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Total Synthesis and Membrane Modifying Properties of the Lipopeptaibol Trikoningin KB II and its Analogues with Acyl Chains of Different Length at the N- and C-Termini

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Abstract: Trikoningin KB II, a ten-amino acid residue lipopeptaibol blocked at the N-terminus by the *n*-octanoyl group and at the C-terminus by the 1,2-amino alcohol L-leucinol, and extracted from the fungus *Trichoderma koningii*, exhibits membrane-modifying properties. We have synthesized by solution-phase methods trikoningin KB II and several analogues with acyl chains of different length at the N- and C-termini. Permeability measurements showed that an appropriate length of the linear acyl chain is a more important characteristic for the onset of significant membrane-modifying activity than its position in the peptide chain. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: fatty acid; lipopeptaibol; membrane activity; peptide synthesis

INTRODUCTION

The *peptaibol* [1] family of antibiotics is a class of molecules that are known to be active on cell membranes, leading to leakage of the cytoplasmic material and eventually to cell death [2]. They are linear peptides containing a large fraction of Aib residues, which strongly promote helix formation, and a C-terminal 1,2-amino alcohol. Sequences of these peptides range from 19 amino acids (alamethicin) to as low as ten (trichogin GA IV [3–12] and trikoningins KB I and KB II [13]) or even six amino acids (trichodecenins) [14–16]. In the case of alamethicin, the length of the helix is such as to allow complete spanning of the membrane bilayer, and channels are believed to form via self-aggregation of several peptide monomers. Interestingly, for membrane activity the shortest peptaibols require the presence of a long acyl chain at the N-terminus (hence, the term *lipopeptaibols*). If the mechanism of channel formation would be operative also for the lipopeptaibols, then trichogin and trikoningin helices would be able to span only about half of the bilayer. In that case membrane activity would require the alignment of two peptide molecules to form a dimeric structural system and the hydrocarbon moiety would allow dimer formation and binding of the peptide to the membrane with the correct geometry of the channels.

In connection with our ongoing study of the conformational properties and mechanism of membrane activity of lipopeptaibols [4–12,16], we describe here the total chemical synthesis and membrane-modifying properties of trikoningin KB II

Abbreviations: CF, carboxyfluorescein; DMAP, 4-dimethylaminopyridine; EDC, *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide; HOAt, 1-hydroxy-7-azabenzotriazole; Iva, isovaline; Lol, leucinol; *n*Oct, *n*-octanoyl; PC, phosphatidylcholine; TOAC, 2,2,6,6-tetramethyl-piperidine-1-oxyl-4-amino-4-carboxylic acid; all chiral protein amino acids and Lol have the L-configuration; on the contrary, the configuration of Iva is D.

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[13] and some decapeptide analogues with acyl chains characterized by a different number of carbon atoms (2, 4 or 8) incorporated either at the N- or at the C-terminus or at both termini. The amino acid sequences of the natural, strictly related trichogin GA IV and trikoningin KB I and KB II molecules are presented below:

Trichogin GA IV: *n*Oct-*Aib*[1]-Gly-*Leu*-Aib-Gly[5]-Gly-*Leu*-Aib-Gly-Ile[10]-Lol
Trikoningin KB I: *n*Oct-*Aib*[1]-Gly-*Val*-Aib-Gly[5]-Gly-*Val*-Aib-Gly-Ile[10]-Lol
Trikoningin KB II: *n*Oct-D-*Iva*[1]-Gly-*Val*-Aib-Gly[5]-Gly-*Val*-Aib-Gly-Ile[10]-Lol

MATERIALS AND METHODS

Peptide Synthesis

Melting points were determined using a Leitz (Wetzlar, Germany) model Laborlux 12 apparatus and are not corrected. Optical rotations were measured using a Perkin-Elmer (Norwalk, CT) model 241 polarimeter equipped with a Haake (Karlsruhe, Germany) model D thermostat. Thin-layer chromatography was performed on Merck (Darmstadt, Germany) Kieselgel 60-F_{254} precoated plates using the following solvent systems: (I) $CHCl_3$/EtOH, 9:1; (II) *n*BuOH/AcOH/H_2O, 3:1:1; (III) toluene/EtOH, 7:1. The chromatograms were developed by quenching of UV fluorescence, chlorine–starch–potassium iodide or ninhydrin chromatic reaction as appropriate. All the new compounds were obtained in a chromatographically homogeneous state. The amino acid analyses were performed on a Carlo Erba (Rodano, Milan, Italy) model 3A 30-amino acid analyzer. The Aib colour yield with ninhydrin is about 20 times lower than those of protein amino acids. Elution of Aib was observed immediately after the Ala peak. HPLC was performed on a Pharmacia (Uppsala, Sweden) model LKB-LCC 2252 liquid chromatograph equipped with an UVICORD model SD detector (226 nm) and a reversed-phase C_{18} Vydac (Hesperia, CA) model 218 TP54 column.

