Pergamon

Tetrahedron 55 (1999) 11495–11510

TETRAHEDRON

Pohlianins A, B and C, Cyclic Peptides from the Latex of *Jatropha pohliana* ssp. *molissima*

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Received 14 March 1999; accepted 29 July 1999

Abstract : From the EtOAc extract of the latex of *Jatropha pohliana* (Euphorbiaceae), two cyclic heptapeptides, pohlianins A (**1**) and B (**2**) and one cyclic octapeptide, pohlianin C (**3**) were isolated by a multi-step chromatography procedure, including HPLC. Their structure were elucidated by chemical degradation, mass spectrometry, homonuclear and heteronuclear NMR experiments. Conformational analysis of these peptides was made by using NMR experiments and distance geometry calculations. Their antimalarial activities were examined.

Keywords : cyclic peptides, *Jatropha*, nmr, molecular modeling, β-bulge.

INTRODUCTION

Natural cyclic peptides are widely distributed compounds which exhibit a large range of biological activities[1-3] and have often been used as models for the studies of structural features in proteins[4]. Conformational determination of such cyclic peptides is an important step to exhibit local interactions which could initiate the folding of native proteins. In addition, since biological activities are known to be related to the three dimensionnal structure, it is of importance to determine their solution conformational preference.

In our continuous investigation of cyclic peptides from the latex of *Jatropha* species[5-7], we isolated from *J. pohliana* collected in the Recife area (Brazil), three new cyclic peptides named pohlianins A (**1**), B (**2**) and C (**3**). Some *Jatropha* sp. are used in folk medicine against malaria. As it has been recently shown that some natural cyclopeptides such as apicidins[8] or cyclosporins[9] have potent antiparasitic effects against *Plasmodium*, we examined the *Plasmodium falciparum* antiproliferative activity of pohlianins. We observed a moderate antimalarial activity for these three peptides with IC_{50} values of 57 μM (**1**), 25 μM (**2**) and 16 μM (**3**), pohlianin C being the more potent.

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PII: S0040-4020(99)00660-2

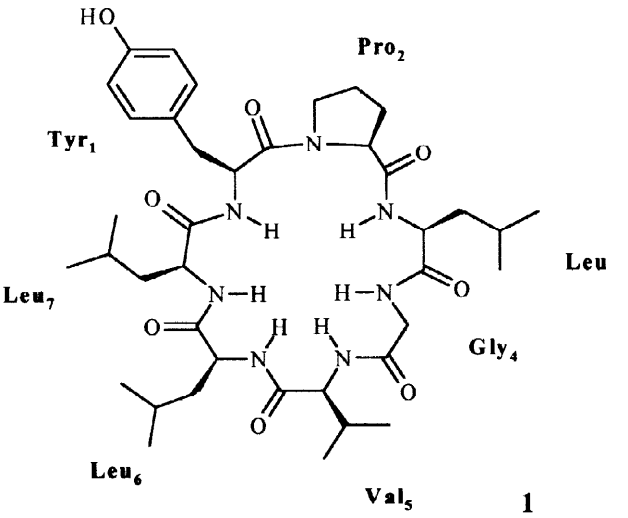
This paper deals with the isolation, structure elucidation and solution conformation of pohlianins A, B and C.

RESULTS AND DISCUSSION

Isolation and characterization of 1, 2 and 3

The latex of *J. pohliana* was partitioned between ethyl acetate and water. The organic phase soluble material was fractionated by exclusion chromatography followed by adsorption chromatography on silica gel. A crude peptidic fraction was yielded which was analysed by C_{18} reversed phase HPLC to reveal three peaks. The mixture was resolved into three pure peptides, pohlianins A (**1**), B (**2**) and C (**3**) by multi-step semi-preparative HPLC. Each peptide proved to be homogeneous by further LSIMS and NMR analyses. They gave a positive reaction with the chlorine/*o*-tolidine reagent in agreement with a peptide structure. A negative reaction with ninhydrin, indicating the absence of free amino group, suggested a cyclic peptide structure.

The amino acid composition was determined by HPLC analysis of the complete acid hydrolysates of the peptides. It was characterised for both **1** and **2**, by the presence of 1 Gly, 3 Leu, 1 Pro and 1 Tyr and the distinction between **1** and **2** arose from the presence of one valine in **1** instead of one leucine in **2**. The amino acid composition of **3**, determined as above, was 3 Gly, 2 Ile, 2 Phe and 1 Thr. The absolute configuration of the chiral amino acids was shown to be L from GC analysis



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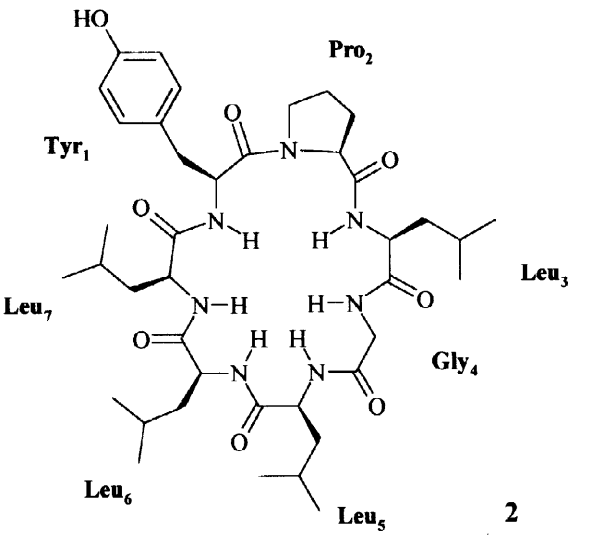

