Trichogin A IV, an 11-Residue Lipopeptaibol from *Trichoderma longibrachiatum*

Catherine Auvin-Guette,[†] Sylvie Rebuffat,[†] Yann Prigent,[‡] and Bernard Bodo*,[†]

Contribution from the Laboratoire de Chimie, URA 401 CNRS, GDR 964 CNRS, Muséum National d'Histoire Naturelle, 63 Rue Buffon, 75231 Paris Cedex 05, France, and Unité Mixte de Recherche Bruker-CNRS, UMR 50, Université Louis Pasteur, 34 Rue de l'Industrie, 67160 Wissembourg, France. *Received July 2, 1991*

Abstract: Trichogin A IV (GA IV) is the main component of the natural trichogin mixture, a new peptide group extracted from in vitro cultures of the fungus *Trichoderma longibrachiatum*. GA IV was isolated by reversed-phase HPLC, and its amino acid sequence was elucidated by FAB mass spectrometry and high-field NMR. Complete ^{1}H and ^{13}C resonance assignments were carried out using HOHAHA, ROESY, ^{1}H–^{13}C COSY, and COLOC two-dimensional spectroscopies. This linear peptide contains an N-terminal extremity acylated by an octanoyl group, 10 amino acids, and a leucinol C-terminal amino alcohol, giving rise to a novel class of peptides we propose to name lipopeptaibols. The methanolic solution conformation of GA IV was examined by a combination of CD data, ^{13}C NMR relaxation measurements, temperature coefficients of NH and CO groups, and NOE data. The structure was found to be helical. The membrane-modifying properties were tested toward liposomes composed of egg phosphatidylcholine with 20 or 30% cholesterol. GA IV revealed permeability modifications similar to those exhibited by a 19-residue acidic peptaibol.

Introduction

Membrane-modifying peptides of fungal origin, which are rich in α-aminoisobutyric acid (Aib), have been the focus of recent investigations.[1-3] These peptides, extracted from *Trichoderma* spp., are characterized by a microheterogeneity which results in a complex mixture of closely related sequence analogues.[4] These linear peptides contain an acetylated N-terminal extremity and a C-terminal amino alcohol and thus form the peptaibol class, which may be broadly grouped into two subclasses: (i) the "long sequences" that contain 18–20 residues, among which are alamethicins,[5] trichosporins,[6] and trichorzianines[7,8] and (ii) the "short sequences" (15–17 residues) as exemplified by emerimicins,[9] zervamicins,[10] and antiamoebins.[11,12]

In continuation of our studies on *Trichoderma* strains, we isolated two new peptide groups from *T. longibrachiatum*. The first one is composed of 19-residue peptaibols, tricholongins B, the structures of which were established previously.[13] The second group, the trichogins (G), exhibits special features that we present in this paper. Trichogins contain 10 amino acids, a C-terminal amino alcohol, and an N-terminal extremity acylated by an octanoyl group (Oc). Therefore, these Aib-containing peptides constitute a new class of hydrophobic peptaibols, bearing a lipidic chain, that we propose to name lipopeptaibols.

We here report on the isolation, sequence determination, and conformational data of trichogin A IV (GA IV), the major component of trichogins of the following sequence:

1 2 3 4 5 6 7 8 9 10 11

Oc-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leuol

Its membrane-modifying properties are discussed as compared to those exhibited by nonadecapeptides such as trichorzianines or tricholongins.

Experimental Section

General Data. The CD spectrum was obtained on a Jobin Yvon CD6 dichrograph. Fluorescence spectra were recorded at 20 °C on an Amino SPF 500 spectrofluorometer.

Cultivation of *T. longibrachiatum* and Isolation of Trichogins. *T. longibrachiatum* (M 3431) was obtained from the Collection de souches fongiques du Muséum National d'Histoire Naturelle, Paris. Lyophilized preparation was transferred to agar slants that contained a seeding medium (2% w/v malt-agar). After an incubation of 10 days at 23 °C, the sporulation of the fungus was complete and the originally white mycelium had taken on a green color. Seventy-five Roux flasks (1 L), each containing 170 mL of synthetic medium (glucose, 5 g; potassium dihydrogen phosphate, 0.8 g; potassium nitrate, 0.72 g; calcium phosphate 0.2 g; magnesium sulfate, 0.5 g; manganese sulfate, 0.01 g; zinc sulfate, 0.01 g; copper sulfate, 0.005 g; iron sulfate, 0.001 g for 1 L of distilled water) were sterilized and inoculated with a suspension of spores of *T. longibrachiatum*.

After an incubation of about 15 days at 23 °C, the mycelium was covered with green spores and the cultivation was stopped. The culture broth (13 L) and the mycelium were separated by filtration. The filtrated broth was extracted two times with 1-butanol (3 L) and the mycelium three times with methanol (300 mL). The solvents were removed under reduced pressure and the extracts combined. The residue was submitted to gel chromatography on Sephadex LH 20 with methanol as eluent. A crude mixture (295 mg) of trichogins G and tricholongins LB was obtained. This peptide mixture was then chromatographed on silica gel with first methylene chloride/methanol 70/30 for trichogins (90 mg) and then with methylene chloride/methanol 50/50 for tricholongins (101 mg); TLC R_f value (SiO_2, Merck 60 F_{254}, 6:2:2 butanol/acetic acid/water) of G was 0.60. The spot gave no reaction with ninhydrin and was detected by spraying anisaldehyde reagent (1:1:50 anisaldehyde/sulfuric acid/acetic acid) and heating.

