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HIGH FIELD NMR STUDY OF ANTIBIOTIC PEPTIDES : ^{1}H ASSIGNMENT OF TRICHORZIANINE A1 SPECTRA BY 2D EXPERIMENTS

D.Davoust*, B.Bodo**, S.Rebuffat** and N.Platzer*

*Laboratoire de Chimie Organique et Structurale, ERA 557, Université P.et M. Curie, 4 place Jussieu, 75230-PARIS CEDEX 05

**Laboratoire de Chimie du Muséum National d'Histoire Naturelle, 63 rue Buffon, 75231-PARIS CEDEX 05 FRANCE

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Abstract - *A new antibiotic peptide, trichorzianine A1, was isolated from a culture of* Trichoderma harzianum. *It contains 19 residues, the* N *terminus is blocked by an acetyl group and the* C *terminus is tryptophanol. As a first part of the structural study of this new peptide, we here present the analysis of the* ^{1}*H NMR spectrum accomplished by 2 DJ resolved and spin echo correlated spectroscopy.*

Trichorzianine A1 (T A1) is the main component of a mixture of antibiotic peptides produced by the fungus *Trichoderma harzianum*. Vigorous acidic hydrolysis allowed detection of 18 amino-acids : Ala (2), Leu (1), Ile (1), Val (1), Ser (1), Pro (1), Glx (3) and Aib (8) (α-amino-isobutyric acid). Furthermore, the UV spectrum showed the presence of a tryptophanyl residue (Trpx).

Since the ninhydrine test was negative and no methylation occurred by treatment with diazomethane, T A1 is likely to have neither *N* nor *C* termini free and must be related to alamethicin (1-3) and suzukacillin (4,5) antibiotic peptides from *Trichoderma viride*. These linear peptides (peptaibophols) (6) have been shown to present remarkable pore forming activity in lipid bilayer membranes (7).

Structure determination of such peptides with no free NH_2 terminal residue is not possible using conventional methods and involves mass spectrometric studies of either intact peptide or oligopeptides obtained from partial hydrolysis (8). This useful technique is of limited value when applied to high weight peptides and does not allow to distinguish between isobaric amino-acids (Ile/Leu). The mass spectrum of intact T A1 gave its molecular mass (1948 u.m.a.) but few sequential informations. Therefore, we have undertaken a study of T A1 by NMR spectroscopy.

The sequential assignment and the study of conformational behaviour of a polypeptide backbone rely on the study of the Nuclear Overhauser Effects between amide proton and H_α proton of neighbouring residues (9,10). Previous-

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ly, resonance lines in the ^1H NMR spectrum, especially those of the amide and H_α protons must be assigned to specific residues. As a first step, this paper describes the use of 2 DJ resolved (11) and 2 D spin echo spectroscopy (SECSY) (12) for the identification of spin systems of non labile protons in the NMR spectra of trichorzianine A1. Assignment of amide protons was performed by conventional double resonance techniques.

METHODS AND MATERIALS : From a culture of *Trichoderma harzianum* Rifaï (ATCC 20672) a mixture of at least 20 related peptides, the trichorzianines, was obtained. Separation of the major component, trichorzianine A1 (T A1) was carried out using intensive low pressure and high performance liquid chromatography (13). T A1 was crystallized from CH_3CN/H_2O : mp. 253-254°C ; $[\alpha]_D^{22} = -25°$ (c = 0.5, EtOH). UV spectrum was recorded on a Beckmann Acta III spectrometer : λ_{max}^{EtOH} nm (ε) = 284 (5400). Molecular weight of T A1 was assigned as 1948 on the basis of its $(M-H)^-$ peak in the negative ion F.A.B. mass spectrum. It was obtained with a Kratos MS 50 spectrometer employing 6 eV xenon atoms directed onto a matrix prepared from the sample dissolved in methanol and mixed with glycerol. Mass measurement was accomplished by using CsI to calibrate the mass marker.

