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Three new abietane diterpenoids from *Podocarpus fleuryi*

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ABSTRACT

Three new abietane diterpenoids, fleuryinols A–C (**1**–**3**), together with fourteen known compounds, were isolated from the twigs and leaves of *Podocarpus fleuryi*. Their structures were established by spectroscopic analysis, including 1D- and 2D-NMR spectroscopic techniques. Compounds **1**–**8** were tested cytotoxic activity against five human cancer cell lines, HL-60, SMMC-772, A-549, MCF-7, and SW480, of which fleuryinol B (**2**) and 19-hydroxyferruginol (**4**) exhibited moderate cytotoxic activity against some cell lines.

1. Introduction

The genus *Podocarpus* (Podocarpaceae) comprises about 80 species mainly distributed in tropical, subtropical, and temperate areas of the Southern Hemisphere and East Asia, of which 13 species were found in southwest of China (Editorial Committee of Flora of China, Chinese Academy of Sciences, 1978). Many of them are used as folk medicine to treat fever, asthma, cough, cholera, arthritis, rheumatism, and venereal diseases (Abdillahi et al., 2010, 2011). *Podocarpus fleuryi* Hickleis, an evergreen tree, is mainly distributed in the southwest of China. Previous chemical investigations of this plant led to the isolation of a number of norditerpenoids and biflavones, some of which showed obvious cytotoxic activity (Fang et al., 1990; Xu and Fang, 1990, 1991). Further study on the twigs and leaves of this plant led to the isolation of three new diterpenoids, fleuryinols A–C (**1**–**3**), as well as fourteen known compounds. In addition, the cytotoxic activity of the diterpenoids (**1**–**8**) were measured in vitro against five human cancer cell lines, HL-60, SMMC-772, A-549, MCF-7, and SW480. In the present paper, we report the isolation, structure elucidation of the isolates, and cytotoxic activity of the selected compounds.

2. Results and discussion

The 70% acetone extract of *P. fleuryi* was suspended in water and partitioned successively with EtOAc. The EtOAc extract was subjected to column chromatography on silica gel, RP-18, MCI, Sephadex LH-20, and semipreparative HPLC, affording three new diterpenoids, fleuryinols A–C (**1**–**3**), as well as fourteen known compounds, 19-hydroxyferruginol (**4**) (Cambie et al., 1984), lambertic acid (**5**) (De Paiva Campello et al., 1975), inumakiol D (**6**) (Sato et al., 2008), totaradiol (**7**) (Ying and Kubo, 1991), 19-hydroxytotarol (**8**) (Ying and Kubo, 1991), 3-acetoxy-methyl-5-[(*E*)-3-acetoxypropen-1-yl)]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran (**9**) (Valcic et al., 1998), divanillyltetrahydrofuran (**10**) (Lima and Braz-Filho, 1997), (+)-pinoresinol (**11**) (Xie et al., 2003), ligballinol (**12**) (Wang et al., 2009), sitostane-3β,5α,6β-triol (**13**) (Carvalho et al., 2010), ergosta-7,22-dien-3β,5α,6β-triol (**14**) (Zhao et al., 2010), stigmast-4-ene-3β,6β-diol (**15**) (Yang et al., 2004), stigmast-5-ene-3β,7α-diol (**16**) (Li and Xiang, 2011), and ergosterol peroxide (**17**) (Krzyczkowski et al., 2009).

Compound **1** was obtained as a colorless oil. The molecular formula $C_{20}H_{30}O_3$ was deduced from HR-EI-MS (*m/z* 318.2193 $[M]^+$, calcd. for $C_{20}H_{30}O_3$, 318.2195), indicating six degrees of unsaturation. The IR absorption bands at 3419, 2926, 1621, and 1420 cm^{-1} revealed the presence of hydroxy and aromatic ring functionalities. An analysis of the ^1H NMR spectrum (Table 1) indicated the typical signals of two olefinic protons [δ_H 6.73 (1H, s), 6.70 (1H, s)], four oxygenated methylene protons at [δ_H 3.78 (1H, dd, J = 10.1, 5.2, H-19a), 3.68 (1H, m, H-16a), 3.61 (1H, m, H-16b),

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Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data for **1**–**3** in acetone-d_6 (δ in ppm and J in Hz).