HPLC Separation of Trichogin A IV (GA IV). High-performance liquid chromatography was carried out on a Waters liquid chromatograph (6000 A and M 45 pumps, a 702 solvent programmer, a WISP 701 automatic injector, and a 481 UV-vis detector).

The HPLC separation was accomplished on a semipreparative C_{18} column (Spherisorb ODS 2, 5 μm, 7.5 mm × 300 mm) (SFCC France)

[†] Muséum National d'Histoire Naturelle.
[‡] Université Louis Pasteur.

(1) Brückner, H.; Kussin, C.; Kripp, T. *Peptides: Chemistry and Biology*; Marshall, G. R., Ed.; ESCOM Science Publishers: Leiden, The Netherlands, 1988; pp 650–652.
(2) King, G. F.; Deegan, J.; Smith, R.; Matouschek, A.; Campbell, I. D. *Biochem. Soc. Trans.* **1987**, *16*, 594–595.
(3) Woolley, G.; Deber, C. M. *Biopolymers* **1989**, *28*, 267–275.
(4) Brückner, H.; Przybylski, M. *J. Chromatogr.* **1984**, *296*, 263–275.
(5) Pandey, R. C.; Cook, J. C., Jr.; Rinehart, K. L., Jr. *J. Am. Chem. Soc.* **1977**, *99*, 8469–8483.
(6) Iida, A.; Okuda, M.; Uesato, S.; Takaishi, Y.; Shingu, T.; Morita, M.; Fujita, T. *J. Chem. Soc., Perkin Trans. 1* **1990**, 3249–3255.
(7) Bodo, B.; Rebuffat, S.; El Hajji, M.; Davoust, D. *J. Am. Chem. Soc.* **1985**, *107*, 6011–6017.
(8) Rebuffat, S.; El Hajji, M.; Hennig, P.; Davoust, D.; Bodo, B. *Int. J. Pept. Protein Res.* **1989**, *34*, 200–210.
(9) Pandey, R. C.; Cook, J. C., Jr.; Rinehart, K. L., Jr. *J. Am. Chem. Soc.* **1977**, *99*, 5205–5206.
(10) Rinehart, K. L., Jr.; Gaudosio, L. A.; Moore, M. L.; Pandey, R. C.; Cook, J. C., Jr.; Barber, M.; Sedgwick, R. D.; Bordoli, R. S.; Tyler, A. N.; Green, B. N. *J. Am. Chem. Soc.* **1981**, *103*, 6517–6520.
(11) Pandey, R. C.; Meng, H.; Cook, J. C., Jr.; Rinehart, K. L., Jr. *J. Am. Chem. Soc.* **1977**, *99*, 5203–5205.
(12) Das, K. M.; Raghothama, S.; Balaram, P. *Biochemistry* **1986**, *25*, 7110–7117.
(13) Rebuffat, S.; Prigent, Y.; Auvin-Guette, C.; Bodo, B. *Eur. J. Biochem.* **1991**, *201*, 661–674.

0002-7863/92/1514-2170$03.00/0

using an eluent of methanol/water 84/16 and a flow rate of 2 mL/min; R_t (GA IV) = 26 min. Purity was evaluated to be about 90% by analytical HPLC on a Spherisorb ODS 2 (3.5 mm × 250 mm) column using an eluent of methanol/water 80/20 and a flow rate of 1 mL/min.

Amino Acid Analysis. Total hydrolysis of GA IV was carried out according to the usual procedure for peptides (6 N HCl at 110 °C in sealed tubes for 24 h). Identification of the amino acids was accomplished by gas chromatography after derivatization.[7,8] Retention times of the *N*-trifluoroacetyl isopropyl ester derivatives were compared with those of standards.

The GC analyses of *N*-trifluoroacetyl isopropyl ester derivatives were performed on a CPSIL 5 capillary column (Chrompack; length, 50 m; i.d., 0.25 mm) with He (1.5 bar) as carrier gas and a temperature program of 100–290 °C at 3 °C/min. Retention times (min): Gly (5.3), Aib (5.6), Leuol (6.0), Leu (8.5), Ile (8.8). Leu, Ile, and Leuol enantiomer analyses were performed on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxan) quartz capillary column (Chrompack; length, 25 m; i.d., 0.2 mm) with He (1.1 bar) as carrier gas and a temperature program of 50–190 °C at 3 °C/min. Retention times (min): L-Ile (19.6), L-Leuol (22.2), L-Leu (24.9).

Mass Spectrometry. Positive ion FAB mass spectra were recorded on a VG Analytical MM ZAB-HF mass spectrometer. Peptide methanol solution was mixed with α-monothioglycerol as matrix on the FAB probe tip. Ions were formed by fast atom bombardment with a 8-keV Xe atom beam generated in a saddle field neutral beam gun (Ion Tech. Ltd., Teddington, U.K.). Resolving power was about 2500. *m/z* (relative intensity): 1104 $(M + K)^+$ (82), 1088 $(M + Na)^+$ (100), 1066 MH^+ (7), 949 (8), 836 (5), 779 (11), 694 (7), 581 (12), 524 (8), 467 (27), 382 (26), 269 (80), 212 (50), 127 (85).

