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CONFORMATIONAL ANALYSIS OF THE CYCLIC PEPTIDE RHIZONIN A IN SOLUTION AND CRYSTALLINE STATE

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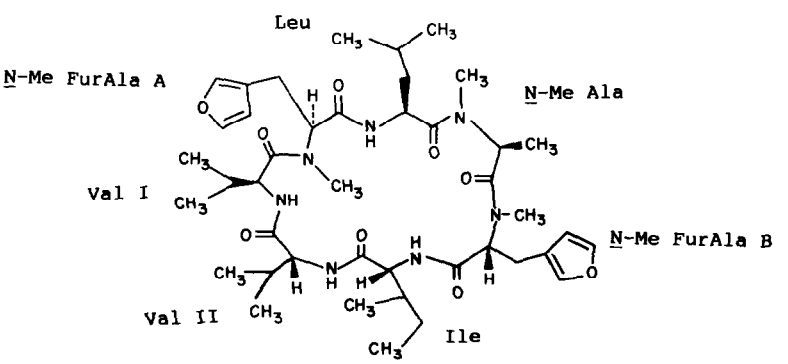
ABSTRACT - The solution conformation of rhizonin A in different solvents has been studied by NMR spectroscopy. The complete assignment of the ^{1}H and ^{13}C resonances in chloroform as well as the ^{1}H resonances in methanol and dimethyl sulphoxide were achieved by the application of various two-dimensional NMR techniques. The spectroscopic evidence indicated that the major conformer of rhizonin A in chloroform and methanol contains all trans amide bonds. The backbone conformation and the orientation of the FurAla B side chain of this conformer correspond closely with those of the crystalline state, as determined by X-ray crystallography.

Rhizonin A (1), a cyclic heptapeptide isolated from the fungus Rhizopus microsporus van Tieghem[1], is a potent non-specific hepatotoxin in rats.[2] Rhizopus species have been identified as a cause of spoilage in fruit and vegetables and as contaminants in grain sorghum. The production of this mycotoxin is of particular concern because of the use of Rhizopus species as enzymatic sources for the production of fermented foods, such as tempeh, in Far Eastern countries.[2] The structure of rhizonin A (1) was determined by the application of mass spectrometry, NMR spectroscopy and amino acid analysis, and was confirmed by X-ray crystallography.[1] The molecule contains three N-methyl amino acids as



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well as two pairs of like amino acids with opposite α-carbon stereochemistry, i.e. L-valine (Val I) and D-valine (Val II), as well as N-methyl-3-(3-furyl)-L-alanine (N-Me FurAla A) and N-methyl-3-(3-furyl)-D-alanine (N-Me FurAla B).

The comparison of the conformation of a biologically active cyclic peptide in the crystalline state with its conformations in a variety of solution environments can be used for mapping of the peptide backbone in terms of its relative mobility. This is valuable information for the extrapolation of available experimental data for the isolated peptide to the unknown situation of complexation with a receptor, because it provides a qualitative indication of the energy required for conformational change in different regions of the molecule.[3,4]

As a first step in the investigation of the structure-activity relationship of rhizonin A, a detailed study of the conformation of the metabolite in different solvents, as well as in crystalline state, was carried out. Some preliminary results of this study were published,[5] and we now present a detailed report of the results obtained.

NMR spectroscopy has become the most powerful technique for the study of the solution conformation of peptides and proteins.[3,4,6] Analysis of the solution conformation of rhizonin A has as a prerequisite the complete assignment of the ^{1}H and ^{13}C NMR spectra of the molecule, and we first want to report the assignments of the ^{1}H NMR spectra of rhizonin A in chloroform, methanol and dimethyl sulphoxide (DMSO) (Table 1) as well as the assignment of the ^{13}C NMR spectrum in chloroform (Table 2).

Rhizonin A exists according to ^{1}H and ^{13}C NMR spectral data predominantly (>9:1) as one conformer in chloroform-d. Results obtained in a (^{1}H,^{1}H) COSY experiment[7] enabled us to assign the ^{1}H NMR signals originating from isolated spin systems, i.e. from the protons of the individual amino acid residues. However, the COSY experiment failed to distinguish between the three methyl resonances (one at δ_H 1.03 and two at δ_H 0.90 p.p.m.) which are correlated with the multiplet at δ_H 1.83 p.p.m. (H_γ-Leu and H_β-Ile), and between the signals arising from the three N-methyl groups or from the pairs of like amino acids. The assignment of the ^{13}C NMR spectrum enabled us to assign these signals unambigiously.

