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3D-structure of cycloreticulin C and glabrin A, cyclopeptides from the seeds of *Annona reticulata*

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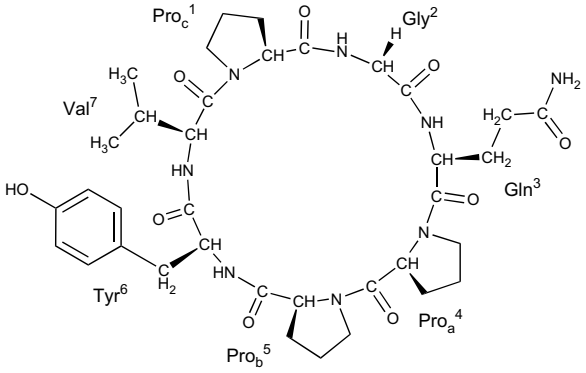
ABSTRACT

Two cyclopeptides, the cycloheptapeptide cycloreticulin C, *cyclo*(Pro[1]-Gly[2]-Gln[3]-Pro[4]-Pro[5]-Tyr[6]-Val[7]) (**1**), and the cyclohexapeptide glabrin A, *cyclo*(Pro[1]-Gly[2]-Leu[3]-Val[4]-Ile[5]-Tyr[6]) (**2**), have been isolated from the methanol extract of the seeds of *Annona reticulata*. Their structures were elucidated on the basis of the MS/MS fragmentation using a Q-TOF mass spectrometer equipped with an ESI source, chemical degradation and extensive 2D-NMR. The solution conformation of cycloreticulin C involves two β-turns, one of type II with *trans*-Pro[1] and Gly[2] at the corners, another of type VIa with *trans*-Pro[4] and *cis*-Pro[5] at the corners, and followed by a β-bulge at the Tyr[6]-Val[7] level. The solid state and solution conformations of glabrin A have been analyzed by X-ray and 2D-NMR studies, respectively, and are characterized by the presence of two β-turns, the first of type VIa and the second intermediary between types I and III at the solid state and a γ-turn in solution.

1. Introduction

Annona reticulata L (Annonaceae) is a small tree, native in tropical America, which has now a widespread pantropical distribution, as it is cultivated for its fruits. Traditional medicine also used this species as antiparasitic, insecticide, antidiarrhoeic and antidysenteric.[1] In continuation of our programme on cyclopeptides from plants,[2–7] we have investigated *A. reticulata*, and recently isolated two cyclooctapeptides, cycloreticulins A and B.[8] Plant cyclopeptides continue to arouse interest due to the questions of structure and bioactivity they ask.[9] We now report on the isolation, the sequence determination based on tandem mass spectroscopy and 2D-NMR and the solution conformation of a new cycloheptapeptide, cycloreticulin C (**1**), together with the X-ray and solution structure of glabrin A (**2**) a cyclohexapeptide isolated for the first time from *A. reticulata*. Glabrin A (**2**) was previously isolated from *A. glabra* by Li et al., but only its sequence was described.[10] Cycloreticulin C (**1**) and glabrin A (**2**) enclose three and one proline residues, respectively. We took in consideration the opportunity of the presence of prolines in these cyclopeptide structures to analyze the effects of this residue on the 3D-structures. In fact, although the backbone of cyclic peptides is generally considered to be flexible, it is interesting to analyze the effects of the presence of one or several prolines on the conformation. Actually, if proline residues constraint the conformation due to the fixed Φ angle they introduce at their level and thus favour stable conformations, on the contrary due to the lack of one intramolecular hydrogen bond that they involve, they increase the flexibility of the ring. In addition, they can be involved in *cis*- or *trans*-Xaa-Pro amide bonds. Thus, when available, it is of interest to analyze and compare the solid and solution conformations of cyclopeptides.



Cycloreticulin C (1)

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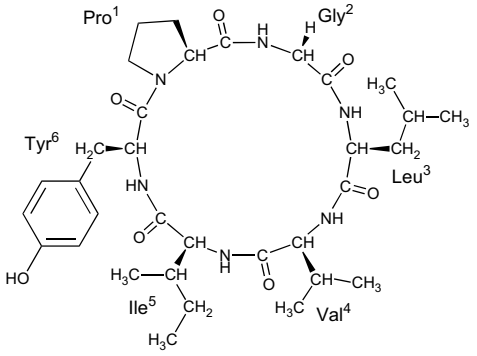

Glabrin A (2)

2. Results and discussion

2.1. Isolation of cyclopeptides

The dried and ground seeds of *A. reticulata* were extracted with methanol and from the ethyl acetate soluble fraction of this extract two peptides, cycloreticulin C (**1**) and glabrin A (**2**), were isolated. They were purified successively by exclusion chromatography, silica gel column chromatography and C_{18} reversed-phase HPLC. Positive reaction with chlorine/*o*-tolidine reagent suggested that they were peptides and the absence of colouration with ninhydrin of the spots on thin layer chromatography suggested that these peptides were cyclic. The total acidic hydrolysis and amino acid analysis of the hydrolyzate after derivatization indicated the presence of Gly (1), Glx (1), Pro (3), Tyr (1) and Val (1) for cycloreticulin C (**1**) and Gly (1), Ile (1), Leu (1), Pro (1), Tyr (1), and Val (1) for glabrin A (**2**). The amino acids in the acidic hydrolyzate were converted into the *n*-propyl esters of their *N*-trifluoroacetyl derivatives. These esters were analyzed by gas chromatography on a chiral capillary column and their retention times were compared with those of standards. All the chiral amino acids were L. The Glx residue in the hydrolyzate was further identified as Gln from the absence of carboxylic acid group and from ^{1}H NMR data for the peptide **1**. Glabrin A has been previously characterized in *A. glabra*,[10] and its sequence is now confirmed from the present X-ray crystallographic study and, in addition, its solution conformation was analyzed and compared to the crystal one, and in addition both solution and crystal structures were compared.

