A New Cyclopeptide from *Clausena anisum-olens*

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A new cyclopeptide, clausenain I (**1**), has been isolated by a multi-step chromatography procedure from *Clausena anisum-olens*. Its structure was elucidated as cyclo (-Gly[1]-Ile[2]-Ile[3]-Val[4]-Leu[5]-Ile[6]-Ile[7]-Leu[8]-Leu[9]-) by extensive 2D-NMR spectroscopic methods and chemical evidence. It is the first time that a natural cyclic peptide has been isolated from the genus *Clausena*.

1. Introduction. – The plants of the genus *Clausena* (Rutaceae) are shrubs widely distributed in the south of China [1]. Previous studies revealed that the plants of the genus *Clausena* mainly contained carbazole alkaloids [2–4] and coumarins [5–7]. *Clausena anisum-olens* (Rutaceae) is a shrub growing in Hekou County of the Yunnan Province. The aerial parts of this plant have been used for the treatment of dysentery and arthritis [1]. The chemical constituents of *C. anisum-olens* have not been investigated until now. During our search for active principles, a new cyclic nonapeptide, clausenain I (**1**; *Figure*), was isolated from *C. anisum-olens* by a multi-step chromatography procedure. Natural cyclic peptides, which are widely distributed in many higher plants, exhibit a large range of biological activities such as antibiotic, anti-inflammatory, and cytotoxic activities and have often been used as models for studies of structural features of proteins [8]. Only a minor number of cyclopeptides have been isolated from the plants of Rutaceae [9]. This is the first time that a natural cyclic peptide has been isolated from the genus *Clausena*. Herein, we describe the isolation and structural elucidation of the new cyclopeptide **1** by extensive 2D-NMR spectroscopic methods.

2. Results and Discussion. – Clausenain I (**1**) was obtained as white amorphous powder by a multi-step chromatography procedure from *C. anisum-olens*. It gave an $[M + Na]^+$ peak in the HR-ESI-MS at *m/z* 970.6681, which was appropriate for the molecular formula $C_{49}H_{89}N_9O_9$. Compound **1** showed a positive reaction with the chlorine/*o*-tolidine reagent, indicating the presence of amide groups, and a negative reaction with ninhydrine, suggesting that **1** is a cyclic peptide. The intense absorptions between 1600–1700 cm^{-1} and between 3100 to 3400 cm^{-1} in the IR spectrum suggested the presence of the amide C=O and NH groups, respectively.

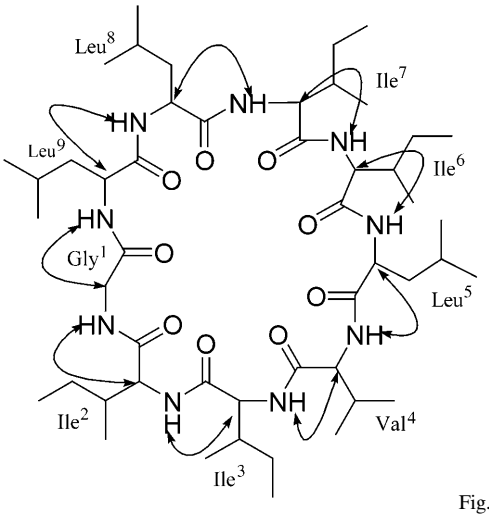

Fig. 1. *The structure and selected NOESY correlations of compound* ***1***

At 300 K, the ^{1}H-NMR spectra (D_5)pyridine) of **1** gave only three broad NH signals and the H–C(α) signals of the amino acid residues overlapped heavily. However, when the temperature was raised to 325 K, a well-resolved ^{1}H-NMR spectrum with sharp proton signals (see the *Table*) was obtained. Assignment of ^{1}H-NMR signals to specific protons in individual residues was obtained by 2D homonuclear COSY and HMQC-TOCSY experiments to show the complete spin systems of the amino acid residues. The corresponding δ(C) were determined on the basis of HMQC and HMBC experiments.

