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Review

# Trichogin: a Paradigm for Lipopeptaibols‡

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Abstract: Lipopeptaibols are members of a novel family of naturally occurring, short peptides with antimicrobial activity, characterized by a lipophilic acyl chain at the *N*-terminus, a high content of turn/helix inducing $\alpha$-aminoisobutyric acid and a 1,2-amino alcohol at the *C*-terminus. Using solution methods, the prototypical lipopeptaibol trichogin GA IV and a large series of appropriately designed analogues were synthesized, which allow: (i) determination of the minimal lipid chain and peptide main-chain lengths for the onset of membrane activity, and (ii) exploitation of a number of physico-chemical techniques aimed at assessing the trichogin preferred conformation under a variety of conditions and at investigating its mechanism of interaction with the phospholipid membranes. 

Keywords: amphiphilicity; antibiotics; conformation; lipopeptaibols; membranes; peptides; trichogin

## INTRODUCTION

Peptaibols [1,2] are a unique group of membrane active compounds of fungal origin. These antimicrobial peptides are characterized by a linear sequence of 10–19 $\alpha$-amino acid residues (excluding lipopeptaibols, see below), a high population of the $C^{\alpha,\alpha}$-disubstituted glycine Aib ($\alpha$-aminoisobutyric acid or $C^{\alpha,\alpha}$-dimethylglycine), an *N*-terminal acetyl group and a *C*-terminal 1,2-(or $\beta$-)amino alcohol (for leading review articles see [3–11]). Because all the first peptaibols sequenced had a phenylalaninol at the *C*-terminus, they were originally classified as peptaibophols [12]. The long-sequence peptaibols, such as alamethicin, are known to form voltage-dependent channels in biological membranes.

More recently, a variety of peptides were sequenced, bringing a new feature to the peptaibol class of antibiotics, namely a fatty acyl moiety linked to the *N*-terminal amino acid [13–21]. Because of the lipophilic character of the *N*-terminal group, these peptides are referred to as lipopeptaibols [14]. Typically, most of them exhibit microheterogeneity, i.e. a series of closely similar peptides with a limited number of conservative variations in the sequence is present in the natural mixture. The amino acid sequences range from 6 (trichodecenins) to 10 (trichogin GA IV, trikoningins and antibiotics LP 237) residues, and the fatty acyl moieties from 8 to 15 carbon atoms. The amino acid sequence of trichodecenin I corresponds to that of the *C*-terminal region of trichogin GA IV. The latter is as follows:

*n*Oct-Aib[1]-Gly-Leu-Aib-Gly[5]-Gly-Leu-Aib
-Gly-Ile[10]-Lol

(where *n*Oct is *n*-octanoyl and Lol is leucinol).

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In addition to the frequently observed, achiral Aib, another residue of the family of $C^{\alpha,\alpha}$-disubstituted glycines (or $C^{\alpha}$-tetrasubstituted $\alpha$-amino acids) [22–28], the chiral Iva (isovaline or $C^{\alpha}$-methyl, $C^{\alpha}$-ethylglycine) is present in the sequence of a naturally occurring trichogin analogue (trikoningin KB II). As for the 1,2-amino alcohol, Lol is a reduced form of the *C*-terminal $\alpha$-amino acid -Leu-OH. It is reasonable to assume that Nature would perform *N*-terminal acylation and *C*-terminal reduction, thereby removing a positive and a negative charge, respectively, from their amino acid sequences, to facilitate membrane interaction of peptaibols and lipopeptaibols. It is also worth emphasizing that a membrane-compatible, *N*-terminal fatty acyl chain is a typical feature of only the peptaibols with the shortest peptide main chain. Interest in lipopeptaibols comes about because of their biophysical properties and antimicrobial activities. For a recent review article on lipopeptaibols the reader is referred to reference [29]. The present review article will focus exclusively on the published data of the extensively investigated trichogin GA IV, the paradigm of lipopeptaibols.

## RESULTS AND DISCUSSION

### Preferred Conformation

To investigate the conformational properties of trichogin GA IV, a number of carefully designed analogues were synthesized by solution methods [30–36] (Figure 1). The most relevant structural feature in the sequence of trichogin GA IV is the presence of the $C^{\alpha,\alpha}$-disubstituted glycine Aib to a remarkable extent (30%). This $\alpha$-amino acid is well known for its very strong tendency to induce $\beta$-turns [37–39] and $3_{10}/\alpha$-helical [40–42] structures in peptides [22–28]. This property is strictly related to the classic '*gem*-dialkyl' (or Thorpe–Ingold) effect of quaternary carbon atoms [43]. It is a reasonable hypothesis that Nature would have exploited this non-coded, hydrophobic amino acid to significantly stabilize folded/helical conformations of short peptides in the membrane environment.

