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Review

Lipopeptaibols, a novel family of membrane active, antimicrobial peptides

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Abstract. Lipopeptaibols are members of a novel group of naturally occurring, short peptides with antimicrobial activity, characterized by a lipophilic acyl chain at the N-terminus, a high content of the turn/helix forming α-aminoisobutyric acid and a 1,2-amino alcohol at the C-terminus. The amino acid sequences range from 6 to 10 residues and the fatty acyl moieties from 8 to 15 carbon atoms. The peptide portion of lipopeptaibols can be shorter than those of the nonlipidated peptaibols that range from 10 to 19 amino acid residues. The longest peptides fold into a mixed $3_{10}/\alpha$ helix, whereas the shortest peptides tend to adopt a β-turn/sheet structure. Using solution methodologies, a series of analogues of trichogin GA IV was synthesized which allowed determination of the minimal lipid chain and peptide main-chain lengths for the onset of membrane activity and exploitation of a number of spectroscopic techniques aimed at determining its preferred conformation under a variety of conditions and investigating in detail its mode of interaction with, and its effect on, the phospholipid membranes.

Key words. Amino acid sequences; amphiphilicity; antibiotics; membranes; peptaibols; peptides; 3D-structure.

Introduction

Peptaibols [1, 2] are a unique group of membrane active compounds of fungal origin. These antibiotic peptides are characterized by a linear sequence of 10–19 α-amino acid residues (excluding lipopeptaibols, see below), a high population of the $C^{\alpha,\alpha}$-disubstituted glycine Aib (α-aminoisobutyric acid), an N-terminal acetyl group and a C-terminal 1,2-(or β-)amino alcohol (for leading review articles see [3–7]). Because all of the first peptaibols sequenced had a phenylalaninol at the C-terminus, they were originally classified as peptaibophols [8]. The long-sequence peptaibols, such as alamethicin, are known to form voltage-dependent membrane channels and to modify the permeability of any kind of natural or model membrane even in the absence of an applied voltage.

More recently, a variety of peptides were sequenced, bringing a new characteristic to the peptaibol class of antibiotics, namely a fatty acyl moiety linked to the N-terminal amino acid [9-17]. Because of the lipophilic character of the N-terminal group, these peptides are referred to as lipopeptaibols [10]. They were isolated from the cultures of the fungi *Trichoderma longibrachiatum* (trichogin), *Trichoderma koningii* (trikoningins), *Trichoderma polysporum* (trichopolyns), *Trichoderma viride* (trichodecenins), *Tolypocladium geodes* (antibiotics LP 237) and *Mycogone rosea* (helioferins).

The primary structures of the lipopeptaibols known to date are listed in figure 1. Typically, most of them exhibit microheterogeneity, i.e. a series of closely similar peptides

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		1 2 3 4 5 6 7 8 9 10
TRICHOGIN	GA IV	FA-**Aib**-Gly-**Leu**-Aib-Gly-Gly-**Leu**-Aib-Gly-Ile-Lol
TRIKONINGINS	KBI	FA-**Aib**-Gly-**Val**-Aib-Gly-Gly-**Val**-Aib-Gly-Ile-Lol
	KBII	FA-**Iva**-Gly-**Val**-Aib-Gly-Gly-**Val**-Aib-Gly-Ile-Lol
ANTIBIOTICS LP237	F5	FA-Aib-Pro-**Tyr**-Aib-Gln-Gln-Aib-**Etn**-Gln-Ala-Lol
	F7	FA-Aib-Pro-**Phe**-Aib-Gln-Gln-Aib-**Aib**-Gln-Ala-Lol
	F8	FA-Aib-Pro-**Phe**-Aib-Gln-Gln-Aib-**Etn**-Gln-Ala-Lol

		1 2 3 4 5 6 7 8 9
TRICHOPOLYNS	I	FA-Pro-AHMO-Ala-Aib-Aib-**Ile**-Ala-Aib-**Aib**-AMAE
	II	FA-Pro-AHMO-Ala-Aib-Aib-**Val**-Ala-Aib-**Aib**-AMAE
	III	FA-Pro-AHMO-Ala-Aib-Aib-**Ile**-Ala-Aib-**Ala**-AMAE
	IV	FA-Pro-AHMO-Ala-Aib-Aib-**Val**-Ala-Aib-**Ala**-AMAE
	V	FA′-Pro-AHMO-Ala-Aib-Aib-**Ile**-Ala-Aib-**Aib**-AMAE

