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RESEARCH ARTICLE

Two new tirucallane triterpenoids from the leaves of *Aquilaria sinensis*

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Abstract Two new tirucallane triterpenoids, aquilacallanes A–B (**1–2**), together with 15 known compounds (**3–17**) were isolated from the leaves of *Aquilaria sinensis*. The structures of these new compounds were elucidated on the basis of extensive spectroscopic analyses. All compounds were evaluated for their cytotoxic activity against five human cancer cell lines. The known compounds, ursolic acid (**7**) and 5,7,4′-trimethoxyflavone (**14**), exhibited weak cytotoxic activity against some cells.

Keywords *Aquilaria sinensis* · Tirucallane triterpenoids · Aquilacallanes A–B · Cytotoxic activity

Introduction

Aquilaria sinensis (Lour) Gilg (Thymelaeaceae), a principal source of the expensive eaglewood, is distributed in the south China such as Hainan, Guangxi, Guangdong, Fujian, and Taiwan provinces. The resin of *A. sinensis* have been used as a traditional sedative, analgesic, and digestive medicine in East Asia (Feng et al. 2011), while the leaves of it is used in China for treatments of inflammation and anaphylaxis (Qi et al. 2009). Unlike the sesquiterpenes and 2-(2-phenylethyl) chromone derivatives isolated from eaglewood of *A. sinensis* (Dai et al. 2010; Gao et al. 2012; Hashimoto et al. 1985; Jain and Bhattacharyya 1959; Maheshwari et al. 1963a, b; Nakanishi et al. 1981, 1984; Shimada et al. 1982; Varma et al. 1965; Yagura et al. 2005; Yang et al. 2012), flavonoid, benzophenone glycoside, and triterpenoids were the main compounds from leaves of *A. sinensis* (Feng et al. 2011; Nie et al. 2009; Qi et al. 2009; Wang et al. 2008). In order to find structurally unique and bioactive natural products, an investigation of the ethanol extract of the leaves of *A. sinensis* was carried out, which led to the isolation of two new tirucallane triterpenoids, aquilacallanes A–B (**1–2**), together with 15 known compounds (**3–17**). In the present study, we describe the isolation, structural elucidation, and cytotoxicity of these compounds.

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Materials and methods

General

Optical rotations were measured with a *JASCO P-1020* polarimeter. UV spectra were recorded with a Shimadzu UV-2401A spectrophotometer. IR spectra were recorded on *Tensor 27* spectrometer with a KBr disk. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer and 2D NMR spectra were recorded on a Bruker Advance III 600 spectrometer. Chemical shifts were reported using TMS as the internal standard. EI-MS and HR-EI-MS spectra were measured with a Waters AutoSpec Premier P776 instrument. Column chromatography (CC) was performed on silica gel (90–200 μm; Qingdao Marine Chemical Inc.), Sephadex LH-20 (40–70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden), and MPLC was performed on a Lisui EZ Purify III System packed

with RP-18 silica gel (40–63 μm, Merck, 71 Darmstadt, Germany) columns. Precoated silica gel GF_{254} and HF_{254} plates (Qingdao Haiyang Chemical Plant, Qingdao, People's Republic of China) were used for thin-layer chromatography (TLC). Preparative and semipreparative HPLC was performed on Shimadzu LC-8A equipped with a Shimadzu PRC-ODS (K) column and Agilent 1100 apparatus equipped with a Zorbax SB-C-18 75 (Agilent, 9.4 mm × 25 cm) column, respectively. Fractions were monitored by TLC and spots were visualized by heating TLC sprayed with 10 % H_2SO_4.

Plant material

The leaves of *A. sinensis* were collected from Xishuangbanna, Yunnan Province, People's Republic of China in July, 2009. The plant was identified by Prof. Xiao Cheng (Kunming Institute of Botany, Chinese Academy of Sciences). And its voucher specimen (200907M) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, CAS.

