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Two cyclopeptides from the seeds of *Annona cherimola*

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Abstract

Bioassay-guided fractionation of cytotoxic of methanol extract of the seeds of *Annona cherimola* provided two novel cyclic peptides, cherimolacyclopeptide E (**1**) and cherimolacyclopeptide F (**2**), which exhibited significant cytotoxic activity against the KB (human nasopharyngeal carcinoma) cell culture system. The peptide **1** and **2** were elucidated by MS/MS fragmentation experiments using a Q-TOF mass spectrometer equipped with an ESI source, extensive 2D NMR analyses and chemical degradation.

Keywords: *Annona cherimola*; Annonaceae; Cyclopeptide; Cherimolacyclopeptide E; Cherimolacyclopeptide F

1. Introduction

The genus *Annona* belongs to the family Annonaceae and consist of more 110 species distributed in subtropical Africa, Asia and America. *Annona cherimola* is a small tree native to Ecuador and Peru. This plant is cultivated for its edible fruits and for traditional uses. Previous publications on *A. cherimola* described the isolation from the seeds of four cyclopeptides namely cherimolacyclopeptides A, B, C, and D, (Wélé et al., 2002, 2004, 2005) Bioassay-guided fractionation of methanol extract provided a cyclohexapeptide, cherimolacyclopeptide E, and a cyclononapeptide, cherimolacyclopeptide F.

2. Results and discussion

2.1. Extraction and isolation

The cytotoxic seed methanol extract was initially fractionated by LH 20 exclusion chromatography and silica gel column chromatography eluted with increasing concentrations of methanol in dichloromethane. Cytotoxic activity was concentrated into a fraction that, by ^{1}H NMR analysis, was predominantly composed of peptides. Final C_{18} reversed-phase HPLC purification of this material provided two novel cyclic peptides, cherimolacyclopeptides E (**1**) and F (**2**). Analysis of the total acidic hydrolysate, after derivatization, indicated the presence of Pro (**1**), Gly (**1**), Leu (**1**), Phe (**1**) and Tyr (**1**) for cherimolacyclopeptide E, and Pro (**2**), Gly (**2**), Met (**2**), Ile (**1**) and Leu (**1**) for cherimolacyclopeptide F. The absolute stereochemistry of **1** and **2** established by gas chromatography analyses indicated

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that all the chiral amino acids were L. The IR spectrum of the compounds displayed bands at 3320 and 1650 cm^{-1} which are typical of peptides, while UV absorption at 242, 254 and 260 indicated the presence of aromatic residues. (Bewley and Faulkner, 1994). All amino acids were of L configuration.

2.2. Mass spectral analysis

ESI-TOF MS of compound **1** provided a pseudomolecular ion $[M+H]^+$ at *m/z* 635 which established its molecular formula as $C_{33}H_{42}N_6O_7$. Fragmentation of $[M+H]^+$ at *m/z* 635 produced a main series of adjacent b_n peaks at *m/z* 472, 325, 268 and 155 corresponding to the successive loss of Tyr, Phe, Gly and Leu yielding the N-terminal dipeptide $[H\text{-}Pro\text{-}Gly]^+$ and suggesting the linear peptide H-Pro^1-Gly^2-Leu^3-Gly^4-Phe^5-Tyr^6. A second series of ions, which were assigned to adjacent a_n ions, with peaks at *m/z* 444, 297 and 240 showed the successive loss of Tyr, Phe, Gly and the N-terminal tripeptide Pro-Gly-Leu. This result is in agreement with the above proposed sequence. (Fig. 1A).

