Hepatitis B Virus Inhibiting Constituents from *Herpetospermum caudigerum*

Hai-Long YUAN,*[,a] Ming YANG,[b] Xian-Yi LI,[a] Rong-Hui YOU,[b] Yi LIU,[c] Jun ZHU,[c] Huan XIE,[c] and Xiao-He XIAO[a]

[a] *302 Hospital of PLA&PLA Institute of Chinese Materia Medica; Beijing 100039, China:* [b] *Key Lab of Modern Preparation of TCM, Ministry of Education; Nanchang 330004, China: and* [c] *Acadamy of Pharmacy, Chengdu University of TCM; Chengdu 610075, China.* Received April 29, 2006; accepted August 22, 2006

From the ethanol extract of the seeds of *Herpetospermum caudigerum* wall, one new lignan compound 1, was isolated and characterized along with three known compounds 2, 3 and 4. The structure elucidation of the isolated new compound was performed on the basis of spectroscopic and chemical evidence. The structures of known compounds were determined by comparison of spectral data and physical data with those previously reported. The activity inhibiting hepatitis b virus was evaluated. Preliminary studies showed that compound 1 and 2 displayed promising inhibitory potential against hepatitis b virus.

Key words hepatitis b virus inhibition; *Herpetospermum caudigerum*; lignan; 2.2.15 cell

Herpetospermum caudigerum grows widely in the southwest of China, Nepal and the northeast of India. In Tibet it is popularly known and used in traditional medicine for the treatment of liver diseases, cholic diseases, and dyspepsia.[1,2] Previously a large amount of lignans have been reported from *Herpetospermum caudigerum*.[3—8] In the previous investigation, a ethanol extract of the seeds of *Herpetospermum caudigerum* showed positive activity in the inhibit hepatitis b virus test.[10,11] Further biological screening of the ethanol extract and ethyl acetate soluble fraction revealed significant inhibitory activity against hepatitis b virus. This prompted us to carry out bioassay-guided isolation studies on the ethyl acetate soluble fraction of this plant. In this paper, we report the isolation and structure elucidation of a new compound 3-benzofuranmethanol-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-4-methoxy-6-[tetrahydro-2-(3-hydroxy-4-methoxyphenyl)-3-methanol]-2-furanmethyl (**1**), and three known compounds (**2**—**4**). The compounds **1** and **2** showed promising inhibitory activity against hepatitis b virus.

The ethyl acetate fraction of the ethanol extract of the seed of *Herpetospermum caudigerum* was subjected to column chromatography over silica gel with different mobile phases. Compounds **1**—**4** were finally obtained and their structures were elucidated by UV, IR, mass and NMR spectroscopy.

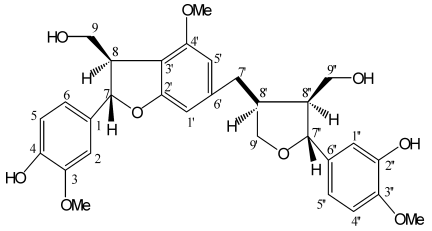
The known compounds **2**, **3**, **4** were determined to be 2-hydroxymethyl-1-(3-methoyl-phenol)-3-(3,4-dimethoyl-benzyl)-tetrahydrofuran (**2**), 2,3-dihydroxymethyl-1,4-(3,4-dimethoyl-benzol)-tetrahydrofuran (**3**) and herpetfluorenone (**4**) by comparison of physical data and NMR spectral data with those previously reported.[3,5,8]

Compound **1** was obtained as a white amorphous solid. The high resolution and fast atomic bombardment mass spectrometry (HR-FAB-MS) established the molecular formula to be $C_{30}H_{34}O_9$, showing a $[M+1]^+$ peak at *m*/*z* 539.1332 (Calcd for $C_{30}H_{35}O_9$, 539.1337) having fourteen degree of unsaturation. The infrared (IR) spectrum of **1** showed the absorption band due to hydroxyl groups (3418 cm^{-1}) and aromatic groups (1605, 1517 cm^{-1}). The ultraviolet (UV) spectrum of **1** showed a conjugated carbonyl absorption band at λ_{max} 230 nm. The ^{1}H- and ^{13}C-NMR spectra of **1** exhibited signals characteristic of a benzofuran-type lignan (Table 1). The ^{1}H-NMR spectrum showed the presence of four hydroxy groups and three methoxyl groups. The signals for aliphatic methylene protons appeared at δ 2.58 (1H, m), 2.79 (1H, m), two aliphatic methine groups at δ 2.80 (1H, m, H-8′), 2.42 (1H, m, H-8″), three oxygenated methylene groups at δ 3.80 (1H, dd, H-9), 3.67 (1H, dd, H-9″), 3.84 (2H, dd, H-9, H-9″), 3.79(1H, dd, H-9′), 4.03 (1H, dd, H-9′), two oxygenated methine groups at δ 5.52 (1H, d, H-7), 4.78 (1H, d, H-7″). The downfield region of the spectrum showed the presence of phenyl groups. The broad-band and distortionless enhancement by polarization transfer (DEPT) ^{13}C-NMR spectra of **1** (Table 1) disclosed 30 carbon singles for three methyl, four methylene, thirteen methane

