PERKIN 2

# Short-chain analogues of the lipopeptaibol antibiotic trichogin GA IV: conformational analysis and membrane modifying properties

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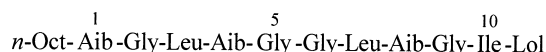
To examine the role of the peptide main-chain length on the conformation and membrane activity of the lipopeptaibol antibiotic trichogin GA IV we have synthesized by solution methods the Leu[11]-OMe analogue and all its short, *N*-octanoylated C-terminal sequences. By FTIR absorption, $^1$H NMR and CD we have shown that largely folded, but not helical, forms characterize the short peptides, while the longest peptides predominantly adopt regular helical structures. Membrane activity is found in main-chain lengths as short as the tetrapeptide and progressively increases up to the undecapeptide.

## Introduction

*Peptaibols*[1] are a unique class of membrane active compounds of fungal origin. These antibiotic *pept*ides are characterized by a linear sequence of 10–19 α-amino acid residues, a high proportion of the $C^{\alpha,\alpha}$-disubstituted glycine, helical inducer[2–4] *Aib* (α-aminoisobutyric acid), an N-terminal acetyl group, and a C-terminal 1,2- (or β-) amino alcoh*ol*. The 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 a voltage.[5,6]

More recently, a variety of peptides were sequenced bringing new characteristics to the peptaibol class of antibiotics, namely a fatty acyl moiety, replacing the acetyl group, linked to the N-terminal amino acid (for a review article see ref. 7). Because of the *lipo*philic character of the N-terminal group, these peptides are referred to as *lipopeptaibols*.[8]

Trichogin GA IV, isolated from *Trichoderma longibrachiatum* and sequenced by Bodo and co-workers,[8] is the most extensively investigated lipopeptaibol. The primary structure of trichogin GA IV is given below, where *n*-Oct is *n*-octanoyl and Lol is leucinol.




We and others have recently shown that trichogin GA IV and its Leu[11]-OMe (OMe, methoxy) analogue are amphiphilic, right-handed, mixed $3_{10}$–α-helical[9] peptides with a remarkable capability to modify membrane permeability.[8,10–13] In these lipopeptides an $N^{\alpha}$-blocking fatty acyl moiety of at least six carbon atoms is required for the onset of significant membrane activity.[14]

To complete our understanding of the structural requirements of trichogin GA IV for membrane activity and, more specifically, to examine the role of peptide main-chain length, we have decided to prepare the Leu[11]-OMe analogue **11** and all its short $N^{\alpha}$-octanoylated C-terminal sequences (peptides **2**–**10**).

This paper describes synthesis, characterization, solution conformational analysis (by FTIR absorption, $^1$H NMR, and CD techniques), and membrane modifying properties of peptides **2**–**11**.

*n*-Oct-Ile-Leu-OMe (**2**)

*n*-Oct-Gly-Ile-Leu-OMe (**3**)

*n*-Oct-Aib-Gly-Ile-Leu-OMe (**4**)

*n*-Oct-Leu-Aib-Gly-Ile-Leu-OMe (**5**)

*n*-Oct-Gly-Leu-Aib-Gly-Ile-Leu-OMe (**6**)

*n*-Oct-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (**7**)

*n*-Oct-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (**8**)

*n*-Oct-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (**9**)

*n*-Oct-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (**10**)

*n*-Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (**11**)

## Experimental

### Peptide synthesis

Melting points were determined using a Leitz model Laborlux 12 apparatus (Wetzlar, Germany) and are not corrected. Thin-layer chromatography (TLC) was performed on Merck Kieselgel 60-F precoated plates (Darmstadt, Germany) using the following solvent systems: (I) chloroform–ethanol 9 : 1; (II) butan-1-ol–acetic acid–water 3 : 1 : 1; (III) toluene–ethanol 7 : 1. The chromatograms were developed by quenching of UV fluorescence or by chlorine–starch–potassium iodide or ninhydrin chromatic reaction as appropriate. All compounds were obtained in a chromatographically homogeneous state. The preparation and characterization of the newly synthesized peptides are described below.

***n*-Oct-Ile-Leu-OMe (2).** This compound was prepared in $CH_2Cl_2$ solution from *n*-Oct-OH, 1-hydroxy-7-aza-1,2,3-benzotriazole (HOAt), *N*-ethyl-*N*′-[3-(dimethylamino)propyl]-carbodiimide (EDC) hydrochloride, *N*-methylmorpholine (NMM), and H-Ile-Leu-OMe [the last compound being obtained *via* catalytic hydrogenation in methanol (MeOH) of the corresponding Z (benzyloxycarbonyl)-derivative[14]]. Yield