The molecular formula of cherimolacyclopeptide F, $C_{45}H_{69}N_9O_{10}S_2$ was deduced by ESI-TOF MS measurements at $[M+H]^+$ *m/z* 960. The CID fragmentation at *m/z* 960 showed two series of adjacent acylium ions (b_n and $b_{n'}$) corresponding to the presence of two proline residues which occurs two specific fragmentations peptidy-prolyl (Xaa-Pro). The first b_n ions series at *m/z* 829, 732, 619, 456, 343, 286 and 155 suggested to the successive loss of Met, Pro, Leu/Ile, Tyr, Ile/Leu, Gly, Met and the N-terminal dipeptide ion $[H\text{-}Pro\text{-}Gly]^+$, and the second $b_{n'}$ ions series at *m/z* 847, 684, 571, 514, 383 and 229 corresponded to the successive loss of Leu/Ile, Tyr, Ile/Leu, Gly, Met, Pro-Gly and the terminal dipeptide ion $[H\text{-}Pro\text{-}Met]^+$. These results suggested the sequence H-Pro^1-Gly^2-Met^3-Gly^4-Ile/Leu^5-Tyr^6-Leu/Ile^7-Pro^8-Met^9 with an ambiguity on the respective position of Leu and Ile. The two adjacent a_n and $a_{n'}$ ions are in agreement with the above proposed sequence. (Figs. 1B and C).

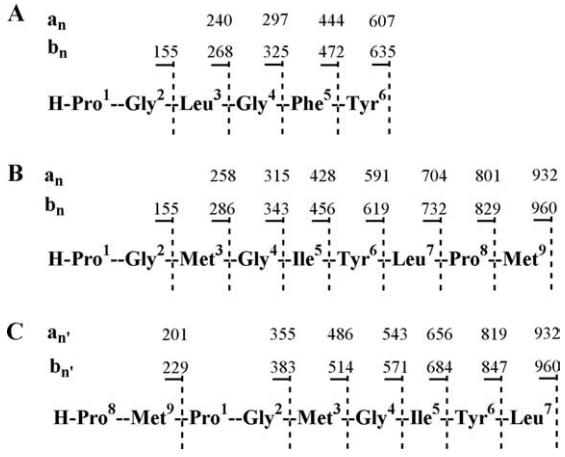


Fig. 1. CID fragmentation of the protonated $[M+H]^+$ ion: (A) fragmentation of **1**; (B) cleavage of the cyclopeptide at the Met^9-Pro^1 amide bond level of **2**; (C) cleavage of the cyclopeptide at the Leu^7-Pro^8 amide bond level of **2**.

2.3. NMR spectral analysis

The peptide nature of cherimolacyclopeptide E was further supported by the presence of six carbonyl signals between δ 169.9 and 173.2 ppm, and five amide NH proton resonances between δ 8.72 and 10.22 ppm in the ^{13}C and ^1H NMR spectra, respectively. The sequence determination was performed from the observation of the connectivities between the carbonyl of residue *i* with the amide and/or the α protons of residue $i+1$ in the HMBC spectrum (Wélé et al., 2002, 2004). In this case, the CO group of Pro^1 at 173.1 ppm was correlated with both $\alpha\alpha'$ protons (4.83, 3.75) and NH amide proton of Gly^2 at 10.22 ppm, the CO of Gly^2 at 169.9 ppm is connected with the NH of Leu^3 at 8.84, the CO of Leu^3 at 173.2 with the NH of Gly^4 at 10.13, the CO of Gly^4 at 170.8 with the NH of Phe^5 at 9.78 and the CO of Phe^5 at 172.4 with the NH of Tyr^6 at 8.72. (Fig. 2) The ROESY spectrum showed strong NOE $d_{NN(i,i+1)}$ between Gly^4 and Leu^3, and between Phe^5 and Tyr^6, and a medium one between Leu^3 and Gly^4. A stretch of $d_{\alpha N(i,i+1)}$ sequential connectivities was observed from Pro^1 to Tyr^6. The chemical shifts of the γ carbon of the proline residue at 25.8 ppm and the NOE correlation between α proton of Tyr^6 and $\delta\delta'$ protons of the proline indicated that the Tyr^6-Pro^1 amide bond is in *trans* configuration (Wüthrich et al., 1982, 1984; Douglas and Bovey, 1973). The whole data agreed with the cyclic structure **1** for cherimolacyclopeptide E, the sequence of which was thus determined as *cyclo* (Pro^1-Gly^2-Leu^3- Gly^4-Phe^5-Tyr^6).