The δ(H) from 6.00 to 10.00 and 3.00 to 6.00 in the ^{1}H-NMR of **1** showed the presence of 7 NH (partly overlapped) and 10 H–C(α), respectively. At higher field, 16 Me signals were present. The ^{13}C-NMR and DEPT spectra of **1** indicated the presence of 9 CH(α) or CH_2(α) groups at δ(C) 30 to 70 and 16 Me groups at δ(C) 10–30. The δ(H) and δ(C) of the amino acid residues (except for the quaternary C-atoms) can be assigned simultaneously by 2D HMQC-TOCSY, because this technique provides not only total H-correlations in the F_2 dimension but also total C-correlations (except for the quaternary C-atoms) in the F_1 dimension [10]. Thus, detailed analysis of the ^{1}H,^{1}H COSY, HMQC-TOCSY, HMBC, and NOESY data of **1** led to the complete assignment of all δ(H) and δ(C) (*Table*).

Careful analysis of the 1D-NMR data together with the ^{1}H,^{1}H COSY and HMQC-TOCSY data identified **1** as a peptide composed of nine amino acid residues; *i.e.* 1 glycine, 1 valine, 4 isoleucine, and 3 leucine residues. Amino acid analysis following hydrolysis of **1** at 120° with 6N HCl confirmed the presence of Gly (1 equiv.), Val (1 equiv.), Ile (4 equiv.), and Leu (3 equiv.). Considering that the 9 identified amino acid residues account for 8 degrees of unsaturation, the remaining unsaturation degree strongly indicated that a cyclic moiety was involved in the structure of **1**. Chemical analyses revealed a negative reaction of **1** with ninhydrine but a positive one after hydrolysis of **1** with concentrated HCl solution, thus establishing the structure of a monocyclic peptide.

Table. *^{1}H- and ^{13}C-NMR Data* ((D_5)pyridine; 325 K) *of Compound* **1**. δ in ppm, J in Hz.

		δ (H)	δ (C)
Gly[1]	$CH_2(\alpha)$	4.68 (*dd*, $J=5.5, 15.5$), 3.88 (*dd*, $J=4.5, 15.5$)	44.2 (*t*)
	NH	9.10 (br. *s*)	
	CO		170.0 (*s*)
Ile[2]	H–C(α)	4.98 (*t*, $J=7.5$)	58.2 (*d*)
	H–C(β)	2.42 (*m*)	37.1 (*d*)
	$CH_2(\gamma)$	1.81, 1.44 (2*m*)	25.2 (*t*)
	Me (δ)	1.02 (*d*, $J=5.5$)	15.8 (*q*)
	Me(δ)	1.17 (*d*, $J=7.0$)	11.2 (*q*)
	NH	8.40 (br. *s*)	
	CO		172.1 (*s*)
Ile[3]	H–C(α)	4.87 (*t*, $J=8.5$)	58.5 (*d*)
	H–C(β)	2.31 (*m*)	37.0 (*d*)
	$CH_2(\gamma)$	1.39 (*m*)	25.4 (*t*)
	Me(δ)	1.02 (*d*, $J=5.0$)	16.1 (*q*)
	Me(δ)	0.86 (*t*, $J=7.5$)	11.6 (*q*)
	NH	9.10 (br. *s*)	
	CO		173.2 (*s*)
Val[4]	H–C(α)	4.53 (br. *s*)	62.3 (*d*)
	H–C(β)	2.86 (1 H, *m*)	29.5 (*d*)
	Me(γ), Me(γ')	1.12 (*d*, $J=8.5$)	19.0 (*q*), 19.8 (*q*)
	NH	8.95 (br. *s*)	
	CO		173.0 (*s*)
Leu[5]	H–C(α)	5.03 (br. *s*)	53.0 (*d*)
	$CH_2(\beta)$	2.12, 2.31 (2*m*)	39.7 (*t*)
	H–C(γ)	1.91 (*m*)	24.7 (*d*)
	Me(δ), Me(δ)	0.92 – 1.02 (*m*)	21.9 (*q*), 23.0 (*q*)
	NH	9.05 (br. *s*)	
	CO		171.9 (*s*)
Ile[6]	H–C(α)	4.56 (br. *s*)	60.6 (*d*)
	H–C(β)	2.53 (*m*)	36.8 (*d*)
	$CH_2(\gamma)$	1.81 (*m*)	25.0 (*t*)
	Me(γ')	0.97 (*d*, $J=7.0$)	15.8 (*q*)
	Me(δ)	1.14 (*t*, $J=7.5$)	11.2 (*q*)
	NH	8.90 (br. *s*)	
	CO		172.7 (*s*)
Ile[7]	H–C(α)	5.03 (br. *t*)	58.5 (*d*)
	H–C(β)	2.23 (*m*)	37.0 (*d*)
	$CH_2(\gamma)$	1.39 (*m*)	25.3 (*t*)
	Me(γ')	1.11 (*d*, $J=7.0$)	16.1 (*q*)
	Me(δ)	1.21 (*t*, $J=7.0$)	11.6 (*q*)
	NH	8.78 (br. *s*)	
	CO		171.6 (*s*)
Leu[8]	H–C(α)	5.15 (br. *dd*, $J=5.5$)	52.0 (*d*)
	$CH_2(\beta)$	2.06 (*m*)	41.9 (*t*)
	H–C(γ)	1.91 (*m*)	25.0 (*d*)
	Me(δ), Me(δ')	0.92 – 1.02 (*m*)	22.2 (*q*), 23.0 (*q*)
	NH	8.90 (br. *s*)	
	CO		173.4 (*s*)
Leu[9]	H–C(α)	4.77 (br. *s*)	53.0 (*d*)
	$CH_2(\beta)$	2.12, 2.06 (2*m*)	39.8 (*t*)
	H–C(γ)	1.91 (*m*)	25.0 (*d*)
	Me(δ), Me(δ')	0.92 – 1.02 (*m*)	22.2 (*q*), 22.8 (*q*)
	NH	9.14 (br. *s*)	
	CO		171.7 (*s*)

