Icetexane Diterpenoids from *Perovskia atriplicifolia*

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Key words
- *Perovskia atriplicifolia*
- Lamiaceae
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- anti-HBV activity

Abstract

Five new icetexane diterpenoids, namely, perovskatones B–D (**1, 3, 4**), 1α-hydroxybrussonol (**2**), and 1α-hydroxypisiferanol (**5**), were isolated from *Perovskia atriplicifolia*, together with a new natural product (**6**) and two known compounds, przewalskin E (**7**) and brussonol (**8**). The structures of the new compounds were elucidated by detailed analyses of their MS, IR, 1D, and 2D NMR data. Compounds **1**–**8** were assayed for their inhibitory hepatitis B virus activities in the HepG 2.2.15 cell line. The results suggested that compounds **1** and **2** possessed noticeable anti-hepatitis B virus activity in *vitro*, suppressing the replication of hepatitis B virus DNA with selectivity index values of 154.3 and 137.7, respectively.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Perovskia atriplicifolia Benth., a perennial shrub belonging to the Lamiaceae family [1], is a folk medicine long been used as a parasiticide and analgesic in Tibet, China. Only a few investigations on this plant have been reported before [2,3]. During our search of anti-hepatitis B virus (HBV) active constituents from a natural source, the 90% ethanol extract of *P. atriplicifolia* was found to possess superior inhibitory HBV activity *in vitro*. A previous study on this plant had led to the isolation of ten compounds, of which perovskatone A was a novel C_{23} terpenoid [4]. As a further phytochemical investigation of this folk medicine, eight icetexane diterpenoids were obtained from the 90% ethanol extract. Based on MS, IR, 1D and 2D NMR data analyses, as well as comparison with the literature, the structures of compounds **1**–**8** were elucidated. Compounds **1**–**5** (**Fig. 1**) were new diterpenoids featuring a icetexane skeleton. Compound **6** [5,6] was a new natural product. Compounds przewalskin E (**7**) [7] and brussonol (**8**) [8] were isolated from this plant for the first time. All isolates were evaluated for their inhibitory HBV potency *in vitro*. Compounds **1** and **2** showed noticeable anti-HBV activity *in vitro* in the HepG 2.2.15 cell line, suppressing the replication of HBV DNA with selectivity index (SI) values of 154.3 and 137.7, respectively. Herein we described the isolation, structural elucidation, and anti-HBV activities of compounds **1**–**8**.

Results and Discussion

Compound **1** was obtained as a yellowish powder. It was assigned the molecular formula $C_{20}H_{26}O_4$ deduced by the positive HRESIMS at *m/z* 353.1721 (calcd. 353.1729 for $C_{20}H_{26}O_4Na^+$). The IR spectrum exhibited characteristic absorption bands due to the *ortho*-quinone carbonyl groups at 1723 and 1680 cm^{-1}. In the ^1H NMR (**Table 1**) spectrum, an olefinic proton signal at δ_H 6.73 (1H, s, H-14), an oxygenated methine at δ_H 4.63 (1H, dd, J = 5.6, 2.0 Hz, H-7), and two singlet methyls at δ_H 0.98, 0.90 (each 3H, Me-19, 18) were observed, together with one isopropyl group at δ_H 2.89 (1H, sept. J = 6.8 Hz), 1.14 (3H, d, J = 6.8 Hz), and 1.12 (3H, d, J = 7.2 Hz). In addition, a pair of characteristic proton signals with a large coupling constant assignable to H-20 appeared at δ_H 2.94 (1H, d, J = 18.4 Hz) and 2.05 (1H, d, J = 18.4 Hz), suggesting that compound **1** possessed an icetexane-type diterpenoid skeleton, with a typical 6/7 carbon ring (rings A and B) [9,10]. The ^{13}C NMR spectrum (**Table 2**) displayed 20 carbon resonances, of which two conjugated carbonyl signals at δ_C 181.8 (s, C-12), 181.0 (s, C-11) were presented. A comparison of the NMR data of compound **1**

