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Three New 18-Oxygenated *ent*-Kaurane Diterpenoids from *Isodon leucophyllus*

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Three new 18-oxygenated *ent*-kaurane diterpenoids, isoleuconins A-C (**1–3**) and ten known diterpenoids were isolated from the aerial parts of *Isodon leucophyllus*. The structures were elucidated by 1D and 2D NMR spectroscopic analysis. All of the compounds were evaluated for their cytotoxicity. Rabdokunmin A (**13**) showed significant cytotoxicity against HT-29 cells, with an IC_{50} value of 6.2 μM.

Keywords: *Isodon leucophyllus*, Labiatae, *ent*-kaurane, diterpenoid, cytotoxicity.

Isodon leucophyllus (Dunn) Kudo (Labiatae/Lamiaceae) is a small shrub mainly distributed in the western districts of Sichuan Province and the north-western regions of Yunnan Province, People's Republic of China [1]. Previous research reported the isolation of 28 diterpenoids (C-20 nonoxygenated and 7, 20-epoxy *ent*-kaurane), 6 flavones and one derivative of ionone [2a-2e]. In continuation of our research for new diterpenoids with antitumor activities, we have reinvestigated the aerial parts of *I. leucophyllus*, collected in Shangri-La County, Yunnan Province. As a result, along with 10 known diterpenoids (rabdoloxin A (**4**) [3a], isoscoparin I (**5**) [3b], 4-*epi*-henryine (**6**) [3c], rabdokunmin C (**7**) [3d], rabdokunmin E (**8**) [3d], rabdoinflexin B (**9**) [3e], excisanin A (**10**) [3f], phyllostachysin H (**11**) [3g], rabdoloxin B (**12**) [3a, and rabdokunmin A (**13**) [3d]), three new 18-oxygenated *ent*-kaurane diterpenoids [isoleuconins A-C (**1–3**)] were isolated from *I. leucophyllus*.

Isoleuconin A (**1**), exhibited a pseudomolecular ion peak (*m/z* 389.1947 $[M+Na]^+$, calcd 389.1940), corresponding to the molecular formula $C_{20}H_{30}O_6$, with six degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl, carbonyl, and *exo*-methylene groups in according with the absorptions at 3373, 1697 and 898 cm^{-1}, respectively. Analyses of the ^1H, ^{13}C and DEPT NMR data (Table 1) provided evidence that **1** possessed one *exo*-methylene group, one carbonyl group, two tertiary methyls, five methylenes (including one oxygenated), seven methines (including four oxygenated), and four quaternary carbons. On the basis of the characteristic signals of three methines (δ_C 44.4, 62.1, and 53.6 assigned to C-5, 9, and 13), three quaternary carbons (δ_C 37.3, 51.4, 39.5 assigned to C-4, 8 and 10), two methyls (δ_C 17.4, 18.0 assigned to C-19 and 20), and an oxygenated methylene (δ_C 70.1, assigned to C-18), together with the *exo*-methylene group (δ_C 149.2 s, 113.6 t, elucidated as C-16 and 17), we presumed that **1** should be an *ent*-kaur-16-ene diterpenoid.

Chemical shift values of some typical carbon signals of **1** were similar to those of the known compound **4** [3a]. The main difference between them was that the conjugated carbonyl group at C-15 of **4** was reduced to a secondary hydroxyl group in **1**. HMBC correlations H-15/C-8, H-15/C-9, H-15/C-7, and H-15/C-16, and the related ^1H-^1H COSY correlations H-15/H_2-17 confirmed the above deduction. ROESY correlations H-15/H-13α, H-15/H-7β indicated that the hydroxyl group located at C-15 adopted a β-orientation, as shown in Figure 1. The signal for C-9 (δ_C 70.1) in **4** shifted upfield to δ_C 62.1 (C-9) in **1**, caused by the γ-steric compression effect between HO-15 and H-9β. That could also support the view that HO-15 in **1** was β-oriented. Consequently, compound **1** was elucidated as 7α,12α,14β,15β,18-pentahydroxy-*ent*-kaur-16-en-11-one, to which the name isoleuconin A was given.