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91%. Oil [from ethyl acetate (EtOAc)–light petroleum (LP)]; $[\alpha]_D^{20}$ −56.6 (*c* 0.5 in MeOH); TLC $R_{F1}$ 0.95, $R_{F2}$ 0.90, $R_{F3}$ 0.75; $\nu_{max}$ (film)/cm$^{-1}$ 3415, 3296, 1751, 1638, 1550; $\delta_H$ (200 MHz; 10 mmol dm$^{-3}$ $CDCl_3$; $Me_4Si$) 6.14 (d, 1H, Leu NH), 6.04 (d, 1H, Ile NH), 4.59 (m, 1H, Leu α-CH), 4.30 (m, 1H, Ile α-CH), 3.73 (s, 3H, OMe $CH_3$), 2.19 (t, 2H, *n*-Oct α-$CH_2$), 1.87 (m, 1H, Ile β-CH), 1.70–1.50 (m, 4H, Leu β-$CH_2$, Leu γ-CH, 1H Ile γ-$CH_2$), 1.32–1.15 [m, 11H, *n*-Oct $(CH_2)_5$, 1H Ile γ-$CH_2$], 0.97–0.87 (m, 15H, Leu 2 δ-$CH_3$, Ile γ-$CH_3$, Ile δ-$CH_3$, *n*-Oct ω-$CH_3$). HPLC $t_r$/min 12.25 [eluants A: 0.05% trifluoroacetic acid (TFA) in $H_2O$, and B: 0.05% TFA in a $CH_3CN$–$H_2O$ 9 : 1 mixture; gradient: from 70 to 90% B in 20 min; reversed-phase $C_{18}$ Phenomenex column; eluant flow rate: 1 ml min$^{-1}$; $\lambda_{abs}$: 226 nm]. Mass spectrometry (MS) found: 385.3 $[M + H]^+$; calc. for $C_{21}H_{40}N_2O_4$: 384.3. Amino acid analysis: Ile 0.93, Leu 1.08.

***n*-Oct-Gly-Ile-Leu-OMe (3).** This compound was prepared as described above for dipeptide **2** using H-Gly-Ile-Leu-OMe obtained *via* catalytic hydrogenation in MeOH of the corresponding Z-derivative.[14] Yield 53%. Mp 120–121 °C (from EtOAc–LP); $[\alpha]_D^{20}$ −41.2 (*c* 0.5 in MeOH); TLC $R_{F1}$ 0.95, $R_{F2}$ 0.90, $R_{F3}$ 0.40; $\nu_{max}$ (KBr)/cm$^{-1}$ 3415, 3280, 1756, 1663, 1632, 1555, 1531; $\delta_H$ (250 MHz; 10 mmol dm$^{-3}$ $CDCl_3$; $Me_4Si$) 6.60 (d, 1H, Ile NH), 6.25 (m, 2H, Ile NH, Gly NH), 4.59 (m, 1H, Leu α-CH), 4.32 (m, 1H, Ile α-CH), 3.95 (d, 2H, Gly α-$CH_2$), 3.72 (s, 3H, OMe $CH_3$), 2.22 (t, 2H, *n*-Oct α-$CH_2$), 1.86 (m, 1H, Ile β-CH), 1.70–1.41 (m, 4H, Leu β-$CH_2$, Leu γ-CH, 1H Ile γ-$CH_2$), 1.32–1.10 [m, 11H, *n*-Oct $(CH_2)_5$, 1H Ile γ-$CH_2$], 1.00–0.80 (m, 15H, Leu 2 δ-$CH_3$, Ile γ-$CH_3$, Ile δ-$CH_3$, *n*-Oct ω-$CH_3$). HPLC $t_r$/min 8.36. MS found: 442.4 $[M + H]^+$; calc. for $C_{23}H_{43}N_3O_5$: 441.3. Amino acid analysis: Gly 1.00, Ile 0.97, Leu 1.03.

***n*-Oct-Aib-Gly-Ile-Leu-OMe (4).** This compound was prepared as described above for dipeptide **2** using H-Aib-Gly-Ile-Leu-OMe obtained *via* catalytic hydrogenation in MeOH of the corresponding Z-derivative.[14] Yield 77%. Mp 154–155 °C [from diethyl ether (DE)–LP]; $[\alpha]_D^{20}$ −20.7 (*c* 0.5 in MeOH); TLC $R_{F1}$ 0.95, $R_{F2}$ 0.85, $R_{F3}$ 0.35; $\nu_{max}$ (KBr)/cm$^{-1}$ 3375, 3301, 1726, 1657, 1648, 1539; $\delta_H$ (250 MHz; 10 mmol dm$^{-3}$ $CDCl_3$; $Me_4Si$) 7.40 (d, 1H, Ile NH), 7.28 (m, 1H, Gly NH), 6.94 (d, 1H, Leu NH), 6.19 (s, 1H, Aib NH), 4.60 (m, 1H, Leu α-CH), 4.30 (m, 1H, Ile α-CH), 3.95 (dd, 2H, Gly α-$CH_2$), 3.72 (s, 3H, OMe $CH_3$), 2.24 (m, 2H, *n*-Oct α-$CH_2$), 2.03 (m, 1H, Ile β-CH), 1.76–1.50 (m, 4H, Leu β-$CH_2$, Leu γ-CH, 1H Ile γ-$CH_2$), 1.54 and 1.50 (2s, 6H, Aib β-$CH_3$), 1.40–1.20 [m, 11H, *n*-Oct $(CH_2)_5$, 1H Ile γ-$CH_2$], 1.04–0.84 (m, 15H, Leu 2 δ-$CH_3$, Ile γ-$CH_3$, Ile δ-$CH_3$, *n*-Oct ω-$CH_3$). HPLC $t_r$/min 11.43. MS found: 527.4 $[M + H]^+$; calc. for $C_{27}H_{50}N_4O_6$: 526.4. Amino acid analysis: Aib 0.92, Gly 1.00, Ile 1.00, Leu 1.10.

