SHORT REPORT

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Rec. Nat. Prod. 7:4 (2013) 346-350

records of natural products

Monoterpenes from *Paeonia sinjiangensis* Inhibit the Replication of Hepatitis B Virus

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(Received December 18, 2011; Revised May 25, 2013; Accepted July 8, 2013)

Abstract: Two new monoterpenes, 4″-hydroxy-3″-methoxyalbiflorin (**1**) and 6′-*O*-*p*-hydroxybenzoyl-4″-hydroxyalbiflorin (**2**), together with six known monoterpenes, which were albiflorin (**3**), oxypaeoniflorin (**4**), paeoniflorin (**5**), paeonins A (**6**), paeonins B (**7**) and benzoylpaeoniflorin (**8**) respectively, were isolated from the root material of *Paeonia sinjiangensis*. The compounds were identified by spectral analysis and comparison with spectroscopic data reported in the literatures. When the *in vitro* anti-hepatitis B virus (HBV) activities of compounds **1**-**8** were evaluated, all the isolated monoterpenes, except **6**, reduced the yield of HBV DNA, suppressed HBsAg and HBeAg protein production from HepG2215 cell culture system, with compound **1** exhibiting the greatest potential.

Keywords: *Paeonia sinjiangensis*; Monoterpene; Hepatitis B Virus; Replication.

1. Plant Source

The root materials of *Paeonia sinjiangensis* was collected in the Altai Mountains, Xinjiang Uygur Autonomous Region and was verified by Associate Professor He Chun-nian (Peking Union Medical College, Beijing) in October 2009. The voucher specimen (No. 091002) has been deposited in Department of Laboratory Medicine, Weihai Woman and Children's Hospital.

2. Previous Studies

Paeonia sinjiangensis is a native peony, endemic to Xinjiang Province of western China. Compared with other species from genus *Paeonia*, *Paeonia sinjiangensis* was a new species firstly discovered in 1979, and fewer studies on the chemistry and bioactivity have been performed [1-3].

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 08/05/2013 EISSN:1307-6167

3. Present Study

Dried and powdered roots of *P. sinjiangensis* (4.5 kg) were extracted with 90% EtOH three times at room temperature. The 90% ethanol solution was concentrated in *vacuo* to give an extract, which was suspended in water and partitioned with cyclohexane, $CHCl_3$, EtOAc and *n*-BuOH successively. The *n*-BuOH extract (106 g) was first subjected to silica gel column chromatography and then eluted with $CHCl_3$/MeOH (90 : 10 → 60 : 40, v/v) gradient to afford several subfractions. The subfractions were further purified on ODS-A C18 reversed-phase silica gel (MeOH–H_2O) and then purified by Sephadex LH-20 column chromatography to give compounds **1** (39 mg), **2** (27 mg), **3** (12 mg), **4** (23 mg), **5** (11 mg), **6** (10 mg), **7** (33 mg) and **8** (7 mg).

Compound **1** was obtained as white amorphous powder with $[\alpha]_D^{20}$ − 26.3° (*c* = 0.186, MeOH) and its molecular formula was determined to be $C_{24}H_{30}O_{13}$ by HRESIMS data (*m*/*z* 525.1546 $[M - H]^-$, calcd 525.1608). The ^1H and ^{13}C NMR spectra of **1** were very similar to those of albiflorin except for the aromatic ring signals at δ_H 7.96 (1H, d, *J* = 1.8 Hz), 7.20 (1H, d, *J* = 8.4 Hz), 8.02 (1H, dd, *J* = 8.4, 1.8Hz) in the ^1H NMR spectrum [6], which were in correspondence with the signals at δ_C 113.9 (CH), 116.7 (CH), 125.2 (CH) in the ^{13}C NMR spectrum. The HMBC spectrum showed the cross peaks between the methoxyl protons at δ_H 3.78 to C-2″ (δ_C 113.9, d), 3″ (δ_C 148.6, s) and 4″ (δ_C 153.6, s). These signals disclosed the presence of a 4-hydroxy-3-methoxybenzoyloxy unit. The locations of these esterifying units were confirmed by the HMBC spectrum, in which long-range correlations were observed from H-1′ (δ_H 5.07, d, *J* = 7.8) to C-1 (δ_C 90.8, s) and H-8 (δ_H 5.08 and 5.21, d, *J* =12.0 Hz) to C-7″ (δ_C 167.1, s). The assignments of the other signals were confirmed by HMQC and HMBC experiments. Therefore, the structure of the compound **1** was elucidated as 4″-hydroxy-3″-methoxyalbiflorin.

