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Sequence and three-dimensional structure of cycloreticulins A and B, new cyclooctapeptides from the seeds of *Annona reticulata*

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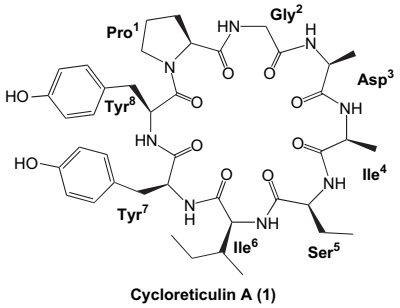
Abstract

Two cyclooctapeptides, cycloreticulin A, *cyclo*(Pro[1]-Gly[2]-Asp[3]-Ile[4]-Ser[5]-Ile[6]-Tyr[7]-Tyr[8]) (**1**) and cycloreticulin B, *cyclo*(Pro[1]-Mso[2]-Tyr[3]-Gly[4]-Thr[5]-Val[6]-Ala[7]-Val[8]) (**2**), have been isolated from the methanol extract of the seeds of *Annona reticulata* L. The sequences were elucidated on the basis of the MS/MS fragmentation using a QTOF mass spectrometer equipped with an ESI source, chemical degradation and extensive 2D-NMR. The solid state conformation of cycloreticulin A, carried out by X-ray study, is characterised by the presence of two β-turns (types II and III) and an inversed γ-turn. Its solution structure appeared quite similar to the crystal one. The cyclic backbone solution structure of cycloreticulin B, close to that of the cyclooctapeptide squamin A, from which its sequence only differs by a Val[8]/Ile[8] substitution, involves three β-turns, two of type I and one of type III, being similar to the crystal structure of squamin A.

Keywords: Cyclopeptides; *Annona reticulata*; Cycloreticulins A and B; Crystal structure; Mass spectrometry; NMR

1. Introduction

Annona reticulata L. (Annonaceae) is a widespread small tree, native in tropical America, which has now a widespread pantropical distribution, because it is cultivated for its fruits. Traditional medicines also used this species as antiparasitic, insecticide, antidiarrhoeic and antidysenteric.[1] In continuation of our programme on cyclopeptides from plants,[2–6] we have isolated from the seeds of *A. reticulata* two novel cyclooctapeptides, cycloreticulins A (**1**) and B (**2**). Although several cyclic octapeptides have been previously isolated from various natural sources,[7] such as agardhipeptin B from cyanobacteria,[8] axinellin C from marine sponges[9] and from plants cyclogossin B,[10] cyclolinopeptides D and E,[11] pohlianin C,[3] cyclosquamosins B-D[12] and squamin,[13,14] only few three-dimensional studies are devoted to such peptides.



Cycloreticulin A (1)

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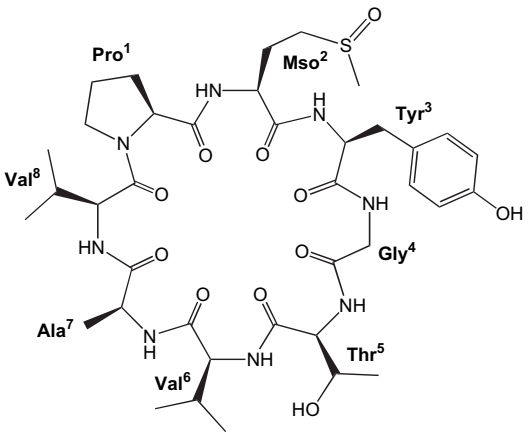

Cycloreticulin B (2)

We now report on the isolation, the sequence determination based on tandem mass spectroscopy and 2D-NMR of cycloreticulins A (**1**) and B (**2**).

The structure of **1** was confirmed by X-ray diffraction analysis and the solution structures of the two compounds have been studied by NMR. The conformations of cyclic peptides are of interest by themselves, as they can also be used as models for the study of recurring structural features of proteins. The very limited number of 3D studies on cyclooctapeptides concern mainly synthetic peptides,[15] and mostly structurally symmetrical compounds.[7,16,17] Cyclic heptapeptides often present a recurrent structure with two β-turns, one of them being followed by a β-bulge. We have previously shown that the solution structure of the cyclic octapeptide cherimolacyclopeptide A was characterised by two β-turns and a new type of β-bulge involving a trifurcated hydrogen bonds between a carbonyl group and three consecutive amide protons.[6] Taking advantage of the two new cyclooctapeptides, we examined their 3D structures in order to know if this conformation was general.

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, cycloreticulins A (**1**) and B (**2**) were purified successively by Sephadex LH-20 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 suggested that they were cyclic. The amino acid analysis of the hydrolysate indicated the presence of Asx (1), Gly (1), Ile (2), Pro (1), Ser (1) and Tyr (2) for cycloreticulin A (**1**), and Ala (1), Gly (1), Mso (1), Pro (1), Thr (1), Tyr (1) and Val (1) for cycloreticulin B (**2**). The Asx residue in the hydrolysate of **1** was further identified as Asp from the presence of a carboxylic acid group. To determine their absolute configuration, the amino acids of the acidic hydrolysate were converted into the *n*-propyl esters of their *N*-trifluoroacetyl derivatives. These ester derivatives were analysed by gas chromatography on a chiral capillary column and their retention times were compared with those of standards. All the chiral amino acids have the L configuration.

2.2. Sequence determination by mass spectrometry

The molecular weight 908 for cycloreticulin A (**1**) was deduced from the (+)ESI-TOF mass spectrum, which displayed the $[M+Na]^+$ and $[M+K]^+$ adduct ions at *m/z* 931 and 947, respectively, and the protonated molecular $[M+H]^+$ ion at *m/z* 909. According to the amino acid composition, the molecular formula $C_{44}H_{60}N_8O_{13}$ was assigned to **1**. Cyclopeptides are not easily sequenced by mass spectrometry due to multiple and indiscriminate ring-opening reactions, which occur during the CID of cyclic peptides, which result in the superimposition of random fragment ions making the interpretation difficult.[18,19] However, 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.[3–5] This specific fragmentation is explained by the more basic nature of the proline nitrogen relative to the other peptide bond nitrogen atoms, which strongly directs the protonation making thus the fragmentation less complex.