No.	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1a	39.8 (t)	2.20 (m)	46.3 (t)	2.29 (dd, 13.6, 3.8)	46.3 (t)	2.30 (dd, 13.4, 3.8)
1b		1.34 (m)		1.73 (overlap)		1.73 (overlap)
2a	19.7 (t)	1.72 (m)	67.5 (d)	4.18 (br s)	67.5 (d)	4.17 (br s)
2b		1.53 (m)				
3a	36.0 (t)	1.94 (overlap)	47.1 (t)	1.74 (overlap)	47.1 (t)	1.74 (overlap)
3b		0.93 (m)		1.45 (dd, 13.8, 3.8)		1.44 (dd, 13.8, 3.8)
4	39.4 (s)		33.3 (s)		33.3 (s)	
5	52.4 (d)	1.39 (m)	50.1 (d)	1.35 (dd, 12.3, 2.1)	50.1 (d)	1.35 (dd, 12.3, 2.1)
6a	20.0 (t)	1.94 (overlap)	20.2 (t)	1.83 (m)	20.1 (t)	1.83 (m)
6b		1.68 (m)		1.72 (m)		1.72 (m)
7a	31.1 (t)	2.77 (m)	30.7 (t)	2.78 (m)	30.7 (t)	2.78 (m)
7b		2.66 (m)		2.68 (m)		2.67 (m)
8	126.3 (s)		125.8 (s)		125.8 (s)	
9	149.0 (s)		149.6 (s)		150.2 (s)	
10	38.2 (s)		38.1 (s)		38.2 (s)	
11	112.2 (d)	6.70 (s)	112.1 (d)	6.70 (s)	112.9(d)	6.70 (s)
12	153.9 (s)		153.1 (s)		153.9 (s)	
13	129.1 (s)		132.5 (s)		128.9 (s)	
14	128.7 (d)	6.73 (s)	126.9(d)	6.75 (s)	128.6(d)	6.72 (s)
15	37.1 (d)	3.14 (m)	27.6 (d)	3.19 (m)	37.0 (d)	3.15 (m)
16a	68.2 (t)	3.68 (m)	23.1 (q)	1.17 (d, 6.6)	68.2 (t)	3.69 (m)
16b		3.61 (m)				3.62 (m)
17	16.9 (q)	1.22 (d, 7.1)	23.0 (q)	1.16 (d, 6.6)	16.8 (q)	1.23 (d, 7.1)
18	27.6 (q)	1.01 (s)	33.7 (q)	0.96 (s)	33.8 (q)	0.96 (s)
19a	64.4 (t)	3.78 (dd, 10.1, 5.2)	24.4 (q)	1.11 (s)	24.3 (q)	1.12 (s)
19b		3.43 (dd, 10.1, 5.2)				
20	26.1 (q)	1.13 (s)	28.4 (q)	1.39 (s)	28.5 (q)	1.40 (s)
2-OH				3.62 (d, 3.3)		3.66 (br s)
12-OH		8.16 (s)		7.76 (s)		8.21 (br s)
16-OH		4.20 (t, 4.9)				4.24 (br s)
19-OH		3.42 (t, 5.2)				