400.13 MHz ^1H NMR spectra were recorded on a Bruker WM 400 spectrometer connected with an Aspect 2000 data system. TA1 was dissolved in CD_3OD, the chemical shifts are reported with respect to internal TMS. For amide group assignment, T A1 was dissolved in CD_3OH : homodecoupling experiments were performed with gated irradiation of the intense OH resonance of the solvent.

The two dimensional NMR measurements were carried out using the Bruker software package. In 2 DJ resolved experiments, the size of the time domain was 8192 x 128 with digital resolution of 0.244 Hz. SECSY experiments were performed using 512 and 2048 data points, with F_1=1000 and F_2 = 2000 Hz. 2 DJ resolved and SECSY spectra were recorded using quadrature detection.

RESULTS AND DISCUSSION

The 400.13 MHz FT 1D spectrum of T A1 shows extensive overlapping resonances. For complete analysis of the proton system 2D spectroscopy is required.

a) 2 DJ resolved spectra of trichorzianine A1

Between 3.5 and 4.5 ppm, the 2 DJ resolved projection spectrum of T A1 contains seventeen well resolved signals which were labelled 1 to 17 from low field to high field. This is the normal chemical shifts range for the expected eleven H_α amino-acid protons, the two β Ser protons and the two δ Pro protons. The occurrence of two supplementary signals suggests that, as peptaibophols, T A1 contains a primary *C* terminal amino-alcohol. The signal multiplicity shown by the cross sections is depicted in Table 1 and, for some of them, in figure 1. Peaks 6 and 7 are quadruplets thus assigned to the H_α Ala protons. Peaks 13 and 16 are each doublet, in agreement with the presence of Val and Ile. All the others signals appear as doublet of doublets, except 3, 12 and 14 which are multiplets.

TABLE I

Signal	δ ppm	Multiplicity *	Assignment	Spin system correlation from SECSY δ ppm	NH groups δ ppm **	$^3J_{NH-CH}$ (Hz)
1	4.49	dd	H_α Leu	H_γ=1.84 $H_{\beta\beta'}$=1.88, 1.88 CH_{3_δ} = 0.88, 0.93	7.93	8.2
2	4.33	dd	H_α Pro	$H_{\beta\beta'}$=2.35, 1.79	-	-
3	4.26	m	H_α Trpol	$H_{\beta\beta'}$=3.07, 2.98 CHO = 3.71 CH'O = 3.67	7.50	8.5
4	4.23	dd	H_α Gln	H_β = 2.14	7.76	7.0
5	4.20	dd	H_α Ser	$H_{\beta\beta'}$=4.06, 3.95	8.06	6.4
6	4.14	q	H_α Ala	CH_{3_β} = 1.41	7.69	6.3
7	4.09	q	H_α Ala	CH_{3_β} = 1.47	8.32	4.0
8	4.06	dd	H_β Ser	H_α = 4.20 $H_{\beta'}$ = 3.95	-	-
9	4.05	dd	H_α Gln	$H_{\beta\beta'}$=2.23, 1.89	8.00	4.3
10	3.95	dd	$H_{\beta'}$ Ser	H_α = 4.20 H_β = 4.06	-	-
11	3.92	dd	H_α Gln	$H_{\beta\beta'}$=2.34, 1.99	7.87	4.8
12	3.87	m	H_δ Pro	$H_{\delta'}$ = 3.75	-	-
13	3.77	d	H_α Ile	H_β=1.98 CH_{3_γ} = 0.96 $H_{\gamma\gamma'}$=1.72, 1.26 CH_{3_δ}=0.89	7.39	4.5
14	3.75	m	$H_{\delta'}$ Pro	H_δ = 3.87	-	-
15	3.71	dd	CHO Trpol	CH'O Trpol = 3.67 H_α = 4.26	-	-
16	3.69	d	H_α Val	H_β = 2.35 CH_{3_γ} = 1.07, 0.97	7.60	8.5
17	3.67	dd	CH'O Trpol	CHO Trpol = 3.71 H_α = 4.26	-	-

* (from CD_3OD solution)

** (from CD_3CH solution)

Assignments for the protons resonances between 3.5 and 4.5 ppm for T A1 in CD_3OD. Spin system correlation from SECSY, amide correlated chemical shifts and $^3J_{NH-CH}$ coupling constants are reported.

