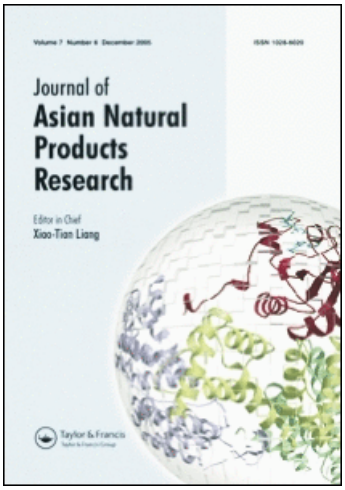

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CYTOTOXIC *ENT*-KAURANOIDS FROM *ISODON LEUCOPHYLLUS*

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Two new compounds, baiyecrystals D and E (**1**, **2**), together with eight known analogues, xerophilusin B (**4**), macrocalin B (**5**), oridonin (**6**), rosthorin A (**7**), lasiocarpanin (**8**), rabdoternin A (**9**) and phyllostachysin A (**10**) and B (**11**), were isolated from the aerial parts of *Isodon leucophyllus*. The structures of **1** and **2** and **4**–**11** were elucidated on the basis of spectroscopic methods, especially the 2D NMR spectral analysis. Compounds **2**, **6**–**8** and **10** were evaluated for their antineoplastic activities *in vitro*. Among them, lasiocarpanin (**8**) showed significant inhibitory activities against K562 and Bcap37 cells, with the IC_{50} values of 0.13 and 1.26 $\mu g\,mL^{-1}$, respectively, which were lower than those of the positive control.

Keywords: *Isodon leucophyllus*; Labiatae; *ent*-kaurane diterpenoids; Baiyecrystal D; Baiyecrystal E; Cytotoxic activities

INTRODUCTION

Isodon leucophyllus (Dunn) Kudo (Labiatae), a small shrub which has been proved to contain a series of *ent*-kauranoids in previous phytochemical investigations [1–3], is mainly distributed in the northwest area of Yunnan Province and the western area of Sichun Province in China. In our systematic search for diterpenoids showing cytotoxicity from the genus *Isodon*, we reinvestigated this plant collected in a different region of Yunnan Province, leading to the isolation of another ten diterpenoids, including two new compounds named baiyecrystal D (**1**) and E (**2**), together with the eight known compounds xerophilusin B (**4**) [4], macrocalin B (**5**) [5], oridonin (**6**) [6], rosthorin A (**7**) [7], lasiocarpanin (**8**) [8], rabdoternin A (**9**) [9], and phyllostachysin A (**10**) [6] and B (**11**) [10]. It was noteworthy that compound (**10**) was obtained as a major constituent (content up to 0.7% in the plant), and that all ten compounds were not obtained in the previous work. Thus we might conclude that the diversity of secondary metabolites of the genus *Isodon* can be ascribed to the diversity of the plant's ecological circumstances, and that the constituents of the same plant may vary according to plant's distribution and the season in which it is gathered.

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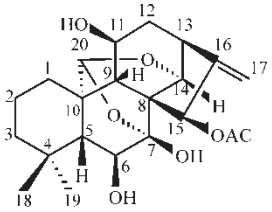
Compounds **2**, **6**, **7**, **8**, **10** were also examined for their bioactivity toward human tumor K562 and Bcap37 cells. This paper mainly reports the isolation and structural elucidation of the new compounds and the cytotoxicity of the tested compounds, as well as the ^{13}C NMR data of **5**, for the first time.



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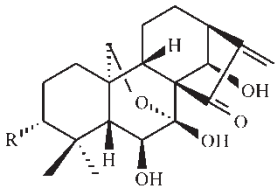
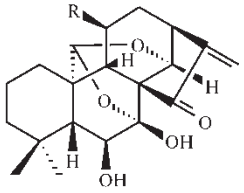
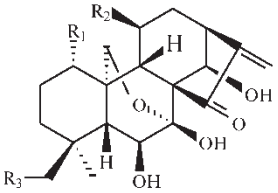


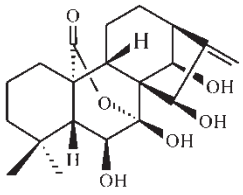
2 : R = OH
3 : R = H



4 : R = H
5 : R = OH



6 : R_1 = OH, R_2 = R_3 = H
7 : R_1 = R_3 = H, R_2 = OH
8 : R_1 = R_2 = H, R_3 = OH



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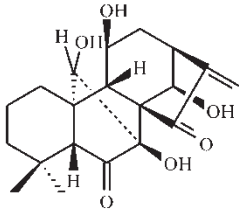

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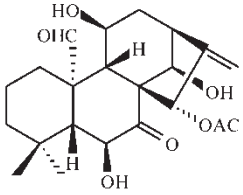

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RESULTS AND DISCUSSION

After repeated chromatographic purification on silica gel, the EtOAc-soluble portion of the Me_2CO extract of *I. leucophyllus* yielded two new diterpenoids, baiyecrystals D and E, together with eight known ones **4**–**11**.