The resonances of the protonated carbon atoms of rhizonin A were assigned by means of a (^{13}C,^{1}H) COSY experiment[8,9] optimized for $^{1}J_{CH}$. However, overlap prevented distinction between the three carbon resonances associated with the methyl proton doublets at δ_H 0.90 and 0.89 p.p.m., i.e. the carbon signals at δ_H 14.5, 19.3 and 23.7 p.p.m. In order to assign these resonances as well as those from the carbonyl and furyl quarternary carbon atoms, a (^{13}C,^{1}H) COSY experiment was performed with a mixing time of 45 ms[9] to detect long range

Table 1 . ^1H NMR data for rhizonin A in $CDCl_3$, $CD_3OH(OD)$ and DMSO-d_6 [a]

AMINO ACID RESIDUE	PROTON	$CDCl_3$ δ(ppm)	J(Hz)	$CD_3OH(OD)$ δ(ppm)	J(Hz)	DMSO-d_6 δ(ppm)	J(Hz)
N-Me Fur-L-Ala A	CH_3N	2 91 s	-	2.98s	-	X 2.92 s Y: 2 70 s	- -
	H-C_α	3 62 dd	10.5,3 9	3 91 dd	10 4, 4 1	X 3 88 dd Y: 4 36 dd	10 1, 4.0 b
	H_1-C_β	3 29 ddd	15 2,3 9,0.9	3 21 dd	15 0,3 9	X· 3.08 dd Y· 2 82 dd	15 1,4 0 14.8,7 7
	H_2-C_β	3 11 dd	15 2,10 5	3 11 dd	15 0,10 4	X 2 97 dd Y 2.59 dd	b 14 8,6 7
	H_A-Fur	6 23 dd	1 6, 0 7	6 37 dd	1 7,0 8	X 6 49 m Y 6 43 m	- -
	H_B-Fur	7 32 t	1 7	7 43 t	1 7	X· 7.53 t Y. 7.53 t	1 3 1.3
	H_C-Fur	7 16 dd	1 5, 0 7	7 26 br s	-	X 7.32 s Y· 7 38 s	- -
L-Leu	NH	7 47 d	9 5	7 62 d	9 4	X· 7 07 d Y 7 31 d	9 4 8 5
	H-C_α	5 24 ddd	11 8,9.5,2 2	5.22 ddd	11 8,9 4,2 3	X 5.05 ddd Y 4 71 ddd	11 2,9.7,2 2 b
	H_1-C_β	1 41 dt	b	1.50 m	-	X· 1 51 m Y 1.57 m	- -
	H_2-C_β	1 23 dt	12 3,2.0	1.26 ddd	13 8,11 1,2 7	X· 1 06 m Y· 1 27 m	- -
	H-C_γ	1 83 m	-	1.86 m	-	X 1 78 m Y 1 57 m	- -
	$CH_3(\delta 2)$	1 03 d	6.4	1.02 d	6.5	X· 0 92 d Y· 0 85 d	6.6 6.4
	$CH_3(\delta 2)$	0 90 d	6 9	0.95 d	6 7	X 0.87 d Y 0.83 d	b b
N-Me-L-Ala[c]	CH_3N	3 11 s	-	3 12 s	-	X 3 01 s Y· 2 99 s	- -
	H-C_α	4 37 q	7 3	4.82 q	3	X· 4 73 q Y· 4 72 q	7 5 7 2
	$CH_3(\beta)$	1 13 d	7.3	1.13 d	7 3	X 1 06 d Y 1 05 d	7.5 7 2
N-Me Fur-L-Ala B[c]	CH_3N	2 99 s	-	3 06 s	-	X 2 94 s Y· 2.94 s	- -
	H-C_α	5 55 dd	12 3, 4 5	5 48 dd	12 2, 4 5	X· 5.26 dd Y 5 26 dd	12 1,3.9 11 9, 3.9
	H_1-C_β	3 38 ddd	15 9,4 5,1 2	3 28 dd	15 8,4 5	X 3.13 dd Y 3 14 dd	15.4,3 9 15 5,3 9
	H_2-C_β	2 76 dd	15 9,12 3	2 83 dd	15 8,12 2	X· 2 72 dd Y· 2 69 dd	15.4,12.1 15 5,11 9
	H_A-Fur	6 21 dd	1 8,0 8	6 27 dd	1 9,0.7	X· 6 26 m Y 6 26 m	- -
	H_B-Fur	7 28 t	1 7	7 37 t	1 7	X· 7 49 m Y 7 49 m	- -
	H_C-Fur	7 19 dd	1 5,0.8	7 33 br s	-	X 7 39 s Y 7 39 s	- -
D-Ile	NH	7 49 d	9 2	7 43 d	9 4	X 7.16 d Y 7 44 d	9 3 9 5
	H-C_α	4 40 t	9 2	4 53 dd	9 4,7 3	X 4 55 dd Y· 4 90 br d	9 3,7 4 9 3,1 2
	H-C_β	1 83 m	-	1 86 m	-	X 1 71 m Y 1 95 m	- -
	$CH_3(\gamma)$	0 90 d	6 8	0 95 d	6 7	X· 0 85 d Y· 0 89 d	6 4 6 9
	H_1-C_γ	1 44 m	-	1.53 m	-	X 1.51 m Y 1 54 m	- -
	H_2-C_γ	1 04 m	-	1 1m	-	X 0.98 m Y. 1.05m	- -
	$CH_3(\delta)$	0.78 t	7 4	0.85 t	7.3	X: 0 80 t Y· 0 92 t	7 4 7 4

Table 1. (continued)

