Effect of N^{α}-Acyl Chain Length on the Membrane-Modifying Properties of Synthetic Analogs of the Lipopeptaibol Trichogin GA IV

C. Toniolo,[1a] M. Crisma,[1a] F. Formaggio,[1a] C. Peggion,[1a] V. Monaco,[1a] C. Goulard,[1b] S. Rebuffat,[1b] and B. Bodo*,[1b]

Contribution from the Biopolymer Research Center, CNR, Department of Organic Chemistry, University of Padova, 35131 Padova, Italy, and the Laboratoire de Chimie, URA 401 CNRS, GDR 1153 CNRS, Muséum National d'Histoire Naturelle, 75231 Paris 05, France

Received December 5, 1995[⊗]

Abstract: Trichogin GA IV, an 11-residue lipopeptaibol blocked at the N-terminus by an *n*-octanoyl group and at the C-terminus by a 1,2-amino alcohol (L-leucinol), extracted from the fungus *Trichoderma longibrachiatum*, exhibits remarkable membrane-modifying properties. We have synthesized trichogin GA IV and several [L-Leu-OMe[11]] analogs carrying at the N-terminus an acyl chain of variable length (C_2−C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}). A succinoylated head-to-head dimer was also prepared. A conformational analysis, carried out by FTIR absorption, CD, and NMR, showed that the right-handed helical structure of the natural lipopeptaibol is essentially preserved in all its analogs. Permeability measurements revealed that at least six carbon atoms in the N^{α}-blocking fatty acyl moiety are required for the onset of significant membrane-modifying properties. Also the head-to-head dimer is remarkably active. Possible models for the mechanism of membrane permeability of trichogin GA IV are discussed.

Introduction

Peptaibols[2] are a unique group of membrane-active peptides biosynthesized by molds, mainly of the genus *Trichoderma*. These antibiotic peptides are usually characterized by a linear sequence of 15−19 amino acid residues, a high proportion of $C^{\alpha,\alpha}$-disubstituted glycines (α-aminoisobutyric acid, Aib, and isovaline, Iva), an N-terminal acetyl group, and a C-terminal 1,2-amino alcohol.[3] These long-sequence peptaibols, such as alamethicin, are known to form voltage-dependent membrane channels and to modify the membrane permeability even in the absence of voltage.[4]

Recently, from several *Trichoderma* species we isolated the following 11-residue peptaibols with an N-terminal *n*-octanoyl group: trichogin GA IV from *T. longibrachiatum*[5,6] and trikoningins KB I and KB II from *T. koningii*.[7] The name *lipopeptaibol* was proposed for such compounds.[6] Subsequently, the list of lipopeptaibols was expanded by Fujita *et al.*[8] who reported the sequences of the 7-residue trichodecenins I and II isolated from *T. viride*.

Trichogin GA IV,[5,6] the main component of the natural trichogin microheterogeneous mixture, is blocked by an *n*-octanoyl moiety at the N-terminus and has a 1,2-amino alcohol, L-Lol (leucinol), at the C-terminus with the following sequence:

*n*Oc-Aib[1]-Gly-L-Leu-Aib-Gly-Gly-L-Leu-Aib-Gly-L-Ile-L-Lol[11]

Despite having a shorter amino acid sequence than the classical peptaibols, this lipopeptaibol exhibits membrane activity. On the basis of a CD and NMR conformational investigation of the natural compound, a right-handed helical structure was proposed. More recently, this 3D-structural assignment was fully supported by an X-ray diffraction analysis on synthetic, racemic trichogin GA IV.[9] These conformational properties are not surprising in view of its relatively high (30%) content in Aib, the strongest known helix-forming amino acid.[10−12]

In order to examine the role of the N-terminal fatty acyl chain length on the membrane-modifying properties of trichogin GA IV, the prototype of lipopeptaibols, we synthesized trichogin GA IV itself and a number of [L-Leu-OMe[11]] analogs with variable acyl chain length, including the C_2−C_8, C_{10},C_{12}, C_{14}, C_{16}, and C_{18} acyl groups. A succinoylated head-to-head dimer was also prepared. In this paper, in addition to the synthesis and characterization, we describe the results of a comparative conformational study (using FTIR, CD, and NMR) and the ability of these peptides to modify membrane permeability as measured on liposomes and erythrocytes. Preliminary data on part of this work have been reported.[13,14]

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

(1) (a) University of Padova. (b) Muséum National d'Histoire Naturelle.

(2) Benedetti, E.; Bavoso, A.; Di Blasio, B.; Pavone, V.; Pedone, C.; Toniolo, C.; Bonora, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7951−7954.

(3) Nagaraj, R.; Balaram, P. *Acc. Chem. Res.* **1981**, *14*, 356−362.

(4) Sansom, M. S. P. *Prog. Biophys. Mol. Biol.* **1991**, *55*, 139−236.

(5) Auvin-Guette, C.; Rebuffat, S.; Prigent, Y; Bodo, B. In *Peptides 1990*; Giralt, E., Andreu, D., Eds.; ESCOM: Leiden, 1991; pp 428−429.

(6) Auvin-Guette, C.; Rebuffat, S.; Prigent, Y; Bodo, B. *J. Am. Chem. Soc.* **1992**, *114*, 2170−2174.

(7) Auvin-Guette, C.; Rebuffat, S.; Vuidepot, I.; Massias, M.; Bodo, B. *J. Chem. Soc., Perkin Trans.1* **1993**, 249−255.

(8) Fujita, T.; Wada, S. I.; Iida, A.; Nishimura, T.; Kanai, M.; Toyama, N. *Chem. Pharm. Bull.* **1994**, *42*, 489−494.

(9) Toniolo,C.; Peggion, C.; Crisma, M.; Formaggio, F.; Shui, X.; Eggleston, D.S. *Nature: Struct. Biol.* **1994**, *1*, 908−914.

(10) Marshall, G. R. In *Intra-Science Chemistry Reports*; Kharasch, N., Ed.; Gordon and Breach: New York, 1971; Vol. 5, pp 305−316.

(11) Karle, I. L.; Balaram, P. *Biochemistry* **1990**, *29*, 6747−6756.

(12) Benedetti, E.; Toniolo,C. *Macromolecules* **1991**, *24*, 4004−4009.

(13) Toniolo, C.; Crisma, M.; Formaggio, F.; Pirrone, L.; Bonora, G. M.; Mammi, S.; Peggion, E. In *Peptides 1992*; Schneider, C. H., Eberle, A. N., Eds.; ESCOM: Leiden, 1993; pp 613−614.