In the crystal structure of trichogin GA IV racemate, as obtained by x-ray diffraction analysis (Figure 2A), both crystallographically independent molecules combine a short (3–4 residues), distorted, right-handed $3_{10}$-helix at the *N*-terminus with a longer segment of irregular, right-handed $\alpha$-helix [44]. The *n*-octanoyl chain is extended and oriented roughly perpendicularly to the helix axis. The structure is amphiphilic with all of the hydrophobic groups (*n*-octanoyl and Leu, Ile and Lol aliphatic side chains) on one helix face and the four Gly residues comprising the hydrophilic face. Aligned on the border between these two helical faces are the Aib methyl groups.

The crystal structure of the [$Ser^{2,5,6,9}$, $Leu^{11}$-OMe] trichogin analogue (Figure 2B) is close to that of the parent antibiotic, but the $\alpha$-helical segment is interrupted by an intramolecular H-bond from the $Ser^{9}$ hydroxyl side chain to the backbone carbonyl of $Ser^{6}$, which maintains the helical structure but shifts the ensuing backbone H-bond into a mixed $3_{10}/\alpha$-helical turn [45]. This structure represents a rare view of a truly amphiphilic helix, with all four Ser residues, significantly more hydrophilic than the corresponding Gly residues of natural trichogin, accommodated on the hydrophilic face. Rather surprisingly, in the crystal state the peptide chain of the non-amphiphilic [$Ser(Bu^{t})^{2,5,6,9}$, $Leu^{11}$-OMe] trichogin analogue is forced into a perfect $3_{10}$-helix [46] (Figure 2C). This observation might be related not only to the steric hindrance brought about by the four bulky *tert*-butyl groups but also, at variance with the structure of trichogin and its Ser-analogue, to the absence of solvation characterizing the structure of this hydrophobic analogue.

Predominantly helical structures with right-handed screw sense are observed for both crystallographically independent molecules of the [$TOAC^{4,8}$, $Leu^{11}$-OMe] trichogin analogue (where TOAC is 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-4-carboxylic acid) [33] (Figure 2D). The structure of one molecule starts as a $3_{10}/\alpha$-helix, but then switches to a pure $\alpha$-helix formed by five consecutive $\alpha$-turns. The backbone conformation of the other molecule differs only at the *C*-terminus.

The x-ray diffraction work on the [$Fmoc^{0}$, $TOAC^{4,8}$, $Leu^{11}$-OMe] trichogin analogue (where Fmoc is fluoren-9-ylmethyloxycarbonyl) (Figure 2E) confirmed our expectations in that the strong helix-forming $C^{\alpha,\alpha}$-disubstituted glycine TOAC [47] may replace Aib residues at given positions in the amino acid sequence without inducing a significant disturbance to the overall peptide secondary structure [48]. The small changes between natural trichogin and its [$Fmoc^{0}$, $TOAC^{4,8}$, $Leu^{11}$-OMe] analogue are seen (i) at the *N*-terminus, where the two consecutive, type-III helical $\beta$-turns in the analogue replace two more irregular $\beta$-turns in the naturally occurring lipopeptaibol, the former of which is a classic type-I $\beta$-turn; and (ii) at the *C*-terminus, where a