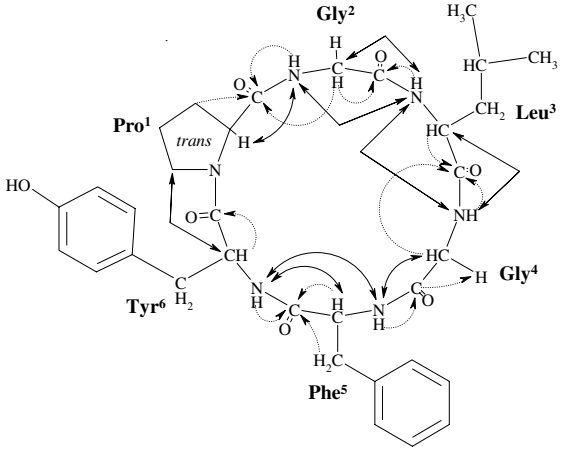
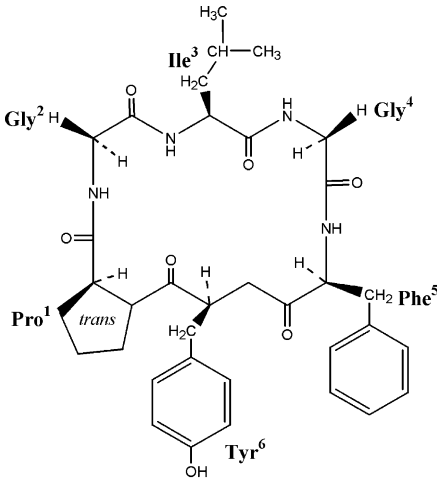
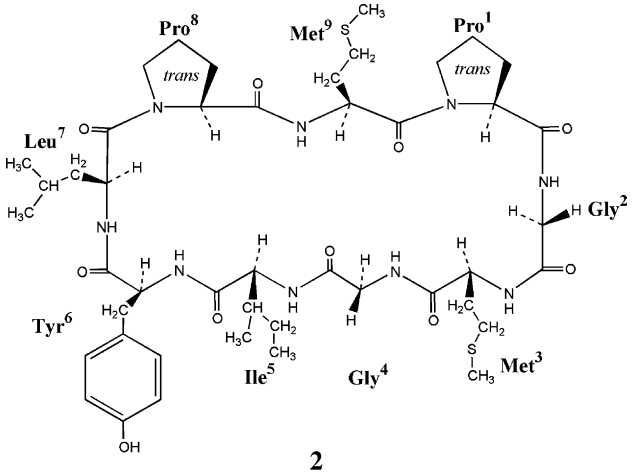


Fig. 2. HMBC (→) and NOEs (↔) correlations for cherimolacyclopeptide G (**1**).



1

The ^{1}H NMR spectrum of cherimolacyclopeptide F (**2**) in DMSO-d_6 solution showed a main stable conformational state where seven amide protons were depicted, two triplets (Gly^2 and Gly^4) and five doublets of two Met (Met^3 and Met^9), one Tyr^6 and two Leu or/and Ile residues at positions 5 and 7. The most distinctive feature in the ^{1}H NMR spectrum of **2** was the presence of two methyl singlets at 1.99 and 2.03 ppm. This is suggested that cherimolacyclopeptide F contained two methionin residues. The $^{3}J_{CH}$ CO(i)/αH($i+1$) correlation between the leucine and the proline 8, and the $^{2}J_{CN}$ CO(i)/NH($i+1$) correlation between the isoleucine and the tyrosine 6 indicated that the Leu is at position 7 and the Ile at position 5. The following correlations between the CO of residue i and the NH group of residue $i+1$ were depicted: Pro^1 to Gly^2, Gly^2 to Met^3, Met^3 to Gly^4, Gly^4 to Ile^5, Ile^5 to Tyr^6, Tyr^6 to Leu^7 and Pro^8 to Met^9. Other connectivities in Fig. 3 are in full agreement with the structure deduced from the mass spectrometry study. The NOE spectrum showed correlations between Gly^2-Met^3 and Gly^4-Ile^5. A stretch of $d_{\alpha N(i,i+1)}$ sequential connectivities were observed between Pro^1-Gly^2, Gly^2-Met^3, Met^3-Gly^4, Gly^4-Ile^5, Ile^5-Tyr^6, Tyr^6-Leu^7 and Pro^8-Met^9. The NOE interaction between the α proton of Met^9 with both δ and δ' protons of Pro^1, and the NOE interaction between the α proton of Leu^7 and the $\delta\delta'$ protons of Pro^8, indicated that the Met^9-Pro^1 and the Leu^7-Pro^8 amide bonds are *trans*. (Fig. 3) This configuration is further confirmed by the γ carbons ^{13}C chemical shifts of both Pro^1 and Pro^8 at 24.8 ppm. The sequence of cherimolacyclopeptide F (**2**) was determined to be *cyclo* (Pro^1-Gly^2-Met^3-Gly^4-Ile^5-Tyr^6-Leu^7-Pro^8-Met^9).