Compound **1** gave a molecular ion peak at *m/z* 406.1971 in its HR-EIMS, in accordance with a molecular formula $C_{22}H_{30}O_7$. From its ^{13}C NMR, DEPT and ^1H NMR spectra, in addition to the signals of an acetoxy group at δ_C 170.3 (s), 21.5 (q), δ_H 2.24 (3H, s), there are twenty carbon signals which consist of those of two tertiary methyls, five methylenes, including one olefinic carbon at δ_C 110.9 (t), eight methines, including four oxygenated ones at δ_C 72.5 (d), 71.2 (d), 71.2 (d), 63.8 (d) and an acetal carbon at δ_C 97.6 (d), and five quaternary carbons, including one hemiketalic carbon at δ_C 100.4 (s), attributable to a diterpenoid. An unconjugated *exo*-methylene in a five-membered ring was discerned from the following spectral data: ^{13}C NMR δ 156.9 (s), 110.9 (t); ^1H NMR δ 5.57, 5.30 (each 1H, s); UV λ_{max} 204.5 (2.59) nm. This spectral evidence and the eight degrees of unsaturation

suggested that **1** had an *ent*-kaur-16-ene basic skeleton, having two extra rings in addition to the regular four. In the ^{1}H NMR spectrum, the absence of the CH_3-20 singlet and the presence of a 1H singlet at δ 5.41 (s) were observed, which hinted that the formation of two rings both involved C-20. This assumption was verified by the HMBC cross-peaks from H-20 (δ 5.41) to C-7 (δ 100.4) and C-14 (δ 71.2). Comparison of the spectral data of **1** with those of xerophilusin B (**4**) [4] and microcalin B (**5**) [5] further confirmed the presence of two epoxy units and established the 7β-hydroxy-7α,20:14α,20-diepoxy-*ent*-kaur-16-ene skeleton substituted by two hydroxyls and an acetoxyl.

Upon carefully comparing the spectra of **1** and xerophilusin B (**4**), one of the hydroxyl groups of **1** was at C-6, as in **4**. The other hydroxyl would be at C-11, judging from the downfield shifts of C-12 (from δ 26.2 in **4** to 36.7 in **1**) in the ^{13}C NMR spectrum and long-range correlations of H-11 (δ 4.34) with C-8, C-10 and C-13 in the HMBC results (Fig. 1). The acetoxy group was assigned to C-15 owing to the HMQC and HMBC experiments: the acetyl carbonyl carbon correlated with H-15 (δ 5.57), which further coupled with C-7, C-8, C-14 and C-16. The β-orientations of OH-11 and OAc-15 were settled by the cross peaks of H-11 with H-1α and H-12α and the upfield shift of C-9 (δ 48.4) [compared with C-9 of **5** (at δ 56.4)] owing to the γ-steric compression effect of 15β-OAc. Moreover, in the ROESY spectrum the positive correlations of H-6 with Me-18 and Me-19 also confirmed the β-configuration of OH-6. Therefore, compound **1** was determined as 6β,7β,11β-trihydroxy-15β-acetoxy-7,20:14,20-diepoxy-*ent*-kaur-16-ene, named baiyecrystal D.

