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Cherimolacyclopeptide D, a novel cycloheptapeptide from the seeds of *Annona cherimola*

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Abstract

In a chemical investigation of the seeds of *Annona cherimola*, a natural cyclic heptapeptide, cherimolacyclopeptide D, were isolated and purified by HPLC with three known cyclic peptides, cherimolacyclopeptides A, B and C. The structure was established by various analyses including MS/MS fragmentation, spectroscopic and chemical evidences.

Keywords: *Annona cherimola*; Annonaceae; Cyclopeptide; Cherimolacyclopeptide D

1. Introduction

The members of the *Annonaceae* family (about 128 genera), generally known as insecticide and parasiticide (Kerharo and Adam, 1974) and mostly distributed in America and Asia, are now cultivated for its edible fruits in Europe. Previous chemical studies revealed many natural compounds of biological interest. Also, *Annona* sp. has been described to produce cyclopeptides which were found to be cytotoxic against tumoral KB cells (Wélé et al., 2002, 2004). Preliminary conformational studies have revealed that cycloheptapeptides are usually organized with two β-turns, one stabilized by a normal hydrogen bond and the second by a bifurcated hydrogen bond forming a β-bulge (Auvin-Guette et al., 1999). In this paper we report the isolation and the structural elucidation based on tandem mass spectroscopy and 2D NMR experiments of a new cyclic heptapeptide, cherimolacyclopeptide D.

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2. Results and discussion

2.1. Isolation and characteristics

The dried and ground seeds of *A. cherimola* were successively extracted with cyclohexane and methanol. Constituents were separated by the combination of column chromatography on silica gel and Sephadex LH-20 gel. Further purification by semi-preparative HPLC gave four compounds, three of them were identified as cherimolacyclopeptides A, B and C (Wélé et al., 2002, 2004). The IR spectrum of cherimolacyclopeptide D gave a negative ninhydrin reaction and showed IR maxima absorptions at 3320 and 1650 cm^{-1} indicated that the compound might be a peptide. Analysis of the total acidic hydrolysate of this compound, after derivatization, showed the presence of Ala (1), Asx (1), Gly (1), Leu (1), Thr (1), Pro (1) and Val (1). The amino acids were converted into *n*-propyl esters of their *N*-trifluoroacetyl derivatives, analysed by gas chromatography on a chiral capillary column. Comparisons of R_t values with those of standards indicated that all the chiral amino acids were L.

2.2. Mass spectral analysis

According to the amino acids composition, the positive ESI-QTOF measurements of cherimolacyclopeptide D gave the molecular formula $C_{29}H_{48}N_8O_7$ corresponding to the molecular weight 652 Da. The $[M + Na]^+$ adducted ion and the protonated molecular $[M + H]^+$ ion occurred respectively at *m/z* 675 and 653. The protonated ion $[M + H]^+$ at *m/z* 653 was subjected to CID experiments (Fig. 1). The ring opening began at Thr–Pro amide bond level and a series of adjacent acylium ions (b_n) at *m/z* 552, 453, 382, 268 and 155 was generated from which the sequence could be deduced: amino acids residue were lost sequentially from the C-terminus to the N-terminus and for cherimolacyclopeptide D was observed the successive loss of Thr, Val, Ala, Asn and Leu, yielding the N-terminal dipeptide Pro–Gly. A second series of ions was observed at *m/z* 524, 425, 354 and 240 which were assigned to adjacent (a_n) ions related to the above b_n ion series. At *m/z* 635 was observed an abundant ion originating from the loss of a molecule of water from the protonated molecular ion $[M + H]^+$. These results suggested the sequence [H-Pro[1]–Gly[2]–Leu[3]–Asn[4]–Ala[5]–Val[6]–Thr[7]]$^+$ for the linearised peptide ion derived from cherimolacyclopeptide D and thus the structure of this peptide for the natural cycloheptapeptide (Figs. 2 and 3).

2.3. NMR study

The ^1H and ^{13}C spectre of cherimolacyclopeptide D were recorded in DMSO-d_6 solution and showed a main conformational state (>95%). The ^1H NMR spectrum of this cycloheptapeptide showed resonances at 7.34 and 8.03 ppm which corresponded to the *syn* and *anti* protons of Asn and six protons in the amide region proton. The ^{13}C NMR spectrum displayed signals for seven carbonyl groups, in agreement with a heptapeptide structure including one proline. The ^1H NMR spectrum was assigned via standard sequential assignment methods developed by Wüthrich et al. (1982, 1984). The COSY and TOCSY spectra assigned the spin systems of the unique Gly, Asn, Ala, Leu, Val, Thr and Pro. All the amino acids were identified using scalar spin–spin couplings determined from ^1H–^1H COSY and TOCSY experiments (Wagner and Akumar, 1981). The ^{13}C NMR assignments of the proton detected heteronuclear HSQC spectrum and combined with the HMBC experiment optimised for long-range *J*-value of 7 Hz for the non-protonated carbons. The carbonyl carbons of the seven amino acids were clearly identified (Table 1). The peptide sequence determination was based on the HMBC experiment. By this way, the sequence determined was done from the connectivities between the carbonyl of residue *i* with the amide and/or a protons of residue *i* + 1. As examples, the CO group of Pro[1] at 171.4 ppm was correlated with the NH, αH and α′H of Gly[2], the CO group of Gly[2] at 168.3 ppm to both