2.2. Sequence determination by mass spectrometry

Cyclopeptides are not easily sequenced even by mass spectrometry. The reason is that multiple and indiscriminate ring-opening reactions occur during the collision-induced dissociation (CID) of cyclic peptides, resulting in the superimposition of random fragment ions and making the interpretation difficult.[11,12] However, we have shown that when a proline is present in the sequence, a specific fragmentation occurs at the peptidyl-prolyl (Xaa-Pro) amide bond, leading to a linear peptide C-ended by an acylium ion (b_n), which undergoes further fragmentation generating series of acylium ions from which the sequence could be deduced.[4,8] This specific fragmentation is explained by the more basic nature of the proline nitrogen, relative to the other peptide bond nitrogens. In this way, the more basic site at proline level strongly directs the protonation making the fragmentation less complex. However, the presence of several prolines in a cyclic peptide could lead to several linearized peptides, the fragmentations of which could make more difficult the interpretation.

The molecular weight 738 for cycloreticulin C (**1**) was deduced from the positive ESI-Q-TOF spectra, which displayed the $[M+K]^+$ adduct ion at *m*/*z* 777, the $[M+Na]^+$ adduct ion at *m*/*z* 761 and the protonated molecular $[M+H]^+$ ion at *m*/*z* 739. According to the amino acid analysis the molecular formula $C_{36}H_{50}N_8O_9$ was assigned to **1**. The CID spectrum of the $[M+H]^+$ ion at *m*/*z* 739 showed a main series of adjacent b_n ions at *m*/*z* 640, 380, 283 and 155, corresponding to the successive loss of Val, Tyr-Pro, Pro and Gln yielding the terminal dipeptide ion $[H\text{-Pro-Gly}]^+$ and suggesting the sequence H-Pro^1-Gly^2-Gln^3-Pro^4-Pro^5-Tyr^6-Val^7 for the linearized peptide (Fig. 1A). A second series of ions with peaks at *m*/*z* 642, 457, 360 and 261 were assigned to a second b_n ion series, showing the successive loss of Pro, Gln-Gly, Pro and Val and yielding the terminal dipeptide ion $[H\text{-Pro-Tyr}]^+$, which indicates the sequence H-Pro^5-Tyr^6-Val^7-Pro^1-Gly^2-Gln^3-Pro^4 (Fig. 1B). A third series of adjacent b_n ion peaks at 611, 457, 358 and 195, corresponding to the successive loss of Gln, Pro-Gly, Val and Tyr yielding the terminal dipeptide ion $[H\text{-Pro-Pro}]^+$ and suggesting the sequence H-Pro^1-Pro^7-Tyr^6-Val^7-Pro^1-Gly^2-Gln^3 (Fig. 1C). This sequence was confirmed by observation of three corresponding a_n ion series. The ESI-Q-TOF spectrum of **1** had the $[M+Na]^+$ adduct ion at *m*/*z* 761, which was further subjected to CID experiments and two a_n ion series, with fragment ions at *m*/*z* 761, 662, 402, 305 and 177 for the first and *m*/*z* 761, 664, 536, 479, 382 and 283 agreed with the sequences [Pro^1-Gly^2-Gln^3-Pro^4-Pro^5-Tyr^6-Val^7] and [Pro^5-Tyr^6-Val^7-Pro^1-Gly^2-Gln^3-Pro^4], respectively (Fig. 1D and 1E). No significant ion fragmentation was observed due the cleavage of Gln-Pro amide bond. All these data were thus in agreement with the linear sequence proposed for cycloreticulin C (**1**). This cyclic peptide enclosing three prolines is thus linearized by three different cleavages at the three possible sites before a proline, but the obtained mass spectrum remains interpretable.

The molecular weight 642 for glabrin A (**2**) was deduced from the positive ESI-TOF spectrum, which displayed the $[M+K]^+$ and $[M+Na]^+$ adduct ions at *m*/*z* 681 and 665, respectively, and the protonated molecular $[M+H]^+$ ion at *m*/*z* 643. According to the amino acid composition, the molecular formula $C_{33}H_{50}N_6O_7$ was assigned to **2**. The protonated molecular ion $[M+H]^+$ of **2** at *m*/*z* 643 was subjected to CID experiment (Fig. 2). The ring opening began at

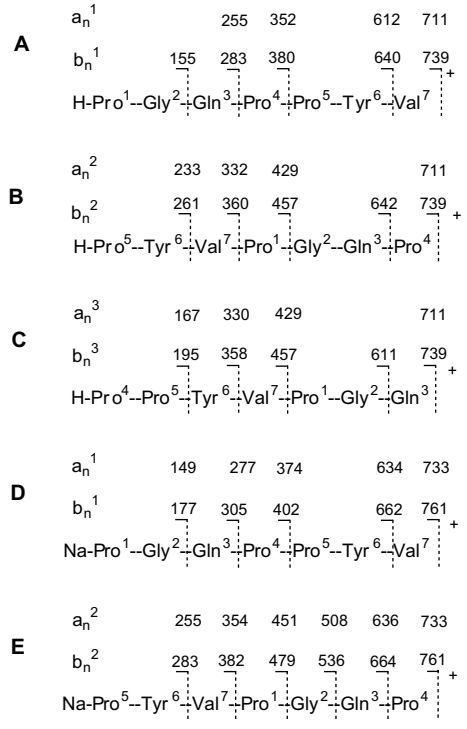

Figure 1. Mass fragmentation for the protonated molecular $[M+H]^+$ (**A**–**C**) and sodium adduct (**D** and **E**) ions of cycloreticulin C (**1**).