(14) Bodo, B.; Rebuffat, S.; Goulard, C.; Peggion, C.; Monaco, V.; Formaggio, F.; Crisma, M.; Toniolo, C. In *Peptides 1994*; Maia, H. L. S., Ed.; ESCOM: Leiden, 1995; pp 749−750.

S0002-7863(95)04081-9 CCC: $12.00 © 1996 American Chemical Society

Experimental Section

Peptide Synthesis. Melting points were determined using a Leitz Model Laborlux 12 apparatus and are not corrected. Optical rotations were measured using a Perkin-Elmer Model 241 polarimeter equipped with a Haake Model D thermostat. Thin-layer chromatography was performed on Merck 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 C. Erba Model 3A27 amino acid analyzer. The Aib color 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 analyses were performed on a Pharmacia Model LKB-LCC 2252 liquid chromatograph equipped with an UVICORD Model SD UV detector (226 nm) and a reversed-phase C_{18} Vydac Model 218 TP54 column.

FTIR Absorption. The FTIR absorption spectra were recorded using a Perkin-Elmer Model 1720X FTIR spectrophotometer, nitrogen-flushed, equipped with a sample-shuttle device, at 2 cm^{-1} nominal resolution, averaging 100 scans. Solvent (baseline) spectra were obtained under the same conditions. Cells with path lengths of 0.1, 1.0, and 10 mm (with CaF_2 windows) were used. Spectrograde deuteriochloroform (99.8% D) was purchased from Fluka.

Circular Dichroism. The CD spectra were obtained on a JASCO Model J-710 spectropolarimeter. Cylindrical fused quartz cells of 10 and 1 mm path lengths were used. The values are expressed in terms of $[\Theta]_T$, the total molar ellipticity (deg cm $dmol^{-1}$). MeOH (Riedel-de-Häen) was used as the solvent.

NMR Spectroscopy. NMR spectra were recorded on a Bruker AC 300 spectrometer equipped with an Aspect 3000 computer. Peptide solutions in CD_3OH (CEA, Saclay, France) were 20 and 50 mM for ^1H and ^{13}C NMR spectra, respectively. Unless otherwise specified, all experiments were run at a temperature of 23 °C. ^1H NMR spectra were obtained by solvent presaturation and referred to the central component of the quintet of the CHD_2 resonance of methanol at 3.313 ppm downfield from TMS. ^{13}C NMR spectra were collected at 75.47 MHz, and chemical shifts were referred to internal CD_3OH taken at 49.00 ppm relative to TMS. The 1D spectra were obtained with 300−700 scans. Standard methods were used to perform the 2D experiments, and pulsed programs were taken from the Bruker software library.

Liposome Leakage Assay. Peptide-induced leakage from egg phosphatidylcholine (PC) vesicles was measured at 20 °C using the carboxyfluorescein (CF)-entrapped vesicle technique as previously described.[15] 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 (0.6 mM), and increasing [peptide]/[lipid] molar ratios (R_i^{-1}) were obtained by adding aliquots of methanolic solutions of peptides, keeping the final methanol 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 (1 nm band pass) with λ_{exc} 488 nm (1 nm band pass). The percentage of released CF at time t was determined as $(F_t - F_o)/(F_T - F_o) \times 100$, with F_o = 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 30 μL of a 10% Triton X-100 solution. The kinetics were stopped at 20 min.

Antimicrobial Activity. The antibacterial activity of trichogin GA IV and some of its N^{α}-acyl-[Leu-OMe[11]] analogs were examined against *Staphylococcus aureus* (strain 209 P) by the agar diffusion test using the Muller−Hinton culture medium and 6 mm diameter pits. The peptide samples were dissolved in DMSO such as to give a 4 mg/mL solution. Eight other concentrations were obtained by successive dilutions, and 50 μL of each solution was deposited into the pits (1.2−200 μg). Inhibition zones were measured after 24 h of incubation at 37 °C.

Hemolytic Activity. Human erythrocytes (O^+) were centrifuged at 600 g for 5 min, washed three times with RPMI 1640, and diluted in RPMI containing 7.5% serum to a final concentration of 10^7 cells/mL. The peptide samples were dissolved in DMSO such as to give a 20 mM solution. Other concentrations were obtained by successive dilutions with DMSO. The peptide solutions (10 μL) were diluted with the 7.5% serum containing RPMI (490 μL), keeping the final DMSO concentration below 1%. The final peptide concentration range was 3−200 μM. A 500 μL sample of the erythrocyte suspension was then added with stirring and the mixture incubated at 37 °C for 3 or 27 h. Absorbance of the supernatant (200 μL) was measured at 405 nm. The 0% hemolysis (OD_0) was determined by incubating 1 mL of the erythrocyte suspension containing 1% DMSO, and the 100% hemolysis (OD_{100}) was measured by incubating the erythrocyte suspension (500 μL) with 490 μL of RPMI and 10 μL of a dilute (1:10) Triton X-100 solution. The percentage of lysis was calculated according to % lysis = $(OD_i - OD_0)/(OD_i - OD_{100}) \times 100$, where OD_i is the optical density for the peptide concentration i.

Results

Peptide Synthesis. The syntheses of trichogin GA IV and its [L-Leu-OMe[11]] undecapeptide analogs were performed step-by-step in solution, beginning from the C-terminal H-L-Leu-OMe residue, *via* the mixed anhydride method with isobutyl chloroformate to incorporate the protein amino acids and the symmetrical anhydride method to incorporate the internal Aib residues (Chart 1). The acylated N-terminal Aib residue was added using the 5(4*H*)-oxazolone method. However, more recently higher yields for the insertion of the N-terminal Aib residue were obtained by treatment of the C-terminal decapeptide with $(Z\text{-Aib})_2O$ (Z = benzyloxycarbonyl), followed by N^{α}-deprotection and treatment with the appropriate carboxylic acid preactivated in CH_2Cl_2 solution with *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide (EDC), and 1-hydroxybenzotriazole. In the last step the N^{α}-octanoyl undecapeptide methyl ester was reduced using $LiBH_4$ to afford the synthetic trichogin GA IV, which was shown by chromatographic and physical methods to be identical to the natural lipopeptaibol.

