A Cytotoxic Cyclic Heptapeptide from the Seeds of *Annona cherimola*

Alassane Wélé,*[,†,‡] Yanjun Zhang,[‡] Idrissa Ndoye,[†] Jean-Paul Brouard,[‡] Jean-Louis Pousset,[‡] and Bernard Bodo[‡]

Laboratoire de Chimie Organique et Thérapeutique, Faculté de Médecine et de Pharmacie, Université Cheikh Anta Diop Dakar-Fann, Sénégal, and Laboratoire de Chimie des Substances Naturelles, ESA 8041 CNRS, Muséum National d'Histoire Naturelle, 63 Rue Buffon, 75005 Paris, France

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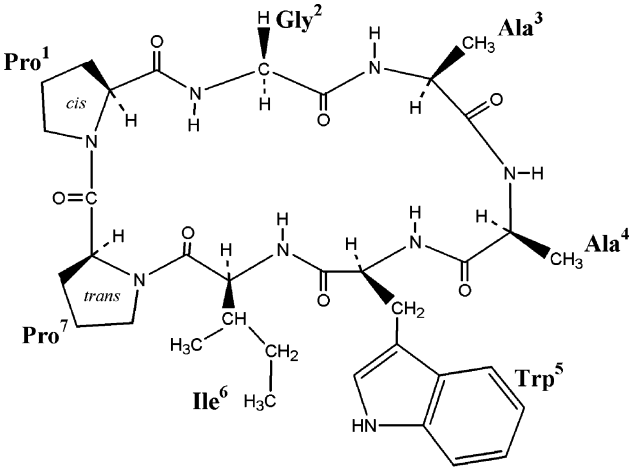
From a methanol extract of the seeds of *Annona cherimola*, a new cyclic heptapeptide, cherimolacyclopeptide C (**1**), has been isolated. The structure was elucidated on the basis of the MS/MS fragmentation using a Q-TOF mass spectrometer equipped with an ESI source, extensive 2D NMR experiments, and chemical degradation. Cherimolacyclopeptide C exhibited significant in vitro cytotoxic activity against KB cells, with an IC_{50} value of 0.072 μM.

Annona cherimola Miller (Annonaceae) is a small tree native to Ecuador and Peru, now widespread in subtropical areas of America, Africa, and Asia and even in the south of Europe, where it is cultivated for its edible fruits. The plant is also used in folk medicine. *A. cherimola* is known to produce many natural compounds of biological interest, such as alkaloids[1] and acetogenins.[2] Several cyclic peptides have been isolated from various natural sources, such as cyclosenegalins A and B,[3] mahafacyclins A and B,[4,5] cyclogossins A and B,[6,7] yunnanins A−C,[8,9] and cherimolacyclopeptides A and B[10] from plants. In continuation of our program on cyclopeptides from the seeds of plants,[3,10] we report herein on the isolation and the sequence determination, based on tandem mass spectroscopy and 2D NMR, of a new cycloheptapeptide from *A. cherimola* seeds, named cherimolacyclopeptide C (**1**).



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The dried and ground seeds of *A. cherimola* were successively extracted with cyclohexane and methanol, and the MeOH extract after concentration was dissolved in ethyl acetate. The EtOAc-soluble fraction was purified by exclusion chromatography, silica gel column chromatography, and C_{18} reversed-phase HPLC to yield a new cyclopeptide, cherimolacylopeptide C (**1**). Positive reaction with chlorine/*o*-tolidine reagent suggested it was a peptide and the absence of coloration of its TLC spot with ninhydrin, that it was cyclic. Analysis of the total acidic hydrolyzate, after derivatization, indicated the presence of Ala (2), Gly (1), Ile (1), Pro (2), and Trp (1). The amino acids were converted into the *n*-propyl esters of their *N*-trifluoroacetyl derivatives, analyzed by gas chromatography on a chiral capillary column, and their retention times compared with those of standards indicated that all the chiral amino acids were L.