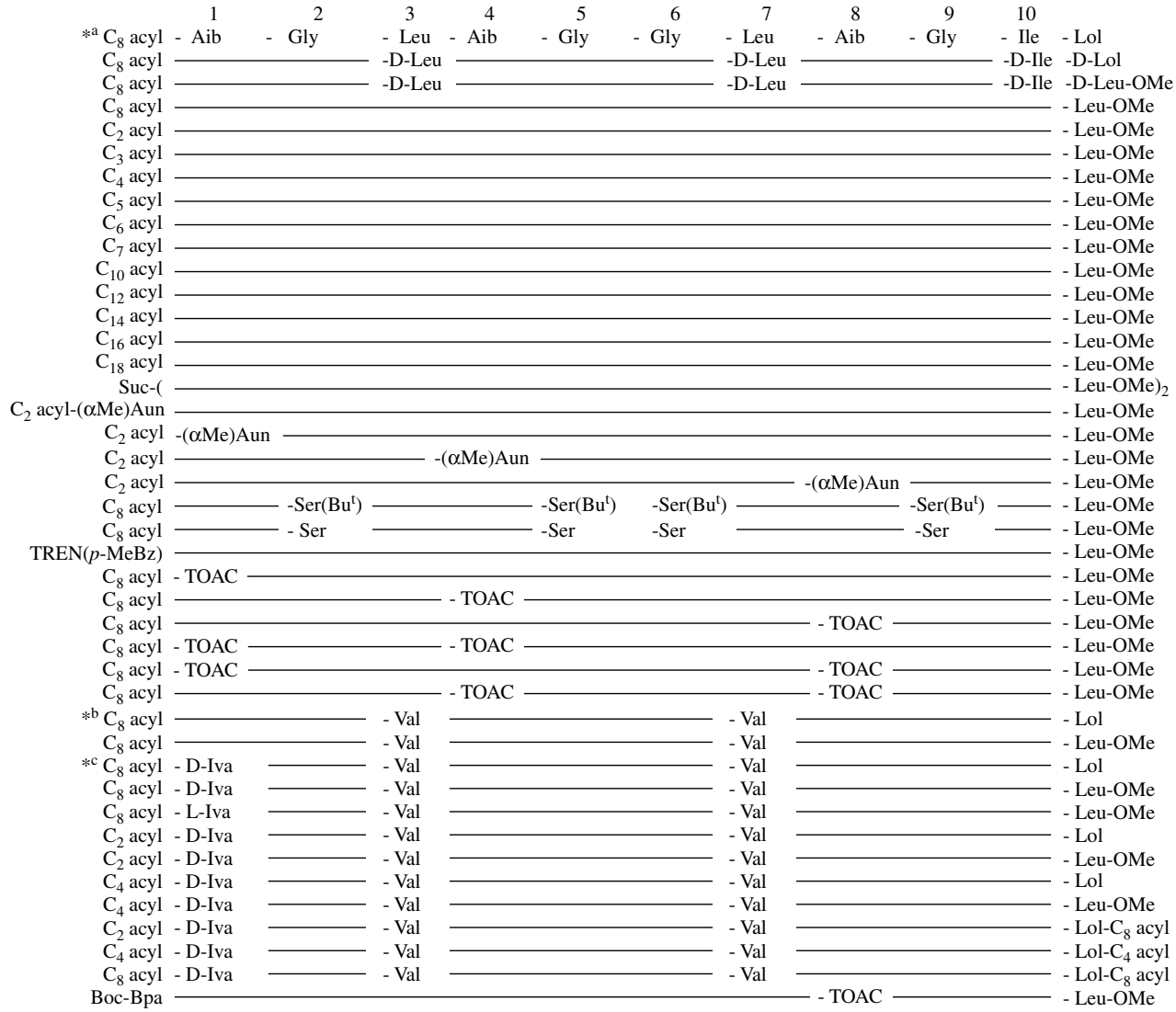



Figure 1 Analogues of trichogin GA IV synthesized and studied to date. The length of the *N*-terminal acyl chain is emphasized (e.g. $C_2$ acyl, acetyl; $C_8$ acyl, octanoyl, etc.). Other abbreviations are OMe, methoxy; Suc, succinoyl; (αMe)Aun, 2-amino-2-methyl-undecanoic acid; $Bu^t$, *tert*-butyl; TOAC, 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-4-carboxylic acid; TREN(*p*MeBz), tris(2-*para*-carboxyphenylmethyl-aminoethyl)amine; Boc, *tert*-butoxycarbonyl; Bpa, 4-benzoylphenylalanine. Starred sequences are those of the natural lipopeptaibols (*a*, trichogin GA IV; *b*, trikoningin KB I; *c*, trikoningin KB II).

regular α-helix in the lipopeptaibol twists into a mixed $3_{10}/\alpha$-helix in the analogue. However, it is relevant to note that in both peptide molecules the central sequence (where the two TOAC residues have been incorporated) is folded in a regular α-helical structure.

Both crystallographic independent molecules of the [Boc-$Bpa^0$, $TOAC^8$, $Leu^{11}$-OMe] (where Boc is *tert*-butoxycarbonyl and Bpa is 4-benzoylphenylalanine) trichogin analogue exhibit mixed helical conformations [49] (Figure 2F). One of the molecules shows a more regular α-helical structure, while the other is characterized by a $3_{10}$-helical structure at the *N*-terminus followed by an α-helical structure.

