

## 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<sup>1</sup>-Ile<sup>2</sup>-Ile<sup>3</sup>-Val<sup>4</sup>-Leu<sup>5</sup>-Ile<sup>6</sup>-Ile<sup>7</sup>-Leu<sup>8</sup>-Leu<sup>9</sup>-) 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*.

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**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 non-peptide, 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.

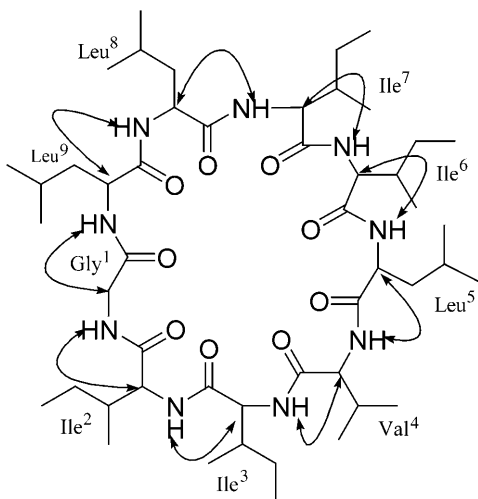


Fig. 1. The structure and selected NOESY correlations of compound **1**

At 300 K, the  $^1\text{H-NMR}$  spectra ( $\text{D}_5$ pyridine) of **1** gave only three broad NH signals and the  $\text{H-C}(\alpha)$  signals of the amino acid residues overlapped heavily. However, when the temperature was raised to 325 K, a well-resolved  $^1\text{H-NMR}$  spectrum with sharp proton signals (see the *Table*) was obtained. Assignment of  $^1\text{H-NMR}$  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  $\delta(\text{C})$  were determined on the basis of HMQC and HMBC experiments.

The  $\delta(\text{H})$  from 6.00 to 10.00 and 3.00 to 6.00 in the  $^1\text{H-NMR}$  of **1** showed the presence of 7 NH (partly overlapped) and 10  $\text{H-C}(\alpha)$ , respectively. At higher field, 16 Me signals were present. The  $^{13}\text{C-NMR}$  and DEPT spectra of **1** indicated the presence of 9  $\text{CH}(\alpha)$  or  $\text{CH}_2(\alpha)$  groups at  $\delta(\text{C})$  30 to 70 and 16 Me groups at  $\delta(\text{C})$  10–30. The  $\delta(\text{H})$  and  $\delta(\text{C})$  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  $^1\text{H},^1\text{H}$  COSY, HMQC-TOCSY, HMBC, and NOESY data of **1** led to the complete assignment of all  $\delta(\text{H})$  and  $\delta(\text{C})$  (*Table*).

Careful analysis of the 1D-NMR data together with the  $^1\text{H},^1\text{H}$  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  $120^\circ$  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. <sup>1</sup>H- and <sup>13</sup>C-NMR Data ((D<sub>5</sub>)pyridine; 325 K) of Compound **1**. δ in ppm, J in Hz.

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

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

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#### Experimental Part

*General.* TLC: commercial silica-gel plates (*Qing Dao Marine Chemical Group Co.*) CC = Column chromatography. Optical rotation: *Jasco 20-MC* polarimeter. IR Spectra: *Nicolet Avatar-360* spectrophotometer;  $\tilde{\nu}_{\text{max}}$  in  $\text{cm}^{-1}$ .  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR Spectra: *Bruker AV-500* spectrometer; chemical shifts  $\delta$  in ppm rel. to  $\text{SiMe}_4$  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 ( $3 \times$ ) with 95% EtOH. The extract was then evaporated to give a brown syrup, which was partitioned in  $\text{H}_2\text{O}$  and extracted with petroleum ether, AcOEt, and BuOH. The AcOEt extract (110.5 g) was subjected to CC (silica gel,  $\text{CHCl}_3/\text{MeOH}$  100:1  $\rightarrow$  1:1, then MeOH); *Fractions I–XIX*. *Fr. XV* was resubmitted to CC (silica gel, then *Sephadex LH-20*): (30 mg).

*Clausenain 1* (= Cyclo(glycyl-L-isoleucyl-L-isoleucyl-L-valyl-L-leucyl-L-isoleucyl-L-isoleucyl-L-leucyl-L-leucyl), **1**). White amorphous powder.  $[\alpha]_{\text{D}}^{25} = -88^\circ$  ( $c = 0.2$ , MeOH). IR (KBr): 3434, 1640.  $^1\text{H}$  and  $^{13}\text{C}$ -NMR *Table*. FAB<sup>+</sup>-MS (pos.): 950 (100 [ $M + 3$ ]<sup>+</sup>). HR-ESI-MS: 970.668 1([ $M + \text{Na}$ ]<sup>+</sup>  $\text{C}_{49}\text{H}_{89}\text{N}_9\text{O}_8^+$ , calc. 970.6680).

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