Cycloleonurinin, a Cyclic Peptide from Leonuri Fructus

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From Leonuri Fructus, a cyclic peptide composed of twelve amino acid residues was isolated. The sequence of the residues was established by mass spectroscopy and by the use of a protein sequencer for the partial hydrolysates obtained by α -chymotrypsin.

Keywords Leonuri Fructus; *Leonurus artemisia*; *Leonurus sibiricus*; cyclic peptide; FAB MS/MS; NMR

Leonuri Fructus (茺蔚子) is a traditional Chinese drug originating from the fruits of *Leonurus artemisia* S. Y. HU (*L. heterophyllus* SWEET) and *L. sibiricus* (Labiateae).^{1,2)} Also the terrestrial part of the plant (益母草) is used as a Chinese drug for invigorating blood circulation, regulating menstrual disturbance and to dispel edema.^{1,2)} In our course of studies on the pharmacological activities of some Chinese medicines, the extracts of the drug showed hypotensive and antiinflammatory effects as well as increased blood flow and relaxation of the aortic smooth muscle.^{3,4)} Further work revealed that the known guanidine alkaloid, leonurine, and several flavonol glycosides were the active constituents.^{3,5)} The fruit, Leonuri Fructus, has been alleged to exhibit

nearly the same activities as the terrestrial part of the same plant but the constituents have not been characterized except for fatty oils, retinol derivatives, and an uncharacterized basic compound (leonurinine).^{1,2)} This work was conducted to make clear the alkaloidal constituent(s) and, as a result, the main Dragendorff-positive component of the seeds was found not to be an alkaloid such as leonurine but a cyclic peptide. The structural elucidation of the peptide will be reported in this paper.

The fruits were first defatted with hexane and then extracted with 70% methanol. The extract, after concentration, was partitioned successively with chloroform, ethyl acetate and butanol. Each fraction was checked by

TABLE I. ¹H-NMR Chemical Shifts of Cycloleonurinin

	α	β	γ	δ	OH	–NH–	–CONH ₂	Arom.H
Thr ¹	4.95	4.40	1.00		5.45	7.60		
Thr ²	4.60	4.00	1.10		5.20	7.40		
Tyr ¹	4.55	2.80, 3.28			9.13 ^{a)}	8.60		{ 6.62 (6H, dd) 6.89 (2H, d) 7.03 (4H, d)
Tyr ²	4.80	2.63, 2.85			9.13 ^{a)}	7.72		
Tyr ³	4.58	2.51, 2.70			9.20 ^{a)}	8.15		
Gly	3.72, 4.58					7.43		
Ala	4.31	1.22				7.43		
Gln	4.10	1.60, 1.95	2.45			8.20	7.18	
Pro ¹	5.00	1.80, 2.18	1.80 ^{b)}	3.5–3.8				
Pro ²	4.28	1.70, 2.35	1.98, 2.18 ^{b)}	3.5–3.8				
Pro ³	4.00	1.51, 1.90	1.92 ^{b)}	3.70, 3.91				
Pro ⁴	4.55	2.0, 2.1	1.98 ^{b)}	3.00, 3.27				

δ ppm in DMSO-*d*₆. a, b) Assignments may be interchanged.

TABLE II. ¹³C-NMR Chemical Shifts of Cycloleonurinin

	α	β	γ	δ	–CONH–	–CONH ₂	Arom.C			
							C-1	C-2, 6	C-3, 5	C-4
Thr ¹	51.6	67.3	17.5		168.1 ^{a)}					
Thr ²	56.1	66.7	18.3		169.0 ^{a)}					
Tyr ¹	58.1	32.5			169.4 ^{a)}		127.1 ^{d)}	130.1 ^{e)}	114.7 ^{f)}	155.3 ^{g)}
Tyr ²	54.1	38.3			169.8 ^{a)}		127.3 ^{d)}	130.1 ^{e)}	114.8 ^{f)}	155.7 ^{g)}
Tyr ³	55.5	35.8			170.1 ^{a)}		128.1 ^{d)}	130.0 ^{e)}	114.9 ^{f)}	155.9 ^{g)}
Gly	40.8				170.3 ^{a)}					
Ala	47.8	17.9			170.6 ^{a)}					
Gln	53.7	26.5	30.1		170.6 ^{a)}	173.7				
Pro ¹	59.2	29.9	21.3 ^{a)}	46.4 ^{b)}	170.7 ^{a)}					
Pro ²	61.7	29.0	24.2 ^{a)}	45.7	171.5 ^{a)}					
Pro ³	60.8	27.6	24.5 ^{a)}	47.8	171.8 ^{a)}					
Pro ⁴	58.1	31.6	25.3 ^{a)}	46.5 ^{b)}	171.9 ^{a)}					

δ ppm in DMSO-*d*₆. a–g) Assignments may be interchanged.