		1 2 3 4 5 6 7 8
HELIOFERINS	A	FA-Pro-AHMO-Ala-Aib-Ile-Ile-Aib-Aib-**AAE**
	B	FA-Pro-AHMO-Ala-Aib-Ile-Ile-Aib-Aib-**AMAE**

		1 2 3 4 5 6
TRICHODECENINS	I	FA-Gly-Gly-Leu-Aib-Gly-**Ile**-Lol
	II	FA-Gly-Gly-Leu-Aib-Gly-**Leu**-Lol

Figure 1. Primary structures of the lipopeptaibols (FA stands for fatty acid). All protein amino acids have the L-(S-) configuration at the 2-(α-)carbon. Residues, where changes in the primary structure of a given group of lipopeptaibols are found, are represented in bold.

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, 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 chemical formulas for the nonprotein α-amino acids N-terminal fatty acids, and 1,2-amino alcohols are given in figure 2. In addition to the frequently observed, achiral Aib, two other residues of the family of $C^{\alpha,\alpha}$-disubstituted glycines (or C^{α}-tetrasubstituted α-amino acids) [18–20], the chiral Iva and Etn residues, were shown to occur, albeit rarely, in the sequences of lipopeptaibols. As for the 1,2-amino alcohols, Lol is a reduced form of the C-terminal α-amino acid -Leu-OH, whereas AMAE and AAE are reduced forms of the C-terminal dipeptide units -Ala-Sar-OH (where Sar is sarcosine or N-methyl glycine) and -Ala-Gly-OH, respectively. 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 mentioning that a membrane- compatible, N-terminal fatty acyl chain is the typical feature of only the peptaibols with the shortest peptide main chain.

Interest in the structure and properties of lipopeptaibols comes about because of their biological activities and possible pharmacological applications. These are summarized in tables 1 and 2.

Three-dimensional structure

Among the various peptaibols conformational analyses were performed only on the decapeptide trichogin GA IV and the hexapeptide trichodecenin I. In particular, to investigate the conformational and membrane-modifying properties (for the latter, see next section) of trichogin GA IV, a number of carefully designed analogues were synthesized by solution methods [21–27] (fig. 3). The most relevant structural feature in the sequences of lipopeptaibols is the occurrence of $C^{\alpha,\alpha}$-disubstituted glycines (Aib, Iva, Etn) to a remarkable extent, from 17% in trichodecenins to as high as 44% in trichopolyns I, II and V. This family of α-amino acids is well known for its very strong tendency to induce β turns [28–30] and $3_{10}/\alpha$-helical [31–33] structures in peptides [18–20]. This property is strictly related to the classic '*gem*-dialkyl' (or Thorpe-Ingold) effect of quaternary carbon atoms [34, 35]. It is a reasonable hypothesis that nature would have exploited these unusual, hydrophobic amino acids to significantly stabilize folded/helical conformations of short peptides in the membrane environment.

In the X-ray diffraction structure of trichogin GA IV racemate 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 α-helix [36]. 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 ali-

