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THREE NEW *ENT*-KAURANOIDS FROM *ISODON PHYLLOSTACHYS*

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Abstract – Three new *ent*-kaurane diterpenoids, phyllostacins C–E (**1**–**3**), together with six known analogues, oridonin (**4**), ponicidin (**5**), effusanin A (**6**), rabdoternin E (**7**), rabdoternin F (**8**), and macrocalyxoformin E (**9**), were isolated from the aerial parts of *Isodon phyllostachys*. The structures of compounds (**1**–**3**) were determined on the basis of spectroscopic methods including extensive 1D and 2D NMR analysis. All compounds were evaluated for their inhibitory activity against K562, HL60 and HepG2 (hepatoma) cell lines.

In previous papers, we have been reported the isolation and biological activity of a series of *ent*-kauranoids in *I. phyllostachys*,[1-5] a shrubbery plant that has been used as an antiphlogistic and antibiotic agent in China, collected from different area. As part of a program aimed at searching for bioactive substances from this species, continuing study on the *I. phyllostachys*, which was collected in Zhongdian County of Yunnan Province, China, led to the isolation nine *ent*-kauranoids including three new compounds, phyllostacins C–E (**1**–**3**), along with six known analogues, oridonin (**4**),[6] ponicidin (**5**),[7] effusanin A (**6**),[8] rabdoternin E (**7**),[9] rabdoternin F (**8**),[9] and macrocalyxoformin E (**9**).[10] All compounds were evaluated for inhibitory activity against K562, HL60 and HepG2 cell lines. In this paper, the isolation, structure elucidation of the new compounds and the results of bioactive testing are described.

