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Two new diterpenoids and other constituents from *Isodon rubescens*

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ABSTRACT

Two new *ent*-kaurene diterpenoids, 15α-acetoxyl-6,11α-epoxy-6α-hydroxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7-olide (**1**), 15α-hydroxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7α(6,11α)-diolide (**2**), together with ten known compounds (**5**–**14**) were isolated from the leaves of *Isodon rubescens*. Their structures were elucidated mainly by various spectroscopic techniques and finally confirmed by single-crystal X-ray diffraction. Compounds **1**, **2**, **8** and **12** were evaluated for their cytotoxicities against EC-1, U87, A549, MCF-7 and Hela cell lines.

1. Introduction

The genus *Isodon* (Labiatae) was widely distributed in China, the leaves of which have been shown to contain some *ent*-kaurene type diterpenoids with cytotoxic activities [1–3]. In a continuation of our research work on diterpenoids, we have recently investigated *Isodon rubescens* (Hemsl.) Hara collected from the northern Henan Province of China. From the AcOEt extract of the leaves, two new *ent*-kaurene diterpenoids (**1** and **2**) and ten known diterpenoid compounds (**5**–**14**) were isolated (Fig. 1). In addition, four of the compounds (**1**, **2**, **8** and **12**) were tested for their cytotoxicity towards cancer cells EC-1, U87, A549, MCF-7 and Hela. This report describes the structure determination of compounds **1** and **2**.

2. Experimental

2.1. General

Melting points were measured using an uncorrected X-4 Digital Display micromelting point apparatus. Optical rotations were taken on a Perkin-Elmer 341 polarimeter. IR spectra were obtained on a Nicolet 170 SX FT-IR spectrometer. ^{1}H, ^{13}C and 2D NMR spectra were measured on a Bruker AM-400 NMR spectrometer using TMS as the internal standard. HR-ESI-MS were recorded on a Waters HPLCQ-Tof HR-MS spectrometer. Column chromatography were carried out on silica gel (200–300 mesh) and TLC on silica gel (GF_{254}, 10–40 μm), and both materials were supplied by Qingdao Marine Chemical Co. 5-Fluorouracil used as a positive control was supplied by Shanghai Xudong pharmaceutical Co. Ltd, (Shanghai, China). Spots were detected on TLC under UV or by heating after spraying with 5% H_2SO_4 in C_2H_5OH.

2.2. Plant material

The plant material, *I. rubescens* (Hemsl.) Hara was collected in Huixian of Henan province, P. R. China, in Aug. 2008, and identified by Professor Changshan Zhu, Henan Agriculture University. A voucher specimen (No. 20080706) is deposited in Pharmacy College, Xinxiang Medical University.

2.3. Extraction and isolation

The air-dried leaves of *I. rubescens* (15 kg) were pulverized and extracted four times with Me_2CO/H_2O (7:3 v/v) at room

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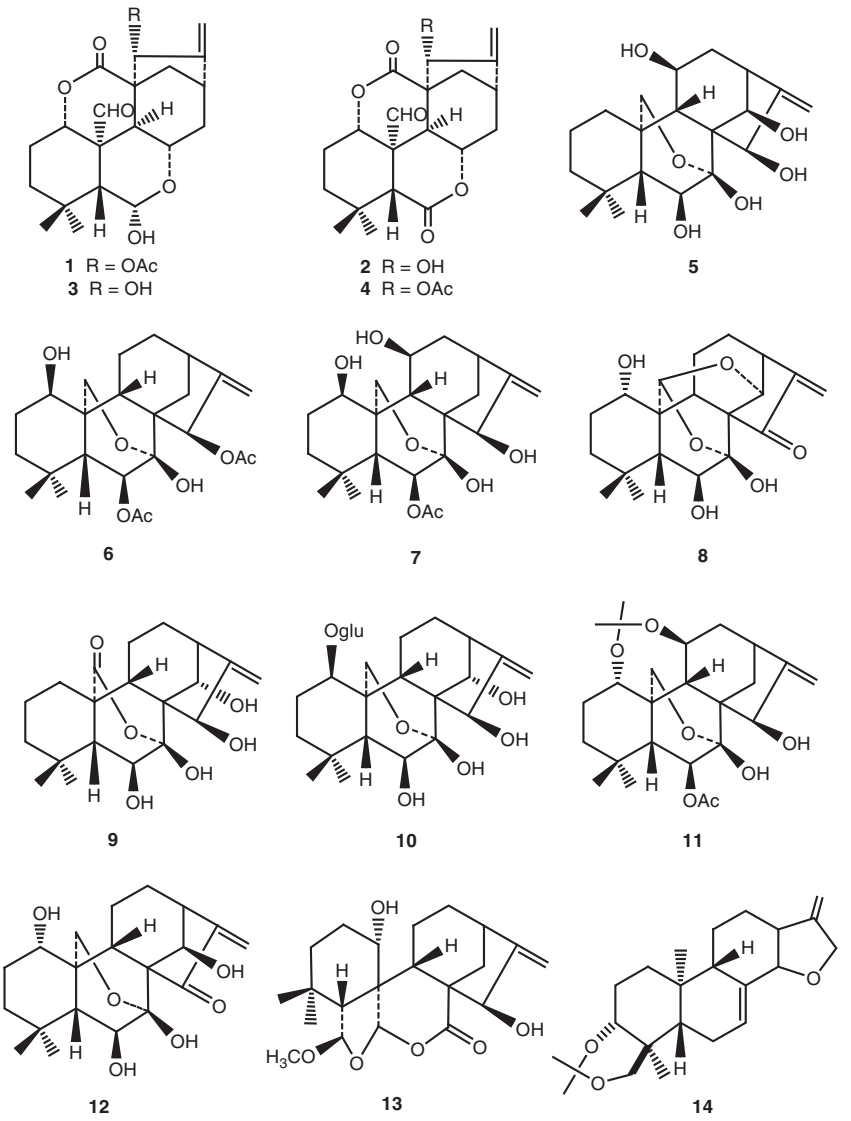