The peptide sequence was determined by a detailed analysis of the ^{1}H,^{1}H-NOESY correlations between the NH and H–C(α) protons (see *Fig.*), which finally allowed us to establish the structure of **1** as cyclo(-Gly^1-Ile^2-Ile^3-Val^4-Leu^5-Ile^6-Ile^7-Leu^8-Leu^9-). It is the first time that a natural cyclic peptide has been isolated from the genus *Clausena*.

This work was financially supported by two grants from the *National Natural Science Foundation of China* (Nos. 39525025 and 30200350). The authors are grateful to the Analysis Group of the Laboratory of Phytochemistry, Kunming Institute of Botany, Chinese Academy of Sciences, for measuring the ^{1}H- and ^{13}C-NMR, FAB-MS, HR-ESI-MS, and IR data.

Experimental Part

General. TLC: commerical silica-gel plates (*Qing Dao Marine Chemical Group Co.*) CC = Column chromatography. Optical rotation: *Jasco 20-MC* polarimeter. IR Spectra: *Nicolet Avatar-360* spectrophotometer; $\tilde{\nu}_{max}$ in cm^{-1}. ^{1}H- and ^{13}C-NMR Spectra: *Bruker AV-500* spectrometer; chemical shifts δ in ppm rel. to $SiMe_4$ as internal standard and coupling constant *J* in Hz. FAB-MS: *VG Autospec-3000* mass spectrometers; in *m/z* (rel. %).

Plant Material. The aerial parts of *C. anisum-olens* were collected in Hekou, Yunnan Province, P. R. China, in April 2002. The plant was identified by Prof. *D. D. Tao* of the Kunming Institute of Botany; a voucher specimen (No. 02041705) is deposited in the Kunming Institute of Botany, Kunming, China.

Extraction and Isolation. The powdered aerial parts of *C. anisum-olens* (22.5 kg) were extracted (3 ×) with 95% EtOH. The extract was then evapoarted to give a brown syrup, which was partitioned in H_2O and extracted with petroleum ether, AcOEt, and BuOH. The AcOEt extract (110.5 g) was subjected to CC (silica gel, $CHCl_3$/MeOH 100:1 → 1:1, then MeOH); *Fractions I–XIX*. *Fr. XV* was resubmitted to CC (silica gel, then *Sephadex LH-20*): (30 mg).

Clausenain I (= *Cyclo(glycyl-*L*-isoleucyl-*L*-isoleucyl-*L*-valyl-*L*-leucyl-*L*-isoleucyl-*L*-isoleucyl-*L*-leucyl-*L*-leucyl)*, **1**). White amorphous powder. $[\alpha]_D^{22} = -88^\circ$ ($c = 0.2$, MeOH). IR (KBr): 3434, 1640. ^{1}H and ^{13}C-NMR *Table*. FAB$^+$-MS (pos.): 950 (100 $[M+3]^+$). HR-ESI-MS: 970.668 1($[M+Na]^+$ $C_{49}H_{89}N_9O_9^+$, calc. 970.6680).

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Received March 9, 2005