The protonated molecular ion $[M+H]^+$ of **1** at *m/z* 909 was subjected to CID experiment (Fig. 1(A)). The ring opening began at the Tyr–Pro amide bond level and a series of adjacent acylium ions (b_n) at *m/z* 746, 583, 470, 383, 270 and 155 was generated from which the sequence could be deduced. The sequential loss of amino acid residues from the C-terminus to the N-terminus of the linearised cycloreticulin A (**1**) was that of Tyr, Tyr, Ile, Ser, Ile and Asp yielding the N-terminal dipeptide Pro–Gly. Two second short series of ions was observed, one at *m/z* 881, 718, 555 and 355 was assigned to adjacent a_n ions related to the above b_n ion series, and the second at *m/z* 891, 728, 565, and 452 was due to the loss from the $[M+H]^+$ ion and the three first b_n fragments of a molecule of water. These data suggested the sequence $[H\text{-}Pro^1\text{-}Gly^2\text{-}Asp^3\text{-}Val^4\text{-}Ser^5\text{-}Ile^6\text{-}Tyr^7\text{-}Tyr^8]^+$ for the linearised peptide ion formed from cycloreticulin A (**1**).

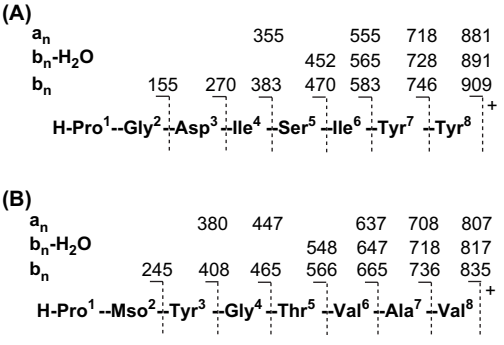

Figure 1. (A) Mass fragmentation for the protonated molecular $[M+H]^+$ ion of cycloreticulin A (**1**). (B) Mass fragmentation for the protonated molecular $[M+H]^+$ ion of cycloreticulin B (**2**).

The molecular weight 834 for cycloreticulin B (**2**) was deduced from the (+)ESI-TOF mass spectrum, which displayed the $[M+K]^+$ adduct ion at *m/z* 873, the $[M+Na]^+$ adduct ion at *m/z* 851 and the $[M+H]^+$ protonated molecular ion at *m/z* 835. According to the amino acid analysis the molecular formula $C_{38}H_{58}N_8O_{11}S$ was assigned to **2**. The CID spectrum of the $[M+H]^+$ ion at *m/z* 835 showed a main series of adjacent b_n peaks at *m/z* 736, 665, 566, 465, 408 and 245, corresponding to the successive loss of Val, Ala, Val, Thr, Gly and Tyr yielding the terminal dipeptide ion $[H\text{-}Pro\text{-}Mso]^+$ and suggesting the sequence H-Pro^1-Mso^2-Tyr^3-Gly^4-Thr^5-Val^6-Ala^7-Val^8 for the linearised peptide (Fig. 1(B)). A second series of ions with peaks at *m/z* 807, 708, 637, 447 and 380 was assigned to the a_n ion series, together with ions at *m/z* 817, 718, 647 and 548 resulting from the loss of a molecule of water from the threonine residue. The proposed sequence for cycloreticulin B (**2**) is thus *cyclo*(Pro^1-Mso^2-Tyr^3-Gly^4-Thr^5-Val^6-Ala^7-Val^8).

2.3. ^1H and ^{13}C NMR sequence assignments for cycloreticulin A (*1*)

The ^1H NMR spectrum of cycloreticulin A (**1**) in pyridine-d_5 (Table 1) showed a main stable conformational state (>90%) where seven amide protons and one carboxylic acid proton were clearly depicted. These data, together with the presence of nine carbonyl groups in the ^{13}C NMR spectrum were in agreement with an octapeptide cyclic structure, including an aspartic acid and a proline. The peptide sequence determination was based on the HMBC experiment. This heteronuclear methodology was preferred, to the homonuclear method described by Wüthtrich and based on $d_{NN(i,i+1)}$ and $d_{\alpha N(i,i+1)}$ connectivities from the ROESY/NOESY spectra,[20–22] because for small size cyclic peptides, conformational information can interfere with sequential ones. All the amino acid spin systems were identified using scalar spin–spin couplings determined from the $^1H-^1H$ COSY and TOCSY experiments.[23] The ^{13}C NMR assignments of the protonated carbons were obtained from the proton detected heteronuclear HSQC spectrum and combined with the HMBC experiment optimised for a long-range *J* value of 7 Hz for the non-protonated carbons and allowing the carbonyl groups to be assigned from the observed correlations with the α and β protons of the same residue. Then, the sequence was determined from the connectivities between the carbonyl of residue i with the amide and/or α protons of residue i+1 (Fig. 2). All the $^3J_{CH}$ CO (i) to NH (i+1) correlations depicted in the HMBC spectrum were in agreement with the structure

Table 1
^{13}C and ^1H NMR data for cycloreticulin A (**1**), in pyridine-d_5 (400 MHz, 298 K)

