Two New Coumarins from *Herpetospermum caudigerum*

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The ethyl acetate extract from the seeds of *Herpetospermum caudigerum* was found to show protective effects on carbon tetrachloride (CCl_4) and thioacetamide (TAA)-induced acute hepatic injuries in mice. From the ethyl acetate extract, two new coumarins, herpetolide A (1) and herpetolide B (2), along with four known compounds, herpetone (3), dehydrodiconiferyl alcohol (4), 2,4-dihydroxypyrimidine (5) and stigmasterol (6) were isolated. The structures of the new coumarins were elucidated on the basis of chemical and physicochemical evidences. Herpetone exhibited protective effects on CCl_4-induced hepatocyte injury.

Key words *Herpetospermum caudigerum*; herpetolide A; herpetolide B; coumarin; herpetone

Herpetospermum caudigerum WALL (*H. pedunculosum* (SER.) BAILL.) (Cucurbitaceae) is distributed in southwest China, Nepal, and northeast India. The dried ripe seeds of *H. caudigerum* have been used for the treatment of liver diseases as a Tibetan medicinal herb in China.[1] The ethyl acetate extract from the seeds of *H. caudigerum* was found to show protective effects on carbon tetrachloride (CCl_4) and thioacetamide (TAA)-induced acute hepatic injuries in mice.[2] From the ethyl acetate extract, a new sesqui-norlignan named herpetone was obtained during our study.[3] The continued study led to the isolation of two new coumarins named herpetolide A (**1**) and herpetolide B (**2**), along with herpetone (**3**), dehydrodiconiferyl alcohol (**4**),[4] 2,4-dihydroxypyrimidine (**5**)[5] and stigmasterol (**6**)[6] from the ethyl acetate extract. The structures of the new coumarins were determined on the basis of chemical and physicochemical evidences.

Compounds **1** was isolated as a white amorphous powder. Its molecular formula $C_{16}H_{14}O_6$ was established from the *quasi*-molecular ion peak at *m/z* 325.0682 $[M+Na]^+$ in the HR-ESI-MS. The IR spectrum suggested the presence of hydroxyls (3384 cm^{-1}), a carbonyl group (1688 cm^{-1}), and benzene rings (1612, 1522, 1453 cm^{-1}). The presence of a 1,2,4,5-tetrasubstituted benzene ring and a 1,2,3,5-tetrasubstituted benzene ring were indicated from the ^{1}H-NMR spectrum (δ 8.02, 1H, s and δ 7.58, 1H, s; δ 7.05, 1H, d, J=2.6 Hz and δ 6.93, 1H, d, J=2.6 Hz), which was supported by the corresponding 12 downfield signals in the ^{13}C-NMR. The ^{1}H- and ^{13}C-NMR spectrum of **1** also suggested the presence of a hydroxymethyl group (δ_H 5.73, 1H, t, J=5.0 Hz, δ_H 4.89, 2H, d, J=5.0 Hz and δ_C 63.8), two methoxyl groups (δ_H 3.96 and 3.83, each 3H, s; δ_C 56.2 and 56.0) and a conjugated carbonyl group (δ_C 160.5). A detailed comparison of ^{1}H- and ^{13}C-NMR data between **1** and reference data of similar compound reported by Tanahashi T. *et al.*[7] implied that **1** possessed the similar coumarin skeleton. The HMBC correlations between CH_2OH (δ 4.89) and C-4a (δ 111.1), C-5 (δ 140.1), C-6 (δ 115.0), along with the NOESY correlation between CH_2OH (δ 4.89) and H-1′ (δ 8.02), H-6 (δ 6.93), indicated that the hydroxymethyl was located at C-5. Moreover, it was deduced that the hydroxy and two methoxyl groups were located at C-7, C-2′ and C-3′ respectively by the HMBC and NOESY experiments (Fig. 2). Consequently, compound **1** was elucidated as the structure showed in Fig. 1, and was trivially named as herpetolide A. Its ^{1}H- and ^{13}C-NMR spectra (Table 1) were completely assigned by detailed DEPT, HMQC, HMBC and NOSEY experiments.

