MEMBRANE ACTIVE PEPTIDES FROM *TRICHODERMA* SPECIES

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Résumé: de diverses espèces de champignons microscopiques du genre Trichoderma, *des peptides amphiphiles de 11 à 20 résidus ont été isolés et leurs séquences déterminées par une étude spectrale (SM, RMN). Ils forment en solution des hélices principalement de type α. Leur action membranaire a été examinée en utilisant des vésicules de phospholipides comme modèle.*

Abstract: *from various* Trichoderma *species, 11- to 20-residue amphiphilic peptides have been isolated and their sequences elucidated from FAB MS and NMR data. They are structured in helices, mainly of the α-type. Their membrane activity has been examined by using SUV as model membranes.*

Introduction

Trichoderma are saprophytic and ubiquitous soil fungi with cellulolytic activity and *T. viride* the type species of the genus, is ranked as one of the most widely distributed of all soil fungi. The genus is characterized by fast growing colonies with conidia more usually green. Rifai (1) distinguished nine species aggregated, based on microscopic characters, but the macroscopic features are very similar for most of them. Among the species we have studied were *T. harzianum*, a very common species described in Africa, America and Europe, *T. koningii*, also common in Europe, Central-America, India, Tahiti, New-Zealand and Japan and *T. longibrachiatum*, quite rare, found in France but also in New-Zealand (2).

Some of the *Trichoderma* show a remarkable antagonistic activity against other microorganisms, bacteria or fungi. This is due, in part, to the production of antibiotic-antifungal compounds. Among them, there is a special class of linear hydrophobic peptides, termed as peptaibols. They are characterized by a C-terminal amino alcohol, an acetylated N-terminal residue and a high content in α-amino isobutyric acid (Aib) which is an α-dialkylated amino acid. The first known peptaibols, alamethicins and suzukacillins which contain twenty residues were isolated from *T. viride* strains. From a strain collected in France of the close species *T. harzianum*, we isolated two groups of nonadecapeptaibols we named trichorzianines A and B (3-5). These peptides show membrane activity, as they modify the membrane permeability and disrupt the bilayer structure at 10^{-6}M (6). At lower concentration alamethicin and trichorzianines form voltage-dependent ionic channels with multiple conductance states. Time life of single channels of trichorzianines differs from that of alamethicin (7).

Recent results related to the isolation, structure elucidation and membrane activity of new peptaibols we obtained from original strains of different *Trichoderma* species are presented below.

Fungal culture and peptide isolation

Single spore cultures were obtained from the "Collection de souches fongiques" du Muséum national d'Histoire naturelle in Paris. Fermentations were performed in Roux flasks on

a synthetic medium. The stationary cultures were incubated at 27°C for about 15 days. The filtered culture broths were extracted several times with *n*-BuOH, and the extracts fractionated by Sephadex LH 20 chromatography with MeOH as eluent. The peptide fraction thus obtained was separated into two peptide groups by silica gel column chromatography. When analyzed by reversed phase HPLC each peptide group appeared as a complex mixture of homologues, as previously observed for trichorzianines; the main components were purified by semi-preparative HPLC (Fig. 1).

