NMR CONFORMATIONAL STUDY OF ITURIN A, AN ANTIBIOTIC FROM *BACILLUS SUBTILIS*

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1. Introduction

The investigations of polypeptide conformations arise not only from the challenge provided by the numerous possible conformations of these molecules but also because they generally possess biological activity as hormones, antibiotics and ionophores. ^1H high field NMR which has the intrinsic potential of revealing something of the spatial arrangement of the neighbour protons in the molecules and ^{13}C NMR which permits investigations of the carbon motions are techniques available in conformational determinations [1]. Thus a great number of peptides, antibiotics and proteins have been studied by NMR with a view toward the eventual elucidation of structure–biological activity relationships [1–7].

In cyclic polypeptides the conformational possibilities are greatly restricted [3,8,9]. In some cases only one of the possible conformations dominates and it is possible to determine which this is from ^1H NMR data in the usual way:

(i) Correlation between coupling constants and peptide backbone conformation [10].
(ii) Temperature dependence of the amide protons.
(iii) Exchange rate for the amide protons.
(iv) ^{13}C T_1 measurements [1].

In this paper we report ^1H and ^{13}C NMR conformational studies of iturin A, a cyclic peptidolipidic antibiotic from *Bacillus subtilis* with the peptide sequence below [11]:

The lipidic moiety is a mixture of 3-amino 12-methyl tridecanoic acid (βNC_{14}) and 3-amino 12-methyl tetradecanoic acid (βNC_{15}) [12].

2. Materials and methods

^1H NMR spectra were recorded on a Varian HR 300 spectrometer operating at 300 MHz in the CW mode and a Brüker WH 270 operating at 270 MHz in the FT mode. The two spectrometers are equipped with decoupler unit and variable temperature accessories. The sample was 40 mg/ml in DMSO d_6. Assignments were made by selective irradiation. In order to increase the resolution of the spectra, convolution difference technique was used at 270 MHz.

^{13}C NMR spectra were recorded on Varian XL 100 spectrometer operating at 25.2 MHz in the FT mode. The sample was 100 mg/ml in DMSO d_6. Assignments were made:

(i) By comparison with the spectrum of the β-amino acid C_{14}–C_{15} fragment recorded in the same conditions.
(ii) Through off-resonance experiment.
(iii) Based on the data in [1].

Spin-lattice relaxation time (T_1) measurements of

L-Ser–βNC_{14},C_{15}–L-Asn–D-Tyr–D-Asn–L-Gln–L-Pro–D-Asn [11]

iturin A were achieved through the 180°–τ–90° sequence. The T_1 values were obtained by regression analysis ($0.95<r<0.99$).

Iturin A was extracted from *Bacillus substilis* and purified by chromatography as in [12].

3. Results

3.1. ^1H NMR

Figure 1 represents the formula of iturin A and its ^{1}H NMR spectrum recorded at 270 MHz in DMSO d_6 solution. Because similar residues occur (e.g., 3 residues Asn) several strong overlaps of the signals arise both in the region of H_α and H_β protons. Consequently the signal at 4.5 ppm may represent three H_α protons (certainly from two Asn and of the lipidic moiety). Only the signals corresponding to the L-Pro, L-Ser and L-Gln were attributed unequivocally from decoupling experiments. The hydroxyl group of L-Ser (4.9 ppm) was attributed unambiguously by exchange with D_2O. Successive irradiations permit assignments of CH_2 (3.7 ppm), CH_α (4.2 ppm) and NH (7.3 ppm) of the L-Ser residue. In the same way, L-Gln and L-Pro protons were assigned. The presence of three Asn residues prevents precise individual assignments of the signals to their own amino acids. Fortunately, among all the amide resonances the NH of L-Gln and L-Ser which are unambiguously attributed are those which exhibit the smallest temperature dependencies ($\Delta\delta_{NH}/\Delta T$). It is important to observe that the slopes ($\Delta\delta_{NH}/\Delta T$) of the different amide protons can be divided in three groups:

(i) L-Gln ($\Delta\delta_{NH}/\Delta T = 4.5 \times 10^{-4}$ ppm × degree^{-1}) and L-Ser ($\Delta\delta_{NH}/\Delta T = 5.4 \times 10^{-4}$ ppm × degree^{-1}). Such small temperature dependencies are consistent with hydrogen bonds or highly buried positions for these NH protons.