Extraction and isolation

The air-dried powder of the plant material (13.0 kg) was extracted three times with 95 % EtOH (50 L × 3, 48 h/time) at room temperature to give a crude extract, which was suspended in water and then extracted with EtOAc (5 L × 3). The EtOAc extract (260.0 g) was decolorized over *MCI* gel (eluted with 90 % MeOH) and then was separated on a silica gel column (100–200 mesh, 10 × 100 cm, 1.5 kg) eluted with petroleum ether (PE)—Me_2CO (100:0→0:100, each 20 L) to give five fractions A–E. Fraction B (35 g) was separated into four subfractions, B1–B4, by MPLC with MeOH/H_2O (60–100 %) as the eluent. Subfraction B1 was subjected to silica gel CC (PE/Me_2CO 95:5), followed by *Sephadex LH-20* ($CHCl_3$/MeOH 1:1) to yield compound **6** (40 mg). Subfraction B2 was subjected to a silica gel CC eluted with $CHCl_3$–MeOH (95:5), then was subjected to *Sephadex LH-20* ($CHCl_3$/MeOH 1:1), and finally purified by preparative HPLC (85 % MeOH in H_2O) to obtain compounds **8** (200 mg), **9** (160 mg) and **10** (75 mg). After the purification of subfraction B3 with *Sephadex LH-20* ($CHCl_3$/MeOH 1:1) and preparative HPLC (90 % MeOH in H_2O), compounds **11** (60 mg) and **12** (66 mg) were isolated. Fraction C (45 g) was subjected to CC (SiO_2, PE/Me_2CO, 10:0→0:10) to provide five subfractions, C1–C5. Repeated crystallization of subfraction C1 from the solvent Me_2CO and gave compound **3** (20 mg). Subfraction C2 was first subjected to CC (SiO_2, $CHCl_3$/MeOH 95:5) and then to semipreparative HPLC (90 % MeOH in H_2O) to give compounds **1** (4 mg), **2** (10 mg), **4** (3 mg). Repeated chromatography of subfraction C3 with CC (SiO_2, $CHCl_3$/MeOH 9:1) yielded compounds **7** (200 mg), **13** (22 mg), **14** (73 mg) and **15** (8 mg). From subfraction C4, compound **5** (30 mg) was obtained after purified by CC (SiO_2, $CHCl_3$/MeOH 8:2) and semipreparative HPLC (35 % MeOH in H_2O). Fraction D (32 g) was divided into four subfractions, D1–D4, by MPLC with MeOH/H_2O (20–100 %) as the eluent. Subfraction D3 was first subjected to CC (SiO_2, $CHCl_3$/MeOH 7:3) and then to *Sephadex LH-20* ($CHCl_3$/MeOH 1:1) to yield compounds **16** (100 mg), **17** (230 mg).

Aquilacallane A (=24-methylenetirucall-7(8)-en-3β,25-diol) (**1**): white amorphous solid; UV ($CHCl_3$) λ_{max} (log ε): 245 (3.51) nm; $[\alpha]_D^{25.4}$ −60.4 (*c* 0.13, $CHCl_3$); IR (KBr) ν_{max} 3420, 2928, 1643, 1374, 1185, 1033 cm^{-1}; ^1H NMR and ^{13}C NMR data see Table 1; positive-ion EI-MS, *m/z* 456, HR-EI-MS, *m/z* 456.3968, calcd for $C_{31}H_{52}O_2$, 456.3967).

Aquilacallane B (=24-methylene-25-methyltirucall-8(9)-en-3β-ol-7,11-dione) (**2**): white amorphous solid; UV (MeOH) λ_{max} (log ε): 272 (3.68), 203 (3.63) nm; $[\alpha]_D^{23.2}$ −17.5 (*c* 0.34, MeOH); IR (KBr): ν_{max} 3431, 2928, 1726, 1668, 1631, 1464, 1411, 1378 cm^{-1}; ^1H NMR and ^{13}C NMR data see Table 1; positive-ion EI-MS *m/z* 482, HR-EI-MS, *m/z* 482.3762, (calcd for $C_{32}H_{50}O_3$, 482.3760).

Cytotoxicity assay

The cytotoxicity of all the compounds against HL-60, SMMC-7721, A-549, MCF-7 and SW-480 cell lines was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) method (Mosmann 1983). Cells were plated in 96-well plates 12 h before treatment and continuously exposed to different concentrations of compounds. After 48 h, 20 μL of MTT solution was added to each well, which was incubated for a further 4 h. Then 20 % SDS (100 μL) was added to each well. After 12 h at room temperature, the OD value of each well was recorded at 595 nm. The IC_{50} value of each compound was calculated by the Reed and Muench method. Cisplatin was induced as a positive control.

