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Ent-kaurane and *ent*-abietane diterpenoids from *Isodon phyllostachys*

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Four new diterpenoids, phyllostachysins I–L (**1**–**4**), along with a known one, hebeiabinin B (**5**), were isolated from the aerial parts of *Isodon phyllostachys* (*I. phyllostachys*). Compounds **1** and **2** feature an *ent*-kaurane backbone, and **3**, **4** and **5** bear an *ent*-abietane skeleton. Their structures were elucidated by means of spectroscopic analysis. Compounds **3** and **4** exhibited significant cytotoxic activity against human tumor cell lines (HL-60, SMMC-7721, A-549, MCF-7 and SW-480), and strongly inhibited NO production in LPS-stimulated RAW264.7 cells.

***Isodon phyllostachys*, diterpenoid, *ent*-kaurane, *ent*-abietane**

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1 Introduction

Isodon phyllostachys (*I. phyllostachys*) (Diels) Kudo, belonging to *Isodon* genus (Labiatae family), is distributed in the northwest district of the Yunnan Province and the southwest district of the Sichuan Province in China. It has been used as an antibiotic and antiphlogistic agent in folk medicine [1]. Previous phytochemical investigations of this species yielded 30 new *ent*-kaurane diterpenoids [2–9]. As a part of our ongoing research for more new diterpenoids with antitumor activity, the aerial parts of *I. phyllostachys* have been reinvestigated. Consequently, four new diterpenoids, phyllostachysins I–L (**1**–**4**, Figure 1), together with one known analogue, hebeiabinin B (**5**) [10], were obtained. Herein, we report the isolation, structure elucidation, and the biological activities of these diterpenoids.

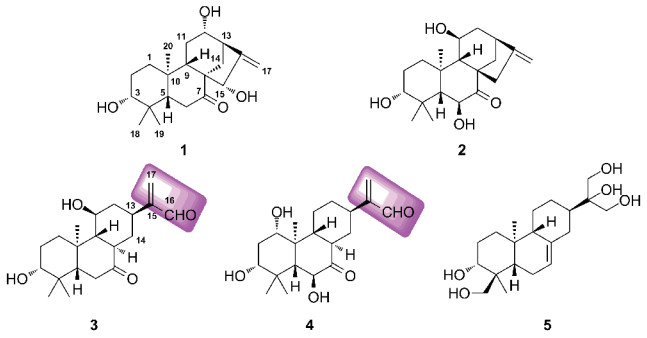

Figure 1 Structures of compounds (**1**–**5**) isolated from *Isodon phyllostachys* (color online).

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2 Experimental

2.1 General procedures

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra: Bruker DRX-600 spectrometers (Germany); at 600/150 MHz resp; δ in ppm, J in Hz; with tetramethylsilane (TMS) as internal standard. High resolution-electrospray ionization-mass spectrometry (HR-ESI-MS): Agilent 6540 QSTAR TOF time-of-flight mass spectrometer (USA). Scanning infrared radiation (IR) spectroscopy: Tenor 27 FT-IR spectrometer (Bruker, Germany); KBr pellets. UV spectra: Shimadzu UV-2401A spectrophotometer (Japan). Optical rotations: JASCO P-1020 polarimeters (Japan). Analytical and semipreparative HPLC: Agilent 1260 or 1100 apparatus; Zorbax SB-C_{18} (Agilent, 4.6 mm×250 mm, 9.4 mm×250 mm, USA) column. Column chromatography (CC): silica gel (Qingdao Marine Chemical Inc., Qingdao, China), Lichroprep RP-18 gel (40–63 μm, Merck, Germany), and MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Japan). Thin-layer chromatography (TLC): silica gel 60 F_{254} on glass plate (Qingdao Marine Chemical Inc., China).

2.2 Plant material

I. phyllostachys were collected in August 2011 from two areas (Muli and Yanyuan county) of the Sichuan Province, China, and identified by Prof. Xiwen Li at the Kunming Institute of Botany (China). A voucher specimen (KIB20110822) has been deposited in the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences.

