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Glaucacyclopeptide A from the seeds of *Annona glauca*

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Abstract

The seeds of *Annona glauca* furnished two cyclopeptides one of which is novel. The structure was elucidated on the basis on mass spectrometry, 2D NMR methods and amino acids analysis.
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Keywords: *Annona glauca*; Annonaceae; Cyclopeptide; Glaucacyclopeptide A

1. Introduction

In the continuation of our search for cyclopeptides in Senegalese medicinal plants (Wélé et al., 2002, 2004a,b,c, 2005), we examined the ethyl acetate extract of the seeds of *Annona glauca* (Annonaceae), a widespread small shrub growing in Senegal, which is used in traditional medicine for various pains (Kerharo and Adam, 1974). Phytochemical studies on this plant led to the isolation of acetogenins (Etcheverry et al., 1995). No previous studies on cyclic peptides on *A. glauca* have been reported. In this paper, we describe the isolation and the structure determination of one new cyclic heptapeptide glaucacyclopeptide A, obtained alongside with the known annomuricatin C (Wélé et al., 2004a,b,c). The cytotoxic activity of this compound was evaluated.

2. Results and discussion

2.1. Extraction and isolation

The dried and ground seeds of *Annona glauca* (1.3 kg) were successively extracted with cyclohexane and methanol. The methanol extract was concentrated to dryness leaving a dark green residue (68.5 g) that was re-extracted with ethyl acetate. The ethyl acetate fraction (23.6 g) was purified by column chromatography on Sephadex LH-20 gel and silica gel. Further purification by semi-preparative HPLC gave two compounds, one of them was identified as annomuricatin C (5 mg) isolated by us (Wélé et al., 2004c). The IR spectrum of the second compound, named glaucacyclopeptide A (8 mg) showed IR maxima absorptions at 3320 and 1650 cm^{-1}. A positive reaction with chlorine-*o*-tolidine reagent and a negative ninhydrin reaction suggested that this compound might be a cyclic peptide. After hydrolysis of this compound, the free amino acids were converted into *n*-propyl esters of their *N*-trifluoroacetyl derivatives and were analysed

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by gas chromatography on chiral capillary column. Comparisons of R_t values with those of standards showed the presence of Ala (1), Gly (2), Leu (1), Pro (1) and Val (2). The configuration of all the chiral amino acids were L.

2.2. Mass spectral analysis

Mass spectrometric of glaucacyclopeptide A was determined by ESI-QTOFMS and gave a pseudo molecular ion $[M+H]^+$ at *m/z* 594 and the $[M+Na]^+$ adduct ion at *m/z* 616. According to the amino acids composition, the molecular formula was $C_{28}H_{47}O_7N_7$. The protonated ion at *m/z* 594 was subjected to CID experiments (ESI-QTOFMSMS) (Fig. 1). The ring opening started at Leu–Pro amide bond giving a series of adjacent acylium ions b_n at *m/z* 481, 382, 283, 226 and 155, and a second ion series at *m/z* 453, 354, 255, 198 and 127 corresponding to the adjacent a_n ions. Amino acids residues were lost sequentially from the C-terminus to the N-terminus and the analysis of the b_n and a_n ion series corresponds to the successive loss of Leu, Val, Val, Gly, Ala and the terminal dipeptide Pro–Gly. These results suggested the sequence $[H\text{-}Pro^1\text{–}Gly^2\text{–}Ala^3\text{–}Gly^4\text{–}Val^5\text{–}Val^6\text{–}Leu^7]^+$ for the linearised peptide derived from glaucacyclopeptide A corresponding to a cycloheptapeptide (Figs. 2 and 3).

2.3. NMR spectral analysis

The heptapeptide nature was evident from its ^{13}C NMR spectrum in DMSO-d_6 indicating seven amide carbonyl groups at 169.1–173.4 ppm. The ^1H NMR spectrum showed six amide protons at 7.26–9.2 ppm. All the amino acids were identified using scalar spin–spin couplings determined from ^1H–^1H COSY and TOCSY experiments (Wagner and Akumar, 1981). The corresponding carbon resonances were elucidated on the basis on the HSQC spectrum. The peptide sequence determination was done from the connectivities between the carbonyl of residue (i) with the amide and/or protons of residue $(i+1)$ on the basis on the HMBC spectrum. All the $d_{CO_iNH_{i+1}}$ correlations were clearly depicted, as shown in Fig. 2. The CO group of

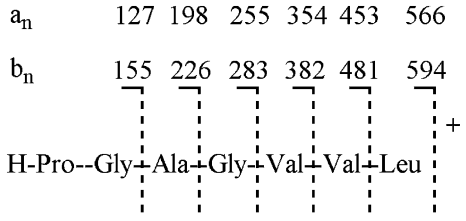

Fig. 1. CID fragmentation of the protonated glaucacyclopeptide A ion.