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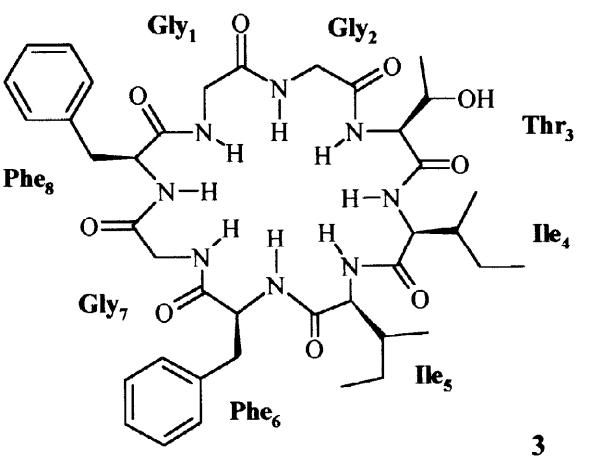

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on a chiral capillary column after derivatization of the acid hydrolysates to N-trifluoroacetyl isopropyl esters.

Sequence determination of pohlianin A

The molecular mass 755 for **1** was deduced from the positive LSIMS spectra which displayed the protonated molecule MH^+ at *m/z* 756 (base peak) and the adduct ions $[M+Na]^+$ and $[M+K]^+$ at *m/z* 778 and 795, respectively (Fig. 1). In the high-resolution spectrum, the protonated molecule MH^+ at *m/z* 756.4670 corresponding to the molecular formula $C_{39}H_{62}N_7O_8$ (calcd 756.4660), was in agreement with the above amino acid composition in a cyclic heptapeptide.

The NMR sequence determination of **1** resulted from the assignments of different spin systems to residue types from ^1H-^1H COSY, TOCSY and ROESY experiments, and sequential assignments were further afforded by exploitation of dNN (i, i+1) and dαN (i, i+1) ROESY connectivities and confirmed by long range ^1H-^{13}C HMBC experiments. NMR data were recorded in DMSO-d_6 because in this polar solvent, the 1D-NMR spectra of **1** gave well resolved sharp signals and though **1** contains one proline residue, only one conformation was observed. Complete ^1H spin systems of 1 Gly, 3 Leu, 1 Pro, 1 Tyr and 1 Val were depicted (Fig. 2a, b) from COSY and

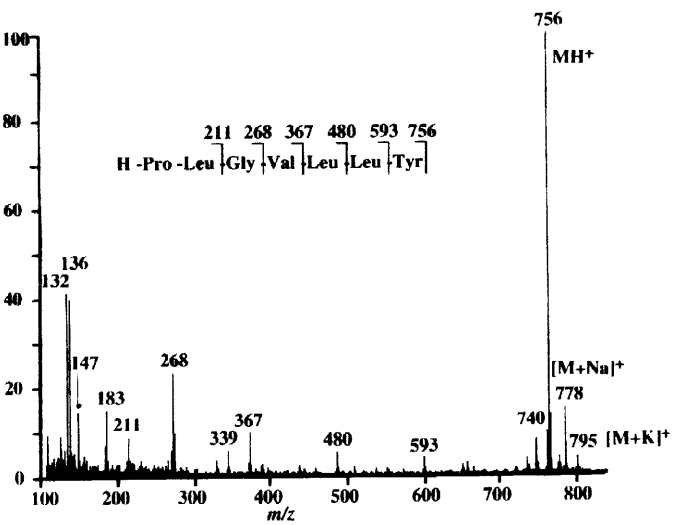

Figure 1 : Positive LSIMS of pohlianin A

TOCSY experiments and the corresponding carbon resonances were determined on the basis of J-modulated ^{13}C, HMQC and HMBC experiments (Table 1). The connectivities between neighbouring amino acids were determined from the ROESY spectrum and were completely carried out using inter-residue dαN (i, i+1) and dNN (i, i+1) connectivities. The lowest field NH proton signal of tyrosine at 9.19 ppm was assigned to Tyr_1. Inter-residue daN (i, i+1) connectivities were found between each adjacent residue extending from Pro_2 to Tyr_1, as well as the ROE connectivity between Tyr_1-Hα and Pro_2-Hα. In addition, dNN (i, i+1) connectivites were observed between Leu_3 and Gly_4 and from Val_5 to Tyr_1, as well as the dδN (i, i+1) connectivity between Pro_2 and Leu_3 (Fig. 3). Accordingly, the structure of pohlianin A was determined as cyclo (-Tyr_1-Pro_2-Leu_3-Gly_4-Val_5-Leu_6-Leu_7).

