FULL PAPER

A Novel C_{22} Terpenoid from the Cultured *Perovskia atriplicifolia*

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A novel terpenoid, named perovskiaol (**1**), was isolated from the cultured *Perovskia atriplicifolia.* Its structure was elucidated by comprehensive spectroscopic analysis as well as by quantum chemical computation of electronic circular dichroism spectra. Perovskiaol (**1**) was a novel C_{22} terpenoid containing a unique *D*-ring simultaneously fused with rings *A*, *B*, and *C*, and encountered in nature for the first time. Cytotoxic bioassay suggested perovskiaol (**1**) possessed significant cytotoxic activity inhibiting NB4, A549, and HepG 2 cell lines with IC_{50} values of 2.35, 1.47, and 0.81 μM, respectively.

Keywords: *Perovskia atriplicifolia*, Terpenoids, Cytotoxic activities, Structure elucidation.

Introduction

The plant *Perovskia atriplicifolia*, a shrub about one and a half meter high, is mainly distributed in Iran, India, Pakistan, Afghanistan, Russia, and south-west of China [1]. Previous investigation suggested there were some bioactive and structural unique compounds in the plant of *Perovskia* genus [2][3]. Our group had also performed a systematic phytochemical study on the medicinal plant *P. atriplicifolia* [4 – 6]. Besides, we had reported the chemical constituents between the cultured *P. atriplicifolia* and wild *P. atriplicifolia* were quite different and two unprecedented terpenoids had been isolated from the cultured *P. atriplicifolia* [7]. To well understand the chemical difference between the cultured *P. atriplicifolia* and wild *P. atriplicifolia*, further investigation on the cultured *P. atriplicifolia* was conducted and a novel C_{22} terpenoid was obtained. Herein, we presented the extraction, isolation, and structure elucidation of compound **1**.

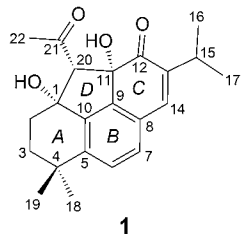

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Fig. 1. The structure of compound **1**.

Results and Discussion

Compound **1** (*Fig. 1*) was obtained as a yellowish gum, with an optical rotation value of + 0.23. The positive ESI-MS gave the quasimolecular ion peak at *m*/*z* 377 $[M + Na]^+$, in agreement with the molecular formula of $C_{22}H_{26}O_4$ revealed by the positive HR-ESI-MS at *m*/*z* 377.1738 $[M + Na]^+$ (calculated for $C_{22}H_{26}O_4Na^+$ 377.1728), indicating ten degrees of unsaturation. The IR spectrum displayed the absorptions of OH at 3449, C=O at 1743, 1695, and aromatic ring at 1603, 1498, 1454 cm^{-1}. The ^{1}H-NMR (*Table 1*) showed a pair of *ortho*-coupled aromatic H-atom signals at δ(H) 7.25 (1 H, *d*, *J* = 8.0 Hz), 7.15 (1 H, *d*, *J* = 8.0 Hz), besides an olefinic H-atom at δ(H) 6.93 (1 H, *d*, *J* = 1.2 Hz) and one isopropyl H-atom signals at δ(H) 2.95 (1 H, sept, *J* = 6.8 Hz), 1.15 (6 H, *d*, *J* = 6.8 Hz). The ^{13}C-NMR (DEPT) spectra (*Table 1*) exhibited 22 C-signals, involving ten quaternary carbons (including two carbonyl, five olefinic, and two oxygenated ones), five CH (including three olefinic ones), two CH_2, and five Me. Detailed analyses of the NMR data suggested compound **1** possessed a basic abietane-type diterpenoid skeleton with an aromatic ring *B*. In the HMBC spectrum (*Fig. 2*), the following

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Table 1. The ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) data of compound **1** in $CDCl_3$; δ in ppm, J in Hz