NMR Spectroscopy. ^1H NMR spectra were recorded on Bruker spectrometers (AC 300, AM 400, and AM 500X) equipped with Aspect 3000 and Aspect X32 calculators. A 0.4-mL amount of 50 mM GA IV in 5-mm tubes (Wilmad, Buena) was used in CD_3OD or CD_3OH (CEA, Saclay, France). All chemical shifts were referred to the central component of the CD_2H resonances of methanol at 3.313 ppm downfield from TMS.

^{13}C NMR data were collected at 75.47 MHz on an AC 300 Bruker spectrometer, using 5-mm tubes; 50 mM sample solution in ^{12}C-enriched CD_3OD (or in CD_3OH for COLOC or ^1H–^{13}C LR COSY experiments) was used. The reference was the central component of CD_3OH at 49.00 ppm downfield from TMS.

(A) COSY (300.13 MHz, 298 K). A total of 512 experiments of 64 scans each were acquired with a sweep width in F_2 of 2702 Hz; size 2K in F_2 and 1K in F_1; zero filling to 4K in F_2 and to 2K in F_1 was applied.

(B) HOHAHA (500.13 MHz, 295 K). The phase-sensitive spectrum was recorded in CD_3OH. A total of 512 experiments of 32 scans each were performed with τ value of 50 ms; spectral width; 4000 Hz in F_2; size 2K in F_1 and F_2; zero filling to 2K in F_1.

(C) ROESY (400.13 MHz, 293 K). The spectrum was recorded in the phase-sensitive mode. A total of 256 experiments of 96 scans each were performed with a mixing time of 250 ms; spectral width, 4424 Hz in F_2; size 2K in F_2 and 1K in F_1.

(D) COLOC (75.47 MHz, CD_3OH, 298 K). Two hundred fifty-six experiments of 136 scans each were run with spectral width 674 Hz in F_2 (size 1K) and 2648 Hz in F_1 (size 2K); zero filling to 2K in F_2 and 4K in F_1; the delay time τ was set to $^1/_2{}^3J_{XH}$ or $^1/_2{}^2J_{XH}$ with J values ranging from 5 to 10 Hz, i.e., different experiments with τ between 50 and 100 ms.

(E) ^1H–^{13}C COSY (75.47 MHz, $^{12}CD_3OD$, 298 K). A total of 256 experiments of 384 scans each were performed with spectral width 5102 Hz in F_2 (size 4K) and 1186 Hz in F_1 (size 1K); the delay time τ was set to $^1/_2{}^1J_{XH}$, i.e., 4 ms for aliphatic region.

(F) ^1H–^{13}C LR COSY (75.47 MHz, 298 K). Two hundred fifty-six experiments of 128 scans each were performed with spectral width 674 Hz in F_2 (size 1K) and 1324 Hz in F_1 (size 2K); the delay time τ was 70 ms.

Membrane Permeability Measurements. Leakage from vesicles was measured, using the carboxyfluorescein (CF)-entrapped vesicle technique. The CF-encapsulated small unilamellar vesicles (egg phosphatidylcholine (egg PC)/cholesterol 70/30 or 80/20) were prepared by sonication. The phospholipid concentration was kept constant (4×10^{-4} M), and increasing [peptide]/[lipid] molar ratios (R_i^{-1}) were obtained by adding aliquots of methanolic solutions of peptide (methanol concentration kept below 0.5% by volume). After rapid and vigorous stirring, the time course of fluorescence change corresponding to CF efflux was recorded at 520 nm (0.5-nm band pass) with λ_{exc} 488 (1-nm band pass). Percentage of released CF at time t was determined as % CF = $(F_t - F_0)/(F_T - F_0)100$, where F_0 is the fluorescence intensity of the vesicle suspension in the absence of peptide, F_t the fluorescence intensity measured at time t in the presence of peptide, and F_T the total fluorescence determined by disrupting the vesicles by addition of 30 μL of a 10% Triton X-100 solution.

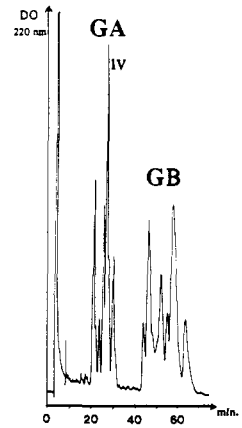

Figure 1. HPLC chromatogram of trichogins. Conditions: mobile phase, CH_3OH/H_2O 84/16; flow rate 2 mL/min; C_{18} reversed-phase Spherisorb ODS2, 5 μm (7.5 × 300 mm); detection UV 220 nm.

