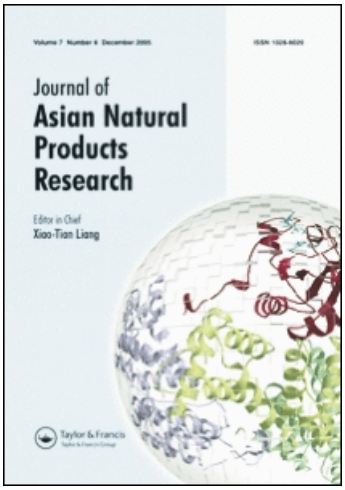

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Cytotoxic *ent*-kaurane diterpenoids from *Isodon macrophyllus*

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Two new *ent*-kaurane diterpenoids, dayecrystals D–E (**1**–**2**), together with nine known compounds, isojaponin A (**3**), rabdosin A (**4**), lushanrubescensin J (**5**), wikstroemioidin B (**6**), maoyecrystal C (**7**), rabdosin B (**8**), isodonal (**9**), shikokianin (**10**), and effusanin A (**11**), were isolated from the leaves of *Isodon macrophyllus*. The structures of the new compounds were elucidated using 1D and 2D NMR spectroscopy. The ^{13}C-NMR spectral data of compound **4** are reported for the first time. All of the compounds were tested for their cytotoxicities against DU145 and LoVo human tumor cells. Compounds **4**, **10**, and **11** showed inhibitory effects on DU145 cells with IC_{50} values 5.90, 4.24, and 3.16 μM, and LoVo cells with IC_{50} values 14.20, 17.55, and 3.02 μM, respectively.

Keywords: *Isodon macrophyllus*; *ent*-kauranoids; diterpene; dayecrystals D–E; cytotoxicity

1. Introduction

Plants belonging to the genus *Isodon* are known to be a rich source of *ent*-kaurane diterpenoids, most of which have been shown to have anti-tumor and anti-inflammatory activities [1]. A series of *ent*-kaurane diterpenoids from *Isodon macrophyllus* (Migo) H. Hara have been previously reported by our research group [2–4]. The continuous research for bioactive diterpenoids from this plant led to the isolation of two new diterpenoids (**1**–**2**) and nine known diterpenoids (**3**–**11**). We now report the isolation and the structure elucidation of these diterpenoids and their biological activities against human tumor DU145 and LoVo cell lines.

2. Results and discussion

Compound **1** exhibited a molecular formula $C_{22}H_{32}O_7$ based on its HRESIMS at *m/z* 431.2049 $[M + Na]^+$. A 6,7-*seco*-6,20-epoxy-1,7-olide-*ent*-kaurane skeleton was evident from the characteristic ^{1}H- and ^{13}C-NMR spectra of **1** showing the presence of two tertiary methyl carbons (δ_C 33.0, 23.7), three non-oxygenated methine carbons (δ_C 53.2, 53.3, and 31.6), three non-oxygenated quaternary carbons (δ_C 57.6, 50.8, and 31.1), an oxygenated methylene carbon (δ_C 74.7), a δ-lactone carbon (δ_C 171.2), a hemiacetal carbon (δ_C 109.1), and a carbonyl carbon (δ_C 212.0). Comparison of the ^{13}C chemical shifts of **1** with those of rabdosin A (**4**) [7] indicated that the difference between them was that the exomethylene in **4** was replaced by a methine at δ_C 56.4 (C-16) and a methoxymethyl group at δ_C 59.0 (OMe) and δ_C 69.2 (C-17) in **1** (Figure 1). The NOESY spectra gave correlation between the H_2-17 and the methoxy proton, which led to the assignment of the methoxy group on C-17. The upfield

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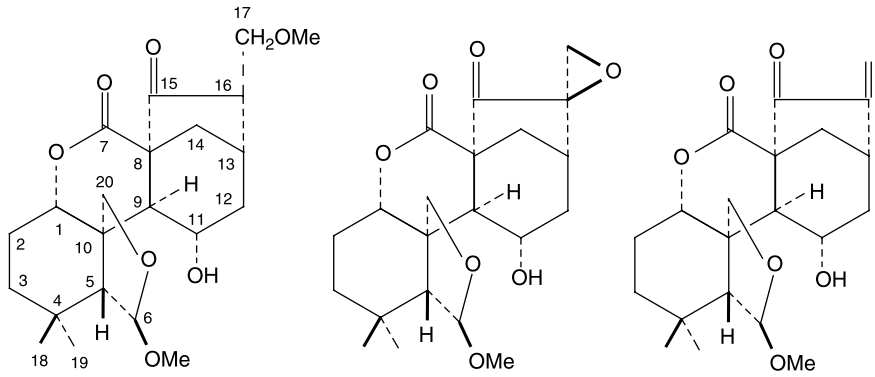