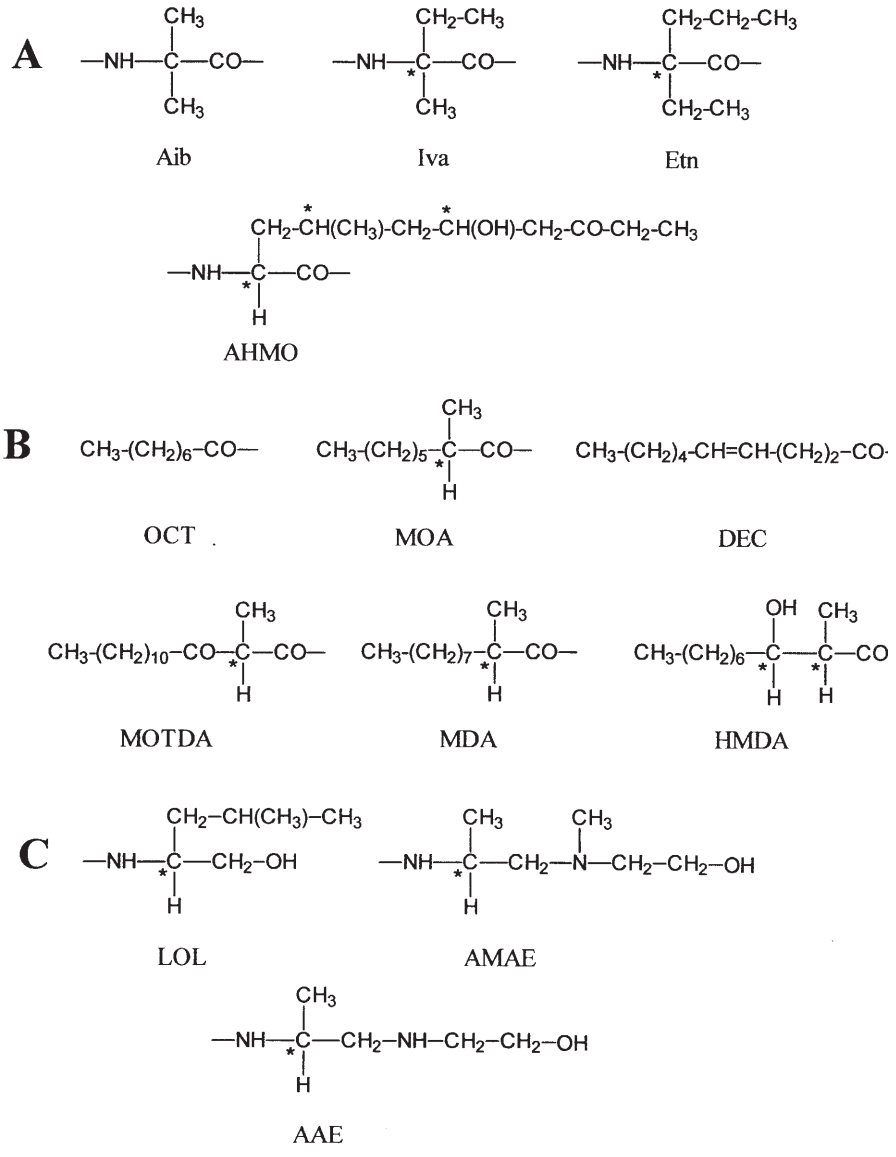

Figure 2. Chemical formulas of nonprotein α-amino acids (*A*), fatty acids (*B*), and 1,2-(or β-)amino alcohols (*C*) found in lipopeptaibols. The abbreviations for the non-protein α-amino acids are: Aib, α-aminoisobutyric acid or $C^{\alpha,\alpha}$-dimethylglycine; Iva, isovaline or C^{α}-methyl, C^{α}-ethylglycine; Etn, α-ethylnorvaline or C^{α}-ethyl, C^{α}-*n*-propylglycine; AHMO, 2-amino-6-hydroxy-4-methyl-8-oxo-decanoic acid. The configurations of Iva and AHMO are (2R) and (2S, 4S, 6S), respectively, whereas that of Etn is not established. The abbreviations for the fatty acids are OCT, *n*-octanoyl; MOA, 2-methyl-octanoyl; DEC, 4-decenyl; MOTDA, 2-methyl-3-oxo-tetradecanoyl; MDA, 2-methyl-decanoyl; HMDA, 3-hydroxy-2-methyl-decanoyl. The configurations of DEC is (4*Z*), those of MOA and MODTA are unknown, whereas those of MDA and HMDA are (2R) and (2S,3S), respectively. The abbreviations for the 1,2-(or β-)aminoalcohols are Lol, leucinol; AAE, 2-(2′-aminopropyl)-aminoethanol; AMAE, 2-[(2′-aminopropyl)-methylamino]-ethanol (or Tdol, trichodiaminol). The configuration of Lol is (2S) and those of AAE and AMAE are both (S).

phatic 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 (fig. 4) is close to that of the parent antibiotic, but the α-helical segment is interrupted by an intramolecular hydrogen 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 [37]. 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 trichogin, accommodated on the hydrophilic face. Rather surprisingly, in the crystal state the peptide chain of the nonamphiphilic [$Ser(Bu^{t})^{2,5,6,9}$, Leu^{11}-OMe] trichogin analogue is forced into a perfect 3_{10}-helix [38]. 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

Table 1. Summary of the Biological Activities of Lipopeptaibols.