Results and discussion

Aquilacallane A (**1**), a white, amorphous solid, showed IR absorptions for hydroxy (3420 cm^{-1}) and double-bond (1643 cm^{-1}) functional groups. The HREIMS exhibited a molecular ion peak at *m/z* 456.3968 $[M]^+$ indicated that **1** had a molecular formula of $C_{31}H_{52}O_2$ (calcd for $C_{31}H_{52}O_2$

Table 1 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **1** and **2** in $CDCl_3$

Pos.	**1**		Pos.	**2**	
	δ_H (J in Hz)	δ_C mult.		δ_H (J in Hz)	δ_C mult.
1a	1.79 (m)	33.7 (t)	1a	2.53 (overlap)	33.8 (t)
1b	1.46 (m)		1b	1.14 (m)	
2a	1.67 (m)	27.6 (t)	2a	1.76 (overlap)	27.3 (t)
2b	1.62 (m)		2b	1.76 (overlap)	
3	3.25 (dd, 11.2, 4.2)	79.2 (d)	3	3.33 (dd, 10.0, 6.1)	77.8 (d)
4		38.9 (s)	4		38.5 (s)
5	1.31 (m)	50.6 (d)	5	1.66 (m)	48.4 (d)
6a	2.14 (m)	23.9 (t)	6a	2.53 (overlap)	35.7 (t)
6b	1.96 (m)		6b	1.63 (m)	
7	5.26 (m)	117.8 (d)	7		199.9 (s)
8		145.8 (s)	8		149.7 (s)
9	2.20 (m)	48.9 (d)	9		154.8 (s)
10		34.9 (s)	10		38.5 (s)
11a	1.52 (overlap)	18.1 (t)	11		202.0 (s)
11b	1.52 (overlap)		12a	2.68 (d, 19.6)	51.4 (t)
12a	1.63 (overlap)	34.0 (t)	12b	2.49 (d, 19.6)	
12b	1.49 (m)		13		45.1 (s)
13		43.5 (s)	14		47.8 (s)
14		34.9 (s)	15a	1.64 (overlap)	35.9 (t)
15a	1.68 (m)	37.1 (t)	15b	1.21 (m)	
15b	1.13 (m)		16a	2.51 (m)	28.0 (t)
16a	2.19 (m)	28.2 (t)	16b	1.64 (overlap)	
16b	1.96 (overlap)		17	1.68 (overlap)	49.2 (d)
17	1.52 (overlap)	52.9 (d)	18	1.27 (s)	29.7 (q)
18	0.82 (s)	21.9 (q)	19	1.33 (s)	17.7 (q)
19	0.75 (s)	13.1 (q)	20	1.45 (overlap)	36.4 (d)
20	1.42 (m)	36.4 (d)	21	0.94 (d, 6.0)	18.4 (q)
21	0.91 (d, 6.4)	18.5 (q)	22a	2.18 (m)	31.8 (t)
22a	1.63 (overlap)	35.5 (t)	22b	1.68 (overlap)	
22b	1.19 (m)		23a	2.13 (m)	27.9 (t)
23a	2.18 (m)	28.0 (t)	23b	1.91 (m)	
23b	1.94 (m)		24		158.5 (s)
24		156.7 (s)	25		36.4 (s)
25		73.6 (s)	26	1.08 (s)	29.3 (q)
26	1.35 (s)	29.3 (q)	27	1.08 (s)	29.3 (q)
27	1.35 (s)	29.3 (q)	28	1.08 (s)	29.3 (q)
28a	5.10 (s)	106.6 (t)	29a	4.87 (s)	105.9 (t)
28b	4.77 (s)		29b	4.68 (s)	
29	0.97 (s)	27.2 (q)	30	1.05 (s)	27.5 (q)
30	0.86 (s)	14.7 (q)	31	0.92 (s)	15.0 (q)
31	0.98 (s)	27.6 (q)	32	1.11 (s)	23.9 (q)

m/z 456.3967), corresponding to 6° of unsaturation. Its ^{1}H-NMR spectrum (Table 1) displayed the presence of one multiplet olefinic proton at δ 5.26, two singlet protons at 5.10 and 4.77, and eight methyl groups at 1.35 (s, 6H), 0.98 (s), 0.97(s), 0.91 (d, $J = 6.4$ Hz), 0.86 (s), 0.82 (s), and 0.75 (s). The ^{13}C-NMR spectrum exhibited 31 carbon resonances due to eight methyls, ten methylenes (one was olefinic carbon), six methines (including an oxymethine at δ 79.2 and one olefinic methine at δ 117.8), and seven quaternary carbons (including one oxygenated at δ 73.6 and two olefinic ones at δ 145.8 and 156.7). The ^{1}H–^{1}H COSY correlations revealed the presence of four fragments