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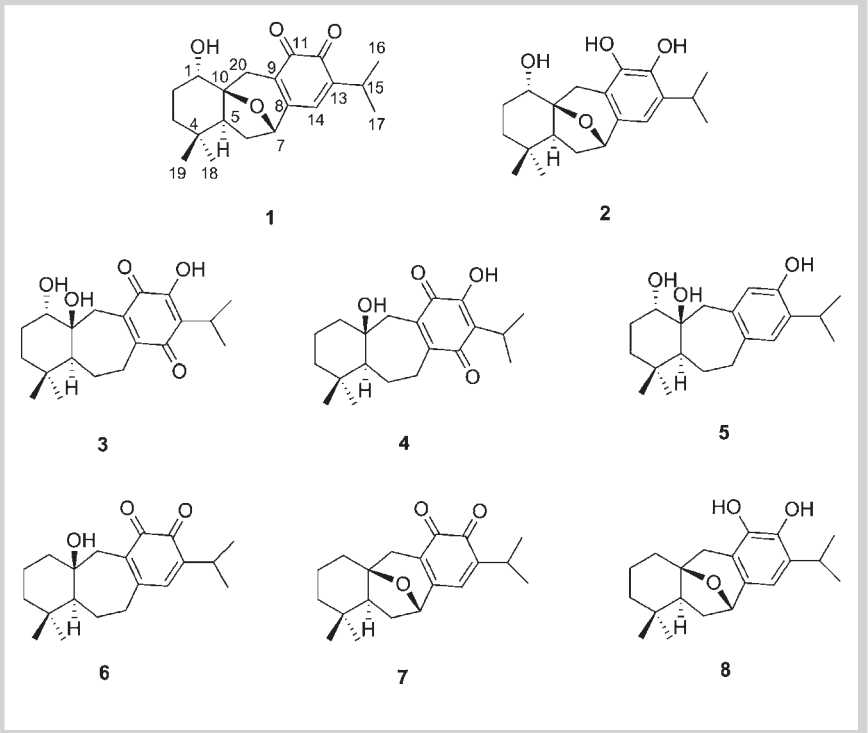

Fig. 1 Structures of compounds **1–8**.

Table 1 ^{1}H NMR (400 MHz, CD_3OD) data of compounds **1–6**. δ in ppm, J in Hz.

Pos.	1	2	3	4	5	6
1α	4.18 (1H, dd, 8.4,	4.17 (1H, dd, 7.6,	3.61 (1H, dd, 7.2,	1.77 (1H, m)	3.54 (1H, m)	1.75–1.83 (1H, m)
β	6.0)	6.0)	6.8)	1.50–1.57 (1H, m)		1.50–1.56 (1H, m)
2α	1.52–1.61 (1H,over-lapped)	1.58–1.65 (1H, m)	1.44–1.50 (1H, m)	1.82–1.86, (1H, over-lapped)	1.45–1.51 (1H, m)	1.82–1.88 (1H, m)
		2.02–2.05 (1H, m)	2.07–2.15 (1H, m)		2.11–2.18 (1H, m)	1.37–1.41(1H, over-lapped)
β	1.93–2.02 (1H,over-lapped)			1.36–1.41 (1H, over-lapped)		
3α	1.52–1.61 (2H,over-lapped)	1.46–1.55 (2H,m)	1.75–1.79 (1H, m)	1.36–1.41 (1H, over-lapped)	1.79 (1H, td, 14.0, 3.6)	1.37–1.41 (1H, over-lapped)
β			1.07–1.12 (1H, m)	1.29–1.31 (1H, m)	1.09 (1H, dt, 13.2, 3.2)	1.29–1.33 (1H, m)
5α	1.93–2.02 (1H, over-lapped)	1.92 (1H, m)	1.69 (1H, dd, 12.2, 2.6)	1.33 (1H, over-lapped)	1.68 (1H, dd, 12.4, 2.8)	1.33 (1H, over-lapped)
6α	2.10–2.13 (2H, m)	2.09–2.15 (1H, m)	1.79–1.83 (1H, m)	1.82–1.86 (1H, over-lapped)	1.93 (1H, m)	1.82–1.88 (1H, over-lapped)
					1.34 (1H, m)	
β		1.82–1.85 (1H, m)	1.28–1.34 (1H, m)	1.33 (1H, over-lapped)		1.33 (1H, over-lapped)
7α	4.63 (1H, dd, 5.6,	4.86 (1H, brd, 6.4)	3.30 (1H, m)	3.33–3.37 (1H, m)	2.68 (2H, m)	2.65 (1H, m)
β	2.0)		2.03 (1H, m)	2.01–2.07(1H, m)		2.54 (1H, m)
11	–	–	–	–	6.53 (1H, s)	–
14	6.73 (1H, s)	6.42 (1H, s)	–	–	6.85 (1H, s)	6.77 (1H, s)
15	2.89 (1H, sept, 6.8)	3.23 (1H, sept, 6.8)	3.22 (1H, sept. 7.2)	3.22 (1H, sept. 7.2)	3.21 (1H, sept. 7.2)	2.87 (1H, sep, 6.8)
16	1.14 (3H, d, 6.8)	1.18 (3H, d, 7.2)	1.22 (3H, d, 7.2)	1.21 (3H, d, 6.8)	1.18 (3H, d, 6.8)	1.13 (3H, d, 7.2)
17	1.12 (3H, d, 7.2)	1.18 (3H, d, 7.2)	1.21 (3H, d, 7.2)	1.21 (3H, d, 6.8)	1.18 (3H, d, 6.8)	1.12 (3H, d, 7.2)
18α	0.90 (3H, s)	0.87 (3H, s)	0.92 (3H, s)	0.91 (3H, s)	0.93 (3H, s)	0.92 (3H, s)
19β	0.98 (3H, s)	0.98 (3H, s)	0.90 (3H, s)	0.89 (3H, s)	0.88 (3H, s)	0.92 (3H, s)
20α	2.05 (1H, d, 18.4)	2.36 (1H, d, 16.8)	3.13 (1H, d, 14.4)	3.13 (1H, d, 14.4)	3.22 (1H, d, 14.4)	3.01 (1H, d, 14.4)
β	2.94 (1H, d, 18.4)	3.19 (1H, d, 16.8)	2.58 (1H, d, 14.4)	2.28 (1H, d, 14.4)	2.42 (1H, d, 14.4)	2.19 (1H, d, 14.4)