AMINO ACID RESIDUE	PROTON	$CDCl_3$ δ(ppm)	J(Hz)	$CD_3OH(OD)$ δ(ppm)	J(Hz)	$DMSO-d_6$ δ(ppm)	J(Hz)
D-Val II[d]	NH	6 18-7.11 d	4 8	8 42d	4.7	X 8.34 d Y· 7.86 d	5 3 5.8
	H-Cα	3 21 dd	8.8,4.8	3 35 dd	9 0,4 7	X 3.40 dd Y. 3 98 dd	8 3,5.3 5 8,3 6
	H-Cβ	1 96 ds	8.8,6 7	1 99 ds	9.0,6.7	X· 1 94 m Y· 2.21 ds	- 6 9,3.6
	CH_3(Υ1)	1.00 d	6.8	1.00 d	6.7	X 0 94 d Y 0.99 d	6.9 6 9
	CH_3(Υ2)	0 97 d	6.7	1 02 d	6.7	X 0 92 d Y· 0.89 d	6 6 6 9
L-Val I[d]	NH	5 59 d	9 8	6 73 d	10 3	X 6.94 d Y· 8 57 d	10 2 9 6
	H-Cα	4.55 t	9.8	4.53 t	10 2	X 4.36 t Y· 4 25 t	9 9 9 6
	H-Cβ	2 12 ds	9 6,6 7	2.12 ds	10 0,6 6	X 2.03 ds Y. 1 97 m	9.9,6 6 -
	CH_3(Υ1)	0 93 d	6 7	0.95 d	6 6	X· 0.85 d Y· 0 73 d	6.6 6 8
	CH_3(Υ2)	0.89 d	6.7	0 89 d	6.6	X 0.76 d Y· 0.55 d	6.6 6.5

[a] Abbreviations used FurAla = 3-(3-furylalanine), Leu = leucine, Ala = alanine, Ile = isoleucine, Val = valine ds = doublet of septets

[b] Not resolved

[c] All the assignments for conformers X and Y in DMSO-d_6 may be interchanged

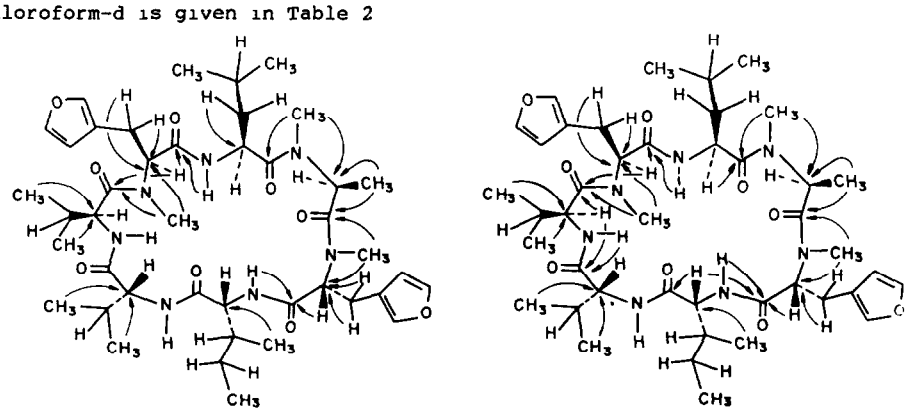
[d] Val I and Val II of conformer Y in DMSO-d_6 may be interchanged

($^{13}C,^{1}H$) couplings. The correlations observed for the ring carbon atoms are shown in Figure 1a. The N-methyl proton resonances were correlated to both the adjacent carbonyl and α-carbon atoms, thereby distinguishing between the N-methyl groups as well as identifying the carbonyl groups of the respective amide bonds. In contrast only three of the amide protons were correlated to adjacent carbonyl groups and none to the adjacent α-carbon atoms. In a supplementary selective population inversion (SPI) experiment[10] irradiation of the fourth amide proton resonance (δ_H 5.59 p.p.m.) effected an intensity change at only the carbonyl resonance at δ_C 170.9 p.p m. The ambiguity regarding a distinction between the two valine units prevented assignment of this carbonyl to either Val II or Ile.

The correlation between the methyl proton signals at δ_H 1.03 p p.m. and the carbon resonances at δ_C 38.9 p.p.m. (C_β -Leu) and δ_C 23.5 p.p.m.(C_γ -Leu) enabled the assignment of the proton resonances at δ_H 1.03 and one of the methyl groups at δ_H 0 90 p.p m to the δ-methyl groups of the leucine residue and the second δ_H 0.90 p.p.m. resonance to the Υ-methyl group of isoleucine. Distinction between the two sets of signals originating from the FurAla residues was also accomplished. For example, correlation of the furylalanyl α-proton signal (δ_H 5 55 p.p.m) with the carbonyl resonance (δ_C 169.1 p.p.m.) which was also correlated to NH-Ile at δ_H 7.49 p.p.m , identified the proton and carbon spin systems associated with H_α-FurAla at δ_H 5.55 p.p.m.

as those of FurAla B. In a complementary result the FurAla α-proton resonance at δ_H 3.62 p.p.m. as well as NH-Leu at δ_H 7.47 p.p.m. were correlated to the carbonyl resonance at δ_C 167.9 p.p.m., thereby identifying it as the carbonyl carbon atom of FurAla A.