δ_H 3.43 (1H, J = 10.1, 5.2, H-19b)], and one phenolic hydroxy (δ_H 8.16, s, 12-OH). The ^{13}C and DEPT NMR spectra (Table 1) exhibited 20 carbon signal resonances due to three methyls, seven methylenes (two oxygenated ones), four methines (two olefinic ones), and six quaternary carbons (four olefinic ones). The above data suggested that compound **1** was a phenolic diterpenoid similar to a known compound 19-hydroxyferruginol (**4**) (Cambie et al., 1984), which was also isolated from this plant in current study. The only difference was that the methyl group at C-16 in **4** was replaced by a hydroxymethyl group in **1**, as deduced from the HMBC correlations (Fig. 2) of H-16a and H-16b with C-13 (δ_C 129.1), C-15 (δ_C 37.1), and C-17 (δ_C 16.9). The relative configuration of **1** was deduced from its ROESY experiment (Fig. 2). The ROESY correlations of CH_3-18/H-5 and CH_2-19/CH_3-20 implied that the hydroxymethyl group at C-19 was β-oriented. On the basis of the above evidence, the structure of **1** was deduced to be as shown in Fig. 1 and named as fleuryinol A.

Compound **2** was isolated as a colorless oil, exhibited a quasi-molecular ion peak at m/z 302.2253 $[M]^+$ (calcd. for $C_{20}H_{30}O_2$, 302.2246) in the HR-EI-MS, corresponding to the molecular formula $C_{20}H_{30}O_3$ with six degrees of unsaturation. The IR spectrum indicated the existence of hydroxy (3428 cm^{-1}) and aromatic (1620, 1466, and 1420 cm^{-1}) groups. The ^{1}H and ^{13}C NMR spectral data (Table 1) of **2** were similar to those of hinokiol (Li et al., 2007) except that downfield shifted of C-1 (+9.3) and C-2 (+39.7) and upfield shifted of C-3 (−30.4) and C-4 (−5.5) respectively, which indicated the hydroxy group was located at C-2 in **2** rather than at C-3 in hinokiol. This finding was confirmed by the HMBC correlations from δ_H 4.18 (br s, H-2) to δ_C 39.8 (t, C-1), 36.0 (t, C-3), 33.3 (s, C-4) and 38.1 (s, C-10), as well as the ^{1}H–^{1}H COSY correlations of H_2-1/H-2 and H-2/H_2-3. In the ROESY spectrum, the correlations of OH-2/CH_3-19, OH-2/CH_3-20, and H-5/CH_3-18 indicated the β-orientation of 2-OH. Thus, compound **2** was established as shown and named as fleuryinol B.

Compound **3**, a colorless oil, had the molecular formula $C_{20}H_{30}O_3$ as deduced from the HR-EI-MS spectrum (calcd. for $C_{20}H_{30}O_3$, 318.2195). Compared the 1D-NMR data (Table 1) of **3** with those of **2** indicated the two compounds were similar, except that one methyl group in **2** was oxidated as hydroxymethyl group in **3**. The hydroxymethyl group was connected to C-15 as inferred from HMBC correlations (Fig. 2) of δ_H 3.69 (m, H-16a) and 3.62 (m, H-16b) with δ_C 128.9 (s, C-13), 37.0 (d, C-15), and 16.8 (q, C-17). The relative configuration of **3** was determined to be same as **2** according to its NMR data and ROESY experiment. Thus, compound **3** was determined as shown and named as fleuryinol C.

Compounds **1**–**8** were evaluated for cytotoxicity against five human cancer cell lines, HL-60 (human myeloid leukemia), SMMC-772 (hepatocellular carcinoma), A-549 (lung cancer), MCF-7 (breast cancer) and SW480 (colon cancer), using the MTT method as reported previously (Mosmann, 1983), with cisplatinas a positive control. The new compound fleuryinol B (**2**) exhibited moderate toxicity effects against HL-60, SMMC-7721, A-549, and MCF-7 cells, with IC_{50} values of 17, 37, 33, and 30 μM, respectively, while the know compound 19-hydroxyferruginol (**4**) showed moderate cytotoxicity against SMMC-7721, and A-549 cell lines, with IC_{50} values of 38 and 14 μM, respectively. The other compounds were inactive in the tested system ($IC_{50} > 40$ μM).