Figure 1. The structures of compounds **1**, **2**, and **4**.

shift of the ^{13}C NMR signal for C-12 (δ_C 33.1 for **1**, δ_C 41.9 for **4**) caused by a γ-gauche shielding effect between MeO-17 and H-12α confirmed the configuration of the methoxymethyl group as α [5]. The above conclusion was also confirmed by the NOESY correlations of H-16β with H-13β (see Figure 2). Therefore, the structure of compound **1** was represented as 11α-hydroxy-6β-methoxyl-16α-methoxymethyl-6, 7-*seco*-6, 20-epoxy-1α, 7-olide-*ent*-kaur-15-one.

Compound **2** was found to have a molecular formula of $C_{21}H_{28}O_7$ by the HRESIMS at *m/z* 415.1735 $[M + Na]^+$. The ^{13}C-NMR spectral data of **2** and **1** were closely coincident, indicating they have the same carbon skeleton. The only difference was that **2** had one oxygenated quaternary carbon (δ_C 75.6) and one oxygenated methylene (δ_C 47.8), instead of a C-16 methoxymethyl unit in **1**. Inspection of the HMBC spectra of **2** showed that the oxygenated methylene proton signals (δ_H 4.46, 4.32) correlated with the quaternary carbon (δ_C 75.6), while the latter carbon signal showed cross-peaks with H-12a (δ_H 2.91) and H-14 (δ_H 2.82, 2.57) (see Table 2). Thus, the epoxy group was assigned to C-16 and C-17, according to the molecular formula of **2** and its unsaturation. The β-orientation of the epoxy ring was ensured by the NOESY correlations of H-17b with H-13β and H-12β (see Figure 2). Therefore, compound **2** was determined to be 11α-hydroxy-6β-methoxyl-6, 7-*seco*-16β, 17: 6, 20-diepoxy-1α,7-olide-*ent*-kaur-15-one.

The structures of compounds **3**–**11** were characterized by comparing their m.p., IR, MS, ^{1}H, and ^{13}C NMR chemical shifts with those reported in literatures [6–13].

Cytotoxicity of compounds **1**–**11** against two kinds of human tumor cells (DU145 and LoVo) was tested according to the previously described method [12]. Compounds **4**, **10**, and

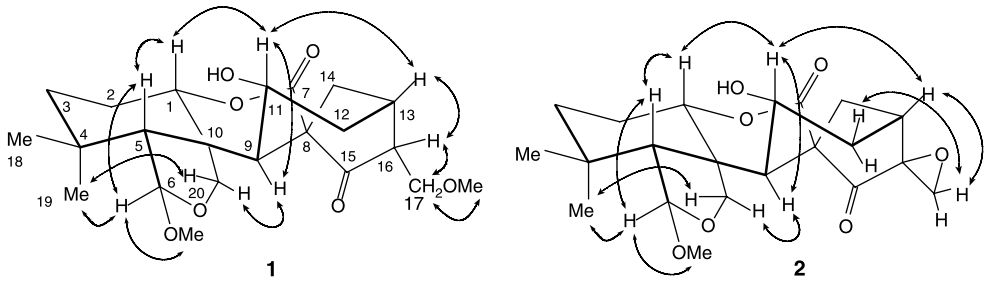

Figure 2. The NOESY correlations of **1** and **2**.

11 were significantly cytotoxic against the DU145 cells with IC_{50} values of 5.90, 4.24, and 3.16 μM, respectively. Compound **11** was most active against the LoVo cells with IC_{50} value of 3.02 μM. The other compounds were almost non-cytotoxic against the two cell lines ($IC_{50} > 20$ μM).

3. Experimental

3.1 General experimental procedures

Melting points were determined with a Kofler melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a Shimadzu UV-2550 instrument. IR spectra were taken on a Nicolet 170SX FT-IR spectrometer. ^1H, ^{13}C, and 2D NMR spectra were recorded on a Bruker AM-400 NMR spectrometer with TMS as internal standard. HRESIMS was obtained on a Waters HPLCQ-Tof HR-MS spectrometer. Silica gel (200–300 mesh) used for column chromatography and silica gel GF_{254} (10–40) for TLC were from Qingdao Marine Chemicals, Qingdao, China. Fractions were monitored by TLC on silica gel and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH (v/v).