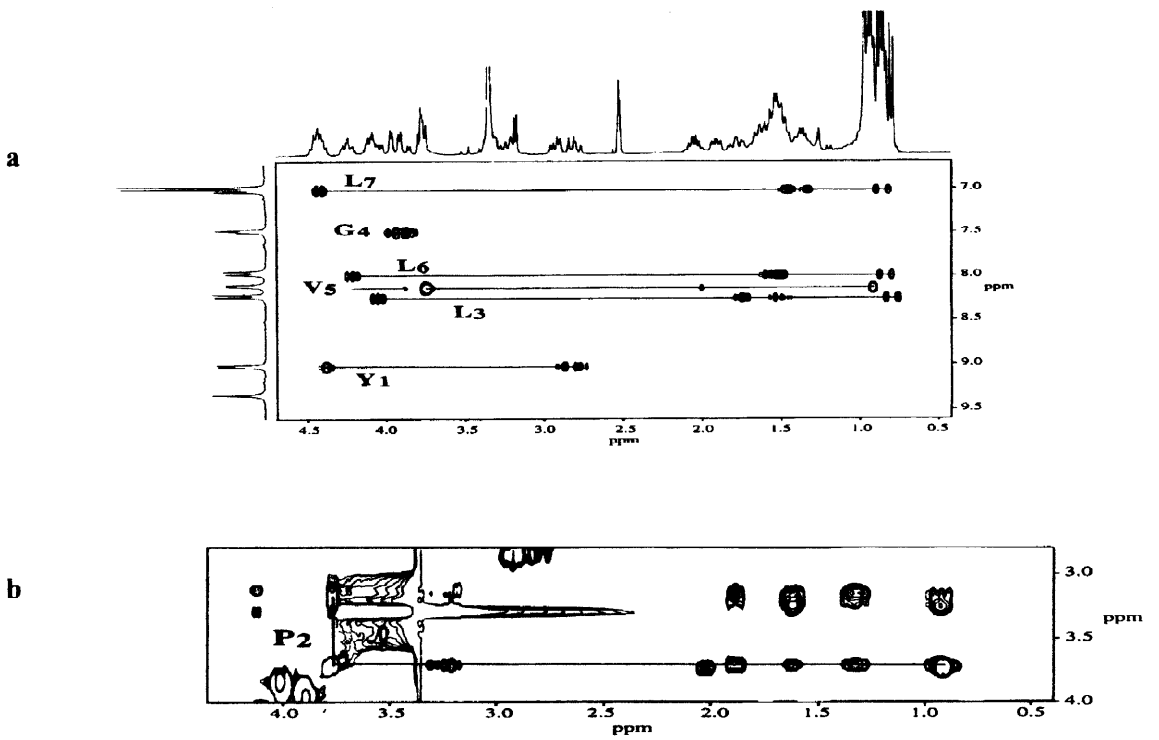

Figure 2 : Expansion of the TOCSY spectrum of **1** in DMSO-d_6 : **a**- ω_2 = 0.4-4.6 ppm, ω_1 = 6.8-9.6 ppm; **b**- ω_2 = 0.4-4.3 ppm, ω_1 = 2.8-4.0 ppm; spin-systems are labelled with the sequential residue positions.

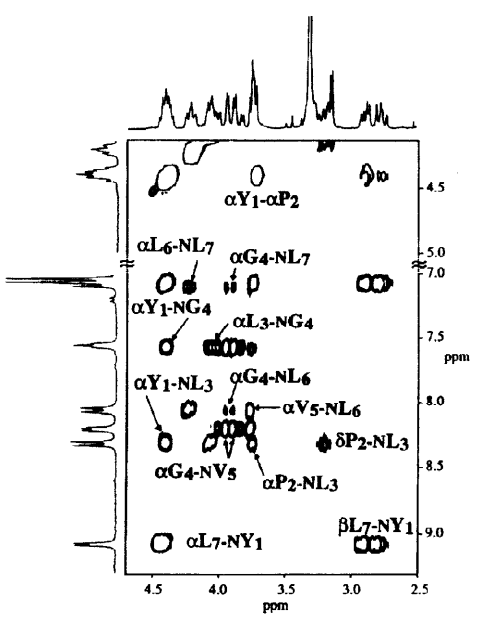

Figure 3 : Parts of the ROESY spectrum of **1** in DMSO-d_6 : ω_2 = 2.5-4.7 ppm, ω_1 = 4.2-9.3 ppm.

Table 1: ^{1}H and ^{13}C NMR spectral data for **1** and **2** (DMSO-d_6. 298 K)

		1				2	
Residue		δ_H (mult, J in Hz)	δ_C	Residue		δ_H (mult, J in Hz)	δ_C
Tyr_1	NH	9.01 (d, 4.9)		**Tyr_1**	NH	9.04 (d, 4.9)	
	α	4.38	53.2		α	4.40	53.2
	β	2.90 (dd, 13.4, 6.0)	36.0		β	2.90	36.1
		2.77 (dd, 13.4, 10.0)				2.78	
	γ		125.9		γ		125.9
	δ	6.99 (d, 8.5)	130.2		δ	7.00 (d, 8.4)	130.2
	ε	6.69 (d, 8.5)	115.3		ε	6.69 (d, 8.4)	115.3
	ζ		156.4		ζ		156.3
	CO		170.3		CO		170.2
Pro_2	α	3.70	61.0	**Pro_2**	α	3.74	60.5
	β	1.87	29.5		β	1.88	29.4
		0.93				0.88	
	γ	1.60	21.2		γ	1.60	21.2
		1.30				1.32	
	δ	3.18	45.8		δ	3.16	45.7
		3.28				3.28	
	CO		170.2		CO		170.3
Leu_3	NH	8.24 (d, 8.2)		**Leu_3**	NH	8.23 (d, 8.6)	
	α	4.05	52.8		α	4.06	52.8
	β	1.75	40.1		β	1.73	40.1
		1.50				1.50	
	γ	1.50	24.6		γ	1.45	24.5
	δ_1	0.82	23.0		δ_1	0.83	23.0
	δ_2	0.74 (d, 6.3)	20.7		δ_2	0.75 (d, 6.3)	20.6
	CO		171.8		CO		171.7
Gly_4	NH	7.50 (t, 4.0)		**Gly_4**	NH	7.48	
	α	3.97 (dd, 3.6, 17.5)	42.5		α	3.92	42.4
		3.85 (dd, 5.1, 17.5)				3.80	
	CO		169.8		CO		169.4
Val_5	NH	8.13 (d, 5.1)		**Leu_5**	NH	8.26 (d, 5.8)	
	α	3.75	60.5		α	3.86	53.7
	β	2.01	29.0		β	1.68	40.0
	γ_1	0.91 (d, 6.5)	19.0			1.45	
	γ_2	0.90 (d, 6.5)	18.1		γ	1,40	24.1
	CO		170.9		δ_1	0.90	22.7
					δ_2	0.84	21.4
					CO		172.0
Leu_6	NH	7.99 (d, 9.2)		**Leu_6**	NH	7.96 (d, 9.2)	
	α	4.22	51.8		α	4.20	51.5
	β	1.58	40.0		β	1.57	40.0
		1.48				1.50	
	γ	1.48	24.3		γ	1,50	24.4
	δ_1	0.86	22.9		δ_1	0.86	23.0
	δ_2	0.77	20.6		δ_2	0.78	20.8
	CO		171.6		CO		171.5
Leu_7	NH	7.02 (d, 10.0)		**Leu_7**	NH	6.97 (d, 7.5)	
	α	4.43	50.4		α	4.42	50.4
	β	1.32	41.0		β	1.32	41.1
		1.45				1.45	
	γ	1.45	24.1		γ	1.40	24.2
	δ_1	0.88	24.6		δ_1	0.89	22.9
	δ_2	0.80	22.4		δ_2	0.81	22.4
	CO		171.4		CO		171.4

