Vol. 113, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1983 Pages 121-126

A 400 MHz ^{1}H NMR STUDY OF FORTUITIN, A NATURAL LINEAR LIPOPEPTIDE

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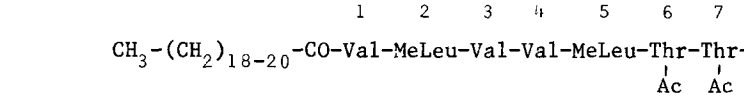
Received March 31, 1983

Fortuitin is a linear acyl nonapeptide methyl ester in which all the peptide residues are hydrophobic. Its 400 MHz ^{1}H NMR spectrum in pyridine-d_5 has been assigned. In this solvent, its conformation is not random and seems to form a hairpin. Conformational equilibria exist in pyridine as well as in less polar solvents such as $CDC\ell_3$ and $CD_2C\ell_2$. As for other lipopeptides, conformation and self-association properties strongly depend on the polarity of the environment. These properties should be related to the interactions of lipopeptides with membranes and to their capacity to induce pore formation.

Lipopeptides from bacterial origin are formed from two well distinct parts : a long aliphatic tail linked to a cyclic or to a linear oligopeptide. Some of them have antibiotic properties (1,2). We have discovered recently that several lipopeptides are able to induce the formation of conducting pores in BLM (Bimolecular Lipid Membranes) (3). Our recent ^{1}H NMR studies (4,5) of conformational and self-association properties of peptidolipin NA displayed the special sensitivity of such compounds to the polarity of the environment. The present paper reports a first ^{1}H NMR investigation of fortuitin, a linear lipopeptide extracted from *Mycobacterium fortuitum*. The structure of this acyl nonapeptide methyl ester was determined by mass spectrometry (6) :



All residues were found to be L, except the two N-MeLeu ones, configuration of which was not established (7). As compared with other lipopeptides (8-10), fortuitin has a pronounced hydrophobic character since the only polar groups are

0006-291X/83 $1.50

amide and ether or ester bonds in the peptide moiety. Taking into account the linearity of the molecule and its composition, one can expect a high flexibility and a dependence of its conformation and self-association on the polarity of the solvent.

EXPERIMENTAL

The sample of fortuitin resulting from previous preparations (7) was used without additional purification. Pyridine-d_5 was used as solvent mainly because of the good resolution of NMR spectra and the existence of a predominant conformation of the solute. Other investigations were then undertaken in $CDC\ell_3$ and $CD_2C\ell_2$. Preliminary experiments were carried out by using Bruker WH 90 and Cameca 250 FT spectrometers. A Bruker WH 400 FT spectrometer was used for recording of final spectra and for *nOe* (nuclear Overhauser effects) and 2D (two dimensional J resolved) measurements. The assignments were based first on a comparison with literature data and on homonuclear decoupling. Difference spectra (with and without decoupling) and some 2D spectra were recorded when it was necessary to resolve multiplets overlaps. *nOe*'s between $C^{\alpha}\underline{H}_i$ and $\underline{NH}_{i+1}$ protons were systematically measured for establishing the peptide sequence (11).