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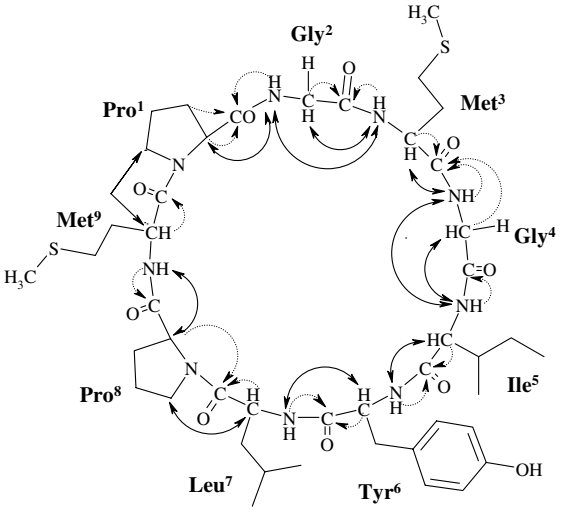

Fig. 3. HMBC (→) and NOEs (↔) correlations for cherimolacyclopeptide H (**2**).

2.4. Bioassays

In a 3-day cytotoxicity bioassay, cyclopeptides **1** and **2** exhibited significant activities in vitro against the KB (human nasopharyngeal carcinoma) cell culture system, with IC_{50} values of 0.017 and 0.06 μM, respectively. Doxorubicin (IC_{50} 0.02 μM) was used as the positive control.

3. Experimental

3.1. General experimental procedure

The melting point was determined on a Büchi melting point B-545 apparatus. The optical rotation was measured on a Perkin–Elmer model 341 polarimeter. Ultraviolet (UV) and infrared (IR) spectra were obtained on Beckman 640 and a Perkin–Elmer 2000 FTIR spectrometer, respectively. HPLC was performed on a Merck apparatus employing a Kromasil C_{18} column (250 × 7.8 mm, 5 μm AIT, Paris) using a flow rate 2 ml/min and UV detection at 220 nm. The ^{1}H (400.13 MHz) and ^{13}C (100.62 MHz) NMR spectra were

recorded in DMSO-d_6 and in Pyridine-d_5 on a Bruker Advance 400 spectrometer operating at 400.13 MHz. Mass spectra were recorded on an API Q-STAR PULSAR *i* of Applied Biosystem. For the CID spectra, the collision energy was 40 eV and the collision gas was nitrogen.

3.2. Plant material

Seeds of the fruits of *A. cherimola* were collected from south of Spain in December 2000. A voucher specimen (VF 10463) for this collection is maintained in the University of Valencia (Spain).

3.3. Isolation of peptides

The dried and powdered seeds (1 kg) was macered successively with cyclohexane and methanol. The methanol extract (43 g) was subjected to Sephadex LH-20 and eluted with methanol. The peptide fraction (11 g) was then repeatedly chromatographed to silica gel column and eluted with increasing concentration of MeOH in CH_2Cl_2. Cytotoxic activity was concentrated into the fraction that eluted with MeOH–CH_2Cl_2 (10:90). Further purification of this fraction by C_{18} reversed phase HPLC using MeOH–H_2O (80–20) yielded in order cherimolacyclopeptide F (40 mg; $t_R = 5.7$ min) and cherimolacyclopeptide E (15 mg; $t_R = 9.9$ min).