Fig. 1. The molecular structures of compounds **1**–**14**.

temperature for 6 days and filtered. The filtrate was concentrated and partitioned successively between AcOEt and H_2O. The AcOEt extract (310 g) subjected to silica gel column (12 × 150 cm, 3000 g, 200–300 mesh) was gradiently eluted with a system of $CHCl_3$—CH_3OH (1:0, 30:1, 20:1, 10:1, 5:1 3:1) to give six factions according to their TLC analysis. From the faction 2 ($CHCl_3$—CH_3OH 30:1) compounds **1** (96 mg), **2** (29 mg) and **6** (56 mg) were obtained by repeated column chromatography over silica gel developing with $CHCl_3$—Me_2CO (20:1, 30:1) and followed by recrystallization. From the faction 3 ($CHCl_3$—CH_3OH 20:1) compounds **5** (14 mg), **7** (45 mg), **8** (60 mg), **9** (67 mg), **11** (14 mg), **12** (230 mg), **13** (110 mg) and **14** (10 mg) were isolated by further purified on silica gel column eluting with $CHCl_3$—MeOH (30:1) and $CHCl_3$—Me_2CO (15:1, 20:1). From the faction 5 ($CHCl_3$—CH_3OH 5:1) compound **10** (110 mg) was separated by repeated column chromatography over silica gel eluted with $CHCl_3$—MeOH (8:1, 10:1) and $CHCl_3$—$(Me)_2CHOH$ (10:1).

2.4. Spectroscopic data

15α-acetoxyl-6,11α-epoxy-6α-hydroxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7-olide (**1**). Colorless crystals from Me_2CO; m.p. 281–283 °C, $[\alpha]_D^{20} -173.1$ (*c* 0.54, Me_2CO); IR (KBr): $\square^{max} = 3516$, 2987, 2967, 2940, 2874, 1743, 1715, 1700, 1660, 1373, 1181, and 919 cm^{-1}; HR-ESI-MS: *m/z* 427.1735 $[M+Na]^+$, requires *m/z* 427.1733 for $C_{22}H_{28}O_7Na$. ^1H- and ^{13}C-NMR data: see Table 1.

15α-hydroxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7α(6,11α)-diolide (**2**). Colorless needles from Me_2CO; m.p. 260–262 °C, $[\alpha]_D^{23} -135.7$ (*c* 0.28, Me_2CO); IR (KBr): $\square^{max} = 3375$, 1754, 1720, 1700, 1365, 1320, 1077, and

Table 1
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compounds **1** and **2** in pyridine-d_5(δ in ppm).