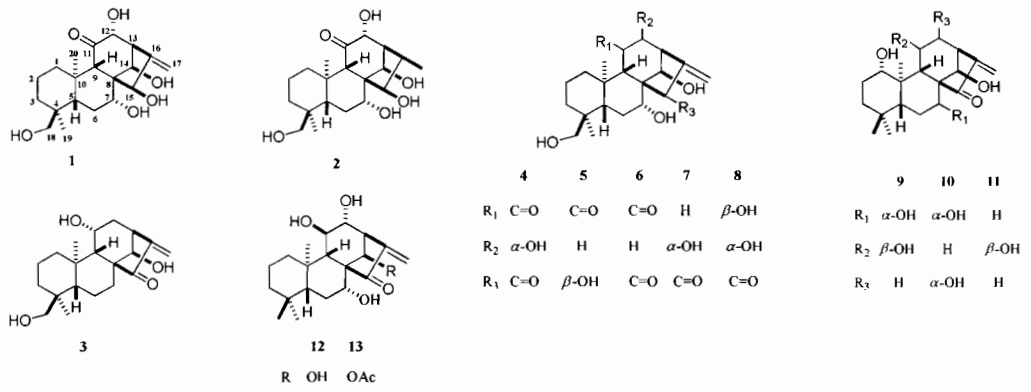

Table 1: ^{1}H and ^{13}C NMR spectroscopic data of isoleuconins A–C (**1–3**) (δ in ppm, *J* in Hz, recorded at 400 MHz and 100 MHz, respectively).

No.	1[a]		2[b]		3[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1α	1.48[c] (d, 13.2)	39.1 t	2.2[c] (m)	40.9 t	1.91 (d, 13.0)	39.5 t
1β	0.97[c] (m)		1.59 (m)		1.07[c] (m)	
2α	1.40 (m)	17.3 t	1.71 (m)	18.5 t	1.70[c] (m)	18.5 t
2β	1.35[c] (m)		1.50 (m)		1.50[c] (m)	
3α	1.02[c] (m)	34.0 t	1.32 (d, 12.7)	35.6 t	1.39 (m)	35.7 t
3β	1.32[c] (m)		1.82[c] (m)		1.81[c] (m)	
4		37.3 s		38.3 s		38.1 s
5β	1.17 (s, overlap)	44.4 d	1.96 (d, 12.0)	46.7 d	1.80[c] (m)	49.0 d
6α	1.61 (m)	28.5 t	2.22[c] (m)	30.8 t	1.45[c] (m)	18.5 t
6β	1.68 (m)		2.49 (d, 9.4)		1.85[c] (m)	
7α		73.2 d		76.4 d	2.61 (d, 13.8)	26.7 t
7β	3.60 (dd, 4.0, 11.2)		4.53 (m)		2.21 (d, 13.8)	
8		51.4 s		54.7 s		57.5 s
9β	2.15 (s)	62.1 d	3.30 (s)	64.5 d	2.18 (s)	66.9 d
10		39.5 s		40.1 s		38.9 s
11β		210.3 s		211.4 s	4.28 (s)	64.9 d
12α		78.4 d		74.9 d	2.34 (m)	41.7 d
12β	3.48 (d, 3.1)		4.50 (s)		2.43 (m)	
13α	2.69 (br. d)	53.6 d	2.85 (d, 5.6)	54.6 d	3.36 (s)	46.4 d
14α	5.07 (br. s)	71.2 d	6.10 (s)	74.3 d	4.90 (s)	73.4 d
15	4.94 (br. s)	72.9 d	5.57 (m)	73.8 d		209.2 s
16α		149.2 s	3.35[c] (m)	35.0 d		150.6 s
17a	5.10 (br.s)	113.6 t	1.22 (d, 7.7)	11.3 q	5.35 (s)	112.8 t
17b	5.24 (br.s)				6.22 (s)	
18a	2.81 (d_{AB}, 11.3)	70.1 t	3.34[c] (m)	71.3 t	3.32 (m)	71.4 t
18b	3.26 (d_{AB}, 11.3)		3.66 (d, 10.3)		3.62 (m)	
19α	0.59 (s)	17.4 q	0.85 (s)	18.2 q	0.80 (s)	18.0 q
20α	1.13 (s)	18.0 q	1.78 (s)	19.3 q	1.08[c] (s)	18.5 q

a: in $CDCl_3$+CD_3OD, **b:** in C_5D_5N, **c:** revealed by HSQC correlations.