Figure 1: HPLC chromatogram of harzianins A, B and C. (Spherisorb ODS2 [5µ]; 2ml/min; detect. UV 220nm; mobile phase MeOH/H$_2$O: 88/12 for HA and 83/17 for HB and HC).
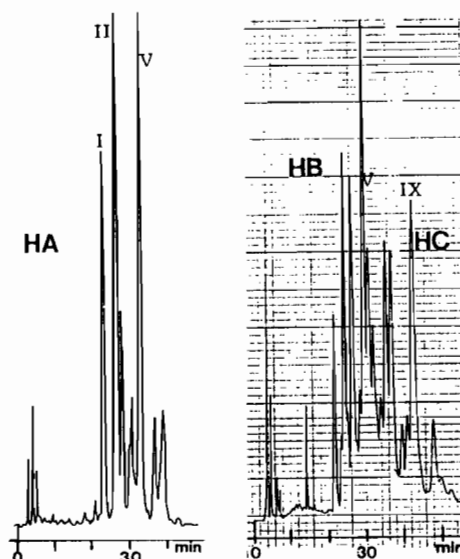
Thus, from a *T. harzianum* strain (MNHN 90-3601) collected in Uruguay, three peptide groups were obtained, harzianins HA with 18 residues, and harzianins HB and HC shorter, having 11 or 14 residues. This strain chemically differed from that collected in France, which produced the 19-residue trichorzianines. From a *T. koningii* strain (MNHN 90-3589) harvested in Central America, two peptide groups were obtained, trikoningins KA with 19 residues, similar to trichorzianins A and the second group, trikoningins KB, having 11 residues and an N-terminal residue acylated by *n*-octanoic acid instead of acetic acid. *T. longibrachiatum* (MNHN 3431) yielded three peptide groups, tricholongins LB with 19 residues, also similar to trichorzianines A, trichogins GA, analogues of trikoningins KB and trichogins GB, longer homologues of GA. At last, *T. saturnisporum* (MNHN 90-3578) also from Central America, produced two kinds of peptaibols: long ones with 20 residues, saturnisporins SA which are longer homologues of trichorzianines and short undecapeptaibols with an acetylated N-terminus, saturnisporins SB.

In sum, the results show the ability of *Trichoderma* species to produce a great variety of peptaibol structures, which can be ranked in two classes: - the long ones with 18 to 20 residues, having an acetylated N-terminal residue, a Pro at 13 (14) position and - the short ones with eleven residues and either an acetylated N-terminal residue and several Pro or a N-terminal residue acylated by *n*-octanoic acid and several Gly (Fig. 2).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
SA II	Ac	Aib	Ala	Aib	Ala	Aib	Ala	Gln	Aib	Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	Iva	Gln	Gln	Pheol
SA IV	Ac	Aib	Ala	Aib	Ala	Aib	Aib	Gln	Aib	Leu	Aib	Gly	Aib	Aib	Pro	Val	Aib	Iva	Gln	Gln	Pheol
TA IIIc	Ac	Aib	Ala	Ala	Aib	Aib	Gln	Aib	Aib	Aib	Ser	Leu	Aib	Pro	Val	Aib	Ile	Gln	Gln	Trpol	
TA VII	Ac	Aib	Ala	Ala	Aib	Iva	Gln	Aib	Aib	Aib	Ser	Leu	Aib	Pro	Val	Aib	Ile	Gln	Gln	Pheol	
TB IIIc	Ac	Aib	Ala	Ala	Aib	Aib	Gln	Aib	Aib	Aib	Ser	Leu	Aib	Pro	Val	Aib	Ile	Gln	Glu	Trpol	
LB I	Ac	Aib	Gly	Phe	Aib	Aib	Gln	Aib	Aib	Aib	Ser	Leu	Aib	Pro	Val	Aib	Aib	Gln	Gln	Leuol	
LB II	Ac	Aib	Gly	Phe	Aib	Aib	Gln	Aib	Aib	Aib	Ser	Leu	Aib	Pro	Val	Aib	Iva	Gln	Gln	Leuol	
KA V	Ac	Aib	Gly	Ala	Aib	Leu	Gln	Aib	Aib	Aib	Ser	Leu	Aib	Pro	Val	Aib	Ile	Gln	Gln	Leuol	
HA V	Ac	Aib	Gly	Ala	Aib	Iva	Gln	Aib	Val	Aib	Gly	Leu	Aib	Pro	Leu	Aib	Iva	Gln	Leuol		

Figure 2 : Selected sequences of long peptaibols

		1	2	3	4	5	6	7	8	9	10	11
HB III	Ac	Aib	Asn	Leu	Leu	Aib	Pro	Aib	Leu	Aib	Pro	Leuol
SB I2	Ac	Aib	Gln	Ala	Leu	Aib	Pro	Leu	Leu	Aib	Pro	Leuol
GA IV	Oc	Aib	Gly	Leu	Aib	Gly	Gly	Leu	Aib	Gly	Ile	Leuol
KB I	Oc	Aib	Gly	Val	Aib	Gly	Gly	Val	Aib	Gly	Ile	Leuol
KB II	Oc	Iva	Gly	Val	Aib	Gly	Gly	Val	Aib	Gly	Ile	Leuol

Figure 2 (continue): Selected sequences of short peptaibols.

