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Cycloleonuripeptides A, B and C, Three New Proline-Rich Cyclic Nonapeptides from *Leonurus heterophyllus* [1)]

Hiroshi Morita, Akira Gonda, Koichi Takeya, and Hideji Itokawa*

Department of Pharmacognosy, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan

Abstract: Three new proline-rich cyclic nonapeptides, cycloleonuripeptides A: *cyclo* (-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met-Ile-), B: *cyclo* (-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met(O)-Ile-), and C: *cyclo* (-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met(O)-Ile-), have been isolated from the fruits of *Leonurus heterophyllus* and their structures were elucidated by extensive 2D NMR methods, chemical degradation and transformation. Cycloleonuripeptides B and C, both of which are epimer, showed cell growth inhibitory activity. Copyright © 1996 Elsevier Science Ltd

On the basis of the existence of many naturally occurring cyclic peptides with unique structures and biological activities, recently we have isolated many cyclic peptides with various bioactivities, such as RAs from *Rubia cordifolia*,[2)] astins from *Aster tataricus*,[3)] pseudostellarins from *Pseudostellaria heterophylla*,[4)] segetalins from *Vaccaria segetalis*,[5)] dichotomins from *Stellaria dichotoma* L. var. *lanceolata* Bge.,[6)] yunnanins from *Stellaria yunnanensis*,[7)] and so on. As a part of our continuing studies in search of new bioactive cyclic peptides from higher plants, we have isolated three new proline-rich cyclic nonapeptides, named cycloleonuripeptides A (**1**), B (**2**) and C (**3**), **2** and **3** showing cell growth inhibitory activity, from the fruits of *Leonurus heterophyllus* (Labiatae), which have been used as a Chinese drug for invigorating blood circulation, regulating menstrual disturbance and to dispel edema. In this communication, we report the structure elucidation of cycloleonuripeptides A, B and C by extensive 2D NMR methods and chemical degradations.

The 70% methanolic extract, after being defatted by *n*-hexane, of the fruits of *L. heterophyllus* was partitioned between n-BuOH and H_2O. The n-BuOH soluble material was subjected to Diaion HP-20 column (H_2O - MeOH), and 80% MeOH eluted fraction was chromatographed on a silica gel column, followed by MPLC and HPLC on ODS to yield three peptidic compounds, named cycloleonuripeptide A (**1**: 0.0035 %), cycloleonuripeptide B (**2**: 0.0020 %) and cycloleonuripeptide C (**3**: 0.0012 %) with a known dodecapeptide, cycloleonurinin.[8)] Compounds **2** and **3** showed cell growth inhibitory activity against p-388 lymphocytic leukemia cells (IC_{50} **2**: 6.0 μg/ml; **3**: 3.7 μg/ml).

Cycloleonuripeptide A (**1**), colorless needles, mp. 216 - 218 °C, $[\alpha]_D$ -175.0° (c 0.36, MeOH), showed a high-resolution FAB-MS spectral quasimolecular ion peak at *m/z* 950.4794 [$(M+H)^+$, Δ -1.6 mmu], corresponding to molecular formula, $C_{47}H_{67}N_9O_{10}S$. The IR absorptions at 3310 and 1638 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The nonapeptide nature of **1** was evident from its ^{13}C NMR spectrum, showing nine amide carbonyl groups, as shown in Table 1. In ^1H NMR spectrum, however, the signals due to four amide protons were only observed. In order to elucidate the amino acid composition, **1** was subjected to complete hydrolysis with 6N HCl by heating at 110 °C for 24 h in a sealed tube. The hydrolysate was then analyzed by HPLC and the amino acid composition was shown to be one glycine (Gly), one isoleucine (Ile), one methionine (Met), one tyrosine (Tyr) and five prolines (Pro) per molecule of **1**. These nine amino acid units accounted for the above observed amide protons and amide carbonyl groups. The absolute stereochemistry of the component amino acids was determined to be L-configuration by derivatization of the acid hydrolysate with Marfey's reagent,9) followed by HPLC analysis. Further, the lack of terminal amino group protons in the ^1H NMR and the observed mass molecular weight suggested that **1** might be cyclic peptide.