with those of przewalskin E (**7**) [7] showed a high similarity except that compound **1** contained one more oxygenated methine than przewalskin E (**7**). Considering that compound **1** had 16 mass units more than przewalskin E, the occurrence of one more hydroxyl in compound **1** was proposed. In the HMBC spectrum (**Fig. 2**), long-range correlations from H-1 (δ_H 4.18) to C-10 (δ_C 84.7)and from H-2 (δ_H 1.52–1.61, 1.93–2.02), H-5 (δ_H 1.93–2.02), and H-20 (δ_H 2.05, 2.94) to C-1 were exhibited, suggesting that the additional hydroxyl should be linked at C-1. The ^{1}H-^{1}H COSY (**Fig. 2**) correlation between H-1 and H-2 further supported the

Table 2 ^{13}C NMR (100 MHz) data of compounds **1**–**6** in CD_3OD.

	δ_C (mult.)					
Position	**1**	**2**	**3**	**4**	**5**	**6**
1	71.1 (d)	71.6 (d)	77.0 (d)	42.8 (t)	77.9 (d)	42.6 (t)
2	26.8 (t)	27.0 (t)	26.4 (t)	19.4 (t)	26.8 (t)	19.5 (t)
3	36.1 (t)	36.0 (t)	35.9 (t)	43.6 (t)	36.2 (t)	43.6 (t)
4	33.7 (s)	34.5 (s)	34.8 (s)	35.3 (s)	34.9 (s)	35.3 (s)
5	54.4 (d)	53.9 (d)	53.1 (d)	59.4 (d)	53.1 (s)	59.4 (d)
6	37.4 (t)	39.2 (t)	20.7 (t)	21.5 (t)	24.7 (t)	21.8 (t)
7	76.4 (d)	78.0 (d)	26.2 (t)	26.5 (t)	36.5 (t)	37.3 (d)
8	154.7 (s)	135.0 (s)	149.6 (s)	149.6 (s)	136.2 (s)	156.3 (s)
9	130.9 (s)	118.4 (s)	139.6 (s)	139.1 (s)	136.5 (s)	135.6 (s)
10	84.7 (s)	84.6 (s)	73.5 (s)	71.5 (s)	74.2 (s)	71.7 (s)
11	181.0 (s)	144.5 (s)	185.0 (s)	185.0 (s)	119.9 (d)	181.1 (s)
12	181.8 (s)	142.4 (s)	153.5 (s)	153.4 (s)	153.3 (s)	181.4 (s)
13	148.6 (s)	133.8 (s)	125.5 (s)	125.5 (s)	133.7 (s)	147.6 (s)
14	134.9 (d)	112.8 (d)	187.9 (s)	188.1 (s)	126.9 (d)	140.4 (d)
15	28.6 (d)	27.9 (d)	25.5 (d)	25.6 (d)	27.8 (d)	28.6 (d)
16	21.8 (q)	23.3 (q)	20.3 (q)	20.4 (q)	23.3 (q)	21.7 (q)
17	21.8 (q)	23.3 (q)	20.4 (q)	20.4 (q)	23.4 (q)	22.1 (q)
18	33.0 (q)	33.1 (q)	32.4 (q)	32.6 (q)	32.8 (q)	32.6 (q)
19	25.0 (q)	24.8 (q)	22.3 (q)	22.1 (q)	22.5 (q)	21.9 (q)
20	31.8 (t)	33.0 (t)	38.1 (t)	40.8 (t)	49.1 (t)	40.9 (t)