The LSIMS spectrum depicted slight but significant ion peak supporting the above sequence. The data were consistent with the initial cleavage of the ring at the Tyr_1/Pro_2 amide bond, yielding a linear N-protonated acylium ion corresponding to the heptapeptide which further sequentially lost the following residues (*m/z*) : Tyr (593), Leu (480), Leu (367), Val (268), and Gly leading to the dipeptide H-Pro_2-Leu_3 acylium ion at *m/z* 211. The mass spectrum of **1** exhibited two additional peaks at *m/z* 643 and 544 which could be assigned to the loss of Leu_6 and Val_5, respectively and resulting from the cleavage of the cyclic peptide between the two Leu (Fig. 1).

Sequence determination of pohlianin B

The molecular formula $C_{40}H_{64}N_7O_8$ for pohlianin B (**2**) was established by HR-LSIMS [*m/z* 770.4812 (MH^+), calcd 770.4816]. The molecular mass difference between **2** and **1** (14 Da) suggested an additional methylene group. The NMR sequence determination was done as for pohlianin A. The result was confirmed by the LSIMS spectrum in which a series of protonated ions were found at *m/z* 607, 494, 381 and 268 resulting from the successive loss of Tyr_1, Leu_7, Leu_6 and Leu_5 and finally leading to the tripeptide H-Pro_2-Leu_3-Gly_4. Thus **2** differs from **1** by the replacement of Val_5 by Leu_5.

Sequence determination of pohlianin C

The LSIMS spectrum of **3** had the protonated molecule MH^+ at *m/z* 793 as the base peak and no adduct ions were observed. Its molecular formula was determined as $C_{40}H_{57}N_8O_9$ by HR-LSIMS (MH^+ *m/z* 793.4255, calcd 793.4248) in agreement with the amino acid composition in a cyclopeptide structure.

The ^1H NMR spectra in DMSO-d_6 at 298 K gave well-resolved sharp signals and the presence of minor conformers was not detected at this temperature. All proton resonances of **3** were assigned by analysis of COSY and TOCSY spectra (Table 2). HMQC and HMBC experiments allowed the assignment of the ^{13}C signals including those of the quaternary carbons (Table 2). Connectivities between neighbouring amino acids which were unequivocally established from the dαN (i, i+1) ROESY cross peaks (Figure 5) and the HMBC CO to NH correlations. The sequence was determined as : *cyclo* (-Gly_1-Gly_2-Thr_3-Ile_4-Ile_5-Phe_6-Gly_7-Phe_8-).

Conformational analysis of cycloheptapeptide pohlianin A (1)

Conformations in solution of cyclic heptapeptides such as segetalins D and E[10], pseudostellarin D[11], yunnanin A[12], evolidine[13] and stylostatin 1[14] were characterized by two β-turns with one β-bulge[15] structure. Generally, when proline is involved in a β-turn at the (i+3) position, this β-turn motif is of type VI. In order to analyze the solution conformational preference of **1**, the temperature coefficients of NH protons, the coupling

Table 2: ^{1}H and ^{13}C NMR spectral data for **3** (DMSO-d_6 298 K)

Residue		δ_H (mult, J in Hz)	δ_C	Residue		δ_H (mult, J in Hz)	δ_C
Gly$_1$	NH	8.91 (dd, 4.8, 5.1)		**Phe$_6$**	NH	8.25 (d, 5.6)	
	α	3.59	43.3		α	4.25	55.5
	CO		169.2		β	2.90	36.2
					γ		137.3[a]
Gly$_2$	NH	8.45 (t, 6.1)			δ	[7.10-7.25]	128.9[b]
	α	3.88 (dd, 16.5, 6.1)	42.0		ε	[7.10-7.25]	128.0[c]
		3.58			ζ	[7.10-7.25]	126.2[d]
	CO		168.7		CO		171.3
Thr$_3$	NH	7.51 (d, 9.7)		**Gly$_7$**	NH	8.23 (dd, 7.6, 3.9)	
	α	4.57	57.2		α	3.82 (dd, 16.8, 7.6)	42.5
	β	4.20	67.9			3.21 (dd, 16.8,3.9)	
	γ	0.97 (d, 6.3)	19.0		CO		169.1
	HO	5.37 (d, 9.6)					
	CO		170.3	**Phe$_8$**	NH	7.98 (d, 8.1)	
					α	4.55	53.6
Ile$_4$	NH	7.68 (d, 8.0)			β	2.85	36.5
	α	4.15 (dd, 8.0, 4.5)	57.7		γ		137.4[a]
	β	2.02	35.5		δ	[7.10-7.25]	129.1[b]
	γ_1	1.30	23.7		ε	[7.10-7.25]	128.1[c]
	γ'_1	1.25			ζ	[7.10-7.25]	126.4[d]
	γ_2	0.85 (d, 7.1)	15.7		CO		172.3
	δ	0.81 (t, 8.3)	11.7				
	CO		170.4				
Ile$_5$	NH	8.05 (d, 8.5)					
	α	4.05 (t, 8.5)	56.3				
	β	1.82	34.8				
	γ_1	1.30	24.3				
	γ'_1	1.06					
	γ_2	0.77 (d, 6.9)	15.1				
	δ	0.70 (t, 7.2)	10.0				
	CO		171.8				

[a,b,c,d] May be reversed within the same column

constant values and the distance geometry (DG) calculations using nuclear Overhauser effect (nOe) constraints were used.