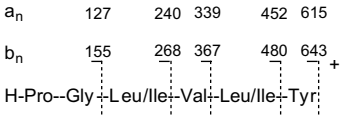

Figure 2. Mass fragmentation for the protonated molecular $[M+H]^+$ ion of glabrin A (**2**).

the Tyr-Pro amide bond level and a series of adjacent acylium ions (b_n) at *m*/*z* 480, 367, 268 and 155 were generated from which the sequence could be deduced: amino acid residues were lost sequentially from the C-terminus to the N-terminus and for glabrin A (**2**) was observed the successive loss of Tyr, Ile/Leu, Val and Ile/Leu yielding the N-terminal dipeptide Pro-Gly. A second significant series of ions were observed at *m*/*z* 615, 452, 339, 240 and 127, which were assigned to adjacent a_n ions related to the above b_n ion series. These results suggested the sequence [H-Pro^1-Gly^2-Leu/Ile^3-Val^4-Leu/Ile^5-Tyr^6]$^+$, further confirmed by 2D-NMR. This method, in addition, allowed the respective positions of Leu and Ile to be unambiguously determined and to identify **1** as glabrin A, a cyclopeptide previously characterized in *A. glabra*.

2.3. ^{1}H and ^{13}C NMR studies

The ^{1}H NMR spectrum of cycloreticulin C (**1**) in pyridine-d_5 solution (Table 1A) and in DMSO-d_6 solution (Table 1B) showed

Table 1
^{13}C and ^{1}H NMR data for cycloreticulin C (**1**) (in pyridine-d_5 (**A**) and in DMSO-d_6 (**B**) at 298 K

Residue	A				B			
	δ_C	δ_H	m	*J* (Hz)	δ_C	δ_H	m	*J* (Hz)
Pro$_c^1$ CO	173.1	—			172.2	—		
α CH	62.7	4.34	dd	9.4, 7.2	61.9	4.09	m	
β CH_2	29.1	2.06	m		28.3	2.11	m	
	—	2.06	m		—	1.75	m	
γ CH_2	25.9	1.87	m		25.3	2.00	m	
	—	1.60	m		—	1.82	m	
δ CH_2	47.8	3.42	m		47.3	3.75	m	
	—	3.37	m		—	3.51	m	
Gly^2 CO	169.8	—			168.6	—		
NH	—	9.77	d	8.4, 4.2	—	8.71	dd	7.9, 4.4
α CH_2	43.9	4.74	dd	17.0, 8.4	42.4	3.98	dd	17.5, 7.9
	—	4.02	d	17.0, 4.2	—	3.50	dd	17.5, 4.4
Gln^3 CO	170.2	—			168.5	—		
NH	—	8.60	d	8.0	—	7.89	d	7.8
α CH	51.8	5.22	ddd	8.0, 7.5, 7.5	50.5	4.43	dd	17.5, 7.8
β CH_2	28.2	2.72	m		31.5	1.95	m	
	—	2.54	m		—	1.60	m	
γ CH_2	33.0	2.84	dd	14.0, 4.6	26.8	2.10	m	
	—	2.69	dd	14.0, 4.6	—	1.98	m	
δ CO	175.0	—			173.6	—		
ε NH_2	—	8.09	s	*anti*	—	7.18	s	
		7.50	s	*syn*	—	6.65	s	
Pro$_a^4$ CO	170.8	—			169.9			
α CH	59.8	4.80	dd	9.4, 7.2	58.8	4.19	m	
β CH_2	23.7	2.01	m		28.0	2.11	m	
	—	1.82	m		—	1.70	m	
γ CH_2	25.0	2.01	m		24.5	1.93	m	
	—	1.40	m		—	1.87	m	
δ CH_2	47.7	3.78	ddd	10.8, 7.0, 3.8	46.8	3.45	m	
	—	3.53	m		—	3.40	m	
Pro$_b^5$ CO	171.5	—			170.4			
α CH	61.4	4.64	d	7.2	60.0	4.35	m	
β CH_2	31.7	2.39	m		30.7	1.98	m	
	—	1.80	m		—	1.80	m	
γ CH_2	21.8	1.38	m		21.0	1.50	m	
	—	1.01	m		—	0.64	m	
δ CH_2	47.0	3.41	m		46.0	3.13	m	
	—	3.41	m		—	2.85	m	
Tyr^6 CO	172.3	—			170.9	—		
NH	—	8.47	d	7.6	—	7.95	d	7.4
α CH	59.4	5.15	ddd	8.0, 7.6, 3.7	58.0	4.17	m	
β CH_2	38.8	3.56	dd	14.0, 3.7	37.0	3.07	dd	4.1, 14.0
	—	3.41	dd	14.0, 8.0	—	2.88	dd	14.0, 10.0
1′	128.4	—	m		127.2	—		
2′6′	130.7	7.35	d	8.5	129.6	6.98	d	6.5
3′5′	116.5	7.07	d	8.5	115.0	6.67	d	6.6
4′	157.9	—			156.0			
OH	—	11.39			—	9.24	s	
Val^7 CO	171.7	—			170.3	—		
NH	—	7.75	d	7.9	—	8.73	d	7.8
α CH	55.7	5.02	dd	7.9, 3.3	54.7	4.39	dd	7.8, 3.2
β CH	32.8	2.38	m		31.4	2.39	m	
γ CH_3	21.1	1.18	d	6.9	20.6	0.91	d	6.9
γ' CH_3	17.9	1.12	d	6.9	17.2	0.74	d	6.9