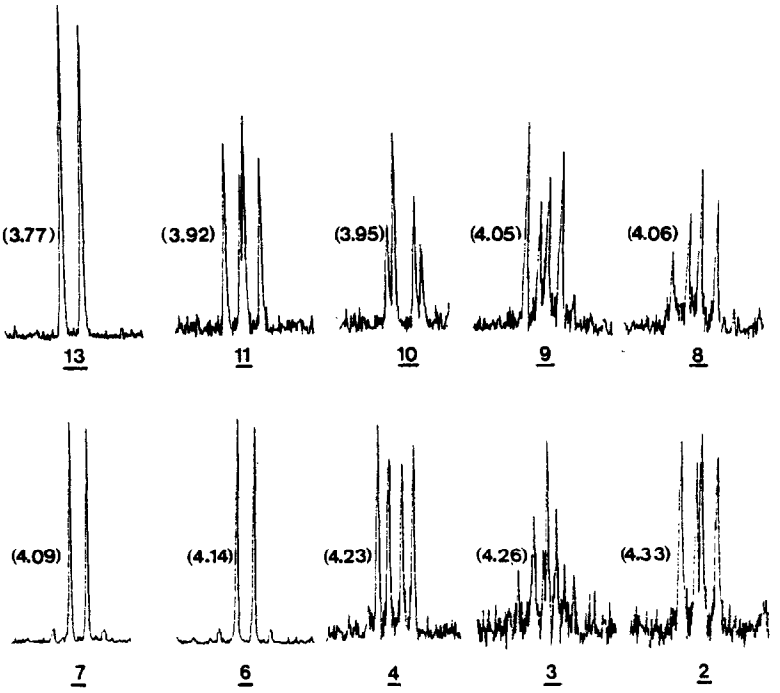

Fig.1 - Cross section representation of selected multiplets in the 400.13 MHz 2 DJ resolved spectrum of T A1. For each cross section the chemical shift of the individual resonance measured on the projection spectrum is given in brackets.

Six signals occur in the region 0.8-1.0 ppm of the 2 DJ resolved projection spectrum. Cross sections display a triplet assigned to the δ CH_3 of Ile and five doublets assigned to the methyl groups of Ile (β CH_3), Leu and Val. Two cross sections of the 1.2-1.6 ppm range exhibit doublet multiplicity corresponding to the two CH_3 Ala. The remaining cross sections are singlets arising from the methyl groups of Aib.

b) 2 D spin echo correlated spectroscopy (SECSY)

The contour plot drawn for the 3.0-4.5 ppm range (Fig.2) shows an AMX system involving signals 5, 8 and 10, which are likely to correspond to H_α and H_β Ser on the basis of their chemical shifts (Table 1) and their multiplicities (see cross sections, Fig.1).

Furthermore, a five spin system is detected including proton 3. This proton is coupled on one hand with the two protons at 3.04 ppm (unambiguously assigned to the CH_2 β Trpx) and on the other hand with 15 and 17.

These results are consistent with the presence of a tryptophanol residue (Trpx = Trpol) as the *C* terminal amino-alcohol. 3 is assigned to the H_α, 15 and 17 to the methylene protons of the primary alcohol group. In 2 DJ resolved spectrum, cross section for 3 is of course a centered symmetrical multiplet (Fig.1).