2.3 Extraction and isolation

The air-dried and powered aerial parts of *I. phyllostachys* (11.0 kg) were extracted with 70% aqueous acetone for five times at room temperature and then filtered. The filtrate was evaporated in vacuo to afford a residue, which was partitioned by liquid-liquid extraction between EtOAc and H_2O. The EtOAc extract (765 g) was chromatographed over silica gel and eluted with $CHCl_3$/acetone (100:0–0:100 gradient) to afford fractions A–G. Fraction C ($CHCl_3$/acetone, 80:20; 120 g) was decolorized on MCI and eluted with 90:10 MeOH/H_2O to yield a yellowish white gum (96 g). The part (47 g) of the gum that was easily soluble in methanol was subjected to RP-18 silica gel CC; four fractions were eluted with MeOH/H_2O (30:70 to 70:30 gradient): C1–C4 (30:70, 40:60, 50:50, and 70:30). Fraction C1 (2 g) was purified by silica gel CC (CH_3Cl/acetone gradient, 90:10–0:100) to yield 5 (22 mg). C2 (18 g) was separated using silica gel CC (petroleum ether/acetone gradient, 90:10 to 0:100) and then by semipreparative HPLC (RP-18, 28%, 30%, 35%, and 27% MeCN/H_2O resp.) to yield compounds **1** (14 mg), **2** (4 mg), **3** (2 mg), and **4** (2 mg).

Phyllostachysin I (**1**): white amorphous powder; $[\alpha]^{23}_{D}$ –41.7 (*c* 0.8, MeOH); UV (MeOH) λ_{max} (logε) 203 (3.1) nm; IR (KBr) ν_{max} 3437, 2966, 2936, 2928, 2877, 1689, 1633, 1443, 1415, 1378, 1092, 1052, 1027, 928 cm^{-1}; positive HRESIMS $[M+Na]^+$ *m/z* 357.2039 (calcd. for $C_{20}H_{30}O_4Na$, 357.2036); ^{1}H and ^{13}C NMR data, see Table 1 and Table 2.

Phyllostachysin J (**2**): white amorphous powder; $[\alpha]^{23}_{D}$ –50.2 (c 0.8, MeOH); UV (MeOH) λ_{max} (logε) 204 (3.2) nm; IR (KBr) ν_{max} 3541, 3389, 2963, 2933, 2873, 1692, 1654, 1633, 1395, 1369, 1302, 1269, 1091, 1042, 1015, 875, 615 cm^{-1}; positive HRESIMS $[M+Na]^+$ *m/z* 357.2038 (calcd. for $C_{20}H_{30}O_4Na$, 357.2036); ^{1}H and ^{13}C NMR data, see Table 1 and Table 2.

Phyllostachysin K (**3**): white amorphous powder; $[\alpha]^{23}_{D}$ –34.3 (*c* 0.47, MeOH); UV (MeOH) λ_{max} (logε) 216 (3.1) nm; IR (KBr) ν_{max} 3411, 2933, 2871, 1699, 1670, 1626, 1447, 1385, 1370, 1254, 1182, 1088, 1055, 1032, 963 cm^{-1}; positive HRESIMS $[M+Na]^+$ *m/z* 357.2044 (calcd. for $C_{20}H_{30}O_4Na$, 357.2036); ^{1}H and ^{13}C NMR data, see Table 1 and Table 2.

Phyllostachysin L (**4**): white amorphous powder; $[\alpha]^{23}_{D}$ –22.1 (*c* 0.8, MeOH); UV (MeOH) λ_{max} (logε) 216 (3.1) nm; IR (KBr) ν_{max} 3448, 2931, 2872, 1700, 1687, 1628, 1445, 1385, 1370, 1248, 1217, 1174, 1091, 1076, 998 cm^{-1}; positive HRESIMS $[M+Na]^+$ *m/z* 373.1990 (calcd. for $C_{20}H_{30}O_5Na$, 373.1985); ^{1}H and ^{13}C NMR data, see Table 1 and Table 2.

Hebeiabinins B (**5**): white amorphous powder; ^{1}H NMR (C_5D_5N) δ_H 5.37 (s, H-7), 4.09 (d, 10.4 Hz, Ha-18), 3.63 (d, 10.4 Hz, Hb-18), 2.78 (d, 13.9 Hz, Ha-14), 2.41 (t, 13.0 Hz, Hb-14), 2.24 (d, 12.2 Hz, Ha-12), 1.53 (q, 12.0 Hz, Hb-12), 1.13 (s, H-19), 0.88 (s, H-20); ^{13}C NMR (C_5D_5N) δ_C 37.9 (t, C-1), 27.7 (t, C-2), 73.5 (d, C-3), 42.9 (s, C-4), 42.9 (d, C-5), 23.3 (t, C-6), 120.1 (d, C-7), 137.9 (s, C-8), 52.8 (d, C-9), 35.1 (s, C-10), 25.9 (t, C-11), 26.6 (t, C-12), 42.2 (d, C-13), 35.7 (t, C-14), 75.3 (s, C-15), 64.8 (t, C-16), 64.9 (t, C-17), 67.5 (t, C-18), 12.9 (q, C-19), 15.8 (q, C-20).