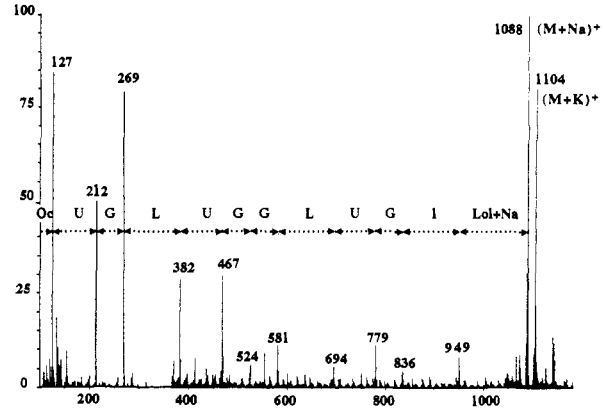

Figure 2. Positive ion FAB mass spectrum of GA IV (U = Aib, Lol = Leuol).

Results and Discussion

Isolation and Amino Acid Composition of Trichogin A IV. When analyzed by TLC, the crude peptide mixture extracted from *T. longibrachiatum* showed two components, corresponding to trichogin and tricholongin groups, that were separated by preparative SiO_2 open-column chromatography. As previously observed for trichorzianines and other peptaibols,[5-8] the C_{18} reversed-phase HPLC analysis of trichogins showed a mixture of closely related peptides. The chromatogram exhibited more than 10 peaks (Figure 1) assembling in two groups, GA and GB. This heterogeneity was confirmed by the positive ion FAB mass spectrum which produced a series of pseudomolecular ions ranging from 1000 to 1500 Da. GA IV, the major GA component, was isolated by semipreparative HPLC and proved to be homogeneous by further FABMS and NMR analysis.

The total acidic hydrolysate of GA IV gave four different amino acids, Aib (3), Gly (4), L-Leu (2), and L-Ile (1), and one amino alcohol, L-Leuol (1) (average rounded values).

Sequence of GA IV by Positive Ion FAB Mass Spectrometry. The positive ion FAB mass spectrum of GA IV was characterized by two pseudomolecular ion species, $(M + Na)^+$ at *m/z* 1088 and $(M + K)^+$ at *m/z* 1104 (Figure 2), from which a molecular weight of 1065 could be deduced leading to the molecular formula $C_{52}H_{95}O_{12}N_{11}$. In addition, extensive series of fragment ions covering the whole molecular weight range were apparent. Acylium fragments at *m/z* 949, 836, 779, 694, 581, 524, 467, 382, 269, 212, and 127 Da arising from amide bond cleavages allowed assignment of the GA IV sequence as X-Aib-Gly-Leu (Ile)-Aib-Gly-Gly-Leu (Ile)-Aib-Gly-Leu (Ile)-Leuol. In this sequence, the respective location of the amino acids Leu and Ile was not

Table I. ^{1}H NMR Specific Assignments (ppm) and Coupling Constants for Resonances of Nonexchangeable Protons of GA IV (CD_3OD, 298 K, 300.15 MHz)[a]

residue		H_α δ, mult, J (Hz)	other groups δ, mult, J (Hz)
Oc	CH_3 (C_8')		0.892 t (7.5)
	CH_2 (C_7' to C_4')		1.32 m
	CH_2 (C_3')		1.60 m
	CH_2 (C_2')		2.256 t (7.6)
Gly 2		3.707 d (17.2)	
		3.876 d (17.2)	
Leu 3		4.303 dd (4.6, 10.4)	β_1 1.64 m; β_2 1.83 m; γ 1.64 m; Me δ_1 0.904 d (6.4); Me δ_2 0.969 d (6.1)
Gly 5		3.806 d (17.3)	
		3.834 d (17.3)	
Gly 6		3.899 d (16.6)	
		3.944 d (16.6)	
Leu 7		4.262 dd (3.6, 9.5)	β_1 1.69 m; β_2 1.76 m; γ 1.69 m; Me δ_1 0.899 d (6.4); Me δ_2 0.949 d (6.0)
Gly 9		3.780 d (16.7)	
		3.848 d (16.7)	
Ile 10		4.213 d (7.5)	β 2.02 m; γ_1 1.29 m; γ_2 1.57 m; Me γ' 0.959 d (6.7); Me δ 0.892 t (7.6)
Leuol 11		4.04 m	β_1 1.34 m; β_2 1.45 m; β'_1 3.474 dd (3.7, 15.1); β'_2 3.514 dd (4.1, 15.1); γ 1.72 m; Me δ_1 0.884 d (6.3); Me δ_2 0.889 d (6.3)

[a] $\Delta\delta$ with three decimal places when obtained from 1D spectra; $\Delta\delta$ with two decimal places when obtained from 2D spectra.