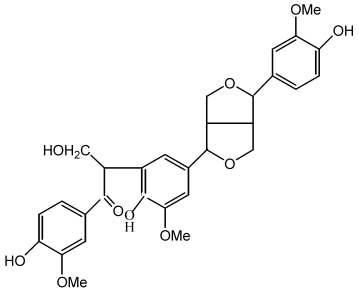
Table 1. ^{1}H- and ^{13}C-NMR Spectral Data and HMBC Correlations for Compound **1** (CD_3OD)

No.	δ_C	δ_H	HMBC (H→C)
1	109.69, d	6.97, d, 1.5	2, 3, 5, 7
2	148.02, s		
3	146.53, s		
4	115.14, d	6.79, d, 8.0	3, 2, 6
5	118.83, d	6.84, dd, 8.0, 1.5	3, 1, 7
6	133.69, s		
7	88.05, d	5.52, d	1, 5, 6, 2′, 8, 3′
8	54.36, d	3.51, m	6, 7, 9, 3′, 2′, 4′
9	63.90, t	3.80, dd; 3.84, dd	7, 8, 3′
1′	113.39, d	6.77, d, 1.5	2′, 3′, 5′, 7′
2′	146.80, s		
3′	129.16, s		
4′	146.06, s		
5′	117.30, d	6.79, d, 1.5	4′, 3′, 1′, 7′
6′	134.51, s		
7′	32.97, t	2.58, m; 2.97, m	1′, 5′, 6′, 8′, 9′, 8″
8′	42.96, d	2.80, m	6′, 7′, 9′, 8″, 7″, 9″
9′	72.53, t	4.03, dd; 0.79, dd	8′, 7′, 7″, 8″
1″	109.59, d	6.93, d, 1.5	2″, 3″, 5″, 7″
2″	144.37, s		
3″	148.10, s		
4″	115.01, d	6.78, d, 8.0	2″, 3″, 6″
5″	118.83, d		1″, 3″, 7″
6″	134.74, s		
7″	83.07, d	4.78, d	1″, 5″, 6″, 9′, 8″, 9″
8″	53.07, d	2.42, m	9′, 7″, 8′, 6″, 9″, 7′
9″	59.48, d	3.67, dd; 0.84, dd	8′, 8″, 7″

* To whom correspondence should be addressed. e-mail: yhlpharm@126.com



A



B

Fig. 1. Structures of Two Compounds Isolated from *Herpetospermum caudigerum*

(A) Compound **1**; (B) compound **2**. The compound **1** structure was elucidated by UV, IR, mass, and NMR spectroscopy and compound **2** was determined by comparison of physical data and NMR spectral data with those previously reported.[3)]

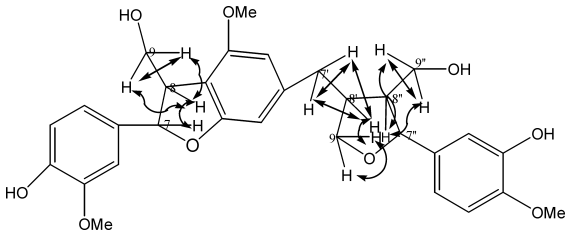


Fig. 2. Key H–H COSY Correlations of Compound **1**

and ten quaternary carbons. The signals for anomeric carbon protons appeared at δ 113.39, 146.80, 129.16, 146.06, 117.30, and 134.51 were assigned to those protons in the benzofuran moiety of **1**. The above data suggested that **1** is a benzofuran-type lignan. It was further confirmed by H–H COSY and heteronuclear multiple-bond connectivity (HMBC) spectral data, and the important correlations are illustrated in Table 1, Figs. 1 and 2. On the basis of these evidence, the structure **1** could be determined to be 3-benzofuranmethanol-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-4-methoxy-6-[tetrahydro-2-(3-hydroxy-4-methoxyphenyl)-3-methanol]-2-furanmethyl.