The three latter observations allowed us to exploit these analogues in the investigation of the solution conformational tendency and the mode of interaction with the membranes of the lipopeptaibol using the stable nitroxide free radical of TOAC and the electron spin resonance (ESR) and fluorescence quenching techniques.

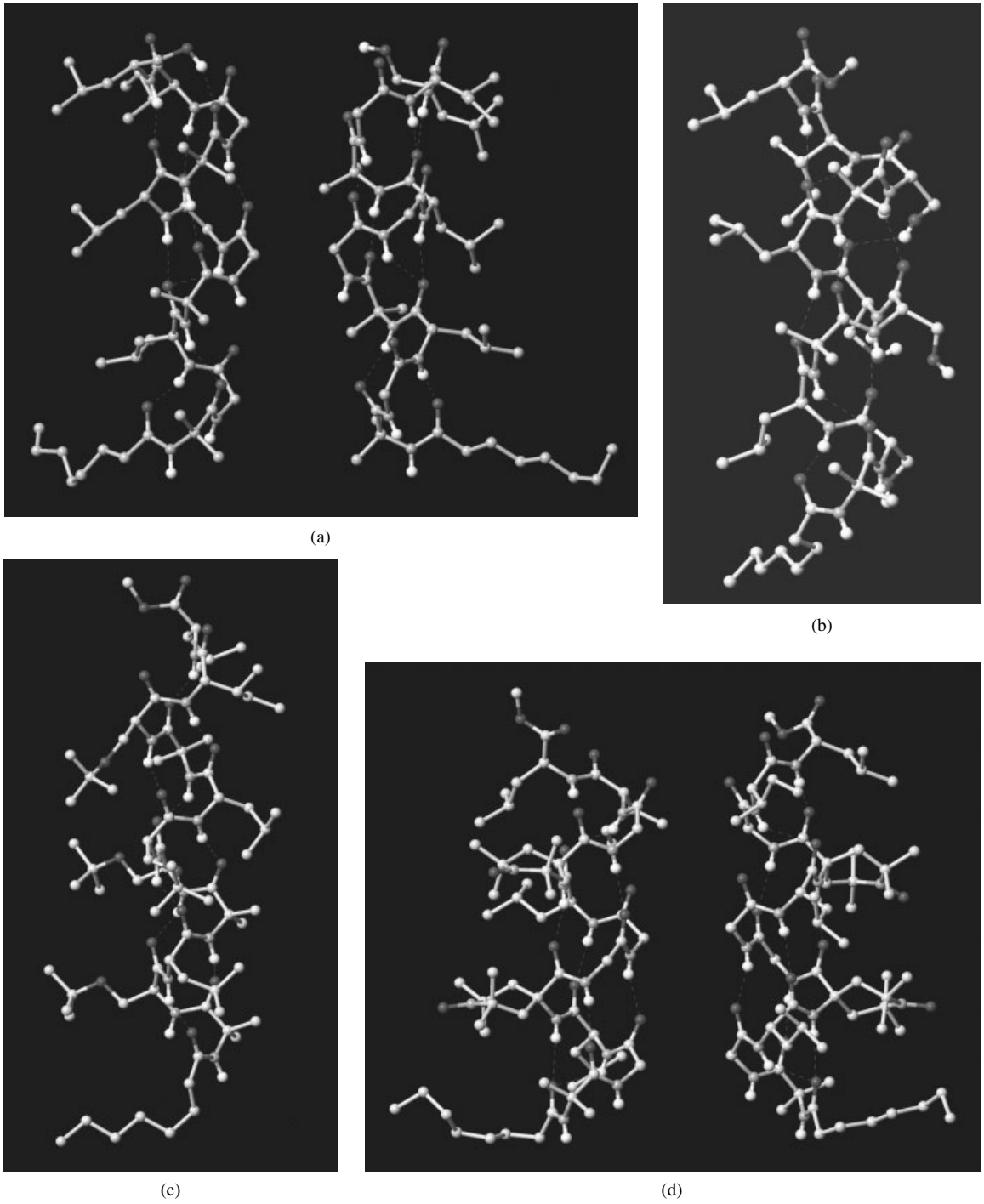
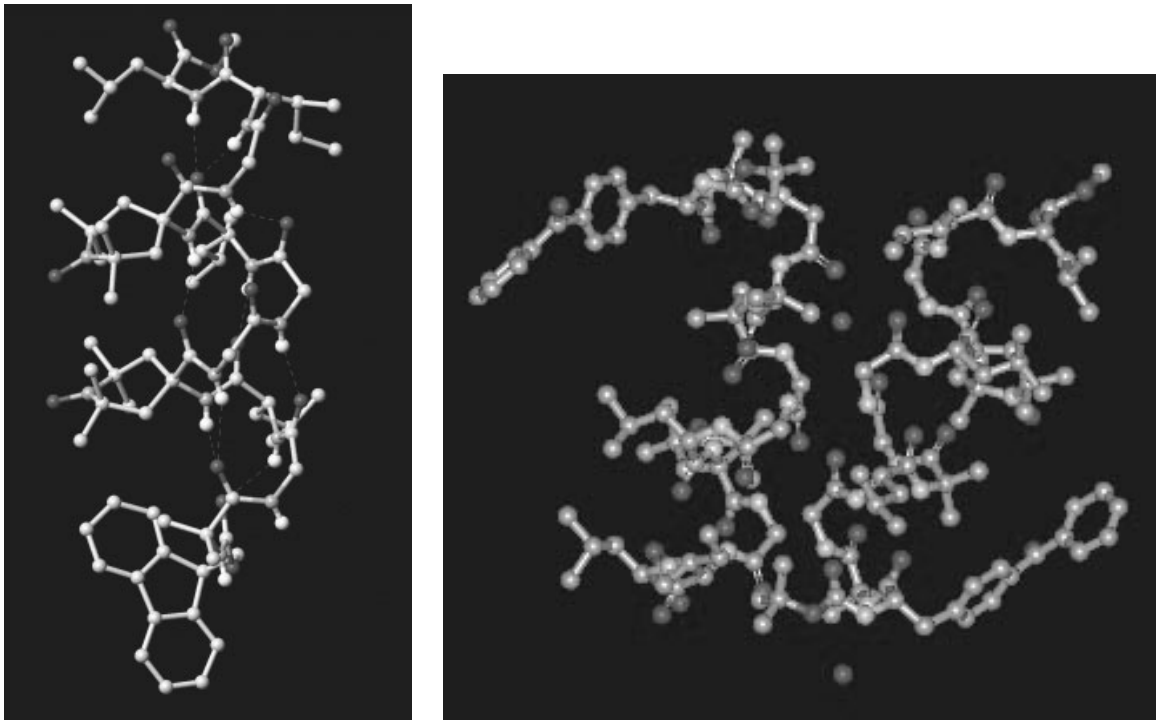