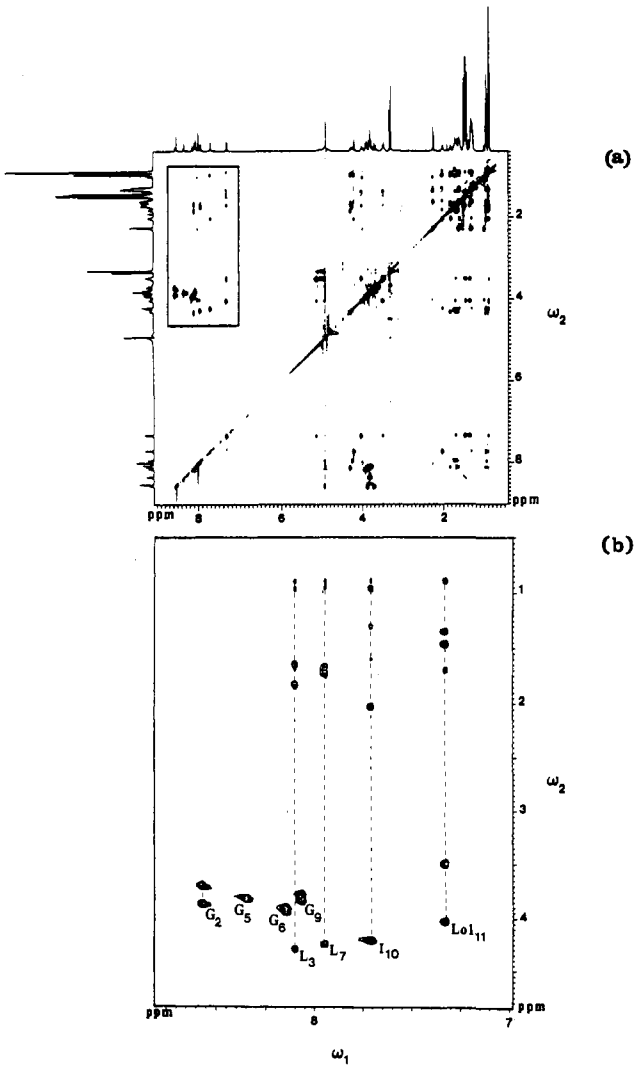

Figure 3. HOHAHA spectrum of GA IV (500.13 MHz, 295 K, CD_3OH). (a) Total spectrum; (b) region of the α proton and aliphatic proton resonances. The spin system of each residue is connected by a dashed line.

assigned and the N-terminal fragment X, at m/z 127, was not identified.

Sequence Determination of GA IV from NMR. At first, sequential assignments were obtained from (^{1}H–^{1}H) 2D experiments (COSY, HOHAHA, ROESY). Then, 2D heteronuclear ^{1}H–^{13}C

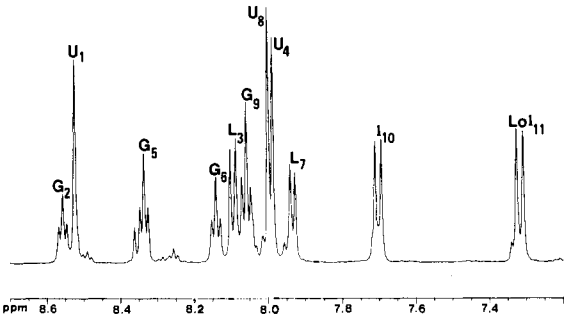

Figure 4. Amide proton region of the 1D spectrum of GA IV (500.13 MHz, 295 K, CD_3OH).

Table II. Chemical Shift Values at 295 K (δ), Vicinal Coupling Constants (^3J, Hz), and Temperature Dependence ($-\Delta\delta/\Delta T$, ppb/K) of Amide Protons and Chemical Shift Values at 298 K (δ) and Temperature Dependence ($-\Delta\delta/\Delta T$, ppb/K) of CO Groups for GA IV

	NH				CO	
residue	δ	mult	$^3J_{NH-C_\alpha H}$	$-\Delta\delta/\Delta T$	δ	$-\Delta\delta/\Delta T$
Oc					176.56	0.2
Aib 1	8.518	s		7.1	178.49	1.5
Gly 2	8.556	dd	4.8/6.0	6.0	173.15	0.8
Leu 3	8.092	d	7.3	2.0	175.83	2.7
Aib 4	8.002	s		4.9	178.31	−0.8
Gly 5	8.332	dd	4.3/5.0	5.7	173.44	1.8
Gly 6	8.144	dd	5.5/6.3	2.4	173.02	0.8
Leu 7	7.933	d	6.4	4.7	175.27	0.9
Aib 8	7.983	s		7.4	178.08	1.6
Gly 9	8.056	dd	5.3/6.5	3.8	172.51	3.0
Ile 10	7.707	d	8.3	2.3	173.62	2.3
Leuol 11	7.317	d	9.0	4.8		

COLOC and ^{1}H–^{13}C LR COSY spectra enabled sequential carbon assignments to be made.

(A) ^{1}H Assignments. The 500-MHz ^{1}H NMR spectrum of GA IV in CD_3OH showed resonances from nearly all of the protons to be well resolved, except for that of Leu, Leuol, and Ile which overlapped. Nevertheless, the 500-MHz HOHAHA experiment (Figure 3) allowed the total assignment of proton resonances (Table I).

In the amide proton region (Figure 4), each Gly amide was readily recognized by its triplet nature due to its coupling to the two resonances between 3.6 and 4.0 ppm. As expected, all Aib amide protons appeared as singlets from the lack of an α-proton. The remaining amide protons showed up as doublets (Table II).