(S: saturnisporin, T: trichorzianine, L: tricholongin, H: harzianin, G trichogin, K: trikoningin; Ac = acetyl, Oc = octanoyl).

Sequence determination

The amino acid composition and chirality were determined from analyses of the complete acid hydrolysates by gaz chromatography on Chirasil-Val capillary column, after derivatization as their N-trifluoroacetyl isopropyl esters. The chiral amino acids were L, except isovaline which was D. The peptide sequences were then determined by combining positive ion FAB Mass Spectroscopy and NMR data. In (+) FAB MS a series of acylium ions was exhibited. The mass difference between two consecutive acylium ions allowed the identification of an amino acid. However, this method do not allow to distinguish between isomeric residues Val/Iva or Leu/Ile.

As an exemple the (+) FAB mass spectrum of the short peptaibol KBI shows the pseudomolecular $[M+Na]^+$ ion at m/z 1060 and a series of acylium ions allowing total sequence determination (Fig. 3). The ion at m/z 127, arose from the n-octanoyl group with was characterized from further ^{13}C NMR studies.
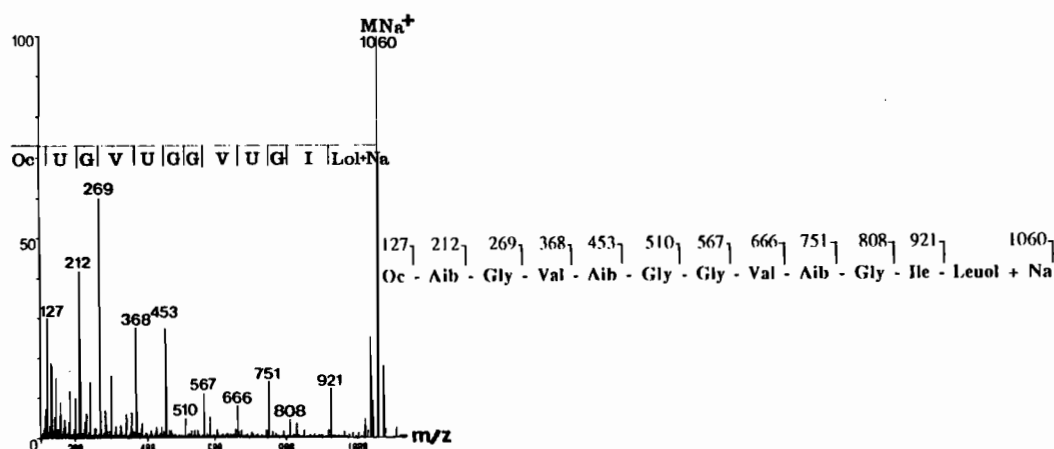

Figure 3 : Positive ion FAB mass spectrum of trikoningin KB I.

