Cordycedipeptide A, a New Cyclodipeptide from the Culture Liquid of *Cordyceps sinensis* (Berk.) Sacc.

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A new cyclodipeptide named as cordycedipeptide A, a new natural compound and two known compound were isolated from the culture liquid of *Cordyceps sinensis* (Berk.) Sacc. Their structures were elucidated as 3-acetamino-6-isobutyl-2,5-dioxopiperazine (1), 3-isopropyl-6-isobutyl-2,5-dioxopiperazine (2) and 3,6-di(4-hydroxy)benzyl-2,5-dioxopiperazine (3) by 1D and 2D-NMR techniques. The cytotoxic assay showed compound 1 had the cytotoxic activities to L-929, A375, and Hela.

Key words *Cordyceps sinensis*; culture liquid; cordycedipeptide A

Cordyceps sinensis (Berk.) Sacc. belongs to the class *Ascomycetes*, mainly distributing in Qinghai, Xizang, Sichuan province, China. It has been regarded as a popular and effective folk medicine for treating various human diseases, such as hepatis, hypertension, hypercholesterolaemia, and gastric cancer, *etc*.[1] More recently, it has been used for medicinal purposes due to its various physiological activities including immunostimulating and anti-tumor activities.[2] But the natural resources of *Cordyceps sinensis* (Berk.) Sacc. are very limited. So *Cordyceps sinensis* (Berk.) Sacc. have been cultured to fulfill the medicinal need.[3] Meanwhile it is necessary to study the chemical constituents of the culture liquid of *Cordyceps sinensis* (Berk.) Sacc. In this paper, we report the isolation and identification of a new cyclodipeptides, along with a new natural product and two known compounds (Fig. 1) from the culture liquid of *Cordyceps sinensis* (Berk.) Sacc. and its antitumor activities.

Compound **1** was obtained as white amorphous powder (MeOH), mp 126 °C, $[\alpha]_D^{20}$ −70.25° (c=0.8, MeOH). Its formula was determined as $C_{10}H_{17}N_3O_3$ by high-resolution EI mass (HR-EI-MS), exhibiting an ion peak at *m*/*z* 227.3642 $[M]^+$ (Calcd 227.3625). The IR spectrum showed absorptions at 3200 (NH), 2960, 1683 (CONH), 1450, 1375, 1322 cm^{-1}. The ^{1}H-NMR(300 MHz, DMSO-d_6) spectrum showed two methyl groups signals at δ 0.93 (3H, d, J=6.9 Hz) and δ 0.85 (3H, t, J=7.3 Hz). In the ^{13}C-NMR (75.5 MHz, DMSO-d_6) and DEPT spectrum, 10 carbon signals were observed, including two methyls, two methylenes, three methines and three quaternary carbons signals. The carbon signals at δ 12.0, 15.1, 24.2, 37.8 were similar with the carbon signals of isoleucine,[4] and the carbon signals at δ 38.7, 171.4 were similar with the carbon signals of asparagine.[5] So we surmised that this compound was a dipeptide. The signals of nine protons in the upfield region (δ 0.85—1.87) were assigned to the fragment CH_3–CH_2–$\underset{|}{C}$H–CH_3 by ^{1}H–^{1}H COSY. In the HMBC spectrum, correlations were observed between 1-NH (δ 7.78) and 6-C (δ 58.7) and 2-CO (δ 167.8), between 4-NH (δ 8.05) and 5-CO (δ 167.0) and 3-C (δ 51.1), between 6-H (δ 3.78) and 5-CO (δ 167.0), between 3-H (δ 4.20) and 2-CO (δ 167.8). From the above data, we could assign 2,5-dioxo piperazine cycle, the fragment of which is *m*/*z* 112 $[C_4H_4N_2O_2^+]$ in EI-MS spectrum. In the HMBC experiment, correlated peaks between 11-H_a (δ 2.31), 11-H_b (δ 2.68) and 12-CO (δ 171.4), between 13-H_a (δ 7.44), 13-H_b (δ 6.91) and 12-CO (δ 171.4) were observed. From the above data, the fragment H_2N–CO–CH_2– was determined. Crossing peaks between 6-H proton (δ 3.78) and carbons at 7-C (δ 37.8) and 8-C (δ 24.2) were observed, which proved that 1-isobutyl was attached to carbon 6-C (δ 58.7). Similarly correlated peaks between 3-H (δ 4.20) and 11-C (δ 38.7) and 12-CO (δ 171.4) were observed, which indicated that the moiety of acetamino group was attached to carbon 3-C (δ 51.1).

The relative stereochemistry of this compound was characterized by NOESY experiment. In NOESY spectrum, the correlation between 3-H (δ 4.20) and 6-H (δ 3.78) suggested a boat conformation of the ring of 2,5-dioxopiperazine, and suggested that 3-H and 6-H should be *cis* conformation. The absolute configuration was determined as follows: Derivatization of these residues obtained from the crude hydrolysate with Marfey's reagent[6] and HPLC analysis with co-injection of standards defined the absolute stereochemistry of ILE as L and ASN as L.

Thus compound **1** was elucidated as 3-acetamino-6-isobutyl-2,5-dioxopiperazine.