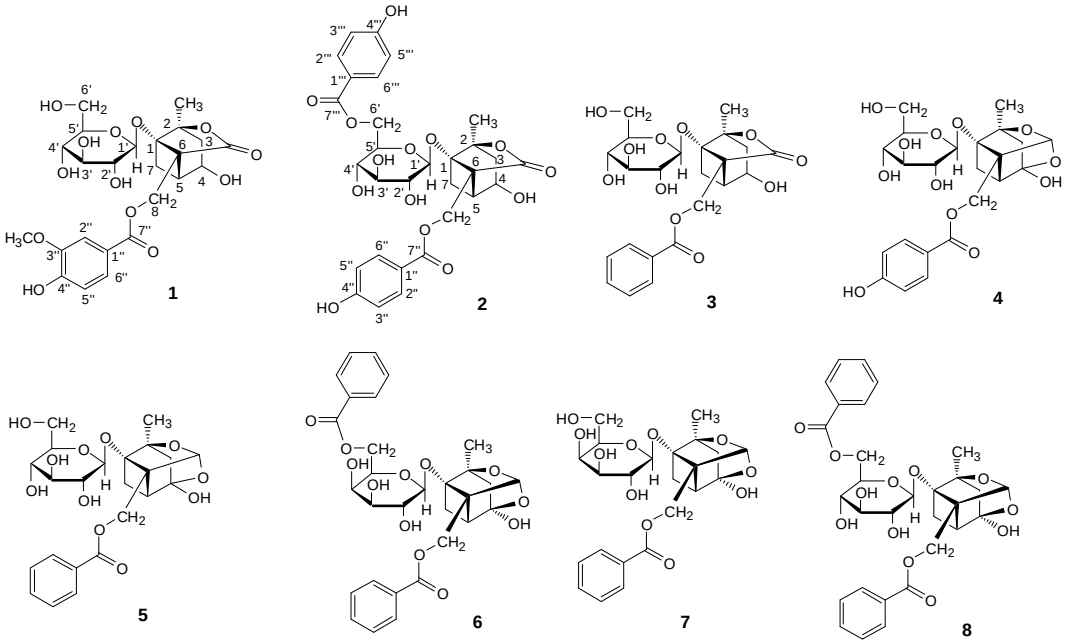

Figure 1. Chemical structures of compounds **1**-**8**.

Compound **2** was obtained as white amorphous powder with $[\alpha]_D^{20}$ − 34.8° (*c* = 0.224, MeOH) and its molecular formula was determined to be $C_{30}H_{32}O_{14}$ by HRESIMS data (615.1763 $[M - H]^-$, calcd 615.1714). The ^1H and ^{13}C NMR spectra of **2** were very similar to those of 6′-*O*-benzoylalbiflorin except for the aromatic ring signals [7]. The ^1H NMR spectra of **1** showed two A_2B_2 signals at δ_H 8.24 (2H, dd, *J* = 9.0, 1.8 Hz) and 7.02 (2H, d, *J* = 9.0 Hz), 8.28 (2H, dd, *J* = 9.0, 1.8 Hz)