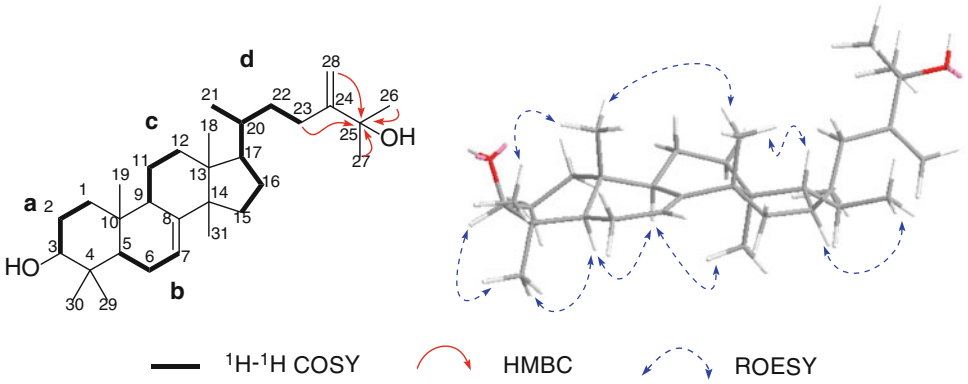

Fig. 1 Key 2D NMR correlations of compound **1**

as show in Fig. 1. The above data suggested **1** were quite similar to that of wallenol (Rios 2002), a known tirucallane triterpenoid. The obvious differences were that **1** have one more quaternary at δ_C 73.6 (s, C-25) and one less methyl group at δ_C 29.2 (one methyl of the t-butyl group) compared with that of wallenol, which indicated the replacement of the methyl belongs to the t-butyl group in wallenol by a hydroxy moiety in **1**. This conclusion can be verified by the HMBC correlations (Fig. 1) from δ 5.10 (s, H-28a), 4.77 (s, H-28b), 2.18 (m, H-23a), 1.94 (H-23b), and 1.35 (s, 6H, Me-26 and Me-27) to δ 73.6 (s, C-25). The relative configuration of **1** was established by the ROESY spectrum. The presence of ROESY correlations of H-3/Me-29, H-5/Me-29, H-5/H-9, and H-9/Me-18 indicated were α-oriented, while the correlations Me-30/Me-19, Me-19/Me-31, and Me-31/H-17 indicated H-17, Me-30 and Me-31 were β-oriented. Meanwhile, the ROESY correlation of Me-21/H-16 in combination with the chemical shift of Me-21 (δ 0.91, d, $J = 6.4$ Hz) suggested compound **1** should be a tirucallane triterpenoid (Arai et al. 1989; Wang et al. 2003), which was further confirmed by the negative optical rotation of **1** (−60.4°) (Mishra et al. 2000). Hence, the structure of **1** was established as 24-methylenetirucall-7(8)-en-3β,25-diol.

Aquilacallane B (**2**) was isolated as a white, amorphous, optical solid (−17.5°). The molecular formula, $C_{32}H_{50}O_3$, was established by the HR-EI-MS (*m/z*: found 482.3762, calcd. for $C_{32}H_{50}O_3$ 482.3760), indicating 8° of unsaturation. Its IR absorption bands showed the presence of hydroxy (3431 cm^{-1}) and α,β-unsaturated carbonyl (1,726 and 1,668 cm^{-1}) groups. Two singlet methylene protons at 4.87 and 4.68, a secondary methyl at 0.94 ($J = 4.5$ Hz, Me-21), and eight tertiary methyl groups at 1.33 (s), 1.27 (s), 1.11 (s), 1.08 (s, 9H), 1.05 (s), and 0.92 (s), were distinctively shown in the ^{1}H-NMR spectrum (Table 1). The ^{13}C- NMR and DEPT spectroscopic data of **2** (Table 1) showed 32 carbon signals consisting of nine methyls, nine methylenes (one sp^2 signal at δ 105.9), four methines (including an oxymethine at δ 77.8), ten quaternary carbons [including three olefinic ones (δ 149.7, 154.8, 158.5) and two carbonyl carbons (δ 199.9, 202.0)]. These data helped to classify **2** as a tirucallane triterpenoid containing a terminal double-bond and a t-butyl group unit, similar to compound **3** (24-methylene-25-methyltirucall -7-en-3-one) (Schun et al. 1986). The most striking differences were that **2** have one hydroxy unit and two carbonyl groups compared with that of **3**. The hydroxyl unit was attached to C-3 as deduced from the HMBC correlations of δ 3.33 (dd, $J = 10.6$, 6.1 Hz, H-3) with δ 33.8 (t, C-1), 25.5 (q, C-30), 15.0 (q, C-31). The two carbonyl groups was connected to C-7 and C-11 as inferred from the HMBC correlations of δ 2.53 (overlap, H-6a), 1.66 (m, H-5), and 1.63 (m, H-6b) with δ 199.9 (s, C-7) and of δ 2.68 (d, $J = 19.6$ Hz, H-12a) and 2.49 (d, $J = 19.6$ Hz, H-12b) with δ 202.0 (s, C-11). Furthermore, the HMBC correlation from δ 1.64 (overlap, H-15a), 1.21 (m, H-15b), and 1.11 (s, Me-32) to δ 149.7 (s, C-9) and from δ 2.53 (overlap, H-1a), 1.33 (s, Me-19), and 1.14 (m, H-1b) to δ 154.8 (s, C-9)