Position	δ(H)	δ(C)
1	–	79.3 (*s*)
2α	2.32 – 2.38 (*m*)	31.5 (*t*)
2β	1.82 – 1.84 (*m*)	
3α	2.40 – 2.42 (*m*)	35.4 (*t*)
3β	1.64 – 1.69 (*dt*, *J* = 14.0, 3.2)	
4	–	33.7 (*s*)
5	–	144.6 (*s*)
6	7.25 (*d*, *J* = 8.0)	128.1 (*d*)
7	7.15 (*d*, *J* = 8.0)	127.5 (*d*)
8	–	127.3 (*s*)
9	–	140.3 (*s*)
10	–	142.5 (*s*)
11	–	79.6 (*s*)
12	–	199.8 (*s*)
13	–	143.4 (*s*)
14	6.93 (*d*, *J* = 1.2)	132.6 (*d*)
15	2.95 (*sept*, *J* = 6.8)	27.4 (*d*)
16	1.15 (*d*, *J* = 6.8)	21.5 (*q*)
17	1.15 (*d*, *J* = 6.8)	21.9 (*q*)
18	1.42 (*s*)	30.3 (*q*)
19	1.12 (*s*)	30.9 (*q*)
20	3.10 (*s*)	64.5 (*d*)
21	–	210.4 (*s*)
22	2.44 (*s*)	31.9 (*q*)
HO–C(1)	3.34 (br. *s*)	–
HO–C(11)	6.01 (br. *s*)	–

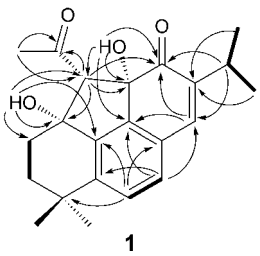

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Fig. 2. Key HMBC (→) and ^{1}H,^{1}H-COSY (▬) correlations of compound **1**.

following long-range correlations from the H-atoms to the C-atoms were observed: H–C(2) to C(1), C(3), C(4), and C(10); H–C(6) to C(4), C(8), and C(10); H–C(7) to C(5), C(9), and C(14); H–C(18), H–C(19), and H–C(3) to C(5). Simultaneously, the HMBCs (*Fig. 2*) between HO–C(1) (δ(H) 3.34) and C(1), C(2), C(10), C(20); HO–C(11) (δ(H) 6.01) and C(9), C(11), C(12), C(20) appeared, demonstrating the presence of HO at C(1) and C(11). In addition, the HMBCs (*Fig. 2*) from H–C(14) to C(9), C(8), C(7), C(12), as well as H–C(15) to C(12), C(13), C(14) were obviously presented, suggesting the existence of ring *C* (*Fig. 1*) in compound **1**. The aforementioned evidences, combined with the correlations H–C(2)/H–C(3), H–C(6)/H–C(7) in the ^{1}H,^{1}H-COSY spectrum (*Fig. 2*), suggested the existence of fragment **1a** (*Fig. 3*). The HMBC spectrum (*Fig. 2*) also exhibited the correlations from H–C(22) (δ(H) 2.44) to C(20), C(21); H–C(20) (δ(H) 3.10) to C(1), C(2), C(11), C(12), and C(21), suggesting there was one methylcarbonyl (CH_3C=O) located at C(20), and the C(20) was linked with C(1) and C(11). Finally, the planar structure of compound **1** was unambiguous identified as shown in *Fig. 1*.

The relative configuration of perovskiaol (**1**) was deduced by the ROESY spectrum analysis (*Fig. 4*). The cross peak between HO–C(1), HO–C(11), and Me–C(22), and the evidence that there was no NOE correlation between the hydroxy groups (HO–C(1), HO–C(11)) and H–C(20) in the ROESY plot suggested the HO–C(1), HO–C(11), and Me–C(22) should be in the same side and be antarafacial with H–C(20). The α-orientation of Me–C(18) and β-orientation of Me–C(19) were deduced by the obvious ROESY correlations of H_β–C(2)/Me–C(19), H_β–C(3)/Me–C(19), and H_α–C(2)/Me-C(18), H_α–C(3)/Me–C(18).

Unfortunately, compound **1** was isolated as a yellowish gum and could not be obtained as crystals in our experiment. In order to identify the absolute configuration of **1**, a comparison of time-dependent density functional theory (TDDFT) calculated electronic circular dichroism (ECD) spectra with experimental CD data was

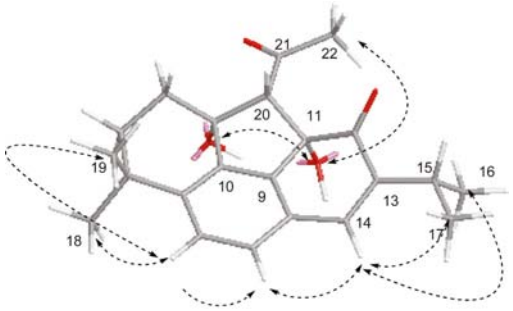

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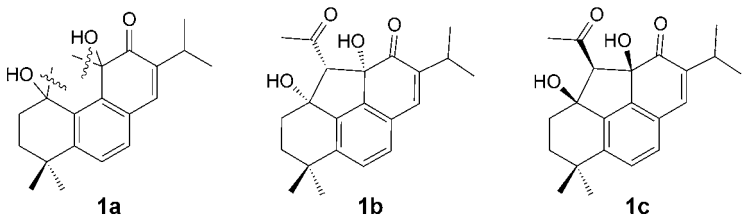
Fig. 4. Key ROESY (↔) correlations of compound **1**.