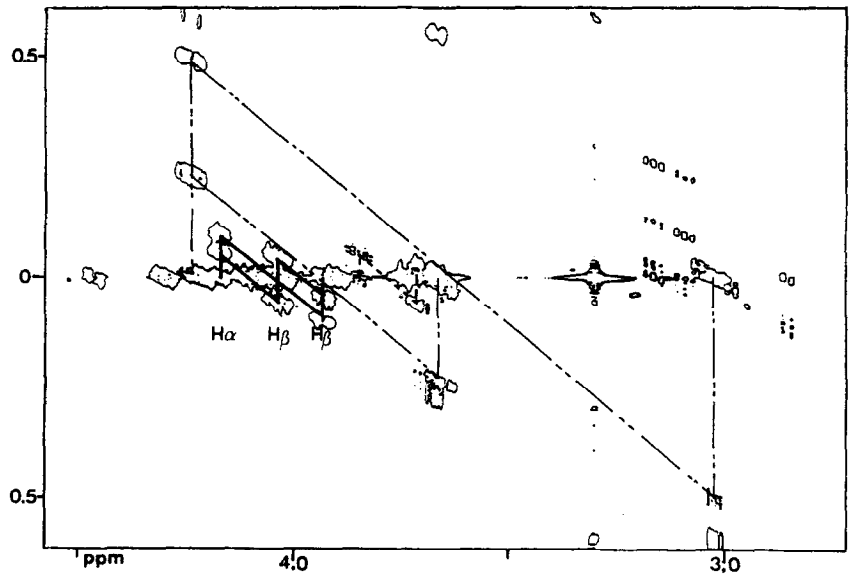

Fig.2 - SECSY spectrum of a 0.02 M solution of T A1 in CD_3OD. Contour plot of the region 3.0-4.5 ppm. (——Trpol), (——Ser), (-------Pro).

Finally, gem coupling between the two δ protons of Pro gives rise to the correlated signals 12 and 14.

Contour plot including the high field region is presented in figure 3. As examples, two coupled spin systems are pointed out for Ile and Val.

As previously outlined by Nagayama and Wüthrich, it is difficult to identify complete spin systems for Pro and Leu owing to the complexicity of the correlation pattern in strongly coupled spin systems (14). The H_α Pro was distinguished from all other H_α signals by comparing the 1 D NMR spectra of T A1 using CD_3OH and CD_3OD as solvents. Apart from the signal of H_β Ser and H_δ Pro, only one signal 2 exhibits the same multiplicity in these two spectra, i.e. is not affected by any exchange phenomenon. This signal at 4.33 ppm is assigned to H_α Pro, devoid of any $^3J_{NH-CH}$ coupling.

The separation between the outer peaks of the quartet in the cross section of 2, 15 Hz, substantiates a *trans* X-Pro peptide bond. A lower value, circa 8 Hz, is characteristic of *cis* X-Pro peptide bond (15,16).

Complete analysis of 2 D SECSY spectra finally lends to assignment of all the 17 signals in the range 3.0-4.5 ppm (Fig.4), of several H_β signals and of all methyl group signals. The results are listed in table 1.

Some data were checked by 1 D homonuclear decoupling experiments.

c) Amide assignments

Assignments of the NH protons were obtained from homonuclear decoupling experiments at each H_α frequency previously determined by 2 DJ resolved experiments. These assignments were checked by homonuclear decoupling throughout