Residue	δ_C	δ_H	m	(*J*, Hz)	Residue	δ_C	δ_H	m	(*J*, Hz)
Pro^1 CO	173.5	—			Ile^6 CO	171.6	—		
α CH	61.6	4.48	dd	8.4, 5.2	NH	—	9.21	d	5.4
β CH_2	29.6	2.03	m		α CH	60.0	4.58	dd	5.4, 3.5
	—	1.91	m		β CH	36.6	1.98	m	
γ CH_2	25.3	1.94	m		γ CH_2	24.9	1.08	m	
	—	1.70	m			—	1.08	m	
δ CH_2	47.8	3.73	m		γ′ CH_3	15.5	0.65	d	7.0
	—	3.60	m		δ CH_3	12.1	0.58	dd	7.4, 7.4
Gly^2 CO	169.9	—			Tyr^7 CO	172.2	—		
NH	—	8.90	dd	6.8, 5.4	NH	—	8.23	d	7.1
α CH_2	44.4	4.43	dd	16.8, 6.8	α CH	58.2	5.02	ddd	11.4, 7.1, 4.2
		4.18	dd	16.8, 5.4	β CH_2	38.1	3.19	dd	14.3, 4.2
Asp^3 CO	176.5	—				—	3.17	dd	14.3, 11.4
NH	—	8.12	d	8.2	1′	129.0	—		
α CH	50.7	5.52	ddd	8.2, 5.3, 2.3	2′,6′	130.8	7.32	m	8.5
β CH_2	42.3	3.78	dd	17.0, 5.3	3′,5′	116.4	7.12	m	8.5
	—	3.01	dd	17.0, 2.3	4′	157.8	—		
γ CO	176.2	—			OH	—	11.3[a]	sl	
δ OH	—	9.30	sl		Tyr^8 CO	172.8	—		
Ile^4 CO	171.9	—			NH	—	7.68	d	9.4
NH	—	8.76	d	6.1	α CH	52.6	5.68	ddd	10.5, 9.4, 3.8
α CH	59.8	4.81	dd	6.1, 4.2	β CH_2	38.2	3.69	dd	14.0, 10.5
β CH	36.4	2.21	m			—	3.50	dd	14.0, 3.8
γ CH_2	25.6	1.63	m		1′	128.3	—		
	—	1.33	m		2′,6′	132.1	7.78	m	8.4
γ′ CH_3	16.0	0.92	d	6.9	3′,5′	116.1	7.18	m	8.4
δ CH_3	12.0	0.69	dd	7.4, 7.4	4′	157.8	—		
Ser^5 CO	173.9	—			OH	—	11.4[a]	sl	
NH	—	8.92	d	7.7					
α CH	56.9	5.24	dd	7.7, 4.2					
β CH_2	64.6	4.66	dd	12.2, 4.2					
	—	4.52	d	12.2					

[a] May be exchanged.

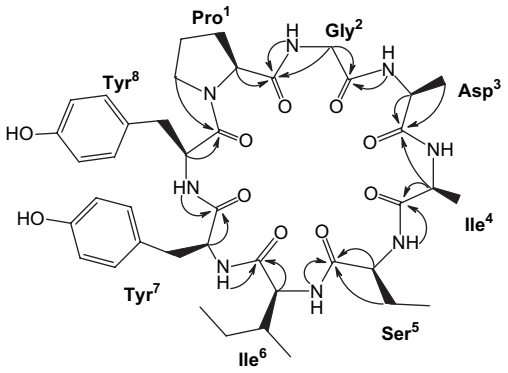

Figure 2. HMBC correlations for cycloreticulin A (**1**).

deduced from the mass spectral data. The chemical shift of γC of Pro[1] at 25.3 ppm clearly agree with a trans conformation of the Tyr[8]–Pro[1] amide bond, which is confirmed from the strong NOE correlations between the αH of Tyr[8] and the δ,δ′H of Pro[1].[24] In addition, the NOESY spectrum showed a set of strong $d_{NN(i,i+1)}$ interactions between Ile[6] and Tyr[7] and between Tyr[7] and Tyr[8] and a weaker one between Gly[2] and Asp[3] (Fig. 3), in agreement with the involvement of these residues in β-turns. All along the sequence were observed strong or moderate $d_{\alpha N(i,i+1)}$ interactions and in the Ser[5] to Tyr[8] region $d_{\beta N(i,i+1)}$ cross-peaks.

2.4. *^{1}H and ^{13}C NMR sequence assignments for cycloreticulin B (**2**)*

The ^{1}H NMR spectrum of cycloreticulin B (**2**) in DMSO-d_6 (Table 2) showed only one stable conformational state and seven amide protons were clearly depicted. These data, together with the presence of eight carbonyl groups in the ^{13}C NMR spectrum were in agreement with an octapeptide cyclic structure including a proline. Two signals in the ratio 3:1were observed for both the amide and the ε-methyl protons of methionine sulfoxide (Mso). This resulted from the presence of two epimers in the ratio 3:1 and due to the chirality of the sulfur atom in sulfoxides. The peptide sequence determination was based on the HMBC experiment showing the connectivities between the carbonyl of residue i with NH and/or αH protons of the i+1 residue (Fig. 4). The respective assignment of the two valines was deduced from the correlation between the carbonyl of Val[8] (170.7 ppm) with αH of Pro[1] and the carbonyl of Val[6] (171.3 ppm) with the NH of Ala[7]. The two carbonyls were previously assigned to each valine from their intra-residue correlations with αH and βH.

The Val[8]–Pro[1] amide bond was trans as indicated by the chemical shift of its γ-carbon (24.4 ppm) together with the strong NOE observed in the ROESY spectrum between αH of Val[8] and δ,δ′H of Pro[1]. The sequence of **2** only differed from that of squamin A,[13,14] a cyclooctapeptide isolated from *Annona squamosa*, by a Val[8]/Ile[8] substitution. All the NMR parameters, excepted those for residue at position 8, were very similar. The structure of squamin A has been confirmed recently from an X-ray crystallographic study by Jiang et al.[14]

2.5. *Crystal structure of cycloreticulin A (**1**)*

The crystal structure analysis of cycloreticulin A (**1**) has been carried out by an X-ray diffraction study. Cycloreticulin A crystallized in orthorhombic system of space group $P2_12_12_1$ with lattice constants a=9.99 Å, b=21.12 Å and c=26.51 Å. Crystal data, torsion angle values and interatomic distances –C=$O\cdots H$–N– values are reported in Tables 3 and 4, respectively. The backbone of this cyclooctapeptide consisting of eight amino acid residues showed the presence of two β-turns and an inversed γ-turn (Fig. 5).

The first β-turn, formed with Tyr[8] at position i and Asp[3] at position i+3, can be classified as a type II β-turn and is characterised by the presence of a *trans*-Pro at position i+1 and a Gly at position i+2. Angles for residues i+1 (Pro[1]: ϕ −62.2°, ψ 134.9°) and i+2 (Gly[2]: ϕ −94.1°, ψ −14.2°) (Table 3) are closed to the standard values: (−60°, +120°; −90°, 0°).[24] This turn is stabilized by a hydrogen bond between the C=O group of Tyr[8] and the NH of Asp[3] with an average $-O\cdots N-$ hydrogen bond distance of 2.98 Å (Table 4). The proximity of the αH of Tyr[8] and the δ,δ′H of Pro[1] showed that the Tyr[8]–Pro[1] amide bond was clearly trans with average distance of 2.33 Å. A second β-turn starting from Ser[5] to Tyr[8] with Ile[6] and Tyr[7] at the corners is formed. The (ϕ, ψ) angles (Table 3) for residues i+1 (Ile[6]:−69.6°, −23.1°) and i+2 (Tyr[7]: −69.3°, −28.8°) suggested that this β-turn is close to type III, but is lightly deformed and roughly go-between type I and type III.