***n*-Oct-Leu-Aib-Gly-Ile-Leu-OMe (5).** This compound was prepared as described above for dipeptide **2** using H-Leu-Aib-Gly-Ile-Leu-OMe obtained *via* catalytic hydrogenation in MeOH of the corresponding Z-derivative.[14] Yield 65%. Oil (from DE–LP); $[\alpha]_D^{20}$ −58.6 (*c* 0.5 in MeOH); TLC $R_{F1}$ 0.95, $R_{F2}$ 0.85, $R_{F3}$ 0.40; $\nu_{max}$ (film)/cm$^{-1}$ 3291, 1747, 1650, 1541; $\delta_H$ (250 MHz; 10 mmol dm$^{-3}$ $CDCl_3$; $Me_4Si$) 7.53 (d, 1H, Leu NH), 7.23 (d, 1H, Ile NH), 6.99 (m, 1H, Gly NH), 6.93 (d, 1H, Leu NH), 6.80 (s, 1H, Aib NH), 4.65 (m, 1H, Leu α-CH), 4.29 (m, 2H, Ile α-CH, 1H Gly α-$CH_2$), 4.18 (m, 1H, Leu α-CH), 3.73 (s, 3H, OMe $CH_3$), 3.46 (m, 1H, Gly α-$CH_2$), 2.10 (m, 2H, *n*-Oct α-$CH_2$), 1.80 (m, 1H, Ile β-CH), 1.70–1.50 (m, 7H, 2 Leu β-$CH_2$, 2 Leu γ-CH, 1H Ile γ-$CH_2$), 1.58 and 1.45 (2s, 6H, Aib 2 β-$CH_3$), 1.30–1.20 [m, 11H, *n*-Oct $(CH_2)_5$, 1H Ile γ-$CH_2$], 0.95–0.84 (m, 21H, 2 Leu 4 δ-$CH_3$, Ile γ-$CH_3$, Ile δ-$CH_3$, *n*-Oct ω-$CH_3$). HPLC $t_r$/min 13.63. MS found: 640.5 $[M + H]^+$; calc. for $C_{33}H_{61}N_5O_7$: 639.4. Amino acid analysis: Aib 0.95, Gly 1.00, Ile 0.93, Leu 2.10.

***n*-Oct-Gly-Leu-Aib-Gly-Ile-Leu-OMe (6).** This compound was prepared as described above for dipeptide **2** using H-Gly-Leu-Aib-Gly-Ile-Leu-OMe obtained *via* catalytic hydrogenation in MeOH of the corresponding Z-derivative.[14] Yield 70%. Mp 87–88 °C (from DE–LP); $[\alpha]_D^{20}$ −63.2 (*c* 0.5 in MeOH); TLC $R_{F1}$ 0.95, $R_{F2}$ 0.85, $R_{F3}$ 0.35; $\nu_{max}$ (KBr)/cm$^{-1}$ 3411, 3303, 1747, 1653, 1543; $\delta_H$ (400 MHz; 10 mmol dm$^{-3}$ $CDCl_3$; $Me_4Si$) 7.68 (d, 1H, Leu NH), 7.58 (t, 1H, Gly NH), 7.25 (d, 1H, Ile NH), 6.90 (t, 1H, Gly NH), 6.69 (d, 1H, Leu NH), 6.51 (s, 1H, Aib NH), 4.50 (m, 1H, Leu α-CH), 4.39 (m, 1H, Ile α-CH), 4.20 (m, 1H, Leu α-CH), 4.02 (m, 2H, Gly α-$CH_2$), 3.72 (s, 3H, OMe $CH_3$), 3.46 (m, 2H, Gly α-$CH_2$), 2.20 (m, 2H, *n*-Oct α-$CH_2$), 1.90 (m, 1H, Ile β-CH), 1.70–1.50 (m, 7H, 2 Leu β-$CH_2$, 2 Leu γ-CH, 1H Ile γ-$CH_2$), 1.55 and 1.40 (2s, 6H, Aib 2 β-$CH_3$), 1.30–1.20 [m, 11H, *n*-Oct $(CH_2)_5$, 1H Ile γ-$CH_2$], 0.95–0.84 (m, 21H, 2 Leu 4 δ-$CH_3$, Ile γ-$CH_3$, Ile δ-$CH_3$, *n*-Oct ω-$CH_3$). HPLC $t_r$/min 10.50. MS found: 697.6 $[M + H]^+$; calc. for $C_{35}H_{64}N_6O_8$: 696.5. Amino acid analysis: Aib 1.00, Gly 2.03, Ile 0.92, Leu 2.02.

***n*-Oct-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (7).** This compound was prepared as described above for dipeptide **2** using H-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe obtained *via* catalytic hydrogenation in MeOH of the corresponding Z-derivative.[14] Yield 58%. Oil (from DE–LP); $[\alpha]_D^{20}$ −37.1 (*c* 0.5 in MeOH); TLC $R_{F1}$ 0.90, $R_{F2}$ 0.80, $R_{F3}$ 0.20; $\nu_{max}$ (film)/cm$^{-1}$ 3299, 1744, 1652, 1538; $\delta_H$ (400 MHz; 10 mmol dm$^{-3}$ $CDCl_3$; $Me_4Si$) 8.01 (t, 1H, Gly NH), 7.68 (t, 1H, Gly NH), 7.50 (m, 2H, Ile NH and Leu NH), 6.77 (d, 1H, Leu NH), 6.63 (t, 1H, Gly NH), 6.48 (s, 1H, Aib NH), 4.40–4.20 (m, 3H, Leu α-CH, Gly α-$CH_2$), 4.03–3.91 (m, 3H, Ile α-CH, Leu α-CH, 1H Gly α-$CH_2$), 3.78–3.72 (m, 1H, Gly α-$CH_2$), 3.72 (s, 3H, OMe $CH_3$), 3.49–3.42 (m, 1H, 1H Gly α-$CH_2$), 3.23–3.14 (m, 1H, 1H Gly α-$CH_2$), 2.25 (m, 2H, *n*-Oct α-$CH_2$), 2.10 (m, 1H, Ile β-CH), 1.78–1.60 (m, 7H, 2 Leu β-$CH_2$, 2 Leu γ-CH, 1H Ile γ-$CH_2$), 1.56 and 1.44 (2s, 6H, Aib 2 β-$CH_3$), 1.35–1.23 [m, 10H, *n*-Oct $(CH_2)_5$], 1.15–1.09 (m, 1H, Ile γ-$CH_2$), 0.98 (d, 3H, Ile γ-$CH_3$), 0.94–0.83 (m, 18H, 2 Leu 4 δ-$CH_3$, Ile δ-$CH_3$, *n*-Oct ω-$CH_3$). HPLC $t_r$/min 9.57. MS found: 753.6 $[M + H]^+$; calc. for $C_{37}H_{67}N_7O_9$: 753.5. Amino acid analysis: Aib 1.00, Gly 3.08, Ile 0.91, Leu 1.95.