Though **1** contains five proline residues, the NMR spectra of **1** in DMSO-d_6 gave well-resolved sharp signals and the presence of minor conformers was not observed. The ^1H NMR spectrum showed one singlet, one doublet and one triplet methyl signals [δ 2.04, 0.69 and 0.76] ascribable to Met and Ile residues. The ^1H-^1H COSY spectrum allowed the coupling sequence of each amino acid resonance and the corresponding carbon resonances were elucidated on the basis of HMQC spectra as shown in Table 1. The gross structure including the sequence of the amino acids for **1** was assembled by connecting the individual amino acids on the basis of connectivities observed in a phase sensitive NOESY experiment (Fig. 1) and was concluded to be *cyclo* (-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met-Ile-). The *cis* amide bond between residues 3 and 4 was substantiated by strong NOE effect between Pro^3-Hα and Pro^4-Hα.

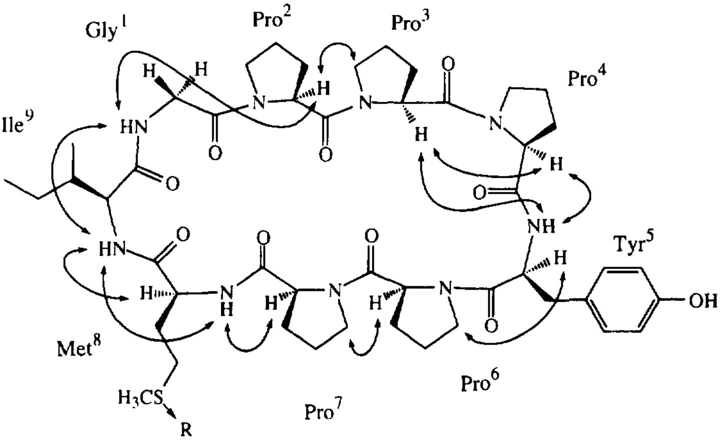

Fig. 1 Structures of cycloleonuripeptides A (**1**), B (**2**) and C (**3**). The arrows show some important sequential NOE relationship in a phase sensitive NOESY spectra. Compounds **2** and **3** are isomers with R=O.

Cycloleonuripeptides B (**2**) and C (**3**),[10,11] have the same molecular formula, $C_{47}H_{67}N_9O_{11}S$ and more molecular weights than **1** at the value of 16 mass unit. It is considered to be stereoisomer each other because both very similar spectroscopic data were observed as follows. In ^{1}H NMR spectra, each singlet methyl signal [**2**: δ 2.53; **3**: δ 2.57], comparing with the methyl signal [δ 2.04] assigned to Met in **1**, was observed. In addition, the ^{13}C signals ascribable to γ and methyl signals of Met in **2** and **3** were resonated at lower field [**2**: δ 49.31 (γ), 37.60 (Me); **3**: δ 49.13 (γ), 37.57 (Me)] than those of **1** [δ 30.20 (γ), 14.52 (Me)]. Therefore, both of compounds **2** and **3** were considered to contain a methionine sulfoxide in stead of methionine in **1**. Reductive transformation of **2** and **3** by thioglycolic acid to **1** provide additional support for the existence of methionine sulfoxide.[12] The absolute stereochemistry of each amino acid in **2** and **3** was determined to be L-configuration except for the configuration of sulfoxide by Marfey's method.[9] The sequencing of **2** and **3** was the same as **1** by the same NOE relationships shown in Fig. 1.

It is conceivable that the inherent constraints present in cycloleonuripeptides A, B and C containing five prolines would reduce the conformational space available to the peptide, allowing one to ascertain the accessible conformations independently from the environment. Studies on the conformational analyses and biological evaluations of cycloleonuripeptides A, B and C are currently being done in our laboratories.

Table 1. ^{1}H and ^{13}C NMR Signal Assignments of Cycloleonuripeptide A (**1**) in DMSO-d_6.