This β-turn is stabilized by a hydrogen bond formed between the C=O group of Ser[5] and the NH of Tyr[8] with an average $-O\cdots N-$ hydrogen bond distance of 3.63 Å (Table 4). A third turn, close to an inversed γ-turn, which involves residues Asp[3], Ile[4] and Ser[5] is stabilized by a loose H-bond

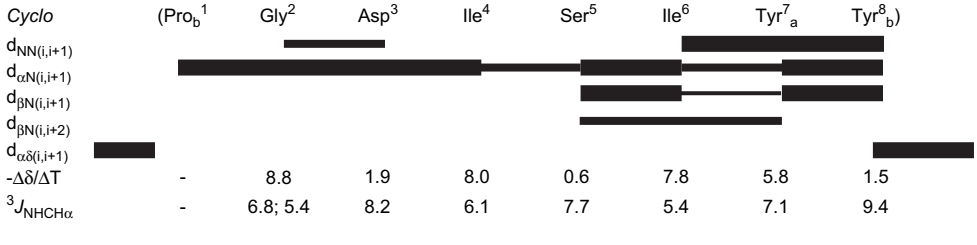

Figure 3. ROESY diagramme showing the repartition of the NOE correlations (weak, medium and strong) between NH, αH, βH, γH and δH, the temperature coefficients of amide protons ($-\Delta\delta/\Delta T$, ppb K^{-1}) and the coupling constants ($^3J_{NHCH\alpha}$, Hz) for cycloreticulin A (**1**) in pyridine-d_5 solution.

Table 2
^{13}C and ^{1}H NMR data for cycloreticulin B (**2**) in DMSO-d_6 (400 MHz, 298 K)

Residue	δ_C	δ_H	m	(J, Hz)	Residue	δ_C	δ_H	m	(J, Hz)
Pro[1] CO	175.5	—			Gly[4] CO	168.9	—		
α CH	62.6	4.58	dd	8.4, 8.4	NH	—	7.91	dd	7.0, 5.6
β CH_2	29.0	2.15	m		α CH_2	43.1	4.01	dd	17.0, 7.0
	—	1.75	m				3.43	dd	17.0, 5.6
γ CH_2	24.4	1.92	m		Thr[5] CO	170.9	—		
	—	1.92	m		NH	—	7.18	d	10.2
δ CH_2	46.7	3.52	m		α CH	55.0	4.87	dd	10.2, 5.0
	—	3.36	m		β CH	68.1	4.50	m	
Mso[2] CO	170.9	—			γ CH_3	19.2	1.00	d	6.2
NH	—	8.84	d	3.7	OH	—	5.00	s	
α CH	54.8	3.95	m		Val[6] CO	171.3	—		
β CH_2	23.8	1.96	m		NH	—	8.09	d	4.1
	—	1.96	m		α CH	62.1	3.49	dd	7.5, 4.1
γ CH_2	48.5	2.57	m		β CH	28.4	1.96	m	
	—	2.52	m		γ CH_3	19.2	0.96	d	6.7
S-CH_3	37.5	2.46	m		γ′ CH_3	19.2	0.88	d	6.8
Tyr[3] CO	171.3	—			Ala[7] CO	172.5	—		
NH	—	7.99	d	9.8	NH	—	7.24	d	6.5
α CH	52.2	4.64	dddd	12.1, 9.8, 2.5, 2.5	α CH	50.5	3.97	dq	6.5, 7.4
β CH_2	35.6	3.42	m		β CH_3	17.4	1.26	d	7.4
	—	2.66	m		Val[8] CO	170.7	—		
1′	128.2	—			NH	—	7.06	d	9.6
2′,6′	128.7	6.90	m	8.5	α CH	55.1	4.09	dd	9.8, 9.6
3′,5′	114.9	6.61	m	8.5	β CH	29.1	2.15	m	
4′	155.5	—			γ CH_3	21.0	0.73	d	6.8
OH	—	9.17	s		γ′ CH_3	18.2	0.66	d	6.5

with a $-O\cdots HN-$ distance of 3.4 Å. It is characterised for the residue Ile[4] at position i+1, by (ϕ, ψ) angle torsion values (−67.4°, −30.6°) somewhat different from the standard values (−80.0°, +60.0°). The crystal structure of cycloreticulin A is characterised by two β-turns, one of type II (Pro[1]–Gly[2]) and another of type III (Ile[6]–Tyr[7]), and a deformed inversed γ-turn centred on Ile[4].

2.6. Solution conformation of cycloreticulin A (*1*)

The chemical shift variations of amide protons with temperature have been recorded between 298 and 318 K. The variations were linear, indicating that no conformational change occurred in this temperature scale. The strong values of temperature coefficients for Tyr[7] (−5.8 ppb K^{-1}), Ile[6] (−7.8 ppb K^{-1}), Gly[2] (−8.8 ppb K^{-1}) and Ile[4] (−8.0 ppb K^{-1}) suggested that they were solvent exposed and not involved in

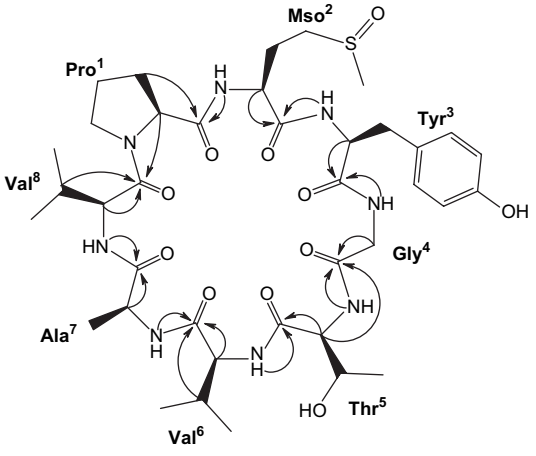

Figure 4. HMBC correlations for cycloreticulin B (**2**).