Compound **2** was obtained as white amorphous powder (MeOH), It showed a yellow spot with 5% $KMnO_4$. HR-EI-MS exhibited an ion peak at *m*/*z* 212.2364 (Calcd 212.2345), consistent with a molecular formula of $C_{11}H_{20}N_2O_2$. In the ^{1}H-NMR spectrum, the proton signals at δ 0.84, 0.93, 1.21, 1.42 and 1.87 and the carbon signals at δ 11.9, 15.1, 24.4, 37.9 in the ^{13}C-NMR spectrum were similar with the ^{1}H and

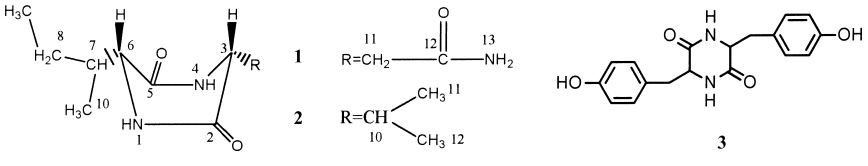

Fig. 1. The Structures of the Compounds

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Table 1. NMR Data of Compound **1** (in DMSO-d_6), δ in ppm, J in Hz

Position	δ_H	δ_C	HMBC correlation	^1H–^1H COSY
4-NH	8.05 (1H, br s)		3-C, 5-CO	
3-CH	4.20 (1H, br s)	51.1	2-CO, 11-C, 12-CO	4-NH, 11-H_a, 11-H_b
2-CO		167.8		
1-NH	7.78 (1H, s)		2-CO, 6-C	6-H
6-CH	3.78 (1H, br s)	58.7	5-CO, 7-C, 8-C	7-H
5-CO		167.0		
7-CH	1.87 (1H, m)	37.8	6-C, 8-C, 9-C, 10-C	6-H, 8-H, 10-H
8-CH_2	H_a (1.22, 1H, m)	24.2	7-C, 9-C, 10-C	7-H, 8-H_b, 9-H
	H_b (1.41, 1H, m)			7-H, 8- H_a, 9-H
9-CH_3	0.85 (t, 7.3)	12.0	7-C, 8-C	8-H_a, 8-H_b
10-CH_3	0.93 (d, 6.9)	15.1	6-C, 7-C, 8-C	7-H
11-CH_2	H_a (2.31, 1H, m)	38.7	3-C, 2-CO, 12-CO	3-H, 11-H_b
	H_b (2.68, 1H, m)			3-H, 11-H_a
12-CO		171.4		
13-NH_2	H_a (7.44, 1H, br s)			
	H_b (6.91, 1H, br s)			

^{13}C signals of isoleucine. The carbon signals at δ 17.3, 18.7, 30.9 were similar with those of valine. Derivatization of these two residues obtained from the crude hydrolysate with Marfey's reagent[6] and HPLC analysis with co-injection of standards defined the absolute stereochemistry of ILE as L and VAL as L. Thus this compound was determined as 3-isopropyl-6-isobutyl-2,5-dioxopiperazine by comparison with the known data.[7]

Compound **3** was determined as 3,6-di(4-hydroxy)benzyl-2,5-dioxopiperazine by comparison with NMR data of references.[8]

The cytotoxicity activities of the compound **1** against L-929, A375 were and tested and Hela IC_{50} (inhibitory concentration, 50%) were determined. Twenty-four hours after drug administration, IC_{50} was 6.30 μg/ml (L-929), 9.16 μg/ml (A375), 61.10 μg/ml (Hela) respectively. The positive control was 5-fluorouracil (5-Fu) dissolved in the same solution, IC_{50} was 6.37 μg/ml (L-929), 4.69 μg/ml (A375), 12.71 μg/ml (Hela).

Experimental

Genaral Procedures Melting points were measured with a Yanaco melting apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 281 spectrophotometer. Optical rotations were on a HITACHI U-3210 polarimeter. The HR-EI-MS was measeured on Zabspec (England) spectrometer. The NMR spectra were recorded at 300 MHz for ^1H- and 75.5 MHz for ^{13}C-NMR spectra on a Bruker ARX-300 spectrometer, and chemical shifts were given on a δ (ppm) scale with tetramethylsilane as an internal standard. Standard pulse sequences were employed for the DEPT, HMQC and HMBC experiments. TLC were performed on precoated Kieselgel 60 F254 plates (Merck). Column chromatography was carried out on silica gel (200—300 mesh), Sephadex LH-20(Pharmacia).

Plant Material *Cordyceps sinensis* (Berk.) Sacc. was obtained as whole herbs from Ganzi in Sichuan Province of China and identified by Professor Qi-shi Sun, Department of Pharmacognosy of Shenyang Pharmaceutical University. The voucher specimen was deposited at the same department.

Liquid Culture Material The liquid medium of *Cordyceps sinensis* include: sugar (1%), yeastextract (0.4%), $CaCl_2$ (0.01%), $MgSO_4 \cdot 7H_2O$ (0.04%), KH_2PO_4 (0.01%). The culture conditions were as follow: temperature: 28±1 °C, light intensity: 58.4 μmol·m^{-2}·s^{-1}, light period: 12 h/d culture time: 72 h 150 rpm/min inoculated density: 10% (150 ml medium in 500 ml flask).

Extration and Isolation The culture liquid of *Cordyceps sinensis* (Berk.) Sacc. (20 l) were extrated with the same volume ethyl acetate and *n*-butanol, respectively. The butanol fraction were concentrated to dry in vacuum. And the butanol fraction was subjected to silical gel column chromatography with chloroform and methanol (1 : 0—0 : 1) as eluent to afford to 28 fractions. The fraction ($CHCl_3$: CH_3OH 6 : 1) was subjected to Sephadex LH-20 (CH_3OH : H_2O 90 : 10) to afford **3** (38 mg) and the mixture of **1** and **2**. The mixture was separated by preparative HPLC [CH_3OH : H_2O 45 : 55, v/v, rate flow, 10 ml/min] to yield **1** (76 mg) and **2** (84 mg).

Cytotoxic Assay Three kinds of cell lines L929, A375 and Hela were selected in the present experiments. Present results suggested that 3-isobutyl-6-acetamino-2,5-dioxopiperazine had a better effect on the L929 cell and A375 cell, but not on Hela cell.

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