None of the α-protons of the consecutive units Val I, Val II and Ile was correlated to adjacent carbons in the initial long-range (^{13}C,^{1}H) COSY experiment (Fig 1a). This observation plus the fact that the amide protons were correlated only to adjacent carbonyl groups prevented distinction between the two valine units. An explanation was found in the expected magnitudes of the coupling constants under consideration. Two-bond heteronuclear couplings, $\underline{H}$-C_α-$\underline{C}O$, have been estimated to be 4-7 Hz[11] and three-bond $\underline{H}$-C_α-N-$\underline{C}O$ couplings are angular dependent, with only a small range of dihedral angles associated with coupling constants that would be large enough to be detected under the experimental conditions used. An increase of the mixing time to 80 ms[9] in a second (^{13}C,^{1}H) COSY experiment led to the detection of five additional correlations of α-protons to adjacent carbonyl groups (Fig 1b). Thus both H_α-FurAla A (δ_H 3.63 p.p.m.) and one of the valine α-protons which resonate at δ_H 4 55 p.p.m. were correlated to the carbonyl carbon of Val I (δ_C 169.9 p.p.m.), thereby unambigiously identifying the proton and carbon resonances of Val I. The correlation of NH-Val I (δ_H 5.59 p p m.) with the carbonyl resonance at δ_C 170.9 p p.m allocated this carbon resonance to Val II. This assignment is supported by the correlation observed between H_α-Ile (δ_H 4.40 p.p.m.) and the resonance at δ_C 171.4 p p.m , which must therefore be the carbonyl carbon atom of Ile. The complete assignment of the ^{13}C NMR spectrum of rhizonin A in chloroform-d is given in Table 2



a b

Figure 1 Long-range heteronuclear shift correlations observed for the ring carbons of rhizonin A, (a) mixing time 45 ms, (b) mixing time 80 ms.

Table 2 ^{13}C NMR data of rhizonin A in $CDCl_3$

AMINO ACID RESIDUE	CARBON	δ_C (p.p.m)	$^1J_{CH}$ (Hz)	AMINO ACID RESIDUE	CARBON	δ_C (p p m)	$^1J_{CH}$ (Hz)
N-Me Fur-	CH_3N	39 9 Q	138 9	N-Me Fur	CH_3N	30 4 Q	138 1
L-Ala A	CO	167.9 S		D-Ala B	CO	169 1 S	
	C_α	66 9 D	131 9		C_α	55 8 D	137 5
	C_β	22.9 T	131 5		C_β	23 0 T	130 0
	C_A-Fur	110.6 D	171 6		C_A-Fur	110 3 D	172 9
	C_B-Fur	142.7 D	201 5		C_B-Fur	142 5 D	201 5
	C_C-Fur	139 8 D	199.7		C_C-Fur	139 2 D	199 5
	C_D-Fur	121 7 S			C_D-Fur	120 3 S	
L-Leu	CO	174 5 S		D-Ile	CO	171 4 S	
	C_α	47 6 D	140.3		C_α	56.5 D	140.1
	C_β	38.9 T	128.2		C_β	37 9 D	128 9
	C_γ	23 5 D	130 3		γ-Me	14 5 Q	126 2
	$C_{\delta 1}$	20 7 Q	125 2		C_γ	24 8 T	124 8
	$C_{\delta 2}$	23.7 Q	126 6		C_δ	10 8 Q	125.2
N-Me	CH_3N	30.7 Q	138.5	D-Val II	CO	170 9 S	
L-Ala	CO	174 9 S			C_α	63 1 D	135 2
	C_α	50 6 D	144 9		C_β	28.7 D	129 6
	C_β	12 9 Q	129.3		$C_{\gamma 1}$	19 5 Q	124 7
					$C_{\gamma 2}$	19 0 Q	127.6
				L-Val I	CO	169.9 S	
					C_α	54 5 D	138 1
					C_β	30 1 D	132 5
					$C_{\gamma 1}$	18 2 Q	129.1
					$C_{\gamma 2}$	19 3 Q	129 2

Rhizonin A exists as a ca. 3:1 mixture of conformers in methanol. Analysis of the most abundant conformer by ^1H NMR spectroscopy (see Table 1) revealed excellent agreement of coupling constants and chemical shifts with the major conformer in the chloroform solution. A single exception was the vicinal coupling constant observed for H_α-Ile, which reflects a modification in the side chain conformation around the C_α-C_β bond. Complete assignment of the proton NMR spectrum necessitated the use of both methanol-d_3 and methanol-d_4 as solvents All the amide resonances were observed in the methanol-d_3, but solvent overlap obscured the region δ_H 4.6-4.9 p p.m , which was therefore analyzed in methanol-d_4 Assignment of resonances was based on a (^1H,^1H) COSY experiment in methanol-d_3 and the use of a solvent gradient of chloroform-methanol mixtures. With the exception of the amide protons, all other proton resonances were virtually unaffected by changes in solvent composition. Both NH-Val I and NH-Val II signals experienced large downfield shifts upon addition of methanol to the chloroform solution, whereas the NH-Leu resonances shifted slowly downfield and NH-Ile was unaffected.