1a **1b** **1c**

Fig. 3. Fragment **1a** and probable structures of compound **1** (**1b**, **1c**).

applied. The experimentally recorded CD spectra of **1**, and the calculated CD spectra for the optimized geometries of α-oriented HO–C(1)/HO–C(11) (**1b**) (*Fig. 3*) and β-oriented HO–C(1)/HO–C(11) (**1c**) (*Fig. 3*) were shown in *Fig. 5*. Results suggested the calculated CD spectrum of α-oriented HO–C(1)/HO–C(11) (**1b**) (*Fig. 3*) was comparable with the experimental CD spectra, indicating that compound **1** included (1*R*)-, (11*S*)-, and (20*S*)-configuration. Finally, compound **1** was proposed the structure of **1b** (*Fig. 3*) and named as perovskiaol (**1**).

Despite the previous discussed samples [8][9] which were derived from the condensation of corresponding metabolites with acetoacetyl-CoA and had been verified to be nonartifact, a HPLC analysis of the raw 90% EtOH extract was still performed. With pure perovskiaol (**1**) as reference, the compound **1** was detected from the petroleum ether (PE) extraction (see Supporting information), giving a convincible evidence that compound **1** was nonartifact during our purification. To well explain the structure of compound **1**, a plausible biosynthesis pathway was proposed (*Scheme*). To the best of our knowledge, no other examples of acetoacetyl-CoA addition on compounds other than *ortho*-quinone were reported, and there were only three examples of acetoacetyl-CoA addition on compounds with *ortho*-quinone skeleton reported before [4][8][9]. Perovskiaol (**1**) could be considered as the first C_{22} terpenoid derived from two-step condensation reactions between 1-oxomiltirone [10] with acetoacetyl-CoA. Recently, *Jiang et al.* [11] had reported a pair of novel abietane-type diterpenoids, (±)-salviaprione, from *Salvia prionitis*. Although the (±)-salviaprione were structurally similar to compound **1**, perovskiaol (**1**) should be considered to be formed by a different biogenetic pathway.

Compound **1** was assayed for its anti-HBV activity in HepG 2.2.15 cell line and cytotoxicity in NB4, A549, HepG 2, PC3, and MCF7 cell lines. Results were summarized in *Tables 2* and *3*. It was concluded that compound **1** possessed slight anti-HBV activity, inhibiting the secretion of HBsAg and HBeAg with selectivity index (SI) values of 3.27 and 3.63, respectively. Interestingly, compound **1** showed remarkable cytotoxicity in NB4, A549, and Hep G 2 cells, with IC_{50} values of 2.35, 1.47, and 0.81 μM, respectively, but exhibited none activity in PC3 and MCF7 cell lines, suggesting further investigation on compound **1** was necessary.

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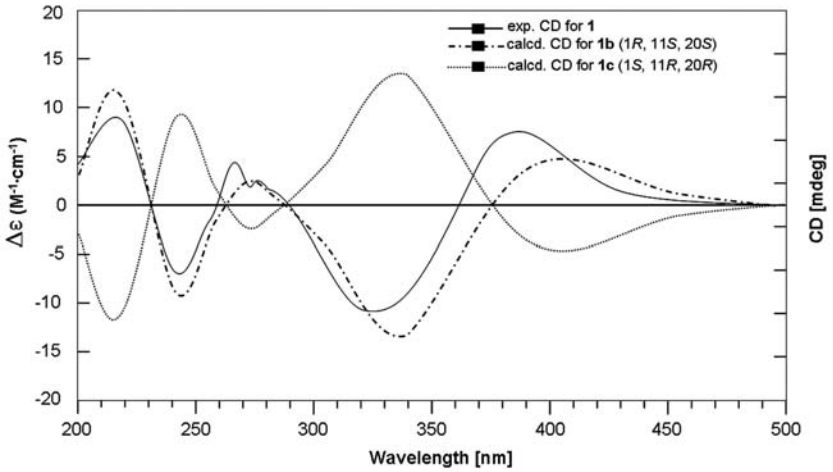

Fig. 5. Comparison of the experimental and calculated ECD spectra of **1** in MeOH at B3LYP/6-311++g(2d,p) level.