(ii) Three NH protons at 7.1 ppm ($\Delta\delta/\Delta T = 1.5 \times 10^{-3}$ ppm × degree^{-1}); 7.7 ppm ($\Delta\delta/\Delta T = 1.7 \times 10^{-3}$ ppm × degree^{-1}) and 8.1 ppm ($\Delta\delta/\Delta T = 2.4 \times 10^{-3}$ ppm × degree^{-1}), respectively, which are probably located in buried solvent regions.

(iii) Two solvent exposed NH resonating both at 8.7 ppm exhibiting strong slopes ($\Delta\delta/\Delta T = 6 \times 10^{-3}$ and 5.2×10^{-3} ppm × degree^{-1}) (fig.2).

Although there still remains a number of unassigned signals, inspection of molecular models (Dreiding and CPK) and literature compilation permit us to propose a model for the secondary structure of iturin A (fig.3). This conformation would be characterized by two β-turns. The first one (turn A) with a

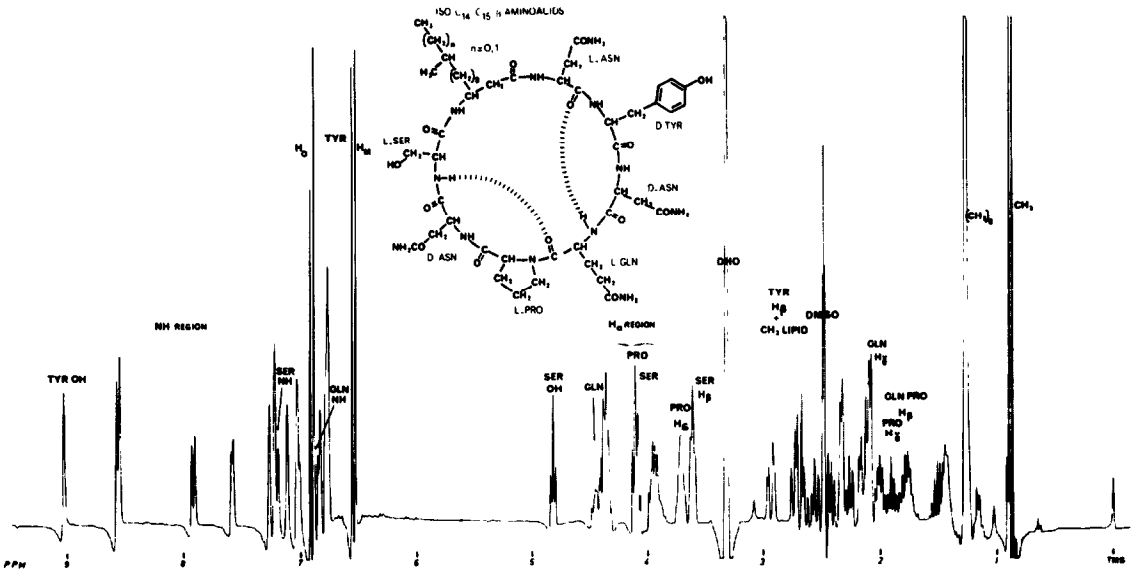

Fig.1. Convolution difference ^{1}H NMR spectrum of iturin A at 270 MHz (20 mg/0.5 ml DMSO d_6).

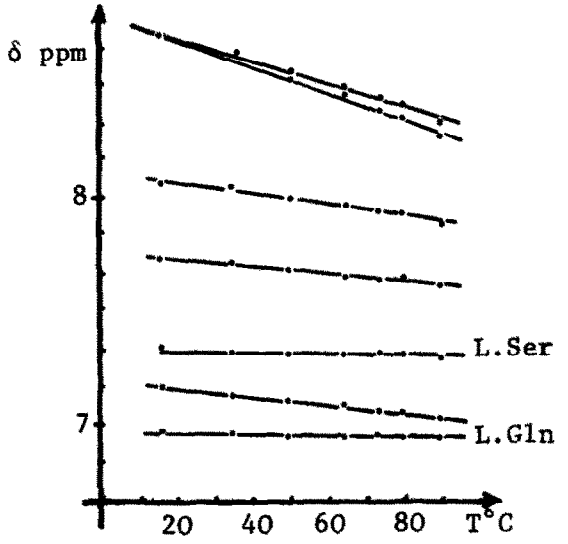

Fig.2. Plots of δNH (ppm) versus temperature.