The Z-protected derivatives were obtained by reacting the pertinent free amino acid with Z-OSu (1-hydroxysuccinimido ester). The L-Leu methyl ester hydrochloride was prepared by the methanol/thionyl chloride method. Removal of the Z group was carried out by catalytic hydrogenation. The Aib 5(4*H*)-oxazolones were synthesized from their N^{α}-acylated free acid precusors and 1 equiv of EDC. Owing to the difficulties in the isolation of the 5(4*H*)-oxazolone from Ac-Aib-OH,[16] for the synthesis of the N^{α}-acetylated undecapeptide methyl ester a different route was designed, namely, the synthesis of the N^{α}-benzyloxycarbonylated undecapeptide analog *via* $(Z\text{-Aib})_2O$, followed by deprotection of the Z group and acetylation of the N^{α}-free derivative with acetic anhydride. The stable, crystalline derivative $(Z\text{-Aib})_2O$[17] was obtained by reacting Z-Aib-OH with 0.5 equiv of thionyl chloride in ethyl acetate.

The synthesis of the N^{α}-succinoylated [L-Leu-OMe[11]] trichogin GA IV dimer was achieved by reacting a large excess of the N^{α}-deprotected undecapeptide methyl ester with succinoyl chloride in acetonitrile in the presence of *N*-methylmorpholine.

The chemical and optical purities of all the intermediates and final synthetic products were assessed by polarimetry, TLC in three different solvent systems, and amino acid analysis (data listed in Table 1), solid-state IR absorption, ^1H NMR, and HPLC, and for the synthetic trichogin GA IV by chiral chromatography and mass spectrometry. It is worth noting that the reversed-phase HPLC retention times of the [L-Leu-OMe[11]]

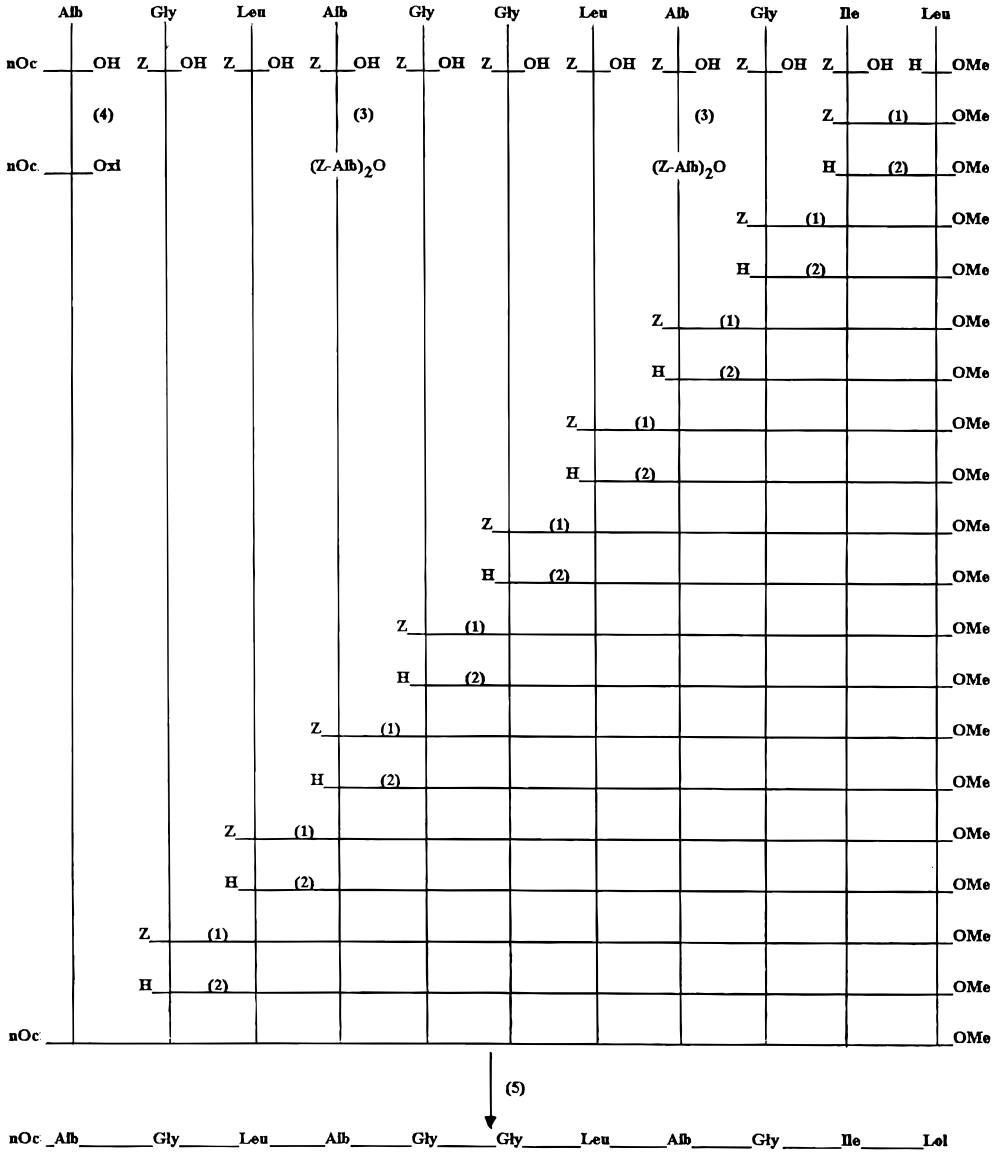
(15) El Hajji, M.; Rebuffat, S.; Le Doan, T.; Klein, G.; Satre, M.; Bodo, B.; *Biochim. Biophys. Acta* **1989**, *978*, 97−104.

(16) Levene, P. A.; Steiger, R. E. *J. Biol. Chem.***1931**, *93*, 581−604.

(17) Valle, G.; Formaggio, F.; Crisma, M.; Bonora, G. M.; Toniolo, C.; Bavoso, A.; Benedetti, E.; Di Blasio, B.; Pavone, V.; Pedone, C. *J. Chem. Soc., Perkin Trans. 2* **1986**, 1371−1376.

(18) Okumura, Y.; Sakurai, A. *Bull. Chem. Soc. Jpn.* **1973**, *46*, 2190−2193.

Chart 1[a]



[a] Conditions: (1) *i*BuOCOCl, NMM. (2) H_2, Pd/C. (3) 0.5 equiv of $SOCl_2$, TEA. (4) EDC (HCl). (5) $LiBH_4$, THF.

trichogin GA IV analogs with an even number of carbon atoms in the acyl chain increase linearly as the number of carbon atoms increases (Figure 1).