The molecular weight of 692 for cherimolacyclopeptide C (**1**) was deduced from the positive ESIQTOFMS, which displayed the $[M + Na]^+$ and $[M + K]^+$ adduct ions respectively at *m/z* 715.35 and 731.34 and the protonated molecular $[M + H]^+$ ion at *m/z* 693.38. According to the amino acid composition, the molecular formula $C_{35}H_{48}N_8O_7$ was assigned to **1**.

The presence of a proline residue in the sequence leads to a specific fragmentation at the peptidyl−prolyl (Xaa-Pro) amide bond and gave a C-terminal linear peptide, which generated a series of acylium ions (b_n) resulting in the successive loss at the amide bond (CO−NH), and a series of adjacent ions (a_n) corresponding to the successive loss at the αC−CO bond. The sequence could be deduced by analyzing the b_n and a_n series ions.[3,10,11]

The protonated molecular ion $[M + H]^+$ of **1** at *m/z* 693.38 was subjected to a CID experiment (Figures 1 and 2) and showed a main series of adjacent b_n peaks at *m/z* 596, 483, 297, 226, and 155 corresponding to the successive loss of Pro, Ile, Trp, Ala, and Ala, yielding the *N*-terminal dipeptide $[H\text{-Pro-Gly}]^+$ and suggesting the sequence H-Pro[1]-Gly[2]-Ala[3]-Ala[4]-Trp[5]-Ile[6]-Pro[7] for the linearized peptide. A second series of ions with peaks at *m/z* 580, 394, 323, 252, and 195 was assigned to a second b'_n ion series, showing the successive loss of Ile, Trp, Ala, Ala, and Gly, yielding the terminal dipeptide ion $[H\text{-Pro-Pro}]^+$, which indicated the sequence H-Pro[7]-Pro[1]-Gly[2]-Ala[3]-Ala[4]-Trp[5]-Ile[6]. The sequence was confirmed by the observation of the two corresponding a_n and a'_n ion series (Figures 1 and 2). The a_n ion series at *m/z* 568, 455, and 269 showed the successive loss of Pro, Ile, and Trp and yielding the terminal tetrapeptide ion $[H\text{-Pro-Gly-Ala-Ala}]^+$, and the a'_n ion series with peaks at *m/z* 552, 366, and 295 indicated the successive loss of Ile, Trp, and Ala and the terminal tetrapeptide ion $[H\text{-Pro-Pro-Gly-Ala}]^+$. These results suggested the sequence $[H\text{-Pro}^1\text{-Gly}^2\text{-Ala}^3\text{-Ala}^4\text{-Trp}^5\text{-Ile}^6\text{-Pro}^7]^+$ for the linearized peptide ion derived from cherimolacyclopeptide C and thus the structure **1** for the natural cyclic heptapeptide.

* Corresponding author. Tel: (221) 865-23-62. Fax: (221) 825-29-52. E-mail: alassanewele@yahoo.fr.
† Université Cheikh Anta Diop Dakar.
‡ Muséum National d'Histoire Naturelle.