The inhibitory effects of compound **1** and **2** on HBV-DNA and the replication and expression of HBsAg and HbeAg were measured in 2.2.15 cell, which was a human hepatoblastoma cell line transfected by HBV.[9)] The 2.2.15 cell line was translated by recombinant HBV-DNA and could express all marks of HBV. It was a good model of chronic cellular viral infection *in vitro*, so the inhibitory effects of compounds **1** and **2** on HBV-DNA and the replication and expression of HBsAg and HBeAg were assayed. In our experiment, different concentration (25—250 μg/ml) of the compounds **1** and **2** was chosen to be incubated with 2.2.15 cells for 48 h, then the inhibitory effect could be seen. Within the concentration range from 25—250 μg/ml, the inhibitory effect of the compounds **1** and **2** on HBsAg and HBeAg was dose-dependent, and at the concentration over 250 μg/ml, as the

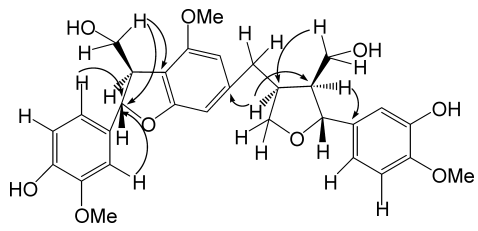


Fig. 3. Key H–C HMBC Correlations of Compound **1**

Table 2. Selective Inhibition Effect of Compounds **1** and **2** on HBV-DNA, the Replication and Expression of HBsAg and HBeAg

Compounds	TC_{50} (μg/ml)	HBeAg		HBsAg		HBV-DNA	
		IC_{50} (μg/ml)	SI	IC_{50} (μg/ml)	SI	IC_{50} (μg/ml)	SI
1	1329.5	159.7	8.32	170.5	7.80	298.1	4.46
2	1732.4	361.3	4.79	347.1	4.99	302.4	5.73

Selection index (SI)=TC_{50}/IC_{50}.

Table 3. Inhibitory Effect of Compound **1** and Compound **2** on HBV-DNA, the Replication and Expression of HBsAg and HBeAg

Dose ($\mu g \cdot ml^{-1}$)	HBV-DNA inhibitory rate (IR) (%)		HBsAg inhibitory rate (IR) (%)		HBeAg inhibitory rate (IR) (%)	
	1	**2**	**1**	**2**	**1**	**2**
25.0	8.3	−0.2	28.6	17.4	21.2	12.4
62.5	17.8	6.9	39.3	22.5	30.6	18.9
125.0	22.4	23.2	51.4	28.7	39.6	36.7
187.5	38.1	42.6	66.8	33.7	51.4	37.5
250.0	47.4	44.3	65.7	36.2	50.6	38.9

P/N=(cpm in test group−cpm in blank control group)/(cpm in positive control group−cpm in blank control group). HBsAg and HBeAg (IR)=[control (P/N)−experiment (P/N)]/[control (P/N)−2.1]×100%. HBV-DNA(IR)=[OD (before treatment)−OD (after treatment)]/OD (before treatment)×100%.

amount of the compounds **1** and **2** increases, the inhibitory effect stopped rising, but only showed a platform phenomenon (Table 3). The inhibitory effect of compounds **1** and **2** (25—250 μg/ml) on HBV-DNA was assessed, and its inhibitory rate (IR) also showed good linear correlation (Table 3). The supernatant of 2.2.15 cells treated with compounds **1** and **2** in different concentrations were collected at day 4 and day 8 respectively. Compared with the control group without compounds **1** and **2**, the 2.2.15 cells treated with compound **1** and **2** showed no obvious difference in toxicity.

The experiment results showed that compounds **1** and **2** is effective in reducing the replication and expression of HBsAg and HBeAg, and has significant inhibitory effect on HBV-DNA. Their antiviral effects are dose-dependent and time-relevant, offering widely research and development prospect.

Experimental

General Mps was measured on NETZSCH DSC 204, with a calefactive speed at 10.0 °C/min from 40 to 300 °C. Optical rotation was recorded at room temperature on a JASCO DIP-370 polarimeter. UV and IR spectra were recorded on a Nicolef Fourier transform infrared spectrometer (Nexus-470) and a HP8452A spectrophotometer respectively. The ^{1}H-NMR (500 MHz), ^{13}C-NMR (500 MHz), and HMBC spectra were recorded on a Bruker Avance-500 instrument. Chemical shifts were reported in δ units (ppm). FAB-MS, HS-MS and EI-MS were acquired with a VG Zabspec spectrometer. Semi-prep HPLC purification were done with a Waters instrument, equipped with a photodiode array detector. Experimental conditions: Hypersil Rp C18 (10.0×250 mm, 25 μm, NO. 2106107); oven temperature, 40 °C, mobile phase: methanol–water 60 : 40 (v/v) (flow rate at 5 ml/min); Alltima Rp C18 (5 mm, Serial 00120175.1), mobile phase, acetonitride–water (1% acetic acid) 24 : 76 (50 min, flow rate at 1 ml/min); detection at 230 nm. CC (column chromatograph) was carried out on silica gel (100—200 mesh, Qingdao china) and RP-Si-gel cc.