Conformational Analysis. A detailed analysis of the preferred conformation of the [L-Leu-OMe[11]] trichogin GA IV analogs with variable N^{α}-acyl chain and the succinoylated head-to-head dimer was performed using FTIR absorption, CD, and NMR in different solvents and compared with that of the parent peptaibol.[6]

The IR absorption spectra of the undecapeptide esters and trichogin GA IV in $CHCl_3$ solution are dominated by strong bands at 3323−3328 cm^{-1} (N—H stretching mode of H-bonded amide groups) and 1655−1657 cm^{-1} (C═O stretching mode of H-bonded amide groups).[19] Additional, but very weak, bands are seen in the 3426−3453 cm^{-1} region (free, solvated amide NH groups) and at 1738−1739 cm^{-1} (ester carbonyl). Obviously, the latter band is absent in the peptaibol. In the N—H stretching region all peptides examined show a more or less pronounced concentration effect in the range 10−0.1 mM, indicating the onset of a nonmarginal amount of intermolecular H bonds. However, at the lowest concentration (0.1 mM) the 3323−3328 cm^{-1} band is still very intense, supporting the view that all these peptides are characterized by an extensive set of intramolecular N—H···O═C H-bonds. The position of the strong C═O stretching band is also in favor of this conclusion.

Auvin-Guette *et al.*[6] have reported the CD spectrum of trichogin GA IV in methanol. The lipopeptaibol exhibits a weak negative maximum at 223 nm (amide n $\rightarrow \pi^*$ transition), followed by more intense amide $\pi \rightarrow \pi^*$ exciton split dichroic bands at 205 nm (negative) and 192 nm (positive). This pattern is reminiscent of those shown by (right-handed) predominantly helical peptides.[20] Figure 2 illustrates the CD curves for three representative [L-Leu-OMe[11]] trichogin GA IV analogs in methanol. Although the spectrum of trichogin GA IV is in general more intense, a similar pattern is found also in all the undecapeptide ester analogs.

The ^1H NMR spectra of the various analogs in methanol are very similar, differing mainly by an increase in the intensity of the polymethylene chain signal at 1.27 ppm. In general, the amide proton and carbonyl carbon chemical shifts and thermal coefficients, and the $^3J_{NH-\alpha CH}$ coupling constants in the ^1H and ^{13}C NMR spectra of the C_2, C_8, C_{14}, and C_{18} N^{α}-acyl analogs

(19) Palumbo, M.; Da Rin, S.; Bonora, G. M.; Toniolo, C. *Makromol. Chem.* **1976**, *177*, 1477−1492.

(20) Beychok, S. In *Poly-α-Amino Acids*; Fasman, G. D., Ed.; Dekker: New York, 1967; pp 293−337.

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

peptide	mp (°C)	crystallizn solvent[a]	$[\alpha]^{20}_{D}$[b] (deg)	TLC R_F(I)	TLC R_F(II)	TLC R_F(III)	amino acid analysis
Z-L-Ile-L-Leu-OMe[f]	116−117	AcOEt/PE	−41.4	0.90	0.95	0.50	Ile 0.96; Leu 1.04
Z-Gly-L-Ile-L-Leu-OMe	119−120	AcOEt/PE	−45.9	0.85	0.95	0.30	Gly 1.00; Ile 1.00; Leu 1.00
Z-Aib-Gly-L-Ile-L-Leu-OMe	136−137	AcOEt/PE	−16.6	0.55	0.95	0.20	Aib 0.97; Gly 1.00; Ile 0.98; Leu 1.00
Z-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	142−143	AcOEt/PE	−46.3	0.55	0.95	0.20	Aib 0.98; Gly 1.00; Ile 1.00; Leu 1.99
Z-Gly-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	95−96	AcOEt/PE	−66.8	0.55	0.90	0.20	Aib 1.03; Gly 1.97; Ile 1.00; Leu 2.00
Z-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	153−154	AcOEt/PE	−36.3	0.40	0.90	0.10	Aib 1.10; Gly 2.90; Ile 0.96; Leu 2.00
Z-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	109−111	AcOEt/PE	−39.1	0.30	0.85	0.10	Aib 2.10; Gly 2.91; Ile 0.97; Leu 2.00
Z-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	144−145	AcOEt/PE	−42.7	0.40	0.90	0.05	Aib 2.02; Gly 3.10; Ile 0.96; Leu 2.92
Z-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	183−184	$CHCl_3$/DE/PE	−42.2	0.35	0.90	0.05	Aib 2.00; Gly 3.95; Ile 1.05; Leu 3.00
Z-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	126−128	$CHCl_3$/PE	−20.4	0.10	0.75	0.70[d]	Aib 3.07; Gly 4.00; Ile 0.97; Leu 3.00
C_2acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	140−141	lyophil.[c]	−20.3	0.00	0.75	0.55[d]	Aib 3.00; Gly 4.06; Ile 0.94; Leu 3.00
C_3acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	167−168	AcOEt/PE	−21.0	0.00	0.75	0.55[d]	Aib 3.00; Gly 4.07; Ile 0.93; Leu 3.00
C_4acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	147−148	AcOEt/PE	−21.1	0.00	0.75	0.55[d]	Aib 3.00; Gly 4.10; Ile 0.92; Leu 3.03
C_5acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	137−139	lyophil.[c]	−19.0	0.00	0.75	0.60[d]	Aib 3.05; Gly 4.00; Ile 0.95; Leu 3.00
C_6acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	130−132	lyophil.[c]	−19.4	0.00	0.75	0.60[d]	Aib 3.10; Gly 4.07; Ile 0.90; Leu 3.00
C_7acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	156−158	AcOEt	−19.4	0.00	0.80	0.60[d]	Aib 3.04; Gly 4.06; Ile 0.90; Leu 3.00
C_8acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	129−130	$CHCl_3$/PE	−21.6	0.00	0.85	0.95[d]	Aib 3.07; Gly 4.04; Ile 0.99; Leu 2.90
C_{10}acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	106−108	$CHCl_3$/PE	−14.5[e]	0.00	0.80	0.95[d]	Aib 3.08; Gly 4.01; Ile 0.98; Leu 2.95
C_{12}acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	128−130	lyophil.[c]	−15.2	0.00	0.85	0.95[d]	Aib 3.03; Gly 4.00; Ile 0.97; Leu 3.00
C_{14}acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	114−115	$CHCl_3$/PE	−16.6	0.00	0.90	0.95[d]	Aib 3.04; Gly 4.04; Ile 0.97; Leu 2.98
C_{16}acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	100−102	$CHCl_3$/PE	−16.3	0.00	0.85	0.95[d]	Aib 3.01; Gly 4.02; Ile 0.99; Leu 2.99
C_{18}acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-OMe	136−138	DE/PE	−15.1	0.10	0.60	0.80[d]	Aib 3.00; Gly 4.04; Ile 1.00; Leu 2.96
$[CH_2CO$-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Leu-$OMe]_2$	171−172	MeOH/DE	−25.7	0.05	0.65	0.50[d]	Aib 3.10; Gly 4.04; Ile 0.91; Leu 3.00
C_8acyl-Aib-Gly-L-Leu-Aib-$(Gly)_2$-L-Leu-Aib-Gly-L-Ile-L-Lol	143−144	$CHCl_3$/PE	−4.8	0.05	0.80	0.95[d]	Aib 3.05; Gly 3.93; Ile 0.99; Leu 1.93
C_3acyl-Aib-OH	154−155	MeOH/DE		0.45	0.80	0.25	
C_3acyl-Aib-OH OXL	oil	AcOEt/PE		0.80	0.80	0.55	
C_4acyl-Aib-OH	160−161	MeOH/DE		0.45	0.80	0.25	
C_4acyl-Aib-OH OXL	oil	AcOEt/PE		0.80	0.80	0.60	
C_5acyl-Aib-OH	149−150	MeOH/DE		0.45	0.85	0.35	
C_5acyl-Aib-OH OXL	oil	AcOEt/PE		0.80	0.85	0.65	
C_6acyl-Aib-OH	142−143	MeOH/DE		0.50	0.85	0.35	
C_6acyl-Aib-OH OXL	oil	AcOEt/PE		0.90	0.90	0.70	
C_7acyl-Aib-OH	135−137	MeOH/DE		0.50	0.85	0.40	
C_7acyl-Aib-OH OXL	oil	AcOEt/PE		0.90	0.90	0.70	
C_8acyl-Aib-OH	139−140	AcOEt/PE		0.50	0.85	0.40	
C_8acyl-Aib-OH OXL	oil	AcOEt/PE		0.90	0.95	0.80	
C_{10}acyl-Aib-OH	132−133	AcOEt		0.30	0.95	0.25	
C_{10}acyl-Aib-OH OXL	oil	AcOEt/PE		0.95	0.95	0.85	
C_{12}acyl-Aib-OH	129−130	DE/PE		0.30	0.95	0.20	
C_{12}acyl-Aib-OH OXL	oil	AcOEt/PE		0.95	0.95	0.85	
C_{14}acyl-Aib-OH	126−127	DE		0.30	0.95	0.20	
C_{14}acyl-Aib-OH OXL	oil	AcOEt/PE		0.95	0.95	0.85	
C_{16}acyl-Aib-OH	120−122	DE		0.35	0.95	0.20	
C_{16}acyl-Aib-OH OXL	oil	AcOEt/PE		0.95	0.95	0.90	
C_{18}acyl-Aib-OH	122−123	AcOEt		0.30	0.95	0.20	
C_{18}acyl-Aib-OH OXL	oil	AcOEt/PE		0.95	0.95	0.90	