Table 3
Main chain torsional angles in degrees for cycloreticulin A (**1**)

Residues	ϕ	ψ	χ_1	ω
Pro[1]	−62.2	134.9	24.0	−175.8
Gly[2]	94.2	−14.2	—	173.9
Asp[3]	−99.1	157.9	166.0	174.4
Ile[4]	−67.4	−30.6	169.0	175.2
Ser[5]	−64.8	151.0	−80.0	−177.6
Ile[6]	−69.6	−23.1	−64.0	−166.7
Tyr[7]	−69.3	−28.8	43.0	−169.0
Tyr[8]	−135.6	118.1	47.0	174.4

Table 4
Distances N···O atoms (Å) of amide bonds for cycloreticulin A (**1**)

N	O							
	Pro[1]	Gly[2]	Asp[3]	Ile[4]	Ser[5]	Ile[6]	Tyr[7]	Tyr[8]
Pro[1]	—	6.48	5.53	8.18	5.28	5.02	4.33	2.28
Gly[2]	2.26	—	4.26	7.65	6.04	7.35	7.64	3.27
Asp[3]	3.64	2.26	—	6.16	4.89	7.48	7.78	2.98
Ile[4]	7.21	4.40	2.28	—	4.37	8.05	9.30	5.21
Ser[5]	8.31	6.83	3.33	2.26	—	6.47	8.07	5.35
Ile[6]	8.93	9.14	5.14	3.73	2.27	—	6.16	5.15
Tyr[7]	8.11	9.60	6.25	6.15	3.19	2.30	—	4.92
Tyr[8]	5.65	7.96	5.66	7.07	3.63	3.34	2.27	—

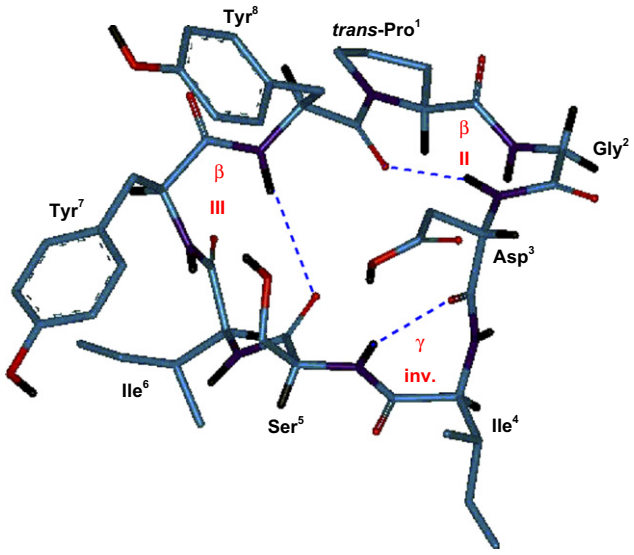

Figure 5. X-ray crystal structure for cycloreticulin A (**1**).

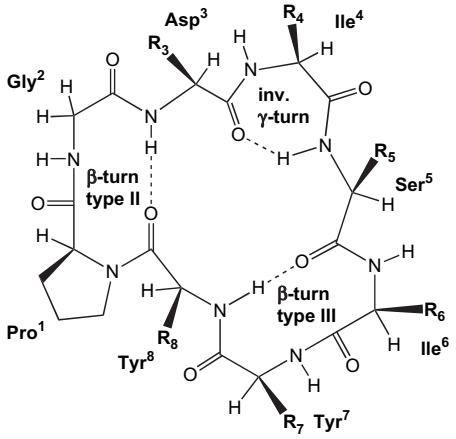

Figure 6. Solution conformation of cycloreticulin A (**1**) (doted lines indicate hydrogen bonds).

intramolecular hydrogen bonds (Table 5, Fig. 3). The three other residues were characterised by weak temperature coefficients, Asp^3 (-1.9 ppb K^{-1}), Tyr^8 (-1.5 ppb K^{-1}), and Ser^5 (-0.6 ppb K^{-1}), indicating that their NH are engaged in intramolecular H-bonds. These results can be rationalised by a solution structure close to the crystal structure. The hydrogen bond formed between the NH of Asp^3 and the CO of Tyr^8 stabilized a type II β-turn with *trans*-Pro^1 at position i+1, the one between the NH of Tyr^8 and the CO of Ser^5 suggested the presence of a type III β-turn and finally the one between the NH of Ser^5 and the CO of Asp^3 is interpreted by an inversed γ-turn.

Calculation of the NH−αH coupling constants for the amide backbone from the crystal structure data (θ) according to Pardi or Donzel equations,[25] are in good agreement with the experimental values. In conclusion, cycloreticulin A in pyridine-d_5 solution appeared to be structured with two β-turns, one of type II with Pro^1 and Gly^2 at the corners, another of type III with Ile^6 and Tyr^7 at the corners, and an inversed γ-turn centred at Ile^4 (Fig. 6).

Table 5
Comparison for cycloreticulin A (**1**) of the calculated $^3J_{NHCH\alpha}$ coupling constants according to Pardi and Donzel with the experimental values (Pyr-d_5)

Residues	θ (°)	$^3J_{NHCH\alpha}$ (Hz)		
		Calculated according to Pardi	Calculated according to Donzel	Observed
Gly^2	+153.2	8.3	8.1	6.8
—	+35.1	5.0	6.2	5.4
Asp^3	−156.4	8.6	8.5	8.2
Ile^4	−129.1	5.4	4.2	6.1
Ser^5	−129.7	5.3	4.2	7.7
Ile^6	−130.7	5.5	4.4	5.4
Tyr^7	−129.0	5.3	4.2	7.1
Tyr^8	−175.8	9.7	10.1	9.4

Calculations were based on the angles θ (H−N−Cα−H) measured on the crystal structure.