Liposome Leakage Assay

Peptide-induced leakage from egg PC vesicles was measured at 20°C using the CF-entrapped vesicle technique [17]. CF-encapsulated small unilamellar vesicles (egg PC/cholesterol, 7:3) were prepared by sonication in Hepes buffer, pH 7.4. The phospholipid concentration was kept constant (0.06 mM), and increasing [peptide]/[lipid] molar ratios (R_i^{-1}) were obtained by adding aliquots of MeOH solutions of peptides, keeping the final MeOH concentration below 5% by volume. After rapid and vigorous stirring, the time course of fluorescence change corresponding to CF escape was recorded at 520 nm (6 nm band pass) with λ_{exc} 488 nm (3 nm band pass). The percentage of released CF at time t was determined as $(F_t - F_0)/(F_T - F_0) \times 100$, with F_0 = fluorescence intensity of vesicles in the absence of peptide, F_t = fluorescence intensity at time t in the presence of peptide, and F_T = total fluorescence intensity determined by disrupting the vesicles by addition of 50 μL of a 10% Triton X-100 solution. The kinetics were stopped at 20 min.

RESULTS

Peptide Synthesis

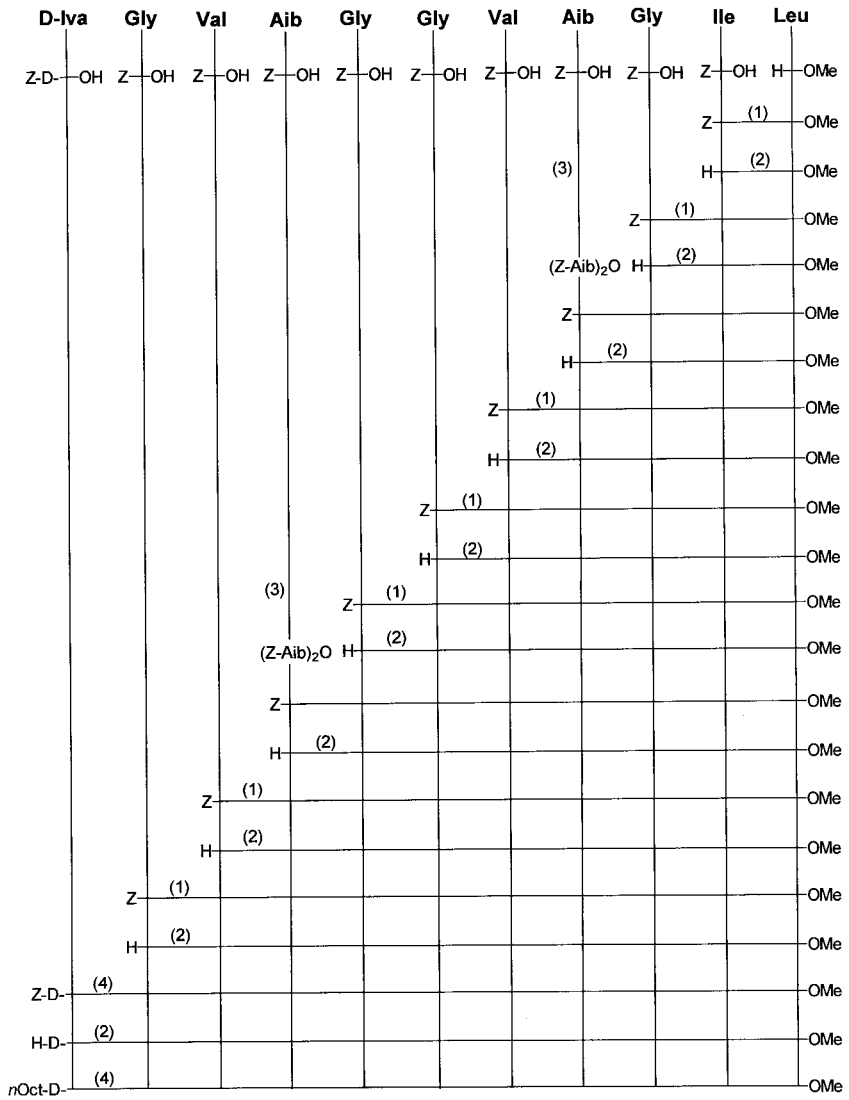
The total chemical syntheses of trikoningin KB II and its analogues were carried out step-by-step in solution, beginning from the C-terminal H-L-Leu-OMe residue, via the mixed anhydride method with *iso*butylchloroformate [18] to incorporate the protein amino acids, the symmetrical anhydride method to add the Aib residues [19,20], and the EDC/HOAt method [21] for the insertion of the D-Iva residues. Removal of the Z N^{α}-protecting group was achieved by catalytic hydrogenation. N^{α}-Acetylation was performed by using acetic anhydride, while N^{α}-acylation with the *n*But and *n*Oct groups was obtained by the EDC/HOAt method. The ester group of the N^{α}-acylated, Leu-OMe[11] undecapeptides was reduced by using $LiBH_4$ [22] to afford the synthetic trikoningin KB II and its analogues with different acyl moieties at the N-terminus. Synthetic and natural [13] trikoningin KB II have identical chromatographic and physical properties. In the last step the alcoholic function of the N^{α}-blocked, Lol[11] decapeptides was acylated by treatment with EDC/HOAt and DMAP as catalyst [23] (Scheme 1).