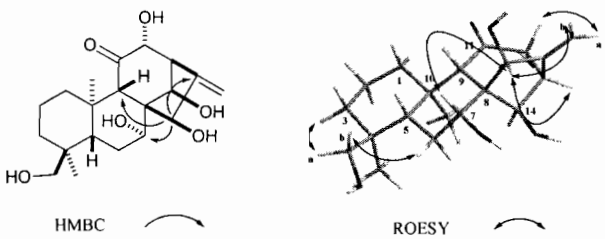

Figure 1: Key HMBC and ROSEY correlations for **1**.

Isoleuconin B (**2**) was obtained as a grey amorphous powder. The HR-ESI-MS gave a pseudomolecular ion peak (*m/z* 391.2109 $[M+Na]^+$, calcd 391.2096), corresponding to the molecular formula $C_{20}H_{32}O_6$,with five degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl and carbonyl groups with absorptions at 3386 and 1698 cm^{-1}, respectively. The absorption for *exo*-methylene disappeared in the IR spectrum of **2**. Typical carbon signals of three methines (δ_C 46.7, 64.5, 54.6 assigned as C-5, 9, and 13), three quaternary carbons (δ_C 38.3, 54.7, 40.1 assigned as C-4, 8 and 10), two methyls (δ_C 18.2, 19.3 assigned as C-19 and 20), and an oxygenated methylene (δ_C 71.3, assigned as C-18) indicated that **2** also should be an *ent*- kaurane diterpenoid. Carefully comparing the ^{13}C NMR data of compound **2** with those of **1**, we found that the *exo*-methylene of compound **1** was reduced to a methyl (δ_C 11.3, q, assigned as C-17) in compound **2**. HMBC correlations H_3-17/C-16, H_3-17/C-13, and H_3-17/C-15, and ^{1}H-^{1}H COSY correlations H_3-17/H-16, H-15/H-16, and H-16/H-13 proved the above elucidation. ROESY correlations H_3-17/H-12β and H-16α/H-13α revealed that the methyl located at C-16 adopted the β-orientation. Thus, the structure of compound **2** was established as 7α,12α,14β,15β,18-pentahydroxy-16β-methyl-*ent*-kaur-11-one, to which the name isoleuconin B was assigned.

The molecular formula for isoleuconin C (**3**) was established as $C_{20}H_{30}O_4$ on the basis of HR-ESI-MS data (*m/z* 357.2040 [M+Na]+, calcd 357.2041). The IR spectrum revealed the presence of hydroxyl, carbonyl, and *exo*-methylene from absorptions at 3371, 1713, 1648, and 928 cm^{-1}, respectively. Analyses of the ^{1}H-, ^{13}C- and DEPT-NMR data (Table 1) provided evidence that **3** possessed one conjugated carbonyl group (δ_C 209.2 assigned to C-15), one *exo*-methylene (δ_C 150.6,

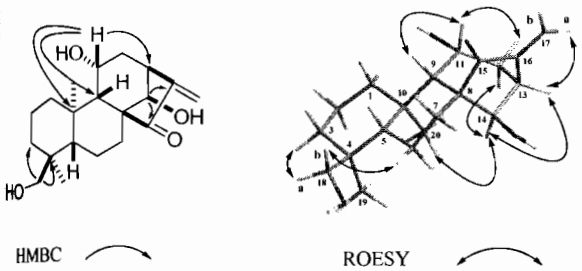

Figure 2: Key HMBC and ROSEY correlations for **3**.

112.8, assigned to C-16 and 17), two oxygenated methines (δ_C 64.9, 73.4, assigned to C-11 and 14), one oxygenated methylene (δ_C 71.4 assigned to C-18) and other typical carbon signals of an *ent*-kaurane diterpenoid. HMBC correlations H_2-17/C-16, H_2-17/C-13, and H_2-17/C-15 indicated that the *exo*-methylene was located at C-16 and conjugated with the carbonyl group (C-15). HMBC correlations H-11/C-8, H-11/C-10, and H-11/C-13, and H-14/C-16, H-14/C-15 disclosed that C-11 and C-14 were substituted by two hydroxyl groups, which was confirmed by ^{1}H-^{1}H COSY correlations H-11/H-12/H-13/H-14 and H-11/HO-11, H-14/HO-14. ROESY correlations H-14/H-20, H-14/H-13α, and H-14/H-12α revealed that H-14 adopted an α-orientation, as shown in Figure 2. ROESY correlations H-11/H-9β and H-11/H-12β revealed that H-11 adopted a β-orientation. HMBC correlations H-18/C-3 and H-18/C-4 indicated that C-18 was substituted by one hydroxyl group. Like compound **1**, this substitution also could be proved by the upfield shifted carbon signals (δ_C 35.7, C-3; δ_C 49.0, C-5) caused by the γ-steric compression effect between HO-18 and H-3β and H-5β in **3** compared with those signals (δ_C 41.8, C-3; δ_C 53.2, C-5) of compound **12**. Therefore, the structure of compound **3** was determined as 11α,14β,18-trihydroxy-*ent*-kaur-16-en-15-one, and named as isoleuconin C.