3.4. Absolute configuration of amino acids

Solution of **1** and **2** containing 1 mg of peptide, in 6 N HCl (1 mL) was heated at 110 °C for 24 h in sealed tubes. After cooling, each 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 and the residue dissolved in CH_2Cl_2 (0.5 mL) and 0.5 mL trifluoracetic anhydride was added. The mixture was kept in a screw-capped at 110 °C for 20 min, the reagents were evaporated, and the mixture 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 t_R (min) values with those of standards amino acids was used: Gly (14.6), DL-Pro (18.0, 18.2), DL-Ile (16.2, 16.9), DL-Leu (18.1, 19.2), DL-Met (27.2, 27.9), DL-Trp (34.2, 36.4) and DL-Tyr (31.7, 31.9).

3.5. Cytotoxicity evaluation

DMSO solutions of chromatographed fractions and aliquots of purified cyclopeptides were assayed for cytotoxic activities in a 3-day in vitro assay against KB cells. Details of the methodology are described in the literature (Chang et al., 1998).

3.6. Cherimolacyclopeptide E (*1*)

Colourless powder, M.p. 213–214 °C (MeOH), $[\alpha]_D^{22} - 56°$ (*c* 0.3, MeOH); λ_{max}^{KBr} cm^{-1}: 3320 and 1650. UV (MeOH): 242, 254, 260 nm; ^{1}H and ^{13}C NMR (Pyridine-d_5, 400 MHz, 298 K, TMS): 4.30 (1H, *dd*, 7.4, 8.0, Pro^1-Hα), 2.08 (1H, *m*, Pro^1-Hβ), 1.82 (1H, *m*, Pro^1-Hβ'), 1.82 (1H, *m*, Pro^1-Hγ), 1.38 (1H, *m*, Pro^1-Hγ'), 3.59 (1H, *m*, Pro^1-Hδ), 3.31 (1H, *m*, Pro^1-Hδ'), 4.83 (1H, *dd*, 17.0, 8.8, Gly^2-Hα), 3.75 (1H, *dd*, 4.0, 17.0, Gly^2-Hα'), 10.22 (1H, *dd*, 4.0, 8.8, Gly^2-NH), 4.95 (1H, *m*, Leu^3-Hα), 2.11 (1H, *m*, Leu^3-Hβ), 1.88 (1H, *m*, Leu^3-Hβ'), 1.75 (1H, *m*, Leu^3-Hγ), 0.90 (3H, *d*, 6.3, Leu^3-δ-CH$_3$), 0.91 (3H, *d*, 6.2, Leu^3-δ'-CH$_3$), 8.84 (1H, *d*, 7.2, Leu^3-NH), 4.42 (1H, *dd*, 13.7, 4.8, Gly^4-Hα), 3.56 (1H, *dd*, 4.2, 13.7, Gly^4-Hα'), 10.13 (1H, *dd*, 5.2, 4.8, Gly^4-NH), 5.28 (1H, *ddd*, 10.9, 8.0, 4.0, Phe^5-Hα), 3.65 (1H, *dd*, 14.7, 10.9, Phe^5-Hβ), 3.10 (1H, *dd*, 14.7, 10.9, Phe^5-Hβ'), 7.32 (2H, *m*, 8.5, Phe^5-2′6′), 7.26 (2H, *m*, 8.5, Phe^5-3′5′), 7.26 (1H, *m*, 8.5, Phe^5-4′), 9.78 (1H, *d*, 8.0, Phe^5-NH), 5.37 (1H, *ddd*, 9.9, 8.2, 4.0, Tyr^6-Hα), 3.50 (1H, *dd*, 14.7, 4.0, Tyr^6-Hβ), 3.40 (1H, *dd*, 14.7, 5.0, Tyr^6-Hβ'), 7.54 (2H, *m*, 8.4, Tyr^6-2′6′), 7.19 (2H, *m*, 8.4, Tyr^6-3′5′), 8.72 (1H, *d*, 8.2, Tyr^6-NH). ^{13}C NMR (Pyridine-d_6, 400 Hz, 298 K, TMS): 173.1 (Pro^1-CO), 62.5 (Pro^1-Cα), 28.7 (Pro^1-Cβ), 25.8 (Pro^1-Cγ), 47.2 (Pro^1-Cδ), 169.9 (Gly^2-CO), 44.3 (Gly^2-Cα), 173.2 (Leu^3-CO), 53.2 (Leu^3-Cα), 42.1 (Leu^3-Cβ), 25.6 (Leu^3-Cγ), 23.3 (Leu^3-Cδ), 22.4 (Leu^3-Cδ'), 170.8 (Gly^4-CO), 45.5 (Gly^4-Cα), 172.4 (Phe^5-CO), 56.3 (Phe^5-Cα), 37.4 (Phe^5-Cβ), 139.0 (Phe^5-C1′), 129.2 (Phe^5-C2′6′), 128.9 (Phe^5-C3′5′), 126.9 (Phe^5-C4′), 171.9 (Tyr^6-CO), 53.4 (Tyr^6-Cα), 37.2 (Tyr^6-Cβ), 128.4 (Tyr^6-C1′), 131.2 (Tyr^6-C2′4′), 116.3 (Tyr^6-C3′5′), 157.8 (Tyr^6-C4′); ESI-QTOF, *m*/*z*: 673 $[M + K]^+$, 657 $[M + Na]^+$, 635 $[M + H]^+$; ESI-QTOF MS/MS on *m*/*z* 635 $[M + H]^+$ (ce 40 eV) *m*/*z* (%): 635 (47), 607 (77), 472 (64), 444 (100), 325 (65), 297 (25), 268 (29), 240 (8), 155 (5).