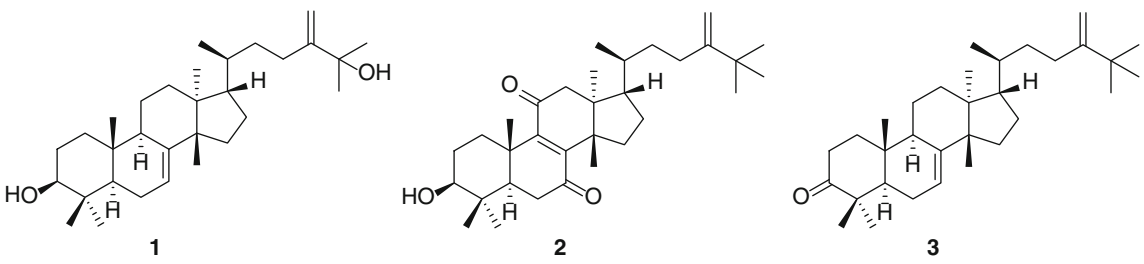

Fig. 2 Structures of compounds **1**–**3**

Table 2 Cytotoxicity of compounds **7** and **14** against tumor cell lines with IC50 (μM)

Compound	HL-60	SMMC-7721	A-549	MCF-7	SW-480
7	14.63	19.78	20.29	>40	21.36
14	25.34	>40	20.33	>40	>40
Cisplatin[a]	3.29	9.62	9.98	15.92	14.43

[a] positive control

suggested the double bond in **2** was located between C-8 and C-9. The other ^{1}H–^{1}H COSY and HMBC correlations supported the planar structure of **2** as shown in Fig. 2. In the ROESY spectrum, the correlations of H-3/H-5 and H-3/Me-30 indicated the β-orientation of 3-OH, that was in good agreement with the coupling constants of H-3. The correlations of Me-19/Me-31, Me-32/H-12a, Me-32/H-17, Me-18/H-20, and Me-21/H-16a as well as the specific rotation between **2** (−17.5°) and **1** (−60.4°) proposed that they have the same stereochemistry. Therefore, the structure of **2** was determined as 24-methylene-25-methyltirucall-8-en-3β-ol-7,11-dione.

The structures of other known compounds (**3**–**17**) were identified to be 24-methylene-25-methyltirucall -7-en-3-one (**3**) (Schun et al. 1986), 11-oxo-β-amyrin (**4**) (Seki et al. 2008), hederagenin (**5**) (Kizu and Tomimori 1982), 3β-acetoxyfriedelane (**6**) (Zhuo et al. 2008), ursolic acid (**7**) (Tundis et al. 2002), luteolin-7,3′,4′-trimethyl (**8**) (Wang et al. 2008), 5-hydroxy-7,4′-dimethoxyflavone (**9**) (Wang et al. 2008), genkwanin (**10**) (Wang et al. 2008), 5,7-dihydroxy-4′-methoxyflavone (**11**) (Yim et al. 2003), luteolin -7,4′-dimethyl (**12**) (Wang et al. 2008), 5,7,3′,4′-tetramethoxy -flavone (**13**) (Sutthanut et al. 2007), 5,7,4′-trimethoxyflavone (**14**) (Sutthanut et al. 2007), 5-hydroxy-3,4′,6,7-tetramethoxyflavone (**15**) (Gadgoli and Mishra 2007), benzophenone C-glycoside (**16**) (Severi et al. 2009), iriflophenone 2-O-α-L-rhamnopyranoside (**17**) (Feng et al. 2011), respectively, by comparison of their spectroscopic data with those reported in the literature.

All the compounds were evaluated for cytotoxicity against human myeloid leukemia (HL-60), hepatocellular carcinoma (SMMC-7721), pancreatic cancer (PANC-1), lung cancer (A-549), and colon cancer (SW-480) cell lines using the MTT method as reported previously [18], with DDP as positive control. The known compound ursolic acid (**7**) exhibited weak toxicity effects against HL-60, SMMC-7721, A-549, and SW-480 cells, with IC_{50} values of 14.63, 19.78, 20.29, and 21.36 μM, respectively, while 5,7,4′-trimethoxyflavone (**14**) showed weak cytotoxicity against HL-60 and A-549 cell lines, with IC_{50} values of 25.34 and 20.33 μM, respectively (Table 2).

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