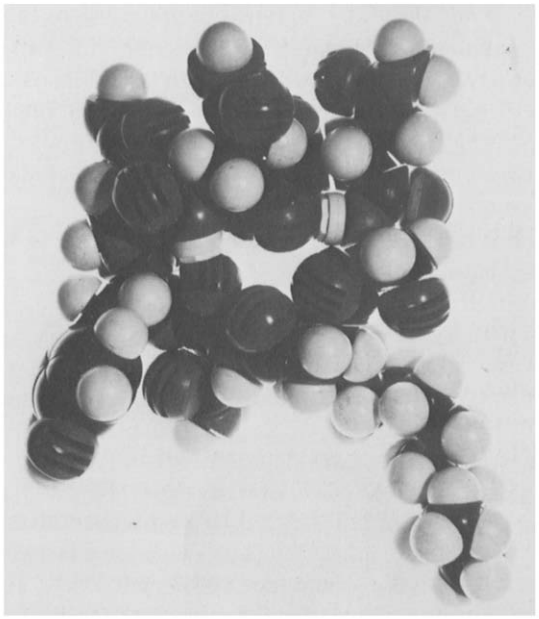

Fig.3. Proposed model for iturin A.

hydrogen bond between the NH of L-Ser (amino acid *i*) and the CO of L-Gln (amino acid *i*+3) would involve the residue L-Pro and D-Asn at the corners of the chain reversal. The second turn(B) would involve an hydrogen bond with the NH of L-Gln and the CO of L-Asn preceding the D-Tyr and D-Asn residues located at the corners of the turn. Some support to this conclusion arise from the well known preference that L-Pro apparently shows for occupying the position *i*+1 in the bends of peptides.

Venkatachalam [13] was the first to characterize three types of turns in a tetrapeptide; other types have been proposed since [14]. It was also shown that, the type of the β-turn involving the CO of an amino acid *i* and the NH of the amino acid *i*+3 is function of the configuration of the amino acids located in the *i*+1 and *i*+2 positions [13,15] (table 1). In our case turn A would preferentially belong to the

Table 1

Predicted values for the ϕ and θ angles and the $^3J_{NH-H_\alpha}$ couplings in the amino acids *i*+1 and *i*+2 of a β-bend involving amino acids *i* to *i*+3 as a function of the β-bend type

β-type	Residue *i*+1				Residue *i*+2			
	Serie	$\phi°$	$\theta°$	$^3J_{NH-H_\alpha}$ (Hz)	Serie	$\phi°$	$\theta°$	$^3J_{NH-H_\alpha}$ (Hz)
I	L (or Gly)	−60	120	~ 4	L	−90	150	8.5–9
I′	D	60	120	~ 4	D (or Gly)	90	150	8.5–9
II	L	−60	120	~ 4	D (or Gly)	80	140	7 –7.5
II′	D (or Gly)	60	120	~ 4	L	−80	140	7 –7.5

type II and turn B to the type I'. ϕ and ψ values for the amino acids i+1 and i+2 as a function of the turn type have been proposed [14]. Then the values of the $^3J_{NH-H_\alpha}$ can be evaluated as a function of the type turn, following a Karplus-type relationship between the dihedral angles θ (related to ϕ) and the ^3J coupling constants [13–16] (table 1).

In iturin A, the turn A (β_{II} type) with a hydrogen bond between the CO of L-Gln and NH of L-Ser involves residues L-Pro and D-Asn as i+1 and i+2 residues. By means of the values reported in table 1, one can expect $^3J_{NH-H_\alpha} \approx 7-7.5$ Hz for the residue D-Asn (i+2). In the same way, in the turn B ($\beta_{I'}$ turn), with a hydrogen bond between the CO of L-Asn and the NH of L-Gln, involving residues D-Tyr and D-Asn as amino acids i+1 and i+2, one can expect for these later $^3J_{NH-H_\alpha} \approx 4$ Hz and $\approx 8.5-9$ Hz, respectively. It is interesting to note that these later values are effectively observed in the spectrum (i.e., at 7.7 ppm, $J \approx 4.5$ Hz, certainly NH of Tyr and at 8.3 ppm, $J \approx 8$ Hz, fig.1). It is noteworthy that the residue L-Pro in general occupies the i+1 position in the β-turns of type II, is in the case here.