Hydrogen bondings

To clarify the presence of intramolecular hydrogen bonds, the temperature dependence of the amide proton chemical shift was examined by NMR. First, for the purpose of investigating whether the molecules were associated or not, NMR spectra were recorded at various concentrations between 0.6 and 12.0 mM. As no

appreciable change in this range was observed, it was concluded that aggregation does not occur under these conditions. The temperature experiments were carried out over the range 298-323 K and a linear dependence of shifts was observed, showing no conformational modification and leading to the temperature coefficients reported in figure 4.

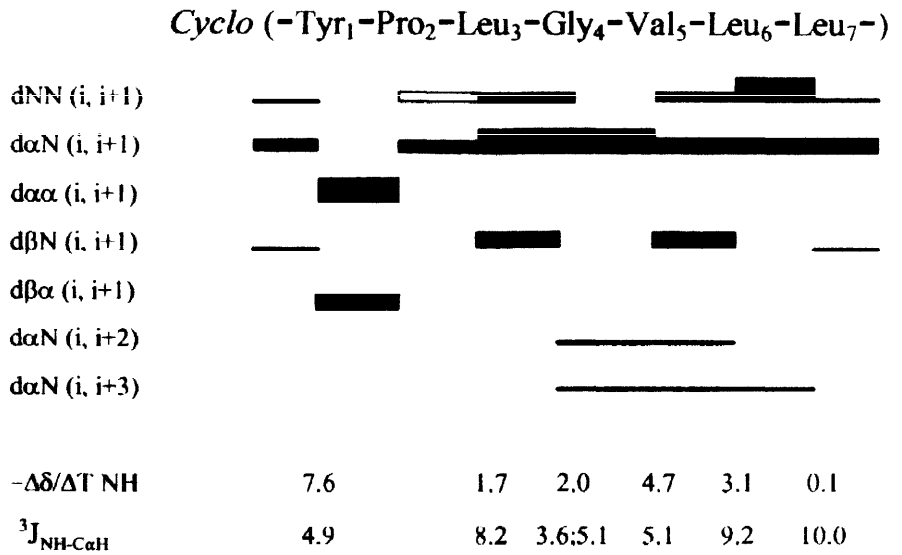

Figure 4 : Amino acid sequence of **1** and survey of the NOE connectivities involving NH and CαH (dαδ connectivities observed for proline are indicated by white boxes), of the $^{3}J_{NH-C\alpha H}$ coupling constants, and of the temperature coefficients of the amide protons. The observed NOEs are classified as strong, medium and weak and shown by thick, medium and thin lines, respectively.

The amide protons in Tyr_1 and Val_5 were clearly exposed to the solvent, whereas the low temperature coefficient of Leu_3, Gly_4 and Leu_7 indicated they were involved in hydrogen bonding. The middle value for Leu_6 may suggest a weak intramolecular hydrogen bond or the shielding by a hydrophobic lateral chain.

Tyr_1-Pro_2 amide bond geometry

Chemical shifts of β and γ carbons of Pro_2 at 29.5 and 21.2 ppm, respectively gave evidence for the presence of a *cis*-tyrosyl-proline amide bond[16]; additional evidence resulted from the strong ROE enhancements observed between α proton of Tyr_1 and α proton of Pro_2 in the ROESY spectrum.

Distance geometry calculations

The solution conformation of **1** was studied by DG calculations using distance constraints derived from ROESY experiments. They were classified into three ranges, 1.86-2.50, 1.86-3.50 and 2.50-4.50 Å corresponding to strong, medium and weak ROEs, respectively. The torsional constraints for amide bonds were taken into consideration, but no hydrogen bonds constraints were used.

The initial structures satisfying the experimental constraints were generated by DG calculations with the program DISTGEOM[17]. Finally, the produced conformers were subjected to energy minimization constraint with the AMBER all-atom force field (Table 3). Among 289 structures embedded by the DG method, 48 structures were converged ; the paiwise root mean square deviations (RMSD) for the backbone heavy atoms was less than 0.2 (Figure 5). Backbone dihedral angles in the mean structure of **1** are summarized in Table 4. RMSD between the individual structures and the mean coordinate position are 0.45Å for the backbone heavy atoms.

Table 3. Distance geometry data for pohlianins A (**1**) and C (**3**).