Fig. 1. FAB MS and MS/MS Spectra and Amino Acid Sequence of the α -Chymotrypsin Hydrolysates

Dragendorff's reagent for the presence of basic substances and the butanol fraction, showing the presence of such compounds, was further separated by column chromatography and high performance liquid chromatography (HPLC). Finally two compounds were isolated; one of them was identified as choline chloride and the other, named cycloleonurinin, was isolated as a colorless powder of mp 222–225 °C in a yield of 0.008%.

The elemental analysis of cycloleonurinin showed a high

content of nitrogen and oxygen atoms and physical properties such as infrared (IR) absorptions (3400, 1650 (br) cm^{-1}) indicated that the compound might be a peptide. Since the compound is negative in ninhydrin reaction and is devoid of ester absorption in the IR spectrum, the compound was suggested not to be an acyclic peptide or a depsipeptide but a cyclic peptide.

Amino acid analysis of the peptide after hydrolysis with 6N hydrochloric acid at 110 °C gave the composition:

Fig. 2. MS/MS Spectrum of m/z 1354 ($M+H^+$) of fr. 4 by FAB MS

proline (Pro) (4 eq), tyrosine (Tyr) (3 eq), threonine (Thr) (2 eq), alanine (Ala) (1 eq), glutamic acid (Glu) (1 eq) and glycine (Gly) (1 eq). The presence of these amino acid units was also proved by ^1H - and ^{13}C -nuclear magnetic resonance (^1H - and ^{13}C -NMR) spectra including those by ^1H - ^1H correlation spectroscopy (H-H COSY), ^{13}C - ^1H COSY (C-H COSY), C-H long-range COSY, distortionless enhancement by polarization transfer (DEPT), and nuclear Overhauser effect spectroscopy (NOESY) as shown in Tables I and II.

The molecular formula of the peptide was established as $\text{C}_{65}\text{H}_{85}\text{N}_{13}\text{O}_{18}$ from fast atom bombardment mass spectrum (FAB MS) and secondary ion MS (SIMS) ($[M+H]^+$ ion at m/z 1336) together with the elemental analysis. The formula suggested the presence of one more acid amide group in the molecule, probably as that in glutamine (Gln), instead of glutamic acid (Glu) detected in the amino acid analysis. Eight $-\text{NH}-$ and one $-\text{NH}_2$ groups shown in the ^1H -NMR spectra and thirteen amide carbonyl groups observed in the ^{13}C -NMR spectra gave support to the assumption. Thus, all the components in the cyclic peptide were proven to be four molecules of proline, three of tyrosine, two of threonine and one each of alanine, glycine and glutamine.

Our attempts to clarify the amino acid sequence by NOESY and C-H long-range COSY gave little success because of the presence of the same amino acid units in the molecules. However, digestion of the peptide with α -chymotrypsin⁶⁾ gave satisfactory results by specific cleavage at the aromatic amino acid units: Four hydrolysates (fr. 1–4) were obtained after reversed-phase HPLC and the FAB MS of the fractions indicated that fr. 2 and fr. 4 retained all the amino acid units ($[M+H]^+$ at m/z 1354), while fr. 1 and fr. 3 corresponded to the products formed by the cleavage at two peptide bonds ($[M+H]^+$ ion at m/z 543 and 830, respectively). The FAB MS and MS/MS spectra⁷⁾ of the four hydrolysates gave the results shown in Fig. 1. Figure 2 shows the MS/MS spectrum of m/z 1354 ($M+H^+$) of fr. 4 by FAB MS. The results obtained by a protein sequencer for the four peptides clearly showed the same results to indicate the sequence shown in Fig. 1. Thus, the structure of the cyclic peptide, cycloleonurinin, was established as shown in Fig. 3.

Enzymatic oxidations of the amino acids after complete hydrolysis indicated that all the amino acids were digested

Fig. 3. The Structure of Cycloleonurinin

by L-amino acid oxidase but not by D-amino acid oxidase,⁸⁾ indicating that all the components are mainly of the L-amino acid series.