and 7.24(2H, d, J = 9.0 Hz). These data, combining with the ^{13}C NMR spectra signals, suggested the presence of two *p*-hydroxybenzoyloxy unit. The locations of these esterifying units were confirmed by the HMBC spectrum, in which long-range correlations were observed from H-1′ (δ_H 5.01, d, J = 7.8) to C-1 (δ_C 91.2, s), H-8 (δ_H 5.10 and 5.23, d, J =12.0 Hz) to C-7″ (δ_C 166.8, s) and H-6′ [(δ_H 5.12, dd, J = 11.4, 5.4Hz), (δ_H 5.23, d, J = 11.4 Hz)] to C-7‴ (δ_C 166.6, s). The assignments of the other signals were confirmed by HMQC and HMBC experiments. Therefore, the structure of the compound **2** was elucidated as 6′-*O*-p-hydroxybenzoyl-4″-hydroxyalbiflorin.

In addition to the two new compounds, six known monoterpenes, which were albiflorin (**3**), oxypaeoniflorin (**4**), paeoniflorin (**5**), paeonins A (**6**), paeonins B (**7**) and benzoylpaeoniflorin (**8**) respectively (Fig. 1), were isolated from the root material of *Paeonia sinjiangensis*. These compounds were identified by spectral analysis and comparison with spectroscopic data reported in the literatures.

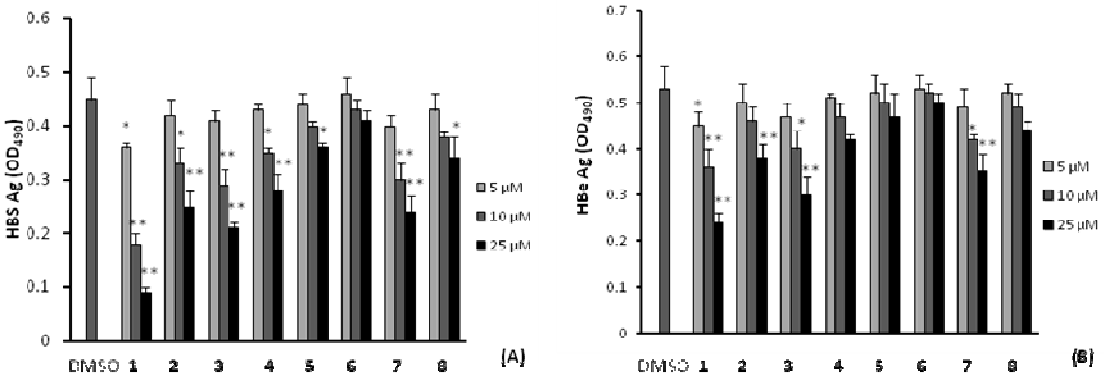

Figure 2. Compounds **1**-**8** treatment suppressed HBsAg and HBeAg proteins production. HepG2215 cells were treated with different concentrations of compounds **1**-**8** for 8 days and the HBsAg (A) and HBeAg (B) levels in the culture medium were determined by ELISA. * $p < 0.05$, ** $p < 0.01$ *versus* DMSO control

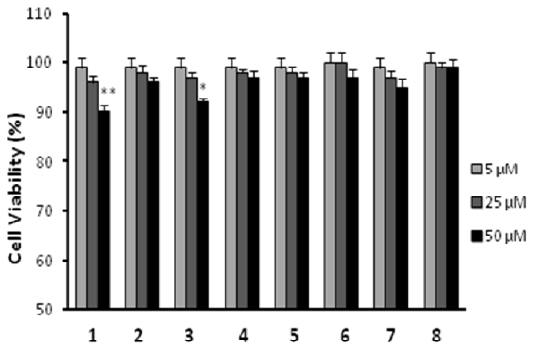

Figure 3. Effects of compounds **1**-**8** on the viability of HepG2215 cells. * $p < 0.05$, ** $p < 0.01$ *versus* DMSO control

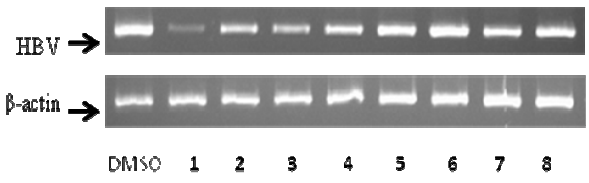

Figure 4. Compounds **1**-**8** treatment reduced the expression levels of HBV mRNA. HepG2215 cells were treated with different concentrations of compounds **1**-**8** for 3 days and total RNA were collected as template to synthesize cDNA. The mRNA levels of HBV were determined by semi-quantitative RT-PCR analysis. Results are representative of three independent experiments.