Position	1 δ_H	1 δ_C	2 δ_H	2 δ_C
1	4.55 (dd, J = 4.0, 6.8 Hz)	77.4 d	4.70 (dd, J = 3.2, 11.2 Hz)	76.8 d
2	1.71,1.99 m	25.8 t	2.02,2.36 m	25.1 t
3	1.42, 2.77 m	40.7 t	1.43 m	39.6 t
4		33.5 s		33.0 s
5	1.91 s	50.1 d	2.84 s	50.8 d
6	5.85 s	92.3 d		169.7 s
7		172.1 s		173.7 s
8		48.7 s		50.8 s
9	3.27 (d, J = 11.6 Hz)	35.0 d	3.14 (d, J = 11.6 Hz)	38.9 d
10		50.6 s		50.4 s
11	4.29 (dt, J = 8.8, 11.2 Hz)	62.6 d	5.02 (dt, J = 8.8,11.2 Hz)	70.2 d
12	1.67,2.71 m	39.5 t	1.71, 2.65 m	37.7 t
13	2.78 m	37.7 d	2.81 m	37.2 d
14	1.14,2.21 (d, J = 12.0 Hz)	32.3 t	1.82,2.10 m	32.0 t
15	6.66 s	80.8 d	5.47 s	81.6 d
16		153.7 s		157.3 s
17	5.03,5.11 brs	111.0 t	5.20, 5.44 brs	110.6 t
18	0.95 s	30.3 q	1.07 s	22.2 q
19	1.15 s	22.1 q	1.14 s	30.2 q
20	10.68 s	202.8 d	10.07 s	199.5 d
21		169.9 s		
22	1.96 s	20.5 q		

898 cm^{-1}; HR-ESI-MS: *m/z* 383.1473 $[M+Na]^+$, requires *m/z* 383.1471 for $C_{20}H_{24}O_6Na$. ^{1}H- and ^{13}C-NMR data: see Table 1.

2.5. Cytotoxicity assay

Cytotoxic activities of the isolated compounds **1**, **2**, **8** and **12** were evaluated using the standard MTT colorimetric method [4]. The cell lines used in this experiment were EC-1, U87, A549, MCF-7 and Hela, and 5-fluorouracil was used as the reference compound.

3. Results and discussion

Compound **1** was obtained as colorless crystals, showed the quasi-molecular ion peak at *m/z* 427.1735 $[M+Na]^+$(caled. 427.1733) in its HR-ESI-MS, corresponding to the molecular formula $C_{22}H_{28}O_7$. The IR (KBr) spectrum exhibited the presence of carbonyl groups (1700, 1715 and 1743 cm^{-1}) and a hydroxyl group (3516 cm^{-1}). The fact that no absorption was recorded in the UV spectrum indicated the absence of any conjugated systems in the molecule. A singlet at δ 1.96 (3 H, s) in the ^{1}H NMR spectrum and two signals at δ 169.9 (s) and 20.5 (q) in the ^{13}C NMR spectrum exhibited the presence of an acetoxyl group. Comparison of other NMR data with those of known compound 6α,15α-dihydroxy-20-oxo-6,7-seco-6,11α-epoxy-1,7-olide-*ent*-kaur-16-ene (**3**) [5], indicated that **1** had the similar skeleton of 6,11α-epoxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7-olide as **3**. The only difference between **1** and **3** was that **1** had one more acetoxyl group. However, some differences were also observed. The signals at δ 5.85 (1H, s, H-15β) and δ 80.2 (d, C-15) in **3** were shifted downfield to δ 6.66 (1H, s, H-15β) and δ 80.8 (d, C-15) in **1**, respectively. Moreover, the signals at δ 49.8 (s, C-8) and δ 158.8 (s, C-16) in **3** were shifted upfield to δ 48.7 (s, C-8) and δ 153.7 (s, C-16) in **1**. These changes of chemical shift value suggested that the position of acetoxyl group should be at C-15. The HMBC correlations of H-22 (δ 1.96, s) and H-15 (δ 6.66, s) with C-21 (δ 169.9 s) further confirmed the above assignment. The relative configuration of 1 was determined by the analysis of a NOESY experiment. In the NOESY spectrum, there were correlations of H-11 (δ 4.29 dt) with H-5 (δ 1.91 d) and H-6 (δ 5.85 s); H-15 (δ 6.66 s) with H-14 (δ 2.21 m) but no correlation with H-9 (δ 3.27 d). Thus, OH-6 and AcO-15 have the α orientation, respectively. On the basis of the above evidence, the structure of compound **1** was determined to be 15α-acetoxyl-6,11α-epoxy-6α-hydroxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7-olide, and named rubescrystal A (Fig. 1).