2.4 Cytotoxic assay

Effects of **1**–**5** on the growth of human tumor cells were evaluated by MTS assay [11]. Five human tumor cell lines, including HL-60 (acute leukemia), SMMC-7721 (hepatie cancer), A-549 (lung cancer), MCF-7 (breast cancer), and SW-480 (colon cancer), obtained from American type culture collection (ATCC), were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium (DMEM) medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere with 5% CO_2. Briefly, cells were seeded into each well of a 96-well cell culture plate. After 12 h of incubation at 37 °C, the test compound (40 μM) was added. After incubating for 48 h, cells were subjected to the MTS assay. Compounds with a growth inhibition rate of 50% were further evaluated at various concentrations in triplicate for 48 h, with *cis*-platin

Table 1 ^{1}H NMR data for compounds **1**–**4** in C_5D_5N

Position	1	2	3	4
1a	1.68 (m)	1.66 (m)	3.14 (d, 14.3)	3.77 (m)
1b	1.01 (m)	1.05 (m)	1.60 (m)	
2a	1.84 (m)	1.85 (m)	1.93 (m)	2.41 (m)
2b	1.77 (m)	1.67 (m)		
3	3.35 (dd, 11.2, 3.1)	3.57 (m)	3.48 (dd, 11.4, 4.4)	3.65 (dd, 10.9, 4.1)
5	1.65 (m)	1.79 (d, 8.9)	1.39 (m)	1.42 (m)
6	2.63 (m)	4.61 (d, 8.9)	2.52 (m)	4.86 (d, 12.3)
8			2.30 (m)	1.36 (m)
9	2.49 (m)	1.67 (m)	1.39 (m)	2.44 (m)
11a	2.29 (m)	4.12 (t, 5.8)	4.02 (br s)	2.32 (d, 12.9)
11b				1.42 (m)
12a	4.36 (m)	2.03 (m)	2.30 (m)	1.81 (m)
12b		1.85 (m)	1.55 (m)	1.12 (m)
13	2.77 (m)	2.98 (d, 4.7)	2.69 (t, 12.5)	2.55 (t, 12.3)
14a	2.49 (m)	2.33 (dd, 11.7, 2.3)	2.30 (m)	3.12 (dd, 13.7, 2.8)
14b	1.62 (m)	2.03 (m)	1.39 (m)	1.50 (m)
15a	4.98 (s)	2.95 (m)		
15b		2.78 (d, 16.4)		
16			9.57 (s)	9.58 (s)
17a	5.32 (s)	5.06 (s)	6.20 (s)	6.16 (s)
17b	5.63 (s)	4.97 (s)	5.88 (s)	5.87 (s)
18	1.01 (s)	1.69 (s)	1.16 (s)	1.81 (s)
19	1.02 (s)	1.30 (s)	1.08 (s)	1.51 (s)
20	1.30 (s)	1.13 (s)	1.34 (s)	1.47 (s)

Table 2 ^{13}C NMR data for compounds **1**–**4** in C_5D_5N

Position	1	2	3	4
1	37.0 (t)	38.1 (t)	39.3 (t)	75.7 (d)
2	27.5 (t)	27.9 (t)	28.5 (t)	39.0 (t)
3	77.8 (d)	78.0 (d)	77.5 (d)	75.3 (d)
4	39.0 (s)	40.3 (s)	40.2 (s)	41.0 (s)
5	50.5 (d)	58.4 (d)	54.5 (d)	58.5 (d)
6	38.1 (t)	73.6 (d)	38.9 (t)	75.2 (d)
7	214.7 (s)	214.6 (s)	210.7 (d)	212.1 (s)
8	53.8 (s)	53.3 (s)	48.6 (d)	56.2 (d)
9	44.0 (d)	54.1 (d)	61.3 (d)	47.9 (d)
10	36.6 (s)	37.2 (s)	39.1 (s)	44.8 (s)
11	38.5 (t)	74.0 (d)	71.4 (d)	32.0 (t)
12	66.1 (d)	27.8 (t)	43.1 (t)	31.8 (t)
13	46.1 (d)	50.5 (d)	32.9 (d)	34.7 (d)
14	21.3 (t)	31.3 (t)	31.6 (t)	29.6 (t)
15	73.7 (d)	49.0 (t)	154.1 (s)	154.9 (s)
16	154.0 (s)	153.5 (s)	194.5 (d)	194.5 (d)
17	110.7 (t)	107.2 (t)	133.0 (t)	132.8 (t)
18	27.6 (q)	30.0 (q)	28.4 (q)	31.1 (q)
19	15.2 (q)	16.6 (q)	15.9 (q)	15.8 (q)
20	14.8 (q)	15.1 (q)	14.1 (q)	10.1 (q)