The inhibitory effects of these monoterpenes on HBV replication were evaluated with the HepG2215 cell culture system, a derivative of the human HepG2 hepatoma cell line that was stably transformed with the HBV DNA. As shown in Fig.2, treatment of the cells with different concentrations of compounds **1**-**8** yielded varied results; the HBsAg protein levels were significantly

Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1** and **2** in pyridine-d_5 (600 MHz for ^{1}H and 125 MHz for ^{13}C)[a,b]

Position	**1**			**2**		
	δ_H	δ_c	HMBC (H→C)	δ_H	δ_c	HMBC (H→C)
1		90.8 s			91.2 s	
2		86.2 s			86.6 s	
3a	2.35 (dd, 12.6, 6.0)	41.6 t	C-2,4	2.33 (dd, 12.6, 6.0)	41.8 t	C-2,4
3b	2.14 (d, 12.6)		C-1,2,4,5	2.12 (d, 12.6)		C-1,4,5
4	4.46 (*dt*, 7.2, 6.0)	66.9 d	C-6	4.45 (*dt*, 7.2, 6.0)	67.3 d	C-6
5	3.13 (*m*)	41.2 d	C-1,3,4,6,7,8	3.13 (*m*)	41.3 d	C-1,4,6,8
6		55.5 s			55.7 s	
7a	3.07 (dd, 10.2, 7.2)	27.3 t	C-1,2,4,5	3.08 (dd, 10.2, 7.2)	27.8 t	C-1,2,4
7b	2.26 (d, 10.2)		C-1,4,6	2.27 (d, 10.2)		C-1,6
8a	5.08 (d, 12.0)	60.6 t	C-1,6,9	5.10 (d, 12.0)	61.5 t	C-1,9
8b	5.21 (d, 12.0)			5.23 (d, 12.0)		
9		176.1 s			175.7 s	
10	1.64 (s)	20.8 q	C-1,2,3	1.67 (s)	20.3 q	C-1,2,3
1′	5.07 (d, 7.8)	100.6 d	C-1	5.01 (d, 7.8)	100.1 d	C-1,2′
2′	4.01 (t, 7.8)	74.8 d	C-1′,3′	4.03 (t, 7.8)	74.6 d	C-1′,3′
3′	4.15 (t, 7.8)	78.2 d	C-2′,4′	4.18 (t, 7.8)	78.0 d	C-2′,4′
4′	4.12 (t, 7.8)	71.6 d	C-3′	4.05 (t, 7.8)	71.6 d	C-3′
5′	4.04 (dd, 7.8, 4.8)	78.2 d		4.08 (dd, 7.8, 4.8)	75.3 d	
6′a	4.32 (dd, 11.4, 5.4)	62.6 t	C-4′	5.12 (dd, 11.4, 5.4)	64.7 t	C-4′,7‴
6′b	4.54 (dd, 11.4, 2.4)		C-5′	5.23 (d, 11.4)		C-5′
1″		122.1 s			121.7 s	
2″	7.96 (d, 1.8)	113.9 d	C-4″,7″	8.24 (dd, 9.0, 1.8)	131.8 d	C-4″,6″,7″
3″		148.6 s		7.02(d, 9.0)	116.4 d	C-1″,2″,4″
4″		153.6 s			164.0 s	
5″	7.20 (d, 8.4)	116.7 d	C-1″,3″	7.02 (d, 9.0)	116.4 d	C-1″,3″,4″,6″
6″	8.02 (dd, 8.4, 1.8)	125.2 d	C-4″,7″	8.24 (dd, 9.0, 1.8)	131.8 d	C-2″,4″,5″,7″
7″		167.1 s			166.8 s	
1‴					121.6 s	
2‴, 6‴				8.28 (dd, 9.0, 1.8)	131.5 d	C-2‴,4‴,5‴,6‴,7‴
3‴, 5‴				7.24(d, 9.0)	116.2 d	C-1‴,2‴,3‴,4‴,6‴
4‴					163.8 s	
7‴					166.6 s	
-OCH_3	3.78 (s)	56.1 q	C-2″,3″,4″			