The mass spectra of the long peptaibols having a proline, as SA II, appeared more complicated because a preferential cleavage occured at the Aib-Pro amide bond (Fig. 4). This resulted in the formation of two N- and C- terminal complementary oligopeptides, which further underwent sequential fragmentations which superimposed. The spectrum presents the pseudomolecular ion $[MH]^+$ at m/z 1937 along with Na and K adducts and abundant fragments at m/z 1149 and 788 corresponding to the N- and C- terminal oligopeptides. Nevertheless, the amino acid sequence can be defined but with an undetermination concerning the respective location of isomeric residues such as Val and Iva.
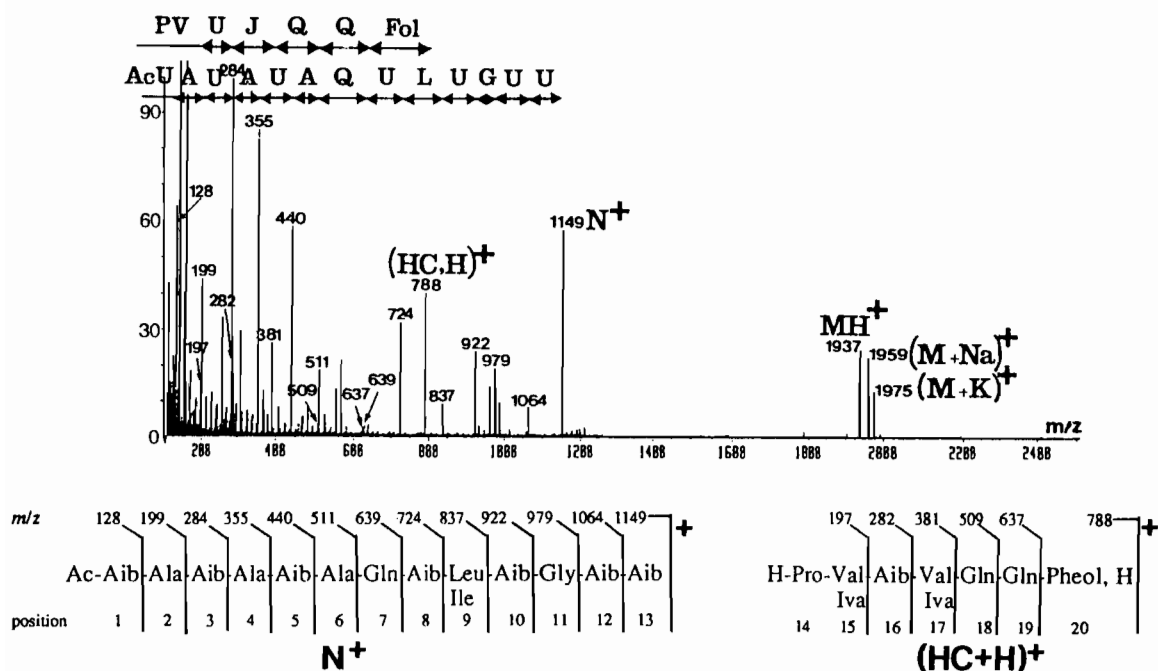
Figure 4: Positive ion FAB mass spectrum of saturnisporin SA II.

To solve such a problem and mainly to get information on the tridimentional structure in solution, NMR studies were undertaken. The sequence determination of a peptide can result either from ^{1}H or ^{13}C NMR data: in the first case information arise from the observation of NOEs between consecutive NH groups or between the αH of a residue and the NH of the following one. Using ^{13}C NMR, information arise from the detection of long range heteronuclear couplings between the carbonyl group of a residue and the NH of the following one. In all cases, the first step is the total assignment of the ^{1}H NMR spectrum which was accomplished by 2D homonuclear experiments including ^{1}H-^{1}H COSY and HOHAHA, to show up through-bond J-coupling connectivities belonging to the same spin systems.

Location of the isomeric residues of SA IV was done by [1]H-^{1}H NOE measurements, which allowed, in addition, assignment of repetitive residues. In a first step, proton assignment was obtained from the HOHAHA spectrum, where all the connectivities of a single amino acid appear on the same line (Fig. 5). Then the ROESY experiment permitted the sequential assignments from observation of NH_i-NH_{i+1} or NH_i-$αH_{i-1}$ connectivities, allowing specific assignment of valine at -15: NH of Val was correlated with both NH-16 and αH-14 (Fig. 6).