RESULTS AND DISCUSSION

The Ala(8) residue was readily assigned : couplings were found between a $C^{\alpha}\underline{H}$ and a $\underline{NH}$ on one hand and a high field $C\underline{H}_3$ on the other one. The two Thr residues were also identified by homonuclear decoupling : each of the $C^{\alpha}\underline{H}$ protons appearing as well resolved quadruplets at 5.29 and 5.19 ppm were coupled to one $\underline{NH}$ and one $C^{\beta}\underline{H}$, the latter being coupled to one $C^{\gamma}\underline{H}_3$. Their relative positions were deduced from *nOe* measurements, irradiation of $\underline{NH}$(7) inducing an enhancement of the $C^{\alpha}\underline{H}$(6) resonance. The two $C\underline{H}_3$ peaks of side chain acetyl groups were found near 1.65 ppm. The assignments of the two NMeLeu residues were more difficult and required *nOe* and 2D experiments. One $C^{\alpha}\underline{H}$ appeared as a well resolved quadruplet located at 5.72 ppm. Inequivalence of couplings of this $C^{\alpha}\underline{H}$ proton with the $C^{\beta}\underline{H}_2$ ones indicates a reduced mobility of the *iso*-butyle side chain. The second $C^{\alpha}\underline{H}$ superimposed to a $C^{\beta}\underline{H}$ of Thr(6) seems to be a triplet. In the same way, the identification of the three Val residues required a careful analysis of difference spectra and *nOe* measurements. Two $C^{\alpha}\underline{H}$ triplets appeared at 5.03 and 5.05 ppm and a third at 4.78 ppm which was slightly broadened by an underlaying signal of residual water. The last $C^{\alpha}\underline{H}$ located at 4.49 ppm was assigned to the Pro residue. The complete sequence assignment was established by measuring $C^{\alpha}\underline{H}_i$ enhancement on irradiation of $\underline{NH}_{i+1}$ and *vice versa* (except for the two NMeLeu residues).

TABLE I

Residue	Thr 7	Val 4	Ala 8	Thr 6	Val 3	Val 1	NMeLeu 2	NMeLeu 5	Pro 9
δ ppm									
NH	9.57	9.46	9.22	9.16	8.77	8.67	NCH_3 3.27 , 3.25		-
$C^{\alpha}H$	5.19	5.03	4.85	5.29	4.78	5.05	5.60	5.72	4.49
$C^{\beta}H$	5.56	2.20	1.28 (CH_3)	5.60	2.09	2.20	1.78 (CH_2)	1.78 (CH_2)	1.78 1.60 (CH_2)
$C^{\gamma}H$	1.22 (CH_3)	0.92		1.22 (CH_3)	0.80 (CH_3)	0.92 (CH_3)			
$J_{NH-C^{\alpha}H}$(Hz)	8.8	8.8	7.3	8.8	8.8	9.0	-	-	-
$\Delta\delta_{NH}/\Delta T$ ppm/°C $\times 10^2$	1.6	1.6	1.3	1.9	1.8	1.5	-	-	-

$COOCH_3$(Pro) : 3.39 ppm ; CH_3-COO-(Thr 6 and 7) : 1.66 and 1.64 ppm.
aliphatic chain : CH_2(2) : 2.30ppm ; CH_2(3) : 1.65ppm ;
CH_3(terminal) : 0.61ppm.
chemical shifts are measured relative to internal TMS.

Most of the spectral parameters are reported in Tables I and II, a decoupling scheme and the proposed assignments are represented on the figure 1. In addition to the peptide protons, the following groups :

$$CH_3-(CH_2)_n-\overset{3}{CH_2}-\overset{2}{CH_2}-\overset{1}{COO}- \qquad (n = 16-18)$$

of the aliphatic tail were also identified. All the assignments were found to be in agreement with the previously proposed formula (6). Dispersion of the

TABLE II

nOe's between NH_{i+1} and $C^{\alpha}H_i$ (all these *nOe*'s are negative).

Irradiated NH_{i+1}	Thr(7)[a]	Val(4)	Ala(8)	Thr (6)	Val(3)	Val(1)
Observed $C^{\alpha}H_i$ (enhancement $\times 10^2$)	Thr(6) 5	Val(3) 16	Thr(7) <5	NMeLeu(5) 5	NMeLeu(2) 8	CH(2)(chain) <5

[a] an enhancement is also observed for the Val 1 NH.