***n*-Oct-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (8).** This compound was prepared as described above for dipeptide **2** using H-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe obtained *via* catalytic hydrogenation in MeOH of the corresponding Z-derivative.[14] Yield 82%. Oil (from DE–LP); $[\alpha]_D^{20}$ −33.0 (*c* 0.5 in MeOH); TLC $R_{F1}$ 0.85, $R_{F2}$ 0.75, $R_{F3}$ 0.10; $\nu_{max}$ (film)/cm$^{-1}$ 3412, 3308, 1746, 1656, 1542; $\delta_H$ (400 MHz; 10 mmol dm$^{-3}$ $CDCl_3$; $Me_4Si$) 8.31 (t, 1H, Gly NH), 8.23 (t, 1H, Gly NH), 7.75 (m, 1H, Leu NH), 7.68–7.50 (m, 4H, Ile NH, Aib NH, Leu NH, Gly NH), 7.35 (s, 1H, Aib NH), 4.44 (m, 1H, Leu α-CH), 4.25 (m, 1H Ile α-CH), 4.12 (m, 1H, Leu α-CH), 3.92–3.60 (m, 6H, 3 Gly α-$CH_2$), 3.72 (s, 3H, OMe $CH_3$), 2.25 (m, 2H, *n*-Oct α-$CH_2$), 2.03 (m, 1H, Ile β-CH), 1.72–1.52 (m, 7H, 2 Leu β-$CH_2$, 2 Leu γ-CH, 1H Ile γ-$CH_2$), 1.45 (m, 12H, 2 Aib 4 β-$CH_3$), 1.32–1.22 [m, 11H, *n*-Oct $(CH_2)_5$, 1H, Ile γ-$CH_2$], 0.94–0.83 (m, 21H, 2 Leu 4 δ-$CH_3$, Ile δ-$CH_3$, Ile γ-$CH_3$, *n*-Oct ω-$CH_3$). HPLC $t_r$/min 10.41. MS found: 839.7 $[M + H]^+$; calc. for $C_{41}H_{74}N_8O_{10}$: 838.6. Amino acid analysis: Aib 2.00, Gly 3.09, Ile 0.92, Leu 1.93.

***n*-Oct-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (9).** This compound was prepared as described above for dipeptide **2** using H-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe obtained *via* catalytic hydrogenation in MeOH of the corresponding Z-derivative.[14] Yield 61%. Oil (from DE–LP); $[\alpha]_D^{20}$ −44.2 (*c* 0.5 in MeOH); TLC $R_{F1}$ 0.90, $R_{F2}$ 0.80, $R_{F3}$ 0.15; $\nu_{max}$ (film)/cm$^{-1}$ 3407, 3311, 1746, 1656, 1542; $\delta_H$ (400 MHz; 10 mmol dm$^{-3}$

$CDCl_3$; $Me_4Si$) 8.21 (t, 1H, Gly NH), 8.10 (t, 1H, Gly NH), 7.98 (s, 1H, NH), 7.68 (s, 1H, NH), 7.65–7.60 (m, 2H, 2NH), 7.55 (t, 1H, Gly NH), 7.48 (s, 1H, NH), 7.38 (d, 1H, NH), 4.50 (m, 1H, Leu α-CH), 4.23 (m, 1H, α-CH), 4.10–4.00 (m, 3H, 3 α-CH), 3.90–3.60 (m, 5H, α-CH), 3.70 (s, 3H, OMe $CH_3$), 2.30 (m, 2H, *n*-Oct α-$CH_2$), 2.05 (m, 1H, Ile β-CH), 1.89–1.60 (m, 10H, 3 Leu β-$CH_2$, 3 Leu γ-CH, 1H Ile γ-$CH_2$), 1.51, 1.50, and 1.45 (3s, 12H, 2 Aib 4 β-$CH_3$), 1.39–1.21 [m, 11H, *n*-Oct $(CH_2)_5$, 1H Ile γ-$CH_2$], 0.99–0.83 (m, 27H, 3 Leu 6 δ-$CH_3$, Ile δ-$CH_3$, Ile γ-$CH_3$, *n*-Oct ω-$CH_3$). HPLC $t_r$/min 12.04. MS found: 952.8 $[M + H]^+$; calc. for $C_{47}H_{85}N_9O_{11}$: 951.6. Amino acid analysis: Aib 1.96, Gly 3.08, Ile 0.95, Leu 2.91.