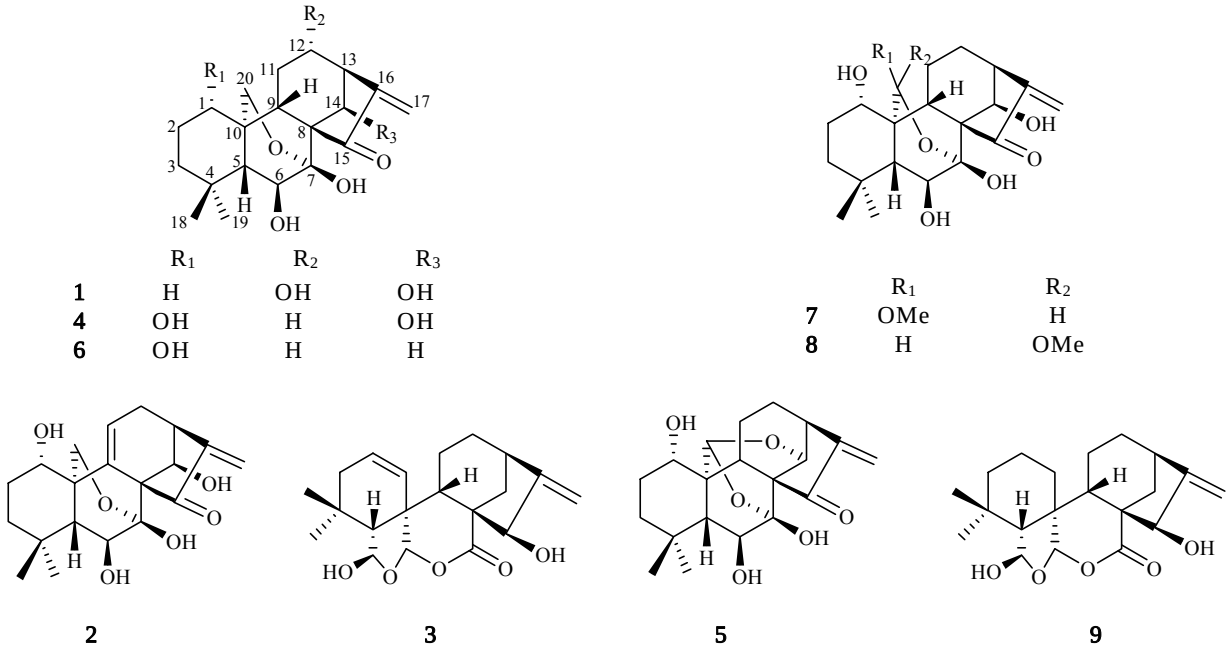

Chart 1

RESULTS AND DISCUSSION

Phyllostacin C (**1**) was isolated as an amorphous powder. The HR-ESIMS measurement gave the molecular ion peak $(M+Na)^+$ at *m/z* 387.1768, corresponding to the molecular formula $C_{20}H_{28}O_6$ (calcd 387.1783) with seven degrees of unsaturation. It comprised a five-membered ring ketone group conjugated with an *exo*-methylene group as a partial structure from the following spectral data: UV (MeOH) λ_{max} (log ϵ) 231 (3.92) nm; IR (KBr) 1714 and 1649 cm^{-1}; ^{1}H-NMR δ 6.36 and 5.55 (each 1H, br s); ^{13}C-NMR δ 208.2 (s), 148.8 (s) and 121.6 (t). In addition, on the basis of the characteristic signals of three methines [δ 60.8, 49.1, 55.9 (C-5, 9, 13)], three quaternary carbons [δ 34.0, 62.6, 36.5 (C-4, 8, 10)], a hemiketal quaternary carbon [δ 98.4 (C-7)], and a noticeable oxygenated methylene [δ 66.2, 4.14 and 4.02 (C-20, each 1H, d, J = 9.9 Hz)] were observed in the ^{13}C-NMR and DEPT spectra, along with 20 signals for the carbons of the diterpenoid skeleton, we assumed that **1** should be a 7β-hydroxy-7α,20-epoxy-*ent*-kaur-16-en-15-one diterpenoid, substituted by three hydroxyl groups, similar to oridonin (**4**).

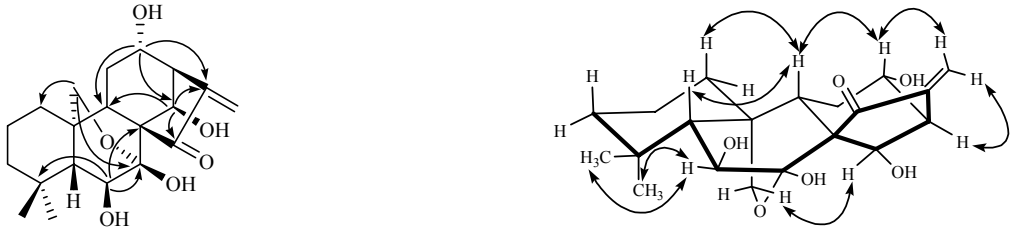

Figure 1. Key HMBC and ROESY correlations of compound (1)

Comparison of the NMR and MS spectra of **1** with those of **4** indicated that the only difference was that

the substitution of one hydroxyl group was at δ 74.2 (C-12) in **1** while it was at δ 74.6 (C-1) in **4**. This was supported from the chemical shifts δ 43.8 (C-13) in **4** downfield to δ 55.9 (C-13) in **1** for the effect of the hydroxyl group at C-12. And it was further confirmed by the HMBC cross-peaks between H-12 with C-9, 14 and 16, respectively (Figure 1). The correlations of H-6α with Me-19, of H-12β with H-9β, H-13α and H-17b, and of H-14α with H-20a in the ROESY spectrum of **1** (Figure 1) deduced that the hydroxyl groups at C-6, C-12 and C-14 were shown to be β, α and β orientations. Thus, compound (**1**) was determined to be 6β,7β,12α,14β- tetrahydroxy-7α,20-epoxy-*ent*-kaur-16-en-15-one.