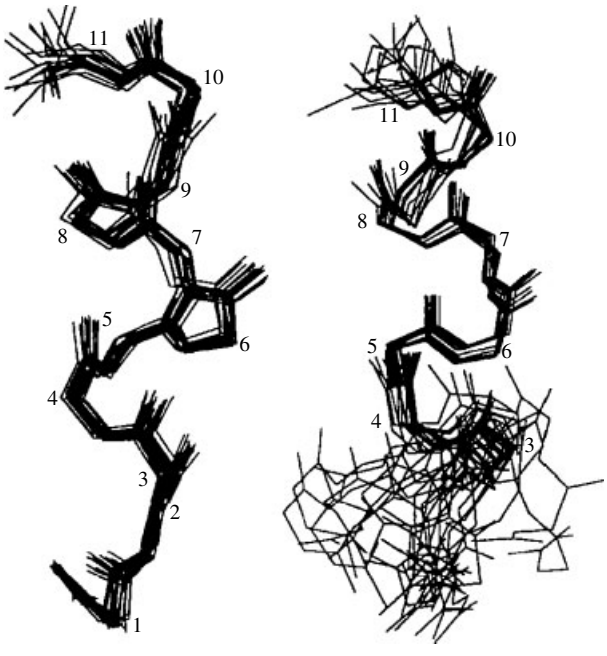

Figure 2 X-ray diffraction structures of: (A) the two crystallographically independent, all-L molecules of trichogin GA IV racemate; (B) the [Ser$^{2,5,6,9}$, Leu$^{11}$-OMe] analogue; (C) the [Ser(Bu$^{t}$)$^{2,5,6,9}$, Leu$^{11}$-OMe] analogue; (D) the two crystallographically independent molecules of the [TOAC$^{4,8}$, Leu$^{11}$-OMe] analogue; (E) the [Fmoc$^{0}$, TOAC$^{4,8}$, Leu$^{11}$-OMe] analogue; (F) the two crystallographically independent molecules of the [Boc-Bpa$^{0}$, TOAC$^{8}$, Leu$^{11}$-OMe] analogue.



(e) (f)

Figure 2 (*Continued*).