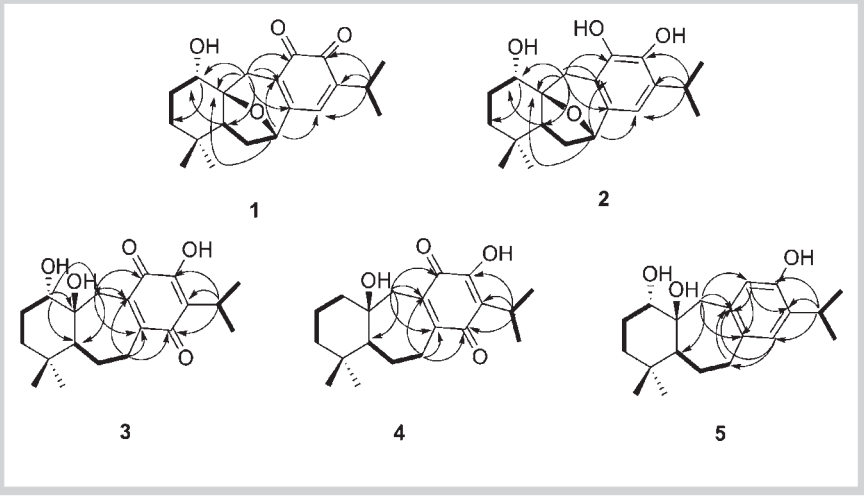

Fig. 2 Key HMBC (→) and ^1H-^1H COSY (—) correlations of compounds **1**–**5**.

above deduction. To establish the hydroxyl orientation at C-1, a ROESY experiment was conducted. The ROESY correlations H-1/H-19, H-1/H_β-2 demonstrated that OH-1 should be in the α-linkage. The other HSQC, HMBC, ^1H-^1H COSY (**Fig. 2**), and ROESY (**Fig. 3**) correlations allowed for the full assignments of proton and carbon signals. Consequently, the structure of compound **1** was determined as shown in **Fig. 1**, and the compound was named perovskatone B (**1**).

Compound **2** was obtained as a yellowish powder and had a molecular formula of $C_{20}H_{28}O_4$ deduced by the HRESIMS at *m/z* 355.1880 (calcd. 355.1885 for $C_{20}H_{28}O_4Na^+$). The characteristic H-20 signals at δ_H 3.19 (1H, d, J = 16.8 Hz), 2.36 (1H, d, J = 16.8 Hz) in ^1H NMR spectrum suggested that compound **2** was also an icetexane diterpenoid. Comparison of its ^{13}C NMR data (**Table 2**) with those of compound **1** demonstrated that both compounds had similar A and B rings. However, there was no carbonyl signal in the ^{13}C NMR spectrum of compound **2**. In addition, the carbon signals ascribable to ring C in compound **2** were identical to those of brussonol (**8**) [8], implying that both compounds had the same ring C. The above deduction was verified by the HMBC correlations (**Fig. 2**) from the oxygenated H-1 (δ_H 4.17) to C-2 (δ_C 27.0), C-3 (δ_C 36.0), C-5 (δ_C 53.9), C-10 (δ_C 84.6), and C-20 (δ_C 33.0), from H-20 to C-11, C-8, and from H-14 to C-9, C-12, C-15. The α-orientation of hydroxyl at C-1 was determined by the H-1/Me-19 correlation in the ROESY plot (**Fig. 3**). Accordingly, the structure of compound **2** was characterized as shown in **Fig. 1**, and the compound was named 1α-hydroxybrussonol (**2**).