The hexane and methanol extracts of the fruits and hexane, chloroform, ethyl acetate, butanol and aqueous fractions of the methanol extract were subjected to pharmacological tests such as hypotensive action on rats and the increasing blood flow and aortic smooth muscle relaxing effects following the previous works.^{3–5)} However, obvious and reproducible effects were not observed in any fractions. Several pharmacological tests on the activity of cycloleonurinin, the new cyclic peptide, were also carried out on the isolated guinea-pig ileum, and on the isolated rat aorta, as well as on blood pressure in urethane anesthetized rats. The peptide did not show any activity in the dose as high as 3×10^{-5} M on isolated organs, and 5 mg/kg, i.v. in the whole animals.⁹⁾

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus. Ultraviolet (UV) and IR spectra were measured with a Shimadzu UV-240 spectrophotometer and a JASCO A-102 infrared spectrophotometer. The ^1H - and ^{13}C -NMR spectra were recorded using a JEOL GSX-400 spectrometer in dimethyl sulfoxide- d_6 with tetramethylsilane as an internal standard. The $[\alpha]_D$ values were determined with a JASCO DIP-140 digital polarimeter. Amino acid analysis was

carried out by a Hitachi amino acid analyser 835 and the amino acid sequence was determined by Protein Sequence 477A RTH Analyzer 120A (Applied Biosystem Co.).

FAB MS and FAB MS/MS spectra were obtained with a JEOL-HX 100 tandem mass spectrometer, which consists of a conventional geometry double-focusing mass spectrometer (MS-I) followed by an electrostatic analyzer used as MS-II. Xenon was used to provide the primary beam of atoms (6 keV). The liquid matrix used for FAB ionization was glycerol-1 N HCl (20:1). MS/MS spectra were obtained by activating the ions in the third field free region by collision with argon gas (sufficient to suppress the precursor ion beam by 25%) and by scanning MS-II.

Kieselgel 60 F₂₅₄ (Merck) precoated plates were used for thin-layer chromatography (TLC) and the spots were detected with Dragendorff's and ninhydrin reagents. Column chromatography was carried out on 70–230 mesh silica gel (Merck). HPLC was performed using a SSC 3100-J pump with an Oyo-Bunko Uvilog 7 UV detector.

Extraction and Isolation of Cycloleonorinin and Choline Chloride Leonuri Fructus (Chinese origin, purchased from Tochimotoenkaido, Osaka, on June 1986) (5 kg) was defatted with hexane (hexane extract, 542 g) and then extracted with 70% MeOH (2.4 l × 3). After removal of the solvent by evaporation, the residue (189 g) was treated with water and the water-insoluble portion was discarded. The aqueous solution was successively extracted with CHCl₃, AcOEt and BuOH saturated with H₂O to give the extracts (7.9, 5.0 and 53.1 g, respectively). The BuOH extract was chromatographed on silica gel (CHCl₃-MeOH (1:0→1:1)) and the fractions containing the substances positive to Dragendorff's reagent were further purified by HPLC (Nucleosil 50-5, eluting with CH₂Cl₂-MeOH (6:1)) to give cycloleonorinin (411 mg, 0.008%) and choline chloride, a pale yellow liquid (35 mg, 0.001%), ¹H-NMR δ ppm: 3.23 (9H, s), 3.52 (2H, t), 4.01 (2H, td), identified by ¹H-NMR and TLC.

Cycloleonorinin White powder of mp 222–225 °C (from MeOH), [α]_D –28.8° (MeOH, c=0.79). UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm: 225, 278. IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3400, 2900, 1650 (br), 1520, 1440, 1220. MS (FAB and SIMS) *m/z*: 1336 [M+H]⁺, 623, 493, 309, 195. MS (EIMS) *m/z*: 445, 339, 338, 308, 274, 260, 256, 242, 230, 220, 216, 212, 204, 202, 194, 168, 154, 107. ¹H- and ¹³C-NMR (Tables I and II). *Anal.* Calcd for C₆₅H₈₅N₁₃O₁₈·5H₂O: C, 54.72; H, 6.71; N, 12.77. Found: C, 54.66; H, 6.39; N, 12.74.

Cycloleonorinin is slightly soluble in MeOH, sparingly soluble in EtOH, dimethylsulfoxide (DMSO) and water and insoluble in acetone and CHCl₃. It gives a positive reaction for Dragendorff's reagent but negative for ninhydrin reagent.