Table 1. ^{1}H- and ^{13}C-NMR Data for Compounds (**1–3**)

	1			**2**		**3**	
	δ_C	δ_H (mult; *J*, Hz)		δ_C	δ_H (mult; *J*, Hz)	δ_C	δ_H (mult; *J*, Hz)
1	30.8	0.85, m	71.5	4.08, (dd, 11.8, 4.7)		126.8	5.87, m, (overlap)
2	19.0	1.35, m	29.5	1.73, m		130.1	5.87, m, (overlap)
				1.85, m			
3	41.5	H_α: 1.24, m	39.8	1.41, m		36.1	2.09, m
		H_β: 1.10, m					
4	34.0		33.7			30.3	
5β	60.8	1.38, br s	52.3	1.78, (d, 9.0)		61.3	2.11, br s
6	74.2	4.23, m, (overlap)	75.3	4.41, (dd, 11.9, 9.2)		103.0	5.75, (d, 6.7)
7	98.4		99.6			174.2	
8	62.6		65.0			52.0	
9β	49.1	1.63, m	140.1			35.2	3.07, (d, 6.7)
10	36.5		44.7			52.8	
11	28.4	1.33, m	120.6	6.30, br s		21.2	1.66, m
12	74.2	4.23, m, (overlap)	35.7	2.78, m		33.7	1.64, m
				2.25, m			
13α	55.9	3.55, br s	44.5	3.22, br s		40.1	2.75, br s
14	72.2	5.53, br s	76.7	4.93, br s		33.5	1.61, m
15	208.2		203.3			76.3	5.67, br s
16	148.8		151.9			156.7	
17	121.6	H_a: 6.36, br s	120.4	H_a: 6.23, br s		106.2	H_a: 5.50, br s
		H_b: 5.55, br s		H_b: 5.63, br s			H_b: 5.14, br s
18	33.6	1.02, s	34.3	1.31, s		30.5	1.11, s
19	22.4	1.22, s	23.6	1.14, s		29.3	0.94, s
20	66.2	H_a: 4.14, (d, 9.9)	68.1	H_a: 4.54, (d, 8.6)		105.8	6.04, s
		H_b: 4.02, (d, 9.9)		H_b: 4.47, (d, 8.6)			

* Assignments were based on the analysis of DEPT, HMQC, HMBC and ROESY spectra.

Phyllostacin D (**2**), a white amorphous powder, was assigned the molecular formula $C_{20}H_{26}O_6$ from HR-ESIMS and NMR with eight degrees of unsaturation. Its IR and NMR data suggested **2** also to be a 6β,7β-dihydroxy-7α,20-epoxy-*ent*-kaur-16-en-15-one diterpenoid, to which one trisubstituted double bond [δ 140.1 (s), 120.6 (d), 6.30 (1H, br s)] and two hydroxyl groups were introduced. Through careful analysis of HMBC spectrum, the correlations of H-5β, H-14α and H-20 with C-9, and of H-11 with C-8, 10 and C-13 enabled us to locate the trisubstituted double bond between C-9 and C-11. The other two hydroxyl groups to the C-1α and C-14β positions were determined on the basis of the HMBC correlations of H-1 with C-5, C-9 and C-20, and of H-14 with C-9, C-15 and C-16, and the ROESY cross-peaks of

H-1β with H-5β and of H-14α with H-20a. Consequently, **2** was determined to be 1α,6β,7β,14β-tetrahydroxy-7α,20-epoxy-*ent*- kaur-9,16-dien-15-one.