2.7. Solution conformation of cycloreticulin B (2)

The sequence of cycloreticulin B (**2**) differs from that of squamin A, *cyclo*(Pro^1-Mso^2-Tyr^3-Gly^4-Thr^5-Val^6-Ala^7-Ile^8), a cyclooctapeptide of *A. squamosa*, only by a Val^8/Ile^8 substitution. Squamin A has been crystallized by Lu et al. who determined its 3D-structure by X-ray crystallography.[13,14] This solid state structure of squamin A is characterised by three β-turns, the first one with Thr^5 at position i and Ile^8 at i+3 and thus with Val^6 and Ala^7 at the two corners, is of type I. The second turn with *trans*-Pro^1 and Mso^2 at the two corners is very close to a type III β-turn (ϕ −64.1°, ψ −18.0°; ϕ −60.6, ψ −19.5°), followed by the third one of type I, with Mso^2 and Tyr^3 at the two corners. These two last turns have the aspect of a 3_{10}-helix fragment. We have recorded the NMR spectra of squamin A in solution in DMSO-d_6 and in pyridine-d_5. The two spectra showed a main conformation (>95%). NOEs data and $^3J_{NH-\alpha H}$ coupling constants for squamin A in the two solvents were very similar and suggested that this cyclic peptide adopts the same solution conformation. We thus analysed the NMR conformational parameters of cycloreticulin B in solution in DMSO-d_6, and compared them with those of squamin A in the same experimental conditions in order to see if the Val^8/Ile^8 substitution modify the conformation state, and if the solution structure of **2** was similar to the crystal structure of squamin A. The ROESY spectrum of cycloreticulin B is very close to that of squamin A. Strong $d_{NN(i,i+1)}$ were observed from Tyr^3 to Thr^5 and from Val^6 to Val^8 and a set of $d_{\alpha N(i,i+1)}$ from Pro^1 to Val^8. Strong $d_{\alpha N(i,i+1)}$ between Tyr^3 and Gly^4, and between Thr^5 and Val^6 and strong $d_{\beta N(i,i+1)}$ and $d_{\beta N(i,i+2)}$ for residues 6−8 are noticed in the two peptides (Fig. 7). The Val^8−Pro^1 amide bond is trans in both cases, as indicated by the strong NOEs between the αH of the residue at position 8 and the δ,δ′H of Pro^1as well as the chemical shift of Pro^1 γH (24.4 ppm). For the two peptides the temperature coefficients of the amide protons have been recorded in the temperature range 298−313 K and they varied linearly. The NH groups of Tyr^3, Gly^4, Thr^5 and Val^8 had low coefficients <-4 ppb K^{-1}, suggesting their involvement in intramolecular

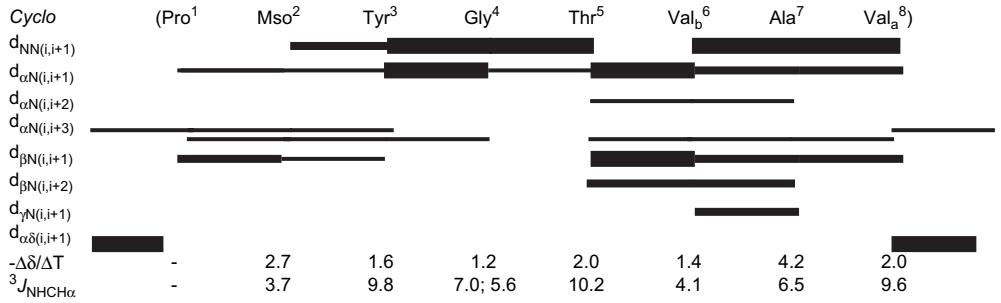

Figure 7. ROESY diagramme showing the repartition of the NOE correlations (weak, medium and strong) between NH, αH, βH, γH and δH, the temperature coefficients of amide protons ($-\Delta\delta/\Delta T$, ppb K^{-1}) and the coupling constants ($^3J_{NHCH\alpha}$, Hz) for cycloreticulin B (**2**) in DMSO-d_6 solution.

hydrogen bonds, whereas the other had strong coefficients indicating that they were solvent exposed (Fig. 7). These data suggested that the two peptides had similar solution structures. Taking into account the atomic coordinates from the crystal structure of squamin A, the NH–αH coupling constants for **2** were calculated using the Pardi and Donzel equations[25] (Table 6). The calculated values were in very good agreement with the observed ones. The solution structure of **2** appeared thus similar to the crystal structure of squamin A, with three β-turns: two of type I, a first with Val^6 and Ala^7 at the corners and stabilized by a H-bond between the CO of Thr^5 and the NH of Val^8, and a second with Mso^2 and Tyr^3 at the two corners and stabilized by a H-bond between the CO of Pro^1 and the NH of Gly^4 (Fig. 8). The third β-turn could be as in the crystal of type III, with Pro^1 and Mso^2 at the two corners and stabilized by a H-bond between the CO of Val^8 and the NH of Tyr^3. The two β-turns, from Val^8 to Tyr^3 give the aspect of a fragment of 3_{10}-helix, which is linked to the first β-turn by the Gly^4–Thr^5 dipeptide (Fig. 8).

The cyclooctapeptide cycloreticulin B (**2**) has thus a solid as well as a solution 3D-structure different from that of cycloreticulin A. Both are also different to the solution structure of cherimolacyclopeptide A previously described,[6] indicating that for the cyclooctapeptides, different 3D structures can be observed depending mostly on the peptide sequences, as solution and solid states structures of a specified peptide are very similar.

Table 6
Comparison for cycloreticulin B (**2**) and squamin A of the calculated $^3J_{NHCH\alpha}$ coupling constants according to Pardi and Donzel with the experimental values measured in DMSO-d_6 (also in Pyr-d_5 between brackets for squamin A)

Residues	θ (°)	$^3J_{NHCH\alpha}$ (Hz)			
		Calculated according to Pardi	Calculated according to Donzel	Observed for squamin A	Observed for cycloreticulin B
Mso^2	−121.2	4.3	2.9	3.8 (3.5)	3.7
Tyr^3	+175.2	9.6	10.0	9.4 (7.7)	9.8
Gly^4	+28.0	5.7	7.2	4.5 (4.5)	5.6
—	+148.7	7.8	7.4	7.1 (6.7)	7.0
Thr^5	−178.2	9.7	10.1	10.2 (10.0)	10.2
Val^6	−120.7	4.3	2.8	4.2 (3.5)	4.1
Ala^7	−140.3	6.8	6.1	6.7 (6.5)	6.5
Ile^8/Val^8	−166.4	9.3	9.6	9.7 (9.0)	9.6

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

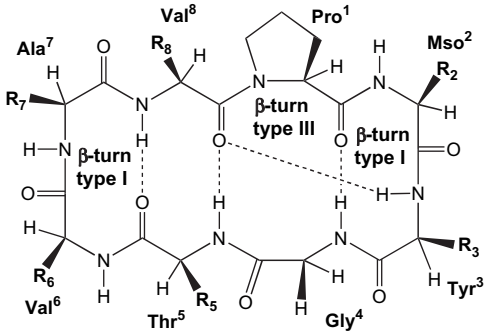

Figure 8. Solution conformation of cycloreticulin B (**2**) (doted lines indicate hydrogen bonds).