The single crystal X-ray diffraction analysis confirms the molecular structure of **1** proposed by spectroscopic methods. The crystal data were as follows: Colorless block crystal from Me_2CO, crystal size: 0.50 × 0.43 × 0.40 mm; crystal data: a = 10.669(3), b = 12.546(3), c = 14.710(3) Å, V = 1969.1(8)Å^3, space group $P2_12_12_1$, Z = 4, D_{cacl} = 1.364 g/cm^3, λ = 0.71073 Å, μ(Mokα) = 0.10 mm^{-1}, F(000) = 864, and T = 103(2) k; a total of 15561 reflections were collected on a AFC10/Saturn724+ with a rotating anode tube. Refinement with 2552 reflections (2399 with I > 2σI) led to final R, R(all), GOF values of 0.0354, 0.0390, and 0.998. (Fig. 2). The crystal structure has been deposited at the *Cambridge Crystallographic Data Centre* and allocated the deposition number CCDC 814320.

Compound **2** was obtained as colorless crystal. The molecular formula of $C_{20}H_{24}O_6$ was deduced from the HR-ESI-MS (*m/z* 383.1473 $[M+Na]^+$). Its IR spectrum showed the absorption bands for carbonyl groups (1754, 1720 and 1700 cm^{-1}) and a hydroxyl group (3375 cm^{-1}). The ^{13}CNMR and DEPT spectra of **2** showed 20 carbon signals which were indicated to be composed of two lactonic carbonyl carbons, one aldehyde group, four quaternary carbon (including an olefinic one), six methines including three oxygenated ones, five methylenes including one olefinic one, and two methyls, which suggested **2** as an *ent*-kauranoid, combined with the consideration of the similar structure of Glaucocalactone (**4**) [6] previously isolated from this genus. The difference between these two compounds was

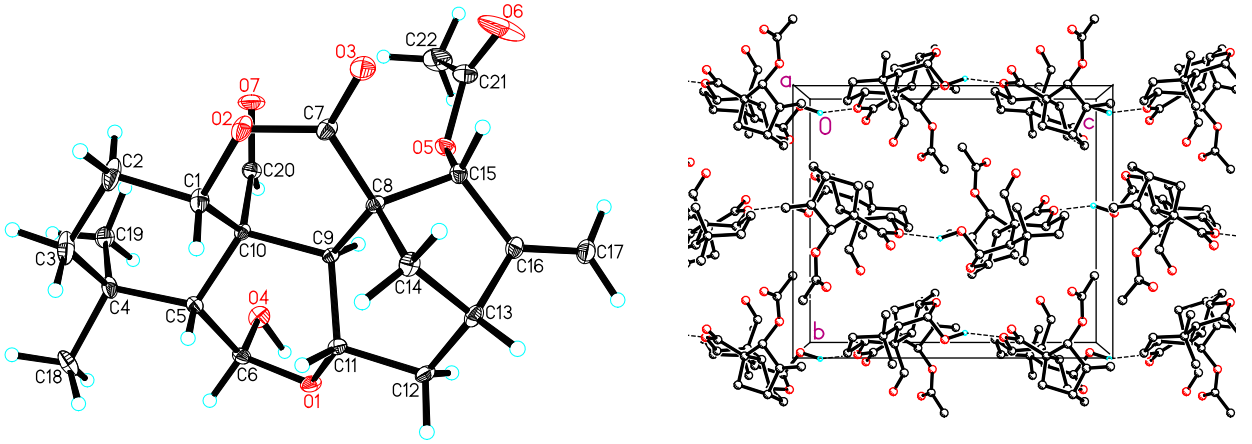

Fig. 2. The crystal structure of compound **1**.

the group at C-15 position. There was a hydroxy group at C-15 in **2** instead of an acetoxyl group in 4. In the NOESY spectrum, H-15 (δ 5.47 s) showed correlation with H-14 (δ 1.82 m) but no correlation with H-9 (δ 3.14 d), it showed that the OH-15 was in α-orientation. Therefore, the structure of **2** was determined to be 15α-hydroxy-20-oxo-6,7-seco-*ent*-kaur-16-en-1,7α(6,11α)-diolide, and named rubescrystal B (Fig. 1).

Its structural assignment including its relative stereochemistry was finally confirmed by X-ray analysis. The crystal data were as follows: Colorless crystal from Me_2CO, crystal size: 0.55×0.21×0.09 mm; crystal data: a=9.453(7), b=6.693(5), c=16.805(13) Å, V=1049.5(14) Å ^{3}, space group $P2_1$, Z=2, D_{cacl}=1.324 g/cm^3, λ=0.71073 Å, μ(Mokα)=0.097 mm^{-1}, F(000)=448, and T=163(2) k; a total of 8093 reflections were collected on a AFC10/Saturn724+ with a rotating anode tube. Refinement with 2552 reflections (1975 with I>2σI) led to final R, R(all), GOF values of 0.0527, 0.0650, and 1.001. (Fig. 3). The crystal structure has been deposited at the *Cambridge Crystallographic Data Centre* and allocated the deposition number CCDC 814321.