In DMSO-d_6 rhizonin A exists as two conformers in comparable concentrations This was evidenced by the presence of eight amide proton doublets in the region δ_H 6.9-8.6 p.p.m and five N-Me singlets (δ_H 2.7-3.0 p.p.m.) of which one has an intensity twice those of the other peaks. This meant that the complete analysis of the ^1H NMR spectrum involved not only the assignment of a hundred and thirty protons to the resonances observed, but also the

differentiation between those of each conformer A (^1H,^1H) COSY experiment proved useful only for the identification of different spin systems. However, a solvent study using a gradient of chloroform-DMSO mixtures, enabled us to distinguish between the resonances of the two conformers (conformers X and Y) This distinction was possible only because of the close similarity between conformer X in DMSO and the chloroform conformer The initial additions of DMSO to a chloroform solution of rhizonin A were done in small aliquots in order to carefully follow the chemical shift changes expected upon strong association of DMSO with dipolar regions of the rhizonin A molecule Major changes in chemical shift were observed for only the valine amide resonances of conformer X, which were shifted downfield [$\Delta\delta(CDCl_3$->DMSO Val I) = -1.35 p.p.m and $\Delta\delta$ ($CDCl_3$->DMSO Val II)=-2 16 p.p.m.], whereas small upfield shifts were observed for the other amide proton signals [$\Delta\delta(CDCl_3$->DMSO)=+0 40 p.p.m for Leu(X) and +0.33 p p.m. for Ile(X)]. The only differences between conformer X and the predominant conformer in chloroform are the modified C_α-C_β side chain conformation of Ile similar to the situation in methanol, and variations in the NH-H_α proton-proton coupling contants of Val II, which represent minor conformational changes in this part of the rhizonin A backbone.

A notable feature of the ^1H NMR spectrum in DMSO was the fact that the N-Me Ala and N-Me FurAla B residues for conformer X and Y had virtually identical chemical shifts and coupling constants, and extensive overlap prevented distinction between the individual conformers for this part of the molecule

CRYSTALLINE STATE CONFORMATION - Before discussing the conformations of rhizonin A in the different solvents, we first want to discuss the crystal conformation as derived from a X-ray crystallographic analysis of rhizonin A.[1] Crystals obtained from ethyl acetate-hexane were orthorhombic, of the space group $P2_12_12_1$, and the structure was refined to a final R_w factor of 0.094. The perspective drawing of the molecule with implied stereochemistry is shown in Figure 2 All amide bonds are trans with a 0-9° deviation from planarity In this conformation NH-Leu is directed towards the ring cavity and the cavity is also crowded somewhat by the methyl group of isoleucine

Analysis of the intramolecular N.. O distances suggests that the molecule may contain two intramolecular hydrogen bonds, the first between the isoleucine amide proton (NH-Ile) and the leucine carbonyl group to form a type II β-loop (N .O = 3 209 Å), and the second between the leucine amide proton (NH-Leu) and the carbonyl group of valine I to form an inverse γ-loop (N .O = 3 055Å) [12] In order to evaluate the potential β-loop the associated torsion angles for rhizonin A were compared with those reported for type II β-loops in the crystal structures of several other cyclic peptides.[12 13] Reasonably good agreement was found for all angles and the values are well within the range of allowed torsion angles for

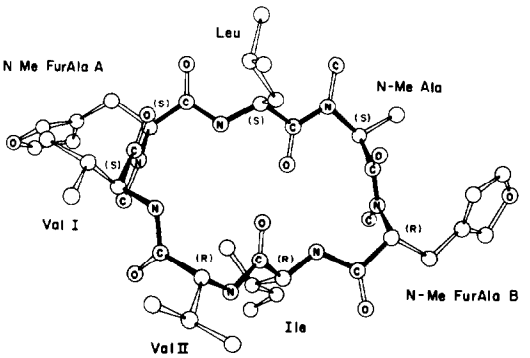


Figure 2 - Perspective drawing of solid state conformation of rhizonin A as determined by X-ray crystallography.

type II β-loops according to the corresponding Venkatachalam plot.[14] Also, the type II' β-loop of iodocyclosporin[15] compares favourably with that of rhizonin A in terms of torsion angles obtained (Table 3), the extent of N-methylation in the loop, and the steric demand of the N-Me Ala and N-Me FurAla B residues. We concluded, therefore, that this backbone arrangement of rhizonin A constitutes a legitimate β-loop.

The situation for the potential inverse γ-loop (γ^i) around the FurAla A residue is quite different. Typical of γ^i-loops is a deviation of 14-24° from planarity in the amide bonds, which is significantly larger than was determined for rhizonin A. In addition, the ϕ and ψ values of rhizonin A do not correspond with the reference data. A final disqualification of this arrangement of backbone atoms as a true γ^i-loop is the fact that the NH-NO angle is 89°, which is much larger than 30°, the upper limit for hydrogen bonding as postulated by Venkatachalam.[14]

Table 3 - Comparison between torsion angles determined for rhizonin A and the corresponding angles from reference data[a].

	Residue 2[b]		Residue 3[b]		N O
Type II β-loop	ϕ_2	ψ_2	ϕ_3	ψ_3	(Å)
Reference	-62°	+140°	+ 91°	-8°	2.86-3 35
Rhizonin A	-59°	+140°	+110°	-37°	3 21
Iodocyclosporin	+56°	-137°	-112°	+53°	3 21
Inverse γ-loop (γ^i)		ω	ϕ	ψ	N O(Å)
Reference		160°	-86°	64°	2 85-2 95
Rhizonin A		175°	53°	47°	3 06

[a] Average values for torsion angles determined by X-ray analysis of other cyclic peptides with corresponding loops[13] were used as reference data

[b] In the case of rhizonin A residue 2 is N-Me-L-Ala and residue 3 N-Me-D-FurAla B, whereas sarcosine and N-Me-L-Leu constitute the corresponding units of iodocyclosporin [15]

SOLUTION CONFORMATION - The NMR data obtained for rhizonin A in chloroform and methanol indicate that a single conformer dominate the equilibrium in these solvents, instead of a set of interconverting conformers. Criteria for conformational homogeneity in solution include large differences between amide temperature gradients and vicinal coupling constants, as well as large differences between the chemical shifts of equivalent protons in like amino acids and between vicinal coupling contants of diastereotopic protons [3] All these requirements are convincingly met for the predominant conformers in chloroform and methanol as well as for both conformers in DMSO.