a main stable conformational state (>95%) where the four amide protons were clearly depicted, one triplet of one Gly, three doublets due to one Tyr, one Gln and one Val. The presence of a Tyr residue was evident, as characteristic signals for a *para*-disubstituted benzene ring were observed at 7.35 and 7.07 ppm (pyridine-d_5) or at 6.98 and 6.67 ppm (DMSO-d_6). Assignment of protons and carbons to amino acid residues was achieved, as usual, from the COSY, TOCSY and HSQC data. The three prolines, Pro_a, Pro_b and Pro_c, were depicted. The peptide sequence determination was based on the data of HMBC experiment. This heteronuclear methodology was preferred, when possible, to the homonuclear method based on $d_{NN(i,\ i+1)}$ and $d_{\alpha N(i,\ i+1)}$ connectivities from the ROESY/NOESY spectra,[13–15] because for small size cyclic peptides, conformational NOE information can interfere with sequential ones. All the amino acid spin systems were identified using scalar spin–spin couplings determined from the ^{1}H–^{1}H COSY and TOCSY experiments.[16]

The ^{13}C NMR assignments of the protonated carbons were obtained from the proton detected heteronuclear HSQC spectrum and combined with the HMBC experiment optimized for a long-range *J* value of 7 Hz, for the non-protonated carbons. This experiment allowed the carbonyl groups to be assigned. By this way, the sequence determination was done from the connectivities between the carbonyl of residue *i* and the amide and/or the α protons of residue *i*+1. Analysis of the long-range correlations in the HMBC spectrum allowed in a first step the assignment of the eight carbonyls to definite residues from the observed intra-residue correlations with αH and βH, and with βH and γH for the ε-carbonyl of the glutamine. Then, the following sequential correlations between the CO of residue and the NH group of residue (*i*, *i*+1) were depicted in HMBC spectrum in pyridine-d_5 solution: Pro^1 to Gly^2, Gly^2 to Gln^3, Pro^5 to Tyr^6 and Tyr^6 to Val^7 (Fig. 3). Two peptide fragments of three residues ended by a proline were thus defined: (i) Pro^1-Gly^2-Gln^3, (ii) Pro^5-Tyr^6-Val^7. They were further connected to each other from the strong NOEs observed in the ROESY spectrum between the αH of Val^7 and the δδ′H of Pro_c, and this Pro thus occupies the position 1. The αH of Pro_b^5 gave a strong NOE correlation with the αH of Pro_a, which was assigned the position 4. In addition, this last NOE clearly indicated that the Pro_a^4-Pro_b^5 amide bond was *cis*. Finally, the NOE interaction between the α proton of Gln^3 and the δδ′ protons of Pro_a^4 allowed to link these two residues and thus to cyclize the peptide. This NOE as well as that between the α proton of Val^7 and δδ′ protons of Pro_b^5 indicated that the Gln^3-Pro_a^4 and Val^7-Pro_b^5 amide bonds were trans. The chemical shift of the γ carbon is expected at 21–22 ppm for *cis*-proline and 24–25 ppm for *trans*-proline. The observed ^{13}C shift values for Pro_c^1, Pro_a^4 and Pro_b^5 at 25.3, 24.5 and 21.0 ppm, respectively, are in concordance with the above proposed amide bond stereochemistry (Table 1A and B). The complete cyclic sequence of **1** was thus defined as *cyclo*(Pro_c^1-Gly^2-Gln^3-Pro_a^4-Pro_b^5-Tyr^6-Val^7).

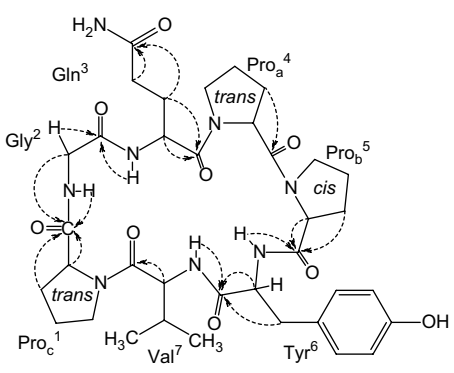

Figure 3. HMBC (H→C) correlations observed for cycloreticulin C (**1**).

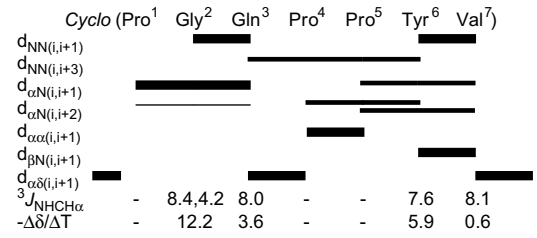

Figure 4. NOESY diagram showing the distribution of the NOE correlations (weak and strong) between NH, αH, βH, γH and δH, the temperature coefficients of amide protons ($-\Delta\delta/\Delta T$, ppb K^{-1}) and the coupling constants ($^3J_{NHCH\alpha}$, Hz) for cycloreticulin C in DMSO-d_6 solution.