as positive controls. The IC_{50} value of each compound was calculated with the Reed and Muench's method [12].

2.5 Nitric oxide production in RAW264.7 cells

RAW264.7 cells were seeded in 96-well cell culture plates (2×10^5 cells/well) and treated with serial dilutions of the compounds at a maximum concentration of 25 μM; this treatment was followed by stimulation with lipopolysaccharide (LPS, 1 μg/mL) for 18 h. NO production in the supernatant was assessed using Griess reagents. The absorbance at 550 nm was measured using a 2104 Envision multilabel plate reader (Perkin-Elmer Life Sciences, Inc., USA). MG-132 was used as a positive control [13]. The viability of RAW264.7 cells was simultaneously evaluated using the MTT assay to exclude the interference of the cytotoxicity of the test compounds. The absorbance was measured at 595 nm.

3 Results and discussion

Phyllostachysin I (**1**) was isolated as a white amorphous powder and exhibited a sodium adduct ion at *m*/*z* 357.2039 ($[M+Na]^+$, calcd. 357.2036), corresponding to $C_{20}H_{30}O_4$ with six indices of hydrogen deficiency. The IR spectrum showed absorption bands for hydroxyl (3437 cm^{-1}), carbonyl (1689 cm^{-1}), and olefinic (1633 cm^{-1}) groups. There were three obvious tertiary methyl singlets and two signals of exocyclic olefinic group in ^{1}H NMR spectrum (Table 1). The ^{13}C NMR and distortionless enhancement by polarization

transfer (DEPT) data of compound **1** (Table 2) indicated the presence of one carbonyl group, three tertiary methyls, one exomethylene, five methylenes, six methines (three oxygenated) and four quaternary carbons. Compound **1** was tentatively assigned as a C-20 non-oxygenated *ent*-kaurane diterpenoid. The ^{1}H-^{1}H COSY revealed three spin systems, H_2-1/H_2-2/H_2-3, H-5/H-6 and H_2-11/H-12/H-13/H_2-14 (Figure 2). The ^{1}H detected heteronuclear multiple bond correlations (HMBC) from H_3-20 to C-1, C-5, C-9 and C-10; H_2-1 to C-3 and C-5; H_3-18 to C-3, C-4, C-5 and C-19; H_2-6 to C-5, C-7 and C-10; H_2-11 to C-8, C-9 and C-12; H-13 to C-11, C-12, C-15, C-16 and C-17; and H_2-17 to C-13, C-15, and C-16, indicated that **1** was a C-20 non-oxygenated *ent*-kauranoid (Figure 2). The HMBC correlations from H_2-1, H_2-2, H-5, H_3-18, and H_3-19 to C-3; H-9, H_2-11, H-13, and H_2-14 to C-12; H-9, H-13, H_2-14, and H_2-17 to C-15 implied OH groups at C-3, C-12, and C-15, respectively. The ROESY correlations of H-3/H_3-18 and H-3/H-5, H-15/H-11β and H-15/H-9, and H-12/H-15, demonstrated that H-3, H-15, and H-12 were β-oriented, and HO-3, HO-15, and HO-12 were α-oriented (Figure 2). Thus, compound **1** was determined as 3α,12α,15α-trihydroxy-*ent*-kaur-16-en-7-one, and given the trivial name *phyllostachysin I*.