The chemical and optical purities of all intermediates and final synthetic products were assessed by TLC in three different solvent systems, polarimetric measurements, solid-state IR absorption data (data listed in Table 1), analytical HPLC and ^{1}H-NMR. A satisfactory amino acid analysis (results not reported) was obtained for the Z- and -OMe protected undecapeptide, the key intermediate in the preparation of all N^{α}-acylated and C-reduced compounds described in this work.

Table 1 Physical and Analytical Properties for the Peptides Discussed in This Work and Their Synthetic Intermediates

Peptide	Melting point (°C)	Recryst. Solvent	$[\alpha]_D^{20}$ (°)[b]	TLC R_{FI}	TLC R_{FII}	TLC R_{FIII}	IR (cm^{-1})[c]
Z-L-Val-Aib-Gly-L-Ile-L-Leu-OMe	89–90	DE/PE	−57.7	0.55	0.90	0.20	3308, 1740, 1652, 1533
Z-Gly-L-Val-Aib-Gly-L-Ile-L-Leu-OMe	91–93	DE/PE	−72.5	0.55	0.90	0.20	3299, 1738, 1647, 1538
Z-$(Gly)_2$-L-Val-Aib-Gly-L-lle-L-Leu-OMe	169–171	DE/PE	−51.0	0.40	0.90	0.10	3315, 1737, 1703, 1655, 1533
Z-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Leu-OMe	104–105	DE/PE	−58.7	0.30	0.90	0.85[d]	3306, 1736, 1658, 1533
Z-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Leu-OMe	125–127	DE/PE	−59.9	0.40	0.90	0.85[d]	3315, 1737, 1657, 1534
Z-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Leu-OMe	158–160	DE/PE	−70.1	0.40	0.90	0.85[d]	3315, 1739, 1655, 1536
Z-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Leu-OMe	132–134	DE/PE	−36.8	0.10	0.95	0.90[d]	3316, 1738, 1656, 1536
Ac-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Leu-OMe	155–156	[f]	−41.6	0.35	0.60	0.85[d]	3320, 1741, 1657, 1538
*n*But-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Leu-OMe	136–138	[f]	−36.9[e]	0.50	0.70	0.85[d]	3320, 1741, 1652, 1539
*n*Oct-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Leu-OMe	140–142	[f]	−36.4	0.10	0.75	0.90[d]	3324, 1741, 1657, 1537
Ac-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Lol	155–156	[f]	−22.7	0.25	0.60	0.75[d]	3321, 1657, 1537
*n*But-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Lol	158–160	[f]	−13.5	0.45	0.65	0.80[d]	3320, 1656, 1538
*n*Oct-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Lol	147–149	[f]	−22.3	0.45	0.70	0.80[d]	3321, 1657, 1537
Ac-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Lol-*n*Oct	127–128	[f]	−21.6	0.40	0.70	0.90[d]	3314, 1722, 1657, 1539
*n*But-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Lol-*n*But	115–116	[f]	−19.3	0.50	0.75	0.90[d]	3326, 1716, 1655, 1539
*n*Oct-D-Iva-Gly-L-Val-Aib-$(Gly)_2$-L-Val-Aib-Gly-L-Ile-L-Lol-*n*Oct	105–106	[f]	−26.1	0.55	0.80	0.90[d]	3327, 1718, 1656, 1538