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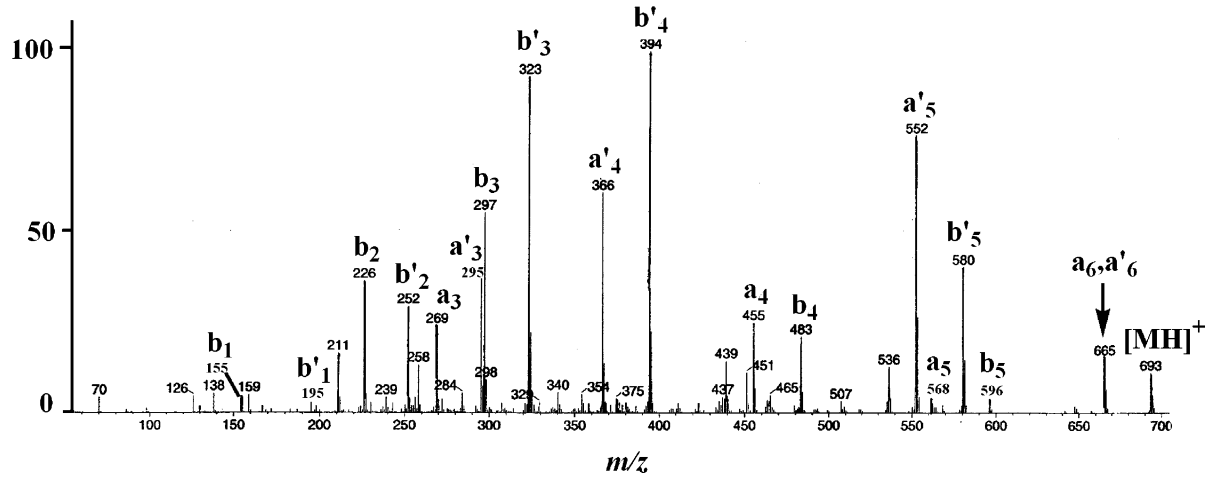

Figure 1. CID mass spectrum of the $[M + H]^+$ ion (m/z = 692) of cherimolacyclopeptide C (**1**).

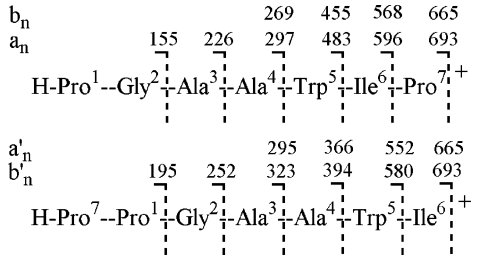

Figure 2. CID fragmentation (m/z 692) of the protonated cherimolacyclopeptide C (**1**) ion.

The ^{1}H NMR spectrum of cherimolacyclopeptide C (**1**) in DMSO-d_6 solution showed a main stable conformational state (>85%) for which the five amide protons were clearly depicted, as well as the presence of seven carbonyl groups in the ^{13}C NMR spectrum, in agreement with a heptapeptide structure including two prolines (Table 1). The peptide sequence determination was based on the HMBC experiment. This heteronuclear methodology was preferred compared to the homonuclear method described by Wüthtrich et al. and based on $d_{NN(i, i+1)}$ and $d_{\alpha N(i, i+1)}$ connectivities from the ROESY/NOESY spectra,[12,13] because for small-size cyclic peptides, conformational information can interfere with sequential information. All the amino acid spin systems were identified using scalar spin−spin couplings determined from the ^{1}H−^{1}H COSY and TOCSY experiments.[14] 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 nonprotonated carbons. This experiment allowed the carbonyl groups to be assigned. In this manner, the sequence determination was carried out from the connectivities between the carbonyl of residue i with the amide and/or α protons of residue i+1. The $^2J_{CH}$, CO (i) to NH (i+1) correlations from Pro^1 to Ile^6, shown in Figure 3, were observed on the HMBC spectrum. The ROESY spectrum clearly showed four $d_{NN(i,i+1)}$ interactions from Gly^2 to Trp^5. A stretch of $d_{\alpha N(i,i+1)}$ sequential connectivities was observed from Pro^1 to Ile^6 (Figure 3). The $d_{\alpha\alpha(i,i+1)}$ connectivities between the two α protons of the proline residues indicated that the second proline is at position 7. Chemical shifts of the γ carbons of Pro^1 and Pro^7 at 21.7 and 24.2 ppm, respectively, indicated the presence of *cis*-Pro^1 and *trans*-Pro^7 amid bonds.[15] The prochiral assignment of the β, γ, and δ protons of Pro resulted in the observed NOEs in the ROESY spectrum. The whole complete NMR data agreed with the cyclic structure **1** for cherimolacyclopeptide C, the sequence of which was thus determined as *cyclo*(Pro^1-Gly^2-Ala^3-Ala^4-Trp^5-Ile^6-Pro^7).