The known compounds **5**–**14** were identified by comparison of their NMR data with literature values. They were identified as Rubescensin C (**5**) [7], Trichokaurin (**6**) [8], Maoyecrystal F (**7**) [**9**], Ponicidin (**8**) [10], Rabdoternin A (**9**) [11], 1α-O-β-D-gluco-pyranosyl enmenol (**10**) [12], Acetonide (**11**) [13], Oridonin (**12**) [14], Rabdonervosin B (**13**) [15], Acetonide of rubescensin J (**14**) [16].

From the anti-tumor activity data, it was found that compounds **8** and **12** have a significant inhibition on cells EC-1, U87, A549, MCF-7 and Hela, compound **2** has inhibition on cells

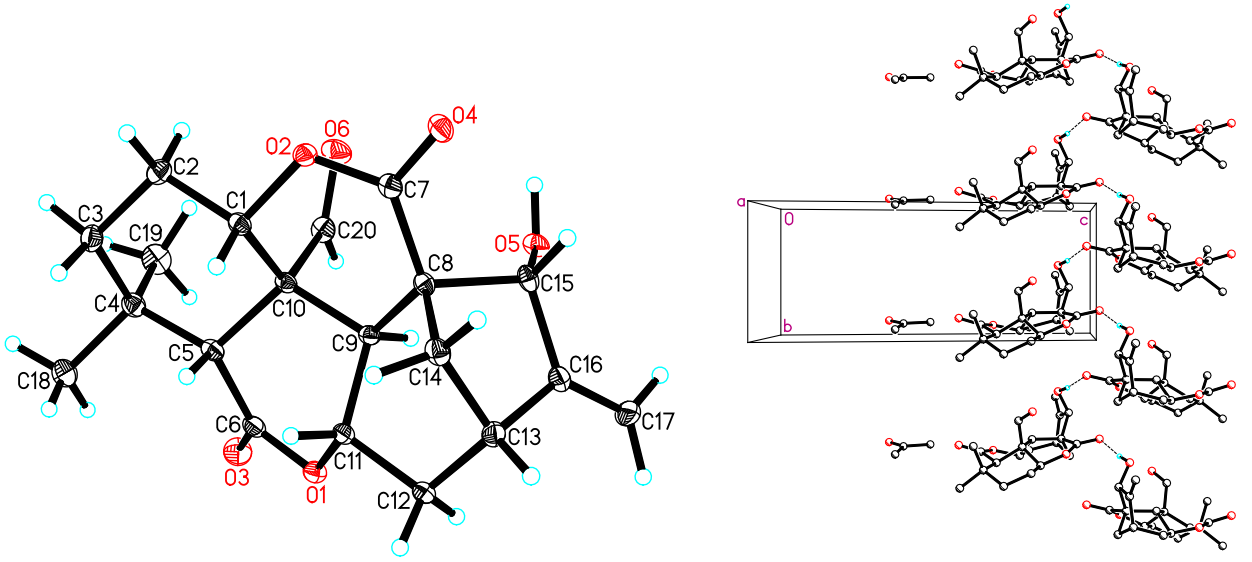

Fig. 3. The crystal structure of compound **2**.

Table 2
In vitro cytotoxicity data of compounds **1**, **2**, **8** and **12** by the MTT assay.

Compound	Cell lines/IC_{50} (μM)					
	EC-1	MCF-7	Hela	K562	A549	U87
1	678.19 ± 39.25	563.90 ± 47.38	365.17 ± 13.48	238.16 ± 15.07	784.39 ± 25.97	487.25 ± 9.01
2	37.69 ± 3.62	79.62 ± 6.33	80.07 ± 6.32	197.35 ± 2.10	462.13 ± 39.06	180.09 ± 11.44
8	43.87 ± 3.05	16.85 ± 2.63	28.14 ± 1.05	5.12 ± 0.46	73.33 ± 5.84	32.19 ± 4.07
12	66.25 ± 3.17	40.78 ± 3.26	37.59 ± 2.00	9.78 ± 1.78	188.75 ± 6.52	80.79 ± 3.40
5-fluorouracil	5.90 ± 0.49	21.08 ± 1.07	300.7 ± 11.28	108.66 ± 5.76	120.36 ± 3.79	28.41 ± 4.55

EC-1, MCF-7 and Hela, respectively; and compound **1** exhibited no significant inhibitory effects against selected cancer cells with IC_{50} values over 100 μM. Cytotoxicity data: see Table 2.

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