The ROESY spectra of GA IV (Figure 5) exhibited all of the connectivities between the amide protons of contiguous residues

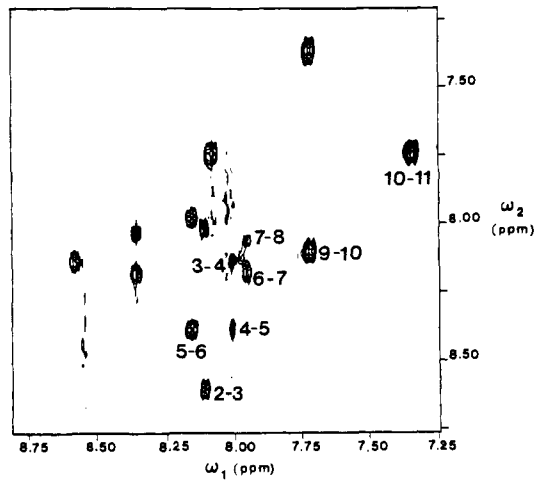

Figure 5. Amide proton region of the ROESY spectrum of GA IV (400.13 MHz, 293 K, CD_3OH).

along the entire chain length, with only two interruptions due to the proximity of the NH protons of Aib 1 and Gly 2 as well as Aib 8 and Gly 9. This result allowed the assignment of isomeric and repetitive residues in the sequence (Table II; Figure 4).

(B) ^{13}C Resonance Assignments. The ^{13}C NMR spectrum of GA IV was analyzed by combining *J*-modulated and two-dimensional heteronuclear ^1H–^{13}C COSY spectra to assign resonances in the spectrum to specific carbon atoms in the molecule (Table III). This allowed the characterization of the N-terminal extremity X to assign the carbonyl groups and to obtain T_1 data for aliphatic resonances.

The carbonyl region was assigned by means of 2D heteronuclear ^1H–^{13}C LR COSY and COLOC experiments, performed with different delays to optimize the connectivities for *J* values ranging between 7 and 10 Hz. Ten of the 11 carbonyl groups were thus assigned to amino acids (Table I). The remaining one at 176.56 ppm was connected to a methylene group at 37.0 ppm. These two carbons, together with the six unassigned resonances in the *J*-modulated spectrum (one methyl group at 14.29 ppm, five methylene groups at 23.57, 26.20, 30.10, 30.30, and 32.81 ppm), allowed the definition of a linear octanoyl chain (Oc) exhibiting a molecular mass of 127 Da.

Examination of the ^1H–^{13}C LR COSY and COLOC experiments providing nearly all of the connectivities between the residues from Oc to Gly 6 and from Leu 7 to Leuol 11 confirmed the sequential assignments.

Conformational Studies. The CD spectrum, interresidue NOE connectivities, ^{13}C NMR relaxation times of $C_\alpha H$, and temperature dependence of NH and CO groups allowed a self-consistent conformational analysis of the GA IV backbone in solution.

The CD spectrum of GA IV in methanolic solution, showing the n–π^* transition at 223 nm (−), accompanied by two π–π^* excimers at 205 (−) and 192 nm (+), was characteristic of a peptide mainly organized in a right-handed helix.[20]

Spin–relaxation measurements of each $C_\alpha H$ were measured to evaluate the motion variations along the peptide backbone. The NT_1 values for the backbone methine carbon atoms lay in the range 0.25–0.37 s (Table III). The greater values observed for the α-carbons of glycines 2, 5, and 9 may be a consequence of the lack of a sterically bulky side chain in this residue allowing faster internal motion,[17] involving a greater conformational flexibility for glycine residues. Nevertheless, the presence of only small variations in the atomic motions of the optically active amino acids along the backbone of GA IV indicated a uniformity of motion throughout the peptide structure.

Table III. ^{13}C Chemical Shifts and Relaxation Data for GA IV ($^{12}CD_3OD$, 298 K)[a]

residue	carbon	δ	T_1, s	NT_1, s
Oc	C_8' CH_3	14.29	1.91	5.73
	C_7' CH_2	23.57	1.52	3.04
	C_6' CH_2	32.81	1.44	2.88
	C_5' CH_2	30.10	1.21	2.42
	C_4' CH_2	30.30	0.88	1.76
	C_3' CH_2	26.20		
	C_2' CH_2	37.00	0.38	0.76
Gly 2	α CH_2	44.76	0.28	0.56
Leu 3	α CH	54.63	0.25	0.25
	β CH_2	40.80	0.22	0.44
	γ CH	26.35*		
	δ_1 CH_3	22.18	0.73	2.19
	δ_2 CH_3	23.43	0.57	1.71
Gly 5	α CH_2	45.08	0.29	0.58
Gly 6	α CH_2	44.36		
Leu 7	α CH	54.85	0.31	0.31
	β CH_2	41.03	0.22	0.44
	γ CH	25.79*		
	δ_1 CH_3	21.99	0.56	1.68
	δ_2 CH_3	23.28	0.49	1.47
Gly 9	α CH_2	44.76	0.28	0.56
Ile 10	α CH	60.52	0.37	0.37
	β CH	37.61	0.33	0.33
	γ CH_2	26.60		
	γ' CH_3	16.04	0.57	1.71
	δ CH_3	11.60	0.9	2.70
Leuol 11	α CH	50.98	0.26	0.26
	β' CH_2	66.00	0.20	0.40
	β CH_2	40.80	0.27	0.54
	γ CH	25.79*		
	δ_1 CH_3	21.78	0.65	1.95
	δ_2 CH_3	23.90	0.65	1.95
Aib 1, 4, 8	α C	57.46	1.64	
		57.95	1.66	
		57.95	1.66	
	β_1 CH_3	24.62	0.33–0.37	
		24.72	0.33–0.37	
		24.92	0.33–0.37	
	β_2 CH_3	26.01	0.26–0.34	
		26.12	0.26–0.34	
		26.35	0.26–0.34	

[a] An asterisk indicates that assignments may be reversed within the same column. *N* is the number of neighboring protons for each carbon nucleus.