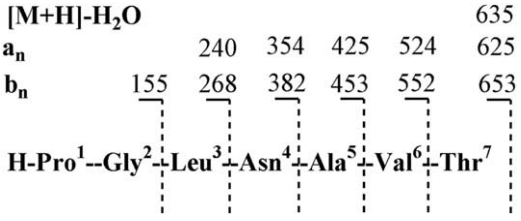

Fig. 1. CID fragmentation of the protonated cherimolacyclopeptide D ion.

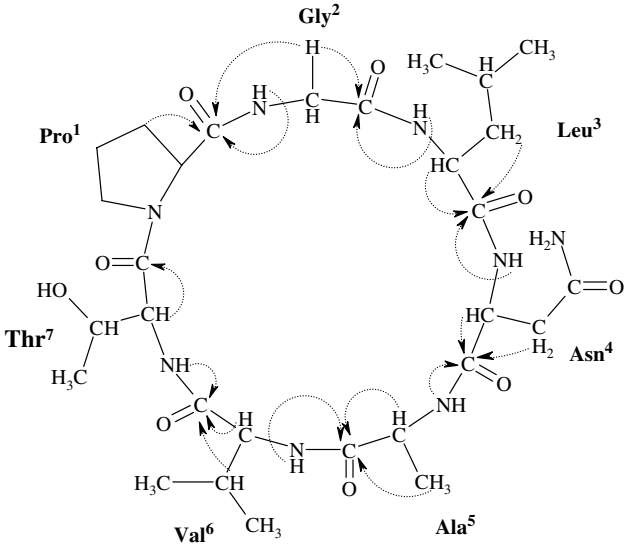

Fig. 2. HMBC correlations for cherimolacyclopeptide D.

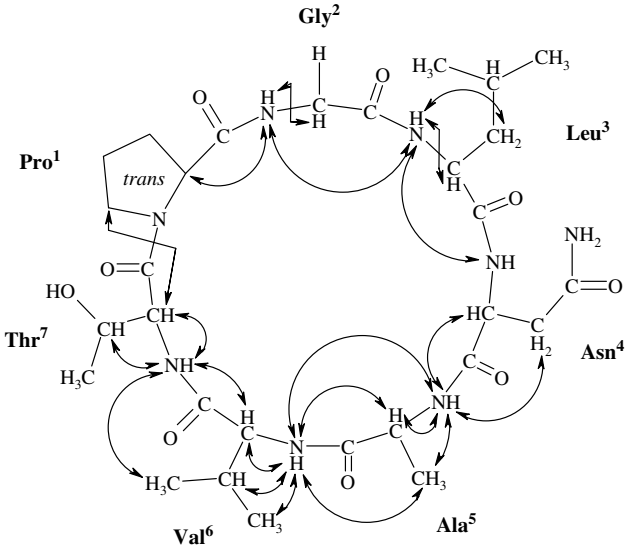

Fig. 3. NOEs correlations for cherimolacyclopeptide D.

Table 1
^{13}C and ^{1}H NMR data for cherimolacyclopeptide D (DMSO d_6, 298 K)

Residue	δ_C	δ_H	m (J Hz)
Pro[1] CO	171.4	–	
α-CH	60.6	4.11	*dd* 8.9, 7.0
β-CH_2	29.1	2.08	*m*
	–	1.78	*m*
γ-CH_2	24.5	2.01	*m*
	–	1.90	*m*
δ-CH_2	48.2	3.99	*m*
	–	3.63	*m*
Gly[2] CO	168.3	–	
NH	–	8.85	*dd* 7.7, 4.9
α-CH_2	42.5	3.96	*dd* 17.1, 7.7
	–	3.32	*dd* 17.1, 4.9
Leu[3] CO	170.6	–	
NH	–	7.90	*d* 10.5
α-CH	53.0	4.53	*m*
β-CH_2	43.3	1.39	*m*
	–	1.17	*m*
γ-CH	24.0	1.41	*m*
δ-CH_3	22.1	0.84	*d* 6.4
δ′-CH_3	22.2	0.79	*d* 6.7
Asn[4] CO	170.0	–	
NH	–	8.20	*d* 5.6
α-CH	49.7	4.33	*m*
β-CH_2	34.5	3.23	*dd* 16.7, 2.7
	–	3.04	*dd* 16.7, 4.5
NH_{syn}	–	7.34	*s*
NH_{anti}	–	8.03	*s*
CO	173.4		
Ala[5] CO	172.2	–	
NH	–	8.14	*d* 3.4
α-CH	51.5	3.86	*dq* 3.4, 7.4
β-CH_3	19.8	1.30	*d* 7.4
Val[6] CO	170.3	–	
NH	–	7.81	*d* 10.4
α-CH	58.8	4.12	*dd* 10.4, 7.3
β-CH	30.3	2.05	*m*
γ-CH_3	19.3	0.81	*d* 5.0
γ′-CH_3	18.4	0.80	*d* 6.5
Thr[7] CO	169.0	–	
NH	–	7.75	*d* 9.0
α-CH	56.5	4.50	*dd* 9.9, 9.0
β-CH	66.5	3.69	*m*
γ-CH_3	19.8	1.04	*d* 6.2