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 for the protonated molecular ion was 40 eV and the collision gas was nitrogen. ^{1}H NMR spectra were recorded either on a Bruker Avance 400 spectrometer operating at 400.13 MHz equipped with XWIN-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 and the spin lock for the ROESY experiments was 150 ms.

3.2. Plant material

Fruits of *A. reticulata* L. (Annonaceae) were collected in Ha Tay (Vietnam) in June 2000. The seeds were collected, immediately washed with distilled water and dried at room temperature. Voucher 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 (3×3 L), the extracts combined and concentrated to dryness to yield an oil (448.0 g), which was discarded. The seeds were then extracted three times with MeOH (3×3 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 over Sephadex LH-20 column with MeOH as eluent. The head fraction (65.0 g) containing peptides and acetogenins was subjected to repeated silica gel column chromatography (Kieselgel 60H Merck) eluted with CH_2Cl_2/MeOH gradient from 5% to 20% MeOH yielding two peptide fractions (I eluted with 10% and then II) and characterised by their R_f on TLC (Silica gel 60 F_{254} Merck, eluent CH_2Cl_2/MeOH: 9/1). Peptides were detected with the Cl_2/*o*-tolidine reagent yielding blue spots with R_f 0.42 for cycloreticulin A (30.0 mg) and R_f 0.27 for cycloreticulin B (47.0 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 hydrolysates were dissolved in anhydrous solution of 3 N HCl in 2-propanol and heated at 110 °C for 30 min. The reagent were 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 screw-capped tubes at 110 °C for 20 min. The reagents were evaporated and the mixtures analysed 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 programme of 50–130 °C at 3 °C min^{-1} and 130–190 °C at 10 °C min^{-1}, with a HEWLETT PACKARD series 5890 apparatus. Comparison of retention time values with those of standard amino acids was used: L-Ala (11.6), L-Asp (25.1), Gly (14.6), L-Ile (16.9), L-Leu (19.2), L-Mso (27.8), L-Pro (18.2), L-Ser (18.8), L-Thr (15.2), L-Tyr (31.9) and L-Val (13.9).

3.5. Cycloreticulin A

$C_{44}H_{60}N_8O_{13}$: colourless solid, mp 269–270 °C (MeOH); $[\alpha]_D^{20}$ −2.7 (*c* 0.1, MeOH). ESI-QTOF *m/z*: 947 $[M+K]^+$, 931 $[M+Na]^+$, 909 $[M+H]^+$. ESI-QTOF MS/MS on $[M+H]^+$ (ce 40 eV) *m/z* (%): 891 (27), 881 (28), 778 (19), 746 (40), 728 (22), 718 (15), 663 (8), 583 (12), 565 (19), 555 (8), 509 (16), 470 (14), 452 (19), 383 (57), 364 (23), 355 (37), 346 (17), 270 (100). Cycloreticulin A was crystallized from methanol at 24 °C to give colourless crystal of prismatic form of dimensions 100 μ×100 μ×200 μ. Because the crystals deteriorated rapidly upon drying, they were sealed in a thin-walled glass capillary containing the mother liquor. 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 654017). Crystal data, orthorhombic system, *a*=9.99 Å, *b*=21.12 Å, *c*=26.51 Å, Space group $P2_1P2_1P2_1$, solvent/temperature of crystallisation, MeOH/298 K.

3.6. Cycloreticulin B

$C_{38}H_{58}N_8O_{11}S$: colourless solid, mp 255–256 °C (MeOH); $[\alpha]_D^{20}$ −7.7 (*c* 2, MeOH). ESI-QTOF *m/z*: 873 $[M+K]^+$, 857 $[M+Na]^+$, 835 $[M+H]^+$. ESI-QTOF MS/MS on $[M+H]^+$ (ce 40 eV) *m/z* (%): 835 (100), 817 (59), 807 (29), 771 (32), 736 (77), 718 (55), 708 (13), 672 (21), 665 (38), 647 (48), 637 (19), 573 (21), 566 (36), 548 (57), 465 (49), 447 (5), 408 (84), 380 (11), 245 (41).