3.7. Cherimolacyclopeptide F (*2*)

Colourless solid, M.p. 139–140 °C (MeOH), $[\alpha]_D^{22} - 68°$ (*c* 0.1, MeOH); λ_{max}^{KBr} cm^{-1}: 3320 and 1650. UV (MeOH): 242, 254, 260 nm; ^{1}H and ^{13}C NMR (DMSO-d_6, 400 MHz, 298 K, TMS): 4.25 (1H, *m*, Pro^1-Hα), 2.10 (1H, *m*, Pro^1-Hβ), 1.70 (1H, *m*, Pro^1-Hβ'), 1.86 (2H, *m*, Pro^1-H$\gamma\gamma'$), 3.68 (1H, *m*, Pro^1-Hδ), 3.55 (1H, *m*, Pro^1-Hδ'), 3.92 (1H, *m*, Gly^2-Hα), 3.44 (1H, *m*, Gly^2-Hα'), 8.38 (1H, *t*, Gly^2-NH), 4.49 (1H, *m*, Met^3-Hα), 2.00 (1H, *m*, Met^3-Hβ), 1.95 (1H, *m*, Met^3-Hβ'), 2.44 (1H, *m*, Met^3-Hγ), 2.30 (1H, *m*, Met^3-Hγ'), 2.03 (1H, *s*, Met^3-S-CH$_3$), 3.99 (1H, *m*, Gly^4-