Compound **3** was obtained as a yellow powder and assigned the molecular formula $C_{20}H_{28}O_5$, in agreement with the HRESIMS at *m/z* 371.1825 (calcd. 371.1834 for $C_{20}H_{28}O_5Na^+$). Compound **3** was deduced to have the icetexane diterpenoid skeleton by the typical H-20 signals at δ_H 3.13 (1H, d, J = 14.4 Hz), 2.58 (1H, d, J = 14.4 Hz) in the ^1H NMR spectrum (**Table 1**). The characteristic carbon shifts at δ_C 185.0 (C-11) and 187.9 (C-14) suggested that compound **3** contained a *para*-quinone ring C [11]. This was supported by the HMBC correlations from H-20 (δ_H 3.13, 2.58) to C-11 (δ_C 185.0), C-8 (δ_C 149.6), and C-9 (δ_C 139.6), as well as the correlations from H-7 (δ_H 3.30, 2.03) to C-14 (δ_C 187.9), C-8, and C-9. Additionally, the ^1H-^1H COSY correlation for H-1/H-2 (**Fig. 2**), together with the long-range HMBC correlations from

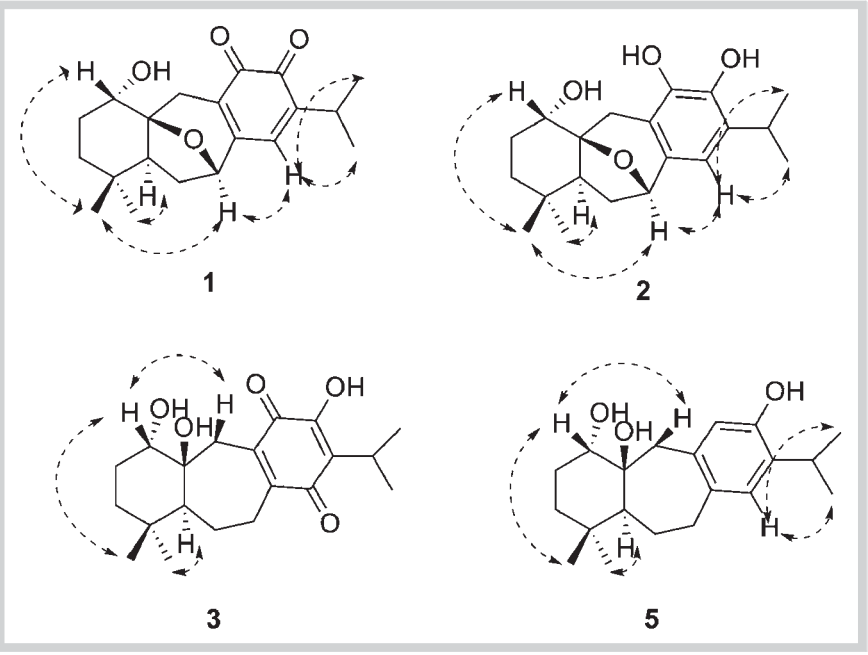

Fig. 3 Key ROESY (←→) correlations of compounds **1**–**3**, and **5**.

H-1 to C-5, C-10, C-20, from H-5 to C-1, C-10, C-20, and from H-6 to C-10 indicated the presence of hydroxyls at C-1 and C-10. Biogenetically, the configurations of C-5 (α-oriented H-5) and C-10 (β-oriented OH-10) in the icetexane diterpenoids from the *Perovskia* genus were 5*S* and 10*S*, which has been extensively discussed and authenticated in some documents [10, 12–15]. The α-orientation of H-5 was verified by the ROESY (**Fig. 3**) correlation of H-5/Me-18 (α-orientation with a carbon chemical shift larger than 30.0 ppm). The α-linked hydroxyl at C-1 could be established by the NOE correlation of H-1/Me-19 in the ROESY experiment (**Fig. 3**). Finally, the structure of compound **3** was determined as shown in **Fig. 1**, and the compound was named perovskatone C (**3**).