***n*-Oct-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (10).** This compound was prepared as described above for dipeptide **2** using H-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe obtained *via* catalytic hydrogenation in MeOH of the corresponding Z-derivative.[14] Yield 69%. Mp 193–194 °C (from DE–LP); $[\alpha]_D^{20}$ −39.3 (*c* 0.5 in MeOH); TLC $R_{F1}$ 0.90, $R_{F2}$ 0.80, $R_{F3}$ 0.10; $\nu_{max}$ (KBr)/cm$^{-1}$ 3316, 1745, 1657, 1542; $\delta_H$ (400 MHz; 10 mmol dm$^{-3}$ [$^2H_6$]DMSO) 8.36 (s, 1H, Aib NH), 8.24 (d, 1H, Leu NH), 8.09–8.06 (m, 2H, Aib NH and Gly NH), 8.02 (d, 1H, Leu NH), 7.92–7.87 (m, 2H, Gly NH, Leu NH), 7.85–7.78 (m, 2H, 2 Gly NH), 7.46 (d, 1H, Ile NH), 4.26–4.16 (m, 4H, Ile α-CH, 3 Leu α-CH), 3.72–3.57 (m, 8H, 4 Gly α-$CH_2$), 3.57 (s, 3H, OMe $CH_3$), 2.11–2.07 (t, 2H, *n*-Oct α-$CH_2$), 1.80 (m, 1H, Ile β-CH), 1.60–1.42 (m, 10H, 3 Leu β-$CH_2$ and γ-CH, 1H Ile γ-$CH_2$), 1.33 and 1.31 (2s, 12H, 2 Aib 4 β-$CH_3$), 1.21 [s, 10H, *n*-Oct $(CH_2)_5$], 1.10–1.03 (m, 1H, 1H Ile γ-$CH_2$), 0.88–0.78 (m, 27H, 3 Leu 6 δ-$CH_3$, Ile γ-$CH_3$, Ile δ-$CH_3$, *n*-Oct ω-$CH_3$). HPLC $t_r$/min 10.63. MS found: 1009.8 $[M + H]^+$; calc. for $C_{49}H_{88}N_{10}O_{12}$: 1008.7. Amino acid analysis: Aib 2.09, Gly 4.05, Ile 0.91, Leu 2.92.

### Polarimetry

The $[\alpha]_D^{20}$ optical rotation values were determined on a Perkin-Elmer model 241 polarimeter (Norwalk, CT) equipped with a Haake model D8 thermostat (Karlsruhe, Germany). A cell with a path length of 10 cm was used.

### Amino acid analyses

The amino acid analyses were performed on a C. Erba model 3A30 amino acid analyzer (Rodano, Milan, Italy). The Aib colour yield with ninhydrin is about 20 times lower than those of protein amino acids. Elution of Aib was observed immediately after the Ala peak.

### Mass spectrometry

Solutions at $1 \times 10^{-6}$ mol dm$^{-3}$ peptide concentration were prepared by dissolving the samples in an $CH_3CN$–water–acetic acid 50 : 50 : 1 mixture. Then, 20 µl injections were performed into a Mariner ESI-TOF mass spectrometer (Perseptive Biosystems, Foster City, CA) at a flow rate of 15 µl min$^{-1}$. Spectra were acquired every five seconds.

### HPLC

HPLC analyses were performed on a Pharmacia model LKB-LCC 2252 liquid chromatograph (Peapack, NJ) equipped with a Uvicord model SD UV detector at 226 nm and reversed-phase $C_{18}$ Vydac (Hesperia, CA) model 218 $TP_{54}$ and Phenomenex (Torrance, CA) model Kromasil 5µ $C_{18}$ 100 A (250 × 4.60 mm) columns.

### FTIR absorption spectra

The solution FTIR absorption spectra were recorded at $1 \times 10^{-3}$ mol dm$^{-3}$ peptide concentration and 293 K 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 recorded under the same conditions. For spectral elaborations the software SpectraCalc provided by Galactic (Salem, MA) was employed. Cells with path lengths of 1.0 and 10 mm (with $CaF_2$ windows) were used. Spectrograde [$^2$H]chloroform (99.8% $^2$H) was purchased from Merck. The solid-state FTIR absorption spectra were recorded in KBr pellets (for solids) or in a film (for oily compounds) using a Perkin-Elmer model 580B spectrophotometer equipped with a Perkin-Elmer model 3600 IR data station.

### $^1$H NMR spectra

$^1$H NMR spectra were recorded at $1 \times 10^{-3}$ mol dm$^{-3}$ peptide concentration and 293 K with Bruker models AC 200, AC 250 and Advance DRX 400 spectrometers (Karlsruhe, Germany), averaging 32 scans. Measurements were carried out in [$^2$H]-chloroform (99.96% $^2$H; Merck) and in [$^2H_6$]DMSO ([$^2H_6$]dimethyl sulfoxide) (99.96% $^2H_6$; Fluka, Buchs, Switzerland).

### CD spectra

CD spectra were recorded at $1 \times 10^{-3}$ mol dm$^{-3}$ peptide concentration and 293 K using a Jasco model J-715 spectropolarimeter (Tokyo, Japan) equipped with a Haake thermostat, averaging 8 scans. Baselines were corrected by subtracting the solvent contribution. Cylindrical, fused quartz cells of 0.5 and 0.2 mm path lengths (Hellma, Müllheim, Germany) were employed. The data are expressed in terms of $[\theta]_R$, the residual molar ellipticity (deg cm$^2$ dmol$^{-1}$). MeOH, 99.9% spectrophotometric grade, was purchased from Acros Organics, Geel, Belgium. Sodium dodecyl sulfate (SDS), 99% purity, was a Pierce Chem. Co. (Rockford, IL) product and was used without recrystallization. Deionized water was further purified using a milliQ reagent grade water system from Millipore (Bedford, MA).