Hα), 3.66 (1H, *m*, Gly^4-Hα′), 7.89 (1H, *t*, Gly^4-NH), 3.89 (1H, *m*, Ile^5-Hα), 1.61 (2H, *m*, Ile^5-Hββ′), 1.09 (1H, *m*, Ile^5-Hγ), 0.98 (1H, *m*, Ile^5-Hγ′), 0.61 (3H, *d*, 6.8, Ile^5-γ CH_3), 0.69 (3H, *d*, 7.3, Ile^5-δ CH_3), 7.81 (1H, *d*, 7.4, Ile^5-NH), 4.24 (1H, *m*, Tyr^6-Hα), 3.00 (1H, *m*, Tyr^6-Hβ), 2.79 (1H, *m*, Tyr^6-Hβ′), 6.99 (2H, *m*, 6.5, Tyr^6-2′6′), 6.63 (2H, *m*, 6.3, Tyr^6-3′5′), 7.90 (1H, *d*, 8.0, Tyr^6-NH), 4.59 (1H, *d*, 8.0, Leu^7-Hα), 1.43 (1H, *m*, Leu^7-Hβ), 1.33 (1H, *m*, Leu^7-Hβ′), 1.56 (1H, *m*, Leu^7-Hγ), 0.86 (3H, *d*, 6.5, Leu^7-δ-CH_3), 0.92 (1H, *d*, 6.3, Leu^7-δ′-CH_3), 7.13 (1H, *m*, Leu^7-NH), 4.40 (1H, *m*, Pro^8-Hα), 2.00 (1H, *m*, Pro^8-Hβ), 1.81 (1H, *m*, Pro^8-Hβ′), 1.86 (2H, *m*, Pro^8-Hγγ′), 3.47 (2H, *m*, Pro^8-Hδδ′), 4.65 (1H, *m*, Met^9-Hα), 1.81 (1H, *m*, Met^9-Hβ), 1.71 (1H, *m*, Met^9-Hβ′), 2.38 (2H, *m*, Met^9-Hγγ′), 1.99 (1H, *s*, Met^9-S-CH_3), 7.55 (1H, *d*, 7.7, Met^9-NH). ^{13}C NMR (DMSO-d_6, 400 MHz, 298K, TMS): 172.0 (Pro^1-CO), 60.4 (Pro^1-Cα), 28.9 (Pro^1-Cβ), 24.8 (Pro^1-Cγ), 47.2 (Pro^1-Cδ), 169.3 (Gly^2-CO), 42.7 (Gly^2-Cα), 171.6 (Met^3-CO), 51.4 (Met^3-Cα), 30.6 (Met^3-Cβ), 29.9 (Met^3-Cγ), 14.6 (Met^3-S-CH_3), 169.2 (Gly^4-CO), 42.7 (Gly^4-Cα), 170.8 (Ile^5-CO), 58.5 (Ile^5-Cα), 35.9 (Ile^5-Cβ), 23.7 (Ile^5-Cγ), 15.2 (Ile^5-Cγ-CH_3), 11.2 (Ile^5-Cδ-CH_3), 170.6 (Tyr^6-CO), 55.6 (Tyr^6-Cα), 35.7 (Tyr^6-Cβ), 127.9 (Tyr^6-C1′), 129.8 (Tyr^6-C2′6′), 119.9 (Tyr^6-C3′5′), 155.8 (Tyr^6-C4′), 171.5 (Leu^7-CO), 48.8 (Leu^7-Cα), 41.6 (Leu^7-Cβ), 24.1 (Leu^7-Cγ), 23.4 (Leu^7-Cδ), 21.4 (Leu^3-Cδ′), 170.7 (Pro^8-CO), 59.9 (Pro^8-Cα), 29.2 (Pro^8-Cβ), 24.8 (Pro^8-Cγ), 46.7 (Pro^8-Cδ), 169.9 (Met^9-CO), 50.1 (Met^9-Cα), 31.3 (Met^9-Cβ), 29.2 (Met^9-Cγ), 14.8 (Met^9-S-CH_3); ESI-QTOF MS, *m*/*z*: 998 $[M + K]^+$, 982 $[M + Na]^+$, 960 $[M + H]^+$; ESI-QTOF MS/MS on *m*/*z* 960 $[M + H]^+$ (ce 40 eV) *m*/*z* (%): 960 (26), 932 (9), 847 (19), 829 (14), 819 (8), 801 (8), 732 (28), 704 (29), 684 (25), 656 (22), 619 (64), 591 (43), 571 (21), 543 (17), 514 (19), 486 (7), 456 (96), 428 (100), 383 (22), 355 (4), 343 (92), 315 (13), 286 (45), 258 (14), 229 (22), 201 (7), 155 (7).

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