A β-turn of type II, with *trans*-Pro_c^1 and Gly^2 at the corners, was suggested from the strong NOE correlation between the α-proton of Pro^1 and the NH of Gly^2 (Fig. 4) and this turn is stabilized by a hydrogen bond between the NH of Gln^3 and the CO of Val^7. A second β-turn involving *trans*-Pro^4 and *cis*-Pro^5 at the corners is stabilized by a hydrogen bond between the NH of Tyr^6 and the CO of Gln^3. It is of type VIa, because strong NOE correlations were observed between the αH of Pro^4 and the αH of Pro^5. The significant NOEs observed between the NH of Val^7 and the NH of Tyr^6 are explained by a β-bulge with a bifurcated hydrogen bond involving the CO of Gln^3 and the NH of Val^7 and Tyr^6. The pattern of the hydrogen bonding, as shown in Figure 5, is in agreement with the thermal coefficient values of the amide protons, measured in DMSO-d_6 solution. The results indicated that the amide protons of Gln^3, Tyr^6 and Val^7 are strongly involved in intramolecular hydrogen bonds, and those of Gly^2 are exposed to solvent (Fig. 4). All the data agreed with a cyclic structure for cycloreticulin C (**1**), with a backbone conformation containing two β-turns, one of type II and the other of type VIa and incorporating a β-bulge involving Val^7 and Tyr^6. This structure, with two β-turns, one of them being adjacent to a β-bulge, seems to be a favourable motif for cyclic heptapeptides, as previously shown.[4,5]

The ^{1}H NMR spectrum of glabrin A (**2**) in DMSO-d_6 solution (Table 2) where the five amide protons were clearly depicted showed a main stable conformational state (>95%). As well, the presence of six carbonyl groups in the ^{13}C NMR spectrum was in agreement with a hexapeptide structure including a proline. In the HMBC spectrum, the CO of Gly^2 at 168.5 ppm was correlated to both αH and α′H of Gly^2 and to both αH and NH of Leu^3, the CO of Leu^3 at 172.0 ppm to ββ′H of Leu^3 and to both αH and NH of Val^4. Similarly, the CO group of Val^4 at 170.7 ppm gave correlations with the αH of Val^4 and both the αH and the NH of Ile^5, the CO of Ile^5 at 171.3 ppm with the αH of Ile^5 and the NH of Tyr^6, the CO of Tyr^6 at 169.7 ppm with the ββ′H of Tyr^6 and the αH of Pro^1, and finally the CO of Pro^1 at 171.3 ppm with the αH, ββ′H of Pro^1 and the αH and NH of Gly^2

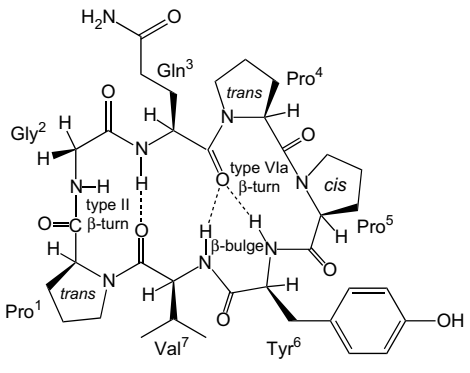

Figure 5. Proposed solution structure of cycloreticulin C (**1**) (dotted lines indicate hydrogen bonds).

Table 2
^{13}C and ^{1}H NMR data, HMBC correlations of glabrin A (**2**) in DMSO-d_6 (400 MHz, 308 K)

Residue	δ_C	δ_H	m	J (Hz)	HMBC correlations with H
Pro^1 CO	171.3	—			α, β,γH Pro^1; αH, NH Gly^2
α CH	60.2	3.12	d	8.1	
β CH_2	29.7	1.90	dd	11.7, 8.1	
	—	0.98	m		
γ CH_2	21.5	1.60	m		
	—	1.30	m		
δ CH_2	46.2	3.23	m		
	—	3.23	m		
Gly^2 CO	168.5	—			αH, α'H Gly^2; αH, NH Thr^3
NH	—	9.08	dd	9.0, 3.2	
α CH_2	41.4	4.09	dd	9.0, 16.6	
	—	3.58	dd	3.2, 16.6	
Leu^3 CO	172.0	—			ββ'H Leu^3; αH, NH Gly^4
NH	—	7.89	d	5.5	
α CH	53.5	3.81	ddd	9.6, 5.5, 5.5	
β CH_2	40.4	1.46	m		
	—	1.37	m		
γ CH_2	24.3	1.67	m		
δ CH_3	22.8	0.88	d	6.5	
δ CH_3	21.3	0.82	d	6.5	
Val^4 CO	170.7	—			αH Val^4; NH, αH Ile^5
NH	—	7.80	d	10.0	
α CH	60.9	3.82	dd	10.0, 10.0	
β CH	29.6	2.01	m		
γ CH_3	19.3	0.86	d	6.5	
γ′CH_3	19.3	0.78	d	6.5	
Ile^5 CO	171.3	—			αH Ile^5; NH Tyr^6
NH	—	7.39	d	9.3	
α CH	55.3	4.15	dt	9.3, 9.7	
β CH	37.0	1.55	m		
γ CH_2	24.3	1.42	m		
	—	1.06	m		
γ′CH_3	14.8	0.83	d	6.7	
δ CH_3	10.4	0.82	t	7.4	
Tyr^6 CO	169.7	—			αH Pro^1; ββH Tyr^6
NH	—	8.74	d	2.2	
α CH	54.2	4.26	ddd	11.0, 4.4, 2.2	
β CH_2	36.4	2.93	dd	12.5, 4.4	
	—	2.67	dd	12.5, 11.0	
1′	125.8	—			
2′6′	130.3	6.95	d	8.4	
3′5′	115.3	6.68	d	8.4	
4′	156.4	—			
OH	—	9.36	s		

(Table 2). All the $^3J_{CH}$, CO (i) to NH (i+1) correlations were depicted in the HMBC spectrum, in agreement with the structure deduced above from the mass fragmentations.