The respective location of Val at position -8 and Iva residues at -5 and -16 in the sequence of HA V was determined by the ^{13}C NMR method: a long-range coupling was observed between the NH of Aib-9 and the carbonyl group of the single Val which was thus located at position -8 (Fig. 7) .

Conformational studies

Conformation in solution in an organic solvent (CD_3OH) of some of these peptaibols was examined by circular dichroism and NMR. All the CD curves agreed with a main right handed helical conformation, but more detailed information occured from NMR data.

Two helix types are expected for Aib-containing peptides: the α-helix and the 3_{10} helix which are stabilized by a hydrogen bond between the carbonyl oxygen atom of residue i and the proton NH group of residue i+4 and of residue i+3 respectively. They can be distinguished by
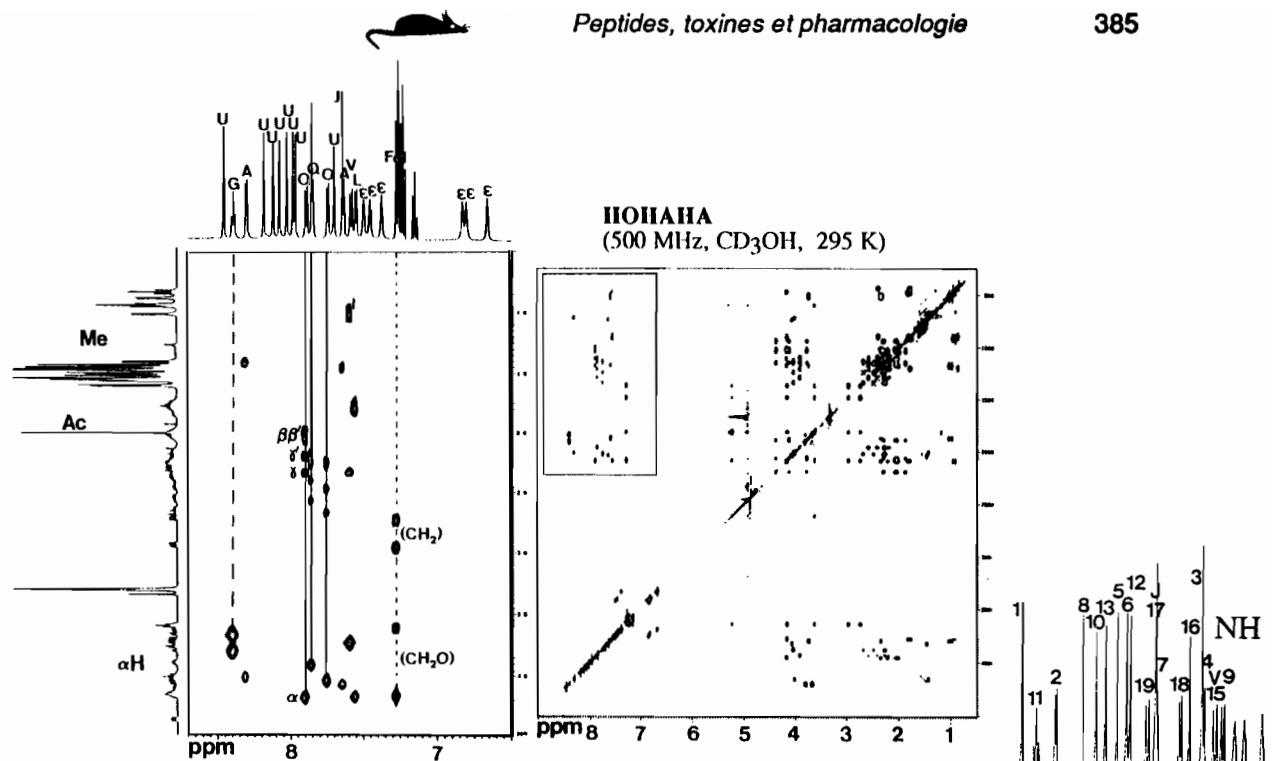
HOHAHA
(500 MHz, CD₃OH, 295 K)