Structural parameters	**1**	**3**
Number of constraints		
distance	58	53
torsion	7	8
Number of converted conformers	48	54
Mean RMS ROE	0.2	0.2
RMSD for backbone heavy atoms of mean structure (Å)	0.45	0.31

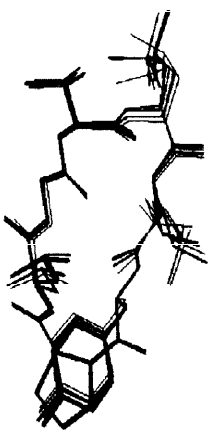

Figure 5 : Superposition of 48 stable conformers of pohlianin A (**1**) from DG calculations

The mean structure adopts a type I β-turn at Val_5-Leu_6 stabilized by a 4—>1 hydrogen bond between Leu_7-NH and Gly_4-CO, in agreement with the low temperature coefficient observed for the Leu_7 amide proton. The proposed solution conformation also showed a turn at Tyr_1-Pro_2 stabilized by a 4—>1 hydrogen bond between Leu_3-NH and Leu_7-CO and a third hydrogen bond between Gly_4-NH and Leu_7-CO, consistent with the low temperature coefficient of the Gly_4-NH, exhibiting a β-bulge motif (Table 5). Pro_2 occupied the i+2 position of a β VIa turn about the *cis* amide bond between residue 1 and 2, as described in axinastatin conformation[18]

Table 4 : Backbone dihedral angles in the mean structures of pohlianins A and C calculated from distance geometry calculations.

Pohlianin A				Pohlianin C			
Residues				Residues			
	Φ	Ψ	ω		Φ	Ψ	ω
Tyr_1	-64	144	-177	Gly_1	65	-170	177
Pro_2	-74	-15	-6	Gly_2	-60	-16	178
Leu_3	-63	-27	-173	Thr_3	-61	-7	175
Gly_4	-172	177	176	Ile_4	54	7	175
Val_5	-56	-39	-177	Ile_5	-60	150	-179
Leu_6	-82	-8	177	Phe_6	-40	99	-175
Leu_7	-157	71	173	Gly_7	70	-13	-179
				Phe_8	-79	72	175

or in other proline containing cyclopeptide[11,19,20]. The α and one the β protons of the proline are significantly shifted upfield relative to other proline containing cyclopeptides, indicating that the proline α proton and one the β protons are positioned close to the center of the Tyr aromatic ring. In the proposed conformation of **1**, the distance from the center of the aromatic ring to the α proton is found to be 2.6 Å and the distance between the center of the aromatic ring and the closest of the two β protons is 3.7 Å (Figure 6). The selective perturbation of the proline ring protons is consistent with a stacking of the pyrrolidine ring of the proline and the aromatic ring of the tyrosine, which have been already observed[21].

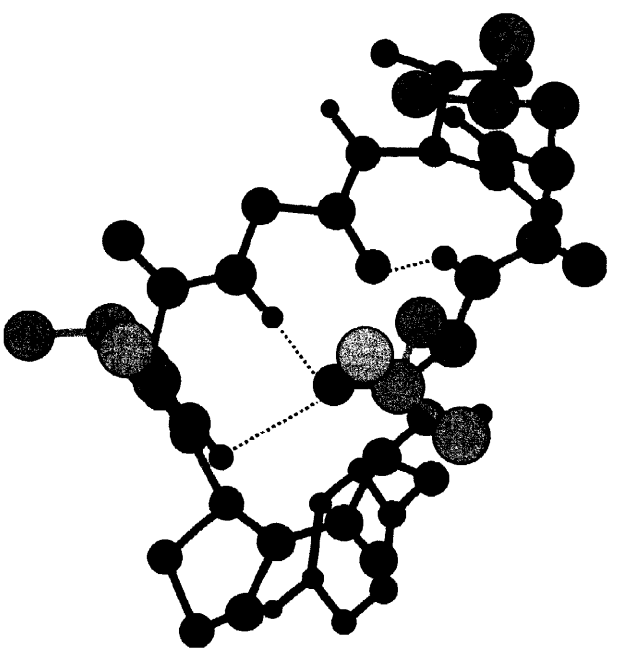

Figure 6 : Proposed conformation of pohlianin A (**1**) in solution. The three broken lines represent intramolecular hydrogen bonds.

Distance geometry calculations which considered ^{1}H-NMR information revealed a structure with a conventional type I β-turn around positions 5 and 6, and a β-bulge motif with a β VIa turn around Tyr_1-Pro_2 stabilized by stacking effect.

Table 5 : Intramolecular hydrogen bonds in mean structures of **1** and **3**.

Compound	From	To	Distance (Å)	Angle (°)[a)]
Pohlianin A	Leu_7-NH	Gly_4-CO	1.99	172
	Leu_3-NH	Leu_7-CO	2.48	160
	Gly_4-NH	Leu_7-CO	2.04	169
Pohlianin C	Ile_4-NH	Gly_1-CO	1.91	170
	Phe_8-NH	Ile_5-CO	1.97	165

[a)] Angles N-H···O

Conformational analysis of the cyclooctapeptide pohlianin C (3)

Usually, cyclooctapeptides exhibit less well-defined conformers than smaller cyclopeptides and are characterized by random coil $^3J_{HN\text{-}Ha}$ coupling constants around 7Hz and by high temperature coefficients[4]. Nevertheless, cyclic octapeptide such as hymenistatin 1[22] showed a dominant conformation in DMSO containing two well-defined β-turns.

In order to determine the conformational preference of **3** in DMSO, the temperature coefficients of amide protons were calculated (Figure 8) and DG calculations were performed. Temperature dependence studies suggested Gly_1, Gly_2, Ile_5, Phe_6 and Gly_7 to be solvent exposed, while Thr_3 and Ile_4 to be involved in intramolecular hydrogen bonds

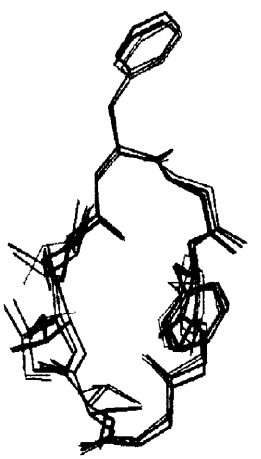

Figure 7 : Superposition of 54 stable conformers of pohlianin C (**3**) from DG calculations

or buried in hydrophobic pocket (Table 3 and Figure 7).