### Liposome leakage assay

Peptide-induced leakage from egg phosphatidylcholine (PC) vesicles was measured at 293 K using the carboxyfluorescein (CF)-entrapped vesicle technique[15] and a Perkin-Elmer model LS 50B luminescence spectrometer. CF-encapsulated small unilamellar vesicles (egg PC–cholesterol, 7 : 3) were prepared by sonication in Hepes buffer, pH 7.4. The phospholipid concentration was kept constant (0.06 mM), and increasing [peptide]/[lipid] molar ratios ($R^{-1}$) were obtained by adding aliquots of MeOH solutions of peptides, keeping the final MeOH concentration below 5% by volume. After rapid and vigorous stirring, the time course of fluorescence change corresponding to CF escape was recorded at 520 nm (6 nm band pass) with $\lambda_{exc}$ 488 nm (3 nm band pass). The percentage of released CF at time *t* was determined as $(F_t - F_0)/(F_T - F_0) \times 100$, with $F_0$ = fluorescence intensity of vesicles in the absence of peptide, $F_t$ = fluorescence intensity at time *t* in the presence of peptide, and $F_T$ = total fluorescence intensity determined by disrupting the vesicles by addition of 50 µL of a 10% Triton X-100 solution. The kinetics experiments were stopped at 20 min.

## Results and discussion

### Synthesis and characterization

The preparation and characterization of Leu$^{11}$-OMe trichogin GA IV (**11**) and all its Z N$^\alpha$-protected, C-terminal truncated sequences have already been reported.[14] The solution synthesis was performed using a racemization-free strategy, including a step-by-step approach from the C-terminal H-Leu-OMe

derivative *via* the mixed anhydride method with isobutylchloroformate to incorporate the Z N$^{\alpha}$-protected protein amino acids, and the symmetrical anhydride method to incorporate the Aib residues. Then, the *n*-Oct N$^{\alpha}$-blocking moiety was introduced in the Z-deprotected peptides using the EDC–HOAt procedure [16] to afford compounds **2**–**11**.

The Z N$^{\alpha}$-protected amino acid derivatives were obtained by reacting the pertinent free amino acid with 1-(benzyloxycarbonyloxy)succinimide in a 1,4-dioxane–alkaline aqueous solvent mixture.[17] The leucine methyl ester hydrochloride (HCl·H-Leu-OMe) was prepared by the MeOH–thionyl chloride method.[18] Removal of the Z group was carried out by catalytic hydrogenation. The stable, crystalline derivative $(Z\text{-Aib})_2O$ [19,20] was obtained by reacting Z-Aib-OH with 0.5 equivalents of thionyl chloride in ethyl acetate.

All peptides were obtained in a chromatographically homogeneous state. They were characterized by melting point determination, polarimetry, thin-layer chromatography (TLC) in three different solvent systems, solid-state IR absorption, $^1$H NMR, high-performance liquid chromatography, and mass spectrometry.

### Solution conformational analysis

An analysis of the preferred conformations of peptides **2**–**11** was performed using FTIR absorption and $^1$H NMR in $CDCl_3$ solution, and CD in MeOH and in a membrane-mimetic environment (SDS micelles).

The 3500–3200 cm$^{-1}$ FTIR absorption spectra of peptides **5**–**11** are dominated by a strong band at 3327–3308 cm$^{-1}$ (N–H stretching mode of strongly H-bonded amide groups)[21–23] (Fig. 1). This absorption is remarkable even at the tetrapeptide level (**4**), but nearly negligible in the di- (**2**) and tripeptides (**3**). Typically, its relative intensity, with respect to those of the free N–H groups at wavenumbers > 3400 cm$^{-1}$, increases as the peptide main chain elongates. We attribute the modest increment exhibited by peptides **6** and **7** to the introduction of two consecutive, flexible Gly residues at those stages. No appreciable differences are seen in the spectra between $1 \times 10^{-3}$ and $1 \times 10^{-4}$ mol dm$^{-3}$ peptide concentration (results not shown). Therefore, the observed H-bonding should be interpreted as arising almost exclusively from intramolecular C=O · · · H–N interactions. In summary, these FTIR absorption results are consistent with the hypothesis that in $CDCl_3$ solution all peptides, beginning from tetrapeptide **4**, are largely folded in intramolecularly H-bonded turn–helical conformations.

For a detailed understanding of the preferred conformations in $CDCl_3$ solution of the peptides under investigation we carried out a 400 MHz $^1$H NMR analysis of two selected sequences. Delineation of solvent shielded (presumably intramolecularly H-bonded) NH groups was achieved by using solvent dependence of NH chemical shifts by adding increasing amounts of a perturbing agent, the H-bonding acceptor DMSO,[24,25] to the $CDCl_3$ solution. Fig. 2A shows the DMSO titration of heptapeptide **7**, the sequence of which corresponds to that of the Leu-OMe analogue of the lipopeptaibol antibiotic trichodecenin I.[26] In Fig. 2B the corresponding titration of the Z-protected undecapeptide analogue of **11**, Z$^0$, Leu$^{11}$-OMe trichogin GA IV, is illustrated. The urethane Z N$^{\alpha}$-protected peptides are more soluble than their acyl (in particular, fatty acyl) blocked counterparts and their NH proton assignments are facilitated by the observation of the N-terminal NH proton at significantly high fields. Unambiguous assignments of all of the NH proton resonances of the heptapeptide were performed *via* analysis of their multiplicities (Aib singlets, Leu and Ile doublets, and Gly triplets) and ROESY experiments. In the undecapeptide we were able to assign with reasonable confidence only the N-terminal urethane proton at high fields, Aib N(1)H, and a NH proton which, by virtue of its multiplicity and position in the spectrum (at very low fields), typical of a N(2)H proton, is attributed to Gly$^2$.