Compound **2**, obtained as colorless crystals, displayed a molecular ion peak in its HR-EIMS spectrum at *m/z* 364.1912, consistent with a molecular formula of $C_{20}H_{28}O_6$. Its ^{13}C NMR and DEPT revealed the presence of two methyls, six methylenes (including an oxygenated one and an olefinic one), six methines (including three oxygenated ones), six quaternary carbons (including a ketonic carbonyl carbon and a hemiketalic carbon). A five-membered ring with a ketone conjugated with an *exo*-methylene was deduced from the spectral data: UV (λ_{max} 235.0 nm; IR ν_{max} 1705, 1640 cm^{-1}; ^{13}C NMR δ 208.8 (s), 152.8 (s) and 119.5 (t); and ^{1}H NMR δ 6.27, 5.50 (each 1H, s). Thus, **2** has an *ent*-kaurene skeleton, as with all diterpenes isolated from the *Isodon* genus so far [11]. In addition to the spectral data seen for *ent*-kaurene skeleton, the H_3-20 singlet was replaced by H_2-20, an AB coupling type of protons at δ 4.24 and 3.97 (each 1H, d, J = 9.7 Hz) in the ^{1}H NMR spectrum of **2**, which together with the demand of unsaturated degrees hinted that one epoxy ring exists in **2**. The correlation between H_2-20 (δ 4.24, 3.97) with hemiketalic carbon C-7 (δ 98.4) in the HMBC spectrum of **2** unambiguously proved the presence of an 7:20-epoxy bridge. Thus, compound **2** had 7β-hydroxy-7α,20-epoxy-*ent*-kaur-16-en-15-one as its basic skeleton. Comparison of the ^{13}C NMR data of **2** with those of longkaurin A (**3**) [12] revealed that they differ only in the A ring signals. With **2**, the downfield shifts of C-2 (δ 28.0 t) and C-4 (δ 40.7 s), and

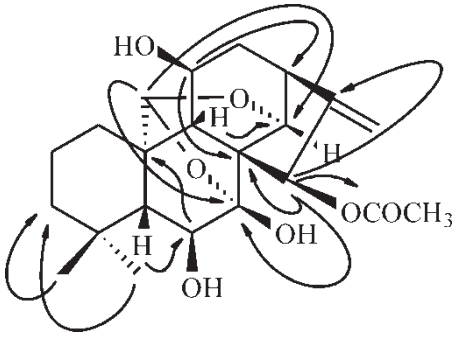

FIGURE 1 Selected HMBC correlations of **1**.

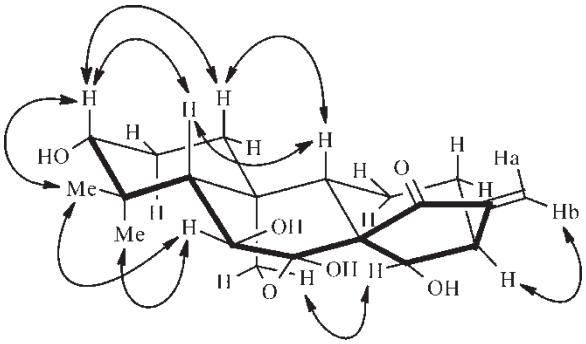

FIGURE 2 Key ROESY correlations of **2**.

the upfield shifts of C-18 (δ 29.3, q) and C-19 (δ 16.3, q), required a hydroxy at C-3 while these effects are absent in longkaurin A (**3**). In the HMBC spectrum, the correlations of H-3 (δ 3.55, br d) with C-1 (δ 29.6, t), C-18 and C-19 and the correlations of C-3 (δ 77.4, d) with H_3-18 (δ 1.32, s) and H_3-19 (δ 1.59, s) were clearly observed, which indicated the presence of OH-3 in **2**. The relative configuration of the OH-3α was revealed by the ROESY correlations of H-3β (δ 3.55, br d) with H-1β (δ 1.06, m) and H-5β (δ 1.53, d) (Fig. 2), and was also established according to the upfield shift of C-18 from δ 34.2 (q) in **3** to δ 29.3 (q) in **2**, and C-19 from δ 23.0 (q) in **3** to δ 16.3 (q) in **2**, due to γ-steric compression between OH-3α and both C-18 and C-19 [13]. Therefore, baiyecrystal E (**2**) was identified as 3α,6β,7β, 14β-tetrahydroxy-7,20-epoxy-*ent*-kaur-16-en-15-one.

Compounds **2**, **6**, **7**, **8** and **10** were assayed for their cytotoxic abilities toward human tumor K562 and BCAP 37 cells; the results are shown in Table II. Compound **8** displayed remarkable sensitivity against two cells, with the IC_{50}s of 0.13 and 1.26 μg mL^{-1}, which are lower than those of the positive reference *cis*-Platin (IC_{50} = 3.84 and 1.54 μg mL^{-1}, respectively). Compounds **2**, **6** and **7** showed distinct activities to K562 cells, with the $IC^{50} < 10\ \mu g\ mL^{-1}$ (6.69, 4.37 and 4.33 μg mL^{-1}, respectively).The major compound **10** exhibited a moderate effect on K562 cells, with an IC_{50} of 10.91 μg mL^{-1}. Compound **5** has been reported to show potent cytotoxic effects against K562, HL-60 and MKN-28 [4].