**Figure 5 : 500 MHz HOHAHA spectrum
of saturnisporin SA IV (CD₃OH)**

**Figure 6: 500 MHz ROESY spectrum
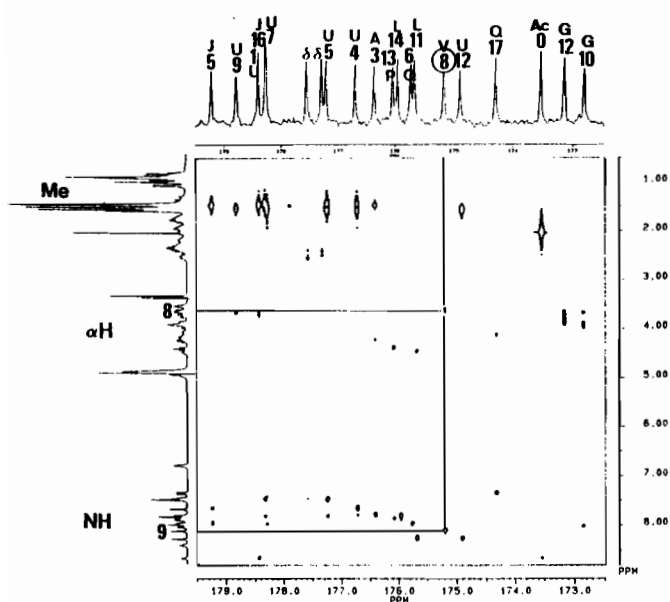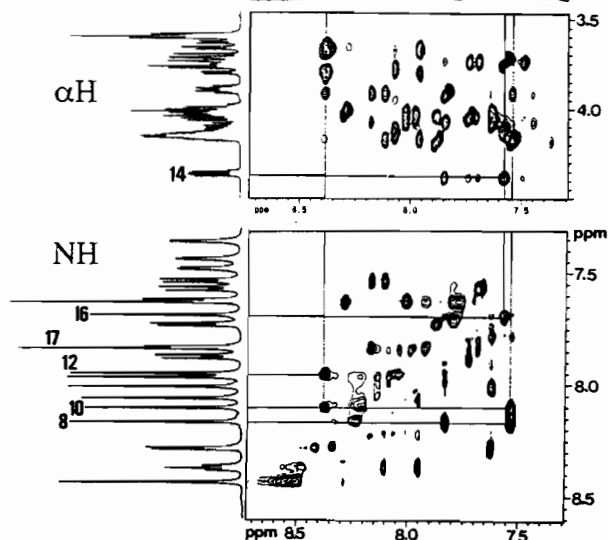of saturnisporin SA IV (CD₃OH)**

Figure 7: ¹H-¹³C long range COSY spectrum of harzianin HA V: CO regio (¹H, 300 MHz; CD₃OH)

the number of free hydrogen amide protons at the beginning of the helix which is 3 in the first case and 2 in the second one, and also by the number of free carbonyl groups at the C-terminus.