Compound **4** was obtained as a yellow powder. It had the molecular formula $C_{20}H_{28}O_4$ deduced by the HRESIMS at *m/z* 355.1877 $[M + Na]^+$ (calcd. for $C_{20}H_{28}O_4Na^+$: 355.1885). The 1D and 2D NMR data of compound **4** were essentially identical to those of compound **3**, implying that compounds **3** and **4** had a similar structure. Compound **4** differed from **3** mainly in ring A where there was no hydroxyl at C-1. This was ascertained by the ^1H-^1H COSY (**Fig. 2**) of H-1 (δ_H 1.77, 1.50–1.57)/H-2 (δ_H 1.82–1.86, 1.36–1.41) and HMBC correlations (**Fig. 2**) from H-1 to C-5, C-10, C-20. Consequently, the structure of compound **4** was elucidated as shown in **Fig. 1**, and the compound was named perovskatone D (**4**).

Compound **5** was obtained as a yellowish powder. It was assigned the molecular formula $C_{20}H_{30}O_3$ by the positive HRESIMS at *m/z* 341.2083 $[M + Na]^+$ (calcd. 341.2092 for $C_{20}H_{30}O_3Na^+$). Detailed analyses of the 1D and 2D NMR data revealed that compound **5** was structurally similar to 1β-hydroxypisiferanol [16]. However, compound **5** included an α-oriented hydroxyl at C-1, which was definitely established by the ROESY correlations for H-1/H-19 and H-1/$H_β$-20 (**Fig. 3**). The other ^1H-^1H COSY and HMBC correlations (**Fig. 2**) further confirmed this deduction. Lastly, compound **5** was deduced as 1α-hydroxypisiferanol (**5**).

Compound **6**, which had been previously synthesized by Moujir [5] and Majetich [6], was obtained as a new natural product in our experiment. The full assignments of proton and carbon signals (**Table 1** and **2**) were firstly performed based on the extensive analyses of 1D and 2D NMR data (see Supporting Information). The known compounds przewalskin E (**7**) and brussonol (**8**), whose structures were determined by comparing the NMR data with those in the literature [7, 8], were obtained from this plant for the first time.

All the isolates were tested for their anti-HBV activities in the HepG 2.2.15 cell line according to the method described in our previous report [17]. Results are summarized in **Table 3**. It was concluded that compounds **1, 2, 4**, and **8** possessed moderate anti-HBV activity *in vitro*, suppressing the secretion of the hepatitis B surface antigen (HBsAg) with SI values ranging from 2.06 to 4.83. Compound **2** could also inhibit the secretion of HBsAg with an SI value of 2.0. Compounds **5**–**7** showed no anti-HBV activity *in vitro*. In addition, compounds **1** and **2** exhibited superior inhibitory HBV DNA replication activity with SI values of 154.3 and 137.7, respectively.

In conclusion, eight icetexane diterpenoids were isolated from the ethanol extract of *P. atriplicifolia*, of which compounds **1**–**5** were new ones and compound **6** was a new natural product. An *in vitro* anti-HBV bioassay suggested that compounds **1** and **2** could moderately inhibit HBV DNA replication in HepG 2.2.15 cells. To the best of our knowledge, icetexane-type diterpenoids were mainly found from the plants of *Salvia* and *Chamaecyparis* genera [12]. Our research illustrated that icetexane diterpenoids were also abundant in the *Perovskia* genus, which could be helpful for exploiting a new use of this medicinal plant.

Material and Methods

General experimental procedures

Optical rotations were measured using a Horiba SEPA-300 high-sensitive polarimeter. UV spectra were obtained on a Shimadzu UV-2401A spectrophotometer. IR spectra were carried out on a Bio-Rad FTS-135 spectrometer with KBr pellets, *v* in cm^{-1}. 1D and 2D NMR spectra were recorded on a Bruker AV-400 (^1H/^{13}C, 400 MHz/100 MHz) spectrometer with tetramethylsilane (TMS)

Table 3 *In vitro* anti-hepatitis B virus activities of compounds **1**–**8**.