3. Experimental procedure

3.1. General experimental procedures

Optical rotation was measured on a Jasco P-1020 polarimeter. UV spectra were obtained on a Shimadzu UV-2401A spectrophotometer. The IR spectrum was obtained with a Bruker Tensor 27

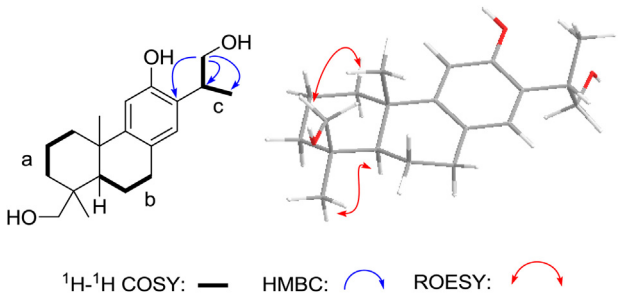


Fig. 2. Key 2D-NMR correlations of compound **1**.

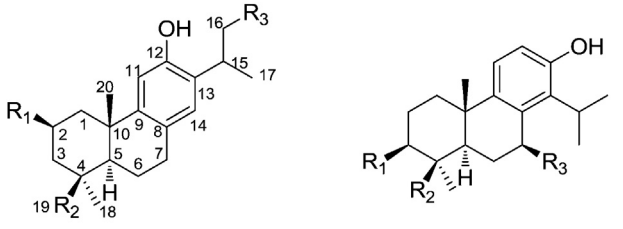
spectrometer KBr desk. ESI-MS were carried out on an API QSTAR Pulsar 1 spectrometer. HREI-MS were performed on a VG Autospec-3000 spectrometer. The NMR spectra were recorded on Bruker AM-400, DRX-500, or Avance III 600 spectrometers (δ in ppm, *J* in Hz). Column chromatography (CC) was performed using silica gel (100–200 and 200–300 mesh, Qingdao Haiyang Chemical Co. Ltd, Qingdao, PR China), Lichropre RP-18 gel (40–63 μm, Merck, Darmstadt, Germany) and MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Japan). Semipreparative reverse-phase (RP) HPLC was performed on an Agilent 1200 series system equipped with a Zorbax SB-C_{18} (5 μm, 9.4 × 250 mm) column. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol. Solvents were distilled before use. All isolated compounds had a degree of purity more than 95% based on TLC, HPLC, and NMR methods.

3.2. Plant material

Twigs and leaves of *P. fleuryi* were collected in Xishuangbanna country of Yannan Province, PR China, in September 2011. The plant was identified by Prof. X. Cheng at Kunming Institute of Botany. A voucher specimen (No. 20110915p) was deposited at the State Key Laboratory of Photochemistry and Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, PR China.

3.3. Extraction and isolation

The air-dired twigs and leaves of *P. fleuryi* (19 kg) were extracted three times (each for 2 days) with 70% acetone at room temperature. The extract was evaporated under reduced pressure to give a residue (750 g), which was suspended in water and partitioned successively with EtOAc. The EtOAc extract (400 g) was chromatographed on a silica gel column with a system of petroleum ether–acetone (1:0→0:1) to yield five fractions A–E. Fraction B was fractioned on a silica gel column using petroleum ether–EtOAc (20:1, 10:1, 9:1) to provided three subfractions B_1–B_3. Subfraction B_1 was chromatographed over silica gel CC, using petroleum ether–acetone (80:1→9:1) as solvent, and further purified by Sephadex LH-20 eluted with $CHCl_3$–MeOH (1:1) to afford **2** (26 mg) and **10** (30 mg). Subfraction B_2 was further chromatographed over silica gel CC eluted with petroleum ether–acetone (50:1→8:2) and then separated by semipreparative HPLC (MeOH–H_2O, 65:35) to give **4** (18 mg) and **7** (15 mg). Fraction C was further chromatographed over MCI gel (MeOH–H_2O, 80:20→100:0) to provide subfractions C_1–C_3. Subfraction C_1 was chromatographed over silica gel CC using petroleum ether–acetone (20:1→7:3) as eluent and further purified by Sephadex LH-20 eluted with $CHCl_3$–MeOH (1:1) to yield **6** (148 mg), **9** (5 mg), and **11** (2 mg). Subfraction C_2 was subjected to Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) followed by chromatographed over repeated silica gel CC and finally purified by semipreparative HPLC (MeOH–H_2O, 50:50) to get compounds **1** (15 mg), **3** (35 mg), and **5** (12 mg). Subfraction C_3 was subjected to a RP-18 gel column eluted with (MeOH–H_2O, 50:50→100:0) followed by Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) to give **12** (2 mg), **13** (5 mg), **14** (5 mg), **15** (14 mg), and **16** (35 mg). Subfraction D was applied to a RP-18 gel column eluted with (MeOH–H_2O, 50:50) and further separated by semipreparative HPLC (MeOH–H_2O, 65:35) to yield **8** (21 mg) and **17** (10 mg).