EXPERIMENTAL

General Experimental Procedures

Melting points were measured on an XRC-1 micromelting apparatus and are uncorrected. IR and UV spectra were obtained on a Bio-Rad FTS-135 infrared spectrometer with KBr pellets and a Shimadzu double-beam 210A spectrometer in MeOH, respectively. Optical rotations were taken on a SEPA-300 polarimeter. The MS spectra were performed on a VG Autospec-3000 spectrometer with 70 eV. ^{1}H, ^{13}C and 2D NMR were recorded on a Bruker AM-400 and DRX-500 spectrometer with TMS as internal standard. The silica gel for TLC and column chromatography were obtained from Qingdo Marine Chemical Inc., China.

Plant Material

The aerial parts of *I. leucophyllus* were collected in Zhongdian Prefecture, northwest of Yunnan Province, China, in October 2001, and were identified by Professor Xi-Wen Li. The voucher specimen (KIB 01-10-183) has been deposited in the Laboratory of Phytochemistry, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation

The air-dried and powdered plants (2.1 kg) were extracted with 70% aq. acetone at room temperature (4 ×, three days for each time). The extract was then concentrated *in vacuo* and filtered to remove pigment; the filtrate was then partitioned with EtOAc. The EtOAc extract (57 g) was subjected to column chromatography on silica gel (200–300 mesh) and eluted gradiently with $CHCl_3$–Me_2CO (from 1:0 to 0:1) to give seven fractions I–VII. Fractions II–VI were repeatedly chromatographed over silica gel with cyclohexane–Me_2CO (4:1, 7:3, 3;2, 1:1), cyclohexane–EtOAc (5:1, 4:1, 3:1, 2:1, 1:1), $CHCl_3$–Me_2CO (7:1, 7:2), $CHCl_3$–MeOH (40:1, 20:1) and cyclohexane–$CHCl_3$–isopropanol (10:0.5:0.5) and purified by recrystallization and preparative TLC to afford compounds **1** (3 mg), **2** (28 mg), **4** (8 mg), **5** (6 mg), **6** (240 mg), **7** (70 mg), **8** (70 mg), **9** (2 mg), **10** (15 g) and **11** (50 mg).

Baiyecrystal D (1)

Colorless needles (MeOH), mp 154–156°C; $[\alpha]_D^{25}$ −49.5 (MeOH, *c* 0.10); UV (MeOH) λ_{max} (log ϵ) (nm): 204.5 (2.59); IR (KBr) ν_{max} (cm^{-1}): 3432, 2930, 2870, 1721, 1630, 1372,1311, 1255, 1099, 1048, 1017, 980; EIMS (70 eV) *m/z* (rel. int. %): 406 $[M]^+$ (1), 364 (3), 346 $[M-AcOH]^+$ (39), 328 $[M-AcOH-H_2O]^+$ (10), 300 (19), 282 (100), 267 (24), 253 (13), 239 (22), 226 (28), 213 (65), 198 (10), 187 (10), 171 (13), 157 (18), 121 (15), 105 (23), 91 (28), 69 (35); HR-EIMS *m/z*: 406.1971 (calcd for $C_{22}H_{30}O_7$, 406.1992); ^1H NMR (400 MHz, C_5D_5N) δ: 5.57 (1H, s, H-15α), 5.57 (1H, s, H-17a), 5.41 (1H, s, H-20), 5.30 (1H, s, H-17b), 5.28 (1H, d, $J = 5.8$ Hz, H-14β), 4.62 (1H, d, $J = 1.8$ Hz, H-6α), 4.34 (1H, d, $J = 4.8$ Hz, H-11α), 2.90 (1H, m, H-13α), 2.83 (1H, br s, H-9β), 2.59 (1H, dt, $J = 13.7$, 4.8 Hz, H-12α), 2.03 (1H, br d, $J = 13.7$ Hz, H-12β), 1.69 (1H, br d, $J = 13.0$ Hz, H-1α), 1.44 (2H, m, H-2), 1.43 (1H, s, H-5β), 1.39 (1H, m, H-3α), 1.10 (1H, m, H-1β), 1.00 (1H, m, H-3β), 0.93 (3H, s, H-19), 0.84 (3H, s, H-18), 2.24 (3H, s, OAc); for ^{13}C NMR spectral data see Table I.