NH of Leu[3] and αα′H of Gly[2], and the CO group of Leu[3] at 170.6 ppm to the NH of Asn[4]. The CO group of Thr was not correlated with an amide proton suggesting that this residue is connected to the proline residue (Fig. 2). Others connectivities are in full agreement with the structure deduced from mass spectrometry study. The ROESY spectrum recorded at 298 K in DMSO-d_6 clearly depicted strong $d_{NN(i,\ i+1)}$ interactions between Asn[4] and Leu[3], Ala[5] and Val[6], and between Gly[2] and Leu[3]. A stretch of $d_{\alpha N(i,\ i+1)}$ sequential connectivities from Pro[1] to Thr[7]. The strong NOE between the amide proton of Gly and α proton of Pro suggested that the proline residue is at position 1 and the glycine residue at position 2. In additional a strong correlation was observed between the α proton of Thr[7] and both δδ′ protons of Pro[1] indicating that the amide bond Thr[7]–Pro[1] is *trans*. The stereochemistry was further confirmed by the γ carbons ^{13}C chemicals shifts of Pro[1] at 24.5 and 29.1 ppm, respectively, in agreement with the presence of *trans*-Pro[1] (Fig. 3) (Douglas and Bovey, 1973).

All the data suggested the cyclic structure for cherimolacyclopeptide D, indicating only one *trans*-amide bond.

In three-days cytotoxicity bioassays, cherimolacyclopeptide D exhibited activity in vitro against KB (human nasophryngeal carcinoma) cell culture systems, with an IC_{50} value of 0.97 μM. Doxorubicin, with an IC_{50} value of 0.02 μM, was taken as the positive control.

3. Experimental section

3.1. General experimental procedures

IR spectra were obtained using KBr discs and the melting point was determined on a Büchi melting point B-545 apparatus. 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}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 (2D spectra). 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.

3.2. Plant material

Fruits of *A. 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 were dried at room temperature. Samples were authenticated by Prof. P. Boiteau (National Museum of Natural History, Paris) and voucher specimen (VF 10463) is deposited in the herbarium of Department of Botany, University of Valencia, Spain.

3.3. Extraction and isolation

Details of the methodology of isolation and purification of cyclic peptides are described in the last papers (Wélé et al. 2002; Wélé et al., 2004). The methanol extract (126.0 g) of powdered seeds of *A. cherimola* (3.0 kg) 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 successively on Sephadex LH-20 and silica gel (Kieselgel 60 H Merck) columns, and the finally purifiedby isochratic reversed phase HPLC (Kromasil C_{18}, 250×7.8 mm, 5 μm, AIT France; flow rate 2 mL/min, detection 220 nm) using MeOH/H_2O:75/25 with 1% TFA to yield cherimolacyclopeptide D (1, t_R 6.3 min, 19.8 mg).

3.4. Absolute configuration of amino acids

Solution of cherimolacyclopeptide D containing 1 mg of peptide, in 6 N HCl (1 ml) was heated at 110 °C for 24 h in sealed tubes. Free amino acids residues were methylated and the methylated amino acids were analysed by CPG (for details see Wélé et al. (2002) and Wélé et al. (2004)). Comparison of retention time (min) values with those of standards amino acids was used: DL-Ala (10.6, 11.6), DL-Asx (24.6, 25.1), Gly (14.6), DL-Leu (18.1, 19.2), DL-Pro (18.0, 18.2), DL-Thr (14.5, 15.2) and DL-Val (13.4, 13.9).

3.5. Bioassays

Aliquot of the purified cherimolacyclopeptide D was dissolved in DMSO solution for cytotoxicity evaluation in vitro, in three days against KB cell. Details of the assay procedure are described in the literature A (Chang et al., 1998)

3.6. Cherimolacyclopeptide D

Colourless solid, M.p. 220–221°C (MeOH), $[\alpha]_D^{22}$ −64° (*c* 0.1, MeOH); λ_{max}^{KBr} cm^{-1}: 3320 and 1650. ^1H and ^{13}C NMR (see Table 1); ESI-QTOF, *m/z*: 691 $[M + K]^+$, 675 $[M + Na]^+$, 653 $[M + H]^+$; ESI-QTOF MS/MS on *m/z* 653 $[M + H]^+$ (ce 40 eV) *m/z* (%): 653 (33), 635 (41), 625 (17), 552 (36), 536 (30), 524 (28), 519 (30), 507 (26), 454 (32), 453 (100), 448 (20), 436 (59), 425 (37), 408 (36), 382 (76), 365 (30), 354 (16), 285 (36), 268 (45), 240 (17), 155 (12).

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