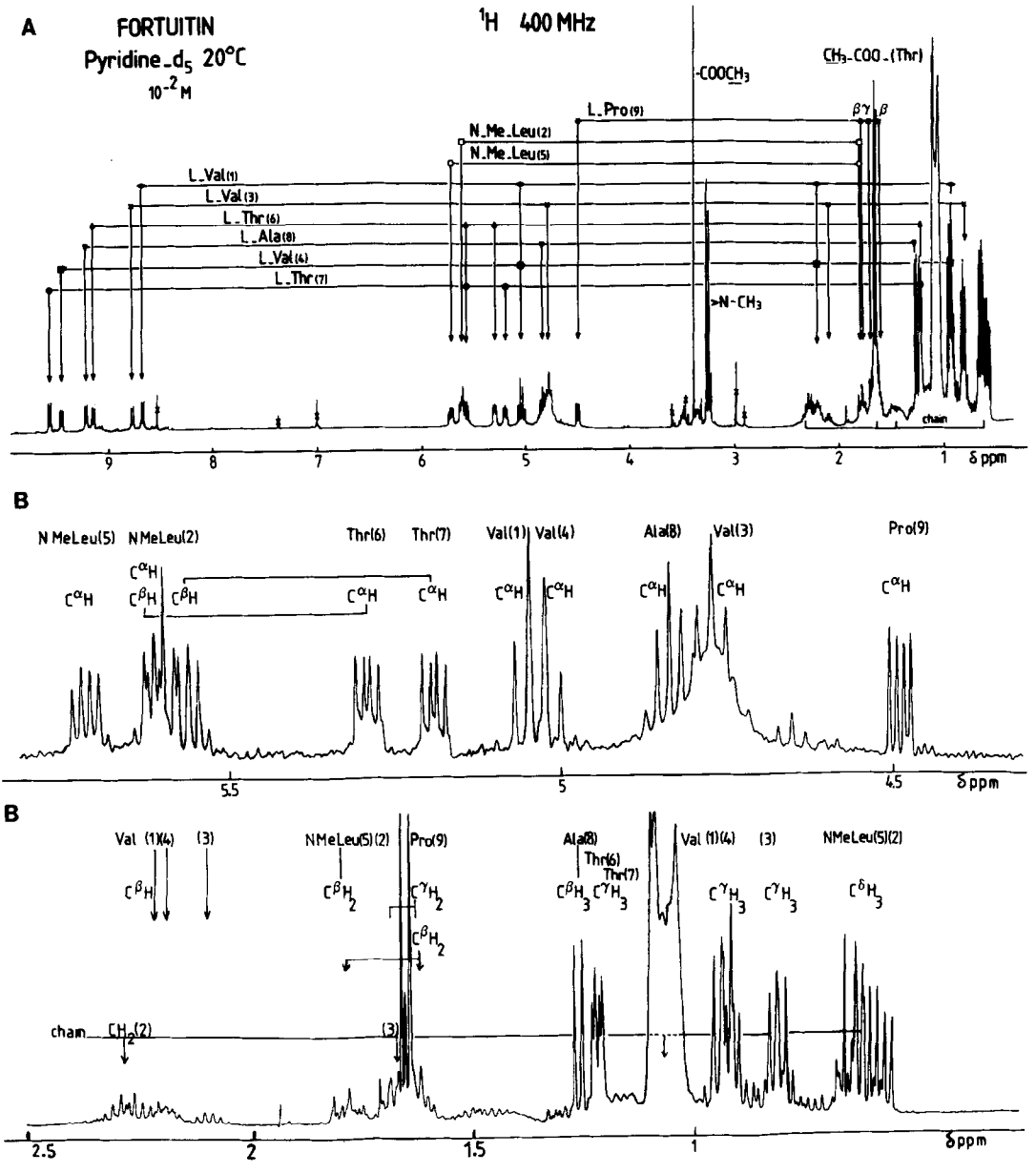


Figure 1 a) 400 MHz ^{1}H spectrum of fortuitin in pyridine-d_5 with the homonuclear decoupling scheme. (x) indicates solvent or impurities. (b) Expanded parts of the previous spectrum. The chemical shifts are measured relative to internal TMS. The resolution was enhanced by a Lorentzian-Gaussian transformation.