Involvement of a NH group in an intramolecular hydrogen bond can be determined by measuring its chemical exchange rate or its temperature coefficient which represents the variation of its chemical shift with the temperature. NOE data were of major significative importance. Informative NOEs were depicted in the TA VII NOESY spectrum, especially dN_i-N_{i+1}, $d\alpha_i$-N_{i+1} and $d\alpha_i$-N_{i+3} which characterize helices and $d\alpha_i$-N_{i+2}, $d\alpha_i$-N_{i+4}, and $d\alpha_i$-β_{i+3} which allow to distinguish between α and 3_{10} helices (Fig. 8).
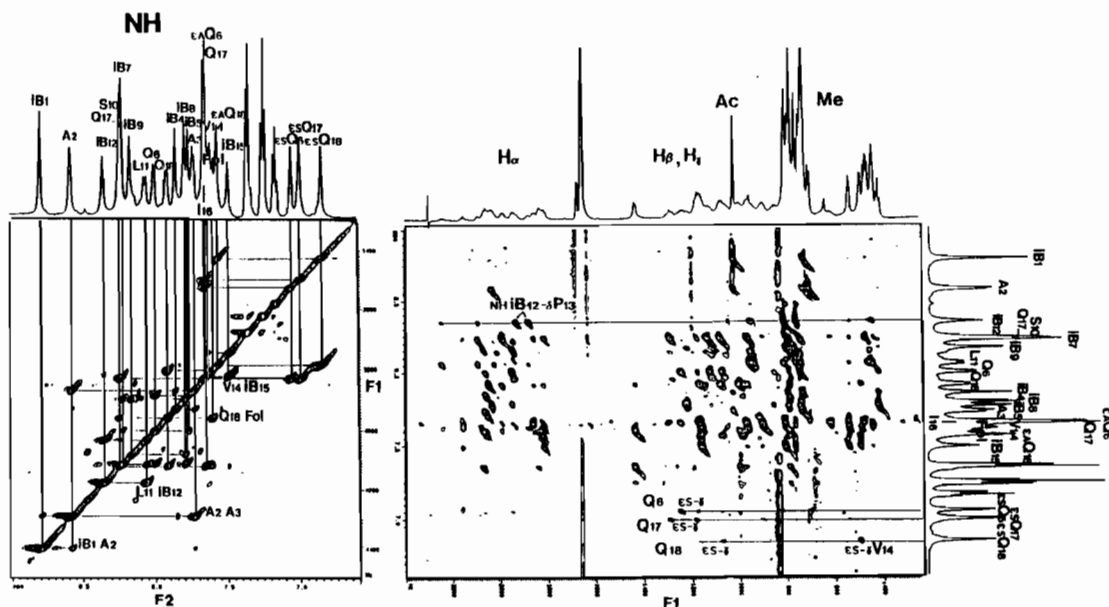

Figure 8: 500 MHz NOESY spectrum of trichorzianine TA VII (CD₃OH)

Analysis of the NMR parameters, coupling constant values, NOE data and temperature coefficients of NH and CO groups, agreed with such a helical structure, as depicted in the following scheme with the hydrogen bonding pattern indicated. Thus the peptide folds in a mainly α-helix, but the proline disrupts the hydrogen bonding, releasing two CO groups 3 and 4 residues before Pro (Ser-10 and Aib-9) and creates a hinge (Fig. 9).
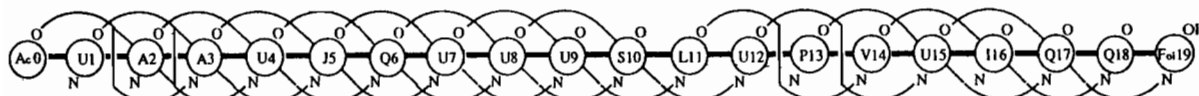

Figure 9: Representation of the secondary structure of trichorzianine TA VII, showing the proposed hydrogen-bonding pattern

Both parts of the α–helix start with 3_{10} or mixed α-3_{10} turns. A more precise representation will be obtained from the molecular dynamics calculation which is now in progress. Such a conformation is very close to that defined for the crystal structure of TA IIIc which was previously determined (8). The angle between the two helix axes is about 22°. All these results show the remarkable stability of the helical structure. We studied the conformation of TAVII in SDS micelles and we obtained comparable NMR spectra, suggesting that the conformation in lipid micelles do not greatly differ from that depicted for the methanol solution.

Membrane activity

We previously described the membrane modifications induced by trichorzianines by measuring the escape of an encapsulated probe (carboxyfluorescein, CF) from egg phosphatidyl choline/cholesterol (70/30) small unilamellar vesicles (6). CF was entrapped at such a concentration that fluorescence self-quenching of the probe occured. By adding increasing amounts of peptides to such a suspension of vesicles, one can observe an increase in the fluorescence intensity upon CF escape and dilution. R_i^{-1} represents the molar ratio [Pept]/[Lip].