Plant Material The dried semen were obtained from Tibet, China, in February 2004, and identified as the semen of *Herpetospermum caudigerum* by Dr. Xiao-he Xiao. A voucher specimen (NO. 040228-5) was deposited in the PLA Institute of Chinese Materia Medica, China.

Extraction and Isolation The dried and powdered semen (5 kg) were extracted with ethanol (2×30 l) at 85 °C, then ethanol was evaporated. The resulting extract was first defatted with petroleum ether (3×900 ml), and then further extracted out with chloroform, ethyl acetate and *n*-buthanol (3×600 ml) respectively. The ethyl acetate extract (60 g) was subjected to cc over silica gel eluted with dichloromethane–acetone (50 : 1, 30 : 1, 10 : 1, 5 : 1, 2 : 1, 1 : 1) to afford six fractions (A—F). The fraction E (19 g) obtained from dichloromethane–acetone (2 : 1) was further applied to a silica gel cc eluted with dichloromethane–acetone (2 : 1) to afford a crude compound **1**. The crude compound **1** was rechromatographed over flash RP-Si-gel and semi-prep HPLC with a mixture of methanol–water and a acetonitride (2% acetic acid) respectively to afford compound **1** (152.6 mg).

Compound **1**: Amorphous powder: mp 163 °C; $[\alpha]_D^{20} = +19.7°$ (0.615, MeOH). Moving spot (*Rf* 0.28). HR-FAB-MS *m/z*: 539.1332 (Calcd for $C_{30}H_{34}O_9$, 539.1337) $[M+1]^+$. UV λ_{max}(MeOH) nm: 230; IR (KBr) cm^{-1}: 3418, 1605, 1517. EI-MS *m/z*: 538 $[M]^+$, 520, 508, 490, 298, 297, 285, 265, 221, 205, 191, 175, 151, 137. ^{1}H-NMR ($CDCl_3$/CD_3OD, 500 MHz), ^{1}C-NMR ($CDCl_3$/CD_3OD, 500 MHz) and HMBC data of **1** are shown in Table 1.

Activity Assay Cell Culture: The 2.2.15 cells were donated by the Medical Sciences. According to Korba's method, confluent cultures bottle in DMEM medium with 10% fetal bovine serum (product of Sigma), 380 μg/ml G418 (product of Sigma), 2×10^5 U/l penicilline–streptomycin (product of Gibco) at 37 °C incubator filled with 5% CO_2. The culture medium was changed every 2 d.

HBsAg and HBeAg Assay: After the passage, 2.2.15 cells were transplanted on 24-well flat-bottomed tissue culture plates with 1 ml per well. The cell concentration was 3×10^5/l. After the cells were attached to the bottom of plates, exchange the supernatant with the medium including Herpetin. Each concentration had 3 replicated wells. Then it was collected after treatment of Herpetin at different concentration. HBsAg and HBeAg were examined by radioimmunoassay (RIA), and the result indicated by P/N.

HBV-DNA Assay: The sample (20 μl) was treated with proteinase K(100 μg/ml) at 55 °C for 1 h. A 0.8 vol of saturated NaI (2.5 g of NaI in 1 ml of water at 100 °C) was added to the sample and heated at 90 °C for 10 min. The DNA was immobilized on nitrocellulose by using a slot-blot apparatus (Schleicher and Schuell). The DNA on the blot was detected with a DNA-specific probe. The intensity of the autoradiographic bands was quantitated by a scanning densitometer.

Acknowledgements The authors thank Dr. Lin Wen-han from peking university for instruction of spectroscopic identification.

References

1) Brend E. K., *J. Antiviral Res.*, **29**, 49—53 (1995).
2) Cheng Y. M., "Flora Xizangica," Science Press, Beijing, 1985, pp. 537—539.
3) Kaouadji M., Favre B. J., Mariotte A. M., *Phytochemistry*, **17**, 2134—2135 (1978).
4) Kaouadji M., Favre B. J., Mariotte A. M., *Biosci.*, **34C**,1129—1132 (1979).
5) Kaouadji M., Favre B. J., *Tetrahedron Lett.*, **24**, 5881—5884 (1983).
6) Kaouadji M., Favre B. J., *Tetrahedron Lett.*, **25**, 5135—5136 (1984).
7) Kaouadji M., Favre B. J., *Tetrahedron Lett.*, **25**, 5137—5138 (1984).
8) Kaouadji M., Favre B. J., *J. Nat. Prod.*, **50**, 1089—1093 (1987).
9) Sells M. A., Chen M. L., Acs G., *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 1005—1008 (1987).
10) Han Y. M., Yuan H. L., Liu Y., Zhao Y. L., *Chin. Tradit. Herb. Drugs*, **36** (Suppl.), 169—171 (2005).
11) Han Y. M., Li Z. M., Yuan H. L., Liu Y., Zhao Y. L., *Chin. J. Exper. Tradit. Med. Form.*, **12**, 42—43 (2006).