Other conformations could be taken into account. For instance an antiparallel β-pleated sheet involving hydrogen bonds between the NH of L-Ser and CO of L-Gln on one side and hydrogen bonds between the NH of L-Gln and CO of L-Ser on the other side could be proposed. However, the observed values of $^3J_{NH-H_\alpha}$ of L-Gln (J=8 Hz) and L-Ser (J=7.5 Hz) do not correspond very well with the values generally reported for an antiparallel β-pleated sheet conformation [4] (^3J>9 Hz). On the other hand, in such a conformation other hydrogen bonds should also be expected. The existence of γ turns could alternately be proposed but according to [17], one should observe among the $^3J_{NH-H_\alpha}$ couplings more than only one small J coupling ($J \approx 4.5$ Hz for NH at 7.7 ppm).

The simple approach to conformational analysis of iturin A by mean of $\Delta\delta$NH slopes versus temperature and correlation between ^3J coupling and dihedral angle remains ambiguous because not all the resonances are assigned to their own amino acid. Exchange rates for amide protons was utilized to compensate for lack of knowledge of the dihedral angle ϕ.

The exchange was followed by adding successive quantities of D_2O because the exchange rates were very slow for all the amide protons of the backbone but not of the side chains. Consequently, these NH_2 groups are not involved in intramolecular hydrogen bonds. Moreover their chemical deshielding in DMSO d_6 solution are in agreement with the values reported in literature for non-bonded resonances and in accordance with their strong $\Delta\delta$NH slopers versus temperature.

3.2. ^{13}C NMR

The proton-decoupled natural abundance ^{13}C Fourier transform NMR spectrum of iturin A is depicted on fig.4. Some of the C_β resonances were not determined exactly due to their overlapping. The resonances assignments of the lipidic moiety were achieved by comparison with the spectrum of the amino acid $C_{14}-C_{15}$ fragment recorded in the same conditions and with the data in [18,19]. From the spectrum, one can evaluate the relative intensities of the terminal methyl groups of the lipidic chain and propose a 1/1 ratio for the mixture C_{14}/C_{15} side chains. The value is in accordance with [12]. As could be expected, the chemical shifts of the proline carbons C_β and C_γ are consistent with a trans-peptide bond [20].

The ^{13}C T_1 values are reported in fig.4 and can be classified in three groups.

(i) The C_α backbone carbons with restricted motion is lying in the range of 0.06 s.

(ii) Essentially the C_β carbons (T_1 = 0.08–0.12 s) whose mobility is still restricted due to their proximity to the backbone and also the other carbons of the amino acid side chains (Gln, Tyr, Pro) (T_1 = 0.15–0.24 s).

(iii) The end of the lipidic moiety which exhibits a large flexibility as shown by the averaged T_1 value of 0.56 s estimated for CH_2, $T_1 \approx 2$ s and CH, $T_1 \approx 3.6$ s.

The effective correlation time for the molecular tumbling may be described from T_1 through the equation [21] $\tau_{eff} = C/NT_1$ where C is a constant = 4.72×10^{-11} s^2, provided τ lies in the extreme narrowing limit $[(\omega_H{}^2 + \omega_C{}^2)\,\tau^2 \ll 1]$. From the averaged C_α backbone T_1, one finds $\tau_{eff} \approx 8 \times 10^{-10}$ s, a value quite in accordance with the overall correlation time (8.8×10^{-10} s) estimated for the nonapeptide oxytocin which contains an hexapeptide cyclic moiety.