	HBsAg			HBeAg		Inhibiting HBV DNA replication	
Compounds	**CC_{50} (mM)[a]**	**IC_{50} (mM)[a]**	**SI[b]**	**IC_{50} (mM)**	**SI**	**IC_{50} (μM)**	**SI**
1	> 2.13	1.03	> 2.06	1.97	> 1.08	13.8	154.3
2	2.85	0.59	4.83	1.42	2.00	20.7	137.7
3	2.13	1.54	1.38	3.67	–	NO	–
4	2.78	0.92	3.02	4.01	–	NT	NT
5	1.85	2.45	–	3.82	–	NT	NT
6	> 2.13	4.08	–	3.68	–	NO	–
7	1.44	2.23	–	1.72	–	NO	–
8	> 3.54	1.39	> 2.55	4.72	–	NO	–
3TC[c]	29.96	23.50	1.27	28.19	1.06	1.12	26750.0

All values are the mean of two independent experiments; [a] IC_{50}: 50% inhibitory concentration; CC_{50}: 50% cytotoxic concentration; [b] SI = CC_{50}/IC_{50}; [c] 3TC: Lamivudine, positive control; NT: not been tested for their trace amount; NO: IC_{50} value was not obtained at the highest tested concentration

as the internal standard. Chemical shifts (δ) are expressed in ppm with TMS as the internal reference. HRESIMS was performed on a VG Autospec-3000 spectrometer. HPLC was performed on an Agilent 1260 liquid chromatograph equipped with a Venusil XBP C18 (10 × 250 mm, 5 μm) column. Column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Marine Chemical, Inc.), Lichroprep Rp-18 gel (40–63 μm, Merck), Sephadex LH-20 (Sigma-Aldrich Co.), or MCI gel (75–150 μm, Mitsubishi Chemical Corporation). Column fractions were monitored by TLC, and the spots were visualized by heating the plates after spraying with 10% H_2SO_4 in EtOH. The positive control lamivudine (3-TC, purity > 99%) was purchased from GlaxoSmithKline (Suzhou) Co., Ltd.

Plant material

The whole plant of *P. atriplicifolia* was collected in Tibet in September 2010, and identified as *P. atriplicifolia* Benth. by Prof. Dr. Li-Gong Lei from Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (TSYJ-201093) was deposited in the Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission & Ministry of Education, School of Chemistry and Biotechnology, Yunnan Minzu University.

Extraction and isolation

The air-dried whole *P. atriplicifolia* plants (9.0 kg) were powdered and extracted with 90% ethanol (54 L) under reflux three times, 2 h each time. After being concentrated *in vacuo*, the extract was suspended in water and successively partitioned with petroleum ether, chloroform, and n-BuOH to give petroleum ether (A), chloroform (B), n-BuOH (C), and aqueous (D) fractions. The petroleum ether (A) (260 g) extract was subjected to silica gel (2.0 kg, 200–300 mesh; 10 × 170 cm) chromatography column (CC) and eluted with gradient petroleum ether/acetone (100 : 0, 98 : 2, 95 : 5, 90 : 10, 80 : 20; v/v; each 5.0 L; 1000 mL/flask; flow rate: 5 mL/min) to afford six fractions (Frs.A.1–6). These fractions were subjected to CC on silica gel, MCI, Rp-18, Sephadex LH-20, or HPLC to afford compounds **1** (23 mg), **2** (44 mg), **3** (16 mg), **4** (4.8 mg), **5** (3.2 mg), **6** (40 mg), **7** (72 mg), and **8** (113 mg). All the isolates had a degree of purity greater than 93%, determined by HPLC.

Perovskatone B (**1**): Yellowish powder; $[\alpha]_D^{18.2}$ +24.0 (*c* 0.50, MeOH); UV (MeOH) λ max(log ε) 272 (1.59), 238 (1.45); IR (KBr) ν_{max} 3447, 2967, 1723, 1680, 1635, 1455, 1368, 1246, 1066, 999, 860 cm^{-1}; ^1H and ^{13}C NMR data, see **Tables 1** and **2**; ESIMS (neg.): *m/z* 329 $[M - H]^-$; ESIMS (pos.): *m/z* 353 $[M + Na]^+$; HRESIMS (pos.): *m/z* 353.1721 (calcd. for $C_{20}H_{26}O_4Na^+$: 353.1729).