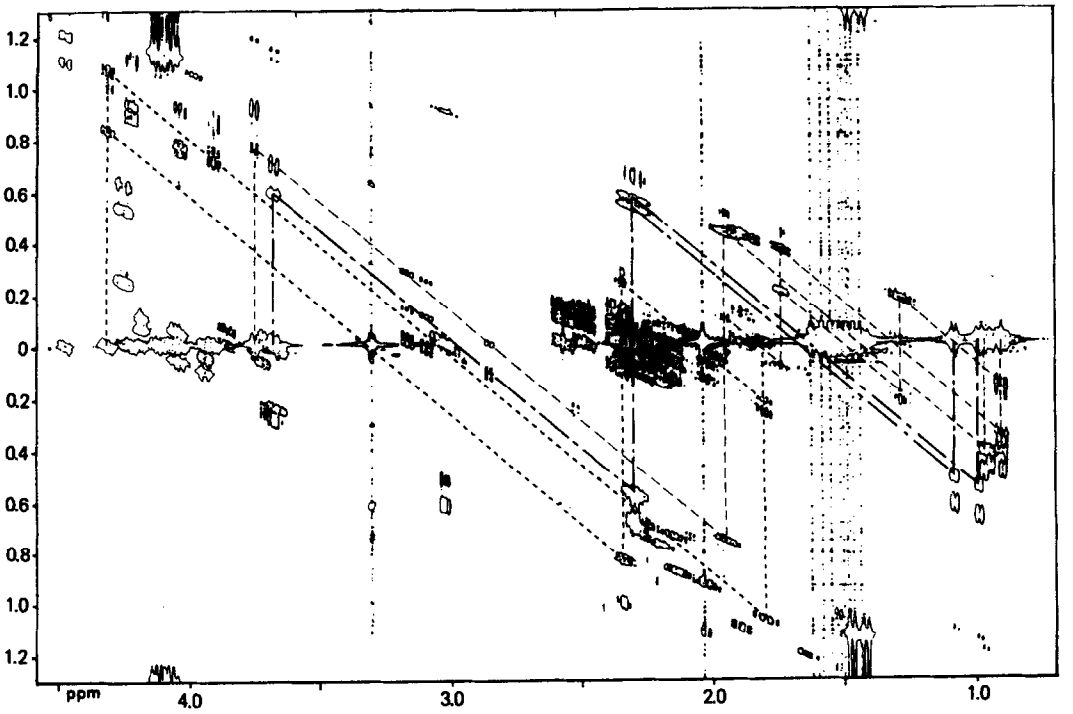

Fig.3 - SECSY spectrum of a 0.02 M solution of T A1 in CD_3OH. Contour plot of the region 0.5-4.5 ppm. (— — —Ile), (——·——Val), (------- Pro).

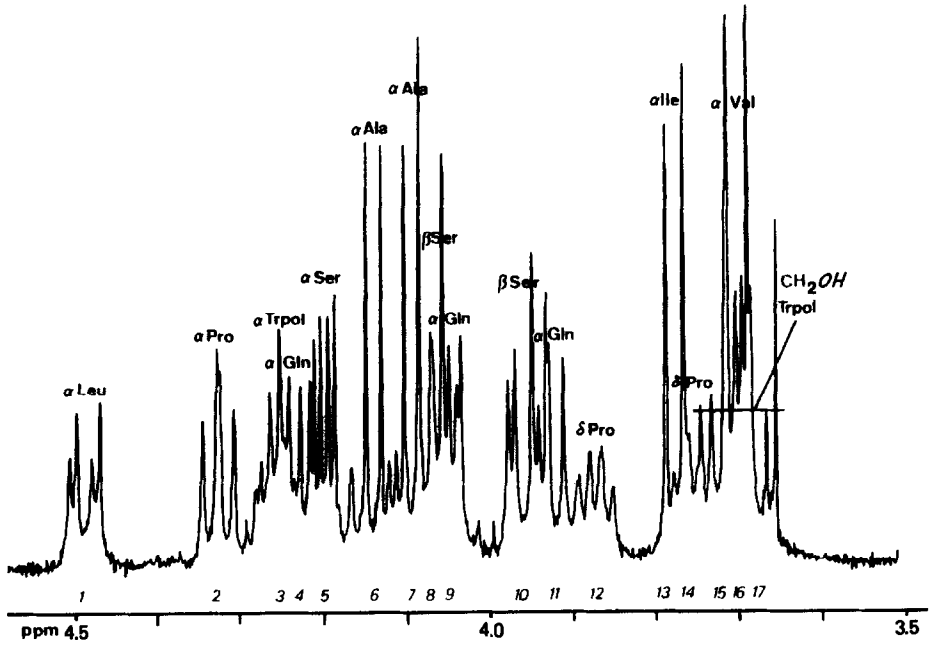

Fig.4 - 3.4-4.6 ppm region of the 400.13 MHz 1 D spectrum of a 0.02 M solution of T A1 in CD_3OD. Assignments of all protons resonances are indicated.