assignment		^{1}H NMR δ_H [int. mult, J(Hz)]	^{13}C NMR δ_C			δ_H	δ_C
Gly^1	α	3.42 (1H, br d, 18.2)	42.63	Pro^6	α	4.59 (1H, t, 7.4)	57.85
		3.96 (1H, br d, 18.2)			β	1.86-2.29 (2H, m)	27.98
	NH	7.58 (1H, br s)			γ	1.86-2.29 (2H, m)	25.38
	C=O		164.42		δ	3.49 and 3.87 (each 1H, m)	46.57
					C=O		170.67*
Pro^2	α	4.67 (1H, dd, 4.3, 8.7)	57.56				
	β	1.87-2.23 (2H, m)	26.98	Pro^7	α	4.73 (1H, dd, 6.3, 8.9)	60.93
	γ	1.87-2.23 (2H, m)	25.28		β	1.84-2.10 (2H, m)	28.98
	δ	3.27 and 3.52 (each 1H, m)	45.55		γ	1.84-2.10 (2H, m)	24.64
	C=O		168.62*		δ	3.67 and 3.75 (each 1H, m)	47.52
					C=O		171.50*
Pro^3	α	4.70 (1H, t, 8.5)	58.73				
	β	1.64-2.27 (2H, m)	27.68				
	γ	1.64-2.27 (2H, m)	24.15	Met^8	α	4.03 (1H, m)	54.01
	δ	3.51 and 3.79 (each 1H, m)	46.62		β	1.96 (1H, m)	30.52
	C=O		169.46*			2.06 (1H, m)	
					γ	2.50 (2H, m)	30.20
Pro^4	α	4.28 (1H, d, 8.0)	60.29		Me	2.04 (3H, s)	14.52
	β	1.64-2.18 (2H, m)	29.39		NH	7.91 (1H, d, 6.3)	
	γ	1.64-2.18 (2H, m)	21.58		C=O		171.71*
	δ	3.09 (1H, br t, 10.0)	45.98				
		3.34 (1H, m)					
	C=O		170.24*	Ile^9	α	4.11 (1H, t, 9.4)	57.48
					β	1.92 (1H, m)	36.01
Tyr^5	α	4.20 (1H, br dd, 2.8, 11.8)	55.61		γ	1.15 and 1.30 (each 1H, m)	24.64
	β	2.56 (2H, m)	32.08		γ	0.69 (3H, d, 6.9)	15.22
	γ		129.00		δ	0.76 (3H, t, 7.3)	9.48
	δ	7.13 (2H, d, 8.5)	129.47		NH	8.02 (1H, d, 10.2)	
	ε	6.64 (2H, d, 8.5)	114.78		C=O		172.47*
	ζ		155.56				
	NH	8.80 (1H, d, 7.1)					
	C=O		170.43*				

* Assignment may be interchanged.