[a] DE, diethyl ether; PE, petroleum ether.
[b] $c = 0.5$, MeOH.
[c] The IR absorption spectra were obtained in KBr pellets (only significant bands in the 3500–3200 and 1800–1520 cm^{-1} regions are reported).
[d] $R_{FIV} = CHCl_3$/MeOH, 9:1.
[e] $c = 0.2$, MeOH.
[f] Lyophylized after HPLC purification.



Scheme 1

Membrane Permeability Measurements

Lipopeptaibols bind to phospholipid bilayers and are able to modify their permeability [3,5,6,10,14]. Therefore, the permeability properties of trikoningin KB II [13] and its analogues with acyl chains of different length at the N- and C-termini were investigated by following fluorimetrically the induced CF leakage from small unilamellar vesicles (egg PC-cholesterol 70:30) for different R_i^{-1} = [peptide]/[lipid] molar ratios [17].

In Figure 1 the membrane modifying properties of trikoningin KB II and its two analogues with a shorter acyl chain at the N-terminus are compared to those of the parent lipopeptaibol trichogin GA IV. An R_i^{-1} value of 80×10^{-3}, allowing the leakage of 50% of the entrapped CF in 20 min, was obtained for trikoningin KB II, whereas the [Ac-D-Iva[1]]- and [*n*But-D-Iva[1]]-analogues are unable to show any membrane permeability effect. As already reported by Bodo and coworkers [13], trichogin GA IV ($R_i^{-1} = 20 \times 10^{-3}$) is significantly more efficient in membrane activity than trikoningin KB II.

Figure 2 compares the membrane permeability results of trichogin GA IV and trikoningin KB II with those of the three trikoningin KB II analogues acylated at both N- and C-termini. It is noteworthy that the *bis*-*n*-octanoylated trikoningin KB II analogue is

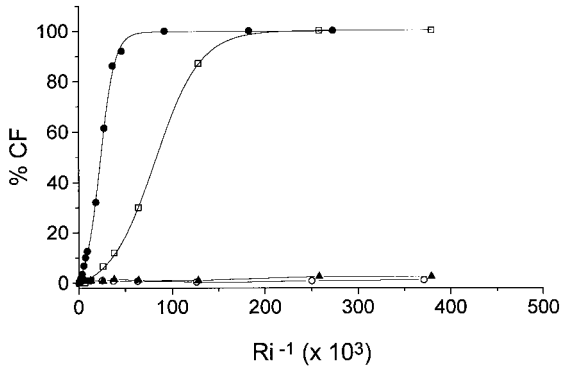


Figure 1 Peptide induced CF leakage at 20 min for different ratios R_i^{-1} = [peptide]/[lipid] from egg PC/cholesterol (70:30) unilamellar vesicles: ●, trichogin GA IV; □, trikoningin KB II; ○, [Ac-D-Iva^1]trikoningin KB II; ▲, [*n*But-D-Iva^1]trikoningin KB II.

much more active ($R_i^{-1} = 17 \times 10^{-3}$) than its parent compound, and even slightly more active than trichogin GA IV itself. However, the N$^\alpha$-acetylated, C-*n*-octanoylated analogue is less efficient ($R_i^{-1} = 120 \times 10^{-3}$). Finally, a striking observation is the lack of any activity exhibited by the *bis*-*n*-butanoylated analogue.

DISCUSSION

Previous detailed FT-IR absorption, NMR, CD and X-ray diffraction conformational studies which emanated from Bodo's and our laboratories [3–12] unequivocally showed that trichogin GA IV is folded in an amphiphilic, right-handed, mixed $3_{10}/\alpha$-helix