Compounds **1–13** were evaluated for their cytotoxic activities against SK-OV-3, BEL-7402 and HT-29 cell lines, using the sulforhodamine B (SRB) method with adriamycin as the positive control [3h]. As may be seen from Table 2, compound **3** exhibited weak activities against SK-OV-3 and BEL-7402 cell lines, while compounds **1** and **2** were found to be inactive against all of these cells. Their analogue **4** exhibited weak inhibitory effects against two kinds of cell lines; more importantly, another analogue, **6**, showed significant activity against these cell lines. Carefully comparing the structure of **6** with that of **4**, we found that the α-hydroxyl group C-12 of compound **4** disappeared in compound **6**. The above results disclosed that in the diterpenoids with a structure like **1**, the α-hydroxy group located at C-12 weakened the cytotoxicity of the *ent*-kaurane diterpenoid. On the other hand, in diterpenoid with a structure like **12** and **13**, esterification with the hydroxyl group located at C-14 could improve the cytotoxicity. The above results further proved that the cyclopentanone conjugated with an *exo*-methylene is the active center of *ent*-kauranoids. [3i]

Table 2: Cytotoxicity bioassay result [a] for compounds **1-13**.

Compd	SK-OV-3	BEL-7402	HT-29
1	>100	>100	>100
2	>100	>100	>100
3	38.0	40.8	82.8
4	38.9	98.9	35.7
5	>100	>100	>100
6	8.6	23.5	6.6
7	55.3	71.3	74.7
8	>100	>100	>100
9	40.0	46.7	29.7
10	>100	30.2	6.2
11	17.1	24.0	17.0
12	9.9	32.8	6.6
13	7.4	26.2	6.2
ADR	0.17	0.067	0.092

[a] Results are expressed as IC_{50} values in μM. Cell lines: SK-OV-3 (human ovarian cancer cell line); BEL-7402 (human lung cancer cell line); HT-29 (human colon cancer cell line).

Experimental

General: Optical rotations, Perkin-Elmer Model 241 polarimeter; UV, Shimadzu UV-2401 PC UV-VIS spectrophotometer; IR, Bio-Rad Fts-135 spectrophotometer; MS, VG Auto spec-3000 spectrometer or Finnigan MAT 90; NMR, Bruker AV-400 or DRX-500 instrument.

Plant material: Aerial parts of *Isodon leucophyllus* (Dunn) Kudo were collected and air dried in Shangri-La county of Yunnan Province in August, 2004. The identity of the plant material was verified by Prof. Xi-Wen Li, and a voucher specimen was deposited in the Herbarium of the Kunming Institute of Botany.

Extraction and isolation: Powdered aerial parts of *I. leucophyllus* (1.8 kg) were extracted with 70% aq. acetone (3× 6 L) at RT for 3 days each time. The extract was evaporated *in vacuo* to remove acetone, then partitioned between H_2O and EtOAc. The EtOAc extract (78 g) was decolored with MCI gel, and then chromatographed over a silica gel column (650g, 100-200 mesh, Qingdao marine chemical factory), eluted with a gradient solvent system [$CHCl_3$–CH_3COCH_3 (1:0, 9:1, 8:2, 7:3, 2:1, 1:1, 0:1)] to afford fractions A–G, monitoring by TLC (volume of each collection was 1000 mL). Fraction E (2:1, 5 g) was submitted to chromatography over a RP-18 column (100 g, 40-63 μm, Merck Company) eluted with 30%→100% MeOH–H_2O to give fractions E1–E7, monitoring by TLC (volume of each collection was 250 mL). Fraction E4 (590 mg) was chromatographed over a silica gel column (200-300 mesh, 20 g) eluted with a gradient solvent system of $CHCl_3$–CH_3OH (60:1→10:1) to afford a mixture of 2 diterpenoids (volume of each collection was 50 mL), which were purified by semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 9.4 ×250 mm,72% MeOH–H_2O, λ_{max} = 210 nm) to yield compound **1** (46 mg) and compound **2** (14 mg). Fraction E2 (105 mg) was subjected to a silica gel CC, eluting