Compound **2** was obtained as a white amorphous powder. Its molecular formula $C_{16}H_{12}O_6$ was established from the *quasi*-molecular ion peak at *m/z* 323.0523 $[M+Na]^+$ in the HR-ESI-MS. The IR spectrum suggested the presence of hydroxyls (3356 cm^{-1}), carbonyl groups (1725, 1674 cm^{-1}), and benzene rings (1622, 1523, 1455 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra of **2** were similar to those of **1**, except for the difference of CHO signals in **2** (δ_H 10.51, 1H, s and δ_C 192.6) and CH_2OH signals in **1** (δ_H 5.73, 1H, t, J=5.0 Hz, δ_H 4.89, 2H, d, J=5.0 Hz and δ_C 63.8). The HMBC of **2** also revealed that the CHO was located at C-5. Thus, compound **2** was determined as the structure showed in Fig. 1, and was trivially named as herpetolide B. Its ^{1}H- and ^{13}C-NMR spectra (Table 1) were assigned by detailed 2D-NMR experiments.

In order to seek the bioactive constituents for the treatment of liver diseases, effects of herpetone, a lignan compound of

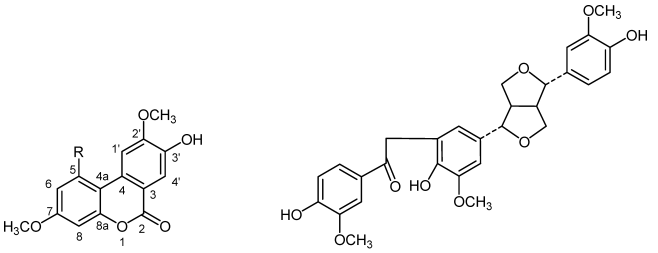

Fig. 1. Structures of Herpetolide A, B and Herpetone from the Seeds of *H. caudigerum*

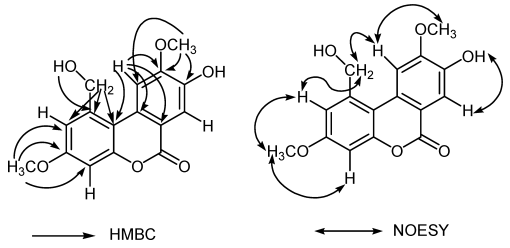

Fig. 2. Important HMBC and NOESY Correlations of Herpetolide A
HMBC: →; NOESY: ↔.

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Table 1. ^{1}H- and ^{13}C-NMR Data of **1** and **2** (600/150 MHz, DMSO-d_6, δ in ppm, J Hz)

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
2		160.5		160.0
3		113.6		113.8
4		128.9		126.5
4a		111.1		112.4
5		140.1		135.9
6	7.05, 1H, d (2.6)	115.0	7.31, 1H, d (2.6)[a)]	114.7
7		159.3		159.6
8	6.93, 1H, d (2.6)	101.4	7.29, 1H, d (2.6)[a)]	106.7
8a		152.8		152.9
1′	8.02, 1H, s	109.7	7.30, 1H, s	110.2
2′		154.3		154.2
3′		147.1		148.4
4′	7.58, 1H, s	114.6	7.61, 1H, s	114.2
–CH_2OH	5.73, 1H, t (5.0)	63.8		
	4.89, 2H, d (5.0)			
7-OCH_3	3.83, 3H, s	56.0	3.91, 3H, s	56.3
2′-OCH_3	3.96, 3H, s	56.2	3.95, 3H, s	56.6
–OH	9.98, 1H, br s		10.26, 1H, br s	
–CHO			10.51, 1H, s	192.6

a) Chemical shift may be exchanged.

Table 2. Effects of Herpetone on the Survival Rate of Rat Hepatocytes Treated with CCl_4

Group	Dose (mg/ml)	OD (mean±S.D.)	Survival rate (%)
Medium		0.545±0.06▲▲	100
CCl_4-treated		0.127±0.02	23.3
Herpetone	0.5	0.257±0.01▲▲	47.1
	0.25	0.168±0.04	30.8
	0.125	0.148±0.01	27.1
	0.0625	0.142±0.01	26.0

Compared with CCl_4-treated, ▲▲$p<0.01$.

relatively high content isolated from the EtOAc extract of *H. caudigerum*, on the survival rate of CCl_4-injured primary cultured rat hepatocytes were investigated (Table 2). The result showed that herpetone significantly improved the cell viability, indicating a protective effect on hepatocytes *in vitro*. In addition, Yuan *et al.* reported that herpetin, another lignan compound from this plant, showed significant inhibitory effects on HBV-DNA and the replication and expression of HBsAg and HBeAg.[8)] These findings suggest that lignan constituents of this plant are worthy of further investigation.