RMSD between the individual structures and the mean coordinate position are 0.3 Å for the backbone heavy atoms. The mean structure adopts a type I β-turn at Gly_2-Thr_3 stabilized by a hydrogen bond between Ile_4-NH and Gly_1-CO, and a type II β-turn at Phe_6-Gly_7 with a hydrogen bond between Phe_8-NH and Ile_5-CO (Table 5). Side chain conformation of Phe_6 and Phe_8 was indicated to be *trans* in the mean structure.

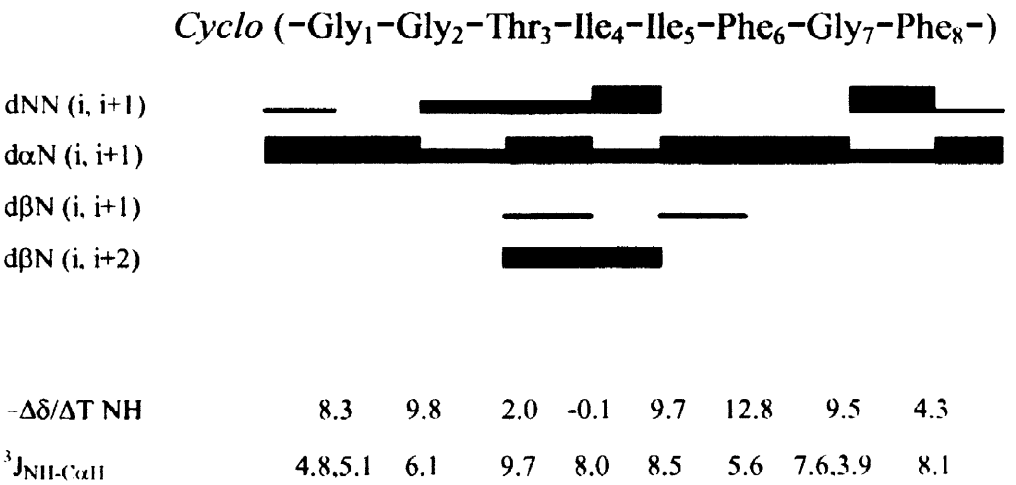

Figure 8 : Amino acid sequence of **3** and a survey of the NOE connectivities involving NH and CαH (dαδ connectivities observed for proline are indicated by white boxes), of the $^3J_{NH-C\alpha H}$ coupling constants, and of the temperature coefficients of the amide protons. The observed NOEs are classified as strong, medium and weak and shown by thick, medium and thin lines, respectively.

EXPERIMENTAL

Plant material

Jatropha pohliana Muell. Arg. ssp. *molissima* Muell. Arg. was collected in September 1997 at Recife in Brasil, and identified by one of us (H.S. Xavier). A voucher specimen was deposited in the herbarium of the Pharmacy Department of the Federal University of Pernambuco, Recife (Brasil). Crude latex was obtained by cutting off leaf stalks, adding a few drops of EtOH to prevent the latex from excessive foaming and stored at -20°C until use.

Extraction and isolation

To 120 ml of crude latex, 60 ml of demineralized H_2O were added and the mixture extracted with 3 x 100 ml of EtOAc. The solvent was removed by evaporation under reduced pressure and the crude residue (561 mg) dissolved in MeOH and chromatographed on Sephadex LH-20 eluted with MeOH. The peptide fraction (336 mg) was then chromatographed over silica gel (Kieselgel 60H Merck) with CH_2Cl_2 / MeOH 85/15. The peptide purification (186 mg) was monitored by TLC (SiO_2, Merck 60 F_{254}) with CH_2Cl_2 / MeOH 85/15 as eluent system. When detected with the Cl_2 / *o*-tolidine reagent, the peptides exhibited two blue spots : R_f 0.74 (**1** and **2**); R_f: 0.67 (**3**). The peptide mixture was purified by reversed phase HPLC (Kromasil C_{18}, 250 x 7.8 mm, 5mm, AIT France; 27% H_2O in MeOH; flow rate 2 cm^3 min^{-1}; detection 220 nm) to yield pohlianin A (**1**) (t_R : 21.5 min, 21.6 mg), pohlianin B (**2**) (t_R : 25.5 min, 26.0 mg) and pohlianin C (**3**) (t_R : 30.0 min, 34.7 mg).

General data

The $[\alpha]_D$ values were determined with a Perkin-Elmer Model 243 B polarimeter.

1 : $[\alpha]_D^{22}$ -122° (c 0.2, MeOH)

2 : $[\alpha]_D^{22}$ -120° (c 0.2, MeOH)

3 : $[\alpha]_D^{22}$ -38° (c 0.2, MeOH)

Amino acid composition and absolute configuration

Pohlianins A, B and C (1 mg) were hydrolyzed with 6 N HCl (0.5 ml) in a sealed tube at 110 °C for 24 h under argon and then, the reagents were removed under reduced pressure. The residues were dissolved in 0.2 M Na-citrate buffer (pH 2.2) and amino acid composition was determined by cation-exchange chromatography on a Liquimat 2 Amino Acid Analyzer (Kontron) and detected with the OPA reagent. The absolute configuration of amino acids was determined on a chiral capillary column by GC. The peptide hydrolysates were dried over KOH pellets and the crude residues dissolved in an anhydrous solution of 3 N HCl in 2-propanol and heated at 110 °C for 20 min. The reagents were evaporated under reduced pressure, the residues were dissolved in CH_2Cl_2 (0.5 ml), and 0.5 ml of trifluoroacetic anhydride was added. The mixtures were kept in a screw-capped tube at 100 °C for 5 min. The reagents were evaporated, and the GC analyses were realized on a Hewlett Packard serie II 5890 gas chromatograph on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column (Chrompack, 25m length, 0.2mm i.d.) with He (1.1 bar) as carrier gas and the following temperature program : 50-130 °C at 3 °C min^{-1}, then 130-190 °C at 10 °C min^{-1}. The retention times of the *N*-trifluoroacetyl isopropyl ester derivatives were compared with those of references.