Complete Hydrolysis of Cycloleonorinin Cycloleonorinin (1 mg) in 6 N HCl (1 ml) was hydrolyzed at 110 °C for 24 h. The solution was evaporated and lyophilized to dryness and the hydrolysates were compared with standard amino acids on TLC developed by BuOH-AcOH-H₂O (7:1:2). The mixture was also applied to the analysis by an amino acid analyzer and the components, Thr, Glu, Gly, Ala, Tyr, Pro (1.86:1.04:1.0:1.0:2.84:3.78), were clarified.

Hydrolysis of Cycloleonorinin with α -Chymotrypsin To cycloleonorinin (10 mg) in NH₄HCO₃ solution (1%, 0.9 ml), α -chymotrypsin (500 μ g dissolved in 50 μ l of 0.001% HCl, Wako Pure Chemical Industries, substrate-enzyme ratio, 400:1) was added and the digestion was performed at 25 °C with the pH maintained at 8.0 by the manual addition of 0.1 N HCl.⁶⁾ After 4 h the reaction mixture showed a negative reaction for Dragendorff's reagent on TLC and the reaction was stopped by adjusting the solution

to pH 2.2 with 1 N HCl. The digestion mixture showed the presence of four hydrolysates (fr. 1–4) by TLC (BuOH-AcOH-H₂O (7:1:2)) and, after lyophilization, the four peptides were separated by HPLC (Nucleosil ODS-5, 8 i.d. × 200 mm, eluted with 0.05% CF₃CO₂H-CH₃CN (4:1)). They were subjected to FAB MS/MS ([M+H]⁺ ions at *m/z* 543, 1354, 830 and 1354, respectively) and to analyses by an automatic amino acid sequencer (Fig. 1).

Enzymatic Oxidation of the Component Amino Acids⁸⁾ To the complete hydrolysates of cycloleonorinin (2.5 mg) in 0.1 M sodium pyrophosphate buffer (pH 8.2, 1 ml), catalase (5 μ g, from bovine liver, Wako Pure Chemical Industries, 6.9 units/ μ g) and nicotinamide adenine dinucleotide (NADH) solution (5 mg/ml, 50 μ l) were added and incubated at 37 °C for 1 h. After equilibration, D-amino acid oxidase (2 mg, from hog kidney, Tokyo Kasei Kogyo Co., 0.02 units/mg) was added and the mixture was incubated at 37 °C for 16 h.

The same amino acid mixture in 0.2 M Tris buffer (pH 7.7, 1 ml) and peroxidase (50 μ g, from horseradish, Wako Pure Chemical Industries, 10 units/mg) were incubated at 37 °C for 1 h. After equilibration, L-amino acid oxidase (0.5 units, from snake venom, Tokyo Kasei Kogyo Co., 5 units/140 μ l) was added and further incubated at 37 °C for 16 h.

The amino acids that remained in both digestion mixtures were subjected to TLC in the solvent system (phenol-H₂O (8:3)) to compare with the hydrolysate mixture and the amino acid standards.

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References

- 1) Jiang Su New Medical College (ed.), "Zhong Yao Da Ci Dian," Vol. 2, Shanghai Science and Technology Press, Shanghai, 1977, pp. 1609–1610.
- 2) Chinese Academy of Medical Science and Others (eds.), "Zhong Yao Zhi," Vol. 3, People's Health Publisher, Beijing, 1984, pp. 513–516.
- 3) C.-E. Wang, S. Yano, K. Watanabe, S. Natori, K. Yamada and Y. Fujimoto, *J. Med. Pharm. Soc. Wakan-Yaku*, **3**, 446 (1986).
- 4) C.-E. Wang, S. Yano, K. Watanabe, K. Kuroda (née Yamada) and S. Natori, in preparation.
- 5) K. Kuroda (née Yamada), K. Kinoshita, S. Natori, C.-E. Wang, S. Yano and K. Watanabe, in preparation.
- 6) Biochemical Society of Japan (ed.), "Seikagaku Jikken Koza, 1, Protein Chemistry, II," Tokyo Kagaku Dojin, Tokyo, 1976, pp. 265–267.
- 7) T. Nakamura, H. Nagaki and T. Kinoshita, *Mass Spectroscopy*, **34**, 307 (1986); T. Kinoshita, K. Nakamura and H. Nagaki, *Nihon Kagaku Kaishi*, **1986**, 1665.
- 8) W. O. Godtfredson, S. Vangedal and D. W. Thomas, *Tetrahedron*, **26**, 4931 (1970).
- 9) Private communication from Professor K. Watanabe, Chiba University.