Experimental

General Melting points were measured on an XRC-1 melting point apparatus and are uncorrected. UV and IR spectra were recorded on a UV-8500 spectrometer and a Nicolet 2000XV FT-IR spectrometer (KBr disk), respectively. Mass spectra were obtained on a VG-7070E mass spectrometer (EI-MS) and a Bruker Daltonics Bio TOF-Q mass spectrometer (HR-ESI-MS). NMR spectra were carried out on a Bruker Avance 600 spectrometer with TMS as the internal standard. Column chromatography was carried out on silica gel (200—300 mesh), Qingdao Haiyang Chemical Group Co., China). Semi-preparative HPLC was carried out using a Shimadzu 10A VP HPLC system (YWG-C_{18} column, 250×10 mm, 10 μm).

Plant Material The seeds of *H. caudigerum* were purchased from Tibetan Shannan Herbal Company and identified by Prof. Minru Jia, College of Pharmacy, Chengdu University of Traditional Chinese Medicine (TCM). Voucher specimens (No. 2001-018a) have been deposited at the phytochemistry laboratory of Chengdu University of TCM.

Extraction and Isolation The dried and powdered seeds of *H. caudigerum* (5.3 kg) were refluxed with 95% ethanol (40 l×4 times, each 2 h). After removal of ethanol under reduced pressure, 370 g of residue was obtained. Mixed well with silica gel (1 kg), this residue was extracted in turns with petroleum ether, ethyl acetate, and 95% ethanol. After removal of solvents under reduced pressure, 90 g of petroleum ether extract, 30 g of ethyl acetate extract, and 45 g of ethanol extract were obtained.

The ethyl acetate extract (30 g) was subjected to silica gel column chromatography (ϕ 6×L 50 cm) and eluted with petroleum ether/ethyl acetate (6 : 1, 3 : 1, 1 : 1, 1 : 2) to give fractions A—D (A, 1.8 g; B, 1.2 g; C, 0.6 g; D, 2.0 g). Compound **1** (12 mg) was obtained by crystallization of fraction A from petroleum ether/ethyl acetate, while the remnant was then subjected to silica gel column (ϕ 3×L 30 cm) and eluted with petroleum ether/acetone to yield compound **2** (9 mg). Compound **3** (31 mg) was obtained by crystallization of fraction B from petroleum ether/ethyl acetate (3 : 1). Compound **4** (5 mg) was obtained by HPLC (C-18 column, CH_3CN : H_2O, 65 : 35) from fraction C. The fraction D was subjected to silica gel column chromatography (ϕ 3×L 30 cm) and eluted with petroleum ether/acetone (2 : 1 to 1 : 1) to give fractions D1, D2. Compound **5** (20 mg) was obtained by crystallization of fraction D1 from petroleum ether/acetone, and compound **6** (15 mg) was obtained by crystallization of fraction D2 from petroleum ether/acetone.

Herpetolide A (**1**): White amorphous powder. mp 95—96.5 °C. ^{1}H- and ^{13}C-NMR data see Table 1. IR (KBr) cm^{-1}: 3384, 1688, 1612, 1522, 1453, 1308, 1155. UV λ_{max} (CH_3OH) nm (log ε): 260 (3.64), 303 (3.18), 335 (2.88). HR-ESI-MS m/z: 325.0682 (Calcd for $C_{16}H_{14}O_6Na$: $[M+Na]^+$, 325.0683). MS m/z: 302 (M^+), 285, 84.

Herpetolide B (**2**): White amorphous powder. mp 105—107 °C. ^{1}H- and ^{13}C-NMR data see Table 1. IR (KBr) cm^{-1}: 3356, 1725, 1674, 1622, 1523, 1455, 1315,1159. UV λ_{max} (CH_3OH) nm (log ε): 247 (3.84), 304 (3.30), 346 (3.22). HR-ESI-MS m/z: 323.0523 (Calcd for $C_{16}H_{12}O_6Na$: $[M+Na]^+$, 323.0527). MS m/z: 300 (M^+), 272, 257, 113.

Protective Effects of Herpetone on Primary Cultured Rat Hepatocytes Treated with CCl_4 Hepatocytes were obtained from rat liver by means of collagenase perfusion method.[9)] Cell suspensions were seeded into 96-well-plates (≥4×10^4 cells/well) and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. CCl_4 was then added to the culture medium at a final concentration of 8 mmol/l to induce hepatocyte injury. After 24 h, herpetone of various concentrations were added to the culture medium and the cells were further cultured for 48 h followed by the addition of 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) into each well. The cells were incubated under the same conditions for additional 4 h. Then, the culture medium was removed. After the addition of 150 μl of DMSO, the optical densities at 570 nm were measured and the survival rates of hepatocytes were calculated [survival rate=OD (sample group)/OD (medium group)×100%].

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