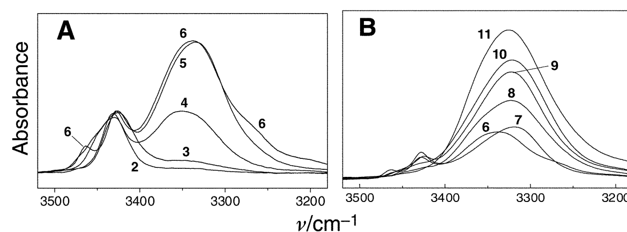


**Fig. 1** FT-IR absorption spectra (3500–3200 cm$^{-1}$ region) of the trichogin GA IV short sequences in $CDCl_3$ solution. (**A**) Peptides **2**–**6**; (**B**) peptides **6**–**11**.

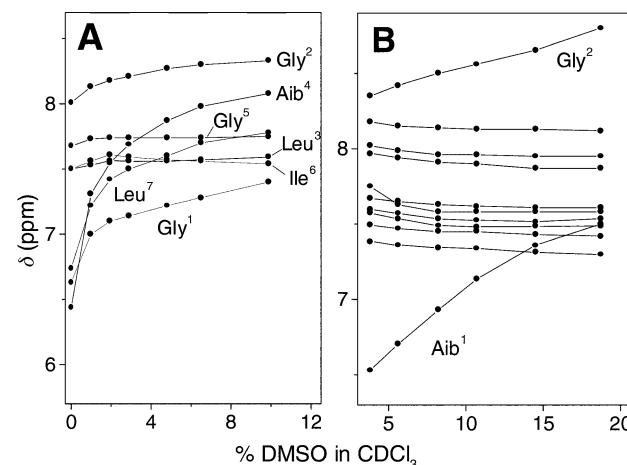


**Fig. 2** Plots of NH proton chemical shifts in the $^1$H NMR spectra of the trichogin GA IV short sequence peptide **7** (**A**) and the Z$^0$, Leu$^{11}$-OMe analogue (**B**) as a function of increasing percentages of DMSO (v/v) added to the $CDCl_3$ solution.

The NMR data for the heptapeptide point to the Leu$^3$, Gly$^5$, and Ile$^6$ NH groups as those involved in the intramolecular H-bonding scheme as donors. Similar results have already been reported for the related Boc (*tert*-butyloxycarbonyl) heptapeptide derivative in the same halohydrocarbon by Gurunath and Balaram,[27] who proposed a multiple, non-helical, β-turn structure,[28–30] with a type-I′ β-turn at -Gly$^1$-Gly$^2$- and two consecutive types-II-I′ β-turns centered at Aib$^4$ (which is constrained to a left-handed helical conformation). More recently, an identical structure for the 1–5 segment of the heptapeptide sequence was found in the crystal state by X-ray diffraction.[31] Interestingly, however, an X-ray diffraction analysis [32] of the isolated, terminally protected pentapeptide showed that the carbonyl group preceding the N-terminal Leu residue acts as the acceptor of two intramolecular H-bonds, giving rise to a -Leu-Aib- type-III′ β-turn and a -Leu-Aib-Gly-Ile- π-turn,[33–35] respectively. A second (type-I′) β-turn encompasses the -Aib-Gly- sequence. Taken together, these findings support the view that the nature of the N-terminal blocking group (*n*-Oct or Boc) does not affect the overall molecular conformation of the heptapeptide and highlight the structural plasticity of the -Leu-Aib-Gly-Ile-Leu- sequence.

Also the NMR data for the Z-protected undecapeptide are indicative of two classes of NH protons. Class (i) (Aib$^1$ and Gly$^2$ NH protons) includes protons whose chemical shifts are remarkably sensitive to the addition of DMSO, while class (ii) (all other NH protons) includes those protons displaying a behaviour characteristic of shielded protons (relative insensitivity of chemical shifts to the $CDCl_3$–DMSO solvent mixture composition). These $^1$H NMR results show that the distribution of the NH protons between the two classes is different in the undecapeptide compared to the heptapeptide sequence, allowing us to define the N(3)H to N(11)H protons

of the undecapeptide as almost inaccessible to the perturbing agent, and therefore, most probably, intramolecularly H-bonded. This situation is indeed that expected either for a regular $3_{10}$-helical or a mixed $3_{10}$–α-helical structure (the latter with the $3_{10}$-helical stretch at the N-terminus). In conclusion, our conformational analysis in $CDCl_3$ strongly suggests that the longest peptides of this series (from octa- to undecapeptides) with 20–30% Aib content are folded in stable helical structures, whereas the shortest peptides (from tetra- to heptapeptides) with <15% Aib content, although characterized by intramolecular H-bonds, do not adopt regular helical conformations.