Figure 10 shows the percent of CF escape at 20 min for increasing R_i^{-1} which is a useful representation for the comparison of peptide activity. The longer neutral peptaibols (18-20 residues) develop such an activity that 100% of CF escapes for R_i^{-1} = 1 to 2×10^{-3}. NA VII, the (1-12) N-terminal fragment of TAVII, is devoided of activity whereas, undecapeptides as GA IV or KBI which have a short octanoyl lipid chain show intermediate activity, which is comparable to that of acidic trichorzianines TB.
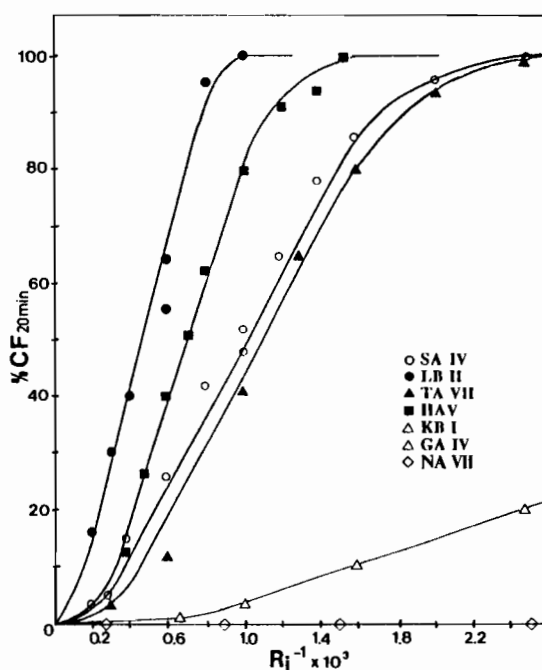

Figure 10: Peptide-induced CF leakage at t = 20 min for different ratios R_i^{-1} = [peptide]/[lipid] from eggPC/cholesterol (70/30) vesicles

It is not determined at the present time if the escape of SUV entrapped material is due to breaks in the bilayer, lipid destabilisation or vesicle fusion. On the other hand, Spach and coll. have shown that all the long peptaibols form voltage-dependent channels, similar to those of alamethicin and trichorzianins and that the shorter ones do not.

By using the variety of the natural peptaibols which we have isolated, we will try to go deeper in the structure/ biological activity/ membrane activity relationship, to investigate the orientation and the state of aggregation of the peptides in the lipid bilayers and the type of modification they induce in the lipids.
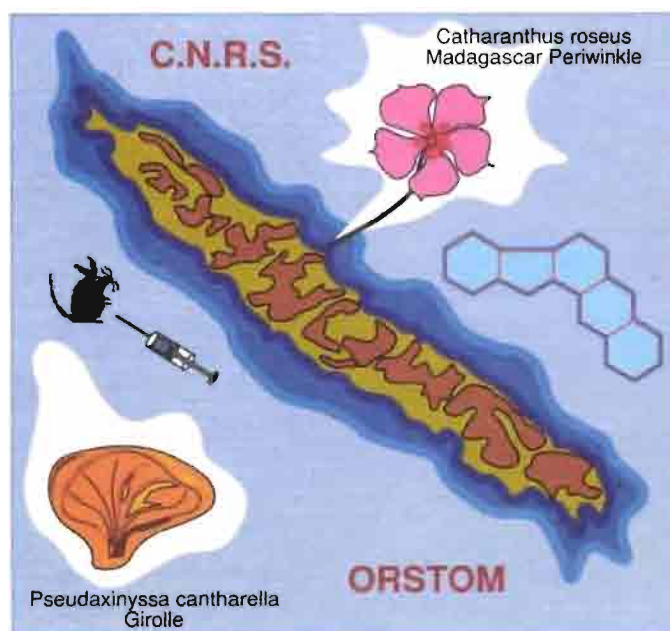
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