[a] AcOEt = ethyl acetate; PE = petroleum ether; DE = diethyl ether; MeOH = methanol. [b] Concentration 0.5 (methanol). [c] Lyophilized after HPLC purification. [d] $CHCl_3$/MeOH, 8:2. [e] Concentration 0.2 MeOH. [f] Reference 18.

Table 2. Chemical Shift Values at 296 K (δ, ppm) and Vicinal Coupling Constants (^3J, Hz) of Amide Protons for Trichogin GA IV and Its [L-Leu-OMe[11]] Analogs

amino acid	GA IV	C_2	C_8	C_{14}	C_{18}	dimer
Aib[1]	8.510(s)	8.541(s)	8.496(s)	8.499(s)	8.540(s)	8.495(s)
Gly[2]	8.544(t, 5.6)	8.523(t, 5.7)	8.528(t, 5.5)	8.530(t, 5.6)	8.495(t, br)	8.393(t, 5.8)
Leu[3]	8.091(d, 7.5)	8.048(d, 6.8)	8.080(d, 7.8)	8.080(d, 8.1)	8.091(d, 7.5)	8.063(d, 7.0)
Aib[4]	7.980(s)	7.999(s)	7.963(s)	7.964(s)	7.965(s)	8.190(s)
Gly[5]	8.327(t, 5.5)	8.261(t, 5.6)	8.289(t, 5.6)	8.293(t, 5.6)	8.287(t, 5.6)	8.179(t, 5.6)
Gly[6]	8.138(t, 5.8)	8.097(t, 6.1)	8.110(t, 5.8)	8.114(t, 5.8)	8.110(t, 5.8)	8.126(t, 5.9)
Leu[7]	7.926(d, 6.4)	7.932(d, 6.8)	7.914(d, 6.8)	7.915(d, 6.9)	7.916(d, 6.6)	8.004(d, 6.9)
Aib[8]	7.987(s)	8.009(s)	7.996(s)	7.995(s)	8.001(s)	8.168(s)
Gly[9]	8.053(t, 5.7)	8.037(t, 5.7)	8.044(t, 5.8)	8.046(t, 6.0)	8.045(t, 6.0)	8.062(t, 6.0)
Ile[10]	7.699(d, 8.4)	7.752(d, 8.4)	7.749(d, 8.4)	7.752(d, 8.5)	7.751(d, 8.5)	7.761(d, 8.4)
Leu-OMe[11]		8.089(d, 7.5)	8.096(d, 7.3)	8.092(d, 7.3)	8.087(d, 7.8)	8.202(d, 7.5)
Lol[11]	7.309(d, 9.0)					