The NOESY spectrum clearly showed strong $d_{NN(i,\ i+1)}$ interaction between Val^4 and Ile^5 (Fig. 6). Similarly, a series of $d_{\alpha N(i,\ i+1)}$ interactions were observed, strong ones between Gly^2 and Leu^3 and between Ile^5 and Tyr^6, and weak ones between Pro^1 and Gly^2 and suggested the presence of a β-turn with the Pro at i+1 position. The αH of Tyr^6 gave strong NOE correlations with the αH of Pro^1

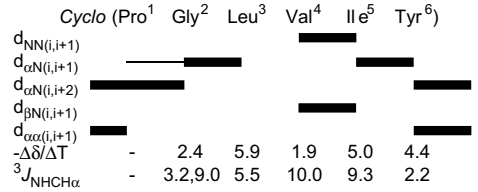

Figure 6. NOESY diagram showing the distribution of the NOE correlations (weak and strong) between NH, αH, βH, γH and δH, the temperature coefficients of amide protons ($-\Delta\delta/\Delta T$, ppb K^{-1}) and the coupling constants ($^3J_{NHCH\alpha}$, Hz) for glabrin A in DMSO-d_6 solution.

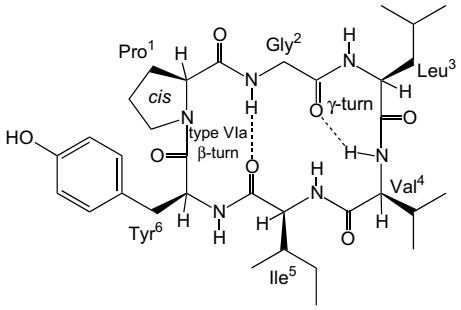

Figure 7. Proposed solution structure of glabrin A (**2**) (dotted lines indicate hydrogen bonds).

indicating that the Tyr-Pro amide bond was cis. Chemical shifts of β and γ carbons of Pro were 29.7 and 21.5 ppm, respectively, giving further evidence of the cis-geometry of this amide bond.[17] The low values of the amide proton temperature coefficients of Gly^2 (2.4 ppb K^{-1}) and Val^4 (1.9 ppb K^{-1}) suggested that they are involved in intramolecular hydrogen bonds (Fig. 6 and 7). The hydrogen bond formed between the NH of Gly^2 and the CO of Ile^5 stabilized a type VIa β-turn with *cis*-Pro at position i+1 and the one between the NH of Val^4 and the CO of Gly^2 suggested the presence of a γ-turn. The large temperature coefficient values found for the amide protons of Leu^3 (5.9 ppb K^{-1}), Ile^5 (5.0 ppb K^{-1}) and Tyr^6 (4.4 ppb K^{-1}), indicated that these protons were solvent exposed.

In conclusion, glabrin A in DMSO-d_6 solution appeared to be structured by a β-turn of type VIa with Tyr^6 and *cis*-Pro^1 at the corners and by a γ-turn centred around Leu^3 (Fig. 7).

2.4. Crystal structure of glabrin A, 2

The conformational analysis of glabrin A (**2**) has been carried out by X-ray diffraction studies. It was crystallized from methanol at 24±1 °C to give colourless crystal of prismatic form of dimensions 100 μm×100 μm×200 μm. Glabrin A crystallized in the orthorhombic space group $P2_1P2_1P2_1$. The lattice constants (a=9.91 Å, b=14.90 Å, c=25.96 Å) and the intensity data collection were obtained on a CAD4 Enraf–Nonuis diffractometer to 0.85 Å resolution. Because the crystals deteriorated rapidly upon drying, they were sealed in a thin-walled glass capillary containing the mother liquor. The backbone of this cyclohexapeptide consisting of amino acids residues showed the presence of two β-turns (Fig. 8). The first

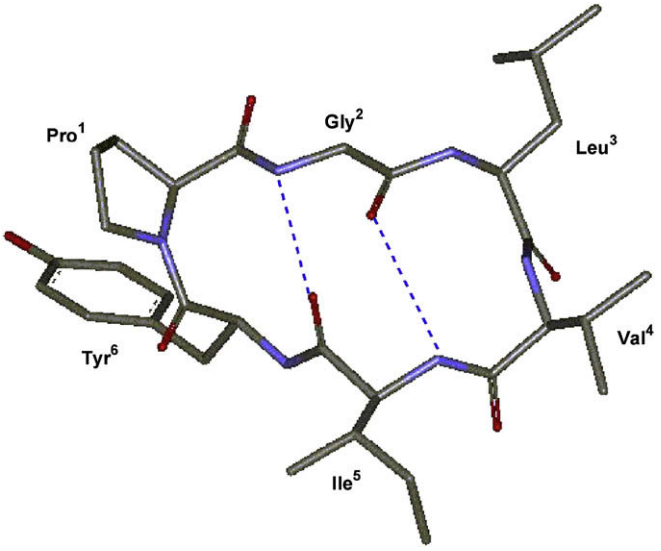

Figure 8. Crystal structure of glabrin A (**2**) (dotted lines indicate hydrogen bonds).