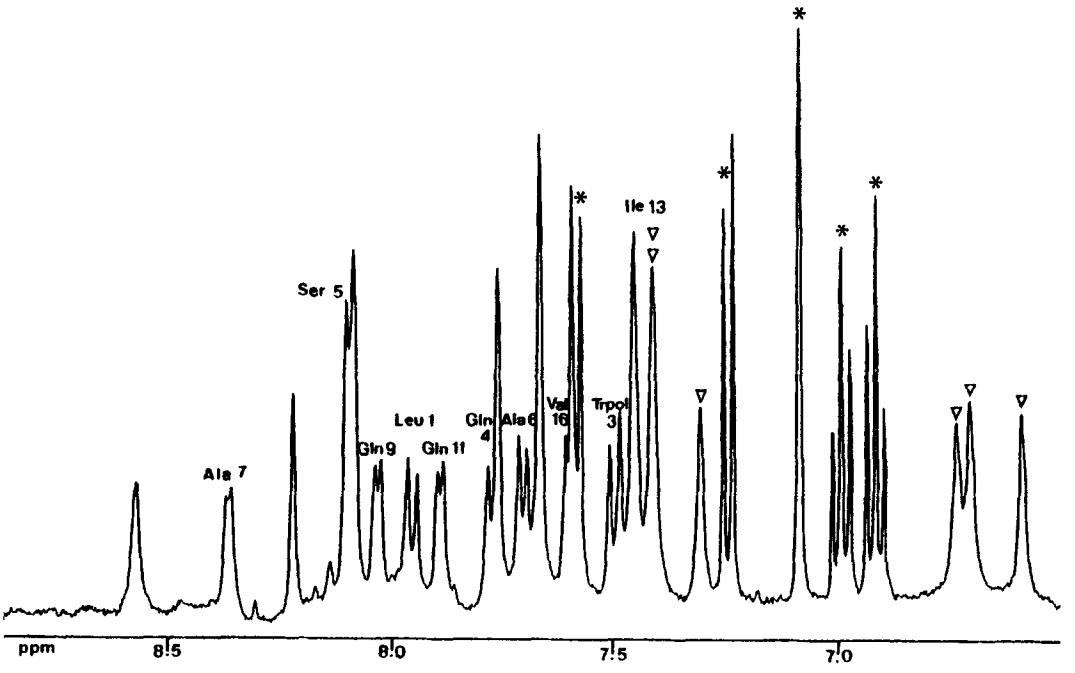

Fig.5 - Low field region of the 1 D spectrum of T A1 in CD_3OH solution (0.05 M). Resolution enhancement (L_B = -1.5, G_B = 0.3). All coupled amide signals have been assigned, numbers refer to the H_α correlated signal (see table 1). Unlabelled signals are due to uncoupled Aib amide protons (∇) and to Gln carboxamide protons. Several peaks (*) arise from tryptophanol.

the amide region and observation of the H_α region. The three one proton signals (Fig.5) occurring at higher field are characteristic of three carboxamide groups (17). Each of them is related to another non equivalent proton signal at lower field (unresolved weak coupling), thus Glx is Gln.

Inspection of $^3J_{NH-CH}$ values (Table 1) yields first insight into the peptide conformation : the great range of values for the $^3J_{NH-CH}$ coupling constants suggests the occurrence of area with β conformation ($^3J_{NH-CH}$ = 7 to 11 Hz) and of area with α helix conformation ($^3J_{NH-CH} \leq 5$ Hz) (9). For a random coil conformation, mean values of 6-7 Hz are expected.

Two experimental results lend further support to the above hypothesis for the conformation of T A1 in CD_3OH solution : - intensities of the amide signals are not significantly altered when the solvent OH signal is strongly irradiated ; - several amide protons exchange very slowly in CD_3OD solution. Lack of saturation transfert and slow exchange rate suggest an important contribution of folded structure resulting in sequestration of NH protons (18).

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