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9. P. Marfey, *Carlsberg Res. Commun.*, **1984**, *49*, 591.
10. Cycloleonuripeptide B (**2**): Colorless powder, [α]$_D$ -153.6° (c 0.98, MeOH); *m/z* 966 (Found: (M+H)$^+$, 966.4740. $C_{47}H_{68}N_9O_{11}S$ requires, 966.4759); ν_{max} (KBr)/cm^{-1} 3436 (NH) and 1636 (amide C=O); λ_{max} (MeOH) / nm 278 (ε 1720). ^{1}H-NMR (DMSO-d$_6$): Gly^1: 3.43 and 3.96 (each d, 18.1, Hα), 7.65 (brs, NH); Pro^2: 4.68 (dd, 4.4, 8.9, Hα), 3.36 and 3.51 (each m, Hδ); Pro^3: 4.71 (t, 7.8, Hα), 3.51 and 3.79 (each m, Hδ); Pro^4: 4.28 (d, 8.2, Hα), 1.90 and 2.12 (each m, Hβ), 1.10 and 1.67 (each m, Hγ), 3.09 (br t, 10.2, Hδ), 3.35 (m, Hδ); Tyr^5: 4.20 (m, Hα), 2.58 (2H, m, Hβ), 7.12 (2H, d, 8.4, Hδ), 6.63 (2H, d, 8.4, Hε), 8.77 (d, 6.9, NH); Pro^6: 4.57 (t, 7.4, Hα), 3.50 and 3.79 (each m, Hδ); Pro^7: 4.80 (dd, 5.1, 8.7, Hα), 3.68 and 3.71 (each m, Hδ); Met(O)^8: 4.01 (m, Hα), 2.18 (m, Hβ), 2.70 and 2.95 (each m, Hγ), 2.53 (3H, s), 8.24 (d, 6.0, NH); Ile^9: 4.13 (t, 8.4, Hα), 1.91 (m, Hβ), 1.12 and 1.33 (each m, Hγ), 0.68 (3H, d, 6.9, Hγ), 0.78 (3H, t, 7.3, Hδ), 8.08 (d, 10.4, NH). ^{13}C-NMR (DMSO-d$_6$): Gly^1: 42.71 (α), 164.37 (C=O); Pro^2: 57.52 (α), 27.04 (β), 25.30 (γ), 45.60 (δ); Pro^3: 58.74 (α), 27.70 (β), 24.17 (γ), 46.64 (δ); Pro^4: 60.29 (α), 30.59 (β), 21.60 (γ), 46.03 (δ); Tyr^5: 55.58 (α), 32.13 (β), 129.00 (γ), 129.49 (δ), 114.80 (ε), 155.57 (ζ); Pro^6: 57.88 (α), 27.97 (β), 25.38 (γ), 46.52 (δ); Pro^7: 60.88 (α), 28.89 (β), 24.67 (γ), 47.42 (δ); Met(O)^8: 53.87 (α), 23.56 (β), 49.31 (γ), 37.60 (Me); Ile^9: 57.52 (α), 36.37 (β), 24.71 (γ), 15.27 (γ), 9.73 (δ); 168.73, 169.48, 170.29 × 2, 170.38, 171.56, 171.59, 172.60 (each C=O).
11. Cycloleonuripeptide C (**3**): Colorless powder, [α]$_D$ -170.5° (c 0.60, MeOH); *m/z* 966 (Found: (M+H)$^+$, 966.4744. $C_{47}H_{68}N_9O_{11}S$ requires, 966.4759); ν_{max} (KBr)/cm^{-1} 3436 (NH) and 1636 (amide C=O); λ_{max} (MeOH) / nm 278 (ε 1560). ^{1}H-NMR (DMSO-d$_6$): Gly^1: 3.43 and 3.95 (each d, 18.1, Hα), 7.65 (brs, NH); Pro^2: 4.68 (dd, 4.4, 8.7, Hα), 1.83 and 2.21 (each m, Hβ), 1.89 and 1.99 (each m, Hγ), 3.29 and 3.51 (each m, Hδ); Pro^3: 4.71 (t, 7.8, Hα), 1.63 and 2.23 (each m, Hβ), 1.91 and 2.02 (each m, Hγ), 3.50 and 3.71 (each m, Hδ); Pro^4: 4.28 (d, 8.0, Hα), 2.10 and 1.87 (each m, Hβ), 1.09 and 1.67 (each m, Hγ), 3.09 (t, 10.0, Hδ), 3.35 (m, Hδ); Tyr^5: 4.19 (dd, 3.0, 11.2, Hα), 2.57 (2H, m, Hβ), 7.12 (2H, d, 8.4, Hδ), 6.63 (2H, d, 8.4, Hε), 8.77 (d, 7.0, NH); Pro^6: 4.57 (t, 7.2, Hα), 1.90 and 2.36 (each m, Hβ), 1.93 and 2.00 (each m, Hγ), 3.50 and 3.80 (each m, Hδ); Pro^7: 4.81 (dd, 5.4, 8.5, Hα), 1.83 and 2.21 (each m, Hβ), 1.86 and 1.95 (each m, Hγ), 3.67 and 3.71 (each m, Hδ); Met(O)^8: 3.99 (m, Hα), 2.10 and 2.24 (each m, Hβ), 2.76 and 2.89 (each m, Hγ), 2.57 (3H, s), 8.30 (d, 5.9, NH); Ile^9: 4.13 (t, 8.5, Hα), 1.91 (m, Hβ), 1.13 and 1.31 (each m, Hγ), 0.67 (3H, d, 6.9, Hγ), 0.77 (3H, t, 7.4, Hδ), 8.06 (d, 10.3, NH). ^{13}C-NMR (DMSO-d$_6$): Gly^1: 42.70 (α), 164.37 (C=O); Pro^2: 57.50 (α), 27.04 (β), 25.31 (γ), 45.60 (δ); Pro^3: 58.74 (α), 27.71 (β), 24.18 (γ), 46.65 (δ); Pro^4: 60.30 (α), 30.59 (β), 21.60 (γ), 46.03 (δ); Tyr^5: 55.60 (α), 32.12 (β), 129.01 (γ), 129.49 (δ), 114.79 (ε), 155.58 (ζ); Pro^6: 57.90 (α), 27.96 (β), 25.42 (γ), 46.54 (δ); Pro^7: 60.87 (α), 28.89 (β), 24.67 (γ), 47.40 (δ); Met(O)^8: 53.84 (α), 23.70 (β), 49.13 (γ), 37.57 (Me); Ile^9: 57.50 (α), 36.38 (β), 24.67 (γ), 15.27 (γ), 9.75 (δ); 168.73, 169.51, 170.29, 170.39, 170.44, 171.60, 171.66, 172.51 (each C=O).
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