Table 3
Main chain torsional angles in degrees for glabrin A (**2**)

Residues	ϕ	Ψ	χ_1	ω
Pro	−83.1	2.6	29.0	168.2
Gly	−71.4	177.0	—	179.6
Leu	−79.7	−24.9	−63.0	177.9
Val	−91.1	−56.1	−62.0	170.6
Ile	−122.3	126.9	−54.0	−176.6
Tyr	−56.7	139.3	−177.0	8.6

one, formed with Ile^5 at position *i* and Gly^2 at position *i*+3, and Tyr^6 and Pro^1 at the corners can be classified as a type VIa β-turn.[17,18] It is characterized by the presence of a *cis*-proline at position *i*+2 and (ϕ, Ψ) angles for residues +1 (ϕ −56.7°, Ψ 139.3°) and *i*+2 (ϕ −83.1°, Ψ 2.6°) (Table 3) close to the standard values: −60°, +120° and −90°, 0°, respectively.[17–19] This turn is stabilized by the hydrogen bond between the C═O group of Ile^5 and the NH of Gly^2 with an average O···HN hydrogen bond distance of 2.2 Å (Table 4). The proximity of the αH of Pro^1 and the αH of Tyr^6 showed that the Tyr^6-Pro^1 amide bond was clearly *cis* with an average distance of 2.99 Å. A second β-turn is formed between Gly^2 and Ile^5, with Leu^3 and Val^4 at the corners. The (ϕ, Ψ) angles (Table 3) for residues *i*+1 (−79.7°, −24.9°) and *i*+2 (−91.1°, −56.1°) showed that this β-turn is go-between types I and III. This turn is stabilized by a hydrogen bond formed between the C═O group of Gly^2 and the NH of Ile^5 with an average O···HN hydrogen bond distance of 3.2 Å (Fig. 5).

Calculation of the NH–αH coupling constants for the amide backbone was done starting by using the crystal structure data, from which the angle θ was measured and the $^3J_{NH-C\alpha H}$ coupling constants calculated with the Pardi and the Donzel equations.[20] The calculated values were very close to those observed (Table 5), which suggested that in the solid state as well as in solution in DMSO-d_6 glabrin A appeared to have a similar well structured conformation involving two intramolecular hydrogen bonds.

The cyclic heptapeptide cycloreticulin C (**1**), the sequence of which has been determined unambiguously by mass spectrometry and 2D-NMR, appears to be structured in solution in DMSO with two β-turns. The first is of type II with *trans*-Pro^1 and Gly^2 at the corners, which can be considered as a canonical structure. The second is of type VIa with *trans*-Pro^4 and *cis*-Pro^5 at the two corners, followed by a β-bulge involving Val^7 and Tyr^6. The sequence of glabrin A (**2**) has been confirmed by spectral and X-ray studies. Its solution conformation appears close to the solid state conformation and the two involve two turns, one being a β-turn of type VIa, with Tyr^6 and *cis*-Pro^1 at the corners. There is some variation between the solution and crystal states for the second turn, which can be either a γ-turn centred on Leu^3 in the first case, or a β-turn intermediate between types I and III with Leu^3 and Val^4 at the corners. This variation is due to a modification of the hydrogen bonding of the CO of Gly^2, which can involve either the NH of Val^4 or the NH of Ile^5. For the two peptides it is interesting to note that, as usually observed, the *cis*-Pro is at the second corner of a type VIa β-turn.

Table 4
Distances O···HN atoms (Å) of amide bonds for glabrin A (**2**)

N	O					
	Pro	Gly	Leu	Val	Ile	Tyr
Pro	—	4.51	8.26	7.73	3.47	2.24
Gly	2.27	—	6.46	6.80	2.80	4.26
Leu	4.25	2.24	—	5.62	4.45	7.17
Val	6.59	3.60	2.28	—	4.52	7.58
Ile	6.92	4.13	4.38	2.25	—	5.57
Tyr	5.44	3.57	5.92	4.43	2.27	—

Table 5
Comparison for glabrin A (**2**) of the calculated $^3J_{NHCH\alpha}$ coupling constants according to Pardi and Donzel with the experimental values

Residues	θ (°)	$^3J_{NH-C\alpha H}$ (Hz)		
		Calculated according to Pardi	Calculated according to Donzel	Observed
Gly^2	−9	6.7	9.0	9.0
—	−129	5.3	4.1	3.2
Leu^3	−141	6.9	6.2	5.5
Val^4	−153	8.2	8.1	10.0
Ile^5	+179	9.7	10.1	9.3
Tyr^6	−60	2.8	2.3	2.2

Calculations used the angles θ (H–N–Cα–H) measured on the crystal structure.

3. Experimental

3.1. General

Optical rotations were measured with a Perkin–Elmer model 341 Polarimeter and the $[\alpha]_D$ values are given in deg cm^2 g^{-1}. Melting points were determined on a Büchi melting point B-545 apparatus. Mass spectra were recorded on an API Q-STAR PULSAR i of Applied Biosystem. For the CID spectra, the collision energy was 40 eV for protonated ions and 70 eV for adduct ions and the collision gas was nitrogen. ^{1}H NMR and 2D-NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400.13 MHz equipped with X-WIN NMR (version 3.1). The coupling constant used to establish the necessary delay for the selection of the proton coupled to the carbon in the HSQC spectrum was 135 Hz, corresponding to a delay of 3.7 ms; the delay for the HMBC spectra was 70 ms corresponding to a long-range coupling constant of 7 Hz.