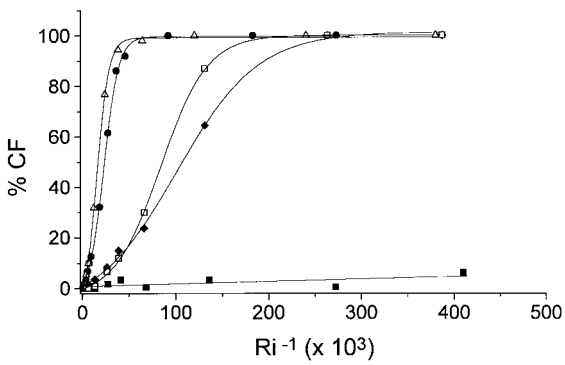


Figure 2 Peptide induced CF leakage at 20 min for different ratios R_i^{-1} = [peptide]/[lipid] from egg PC/cholesterol (70:30) unilamellar vesicles: ●, trichogin GA IV; □, trikoningin KB II; ■, [*n*But-D-Iva^1, Lol-*n*But^{11}]trikoningin KB II; ◆, [Ac-D-Iva^1, Lol-*n*Oct^{11}]trikoningin KB II; △, [*n*Oct-D-Iva^1, Lol-*n*Oct^{11}]trikoningin KB II.

[24] in structure-supporting organic solvents and in membrane-mimetic environments. The very limited differences in the amino acid sequences between trichogin GA IV and the trikoningins are not expected to modify significantly the preferred conformation of the latter lipopeptaibols [13].

Published membrane permeability measurements on trichogin GA IV and its analogues, and on trikoningins KB I and KB II [3–13] clearly indicated that: (i) replacement of the C-terminal Lol moiety with Leu-OMe does not affect at all the membrane activity; (ii) at least six carbon atoms in the N$^\alpha$-blocking fatty acyl moiety are essential for the onset of significant membrane modifying properties. Among the monomeric trichogin analogues the most active is the C_{10} peptide. However, the head-to-head succinoyl dimer is the most effficient trichogin analogue synthesized to date; (iii) substitution of the more hydrophobic Leu^3 and Leu^7 residues (trichogin GA IV) by the less hydrophobic Val^3 and Val^7 residues (trikoningins) induces a remarkable decrease in membrane activity; (iv) conversely, a significant increase in membrane activity was observed upon replacement of the four Gly^{2,5,6,9} residues, forming the more polar face of the amphiphilic helical structure, with the more hydrophilic Ser residues; (v) replacement of the Aib residue (positions 1, 4, and 8) with other members of the family of the strongly helicogenic C$^\alpha$-tetrasubstituted α-amino acids (D-Iva, TOAC [25,26]) affects only marginally the membrane activity; (vi) the same observation holds true upon moving the long acyl moiety from the N$^\alpha$-blocking group to the side-chain of an N-terminal extra-residue.

In the present work we have described the *first total chemical synthesis* of the naturally occurring lipopeptaibol trikoningin KB II and a variety of analogues with acyl chains of different length (C_2, C_4, and C_8) at the N- and C-termini by using the step-by-step solution approach.

From the membrane permeability measurements on the trikoningins performed in this work in comparison with our synthetic trichogin GA IV, we were able to confirm the results obtained by Bodo's and our groups [5,13] pointing to the strict requirement for an N$^\alpha$-blocking acyl chain of more than four carbon atoms for a significant membrane activity of lipopeptaibols and to the beneficial effect of the presence of two Leu residues (compared to two Val residues) on the hydrophobic face of the amphiphilic helix.

New relevant *information* extracted from this work may be summarized as follows: (i) in striking con-

trast to the effect produced by *one long* (C_8) acyl chain, *two short* (C_4) acyl chains, each with *half* number of carbon atoms, concomitantly linked at the two termini of the peptide chain, are not able to induce any appreciable membrane activity; (ii) the position of the long acyl chain does have some influence, albeit not dramatic, on membrane activity (it is higher when the C_8 acyl chain is located at the N-terminus of the peptide chain); (iii) the presence of two C_8 acyl chains, concomitantly linked at the two termini of the peptide chain, produces a significant increase in membrane activity.

Despite recent significant advancements in our understanding of the mechanism of action of these lipopeptaibols in the membrane environment, at the present stage of our research we are not yet in the position of unambiguously discriminating between the *barrel-stave* (channel formation) mechanism, which implies that the peptide molecule would be parallel to the normal to the bilayer, and the *carpet-like* mechanism, which requires the helical peptide floating on the bilayer with the hydrophobic face buried into the membrane [27,28]. An investigation is currently underway in our laboratory aiming at unraveling the precise mechanism of action of these lipopeptaibols in the membrane environment by exploiting a series of synthetic, monolabeled trichogin GA IV analogues, designed to incorporate at selected positions (1, 4, 8) of the peptide chain the nitroxide-based amino acid TOAC [26], and the ESR technique, which will provide us with the immersion depth and orientation of the free radical moiety with respect to the lipid bilayer [29,30].

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