3.2 Plant material

The leaves of *I. macrophyllus* (Migo) C.Y.WU. et H. W. Li were collected from Tongbai Prefecture of Henan province, China, in August 2003. The plant material was identified by Prof. Zhong-wen Lin, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (200308-XXMU-Lin) is deposited in the herbarium of the College of Pharmacy, Xinxiang Medical University.

3.3 Extraction and isolation

The dried and powdered leaves (10.0 kg) of *I. macrophyllus* were extracted three times with 70% Me_2CO (3 × 40 l) at room temperature for 3 days and filtered. The filtrate was concentrated and partitioned with EtOAc (4 × 10 l). The EtOAc layer was evaporated under reduced pressure to give 360 g of residue, which was absorbed on 560 g silica gel and subjected to silica gel column (10 × 100 cm, 200–300 mesh, 3000 g) gradiently eluted with $CHCl_3$ (13 l), $CHCl_3$–MeOH (30:1, 10:1, 9:1, 8:2, 7:3, 6:4, 0:1, each in 20 l) to yield eight fractions (1–8). Fraction 1 (31 g) was chromatographed on silica gel column chromatography (5 × 60 cm, 200–300 mesh, 700 g) gradiently eluted with PE-Me_2CO (20:1, 15:1, 10:1, 5:1, each in 4000 ml) to afford compounds **7** (11 mg), **8** (16 mg), **9** (83 mg), and **10** (32 mg). Fraction 2 (104 g) was further separated on a silica gel column chromatography (8 × 80 cm, 200–300 mesh, 1600 g) into six fractions (2a–2f) by eluting with $CHCl_3$–MeOH (1:0, 9:1, 8:2, 7:3, 6:4, 0:1, each in 10 l). Fraction 2b (13 g) was subjected to silica gel column chromatography (4 × 50 cm, 200–300 mesh, 300 g) and eluted with $CHCl_3$–MeOH (100:1, 70:1, 50:1, 25:1, 10:1, each in 2000 ml) to afford three parts. Compound **1** (54 mg) was obtained from the third part by column chromatography with $CHCl_3$–Me_2CO (50:1). Compound **2** (16 mg) was acquired from the first part by column chromatography with $CHCl_3$–Me_2CO (50:1) as eluent. The second part was repeatedly chromatographed by silica gel column and purified by recrystallizing in $CHCl_3$–Me_2CO (10:1) to afford compound **4** (33 mg). Fraction 2d was repeatedly chromatographed over silica gel column and purified by recrystallizing in $CHCl_3$–Me_2CO (10:1) to afford compound **5** (17 mg). Compounds **3** (45 mg), **6** (8 mg), and **11** (5 mg) were obtained from fraction 2c by column chromatography with $CHCl_3$–MeOH (100:1) as eluent and recystallization in MeOH.

3.3.1 Dayecrystal D (*1*)

Colorless needles (Me_2CO), $C_{22}H_{32}O_7$, m.p.: 148–150°C; $[\alpha]_D^{20}$: −18.0 (*c* 0.24, MeOH); IR (KBr) ν_{max}: 3394, 2951, 2914, 2851, 1762, 1726, 1492, 1460, 1390, 1288, 1260, 1196, 1110, 1053, 1026, 973, 922 cm^{-1}; UV λ_{max} (MeOH): no absorption; ^1H-NMR (C_5D_5N,

400 MHz): δ5.00 (1H, s, H-6α), 4.83 (1H, dd, $J = 6.8$, 10.8 Hz, H-1β), 4.35 (1H, m, H-11β), 4.25 (2H, s, H_2-20), 3.69 (1H, dd, $J = 4.4$, 10.4 Hz, H-17a), 3.61 (1H, d, $J = 10.4$ Hz, H-17b), 3.32 (1H, s, H-5β), 3.20 (3H, s, OMe), 3.15 (3H, s, OMe), 2.96 (1H, m, H-16β), 2.76 and 1.94 (each 1H, m, H-14 a/b), 2.73 (1H, m, H-13β), 2.60 and 1.78 (each 1H, m, H-12 a/b), 2.50 (1H, d, $J = 10.8$ Hz, H-9α), 1.83 (2H, m, H_2-2), 1.29 (2H, m, H_2-3), 0.94 and 0.90 (each 3H, s, 2 × Me); ^{13}C-NMR (DEPT) spectral data see Table 1; HRESIMS: *m/z* 431.2049 $[M + Na]^+$ (calcd for $C_{22}H_{32}O_7Na$, 431.2046) (Table 2).