Table 1. ^{13}C and ^{1}H NMR Data for Cherimolacyclopeptide C (**1**), in DMSO-d_6 (400 MHz, 293 K; s, singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet)

residue		δ_C	δ_H, multiplicity (J Hz)
Pro^1	CO	172.2	
	α CH	60.0	4.33 d (4.0)
	β CH_2	31.6	2.14 m
			2.00 m
	γ CH_2	21.7	1.80 m
			1.65 m
	δ CH_2	46.5	3.54 m
			3.32 m
Gly^2	CO	167.7	
	NH		8.39 dd (5.5, 6.2)
	α CH_2	43.7	3.78 dd (6.2, 16.7)
			3.57 dd (5.5, 16.7)
Ala^3	CO	173.1	
	NH		7.50 d (8.4)
	α CH	46.7	4.52 dq (8.4, 7.1)
	β CH_3	19.3	1.24 d (7.1)
Ala^4	CO	172.9	
	NH		8.36 d (3.2)
	α CH	51.5	3.70 dq (3.2, 7.2)
	β CH_3	16.1	1.08 d (7.2)
Trp^5	CO	171.1	
	NH		7.84 d (8.0)
	α CH	54.3	4.37 td (4.6, 8.0)
	β CH_2	26.4	3.22 m
	2′ CH	123.0	7.02 m
	3′ C	110.6	
	4′ CH	118.0	7.47 m
	5′ CH	118.3	6.98 m
	6′ CH	121.0	7.04 m
	7′ CH	111.4	7.30 m
	3′a C	127.0	
	7′a C	136.1	
	1′ NH		10.79 s
Ile^6	CO	167.8	
	NH		6.81 d (8.5)
	α CH	54.8	4.30 m
	β CH	36.4	1.82 m
	γ CH_2	23.9	1.50 m
			1.01 m
	γ' CH_3	15.8	0.81 d (7.3)
	δ CH_3	11.6	0.83 t (6.6)
Pro^7	CO	170.2	
	α CH	58.6	4.35 d (8.5)
	β CH_2	28.1	2.20 m (8.5)
			1.72 m
	γ CH_2	24.2	1.92 m
			1.88 m
	δ CH_2	46.9	3.52 m
			3.37 m

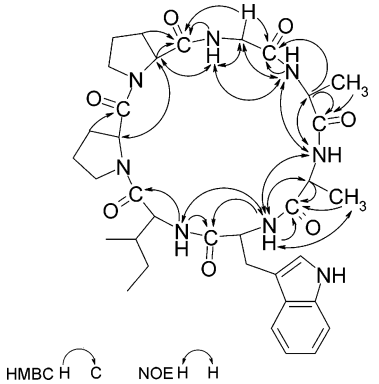

Figure 3. HMBC and NOE correlations for cherimolacyclopeptide C (**1**).

In a 3-day cytotoxicity bioassay, cherimolacyclopeptide C (**1**) exhibited significant activity in vitro against the KB (human nasophryngeal carcinoma) cell culture system, with an IC_{50} value of 0.072 μM. Doxorubicin, with an IC_{50} value of 0.02 μM, was used as the positive control.

Experimental Section

General Experimental Procedures. The melting point was determined on a Büchi melting point B-545 apparatus. The optical rotation was measured with a Perkin-Elmer model 341 polarimeter, and the $[\alpha]_D^{22}$ value is given in deg cm^2 g^{-1}. ^{1}H and ^{13}C NMR spectra were recorded either (1D ^{13}C) on a Bruker AC 300 spectrometer, equipped with an Aspect 3000 computer using DISNMR software, or (2D spectra) on a Bruker Avance 400 spectrometer, operating at 400.13 MHz. The coupling constant used to establish the necessary delay for the selection of the protons 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. A phase-sensitive ROESY experiment was carried out with a mixing time of 150 ms. Mass spectra were recorded on an API Q-STAR Pulsar i mass spectrometer (Applied Biosystems). For the CID spectra, the collision energy was 40−60 eV and the collision gas was nitrogen.