Lipopeptaibols	References	Description of biological activities
Trichopolyns	9, 17	Immunosuppressants with a mode of action different from that of cyclosporin A (in particular, they do not affect the production of interleukin 4). IC^{*}_{50} (nM) for proliferation of lymphocytes in mouse allogeneic MLR: trichopolyn I 5.2, trichopolyn II 10.7 (for comparison, cyclosporin A 7.5).
Trichogin GA IV	10, 21	Antibacterial activity against *Staphylococcus aureus*; inhibition diameter: 13 mm at 3 µg/pit, 9 mm at 1.5 µg/pit.
Trikoningins	11	Antibacterial activity against *Staphylococcus aureus* (strain 209 P); inhibition diameter: 11 mm at 6.2 µg/pit. Inactive against *Escherichia coli*.
LP 237s	15, 16	Cytotoxicity (antitumor activity) against mouse leukemia cells ($IC_{50} \approx 0.5$ µg/ml) and a number of carcinoma cell lines (lung, ovarian, colon, breast) ($IC_{50} = 0.2–0.5$ µg/ml). The cytotoxicity of the single components is lower than that of the naturally occurring mixture of peptides. Therefore, it is reasonable to assume that the most potent component of the mixture remains to be identified (assuming that several components are not acting synergistically).
Helioferins	13, 14	Hemolysis at >100 µg/ml. Cytostatic activity ($IC_{50} = 0.01–0.04$ µg/ml) against leukemia and mouse fibroblast cell lines. Protonophoric activity (moderate) causing uncoupling of oxidative phosphorylation in mitochondria. Strong antibacterial activity against Gram-positive bacteria and mycobacteria, and strong antifungal activity (see table1).

* Concentration of peptide required to produce 50% of the indicated response.

Table 2. Minimal Inhibitory Concentrations (MIC) of Helioferins.

Bacteria	Gram +/–	MIC (µg/ml)	Fungi	MIC (µg/ml)
Bacillus subtilis	+	3.0	*Rhodotorula rubra*	0.78
Staphylococcus aureus	+	1.5	*Kloeckera brevis*	3.12
Micrococcus luteus	+	1.5	*Fusarium culmorum*	6.25
Mycobacterium phlei	+	0.8	*Penicillium notatum*	1.56
Mycobacterium smegmatis	+	3.1	*Candida albicans*	5.00
Escherichia coli	–	>100		
Pseudomona aeruginosa	–	>100		

of solvation characterizing the structure of this hydrophobic analogue. The X-ray diffraction work on the [$Fmoc^0$, $TOAC^{4,8}$, Leu^{11}-OMe] trichogin analogue (where Fmoc is fluoren-9-ylmethyloxycarbonyl and TOAC is 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl-4-carboxylic acid) confirmed our expectations in that the strong helix-forming $C^{\alpha,\alpha}$-disubstituted glycine TOAC [39] may replace Aib residues at given positions in the amino acid sequence without inducing a significant disturbance to the overall peptide secondary structure [40]. The small changes between 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 β turns in the analogue replace two more irregular β turns in the naturally occurring lipopeptaibol, the former of which is a classic type-I β turn; and (ii) at the C-terminus, where a 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. This observation allowed us to exploit this analogue to investigate 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 ESR and fluorescence-quenching techniques (see below).

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 under a variety of conditions by Fourier transform-infrared (FT-IR) absorption, circular dichroism (CD), nuclear magnetic resonance (NMR), electron spin resonance (ESR) and related techniques [10, 21–26, 41, 42]. A pioneering study suggested an amphiphilic, right-handed, mixed $3_{10}/\alpha$-helical structure of relatively low stability in MeOH solution [10]. A similar conformation was described for the [Leu^{11}-OMe] analogue in the same solvent [21]. Mixed $3_{10}/\alpha$-helical structures were reported for the [Leu^{11}-OMe] [23], [Ac-(αMe)Aun^0, Leu^{11}-OMe] [22], [$Ser^{2,5,6,9}$, Leu^{11}-OMe] [23] and single TOAC-based [26] trichogin analogues in a number of alcohols and in a membrane-mimetic environment. The helical structure of