3.3.2 *Dayecrystal E (2)*

Colorless needles (Me_2CO), $C_{21}H_{28}O_7$, m.p.: 270–273°C; $[\alpha]_D^{20}$: −8.2 (*c* 0.40, MeOH); IR (KBr) ν_{max}: 3453, 3290, 2921, 2855, 1770, 1735, 1462, 1315, 1263, 1197, 1108, 1050, 1028, 978, 919, 801 cm^{-1}; UV λ_{max} (MeOH): no absorption; ^{1}H-NMR (C_5D_5N, 400 MHz): δ4.99 (1H, s, H-6α), 4.95 (1H, m, H-1β), 4.46 and 4.32 (each 1H, d, $J = 13.2$ Hz, H-17a/b), 4.41 (1H, m, H-11β), 4.26 and 4.17 (each 1H, ABd, $J = 10.0$ Hz, H_2-20), 3.24 (1H, s, H-5β), 3.06 (3H, s, OMe), 2.91 and 1.98 (each 1H, m, H-12 a/b), 2.86 (1H, m, H-13β), 2.82 and 2.57 (each 1H, m, H-14 a/b), 2.62 (1H, d, $J = 10.4$ Hz, H-9α), 1.85 (2H, m, H_2-2), 1.35 (2H, m, H_2-3), 0.95 and 0.91 (each 3H, s, 2 × Me); ^{13}C-NMR (DEPT) spectral data, see Table 1; HRESIMS: *m/z* 415.1735 $[M + Na]^+$ (calcd for $C_{21}H_{28}O_7Na$, 415.1732).

Table 1. ^{13}C (100 MHz) NMR spectral data of compounds **1**, **2**, and **4** in C_5D_5N (δ in ppm, *J* in Hz).

	1	2	4
1	77.3 d	77.1 d	77.0 d
2	24.1 t	23.6 t	24.2 t
3	37.1 t	36.6 t	37.1 t
4	31.1 s	31.3 s	31.7 s
5	53.2 d	52.9 d	53.3 d
6	109.1 d	109.2 d	109.7 d
7	171.2 s	169.7 s	171.3 s
8	57.6 s	58.3 s	57.2 s
9	53.3 d	54.4 d	53.2 d
10	50.8 s	51.0 s	51.4 s
11	64.0 d	63.1 d	64.4 d
12	33.1 t	31.4 t	41.9 t
13	31.6 d	41.2 d	35.8 d
14	35.1 t	34.2 t	33.9 t
15	212.0 s	204.0 s	201.0 s
16	56.4 d	75.6 s	151.0 s
17	69.2 t	47.8 t	118.1 s
18	33.0 q	32.5 q	33.1 q
19	23.7 q	23.3 q	23.6 q
20	74.7 t	74.2 t	74.5 t
OMe	54.7 q	54.4 q	54.8 q
OMe	59.0 q		

Table 2. HMBC correlations of compounds **1** and **2**.

No.	1	2
C-1	H-2a/b, 3a/b, 20a/b	H-3a/b, 9α, 20a
C-2	H-1β, 3a/b	H-1β, 3a/b, 5β
C-3	H-18, 19	H-18, 19
C-4	H-2a/b, 3a/b 5β, 6α, 18, 19	H-5β, 6α, 18, 19
C-5	H-1β, 3a/b, 9α, 18, 19, 20a/b	H-6α, 18, 19, 20a/b
C-6	H-3a/b, 5β, OMe, 20a/b	H-5β, OMe, 20a/b
C-7	H-9α, 14b	
C-8	H-9α, 14b	H-9α, 13β
C-9	H-1β, 5β, 12b, 14a/b, 20a/b	H-1β, 5β, 12b, 14a
C-10	H-2a/b, 5β, 6α, 9α, 20a/b	H-1β, 6α, 9α, 20a/b
C-11	H-9α, 12a, 13β, 20a/b	H-6α, 9α, 12a/b, 13β
C-12	H-9α, 14b	H-9α, 13β, 14b
C-13	H-12a, 14a, 16β, 17a/b	H-12a/b, 14a, 17b
C-14	H-12a, 16β	H-12a
C-15	H-9α, 12b, 14a, 16β, 17a/b	H-9α, 13β, 14a/b
C-16	H-12a, 13β, 14a, 17a/b	H-12a, 13β, 14a/b, 17a/b
C-17	H-16β, OMe	
C-18	H-3a/b, 5β, 19	H-5β, 19
C-19	H-3a/b, 5β, 18	H-5β, 18
C-20	H-1β, 5β, 6α, 9α	H-1β, 5β, 6α, 9α
OMe	H-6α	H-6α
OMe	H-17a/b	

3.4 *Tests of cytotoxicity against human tumor DU145 and LoVo cells*

Previously described bioassay methods were adopted [14].

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