3.7. Squamin A

$C_{39}H_{60}N_8O_{11}S$: colourless solid, mp 260–261 °C (MeOH); $[\alpha]_D^{20}$ −8.0 (*c* 0.3, MeOH). ESI-QTOF *m/z*: 849 $[M+K]^+$, 871 $[M+Na]^+$, 887 $[M+H]^+$. ^{13}C NMR (pyridine-d_5): 176.8 (Pro^1-CO), 64.0 (Pro^1-Cα), 30.2 (Pro^1-Cβ), 25.5 (Pro^1-Cγ), 48.1 (Pro^1-Cδ); 173.2* (Mso^2-CO), 55.8 (Mso^2-Cα), 24.9 (Mso^2-Cβ), 49.0 (Mso^2-Cγ), 37.3 (Mso^2-Cε); 173.0 (Tyr^3-CO), 53.1 (Tyr^3-Cα), 37.0 (Tyr^3-Cβ), 129.3 (Tyr^3-1′), 129.8 (Tyr^3-2′,6′), 116.2 (Tyr^3-3′,5′), 157.4 (Tyr^3-4′); 170.6 (Gly^4-CO), 44.6 (Gly^4-Cα); 172.4 (Thr^5-CO), 56.8 (Thr^5-Cα), 70.8 (Thr^5-Cβ), 19.7 (Thr^5-Cγ); 172.4 (Val^6-CO), 63.0 (Val^6-Cα), 29.8 (Val^6-Cβ), 19.5 (Val^6-Cγ), 19.7 (Val^6-Cγ′); 173.8 (Ala^7-CO), 52.0 (Ala^7-Cα),18.3 (Ala^7-Cβ); 172.1* (Ile^8-CO), 56.0 (Ile^8-Cα), 36.7 (Ile^8-Cβ), 24.5 (Ile^8-Cγ), 17.4 (Ile^8-Cγ′), 11.2 (Ile^8-Cδ). ^1H NMR (pyridine-d_5): 5.28 (dd, 8.5, 8.5, Pro^1-Hα), 1.95 and 2.36 (m, Pro^1-H_2β), 1.85 and 2.14 (m Pro^1-H_2γ), 3.93 and 4.04 (m, Pro^1-H_2δ); 9.69 (d, 3.8, Mso^2-NH), 4.57 (m, Mso^2-Hα), 2.35 and 2.59 (m, Mso^2-H_2β), 2.74 and 2.87 (m, Mso^2-H_2γ), 2.49 (s, Mso^2-H_3ε); 8.70 (d, 9.5, Tyr^3-NH), 5.67 (m, Tyr^3-Hα), 4.28 (ddd, 15.8, 5.4, 2.7, Tyr^3-H_2β), 3.30 (dd, 15.8, 12.2, Tyr^3-H_2β), 7.39 (m, 8.4, Tyr^3-2′,6′), 7.16 (m, 8.4, Tyr^3-3′,5′); 8.76 (dd, 6.7, 5.0, Gly^4-NH), 4.69 (dd, 17.0, 6.7, Gly^4-Hα), 3.97 (dd, 17.0, 4.5, Gly^4-Hα); 8.06 (d, 10.0, Thr^5-NH), 5.59 (dd, 10.0, 2.8, Thr^5-Hα), 4.90 (dq, 2.8, 6.2, Thr^5-Hβ), 1.38 (d, 6.2, Thr^5-H_3γ); 9.02 (d, 4.0, Val^6-NH), 4.16 (ddd, 6.5, 3.5, 3.2, Val^6-Hα), 2.30 (m, Val^6-Hβ), 1.10 (d, 6.9, Val^6-H_3γ), 1.12 (d, 6.9, Val^6-H_3γ′); 7.76 (d, 6.5, Ala^7-NH), 4.82 (dq, 6.5, 7.4, Ala^7-Hα), 1.56 (d, 7.4, Ala^7-H_3β); 7.62 (d, 9.5, Ile^8-NH), 4.87 (m, Ile^8-Hα), 2.24 (m, Ile^8-Hβ), 1.28 & 1.50 (m, Ile^8-H_2γ), 0.98 (d, 6.2, Ile^8-H_3γ′), 0.62 (t, 7.6, Ile^8-H_3δ). ^{13}C NMR (DMSO-d_6): 175.6 (Pro^1-CO), 62.7 (Pro^1-Cα), 29.0 (Pro^1-Cβ), 24.5 (Pro^1-Cγ), 46.8 (Pro^1-Cδ); 171.0 (Mso^2-CO), 54.9 (Mso^2-Cα), 23.8 (Mso^2-Cβ), 48.6 (Mso^2-Cγ), 37.6 (Mso^2-Cε); 171.5 (Tyr^3-CO), 52.0 (Tyr^3-Cα), 35.4 (Tyr^3-Cβ), 128.1 (Tyr^3-1′), 18.4 (Tyr^3-2′,6′), 114.9 (Tyr^3-3′,5′), 155.6 (Tyr^3-4′); 169.0 (Gly^4-CO), 43.1 (Gly^4-Cα); 171.0 (Thr^5-CO), 55.1 (Thr^5-Cα), 68.2 (Thr^5-Cβ), 19.3

(Thr^5-Cγ); 171.4 (Val^6-CO), 62.1 (Val^6-Cα), 28.4 (Val^6-Cβ), 19.2 (Val^6-Cγ), 19.3 (Val^6-Cγ′); 172.6 (Ala^7-CO), 50.6 (Ala^7-Cα), 17.4 (Ala^7-Cβ); 170.7 (Ile^8-CO), 54.3 (Ile^8-Cα), 35.1 (Ile^8-Cβ), 23.4 (Ile^8-Cγ), 16.8 (Ile^8-Cγ′), 11.2 (Ile^8-Cδ). ^{1}H NMR (DMSO-d_6): 4.58 (dd, 9.0, 8.0, Pro^1-Hα), 1.18 and 2.16 (m, Pro^1-H$_2$β), 1.84 and 1.90 (m, Pro^1-H$_2$γ), 3.32 and 3.53 (m, Pro^1-H$_2$δ); 8.83 (d, 3.5, Mso^2-NH), 3.96 (m, Mso^2-Hα), 1.93 (m, Mso^2-H$_2$β), 2.48 and 2.58 (m, Mso^2-H$_2$γ), 2.45 (s, Mso^2-H$_3$ε); 7.96 (d, 9.4, Tyr^3-NH), 4.68 (ddd, 10.7, 9.4, 2.5, Tyr^3-Hα), 3.42 and 2.68 (m, Tyr^3-H$_2$β), 6.89 (m, 8.5, Tyr^3-2′,6′), 6.61 (m, 8.5, Tyr^3-3′,5′), 9.20 (s, Tyr^3-OH); 7.95 (dd, 7.1, 4.5, Gly^4-NH), 4.01 (dd, 17.1, 7.1, Gly^4-Hα), 3.43 (dd, 17.1, 4.5, Gly^4-Hα); 7.17 (d, 10.2, Thr^5-NH), 4.87 (dd, 10.2, 3.0, Thr^5-Hα), 4.48 (dq, 3.0, 6.2, Thr^5-Hβ), 1.00 (d, 6.2, Thr^5-H$_3$γ); 8.10 (d, 4.2, Val^6-NH), 3.50 (dd, 7.4, 4.2, Val^6-Hα), 1.95 (m, Val^6-Hβ), 0.95 (d, 6.7, Val^6-H$_3$γ), 0.87 (d, 6.8, Val^6-H$_3$γ′); 7.22 (d, 6.7, Ala^7-NH), 3.97 (dq, 6.7, 7.4, Ala^7-Hα), 1.25 (d, 7.4, Ala^7-H$_3$β); 7.03 (d, 9.7, Ile^8-NH), 4.13 (dd, 10.1, 9.7, Ile^8-Hα), 1.91 (m, Ile^8-Hβ), 0.87 and 1.32 (m, Ile^8-H$_2$γ), 0.56 (d, 6.5, Ile^8-H$_3$γ′), 0.77 (t, 7.2, Ile^8-H$_3$δ). (*Can be exchanged).

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