Scheme. Proposed biogenetic pathway of compound **1.**

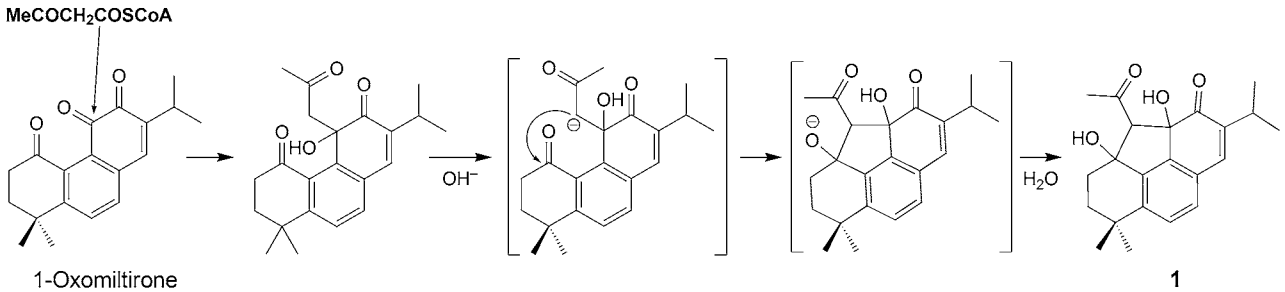

Table 2. *In vitro* anti-HBV activities of compound **1**[a])

Compounds	CC_{50} [mM][b])	HBsAg		HBeAg	
		IC_{50} [mM][b])	*SI*[c])	IC_{50} [mM]	*SI*
1	> 3.30	1.01	> 3.27	0.91	> 3.63
3TC[d])	29.96	23.50	1.27	28.19	1.06

[a]) All values are the mean of two independent experiments. [b]) IC_{50}: 50% Inhibitory concentration; CC_{50}: 50% cytotoxic concentration. [c]) $SI = CC_{50}/IC_{50}$. [d]) 3TC: Lamivudine, positive control.

Table 3. Cytotoxicity of compound **1** in the five tested cell lines[a])

Compounds	IC_{50} [μM]				
	NB4	A549	HepG 2	PC3	MCF7
1	2.35	1.47	0.81	> 10	> 10
Paclitaxel	0.03	0.02	0.20	0.20	0.12

[a]) All values are the mean of two independent experiments. Paclitaxel was used as the positive control.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary data (^{1}H- and ^{13}C-NMR, DEPT, HSQC, HMBC, ^{1}H,^{1}H-COSY, ROESY, HR-ESI-MS spectra, as well as the ECD method of compound **1**) associated with this article can be found at *Wiley Online*.

Experimental Part

General

Column chromatography (CC): Silica gel (SiO_2; 200 – 300 mesh; *Qingdao Meigao Chemical Company*, Qingdao, P. R. China); *Lichroprep Rp-18* gel (40 – 63 μm; *Merck*, Darmstadt, Germany); *MCI* gel (75 – 150 μm; *Mitsubishi Chemical Corporation*, Tokyo, Japan). HPLC: *Agilent 1260* liquid chromatograph (*Agilent,* Palo Alto, CA, USA) equipped with a *Venusil XBP C18* (10 × 250 mm, 5 μm; *Bonna-Agela Technologies Inc.*, Tianjin, China) column. Optical rotation: *Horiba SEPA-300* highsensitive polarimeter (*Horiba Seisakusho*, Tokyo, Japan). UV Spectra: *Shimadzu UV-2401A* spectrophotometer (*Shimadzu*, Tokyo, Japan). CD Spectra: *Jasco J-720* spectrometer (*Jasco, Inc.*, Tokyo, Japan). IR Spectra: *Bio-Rad FTS-135* spectrometer (*Bio-Rad*, Berkeley, CA, USA) with KBr pellet, $\tilde{\nu}$ in cm^{-1}. 1D- and 2D-NMR spectra: *Bruker AV-400* (^{1}H/^{13}C, 400 MHz/100 MHz) spectrometer (*Bruker Corp.*, Zurich, Switzerland) with tetramethylsilane (TMS) as internal standard; chemical shifts (δ) were expressed in ppm. HR-ESI-MS: *VG Autospec-3000* spectrometer.

Plant Material

The whole plant of the cultured *P. atriplicifolia* was purchased from *Sichuan Luxi Flower Gardening Co., Ltd.* (Chengdu, P. R. China) in May 2013, and was identified as *P. atriplicifolia* Benth. by Prof. Dr. *Li-Gong Lei* from Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (TSYJ-201358) was deposited with the Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission & Ministry of Education, School of Ethnomedicine & Ethnopharmcy, Yunnan Minzu University.