1α-Hydroxybrussonol (**2**): Yellowish powder; $[\alpha]_D^{18.4}$ – 18.9 (*c* 0.10, MeOH); UV (MeOH) λ max(log ε) 275 (1.02), 231 (2.15); IR (KBr) ν_{max} 3545, 2967, 1643, 1635, 1596, 1501, 1453, 1370, 1266, 1128, 1066, 957, 803 cm^{-1}; ^1H and ^{13}C NMR data, see **Tables 1** and **2**; ESIMS (neg.): *m/z* 331 $[M - H]^-$; ESIMS (pos.): *m/z* 355 $[M + Na]^+$; HRESIMS (pos.): *m/z* 355.1880 $[M + Na]^+$ (calcd. for $C_{20}H_{28}O_4Na^+$: 355.1885).

Perovskatone C (**3**): Yellow powder; $[\alpha]_D^{18.0}$ + 15.8 (*c* 0.29, MeOH); UV (MeOH) λ max(log ε) 284 (3.09); IR (KBr) ν_{max} 3448, 2960, 1660, 1643, 1456, 1376, 1128, 1065 cm^{-1}; ^1H and ^{13}C NMR data, see **Tables 1** and **2**; ESIMS (neg.): *m/z* 347 $[M - H]^-$; ESIMS (pos.): *m/z* 371 $[M + Na]^+$; HRESIMS (pos.): *m/z* 371.1825 $[M + Na]^+$ (calcd. for $C_{20}H_{28}O_5Na^+$: 371.1834).

Perovskatone D (**4**): Yellow powder; $[\alpha]_D^{16.8}$ + 65.0 (*c* 0.60, MeOH); UV (MeOH) λ max(log ε) 284 (3.02); IR (KBr) ν_{max} 3448, 2961, 1660, 1641, 1450, 1384, 1126, 1070 cm^{-1}; ^1H and ^{13}C NMR data, see **Tables 1** and **2**; ESIMS (neg.): *m/z* 331 $[M - H]^-$; ESIMS (pos.): *m/z* 355 $[M + Na]^+$; HRESIMS (pos.): *m/z* 355.1877 $[M + Na]^+$ (calcd. for $C_{20}H_{28}O_4Na^+$: 355.1885).

1α-Hydroxypisiferanol (**5**): Yellowish powder; $[\alpha]_D^{17.9}$ + 115.0 (*c* 0.31, MeOH); UV (MeOH) λ max(log ε) 272 (1.13), 230 (1.98); IR (KBr) ν_{max} 3530, 2941, 1606, 1498, 1451, 1375, 1126, 1064 cm^{-1}; ^1H and ^{13}C NMR data, see **Tables 1** and **2**; ESIMS (neg.): *m/z* 317 $[M - H]^-$; ESIMS (pos.): *m/z* 341 $[M + Na]^+$; HRESIMS (pos.): *m/z* 341.2083 $[M + Na]^+$ (calcd. for $C_{20}H_{30}O_3Na^+$: 341.2092).

Anti-hepatitis B virus assays

The inhibitory potency for the secretion of HBsAg and hepatitis B envelope antigen (HBeAg) was conducted according to the method described in our previous report [17]. All the evaluated compounds were dissolved in DMSO (Gibco Invitrogen). The concentration of DMSO in the media was maintained at less than 2.5 μL/mL to ensure that it did not affect the growth of HepG 2.2.15 cells. HBV DNA extraction was also conducted in HepG 2.2.15 cells. Briefly, the HepG 2.2.15 cells were seeded in 24-well culture plates at a density of 5×10^5 cells/mL. After two days, the culture medium was replaced with fresh medium supplemented with (or without) the tested compounds; this was repeated every other day for an additional five days. Cells were collected, and total DNA was extracted with a commercial kit (Qiagen) following the manufacturer's instructions. The real-time PCR assay was used to detect the HBV DNA according to the literature reported [18]. An

antiviral agent, 3 TC [lamivudine, GlaxoSmithKline (Suzhou) Co., Ltd.], was used as a positive control.

Cytotoxicity assays

The toxicities of the compounds were assayed by a modified MTT method [19]. In brief, the test samples were prepared at different concentrations. After seeding HepG 2.2.15 cells in 96-well microplate for 4 h, the samples (20 µL) were placed in each well and incubated for three days at 37 °C, then 0.1 mL MTT (400 µg/mL) was added for 4 h. After removal of the MTT medium, DMSO (100 µL/well) was added to the microplate for 10 min. The formazan crystals were dissolved, and the absorbance was measured on a microplate reader at 490 nm.

Supporting information

The detailed isolation procedure and 1D and 2D NMR spectra of the new compounds **1**–**6** are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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