Composition and absolute configuration of amino acids (number) **1** : Gly (1), *L*-Leu (3), *L*-Pro (1) *L*-Tyr (1) and *L*-Val (1); **2** : Gly (1), *L*-Leu (4), *L*-Pro (1) and *L*-Tyr (1); **3** : Gly (3), *L*-Ile (2), *L*-Phe (2), and *L*-Thr (1).

Secondary ion mass spectrometry

Positive LSIMS spectra were recorded on a ZAB2-SEQ (VG Analytical, Manchester, UK) mass spectrometer equipped with a standard FAB source and a Cs ion gun operating at 35 kV. The peptide methanolic solution was mixed with a-monothioglycerol as matrix. Positive HR-LSIMS were recorded on a ZAB-HF spectrometer ; the resolution was 2000.

Pohlianin A : *m/z* (relative intensity) 211 (10), 268 (25), 339 (5), 367 (13), 480 (8), 544 (3), 593 (7), 643 (4), 740 (8), 756 (MH^+, 100), 778 ($[M+Na]^+$, 15), 794 ($[M+K]^+$, 4).

Pohlianin B : *m/z* 268 (12), 293 (33), 381 (5), 494 (3), 507 (3), 607 (4), 663 (3), 743 (3), 754 (23), 770 (MH^+, 100)

Pohlianin C : *m/z* 262 (4), 293 (9), 777 (10), 793 (MH^+, 100)

NMR Spectroscopy

^1H and ^{13}C NMR spectra were run in DMSO-d_6 using a Bruker AC 300 spectrometer, equipped with an Aspect 3000 computer using DISNMR software. A 10 mg sample of **1**, **2** and **3** in a 5-mm tube (0.5 ml of degassed DMSO-d_6) was used for homonuclear and heteronuclear measurements. The one dimensional ^1H NMR spectra (300.13 MHz, DMSO-d_6, 298 K) were obtained with 64-186 scans.

For **1**, the COSY spectrum was run with a total of 256 experiments of 40 scans each with a sweep width in F_2 of 2994 Hz (size 2K), zero filling in F_1 (size 1K). For the TOCSY spectra, 256 experiments of 128 scans were performed with a sweep width in F_2 of 3311 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing functions shifted by $\pi/2$ in F_1 and F_2 were applied. A phase-sensitive ROESY NMR experiment was obtained with mixing time of 150 ms. A total of 256 experiments of 112 scans each were acquired with a sweep width in F_2 of 3311 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing functions shifted by $\pi/2$ in F_1 and F_2 were applied.

For **2**, the COSY spectrum was run with a total of 256 experiments of 20 scans each with a sweep width in F_2 of 2941 Hz (size 2K), zero filling in F_1 (size 1K). For the TOCSY spectra, 256 experiments of 112 scans were performed with a sweep width in F_2 of 3311 Hz (size 2K) zero filling in F_1 (size 1K); sine bell weighing functions shifted by $\pi/2$ in F_1 and F_2 were applied. A phase-sensitive ROESY NMR experiment was obtained with mixing time of 150 ms. A total of 256 experiments of 112 scans each were acquired with a sweep width in F_2 of 3311 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing functions shifted by $\pi/2$ in F_1 and F_2 were applied.

For **3**, the COSY spectrum was run with a total of 256 experiments of 16 scans each with a sweep width in F_2 of 2825 Hz (size 2K), zero filling in F_1 (size 1K). For the TOCSY spectra, 256 experiments of 112 scans were performed with a sweep width in F_2 of 3144 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing

functions shifted by $\pi/2$ in F_1 and F_2 were applied. A phase-sensitive ROESY NMR experiment was obtained with a mixing time of 150 ms. A total of 256 experiments of 112 scans each were acquired with a sweep width in F_2 of 3144 Hz (size 2K), zero filling in F_1 (size 1K); sine bell weighing functions shifted by $\pi/2$ in F_1 and F_2 were applied.

The ^{13}C NMR experiments were acquired at 75.47 MHz using a ^{1}H-^{13}C Dual probehead. The delay preceding the ^{13}C pulse for the creation of multiple quanta coherences through several bonds in the HMBC was set to 70 ms. Heteronuclear coupling constant value used in the HMQC experiment to establish the delay needed to select the protons coupled to carbon was 135 Hz.

Computational Methods

Computer modeling and all calculations were carried out using the molecular-modeling software package TINKER. DG, SA and molecular mechanics calculations were performed with the AMBER all-atom force field. Solvent molecules were not included in the calculations. The distance geometry structures were regularized in DISTGEOM. Further refinement of the structures employed simulated annealing. The structures were initially equilibrated at 200°C for 1000 steps of molecular dynamics at a time-step of 0.04 ps, followed by 10000 dynamics steps of cooling to 0°C at a time-step of 0.02 ps. Each energy minimization was carried out until the derivatives became less than 0.01 $kcal.mol^{-2}$ A^{-1} using the OPTIMIZE program.

ACKNOWLEDGEMENTS

We thank Pr. Ph. Grellier (Laboratoire de Biologie Parasitaire, MNHN) for antimalarial tests and Dr. M. Becchi (Centre de Spectroscopie du CNRS, Lyon) for LSIMS measurements.

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