leu-Ile-Aib-OMe (140 mg, 0.24 mmol) in MeOH (1.4 cm^3) was cooled in an ice-bath and 1.0 cm^3 of 1 M NaOH was added. The mixture was stirred at room temperature until TLC analysis indicated complete consumption of the methyl ester. The cooled mixture was then neutralized with 1 M HCl, and evaporated *in vacuo*. The crude product was purified using Sephadex LH-20 with MeOH as eluent, to yield 116 mg (85%) of a solid; LSIMS *m/z* 593 $[M + Na]^+$ (100), 468 (7), 355 (14), 242 (23) and 128 (18); HR-LSIMS $[M + Na]^+$ 593.3293 (Calc. for $C_{26}H_{46}N_6NaO_8$: *m/z* 593.3275); $[\alpha]_D^{22}$ −10 (*c* 0.1, MeOH); δ_H(300 MHz; CD_3OH) 0.87 (m, 6 H, Me^{δ} Ile, δ Leu), 0.94 (d, *J* 6.8, 3 H, Me^{γ} Ile), 0.95 (d, *J* 6.5, 3 H, δ Leu), 1.21 (m, 1 H, γ′ Ile), 1.42 (s, 3 H, β Aib), 1.45 (s, 3 H, β Aib), 1.46 (s, 3 H, β Aib), 1.47 (s, 3 H, β Aib), 1.54 (m, 1 H, γ Ile), 1.57 (m, 1 H, β′ Leu), 1.73 (m, 1 H, γ Leu), 1.81 (m, 1 H, β Leu), 1.95 (m, 1 H, β Ile), 1.99 (s, 3 H, Ac), 2.73 (ABX system, *J* 4.9 and 15.4, 1 H, β′ Asn), 2.81 (ABX system, *J* 6.3 and 15.4, 1 H, β Asn), 4.16 (m, 1 H, α Ile), 4.28 (m, 1 H, α Leu), 4.45 (m, 1 H, α Asn), 6.93 (s, 1 H, δ *syn* Asn), 7.45 (d, *J* 9.1, 1 H, NH Ile), 7.69 (s, 1 H, δ *anti* Asn), 7.78 (s, 1 H, NH Aib^5), 8.12 (d, *J* 7.5, 1 H, NH Leu), 8.45 (d, *J* 6.5, 1 H, NH Asn) and 8.53 (s, 1 H, NH Aib^1).

Ac-Aib-Asn-Leu-Ile-Aib-Pro-Iva-Leu-Aib-Pro-Leu-OMe. Boc-Pro-Iva-Leu-Aib-Pro-Leu-OMe (100 mg, 0.13 mmol) was deprotected according to procedure B. The TFA salt and Ac-Aib-Asn-Leu-Ile-Aib-OH (50 mg, 0.087 mmol) were treated with BOP (38.5 mg, 0.087 mmol) and DIEA (45 mm^3, 0.26 mmol) according to procedure A, to yield, after purification by silica gel chromatography (CH_2Cl_2–MeOH, 85:15), 42 mg (41%) of a powder; TLC R_f (CH_2Cl_2–MeOH, 85:15) 0.42; LSIMS *m/z* 1195 $[M + Li]^+$ (100), 798 (6), 641 (27), 628 (6), 531 (13), 446 (16), 333 (9) and 291 (8); HR-LSIMS $[M + Li]^+$ 1195.7598 (Calc. for $C_{58}H_{100}LiN_{12}O_{14}$: *m/z* 1195.7642); δ_H(300 MHz; CD_3OH) 0.83 (m, 3 H, Me^{γ} Iva), 0.87 (m, 3 H, Me^{δ} Ile), 0.89 (m, 18 H, δ Leu^3, δ Leu^8, δ Leu^{11}), 0.95 (m, 3 H, Me^{γ} Ile), 1.30 (m, 1 H, γ′ Ile), 1.45 (s, 3 H, Me^{β} Iva), 1.46 (s, 6 H, β Aib), 1.49 (s, 9 H, β Aib), 1.50 (s, 3 H, β Aib), 1.55 (m, 1 H, γ Ile), 1.76 (m, 9 H, β and γ Leu^3, β and γ Leu^8, β and γ Leu^{11}), 1.77 (m, 1 H, β′ Iva), 1.80 (m, 1 H, β′ Pro^6), 1.83 (m, 1 H, β′ Pro^{10}), 1.86 (m, 2 H, 2 × γ Pro^{10}), 1.92 (m, 1 H, γ′ Pro^6), 1.96 (m, 1 H, β Ile), 2.02 (s, 3 H, Ac), 2.12 (m, 1 H, γ Pro^6), 2.20 (m, 1 H, β Pro^{10}), 2.32 (m, 1 H, β Pro^6), 2.46 (m, 1 H, β Iva), 2.76 (d, *J* 5.7, 2 H, β Asn), 3.42 (m, 2 H, δ′ Pro^6, δ′ Pro^{10}), 3.69 (s, 3 H, ester), 3.81 (m, 2 H, δ Pro^6, δ Pro^{10}), 4.20 (m, 3 H, α Leu^3, α Ile^4, α Pro^6), 4.34 (m, 3 H, α Asn^2, α Leu^8, α Leu^{11}), 4.50 (m, 1 H, α Pro^{10}), 7.04 (s, 1 H, δ *syn* Asn), 7.35 (d, *J* 8.8, 1 H, NH Ile^4), 7.49 (s, 1 H, NH, Iva), 7.62 (d, *J* 8.5, 1 H, NH Leu^8), 7.76 (s, 2 H, NH Aib^5, NH Aib^9), 7.80 (s, 1 H, δ *anti* Asn), 8.11 (d, *J* 7.1, 1 H, NH Leu^3), 8.16 (d, *J* 7.8, 1 H, NH Leu^{11}), 8.61 (d, *J* 5.5, 1 H, NH Asn) and 8.67 (s, 1 H, NH Aib^1).

Ac-Aib-Asn-Leu-Ile-Aib-Pro-Iva-Leu-Aib-Pro-Leuol: HB I. A solution of Ac-Aib-Asn-Leu-Ile-Aib-Pro-Iva-Leu-Aib-Pro-Leu-OMe (32 mg, 0.027 mmol) in EtOH (1 cm^3) was cooled in an ice-bath and $NaBH_4$ (6 mg, 0.162 mmol) was added. The mixture was stirred at 50 °C for 8 h, and the solvent was evaporated off *in vacuo*. The residue was dissolved in ethyl acetate and washed with water. The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo* to yield 25 mg (80%) of HB I; TLC R_f (CH_2Cl_2–MeOH, 85:15) 0.37; LSIMS *m/z* 1167 $[M + Li]^+$ (55), 947 (64), 553 (81), 468 (34), 395 (28), 355 (63), 310 (25), 242 (100), 197 (69) and 128 (70); HR-LSIMS $[M + Na]^+$ 1183.7333 (Calc. for $C_{57}H_{100}N_{12}NaO_{13}$: *m/z* 1183.7430); $[\alpha]_D^{22}$ +7 (*c* 0.1, MeOH); ^{1} NMR data were identical with those described for natural HB I.

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