A comparison of the ^1H NMR data for the predominant conformers in chloroform and methanol and conformer X in DMSO (see Table 1) reveals close agreement in all aspects except the chemical shifts of the amide protons. These values are expected to be sensitive to the solvent environment and do not necessarily reflect conformational changes. Differences which are apparent, however, are the larger vicinal coupling constant for NH-Val II[^3J(DMSO) 5.2 Hz as compared to $^3J(CDCl_3)=^3J(CD_3OH)=4.5$ Hz] indicative of a small change in the dihedral angle θ (HN-C_αH), and a change in the coupling pattern of H_α-Ile owing to a modified side chain conformation in methanol and DMSO. Since no significant changes in backbone conformation were detected in the different solution environments the three sets of NMR data are interpreted collectively

The backbone conformation of a small cyclic peptide, such as rhizonin A, depends mainly on the geometry of the amide bonds, and the presence of hydrogen bonds between the amide protons and the carbonyl groups. A NOESY[16] experiment proved to be particularly useful in establishing the geometry of the N-methyl amide bonds of rhizonin A in chloroform In all three cases under consideration nuclear Overhauser effects were observed between the N-methyl protons and the α-protons of the preceding residues, thereby establishing the trans amide bond configuration (Figure 3) It can be concluded, therefore, that rhizonin A exists in an all trans configuration in chloroform solution similar to the crystalline

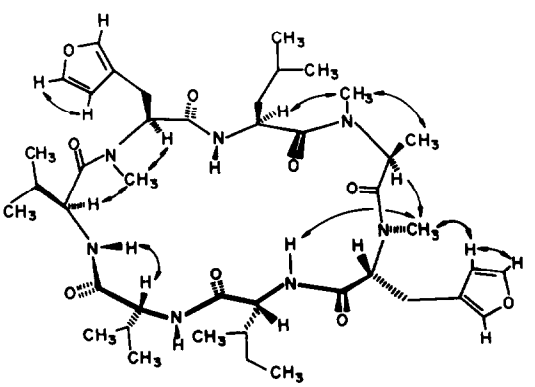


Figure 3 · NOE's observed for rhizonin A in $CDCl_3$ in a NOESY experiment

state. No n.O.e.'s was observed in DMSO, and therefore conclusions concerning the geometry of the amide bonds of conformer Y could not be made.

A knowledge of the chemical environment of the amide protons provided further useful information regarding the conformation of the molecule. This was achieved by the evaluation of a variety of indicators such as amide chemical shifts, rates of H/D exchange and the chemical shift dependance of changes in temperature, solvent and concentration (Table 4). In DMSO solution the solvent exposed amide protons are observed at lower field (δ_H 6,4 - 8,0 p.p.m.),[3,12,17,18] the reason being that the hydrogen bonding to the carbonyl group resulted in less deshielding of the amide protons than hydrogen bonding to the DMSO solvent matrix[17]. The situation is reversed in chloroform where solvent exposed amide protons resonate upfield relative to those that are solvent shielded or hydrogen-bonded. Chemical shift data for methanol solutions are more difficult to interpret because of the possibility of carbonyl protonation which will result in the deshielding of neighboring amide protons[3]. For rhizonin it appears to behave similar to DMSO

Table 4 NMR data for amide protons in various solvents

	NH-Leu		NH-Ile		NH-Val I		NH-Val II	
Chloroform								
δ (ppm)	7.47		7 49		5 59		6 52-7 11	
$^3J(\underline{HNC_\alpha H})$(Hz)	9.3		9 2		9 9		4.6	
$\Delta\delta/\Delta T \times 10^3$ (ppm/ K)[a]	-4 0		-1.0		+0 9		-7 4	
D_2O exchange	> 24h		> 24h		>24h		<2 min	
Methanol	X	Y	X	Y	X	Y	X	Y
δ (ppm)	7 62	7 58	7.43	7 78	6 73	8.48	8 42	7 86
$^3J(\underline{HNC_\alpha H})$(Hz)	9 3	8 5	9.3	9 6	10 0	9 7	4 5	5 4
$\Delta\delta/\Delta T \times 10^3$ (ppm/ K)[a]	-2.7	-2 5	-0 4	-2 1	-8 4	-7.8	-6.0	-7 2
DMSO	X	Y	X	Y	X	Y	X	Y
δ(ppm)	7.07	7 31	7 16	7 44	6 94	8 57	8 34	7 86
$^3J(\underline{HNC_\alpha H})$(Hz)	9 4	8 5	9 5	9.5	10 2	9 7	5 2	5 8
$\Delta\delta/\Delta T \times 10^3$ (ppm/ K)[a]	-2 1	-0 2	-0 7	-0 2	-6.4	-3.4	-3 4	-4.0

[a] Obtained for the temperature range 30-60°C Negative values represent a shift to higher field.