with a gradient solvent system [$CHCl_3$–CH_3OH (40:1→5:1)] to afford a mixture of diterpenoids, which was purified by semi-preparative HPLC (45% MeOH–H2O, λ_{max} = 210 nm) to obtain compound **5** (45 mg). Fraction C (8:2, 11 g) was submitted to CC over a RP-18 column (200 g, 40-63 μm, Merck Company, 30%→100% MeOH–H_2O) to give fractions C1–C5, monitoring by TLC (volume of each collection was 250 mL). In the sixth, seventh and eighth bottles of elution solvent belonging to fraction C1, compound **4** (3.1 g) was separated as needle crystals. The mother liquid of compound **4** was subjected to RP-18 CC (100 g, 40-63μm, Merck Company, 30%→100% MeOH–H_2O) to obtain compounds **6** (434 mg) and **13** (78 mg). Fraction C2 (2.3 g) was subjected to silica gel CC (200-300 mesh, 40g) eluting with a gradient solvent system of light petroleum–CH_3COCH_3 (1:0 to 0:1, volume of each collection was 50 mL). Compound **9** (509 mg) was separated out as needle crystals from the first bottle of fraction C2. Compound **7** (3.1 mg) and compound **8** (1.7 mg) were isolated by semi-preparative HPLC (40% MeOH–H_2O, 45% MeOH–H_2O, λ_{max} = 230 nm) from the fourth and fifth bottle of elution solvent. The tenth bottle (23 mg) of the above elution was subjected to RP-18 CC (5 g, 40-63 μm, Merck Company, 30%→100% MeOH–H_2O) to obtain a mixture mainly contained two diterpenoids. The mixture was separated by semi-preparative HPLC (24% ACN–H_2O, λ_{max} = 230 nm) to obtain compound **10** (5 mg) and one unpurified diterpenoid, and then purified by semi-preparative HPLC (38% MeOH–H_2O, λ_{max} = 230 nm) to obtain compound **3** (3 mg). Compound **11** (4.0 mg) was isolated from fraction F (1:1) after continued CC on RP-18, and then purified by semi-preparative HPLC (42% MeOH–H_2O, λ_{max} = 238 nm).Compound **12** (2.4 g) separated as granular crystals from the EtOAc extract when dissolved in acetone.

Isoleuconin A (1)

Grey tablet-like crystals
$[\alpha]_D^{23.6}$: +52.0 (*c* = 12.60 mg/mL, MeOH).
IR (KBr): 3373, 2930, 2873, 1697, 1455, 898, cm^{-1}.
UV λ_{max} (MeOH) nm (log ε): 204 (3.66).
^1H and ^{13}C NMR: Table 1.
HR-ESI MS: *m/z* $[M+Na]^+$, calculated for $C_{20}H_{30}O_6Na$ (calcd. 389.1940); found: 389.1947.

Isoleuconin B (2)

Grey powder
$[\alpha]_D^{23.8}$: +12.0 (*c* = 5.82 mg/mL, MeOH).
IR (KBr): 3386, 2931, 2874, 1697, 1453 cm^{-1}.
UV λ_{max} (MeOH) nm (log ε): 203 (3.23).
^1H and ^{13}C NMR: Table 1.
HR-ESI MS: *m/z* $[M+Na]^+$, calculated for $C_{20}H_{32}O_6Na$ (calcd. 391.2096); found: 391.2109.

Isoleuconin C (3)

White powder
$[\alpha]_D^{18.9}$: -76.0 (*c* = 0.79 mg/mL, MeOH).
IR (KBr): 3443, 3371, 2935, 2855, 1713, 1648, 928 cm^{-1}.
UV λ_{max} (MeOH) nm (log ε): 236 (3.81).
^1H and ^{13}C NMR: Table 1.
HR-ESI MS: *m/z* $[M+Na]^+$, calculated for $C_{20}H_{30}O_4Na$ (calcd. 357.2041); found: 357.2040.

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