a) Chemical shift values were in ppm and *J* values (in Hz) were presented in parentheses.
b) The assignments were based on HMQC and HMBC.

attenuated in cells that were treated with compounds **1**, **3** and **7** at concentrations of 10 and 25 μM; compounds **2** and **4** could also significantly inhibit the HBsAg production at the highest concentration of 25 μM; whereas HBsAg levels were marginally affected when the cells were treated with **6**. Among

all the effective compounds, **1** showed the greatest potential, with its IC_{50} being less than 10 μM. Of note, the inhibitory effect of all the compounds on the replication of Hepatitis B virus was in a dose-dependent manner. ELISA analysis of the HBeAg levels in the culture medium showed similar results (Fig. 2B). Since the reduction in HBsAg and HBeAg levels may be due to direct impairment of cell functions, the cytotoxicity of these compounds on HepG2215 cells was next performed. The results revealed that while **1** and **3** at concentration of 50 μM could reduce the cell viability, all the compounds at concentrations of 5 μM and 25 μM did not show any cytotoxicity (Fig. 3). The followed RT-PCR analysis of HBV mRNA provided direct evidence that these compounds inhibited the virus replication in HepG2215 cells (Fig. 4). These results, taken together, suggested that the isolated monoterpenes inhibited the HBV replication in HepG2215 cells without affecting the cells' viability.

Compound 1: white amorphous powder, $[\alpha]_D^{20}$ – 26.3° (*c* = 0.186, MeOH); Negative-ion HRESIMS *m/z* 525.1546 $[M - H]^-$ (calcd for $C_{24}H_{29}O_{13}$: 525.1608); UV λ_{max} (MeOH) (log ε) at 203 (4.09), 224 (3.93), 260 (3.47); ESI-MS^n (positive ion) *m/z*: 527 $[M + H]^+$, 365 [M – Glu + H]$^+$, 213 [M – Glu – 3-methoxy-4-hydroxybenzyl + H]$^+$; The IR spectrum (3419, 2921, 2865, 1753, 1714, 1596, 1385, 1281, 1272, 1166, 1118, 1078, 823, 762, 715 cm^{-1}); The ^{1}H-NMR (600 MHz, CD_3OD) and ^{13}C-NMR data (125 MHz, CD_3OD) see Table 1.

Compound 2: white amorphous powder, $[\alpha]_D^{20}$ – 34.8° (*c* = 0.224, MeOH); Negative-ion HRESIMS *m/z* 615.1763 $[M - H]^-$ (calcd for $C_{30}H_{31}O_{14}$: 615.1714); UV λ_{max} (MeOH) (log ε) at 203 (4.59), 224 (4.39), 260 (3.76); ESI-MS^n (positive ion) *m/z*: 617 $[M + H]^+$, 481 [M – 4-hydroxybenzyl + H]$^+$, 345 [M – 2×4-hydroxybenzyl + H]$^+$, 198 [M – 2×4-hydroxybenzyl – Glu + H]$^+$; The IR spectrum (3417, 2922, 2872, 1757, 1716, 1573, 1381, 1284, 1276, 1170, 1115, 1079, 943, 772, 714 cm^{-1}); The ^{1}H-NMR (600 MHz, CD_3OD) and ^{13}C-NMR data (125 MHz, CD_3OD), see Table 1.

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