Plant Material. Fruits of *Annona cherimola* Miller (Annonaceae) were collected in the south of Spain in December 2000. The seeds were collected and were immediately washed with distilled water and dried at room temperature. Samples were authenticated by Prof. P. Boiteau (National Museum of Natural History, Paris), and a voucher specimen (VF 10463) is deposited in the herbarium of Department of Botany, University of Valencia (Spain). The dried and powdered seeds of *A. cherimola* (3.0 kg) were macerated three times with cyclohexane (3 L), and the combined extracts yielded an oil (531 g), which was discarded. The seeds were then extracted three times with MeOH (3 L) at room temperature to give after concentration under reduced pressure the MeOH extract (126 g), which was partitioned between EtOAc and water. The organic phase was concentrated to dryness, and the residue (61.5 g) was dissolved in MeOH and chromatographed on a Sephadex LH-20 column with MeOH. The peptide fraction (33.6 g) was then repeatedly subjected to silica gel column chromatography (Kieselgel 60 H Merck) and eluted with CH_2Cl_2 containing increasing amounts of MeOH from 5% to 20%. The peptide purification was monitored by TLC (silica gel 60 F_{254} Merck) with CH_2Cl_2−MeOH (9:1) as eluent system, and the peptides were detected with the chlorine/*o*-tolidine reagent exhibiting a characteristic blue spot with $R_f = 0.43$. The corresponding peptide mixture was finally purified by isochratic reversed-phase HPLC (Kromasil C_{18}, 250 × 7.8 mm, 5 μm, AIT Paris, France; flow rate 2 mL/min, detection 220 nm) using MeOH−H_2O (55:45) with 1% TFA, to yield cherimolacyclopeptide C (**1**, t_R = 17.4 min, 19.8 mg).

Absolute Configuration of Amino Acids. A solution of **1** containing 1 mg of peptide, in 6 N HCl (1 mL), was heated at 110 °C for 24 h in a sealed tube. After cooling, the solution was concentrated to dryness. The hydrolysate was dissolved in an anhydrous solution of 3 N HCl in 2-propanol and heated at 110 °C for 30 min. The reagents were evaporated under reduced pressure, the residue was dissolved in CH_2Cl_2 (0.5 mL), and 0.5 mL of trifluoracetic acid anhydride was added. The mixture was kept in a screw-capped vial at 110 °C for 20 min, the reagents were evaporated, and the mixture was 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 a 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 t_R (min) values with those of standards amino acids was used: DL-Ala (10.6, 11.6), Gly (14.6), DL-Pro (18.0, 18.2), DL-Ile (16.2, 16.9), and DL-Trp (34.2, 36.4).

Cherimolacyclopeptide C (1): colorless solid; mp 233−234 °C (MeOH); $[\alpha]^{22}_D$ −8.4° (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR (see Table 1); ESIQTOFMS, *m/z* 731.34 $[M + K]^+$, 715.35 $[M + Na]^+$, 693.38 $[M + H]^+$; ESIQTOFMS-MS *m/z* 693.38 $[M + H]^+$ (40 eV) *m/z* 693 (11), 665 (15), 596 (2), 580 (40), 568 (1), 552 (76), 483 (21), 455 (25), 394 (100), 366 (61), 323 (93), 297 (56), 295 (37), 269 (25), 226 (37), 252 (30), 195 (2), 155 (2).

Bioassays. An aliquot of purified cherimolacyclopeptide C (**1**) was dissolved in DMSO solution for cytotoxicity evaluation against KB cells. Details of the assay procedure are described in the literature.[16]

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