TABLE I ^{13}C NMR chemical shifts of compounds **1** and **2** in C_5D_5N (100 MHz, δ in ppm)

Carbon	***1***	***2***	***5***
1	28.4 (t)	29.6 (t)	28.2 (t)
2	18.8 (t)	28.0 (t)	18.9 (t)
3	40.9 (t)	77.4 (d)	41.3 (t)
4	33.6 (s)	40.7 (s)	33.6 (s)
5	61.7 (d)	60.9 (d)	63.2 (d)
6	71.2 (d)	73.5 (d)	72.8 (d)
7	100.4 (s)	98.4 (s)	101.6 (s)
8	50.8 (s)	62.5 (s)	56.1 (s)
9	48.4 (d)	52.0 (d)	56.4 (d)
10	42.5 (s)	36.3 (s)	43.1 (s)
11	63.8 (d)	16.7 (t)	63.5 (d)
12	36.7 (t)	30.0 (t)	35.3 (t)
13	41.6 (d)	43.7 (d)	39.7 (d)
14	71.2 (d)	73.6 (d)	70.4 (d)
15	72.5 (d)	208.8 (s)	199.4 (s)
16	156.9 (s)	152.8 (s)	150.6 (s)
17	110.9 (t)	119.5 (t)	115.2 (t)
18	31.3 (q)	29.3 (q)	31.5 (q)
19	23.0 (q)	16.3 (q)	23.2 (q)
20	97.6 (d)	66.1 (t)	98.5 (*d*)
OAc	170.3 (s)		
	21.5 (q)		

TABLE II Cytotoxicities of compounds **2**, **6**, **7**, **8** and **10**

		IC_{50} ($\mu g\, mL^{-1}$)	
Compound	*MW*	*K562*	*BCAP37*
2	364	6.69	78.68
6	364	4.37	8.32
7	364	4.33	52.20
8	364	0.13	1.26
10	362	10.91	91.81
cis-Platin		3.84	1.54

Baiyecrystal E (2)

Colorless cubic crystals (MeOH), mp 225–228°C; $[\alpha]_D^{25}$ −85.0 (MeOH, *c* 0.10); UV (MeOH) λ_{max} (log ϵ) (nm): 235.0 (3.00); IR (KBr) ν_{max} (cm^{-1}): 3505, 2948, 2878, 1705, 1640, 1450, 1080, 1060, 1053, 1039; EIMS (70 eV) *m/z* (rel. int. %): 364 $[M]^+$ (100), 346 $[M-H_2O]^+$ (4), 328 $[M-2\times H_2O]^+$ (4), 318 (9), 303 (5), 285 (16), 261 (10), 243 (12), 215 (13), 201 (11), 183 (13), 167 (19), 149 (34), 121 (21), 105 (33), 95 (25), 85 (42), 67 (29), 55 (50); HR-EIMS *m/z*: 364.1912 (calcd for $C_{20}H_{28}O_6$, 364.1886); ^{1}H NMR (400 MHz, C_5D_5N) δ: 6.27 (1H, s, H-17a), 5.50 (1H, s, H-17b), 5.13 (1H, s, H-14α), 4.39 (1H, dd, $J = 11.2$, 5.8 Hz, H-6α), 4.24 (1H, ABd, $J = 9.7$ Hz, H-20a), 3.97 (1H, ABd, $J = 9.7$ Hz, H-20b), 3.55 (1H, br d, $J = 11.4$ Hz, H-3β), 3.16 (1H, d, $J = 9.3$ Hz, H-13α), 2.34 (1H, m, H-12), 1.77 (1H, m, H-2α), 1.66 (1H, overlap, H-11α), 1.63 (1H, overlap, H-9β), 1.59 (3H, s, H-19), 1.53 (1H, d, $J = 5.8$ Hz, H-5β), 1.52 (1H, m, H-1α), 1.32 (3H, s, H-18), 1.25 (1H, m, H-1α), 1.20 (1H, m, H-11β), 1.06 (1H, m, H-1β); for ^{13}C NMR spectral data see Table I.

Bioactivity Assay

The cytotoxicity evaluation towards K562 and Bcap37 tumor cells (Table II) were examined by MTT and SRB methods, for which the experimental detail has been reported previously [14].

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