The NOE effects detected by ROESY experiments exhibited a stretch of strong sequential $d_{NN(i,i+1)}$ connectivities and one $d_{\alpha N(i,i+3)}$ between $C_\alpha H$ Leu 3 and NH Gly 6 that agreed with a generally helical structure. The lack of many $d_{\alpha N(i,i+4)}$, $d_{\alpha N(i,i+3)}$, or $d_{\alpha N(i,i+2)}$ connectivities also suggested flexibility, as expected for a short peptide.[18]

Involvement of NH groups in intramolecular hydrogen bonds can be examined with respect to two criteria: (i) temperature dependence of NH chemical shifts; (ii) hydrogen–deuterium (H–D) exchange study. The absence of concentration dependence over the range 0.5–50 mM indicated that intermolecular interactions were negligible; therefore, the temperature coefficients were representative of the relative involvement of amide protons in intramolecular hydrogen bonds.

The temperature dependence of chemical shifts was linear between 263 and 308 K for all of the NH groups of GA IV in CD_3OH, indicating no conformational modification in the temperature range (Table II). Three backbone amide resonances, Leu 3, Gly 6, and Ile 10, exhibited low ($-\Delta\delta/\Delta T$) values (<3.5 ppb/K) characteristic of bound NH groups. Four resonances, Aib 1, Gly 2, Gly 5, and Aib 8, had high ($-\Delta\delta/\Delta T$) values (>5.5

(14) El Hajji, M.; Rebuffat, S.; Le Doan, T.; Klein, G.; Satre, M.; Bodo, B. *Biochim. Biophys. Acta* **1989**, *978*, 97–104.
(15) Motta, A.; Picone, D.; Tancredi, T.; Temussi, P. A. *Tetrahedron* **1988**, *44*, 975–990.
(16) Chandrasekhar, K.; Das, M. K.; Kumar, A.; Balaram, P. *Int. J. Pept. Protein Res.* **1985**, *32*, 167–174.
(17) Deslauriers, R.; Grzonka, K.; Schaumburg, K.; Shiba, T.; Walter, R. *J. Am. Chem. Soc.* **1975**, *97*, 5093–5100.
(18) Price, W. S.; Mendz, G. L.; Martenson, R. E. *Biochemistry* **1988**, *27*, 8990–8999.
(19) Wagner, G.; Wüthrich, K. *Nature (London)* **1978**, *275*, 247–248.
(20) Jung, G.; Dubischar, N.; Leibfritz, D. *Eur. J. Biochem.* **1975**, *54*, 395–409.

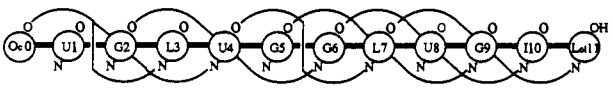

Figure 6. Proposed intramolecular hydrogen-bonding scheme in GA IV, consistent with NMR data.

ppb/K) indicative of their exposure to solvent. The remaining NH groups Aib 4, Leu 7, Gly 9, and Leuol 11 had moderate ratio values and appeared to be partially solvent-exposed or involved in labile hydrogen bonds.

The study of hydrogen–deuterium exchange of the amide protons at 278 K revealed all of the resonances except that of Leuol to be exchanged out in less than 7 min. This result denoted a low stability of the GA IV helix, in accordance with previous studies indicating a correlation between the rate of exchange of the backbone amide protons and the thermal stability.[19]

To obtain information about the involvement of carbonyl groups in intramolecular hydrogen bonds, the temperature dependence of CO chemical shifts in ^{13}C NMR experiments was studied. Their chemical shift variations were linear between 263 and 308 K. The measured values of $(-\Delta\delta/\Delta T)$ (Table II) showed those coefficients to be separated into three classes: the class with the highest values (>2 ppb/K) showing clearly solvent-exposed CO groups (Leu 3, Gly 9, and Ile 10), the class with the lowest values (<1 ppb/K) characterizing the bound CO groups (Oc, Gly 2, Aib 4, Gly 6, and Leu 7, and the class of the intermediate values (Aib 1, Gly 5, and Aib 8). The solvent-exposed character can be modulated by the more or less hydrophobic environment in the helix.

Conformational Representation. The CD and NMR results agreed with a general helical conformation for trichogin A IV in solution. The proposed conformational structure (Figure 6), which contains mixed hydrogen bonds as previously observed in Aib-containing peptides in solution[12] and in the solid state,[21,22] represents a mainly α-helical structure. The high values of Leu 3 CO temperature coefficient and Aib 8 NH temperature coefficient suggest a 3_{10} bond between Aib 4 and Leu 7. This hydrogen-bonding pattern for GA IV exhibits a highly apolar face containing the total hydrophobic residues Leu, Ile, Leuol, and the lipidic chain and a less hydrophobic face including the four Gly residues. This amphiphilic character of the helix would account for the membrane properties of the lipopeptide.[23]

Membrane-Modifying Properties of GA IV. As shown in previous studies, alamethicins,[24,25] trichorzianines,[14,26] and tricholongins[13] bind to phospholipid bilayers and modify membrane permeability. The peptaibol activity was favored by the presence of a neutral helix corresponding to 19 or 20 residues. Comparison between the acidic trichorzianine TB IIIc and the neutral trichorzianine TA IIIc (or tricholongin LB II) showed the permeability induced by TB IIIC to be weaker than that induced by TA IIIc (or LB II). In addition, TB IIIc activity strongly depended upon the cholesterol amount in the bilayer.[14] Identical studies on NA VII showed that a 12-residue peptide was devoid of membrane activity (Figure 7).