	1	2	3	4	5	6	7	8	9	10	
*a C_8 acyl	- Aib	- Gly	- Leu	- Aib	- Gly	- Gly	- Leu	- Aib	- Gly	- Ile	- Lol
C_8 acyl			-D-Leu				-D-Leu			-D-Ile	-D-Lol
C_8 acyl			-D-Leu				-D-Leu			-D-Ile	-D-Leu-OMe
C_8 acyl											- Leu-OMe
C_2 acyl											- Leu-OMe
C_3 acyl											- Leu-OMe
C_4 acyl											- Leu-OMe
C_5 acyl											- Leu-OMe
C_6 acyl											- Leu-OMe
C_7 acyl											- Leu-OMe
C_{10} acyl											- Leu-OMe
C_{12} acyl											- Leu-OMe
C_{14} acyl											- Leu-OMe
C_{16} acyl											- Leu-OMe
C_{18} acyl											- Leu-OMe
Suc-(											- Leu-OMe)$_2$
C_2 acyl-(αMe)Aun											- Leu-OMe
C_2 acyl	-(αMe)Aun										- Leu-OMe
C_8 acyl		-Ser(Bu^t)			-Ser(Bu^t)	-Ser(Bu^t)			-Ser(Bu^t)		- Leu-OMe
C_8 acyl		- Ser			- Ser	- Ser			- Ser		- Leu-OMe
TREN(*p*-MeBz)											- Leu-OMe
C_8 acyl	- TOAC										- Leu-OMe
C_8 acyl				- TOAC							- Leu-OMe
C_8 acyl								- TOAC			- Leu-OMe
C_8 acyl	- TOAC			- TOAC							- Leu-OMe
C_8 acyl	- TOAC							- TOAC			- Leu-OMe
C_8 acyl				- TOAC				- TOAC			- Leu-OMe
*b C_8 acyl			- Val				- Val				- Lol
C_8 acyl			- Val				- Val				- Leu-OMe
*c C_8 acyl	- D-Iva		- Val				- Val				- Lol
C_8 acyl	- D-Iva		- Val				- Val				- Leu-OMe
C_8 acyl	- L-Iva		- Val				- Val				- Leu-OMe
C_2 acyl	- D-Iva		- Val				- Val				- Lol
C_2 acyl	- D-Iva		- Val				- Val				- Leu-OMe
C_4 acyl	- D-Iva		- Val				- Val				- Lol
C_4 acyl	- D-Iva		- Val				- Val				- Leu-OMe
C_2 acyl	- D-Iva		- Val				- Val				- Lol-C_8 acyl
C_4 acyl	- D-Iva		- Val				- Val				- Lol-C_4 acyl
C_8 acyl	- D-Iva		- Val				- Val				- Lol-C_8 acyl

Figure 3. 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; (α)Me)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. Starred sequences are those of the natural lipopeptaibols (*a*, trichogin GA IV; *b*, trikoningin KBI; *c*, trikoningin KBII).

the Ser analogue is remarkably the least flexible. Different acyl moieties at the N-terminus do not influence the overall peptide conformational preference [21]. In those cases where it was possible to define the direction of the N-terminal *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 covalently linked head-to-head succinoyl dimer [21]. In the *bis*-TOAC trichogin analogues in alcohol solutions, the N-terminal region of the peptides folds in a 3_{10}-helix, whereas the central and C-terminal regions preferentially adopt the α-helical conformation [24, 25], as observed in the crystal state. However, the C-terminal region seems to be in equilibrium with unfolded conformers. It is suggested that the flexible -Gly^5-Gly^6- stretch creates a hinge point between the two helical regions.

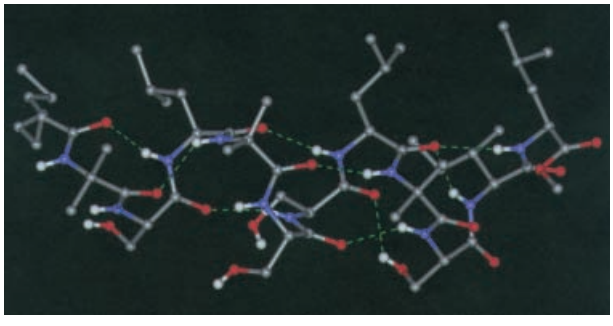

Figure 4. X-ray diffraction structure of the [Ser^{2,5,6,9}, Leu^{11}-OMe] trichogin GA IV analogue.