Chemical shift considerations indicated that NH-Val II of conformer X (and also the predominant conformer in chloroform and methanol) is solvent exposed, whereas NH-Leu and NH-Ile are solvent shielded and possibly also intramolecularly hydrogen-bonded. The conclusion regarding NH-Val II(X) is supported by the large temperature coefficients obtained in all three solvents, as well as its large concentration dependence and rapid deuterium exchange in chloroform. The chemical

environments of amide protons in conformer Y are similarly deduced: while both valine amide protons are solvent exposed (large temperature dependence associated with low-field chemical shifts in DMSO), the solvent and temperature dependencies of both NH-Leu(Y) and NH-Ile(Y) indicated their involvement in intramolecular hydrogen bonds. The small $\Delta\delta/\Delta T$ values obtained for NH-Ile are consistent with the interpretation that it is involved in an intramolecular hydrogen-bond in conformer X. The Leu amide proton, however, seems to be only solvent shielded ($\Delta\delta/\Delta T > 2 \times 10^{-3}$ p.p.m./K) in conformer X and is probably directed towards the ring cavity similar to its orientation in the crystal conformation of rhizonin A (Figure 2).

Several contradictions are evident upon inspection of the data for NH-Val I(X), i.e. its large temperature dependance in DMSO and MeOH, and high-field chemical shift in $CDCl_3$ are indicative of solvent exposure, whereas the small temperature dependence in chloroform and high-field chemical shift in DMSO suggest solvent shielding. In order to facilitate the interpretation of these results, solvent studies were undertaken in gradients of chloroform/methanol and chloroform/DMSO. The results show similar trends in the behaviour of the valine amide protons upon addition of either methanol or DMSO to a chloroform solution of rhizonin A. The chemical shift of NH-Val I(X) is initially slow to change, but becomes strongly solvent dependent when the polar solvent constitutes 25% or more of the solvent composition The chemical shift of NH-Val II(X), in turn, is strongly affected by the addition of small amounts of polar solvents. We have concluded that both valine protons are solvent exposed, although NH-Val I may be to a somewhat lesser extent The amide proton involved in the single intramolecular hydrogen bond of conformer X has been identified as NH-Ile and the question regarding which carbonyl group acts as receptor may be considered next.

NH-Ile of conformer X can be associated with either C=O (Leu) in a type II β-turn or with C=O (N-Me Ala) in a γ-turn based on the α-carbon stereochemistry of the residues involved.[12] The results obtained from the NOESY experiment in chloroform (see Figure 3) support the first option rather than the second. The n.O.e.'s observed between NH (Ile) and N-Me (FurAla B) as well as between N-Me (FurAla B) and H_α (N-Me Ala) restricts $\phi_{FurAla\ B}$ to approximately 120° rather than 70°, which is required for a γ-loop. Since no difference in backbone conformation could be detected for the major conformers in chloroform, methanol and conformer X in DMSO, this hydrogen-bond is assumed for all three solvents.

It is clear that the backbone conformation of conformer X corresponds very well with that of the crystal conformation. The solvent shielding of NH-Leu (X) corresponds with the internal orientation of this group in the crystalline state. A comparison of the torsion angles, ϕ, obtained from NMR data[11] and those obtained by X-ray analysis indicate excellent agreement for the Leu, Ile and Val I

residues (Table 5). However, the Karplus-type relationship between the vicinal coupling constant of NH-Val II and ϕ generated four regions of possible torsion angles. Inspection of models which accommodated the conformational restraints on the peptide backbone discussed so far indicates that two of the four possibilities can be ruled out on the basis of induced steric strain, i.e. ϕ_D=170->175° and -95->-105°. Indirect evidence regarding the magnitude of heteronuclear $\underline{H}C_\alpha$-N-$\underline{C}O$ couplings for the two valine and the Ile residues obtained from long-range ($^{13}C,^1H$) COSY experiments allows the evaluation of the two remaining possiblities. The experimental conditions chosen for smaller couplings did produce correlations between H_α (Val I) and C=O (Val II) and H_α (Ile) and C=O(FurAla B), respectively, for which the torsion angles ϕ_L(Val I)=-120° and ϕ_D(Ile) +120° correspond to a 4 Hz three-bond coupling.[11] The corresponding heteronuclear correlation was not observed for H_α(Val II) which indicates that the value of its $^3J_{CH}$ coupling constant must be smaller than 4Hz. This is only possible for ϕ_D(Val II)=65->70°, the torsion angle in the crystal conformation of the molecule

Table 5. Comparison of torsion angles, ϕ, obtained from NMR data for conformer X and those obtained by X-ray analysis

Amino Acid	$^3J_{corr}$, Hz [a]	ϕ, degrees [b]	crystalline state [c]
L-Leu	10.2	-100 - -150	-135°
D-Ile	10 1	+95 - +150	+124°
L-Val I	10 8	-105 - -140	-121°
D-Val II	5.0	+170 - +175	
		+65 - +70	+ 80°
		-15 - -25	
		-95 - -105	

[a] Corrected values to accommodate electronegativity effects[11]
[b] Allowed range of values deduced from vicinal coupling constants, $^3J_{NHC\alpha H}$, reference 11.
[c] Reference 1.