The preferred conformation and self-assembling properties of trichogin GA IV and its synthetic analogues were also examined in solution (and in the glassy liquid state as well) under a variety of conditions by Fourier transform-infrared (FT-IR) absorption, circular dichroism (CD), nuclear magnetic resonance (NMR), ESR and related techniques [14, 30–35, 50–55]. A pioneering study suggested an amphiphilic, right-handed, mixed $3_{10}/\alpha$-helical structure in MeOH solution [14]. A similar conformation was described for the [Leu$^{11}$-OMe] analogue in the same solvent [30]. Mixed $3_{10}/\alpha$-helical structures were reported for the [Leu$^{11}$-OMe] [32], [Ac-($\alpha$Me)Aun$^0$, Leu$^{11}$-OMe] [31], [Ac$^0$, ($\alpha$Me)Aun$^1$, Leu$^{11}$-OMe] [51], [Ac$^0$, ($\alpha$Me)Aun$^4$, Leu$^{11}$-OMe] [51], [Ac$^0$, ($\alpha$Me)Aun$^8$, Leu$^{11}$-OMe] [51], [Ser$^{2,5,6,9}$, Leu$^{11}$-OMe] [32], [Val$^{3,7}$: trikoningin KB I] [50], [D-Iva$^1$, Val$^{3,7}$: trikoningin KB II] [50], its diastereomer [L-Iva$^1$, Val$^{3,7}$] [50] and single TOAC-based [35] trichogin analogues in a number of alcohols and in a membrane-mimetic environment (Figure 3). The helical structure of the Ser-analogue is remarkably the least flexible. Different acyl moieties at the *N*-terminus do not influence the overall peptide conformational preference [30]. In those cases where it was possible to define the direction of the *N*-terminal




(a) (b)

Figure 3 Overlay of the 27 minimized structures of [Ser$^{2,5,6,9}$, Leu$^{11}$-OMe] trichogin GA IV (A), and of 19 structures of [Leu$^{11}$-OMe] trichogin GA IV selected among the 29 minimized structures (B).

*n*-octanoyl chain, a perpendicular orientation with respect to the helix axis was observed. The helical structure of trichogin seems to be maintained in the symmetrical conformation adopted by the head-to-head succinoyl dimer [30]. In the *bis*-TOAC trichogin analogues in alcohol solutions the *N*-terminal region of the peptides folds in a $3_{10}$-helix, while the central and *C*-terminal regions preferentially adopt the $\alpha$-helical conformation [33,34], as observed in the crystal state. However, the *C*-terminal region seems to be in equilibrium with unfolded conformers. It was suggested that the flexible -Gly$^5$-Gly$^6$- stretch creates a hinge point between the two helical regions.

## Membrane Activity

The major reason for interest in trichogin GA IV is the finding that this lipopeptaibol has a considerable membrane perturbing activity [14]. Relatively minor changes in the amino acid sequence of this lipopeptaibol can have large effects on the membrane activity. Trikoningin KB I has the same structure as trichogin, except that the Leu residues at positions 3 and 7 in trichogin are substituted for Val in trikoningin. This replacement results in a 25-fold reduction in the potency of the peptide to release the aqueous contents of small unilamellar liposomes of phosphatidylcholine and cholesterol [15]. The substitution of Aib$^1$ (trikoningin KB I) with D-Iva (trikoningin KB II) or L-Iva [50] does not modify the peptide–bilayer interaction [15, 36, 50].

Both trichogin and trikoningins have an *n*-octanoyl group at the *N*-terminus. Lipidation of these peptides has been shown to be essential for the membrane activity. Trichogin analogues acylated with chains shorter than four carbon atoms are inactive and there is an increased membrane activity with increasing acyl chain length for both trichogin [30] and trikoningins [36]. A possible role for acylation is that it facilitates the partitioning of the lipopeptide from water into the membrane. However, moving the *n*-octanoyl group to the *C*-terminus of trikoningin slightly reduces the membrane activity [36], suggesting that the activity of the lipopeptide is not determined exclusively by its overall hydrophobicity. Nevertheless, partitioning is likely to play a significant role, since incorporating *n*-octanoyl groups at both ends of the peptide increases activity [36]. On the other hand, the membrane activity is not sensitive to moderate changes in the location of the lipidic group since moving the acyl chain from the *N*-terminal amino group of trichogin to the side chain of residue 1, 4 or 8 results in similar activity [31,51]. There may also be other roles for the acyl group, including orienting the peptide in the membrane in a particular manner, or the acyl chain itself may contribute to the destabilization of the membrane.