In the 1–5 segment of a synthetic precursor of trichodecenin I the crystallographic conformation is virtually identical to that reported from an nuclear magnetic resonance (NMR) investigation in $CDCl_3$ solution of a similar protected sequence [43], and it is characterized by multiple, nonhelical β turns [44]. In the crystal structure

a β-sheet arrangement is seen at the C-terminus. A conformational transition to a conventional, continuous helical conformation is observed in dimethylsulfoxide [43]. Interestingly, the molecular conformation in the poorly interactingly solvent $CDCl_3$ is significantly different from those of known Aib-based short peptides [43] and from the conformation observed from the *same* sequence in trichogin (see above). This finding highlights the flexibility inherent in Gly-rich sequences, even those containing the rigid Aib residues. It also speaks to the issue of critical peptide chain length for helix formation in the absence of a predominant percentage of $C^{\alpha,\alpha}$-dimethylglycines (only 17% in trichodecenin). A further type of structural motif (a π-turn, including two different, nonhelical β turns) was reported for the terminally protected, C-terminal pentapeptide sequence of the [Leu^{11}-OMe] analogue of trichodecenin I in the crystal state [45], again showing the plasticity of this region of the trichogin molecule.

Membrane activity

The major reason for interest in trichogin GA IV is the finding that this lipopeptaibol has a considerable membrane-perturbing activity [10]. It is even more lytic to small unilamellar vesicles of phosphatidylcholine and cholesterol than are several longer nonlipidated peptaibols. Relatively minor changes in the amino acid sequence of this lipopeptaibol can have large effects on membrane activity. Trikoningin KBI 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 [11]. The substitution of Aib^1 (trikoningin KBI) with an (R)-Iva residue (trikoningin KBII) does not modify the peptide-bilayer interaction [11, 27].

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 lytic activity. Trichogin analogues acylated with chains shorter than four carbon atoms are inactive, and there is an increased membrane-lytic potency with increasing acyl chain length for both trichogin [21] and trikoningin [27]. A possible role for acylation is that it facilitates the partitioning of the lipopeptide from water into the membrane. However, moving the octanoyl group to the C-terminus of trikoningin slightly reduces the membrane lytic activity [27], suggesting that the activity of the lipopeptide is not determined exclusively by its overall hydrophobicity. Nevertheless, partitioning likely plays a significant role, since incorporating octanoyl groups at both ends of the peptide increases activity [27]. On the other hand, the lytic activity is not sensitive to small 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 results in similar membrane lytic activity [22]. 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.

Little is known about the mechanism of action of trichogin. Its short length of 10 amino acids would suggest that it is not capable of spanning a membrane. This is in contrast to the well-known action of the 19-amino acid, nonlipidated peptaibol alamethicin that is sufficiently long to span a membrane [3–7]. The ability of alamethicin to form nonselective 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 [46].

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 6 amino acids in length and whose N-terminus is acylated with 4-decenoic acid [12]. Long-sequence peptaibols have only an acetyl group on the N-terminal function [3–7]. All of the lipopeptaibols that are N^{α}-acylated with an 8–15 carbon fatty acid have between 6 and 10 amino acids (see above). Membrane-modifying properties, albeit modest, are found at the level of a synthetic trichogin sequence as short as the N^{α}-octanoylated C-terminal tetrapeptide [47]. Membrane activity progressively increases from tetra- to 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. Thus, 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 α helix. Even if the peptaibols do not fold into a perfect 3_{10} helix, the presence of the Aib residues would likely 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 [21].

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. As mentioned in the preceding section, the nitroxide chosen, TOAC, is itself an α-amino acid that can substitute for Aib residues without a change in the conformation of the peptide or in the functional properties. 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 [26] and fluorescence-quenching [48] 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 (comprised of the fatty acyl chain and the $Leu^{3,7}$, Ile^{10} and Lol side chains) oriented toward the membrane and the polar face (comprised primarily of Gly residues) facing the water (fig. 5). 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 slightly more efficient for the all-R enantiomer [49].

One test of the mechanism of membrane lysis induced by trichogin may come from its lipid dependence. The lysis induced by trichogin is not affected by the presence of cholesterol in the membrane [10], in contrast to the lower potency observed with nonlipidated 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 nonlipidated 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. [50] 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 [51]. Although the self-association of alamethicin is independent of the amount of cholesterol in the membrane, binding of this peptide to a membrane is inhibited by the presence of cholesterol [52]. 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 nonselective ionophoric activity that is independent of the amount of cholesterol in the membrane of different mollicutes [53]. 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 lytic action by cholesterol. Mammalian cell membranes are rich in cholesterol, whereas those of bacteria are devoid of this lipid. This factor has been suggested to contribute to the bacterial specificity of magainin [54]. Thus, lipopeptaibols may exhibit less antimicrobial specificity than nonlipidated peptides. In addition, lipopeptides and lipoproteins with saturated acyl chains are sequestered into cholesterol-rich domains, or 'rafts' in mammalian membranes [55]. 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 saturated acyl chains sequester into rafts, whereas 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.