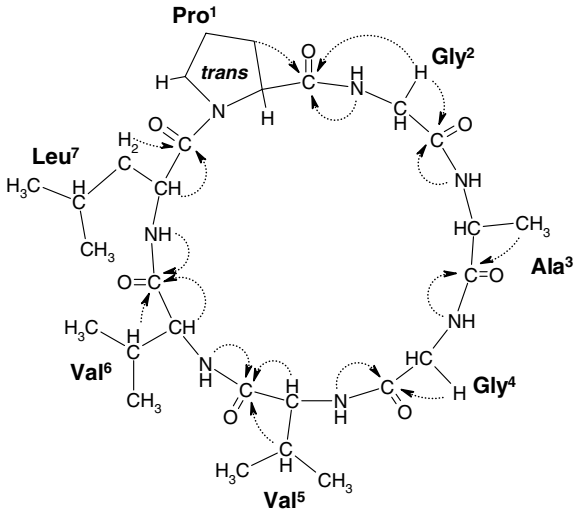

Fig. 2. HMBC correlations for glaucacyclopeptide A.

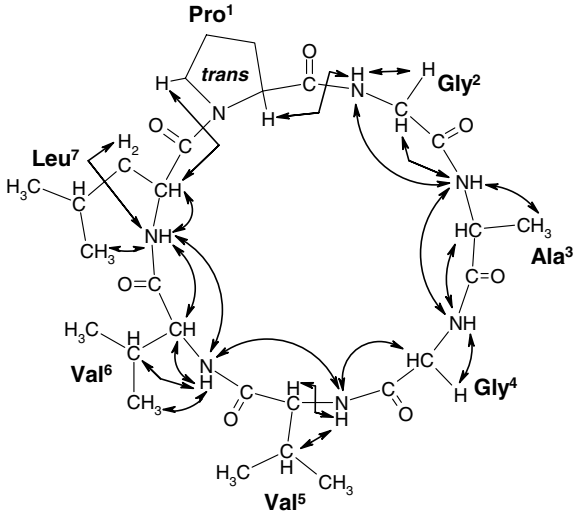

Fig. 3. NOEs correlations for glaucacyclopeptide A.

Leu was not correlated with an amide proton suggesting that this residue is connected to the proline residue. These results are in full agreement with the structure deduced from mass spectrometry study. The ROESY spectrum recorded at 298 K in DMSO-d_6 clearly showed $d_{NN(i,i+1)}$ and $d_{\alpha N(i,i+1)}$ sequential connectivities from Pro^1 to Leu^7 (Fig. 3). A strong correlation between $\delta\delta'$ protons of Pro^1 was observed indicating that the amide bond Leu^7–Pro^1 is *trans*. In addition, the ^{13}C signals ascribable to γ and β carbons of Pro^1 were resonated at 24.7 and 29.5 ppm, respectively, in agreement with the presence of *trans*-Pro^1 (Douglas and Bovey, 1973). Therefore, the structure of glaucacyclopeptide A was unequivocally established to be *cyclo* (Pro–Gly–Ala–Gly–Val–Val–Leu), including only one *trans* amide bond.

2.4. Bioassays

As the most of the cyclopeptides isolated from the seeds of *Annona* sp., glaucacylopeptide A exhibited cytotoxic activity in vitro against tumoral KB cells culture systems with an IC_{50} value at 0.73 μM. Doxorubicin (IC_{50} 0.02 μM) was used as positive control.

3. Experimental section

3.1. General

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}. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer, mass spectra were recorded on an API Q-STAR Pulsar i mass spectrometer (Applied Biosystems) at 40–60 eV.

3.2. Extraction and isolation

Seeds of *Annona glauca* were obtained from fruits collected in October 2001 by Dr Modou Lô from Dakar. A voucher specimen (AL 301) is deposited at Jardin des Plantes Utiles, Faculté de Médecine et de Pharmacie, Dakar, Sénégal.

Details of the methodology of isolation and purification of cyclic peptides are described in the last papers (Wélé et al., 2002, 2004a,b,c). The methanol extract of powdered seeds were treated with ethyl acetate which was concentrated and successively chromatographied on Sephadex LH-20 and silica gel columns. The final purification on reversed-phase HPLC (Kromasil C18, 250 × 7.8 mm, 5 μm, AIT France; flow rate 2 mL/min, detection 220 nm) using MeOH/H_2O: 60/40 yielded glaucacyclopeptide A (t_R 6.5 min, 8 mg).