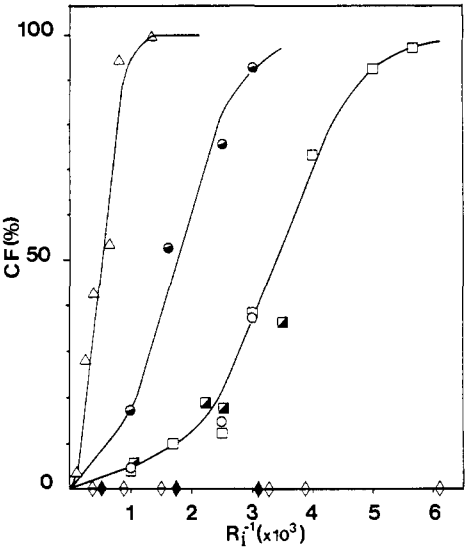

Figure 7. Peptide-induced CF leakage at 20 min for different ratios R_i^{-1} = [peptide]/[lipid] from egg PC/cholesterol (70/30) vesicles [(□) GA IV, (○) TB IIIc, (△) TA IIIc, LB II, (◇) NA VII] and from egg PC/cholesterol (80/20) [(◪) GA IV, (◒) TB IIIc, (◆) NA VII]. TA IIIc: Ac-Aib-Ala-Ala-Aib-Aib-Gln-Aib-Aib-Aib-Ser-Leu-Aib-Pro-Val-Aib-Ile-Gln-Gln-Trpol. TB IIIc: Ac-Aib-Ala-Ala-Aib-Aib-Gln-Aib-Aib-Aib-Ser-Leu-Aib-Pro-Val-Aib-Ile-Gln-Glu-Trpol. LB II: Ac-Aib-Gly-Phe-Aib-Aib-Gln-Aib-Aib-Aib-Ser-Leu-Aib-Pro-Val-Aib-Iva-Gln-Gln-Leuol. NA VII: Ac-Aib-Ala-Ala-Aib-Iva-Gln-Aib-Aib-Aib-Ser-Leu-AibOCH$_3$.

To compare the membrane-modifying properties of GA IV and those of 19-residue peptaibols, we studied the induced carboxyfluorescein (CF) leakage from small unilamellar vesicles composed of egg phosphatidylcholine (egg PC) containing 20 and 30% cholesterol, for different ratios R_l^{-1} = [peptide]/[lipid], as described previously.[14] For 30% cholesterol containing vesicles, GA IV and TB IIIc induced similar CF leakage. When the cholesterol amount in the bilayer was decreased to 20%, GA IV activity was not affected, contrary to TB IIIc which exhibited a greater leakage. Along with the neutral 19-residue peptaibols TA IIIc or LB II, GA IV did not exhibit a close dependent activity upon cholesterol amount.

In spite of its short 11-residue chain length, the first natural lipopeptaibol GA IV exhibits significant membrane-modifying properties.

Acknowledgment. We are indebted to Dr. M. F. Roquebert (Laboratoire de Cryptogamie du Muséum National d'Histoire Naturelle), who provided the *T. longibrachiatum* strain, and Dr. D. Brault (Laboratoire de Biophysique du Muséum National d'Histoire Naturelle) and Dr. H. Lam Thanh (CEA, Saclay) for access to the spectrofluorometer and the dichrograph, respectively. We thank Dr. D. Fraisse (Centre de Spectrométrie de Masse, CNRS, Lyon, France) for FAB mass spectra, and Dr. P. Richardin (Laboratoire de Conservation des Documents Graphiques du Muséum National d'Histoire Naturelle) for GC chromatograms. This work was supported by a grant from the Ministère de la Recherche et de l'Enseignement Supérieur (88 T 0101).

Supplementary Material Available: Various spectra of GA IV (25 pages). Ordering information is given on any current masthead page.

(21) Bosch, R.; Jung, G.; Schmitt, H.; Winter, W. *Biopolymers* **1985**, *24*, 979–999.
(22) Karle, I. L.; Sukumar, M.; Balaram, P. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 9284–9288.
(23) Takahashi, S. *Biochemistry* **1990**, *29*, 6257–6264.
(24) Knoll, W. *Biochim. Biophys. Acta* **1986**, *863*, 329–331.
(25) Schwarz, G.; Stankowski, S.; Rizzo, V. *Biochim. Biophys. Acta* **1986**, *861*, 141–151.
(26) Le Doan, T.; El Hajji, M.; Rebuffat, S.; Rajesvari, M. R.; Bodo, B. *Biochim. Biophys. Acta* **1986**, *858*, 1–5.