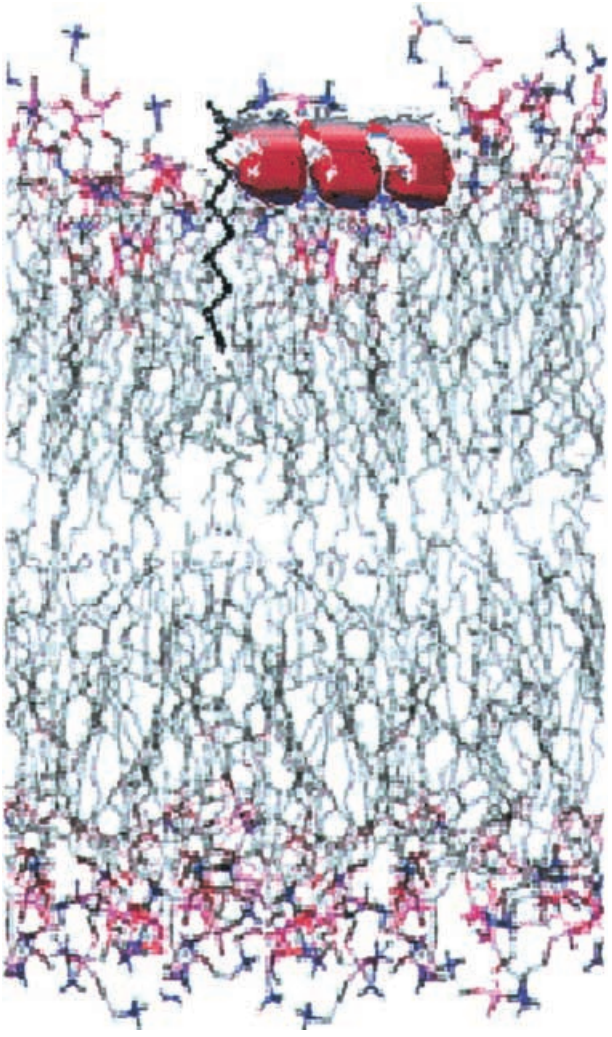

Figure 5. Proposed mode of action, in cartoon format, of trichogin GA IV on membranes according to the carpetlike mechanism. The hydrophobic portion of the peptide is shown in a deeper shade of red and faces the membrane.

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 [56].

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 [57]. 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. In addition, certain suggested mechanisms for antimicrobial activity require the formation of intermediates with specific changes in membrane curvature. An example is the pore mechanism developed by Matsuzaki [58], which suggests that there is an aqueous pore lined with both peptide and lipid that has acquired positive curvature. Magainin is an antimicrobial peptide that is believed to function by a pore-forming mechanism [59], and the sensitivity of its lytic action has been shown to be increased when the target membrane has more positive curvature tendency [60], in accord with a pore-forming mechanism. Trichogin also increases positive membrane curvature [56], which would be consistent with a pore-forming mechanism. 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 [56].

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 a more hydrophobic Ser(Bu^t). The resulting trichogin analogue promotes negative, rather than positive, curvature. It is less potent than trichogin in promoting leakage, and the observed leakage from liposomes is relatively insensitive to the lipid composition of the membrane [56]. However, this Ser(Bu^t) analogue is effective at promoting liposomal fusion. This activity was observed only with liposomes comprised of lipid which themselves had an intrinsic negative curvature [56]. It is known that 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) make an interesting comparison. The two 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, in accord with the pore mechanism of leakage. With the Ser(Bu^t) analogue the result of membrane interaction is the promotion of membrane fusion.

Finally, we also succeded 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 [61]. Likely, this is due to a conformational change of the template-peptide conjugate in the membrane biased by the formation of the metal complex.

Lipopeptaibols can thus have a variety of effects on membranes, including the induction of membrane leakage. Several lipopeptaibols have been shown to have antibacterial activity. Most of the lipopeptaibols are present in nature as complex mixtures of sequence-related compounds, only a few of which have been sequenced. It is likely that additional lipopeptaibols or mixtures with more potent antimicrobial activity will be discovered in the future. In addition, carefully designed analogue of some lipopeptaibols, such as trichogin GA IV, are excellent, short and easy-to-synthesize templates for biophysical studies of lipopeptide-membrane interactions as well as for designing novel antimicrobial agents with improved properties.

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