The short length (10 amino acid residues) of trichogin would suggest that it is not capable of spanning a membrane. This property is in contrast to the well-known action of the 19-amino acid, non-lipidated peptaibol alamethicin that is sufficiently long to span a membrane bilayer [3–11]. The ability of alamethicin to form channels is thought to be a result of it forming an aggregate of amphipathic helices surrounding an aqueous channel and oriented along the bilayer normal. The ability to form this barrel stave arrangement is dependent on the concentration of the peptide in a membrane [56].

There are other lipopeptaibols with peptide moieties that are too short to span the bilayer. The shortest examples are trichodecenins I and II which are only six amino acids in length and whose *N*-terminus is acylated with 4-decenoic acid [16]. Long-sequence peptaibols have only an acetyl group on the *N*-terminal function [3–11]. All of the lipopeptaibols that are N$^\alpha$-acylated with an 8–15 carbon fatty acid have between six and ten amino acids. Membrane activity, albeit modest, is found at the level of a synthetic trichogin sequence as short as the N$^\alpha$-octanoylated, Leu$^4$-OMe *C*-terminal tetrapeptide [57]. Membrane activity progressively increases from the tetra- to the Leu$^{11}$-OMe undecapeptide. It should be remembered, however, that peptaibols are rich in Aib residues that frequently form $3_{10}$-helices, rather than $\alpha(3.6_{13})$-helices. As the $3_{10}$-helix is more elongated and thinner than the $\alpha$-helix, to span a membrane bilayer a $3_{10}$-helical peptide of only ~15 amino acids would be required, rather than the ~20 amino acids required for an $\alpha$-helix. Even if the peptaibols do not fold into a perfect $3_{10}$-helix, the presence of the Aib residues is likely to lower the activation energy for the transient formation of such an ordered structure. Nevertheless, the shorter lipopeptaibols would still be too short to span the bilayer unless they dimerize end-to-end. Indeed, a synthetic, trichogin dimer, covalently linked head-to-head through a short spacer (a succinoyl moiety), proved to be remarkably active [30].

Several studies have indicated where trichogin is located within a membrane. The approach used has been to substitute a nitroxide spin label for the Aib residues. The nitroxide chosen, TOAC, is

itself an $\alpha$-amino acid that can substitute for Aib residues without a change in the conformation of the peptide or in the functional properties [47]. The probe is substituted, one at a time, for each of the three Aib residues in trichogin. The extent of insertion of the peptide portion of trichogin was assessed by the burial of the TOAC residues using both ESR [35] and fluorescence quenching [58] techniques. Both approaches led to similar conclusions, i.e. the peptide portion of the molecule is helical and the long helix axis is oriented in the plane of the bilayer (carpet-like mechanism) with the hydrophobic face (comprising the fatty acyl chain and the $Leu^{3,7}$, $Ile^{10}$ and Lol side chains) oriented toward the membrane and the polar face (comprising primarily Gly residues) facing the water. This finding is difficult to reconcile with a barrel stave model of pore formation. Therefore, other modes of action must be considered. A preliminary investigation of membrane activities of the two trichogin enantiomers suggested that their diastereomeric interactions with chiral lipid environments are comparable, but apparently are slightly more efficient for the all-D enantiomer [59].

One test of the mechanism of membrane activity induced by trichogin may come from its lipid dependence. The activity induced by trichogin is not affected by the presence of cholesterol in the membrane [14], in contrast to the lower potency observed with non-lipidated peptaibols with membranes containing cholesterol. These leakage experiments were performed with sonicated liposomes. Such preparations are known to be intrinsically unstable because of the high curvature required. The extent of the curvature strain is also likely to be increased by the presence of high mole fractions of cholesterol that would reduce the flexibility of the membrane. Nevertheless, the inhibition of the leakage induced by the non-lipidated peptaibols is not unexpected. Cholesterol is known to tighten the packing of lipid molecules, thus restricting access to other substances. In agreement with this result, Duval *et al.* [60] found that the 17-residue trichorzins are more deeply embedded in membranes not containing cholesterol. Also the shorter 13-residue harzianins, which do not appear to function via a barrel stave mechanism, are inhibited by cholesterol [61]. Although the self-association of alamethicin is independent of the amount of cholesterol in the membrane, the binding of this peptide to a membrane is inhibited by the presence of cholesterol [62]. However, the relevance of cholesterol effects to the biological action of these peptides is questioned by the observation that alamethicin, as well as peptaibols of the trichorzin family of 17-residue peptides, have ionophoric activity that is independent of the amount of cholesterol in the membrane [63]. The question is of particular relevance with regard to antibacterial activity, since selectivity of the lipopeptide toward bacterial cells compared with mammalian cells would be achieved if there were inhibition of the membrane action by cholesterol. Mammalian cell membranes are rich in cholesterol while those of bacteria are devoid of this lipid. This factor has been suggested to contribute to the bacterial specificity of magainin [64]. Thus, lipopeptaibols may exhibit less antimicrobial specificity than non-lipidated peptides. In addition, lipopeptides and lipidated proteins with longer saturated acyl chains are sequestered into cholesterol-rich domains, or 'rafts', in mammalian membranes [65]. This property might be used to advantage by substituting the acyl chain of the lipopeptaibol so as to target it to particular domains of biological membranes. This can be done because lipopeptides with longer saturated acyl chains sequester into rafts, while those with unsaturation do not. The location of peptaibols in the membrane is likely to affect its toxicity as well as to have other biological consequences.