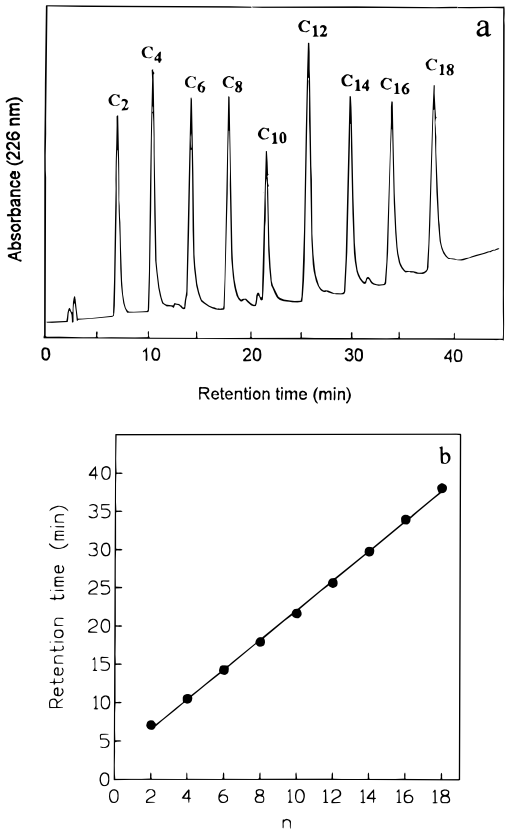


Figure 1. (a) Reversed-phase HPLC chromatography and (b) plot of HPLC retention times of the N$^\alpha$-acylated [L-Leu-OMe[11]] trichogin GA IV analogs as a function of *n*, the number of carbon atoms in the acyl chain (only even numbers are reported). Mobile phase: gradient from 50% to 100% of **B** in **A** during 40 min (**A** is 0.05% TFA in H_2O; **B** is 0.05% TFA in a 9:1 MeCN/H_2O mixture). Flow rate: 1 mL/min. Column: reversed-phase C_4 Vydac Model 214TP54 (250 × 4.6 mm). Detection: UV, 226 nm.

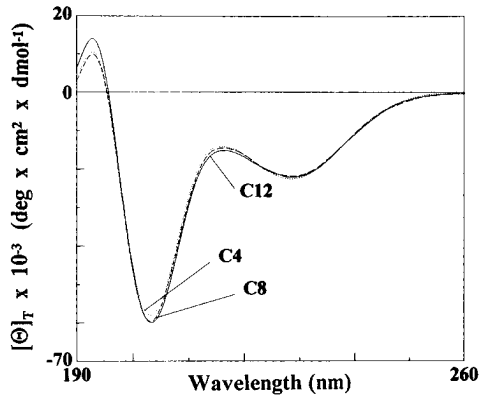


Figure 2. CD spectra in methanol of the [L-Leu-OMe[11]] trichogin GA IV analogs with C_4, C_8, and C_{12} atoms in the N^α-acyl chain (concentration 1 mM).

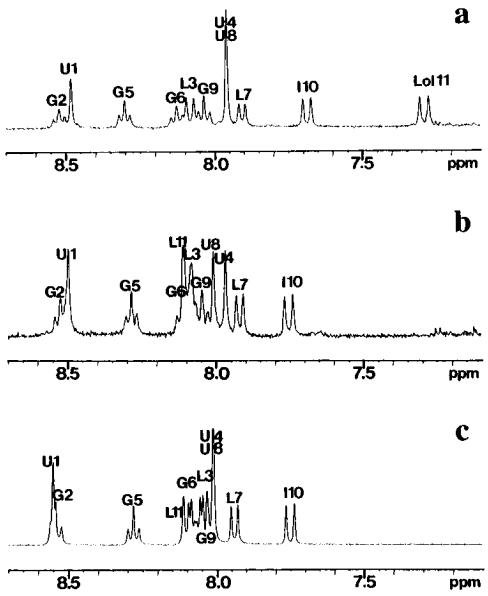


Figure 3. Amide proton region of the ^{1}H NMR spectrum of trichogin GA IV (a) and the [L-Leu-OMe[11]] trichogin GA IV analogs with C_8 (b) and C_2 (c) atoms in the N^α-acyl chain (300 MHz, 295 K, CD_3OH, 20 mM).

and the succinoyl dimer are close to those of trichogin GA IV (Tables 2 and 3 and Figure 3). However, some differences are seen near the C-terminus: (i) The $^3J_{NH-\alpha CH}$ value is smaller for the C-terminal residue of the [Leu-OMe[11]] analogs as compared to the value for Lol[11] in trichogin GA IV. (ii) The thermal coefficients of the Lol[11] NH proton and Ile[10] carbonyl carbon in trichogin GA IV are lower than the corresponding values for Leu[11] and Ile[10] in the three [Leu-OMe[11]] peptides. These findings can be interpreted in terms of a preferential structure with a more extensive set of intramolecular H bonds (C-terminal helical cap) for the natural antibiotic. Interestingly, the spectrum of the succinoyl dimer exhibits signals for only half of the protons, and the succinoyl methylene gives an AB system centered at 2.54 ppm, thus indicating that this molecule is symmetrical. This result is confirmed by the ^{13}C NMR spectrum which displays only 12 carbonyl carbon signals (Table 3). More generally, the chemical shifts for the carbonyl groups of the C_2 N^α-acyl analog and the succinoyl dimer, which were assigned from ^{1}H−^{13}C long-range COSY experiments, show great similarities with those of trichogin GA IV. All these data, taken together, strongly suggest that all the peptide analogs should have similar helical conformations. In summary, the helical structure, previously determined for trichogin GA IV in methanol solution[6] and in the crystal state,[9] appears to be maintained in the synthetic analogs.

Table 3. Temperature Dependence ($-\Delta\delta/\Delta T$, ppb/K) of Amide Protons, Chemical Shift Values at 296 K (δ, ppm), and Temperature Dependence ($-\Delta\delta/\Delta T$, ppb/K) of Carbonyl Carbons for Trichogin GA IV and Its [L-Leu-OMe[11]] Analogs

	$-\Delta\delta/\Delta T$ **NH**				δ **CO**			$-\Delta\delta/\Delta T$ **CO**		
amino acid	GA IV	C^2	C^8	dimer	GA IV	C^2	dimer	GA IV	C^2	dimer
O/A/ S[a]					176.6[O]	173.5 [A]	175.0[S]	0.2	2.0	1.7
Aib[1]	7.1	8.2	8.6	7.7	178.5	178.2	178.0	1.5	1.0	3.1
Gly[2]	6.0	6.8	6.9	5.7	173.1	173.0	172.7	0.8	0.1	−0.1
Leu[3]	2.0	2.5	2.6	3.2	175.8	175.7	175.3	2.7	1.3	−0.9
Aib[4]	4.9	6.1	4.9	6.6	178.3	178.1	177.9	−0.8	−1.0	−2.3
Gly[5]	5.7	6.2	6.2	5.1	173.4	173.1	172.9	1.8	0.9	−1.8
Gly[6]	2.4	2.5	2.7	3.0	173.0	172.7	172.5	0.8	−1.2	−0.5
Leu[7]	4.7	5.1	4.9	5.1	175.3	174.8	174.8	0.9	2.2	3.3
Aib[8]	7.4	8.6	8.8	9.6	178.1	177.6	177.5	1.6	1.8	2.4
Gly[9]	3.8	5.6	5.5	5.5	172.5	172.1	172.1	3.0	0.9	0.0
Ile[10]	2.3	3.7	3.7	3.4	173.6	173.8	173.9	2.3	5.6	6.0
Leu[11]		8.8	9.0	9.1		174.2	174.2		0.2	0.1
Lol[11]	4.8									

[a] O, A, and S refer to octanoyl, acetyl and succinoyl groups, respectively.