NH chemical shifts, high values of $^{3}J_{\underline{NH}-C^{\alpha}\underline{H}}$ coupling constants and existence of relatively high *nOe*'s especially for Val(3) and (4) residues clearly prove the conformation of fortuitin in pyridine-d_5 is not random. A 1-5 sequence including three Val and two Leu bulky residues excludes the existence of a helical conformation. More certainly, most of the residues should have a

nearly extended conformation for which the $C^{\alpha}\underline{H}_i$ and $\underline{NH}_{i+1}$ protons are neighbouring. The detection of an interaction between NH(1) and NH(7) groups suggests that two molecules interact head to tail as in a β anti // structure or that one molecule is folded in order to bring together the two peptide ends.

The absence of significant broadening of the spectrum and limited concentration effects indicate that there are no large aggregates formed by β structures. One could then consider a folding stabilized by CO(1)...NH(7) and NH(1)...CO(7) hydrogen bonds in which NH(1) and (7) groups are neighbouring. The nature of the bend involving the Val(3) and (4) residues depends on the configuration of N-methylated peptide bonds of the two Leu(2) and (5) residues which can be either *cis* or *trans* (12). The high value of the *nOe* found for the NH(3) (Table II) shows that the conformation is rigid and compact in the vicinity of the Val(3) and (4) residues. This folded conformation should be in equilibrium with extended forms as suggested by the existence of additional small NH resonances for the Val(1) and Thr(6) residues and of a $C^{\alpha}\underline{H}$ resonance for the Val(3) one (all these resonances are upfield shifted)(fig.1). The values of the $\Delta\delta_{NH}/\Delta T$ temperature coefficients (Table I) (1.3 to 1.9×10^{-2} ppm/°C) are rather higher than those of N-methyl-acetamide ($\simeq 1.1 \times 10^{-2}$ ppm/°C) usually taken as reference for an accessible NH group. This confirms the existence of conformational equilibria and prevents to relate directly these coefficients to the existence of intramolecular hydrogen bonds. Preliminary experiments have been carried out in less polar solvents such as $CDC\ell_3$ and $CD_2C\ell_2$. In chloroform, the NH group appearing at the highest field is split into two unequal peaks. That demonstrates the existence of two unequally populated states in slow equilibrium on the NMR time scale and confirms the dependence of the conformation on the polarity of the solvent. The existence of multiple forms is still more clearly revealed in $CD_2C\ell_2$ in which all the NH resonances are split . The CD (Circular Dichroism) spectrum of fortuitin in hexafluoroisopropanol is complex. A negative minimum at 225 nm suggests the presence of some β-bends (13), but the global spectrum corresponds more to a mixture of conformations with a predominance of unordered or of extended forms.

CONCLUSION

We have established the necessary basis for further NMR investigations of conformational and self-association properties of fortuitin. In pyridine, the conformation of this linear lipopeptide is not random but more probably it is folded around the Val(3) and (4) residues. The conformation and self-association of fortuitin strongly depend on the polarity of its environment. It should be particularly interesting to relate these properties with the interactions of fortuitin with membranes. Indeed, as previously mentioned, we have demonstrated very recently that several lipopeptides are able to induce the formation of conducting pores in BLM (3). Fortuitin belongs to this family of compounds although it is not the more efficient inasmuch as the formed pores are irregular in size and in life times for a BLM of glyceryl monooleate. We are developing now in parallel permeability studies and NMR studies in order to correlate the pores forming capacity of such small amphiphiles and their behaviour in homogeneous solution.

ACKNOWLEDGMENTS : We thank C. Merienne from the "Laboratoire de RMN, Institut d'Electronique d'Orsay (France)" for his help in recording numerous 250 MHz ^{1}H spectra. We thank A. Heitz for her help and fruitful discussions.

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