We extended our conformational study to MeOH solution and a SDS micellar environment using CD spectroscopy. Fig. 3 shows the CD spectra of peptides **2**–**11** in MeOH. The curves of peptides **2**–**7** are similar and typical of an unordered conformation.[36] Conversely, the curve of peptide **11**, displaying negative Cotton effects at 203 nm and 228 nm, resembles those of right-handed $3_{10}$–α-helices.[36–39] However, the ratio ($R$) between the intensities of the 228 nm *versus* 203 nm band is about 0.4, closer to the value theoretically predicted[37] and experimentally found[38,39] for $3_{10}$-helical peptides than to that (about 1.0) of α-helical peptides. The curves of peptides **8**–**10** are indicative of a more or less pronounced transition from unordered to helical conformations. In 300 mM aqueous SDS the CD curves (not shown) suggest the onset of an ordered structure, albeit to a modest extent, even at the level of peptide **4**. The curve of **10** in SDS resembles that of **11** in MeOH solution. Interestingly, a pronounced increment of the α-helical form compared to the amount of $3_{10}$-helix is exhibited by peptide **11** in SDS, as revealed by the increase of the $R$ ratio to ≅ 0.65. In conclusion, our CD analysis supports the view that in a membrane-like environment (SDS micelles): (i) even very short trichogin GA IV sequences tend to adopt folded structures, and (ii) the undecapeptide trichogin GA IV analogue is in a mixed $3_{10}$–α-helical form, as found in the crystal state.

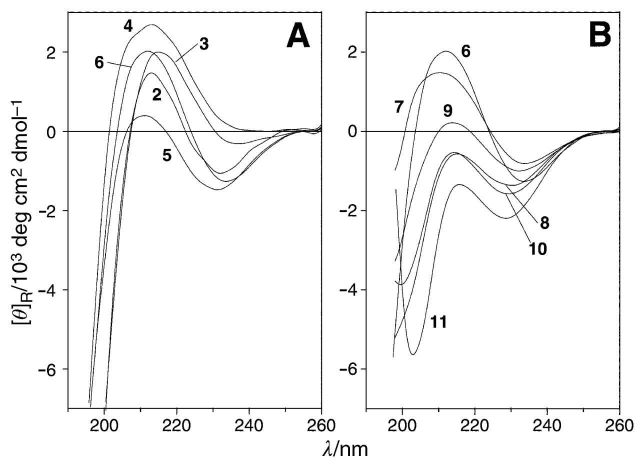


**Fig. 3** CD spectra (190–260 nm region) of the trichogin GA IV short sequences in MeOH solution. (**A**) Peptides **2**–**6**; (**B**) peptides **6**–**11**.

### Membrane permeability properties

The membrane modifying properties of peptides **2**–**11** were tested in comparison with those of the natural lipopeptaibol by measuring the induced leakage of CF entrapped in egg PC–cholesterol (7 : 3) small unilamellar vesicles[15] (Fig. 4). The undecapeptide analogue **11** has the same activity as trichogin GA IV. Peptides **2** and **3** are almost inactive. By increasing the peptide main-chain length from **3** to **11** a steady increase in the activity is seen. In summary, a threshold in the activity is observed at the level of the tetrapeptide **4**. This latter finding favours the conclusion that a relatively stable, folded structure is a prerequisite for the onset of membrane activity. This 3D-structural property, in turn, requires a minimal peptide main-chain length (four residues) and presence of a bend stabilizing amino acid (Aib). However, the full trichogin sequence with its three Aib residues is needed for a membrane activity as high as that of trichogin GA IV.

## Conclusions

The results accumulated in the present investigation considerably expand our knowledge of the minimal structural requirements for membrane activity of trichogin GA IV, the most extensively studied lipopeptaibol antibiotic.[7] The $N^{\alpha}$-blocking fatty acyl moiety has already been shown to play a major role in the membrane modifying properties of the undecapeptide esters.[14] More specifically, at least six carbon atoms in the aliphatic chain are required for a significant activity. Here, by using a set of $N^{\alpha}$-*n*-octanoylated synthetic peptides of increasing main-chain length, we established that membrane permeability properties, found at main-chain lengths as short as the tetrapeptide, progressively increase up to the undecapeptide. A significant influence of the three, strategically positioned in the amino acid sequence, Aib residues is also evident. In addition, from our analysis it is clear that membrane activity and amount of folding in chloroform run roughly parallel in this peptide series of variable backbone length, in the sense that largely folded, but not helical, forms are typically present in the short peptides, while the longest peptides exhibit regular helical stretches.


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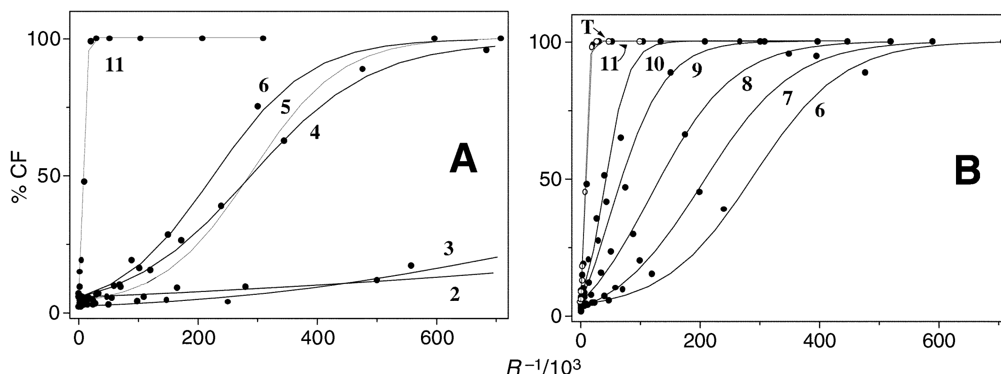


**Fig. 4** Peptide-induced CF leakage at 20 min for different ratios $R^{-1}$ = [peptide]/[lipid] from egg PC–cholesterol (70 : 30) vesicles. (**A**) Peptides **2**–**6** and **11**; (**B**) peptides **6**–**11** and trichogin GA IV (**T**).

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