Phyllostacin E (**3**) was obtained as an amorphous powder, and the molecular formula was established to be $C_{20}H_{26}O_5$ by its HR-ESIMS data ($(M+Na)^+$, *m/*z 369.1668). Careful analysis of the 1D and 2D NMR spectra of **3** and considering the structures of diterpenoids previously isolated from the genus *Isodon*, along with one characteristic lactonic carbonyl signal at δ 174.2 assignable to C-7 and three oxymethines [one at δ 76.3, 5.67 (1H, br s) attributable to C-15 and H-15, δ 103.0, 5.75 (1H, d, J = 6.7 Hz) assignable to C-6 and H-6 and the other resonating relation at δ 105.8, 6.04 (1H, s) ascribable to C-20 and H-20], compound (**3**) should possess the skeleton of 6,7-*seco*-6,20-epoxy-7,20-olide-*ent*-kauranoid. Comparison of the NMR data of **3** with those of macrocalyxoformin E (**9**) suggested that **3** resembled **9**, except for the additional existence of one inner disubstituted double bond which was assigned between C-1 and C-2 unambiguously for the signal δ 48.9 (C-10) in **9** downfield to δ 52.8 (C-10) in **3** due to the effect of this double bond, which was confirmed by the HMBC results (Figure 2). The β-orientation of OH-6 group was evident from the ROESY correlations of H-6α with H-20. And the β-orientation of OH-15 was determined by the significant upfield signal of δ 35.2 (C-9), which was caused by the γ-gauche steric compression effect between the OH-15 group and H-9β. Therefore, **3** was assigned to 6β,15β-dihydroxy-6,7-*seco*-6,20-epoxy-7,20α-olide-*ent*-kaur-1,16-dien.

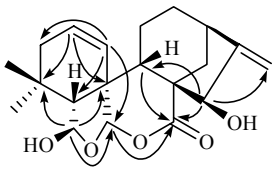

Figure 2. Key HMBC correlations of compound (3)

Compounds (**4**–**9**) were known compounds, whose structures were elucidated by comparisons with the literature.

Table 1. Inhibitory effects of all compounds against K562, HL60 and HepG2 cell lines

compound	IC_{50}(μg/mL)			compound	IC_{50}(μg/mL)		
	K562	HL60	HepG2		K562	HL60	HepG2
cis-platin	2.93	0.21	1.47		3.52	1.88	2.12
1	> 100	> 100	> 100	**6**	4.12	1.52	2.58
2	> 100	> 100	> 100	**7**	3.10	0.28	8.2
	28.53	18.56	15.58	**8**	0.12	0.02	0.39
4	3.28	2.47	3.58	**9**	> 100	> 100	> 100

Compounds (**1**–**9**) were tested for cytotoxicity against K562, HL60 and HepG2 cell lines using the

method described in the literature.[11] Compound (**8**) exhibited significant inhibitory activity against those tumor cell lines with IC_{50} values of 0.12, 0.02 and 0.39 μg/mL, respectively. Compounds (**3**–**7**) showed less activity, while compounds (**1**–**2**) and (**9**) displayed noncytotoxicity with IC_{50} values of > 100 μg/mL. Although compounds (**1**–**2**) have an active center of cyclopentanone conjugated with an exomethylene group, these two compounds were completely inactive which suggests that the substituents in the molecules also affect their cytotoxicity.

EXPERIMENTAL

General Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. IR spectroscopy was scanned by a Tenor27 spectrophotometer using KBr pellets. MS spectra were performed on a VG Autospec-3000 spectrometer and a Finnigan MAT 90 instrument. 1D and 2D NMR spectra were recorded on Bruker AM-400 and DRX-500 spectrometers with TMS as internal standard. The chemical shifts (δ) were expressed in ppm with reference to the solvent signals. Semipreparative HPLC was performed on an Agilent 1100 apparatus equipped with a UV detector and a Zorbax SB-C-18 column. Column chromatography was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, P. R. China), Lichroprep RP-18 gel (40–63 μm, Merk, Darmstadt, Germany) and MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Japan).