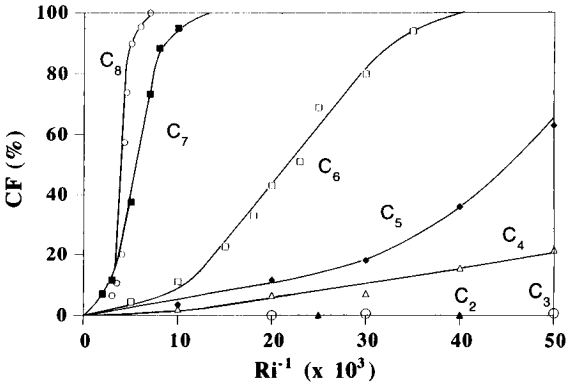


Figure 4. Peptide-induced CF leakage at 20 min for different ratios R_i^{-1} = [peptide]/[lipid] from egg PC/cholesterol (70:30) vesicles. The number of carbon atoms (2−8) in the N^{α}- acyl chain of the [L-Leu-OMe[11]] trichogin GA IV analogs is indicated.

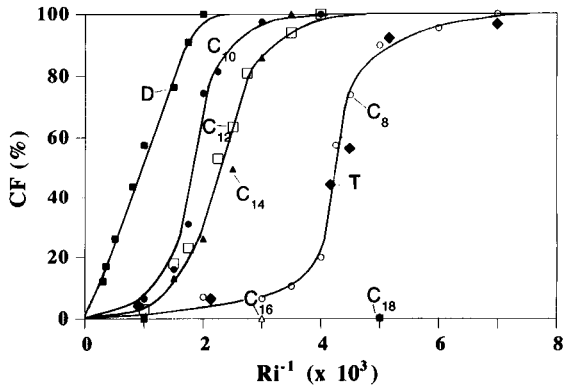


Figure 5. Peptide-induced CF leakage at 20 min for different ratios R_i^{-1} = [peptide]/[lipid] from egg PC/cholesterol (70:30) vesicles. The number of carbon atoms (8, 10, 12, 14, 16, 18) in the N^{α}- acyl chain of the [L-Leu-OMe[11]] trichogin GA IV analogs is indicated. **T** and **D** refer to trichogin GA IV and the [L-Leu-OMe[11]] trichogin GA IV succinoyl dimer, respectively.

Membrane-Modifying Properties from Liposome Leakage Measurements. The membrane-modifying properties of the different analogs were tested in comparison to those of the natural lipopeptide by measuring the induced leakage of CF entrapped in egg PC/cholesterol (7:3) small unilamellar vesicles (Figures 4 and 5). The C_8 [Leu-OMe[11]] analog has similar activity as the natural lipopeptaibol, both compounds leading to 50% CF leakage at 20 min for $R_i^{-1} = 4.2 \times 10^{-3}$ and 100% for $R_i^{-1} = 7 \times 10^{-3}$. The C_2 analog is almost inactive. By increasing the lipid chain length from C_3 to C_{10}, a continuous increase in the activity is observed. A threshold in the increase is observed from an acyl chain length of six carbon atoms. The most active analog is the C_{10} peptide which induces, at 20 min, 50% leakage for $R_i^{-1} = 1.8 \times 10^{-3}$, and 100% for $R_i^{-1} = 4 \times 10^{-3}$. Upon further increasing the chain length from C_{10} to C_{14} atoms, a slight decrease in the membrane activity is noticed. Surprisingly, the activity dramatically falls for the C_{16} and C_{18} analogs, which are found inactive, even for R_i^{-1} ratios higher than 4×10^{-3}. This apparently lower or null activity for the $C_{14}-C_{18}$ analogs can be explained on the basis of a comparison of their kinetic profiles with those of the more active peptides (Figure 6). While the natural peptide (not shown) and the efficient analogs exhibit kinetics with steep slopes at the origin, the $C_{14}-C_{18}$ analogs develop a lag time (possibly due to self-association of these lipopeptides) which increases when increasing the lipid chain length. The succinoyl dimer is more efficient than all the undecapeptides (Figure 5).

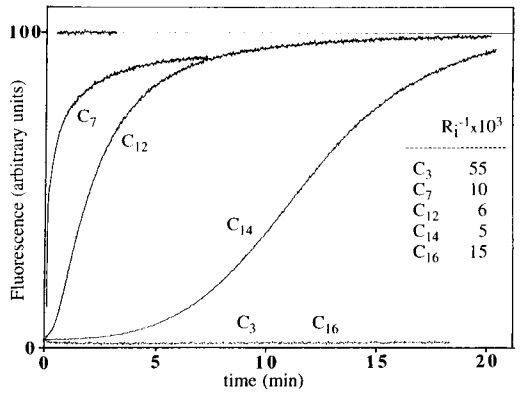


Figure 6. Kinetic profile for the peptide-induced CF leakage from egg PC/cholesterol (70:30) vesicles. The R_i^{-1} = [peptide]/[lipid] values are listed for each peptide. The number of carbon atoms (3, 7, 12, 14, 16) in the N^{α}- acyl chain of the [L-Leu-OMe[11]] trichogin GA IV analogs is indicated.

Biological Activities. The antibacterial activity of the C_2, C_8, and C_{16} N^{α}-acyl and succinoyl dimer analogs were examined against *S. aureus* by the agar diffusion method using 6 mm pits and compared to the natural lipopeptide (Table 4). The C_8 [Leu-OMe[11]] analog has activity similar to that of the natural compound, whereas the observed inactivities of the C_2 and C_{16} analogs are explained by the absence of a lipidic chain for the former analog and by a lack of diffusion (probably due to a greater hydrophobicity) for the latter analog.