	R_1	R_2	R_3
1	H	CH_2OH	OH
2	OH	CH_3	H
3	OH	CH_3	OH
4	H	CH_2OH	H
5	H	COOH	H
6	H	COOH	OH
7	OH	CH_3	H
8	H	CH_2OH	H

Fig. 1. Structures of compounds **1**–**8** isolated from *P. fleuryi*.

3.3.1. Fleuryinol A (**1**)

Colorless oil; $[\alpha]_D^{16.7}$ +44.0 (*c* = 0.34, MeOH); UV (MeOH) λ_{max} (logε): 284 (3.54), 202 (4.47) nm; IR (KBr) ν_{max}: 3419, 2926, 2872, 1697, 1621, 1498, 1454,1420, 1376, 1269, 1024, 892 cm^{-1}; ^{1}H and ^{13}CNMR data, see Table 1; ESI-MS (positive) *m/z*: 341 $[M+Na]^+$; HR-EI-MS *m/z* 318.2193 $[M]^+$ (calcd. for $C_{20}H_{30}O_3$, 318.2195).

3.3.2. Fleuryinol B (**2**)

Colorless oil; $[\alpha]_D^{22}$ +116.7 (*c* = 0.26, MeOH); UV (MeOH) λ_{max} (logε): 284 (3.60), 205 (4.46) nm; IR (KBr) ν_{max}: 3428, 2959, 2868,1620, 1510, 1466, 1420, 1366, 1325, 1237, 1189, 1037 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS (positive) *m/z*: 325 $[M+Na]^+$; HR-EI-MS *m/z* 302.2253 $[M+Na]^+$ (calcd. for $C_{20}H_{30}O_2$, 302.2246).

3.3.3. Fleuryinol C (**3**)

Colorless oil; $[\alpha]_D^{16.7}$ +77.6 (*c* = 0.29, MeOH); UV (MeOH) λ_{max} (logε): 284 (3.53), 202 (4.56) nm; IR (KBr) ν_{max}: 3427, 2957, 2932, 2873, 1641, 1631, 1622, 1501, 1469, 1420, 1366, 1326, 1231, 1031 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS (positive) *m/z*: 341 $[M+Na]^+$; HR-EI-MS *m/z* 318.2198 $[M]^+$ (calcd. for $C_{20}H_{30}O_3$, 318.2195).

3.4. Cytotoxicity assay

The cytotoxicity of the diterpenoids (**1–8**) against HL-60, SMMC-7721, A-549, MCF-7, and SW480 cell lines were measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method in 96-well culture plates (Mosmann, 1983). All cells were cultured in RMPI-1640 or DMEM medium, supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. Briefly, 100 μL adherent cells were seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while suspended cells were seeded immediately prior to drug addition, with an initial density of 1×10^5 cells/mL. Each tumor cell line was exposed to the tested compounds at different concentration. After 48 h, 20 μL of MTT solution was added to each well, which was incubated for a further 4 h. Then 10% SDS solution

(200 μL) was added to each well. After compound treated, cell viability was detected and the cell growth curve was graphed. The IC_{50} value was calculated by the Reed and Muench method (Reed and Muench, 1938).

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