Table 2
^{13}C chemical shifts of iturin A in DMSO d_6

Residue	C_α	C_β	C_γ	C_δ
L-Ser	56.2	61.3		
L-Asn	50.8	34.9		
D-Tyr	56.2	34.9		
D-Asn	49.8	36.3		
L-Gln	50.8	28.	30.6	
L-Pro	60.8	29.3	25.3	45.5
D-Asn	49.8	36.3		
βNC_{14}	47.2	$(CH_2)_n$ 28.8	$-CH_2-$ 31.2 $-CH<$ 26.7 CH_3 22.5 CH_3	22.5
βNC_{15}	47.2	28.8	$-CH_2-$ 38.5 $-CH<$ 26.7 CH_3 22.5 $-CH_2-$ 31.2 CH_3 13.8	

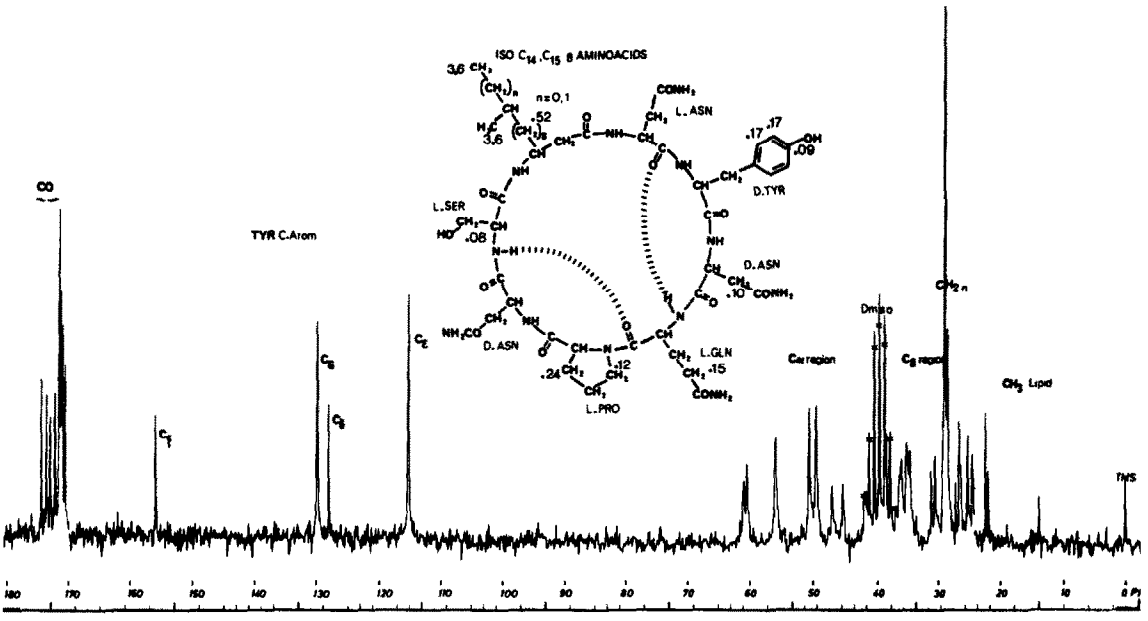

Fig.4. ^{13}C NMR of iturin A. The ^{13}C T_1 values are reported in the formula. C_α T_1 values averaged 0.06 s (not reported).

4. Conclusion

In conclusion, we propose that, in DMSO d_6 solution, iturin A populates preferentially a highly folded conformation characterized by two β-turns involving:

(i) The residues L-Gln, L-Pro, D-Asn and L-Ser with an hydrogen bond between the CO of L-Gln and NH of L-Ser for the type II β-bend.

(ii) The residues L-Asn, D-Tyr, D-Asn and L-Gln with a hydrogen bond between the CO of L-Asn and NH of L-Gln for the type I' β-bend.

The small temperature dependencies of several amide protons indicate a large steric hinderance to the solvent access, certainly due to the mobility of the side chains. All of these results are in favour of a relatively rigid structure of the backbone with a small cavity (<2 Å diam.) in which the backbone carbonyls are directed inside. This type of structure is generally observed in ionophores [22] and may be related with the antibiotic role of iturin A. Moreover the side chains of α-amino acids are not involved in hydrogen bonds and they keep a large degree of freedom allowing a possible interaction with microbial envelope. Such an interaction was recently found with the bacterial membrane of *M. luteus* protoplasts [23]. Other results have pointed out the influence of the amino acid residues surrounding the hydrophobic β-amino acid on the lytic activity of antibiotics from iturin groups on the lysis of *M. luteus* protoplasts [24].

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