3.3. Absolute configuration of amino acids

Glaucacyclopeptide A (1 mg) was dissolved in 1 ml of 6N HCl and was heated at 110 °C for 24 h in sealed tube. Free amino acids were methylated into *n*-propyl esters of their *N*-trifluoroacetyl derivatives and methylated amino acids were analysed by GC (for details see Wélé et al., 2002, 2004a,b,c). The retention time values (min) were compared with those of standards amino acids: DL-Ala (10.6, 11.6), Gly (14.6), DL-Leu (18.1, 19.2), DL-Pro (18.0, 18.2) and DL-Val (13.4, 13.9).

3.4. Bioassays

Cytotoxic assays were carried out in three days on human cancer KB line. Details of the assays procedure expressed as IC_{50} (μM) are described in the literature (Chang et al., 1998).

3.5. Glaucacyclopeptide A

Colourless solid, m.p. 174–175 °C (MeOH), $[\alpha]_D^{22}$ −57° (c 0.2, MeOH), λ_{max}^{KBr} cm^{-1}: 3320 and 1650. ^1H NMR (DMSO-d_6, TMS): 4.43 (1H, *dd*, 7.2, 8.3, Pro^1-Hα), 1.89 (1H, *m*, Pro^1-Hβ), 1.70 (1H, *m*, Pro^1-Hβ), 1.79 (2H, *m*, Pro^1-Hγ), 3.43 (1H, *m*, Pro^1-Hδ), 3.80 (1H, *m*, Pro^1-Hδ′), 4.10 (1H, *dd*, 16.5, 4.2, Gly^2-Hα), 3.76 (1H, *dd*, 8.3, 16.5, Gly^2-Hα′), 8.86 (1H, *dd*, 4.2, 8.3, Gly^2-NH), 3.70 (1H, *dd*, 2.9, 6.8, Ala^3-Hα), 1.10 (3H, *d*, 7.7, Ala^3-Hβ), 8.20 (1H, *d*, 2.9, Ala^3-NH), 3.90 (1H, *dd*, 16.9, 3.8, Gly^4-Hα), 3.60 (1H, *dd*, 7.5, 16.9, Gly^4-Hα′), 9.20 (1H, *dd*, 3.8, 7.5, Gly^4-NH), 4.53 (1H, *dd*, 9.3, 6.1, Val^5-Hα), 1.97 (1H, *m*, Val^5-Hβ), 0.84 (3H, *d*, 5.7, Val^5-Hγ), 0.79 (3H, *d*, 6.2, Val^5-Hγ′), 7.70 (1H, *d*, 9.3, Val^5-NH), 4.30 (1H, *dd*, 10.7, 7.5, Val^6-Hα), 2.01 (1H, *m*, Val^6-Hβ), 0.82 (3H, *d*, 5.5, Val^6-Hγ), 0.84 (3H, *d*, 6.5, Val^6-Hγ′), 7.26 (1H, *d*, 10.7, Val^6-NH), 4.60 (1H, *m*, Leu^7-H α), 1.30 (1H, *m*, Leu^7-Hβ), 1.26 (1H, *m*, Leu^7-Hβ′), 1.37 (1H, *m*, Leu^7-Hγ), 0.80 (1H, *d*, 6.3, Leu^7-Hδ), 0.77 (1H, *d*, 6.5, Leu^7-Hδ′), 7.54 (1H, *d*, 9.3, Leu^7-NH). ^{13}C NMR (DMSO-d_6): 170.9 (Pro^1-CO), 60.5 (Pro^1-Cα), 29.5 (Pro^1-Cβ), 24.7 (Pro^1-Cγ), 47.9 (Pro^1-Cδ), 169.1 (Gly^2-CO), 42.4 (Gly^2-Cα), 173.4 (Ala^3-CO), 50.7 (Ala^3-Cα), 18.3 (Ala^3-Cβ), 169.6 (Gly^4-CO), 41.9 (Gly^4-Cα), 171.7 (Val^5-CO), 57.5 (Val^5-Cα), 29.8 (Val^5-Cβ), 19.6 (Val^5-Cγ), 19.7 (Val^5-Cγ′), 172.5 (Val^6-CO), 57.3 (Val^6-Cα), 30.2 (Val^6-Cβ), 18.9 (Val^6-Cγ), 19.1 (Val^6-Cγ′), 170.3 (Leu^7-CO), 52.6 (Leu^7-Cα), 42.7 (Leu^7-Cβ), 23.8 (Leu^7-Cγ), 21.9 (Leu^7-Cδ), 22.0 (Leu^7-Cδ′); ESI-QTOF, *m/z*: 594 $[M+H]^+$, 616 $[M+Na]^+$; ESI-QTOF MS/MS on *m/z* 594 $[M+H]^+$ (ce 45 eV) *m/z* (%): 594 (41), 566 (58), 481 (29), 453 (74), 382 (45), 354 (47), 283 (67), 255 (85), 226 (100), 198 (27), 155 (16), 127 (5).

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