As mentioned above, the peptide portion of trichogin lies along the membrane–water interface parallel to the plane of the bilayer. This property is a consequence of the helical conformation of the peptide that positions the hydrophobic residues on one face of the helix. However, the opposite hydrophilic face is not very polar, being composed largely of Gly residues. When the four Gly residues in the peptide are substituted by Ser residues, the hydrophobic moment is greatly increased and the peptide is stabilized at the membrane–water interface. This substitution leads to a modest increase in bacteriostatic activity [66].

There is also an effect of the curvature properties of lipids in the target membrane on the effects of peptides and lipopeptides. There are two aspects of membrane curvature that can affect membrane bilayer stability. One is the intrinsic tendency of a particular membrane to form a curved structure and the other is the elasticity of the membrane that determines the energy that is required to alter the curvature. Peptides can affect membranes so as to increase positive curvature, i.e. curvature with the headgroups having a larger cross-sectional area, as occurs in water-soluble micelles. Peptides can also affect membranes so as to increase negative

curvature, i.e. curvature with the methyl ends of the acyl chains having a larger cross-sectional area, as occurs in the inverted hexagonal phase ($H_{II}$). Membrane curvature is a property that is biologically regulated so as to maintain homeostasis [67]. A substance that partitions into the membrane and has a marked effect on membrane curvature is likely to be cytotoxic because it will destabilize the bilayer structure. Trichogin increases positive membrane curvature [66]. Substitution of the four Gly residues of trichogin with Ser enhances the promotion of positive curvature by the peptide, increasing the leakage of liposomes as well as the bacteriostatic activity [66].

It is interesting to compare the consequences of increasing the hydrophilicity of residues 2, 5, 6 and 9 by substituting Ser for Gly to an analogue in which the hydrophilicity of these residues is decreased. This was accomplished by substituting the Gly residues with the more hydrophobic Ser($Bu^t$) residues. The resulting trichogin analogue promotes negative, rather than positive, curvature. It is less potent than trichogin in inducing leakage. The observed leakage from liposomes is relatively insensitive to the lipid composition of the membrane [66]. However, this Ser($Bu^t$) analogue is effective at promoting liposomal fusion. This activity was observed only with liposomes composed of lipids which themselves had an intrinsic negative curvature [66]. It is known that the initiation of membrane fusion to form a stalk intermediate requires increased negative curvature, so that these results are explicable in terms of the effect of this peptide on membrane curvature. The more hydrophilic analogue with Ser does not promote membrane fusion. Thus, the membrane effects of the two trichogin analogues with Ser or with Ser($Bu^t$) residues make an interesting comparison. The two analogues have opposing effects on membrane curvature and they destabilize membrane bilayers in different ways. In the case of the Ser-analogue the consequence is membrane leakage. With the Ser($Bu^t$) analogue the result of membrane interaction is the promotion of membrane fusion.

Finally, some of us also succeeded in the synthesis of a tripodal trichogin analogue able to alter membrane permeability and showed that the observed effect can be controlled by the addition or removal of Zn(II) ions [68]. It is likely that this is due to a conformational change of the template–peptide conjugate in the membrane biased by the formation of the metal complex.

Trichogin GA IV and its appropriately designed analogues can thus have a variety of effects on membranes, including the induction of leakage. Some of them have been shown to have antibacterial activity. In addition, trichogin is an excellent, short and easy-to-synthesize template for biophysical studies of lipopeptide–membrane interactions as well as for designing novel antimicrobial agents with improved properties.

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