Extraction and Isolation

The air-dried whole cultured *P. atriplicifolia* (4.0 kg) were powdered and extracted with 90% EtOH under reflux for three times, 2 h each time. After concentrated *in vacuo*, the extract was suspended in H_2O, and successively partitioned with PE, $CHCl_3$, and BuOH to give PE (*A*), $CHCl_3$ (*B*), BuOH (*C*), and aq. (*D*) fractions. By HPLC analysis, the extract of the cultured *P. atriplicifolia* in Sichuan province and wild *P. atriplicifolia* collected in Tibet, the chemical constituents in both PE (*A*) extract showed some different. The PE (*A*) (103 g) extract was then subjected to SiO_2 chromatography column (CC) and eluted with gradient PE/acetone (100:0, 98:2, 95:5, 90:10, 80:20) to afford six fractions (*Frs. A.1 – A.6*). The *Fr. A.4* (2.3 g, eluted by PE/acetone 80:20) was separated on a SiO_2 CC, eluting with gradient PE/AcOEt (90:10, 80:20, 70:30) to provide three fractions (*Frs. A.4.1 – A.4.3*). The *Fr. A.4.2* (1.56 g) was decolored on a *MCI* CC (MeOH/H_2O 40:60, 60:40, 80:20, 90:10, 100:0) and further purified on HPLC (MeCN/H_2O 40:60) to give compound **1** (16 mg).

Perovskiaol (= **(3a*S*,4*S*,4a*R*)-4-Acetyl-3a,4a-dihydroxy-7,7-dimethyl-2-(propan-2-yl)-3a,4,4a,5,6,7-hexahydro-3*H*-cyclopenta[*def*]phenanthren-3-one**; **1**). Yellowish gum. $[\alpha]_D^{13.3}$ = + 0.23 (*c* = 0.29, MeOH/$CHCl_3$ 1:1). CD (*c* = 0.17 mg/ml, MeOH, 20.1 °C): $\lambda(\Delta\varepsilon)$ = 218 (+ 8.7), 264 (+ 4.6), 324 (– 10.3), 382 (+ 6.7). UV (MeOH): 248 (3.49), 366 (2.35), 434 (2.23). IR (KBr): 3449, 1743, 1695, 1603, 1498, 1454, 1260, 1128, 1069, 993, 865. ^{1}H- (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$): see *Table 1*. ESI-MS (pos.): 377 ($[M+Na]^+$). HR-ESI-MS (pos.): 377.1738 ($[M+Na]^+$, $C_{22}H_{26}NaO_4^+$; calc. 377.1723).

Computational Methods

The geometries of **1b** and **1c** were fully optimized at the B3LYP/6-311++g(d,p) level in the gas phase; they were further checked by frequency calculation, which resulted in all positive frequencies. ECD calculations were performed with the TDDFT calculations at TD-DFT-B3LYP/6-311++g(2d,p) level based on B3LYP/6-311++g (2d,p) optimized geometries, and ECD spectra were then simulated using SpecDis software [12 – 14]. The final

ECD spectrum was obtained based on *Boltzmann* weighting of each conformer. All calculations were performed by *Gaussian* 03 programs [15].

Anti-HBV Assay

The inhibitory potency for the secretion of HBsAg and HBeAg was conducted according to the method as our previous report [16]. An antiviral agent, 3TC (lamivudine; *GlaxoSmithKline, Suzhou Co., Ltd.*, Suzhou, P. R. China) was used as a positive control.

Cytotoxicity Assay

Cytotoxic activities were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method in NB4, A549, HepG 2, PC3, and MCF7 cell lines. Briefly, the cell suspensions (200 ml) at a density of 5×10^4 cells/ml were plated in 96-well microtiter plates and incubated for 24 h at 37 °C in a humidified incubator at 5% CO_2. The tested compound (2 ml in DMSO) soln. at different concentrations was added to each well and further incubated for 72 h under the same conditions. Then, 20 ml of the MTT soln. was added to each well and incubated for 4 h. The old medium (150 ml) containing MTT was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a *Spectra Max Plus* plate reader (*Molecular Devices*, Sunnyvale, CA, USA) at 540 nm. Dose–response curves were generated and the IC_{50} values were defined as the concentration of compound required to inhibit cell proliferation by 50%. Paclitaxel (purity > 98%, purchased from *Sigma–Aldrich Trading Co, Ltd*., Shanghai, China), an approved agent for the treatment of many tumors, was used as the positive control.

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