The hemolytic activity of trichogin GA IV and its C_2, C_8, and C_{16} [Leu-OMe[11]] analogs was examined on human erythrocytes. The natural compound (not shown) and its C_8 analog exhibit similar hemolytic activity, showing a threshold effect for a concentration around 1.6×10^{-5} M after 3 h of incubation (Figure 7). The C_{12} analog is slightly more active, whereas

Table 4. Antibacterial Activity of Trichogin GA IV and Its [L-Leu-OMe[11]] Analogs against *S. aureus* As Determined by the Agar Diffusion Method (Inhibition Diameters in Millimeters)

peptide	200 μg/pits	100 μg/pits	50 μg/pits	25 μg/pits	12.5 μg/pits	6.25 μg/pits	3 μg/pits	1.5 μg/pits
trichogin GA IV	17	17	16	15	15	14	13	9
C_2 [Leu-OMe[11]]	–	–	–	–	–	–	–	–
C_8 [Leu-OMe[11]]	17	16	15	15	16	14	12	8
C_{16} [Leu-OMe[11]]	–	–	–	–	–	–	–	–
succinoyl dimer	–	–	–	–	–	–	–	–

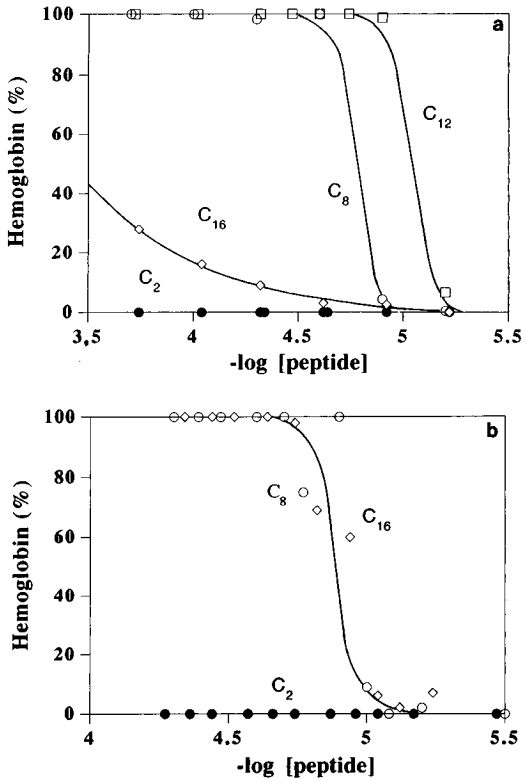


Figure 7. Hemolytic activity of selected [L-Leu-OMe[11]] trichogin GA IV analogs at 3 h (a) and 27 h (b). The number of carbon atoms in the N^{α}- acyl chain is indicated.

the C_{16} analog is found much less active and the C_2 analog completely inefficient. Incubating for 27 h allows a characteristic distinction between the C_{16} and the C_2 analogs, as the hemolytic activity increases with increasing incubation time only for the C_{16} analog. These results parallel those observed with liposomes, suggesting that the trichogin GA IV analogs, having a lipidic chain with more than 15 carbon atoms, take a much longer time to reach the membrane target.

Discussion

In addition to the first total synthesis of the lipopeptaibol trichogin GA IV, we have reported here the preparation and characterization of 12 [L-Leu-OMe[11]] undecapeptide analogs and the succinoylated head-to-head dimer. We have also demonstrated that a right-handed helix is a characteristically common structural element for all these peptide molecules. The N^{α}-blocking fatty acyl moiety has been shown to play a major role in the membrane-modifying properties of the undecapeptide esters. More specifically, at least six carbon atoms in the aliphatic chain are required for a significant activity. Full membrane activity is also exhibited by the succinoylated head-to-head dimer.

A considerable body of evidence has been accumulated to support the view that 15−19 amino acid peptaibols are helical and capable of forming channels in biological membranes through which water molecules and ions may readily pass.[3,4,6] Very different patterns of helix aggregation have been observed. For these long peptaibols the helix length is sufficient to span the lipid bilayer. In contrast, the helix length of trichogin GA IV is approximately half that of alamethicin.[9] It is reasonable to conclude that, if the mechanism of action for this latter class of peptaibols involves channel formation in bilayers, the arrangement of molecules required to bring about such disruptions must be quite different from the longer sequences. The crystal structure observed for the trichogin GA IV racemate was revealing in light of this unique requirement. Trichogin GA IV is able to pack with other like molecules to form a channel in which water molecules can clearly be accommodated. Molecular aggregation is enhanced by the amphiphilic nature of the helical structure. Noncovalent linkage of two trichogin molecules ("dimer" formation) at the membrane surface *via* the orthogonally-oriented fatty acyl chains covalently bound to the α-amino groups, and subsequent insertion into the membrane, could lead to a molecular aggregate with Gly-rich faces at its interior to generate a hydrophilic channel of sufficient length to span the bilayer. The results described in this paper, in particular the requirement for a minimal aliphatic chain length for membrane activity and the full activity exhibited by the head-to-head dimer, give some support to this hypothesis. In this model the succinoyl covalent linker of the trichogin GA IV dimer may replace the two sticky fatty acyl chains.

However, it is fair to point out that the orientation of a peptide in the liposome lipid is not necessarily the same as in a voltage-dependent channel; *i.e.*, a peptide chain is not necessarily parallel to the normal to the bilayer. With liposomes, a situation where the trichogin GA IV undecapeptide would float on the bilayer, with the hydrophobic moieties (octanoyl group and Leu, Ile, and Lol side chains) buried into the membrane and the Gly-rich face exposed to the bulk water, cannot be discarded on the basis of the results presented in this work. Alternatively, an oblique disposition of the peptide, with the anchoring octanoyl chain deeply buried into the membrane, may also represent a plausible model. A study is currently underway in our laboratories aiming at discriminating among the different possibilities discussed above by exploiting two series of synthetic analogs of trichogin GA IV, each designed to incorporate at selected positions of the peptide chain either a paramagnetic or a fluorescent amino acid residue. We are also examining the properties of trichogin GA IV and selected analogs under the conditions of voltage-controlled experiments, a prerequisite to probe the nature of a voltage-dependent channel. Due to their short chain length and low dipole moment, one would expect a burstlike behavior with short-living single pores, as already found for sequential Ala/Aib model peptides of comparable length.[21,22]

JA954081O

(21) Menestrina, G.; Voges, K. P.; Jung, G.; Boheim, G. *J. Membr. Biol.* **1986**, *93*, 111−132.
(22) Vogel, H.; Nilsson, L.; Rigler, R.; Meder, S.; Boheim, G.; Beck, W.; Kurth, H. H.; Jung, G. *Eur. J. Biochem.* **1993**, *212*, 305−313.