Plant Material The aerial parts of *I. phyllostachys* were collected in Zhongdian County of Yunnan Province, P.R. China, in August 2004 and identified by Prof. Xi-wen Li. A voucher specimen (KIB 04081916) was deposited in State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation The air-dried and powdered aerial parts of *I. phyllostachys* (3.5 kg) were extracted with 70% acetone (3 × 10 L, 24 h) at rt and filtered. The filtrate was evaporated to give a residue, which was suspended in H_2O (2.5 L) and then extracted with EtOAc (3 × 3 L). The EtOAc extract (95.0 g) was decolorized on MCI gel, eluted with 90% MeOH–H_2O, to yield an yellowish gum (80 g). The gum was subjected to column chromatography over a silica gel (200-300 mesh, 1.0 kg) column, eluted with $CHCl_3$–Me_2CO (1:0–0:1 gradient system), to obtain fractions A–E. Fraction B (9.5 g) provided **1** (10 mg) and **4** (820 mg) after being chromatographed over silica gel developing with $CHCl_3$–MeOH (40:1). Fraction C (10.0 g) was separated by RP–18 (40%–90% gradient system) to provide fractions C_1–C_2. Fraction C_1 was separated by repeated column chromatography (silica gel, petroleum ether–EtOAc 3:2) to afford **5** (20 mg), **6** (60 mg). Fraction C_2 provided **3** (20 mg) and **9** (8 mg) after being chromatographed over silica gel developing with petroleum ether–EtOAc (3:2), followed by semipreparative HPLC with 35% MeOH–H_2O. Fraction D (14.0 g) was passed through RP–18 to yield

two main fractions D_1–D_2, eluted with 40%–90% gradient system. Fraction D_1 was subjected to a silica gel column with a gradient elution ($CHCl_3$–2-propanol, 30:1–10:1), followed by semipreparative HPLC (40% MeOH–H_2O) to isolate **7** (25 mg) and **8** (33 mg). Fraction D_2 was purified by a silica gel column eluted with $CHCl_3$–MeOH (20:1), followed by semipreparative HPLC (38% MeOH–H_2O) to get **2** (6 mg).

Compound (**1**): white amorphous powder; $[\alpha]_D^{22}$ –12.5° (*c* 0.826, C_5H_5N); UV (MeOH) λ_{max} nm (log ε): 231 (3.92); IR (KBr): 3397, 3227, 2936, 1714, 1649, 1064 cm^{-1}; HR-ESI-MS *m/z*: 387.1768 ($(M+Na)^+$, calcd 387.1783 for $C_{20}H_{28}O_6$); ^1H- and ^{13}C-NMR: see Table 1.

Compound (**2**): white amorphous powder; $[\alpha]_D^{23}$ +61.1° (*c* 0.590, C_5H_5N); UV (MeOH) λ_{max} nm (log ε): 223 (3.95); IR (KBr): 3395, 1713, 1644, 1454, 1021, 915, 747 cm^{-1}; HR-ESI-MS *m/z*: 385.1640 ($(M+Na)^+$, calcd 385.1627 for $C_{20}H_{26}O_6$); ^1H- and ^{13}C-NMR: see Table 1.

Compound (**3**): white amorphous powder; $[\alpha]_D^{23}$ –204.8° (*c* 0.420, C_5H_5N); UV (MeOH) λ_{max} nm (log ε): 203 (3.87); IR (KBr): 3476, 3401, 2949, 1738, 1632, 1251, 1054 cm^{-1}; HR-ESI-MS *m/z*: 369.1668 ($(M+Na)^+$, calcd 369.1677 for $C_{20}H_{26}O_5$); ^1H- and ^{13}C-NMR: see Table 1.

Cytotoxicity experiments Cytotoxicity evaluation was performed by MTT method for the human tumor K562 cells. Briefly, 4×10^4/ml cells were added to each well (90 μL/well), and incubated with various concentrations of drugs (100, 30, 10, 3, 1, 0.3 μg/L) or without drugs in three replicates for 48 h at 37°C in a humidified atmosphere of 5% CO_2. After 48 h, 10 μl of MTT solution (5 mg/mL) were added to each well, which were incubated for another 4 h. Then 10% SDS-5% Isobutanol-0.012mol/L HCl was added to each well (100 μL/well). After 12 h at rt, the OD value of each well was recorded on Model680 reader at 570nm.

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