Although flexibility can be assumed for the side chains of rhizonin A in solution, preferred rotamers are encountered in many cases as indicated by the fact that coupling constants which deviate significantly from the mean value of ca. 7 Hz for free mobility were obtained and large differences between chemical shift and coupling constant values of diastereotopic protons were observed. The preferred conformations of the FurAla side-chains are of particular interest in the interpretation of the NMR data because of the magnetic anisotropy associated with furyl groups. The vicinal coupling constants of H_α(FurAla B) (J 12.3 and 4.5 Hz) suggest gauche and antiperiplanar arrangements between the α-proton and the two β-protons of the residue To satisfy this requirement the furyl group can be directed either towards N-Me Ala as in the crystal conformation or towards Ile, a position where the adjacent carbonyl group will prevent free rotation of the furyl

group. H_α (FurAla B) is strongly deshielded relative to similar protons in a cyclic tetrapeptide [$\delta(H_\alpha$-Phe DMSO) 4.34 ppm],[19] which can be expected for α-protons in the plane of the carbonyl group of the adjacent amino acid.[3 19] The orientation of the furyl group of FurAla B towards N-Me Ala is indicated by the n O.e observed between H_α-Fur B and N-Me (FurAla B) (Fig. 3).

The favoured rotamer of the side-chain of FurAla A in conformer X positions the furyl group over the peptide ring towards the valine residues (Figure 4). This is indicated by a number of observations, i e. the so far unexplained high-field occurence of the NH-Val I resonance in all three solvents despite its known solvent exposure, shielding of H_α-Val II and the vicinal coupling constants of H_α (FurAla A), which agree with an eclipsed conformation around the C_α-C_β bond. Figure 4 shows the conformation postulated for rhizonin A in chloroform, methanol and conformer X in DMSO.

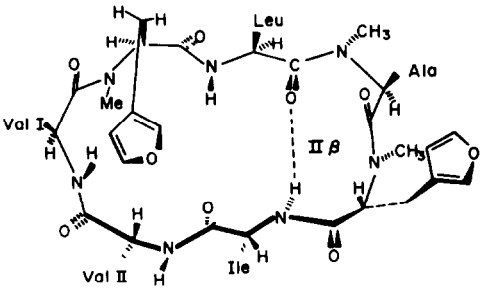


Figure 4 - Backbone conformation postulated for conformer X of rhizonin A.

Since no n.O.e.'s was observed for rhizonin A in DMSO solution, no definite conclusions concerning the geometry of the amide bonds of conformer Y could be made. The chemical environments of amide protons in conformer Y are deduced similarly to those in conformer X Both valine amide protons are solvent exposed (large temperature dependence associated with low-field chemical shifs in DMSO), whereas the solvent and temperature dependencies of both NH-Leu(Y) and NH-Ile(Y) indicated their involvement in intramolecular hydrogen bonds. A notable characteristic of conformers X and Y in DMSO solution is the extensive overlap in the NMR signals associated with the N-Me Ala and N-Me FurAla B residues. The similarity in chemical shifts and coupling patterns of these resonances as well as the fact that NH-Ile(Y) is hydrogen bonded, are considered to be convincing evidence for the existence of the same β-loop in both conformers. Differences between the backbone conformations of conformers X and Y are observed in the vicinal coupling constants for NH-Leu and NH-Val II Also, the shielding of NH-Val I and H_α in conformer X caused by the conformation of FurAla A, is absent in conformer Y However, lack of data prevented us from making further postulations concerning the conformation of conformer Y

We therefore conclude that the backbone conformation of rhizonin A in crystalline state corresponds very well with that of the major conformers in chloroform and methanol, as well as with conformer X in DMSO. In both conformers the molecule contains all trans amide bonds as well as a type II β-turn enveloping the L-Ala and D-FurAla B residues. The conformers existing in the polar solvents offer the best indication of those adopted by rhizonin A under biological conditions. As a first approximation it may be assumed that the β-loop encountered in the crystalline state as well as in both solution conformers will be conserved. The left hand portion of the molecule is more flexible and under biological conditions conformational variations can probably be expected in this region of the peptide backbone.

EXPERIMENTAL

All experiments were performed on a Bruker WM-500 spectrometer operating at 500.13 MHz for proton and 125.76 MHz for carbon-13. Solutions of rhizonin A were analyzed in a 5mm tubes at 30°C. The ($^{13}C,^{1}H$) COSY experiments were performed on a 0.25M solution of rhizonin A; 128 experiments on 2K data points were transformed in a matrix of 256 x 2048 points using the following experimental conditions spectral width F_1=2000 Hz and F_2=22723 Hz, acquisition time 0,045s and relaxation delay 2s. The different mixing times employed for the respective long-range ($^{13}C,^{1}H$) correlation experiments are discussed in the text. The ($^{1}H,^{1}H$) COSY experiment was performed on a 0.05M solution of rhizonin A; 512 experiments on 2K data points were transformed in a matrix of 512 x 2048 points using the following experimental conditions: spectral width F_1=1968 5 Hz and F_2=3937 Hz, acquisition time 0,25s and relaxation delay 4s.

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