3.2. Plant material

Fruits of *A. reticulata* L. (Annonaceae) were collected in Ha Tay (Vietnam) on June 2000. The seeds were collected and were immediately washed with distilled water and were dried at room temperature. Samples were deposited in the Herbarium of the National Museum of Natural History (Paris).

3.3. Extraction and isolation

The dried and powdered seeds of *A. reticulata* (1.3 kg) were macerated three times with cyclohexane (3 L), the combined extracts yielded an oil (448.0 g), which was discarded. The seeds were then extracted three times with MeOH (3 L) at room temperature to give after evaporation of the solvent under reduced pressure the MeOH extract (128.5 g), which was partitioned between EtOAc and water. The organic phase was concentrated to dryness and the residue (78.0 g) was dissolved in MeOH and chromatographed on Sephadex LH-20 column with MeOH. The head fraction (65.0 g) containing peptides and acetogenins was subjected to repeated silica gel column chromatography (silica gel 60 H Merck) eluted with CH_2Cl_2 containing increasing amount of MeOH from 5% to 20% yielding two peptide fractions (I and II), characterized by TLC on silica gel 60 F_{254} Merck, with CH_2Cl_2/MeOH 9:1 as eluent system. The peptides were detected with Cl_2/*o*-tolidine reagent as blue spots with R_f 0.29, which gave glabrin A (**2**) fraction (219.0 mg) and with R_f 0.50 corresponding to cycloreticulin C (**1**) fraction (176.0 mg).

3.4. Absolute configuration of amino acids

Solutions of **1** and **2** (each containing 1 mg of peptide) in 6 N HCl (1 ml) were heated at 110 °C for 24 h in sealed tubes. After cooling,

the solutions were concentrated to dryness. The hydrolyzates were dissolved in anhydrous solution of 3 N HCl in 2-propanol and heated at 110 °C for 30 min. The reagent was evaporated under reduce pressure. The residues were dissolved in CH_2Cl_2 (0.5 ml) and 0.5 ml trifluoracetic anhydride was added. The mixtures were kept in a screw-capped tubes at 110 °C for 20 min. The reagents were evaporated and the mixtures analyzed on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column with helium (1.1 bar) as carrier gas and temperature program of 50–130 °C at 3 °C/min and 130–190 °C at 10 °C/min, with a HEWLETT PACKARD series 5890 apparatus. Comparison of retention time values (t_R, min) with those of standard amino acids was used: Gly (14.6), L-Glx (29.3), L-Ile (16.9), L-Leu (19.2), L-Pro (18.2), L-Thr (15.2), L-Tyr (31.9) and L-Val (13.9).

*3.4.1. Cycloreticulin C (**1**)*

$C_{36}H_{50}N_8O_9$: colourless solid, mp 216–217 °C (MeOH); $[\alpha]_D^{22}$ −6.3 (*c* 2, MeOH). ESI-Q-TOF, *m*/*z*: 777 $[M+K]^+$, 761 $[M+Na]^+$, 739 $[M+H]^+$. ESI-Q-TOF MS/MS on $[M+H]^+$ (ce 40 eV) *m*/*z* (%): 739 (4), 711 (15), 642 (5), 640 (17), 612 (6), 611 (4), 457 (100), 429 (67), 380 (20), 360 (16), 358 (63), 352 (5), 332 (8), 330 (10), 283 (39), 261 (23), 255 (10), 195 (19), 167 (7), 155 (1). ESI-Q-TOF MS/MS on $[M+Na]^+$ (ce 70 eV) *m*/*z* (%): 761 (21), 733 (47), 664 (3), 662 (2), 650 (49), 636 (11), 634 (39), 536 (3), 508 (8), 479 (4), 451 (100), 402 (5), 382 (7), 376 (51), 374 (12), 354 (12), 352 (68), 305 (21), 283 (2), 277 (2), 255 (2), 177 (2), 155 (1).

*3.4.2. Glabrin A (**2**)*

$C_{33}H_{50}N_6O_7$: colourless solid, mp 225–226 °C (MeOH); $[\alpha]_D^{22}$ −17.2 (*c* 0.1, MeOH). ESI-Q-TOF, *m*/*z*: 681 $[M+K]^+$, 665 $[M+Na]^+$, 643 $[M+H]^+$. ESI-Q-TOF MS/MS on $[M+H]^+$ (ce 40 eV) *m*/*z* (%): 643 (41), 615 (31), 598 (11), 544 (10), 502 (22), 485 (17), 480 (45), 452 (17), 403 (18), 386 (12) 367 (100), 339 (56), 318 (13), 268 (52), 240 (10), 213 (6) 155 (32), 127 (3). Glabrin A was crystallized from methanol at 25 °C to give colourless crystal of prismatic form. Data collection was performed on a CAD4 Enraf–Nonuis diffractometer equipped with a rotating copper anode. The structure was determined by the direct methods using the SIR97 program and the refinement was carried out by the block-diagonal matrix least-squared method. Data have been deposited at the Cambridge Crystallographic Data Centre (CCDC 674029). Crystal data, orthorhombic system, *a*=9.91 Å, *b*=14.90 Å, *c*=25.96 Å, space group $P2_1P2_1P2_1$, solvent/temperature of crystallization, MeOH/298 K.

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