Phyllostachysin J (**2**) was determined to have the molecular formula $C_{20}H_{30}O_4$ based on HRESIMS and ^{13}C NMR data, corresponding to six indices of hydrogen deficiency. There were three tertiary methyl singlets in ^{1}H NMR spectrum (Table 1). The ^{13}C NMR and DEPT data of compound **2** (Table 2) showed the presence of one carbonyl group, three tertiary methyls, one exomethylene, five methylenes, six methines (three oxygenated) and four quaternary carbons (one olefinic). Comparisons of the ^{1}H and ^{13}C NMR data of **2** with those of compound **1** indicated that both compounds had same skeleton and similar structure. The ^{1}H-^{1}H COSY revealed three spin systems, H_2-1/H_2-2/H-3, H-5/H-6 and H-9/H-11/H_2-12/H-13/H_2-14. The HMBC correlations from H_2-2, H_3-18 and H_3-19 to C-3, from H-5 to C-6, and from H-12, H-13 and H-14 to C-11, implied an OH group at C-3, C-6, and C-11, respectively. The ROESY correlations of H-3/H_3-18β and H-3/H-5, H-6/H_3-19α and H-6/H_3-20α, and H-11/H-13α demonstrated that H-3, H-6, and H-11 were β, α,α-oriented, respectively. Therefore, the structure of compound **2** was identified as 3α,6β,11β-trihydroxy-*ent*-kaur-16-en-7-one, and given the trivial name phyllostachysin J.

Phyllostachysin K (**3**) was obtained as a white amorphous powder, and its molecular formula was established as $C_{20}H_{30}O_4$ based on HRESIMS and ^{13}C NMR data, showing six indices of hydrogen deficiency. The IR spectrum showed absorption bands for hydroxyl (3411 cm^{-1}), carbonyl (1699 cm^{-1}), and α,β-unsaturated aldehyde (1670 and 1626 cm^{-1}) groups. There were three tertiary methyl singlets and two signals of olefinic group in ^{1}H NMR spectrum (Table 1). ^{13}C NMR and DEPT data (Table 2) of **3** exhibited 20 resonances including three methyls, six methylenes (one olefinic), six methines (two oxygenated), three quaternary carbons (one olefinic), a conjugated carbonyl, and a carbonyl carbon. Compound **3** was tentatively assigned as a C-20 non-oxygenated *ent*-abietane diterpenoid. The ^{1}H-^{1}H COSY revealed three spin systems, H_2-1/H_2-2/H-3, H-5/H_2-6, and H-8/H-9/H-11/H_2-12/H-13/H_2-14 (Figure 3). The HMBC correlations from H_3-20 to C-1, C-5, C-9 and C-10; Ha-1 to C-3 and C-5; H_3-18 to C-3, C-4, C-5 and C-19; H_2-6 to C-5, C-7, and C-10; H-8 to C-7, C-9, C-11, and C-13; and H_2-17 to C-13, C-15, and C-16, indicated that **3** had an abietane skeleton (Figure 3). The ROESY correlations of H_3-20/H_3-19α and H_3-20/H-11/H-13 suggested that C-20, H-11, and H-13 were α-oriented, respectively, and compound **3** was an *ent*-abietanoid. The HMBC correlations from H-1, H-2, H_3-18 and H_3-19 to C-3, and from H-8, H-9 and H-12 to C-11 implied an OH group at C-3 and C-11, respectively. The ROESY correlations of H-3/H_3-18β and H-3/H-5 demonstrated that H-3 was β-oriented and HO-3 was α-oriented. Thus, compound **3** was established as 3α,11β-dihydroxy-7-oxo-*ent*-abiet-15-en-17-al and given the trivial name phyllostachysin K.

Phyllostachysin L (**4**) had the molecular formula $C_{20}H_{30}O_5$ by HRESIMS and ^{13}C NMR data, corresponding to six indices of hydrogen deficiency. The ^{1}H NMR data (Table 1) showed characteristic resonances of three tertiary methyl group and two signals of olefinic group. The ^{13}C NMR and DEPT data (Table 1) of **4** exhibited 20 carbon signals, including three methyls, five methylenes (one olefinic), seven methines (three oxygenated), three quaternary carbons (one olefinic), a conjugated carbonyl, and a carbonyl carbon. Careful comparisons of the ^{1}H and ^{13}C NMR data of **4** with those of **3** indicated that both compounds had same skeleton

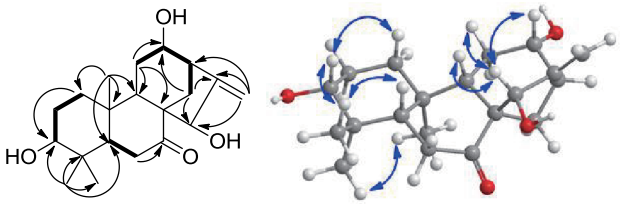

Figure 2 ^{1}H-^{1}H COSY (bold), selected HMBC (arrow) and key ROESY correlations of compound **1** (color online).

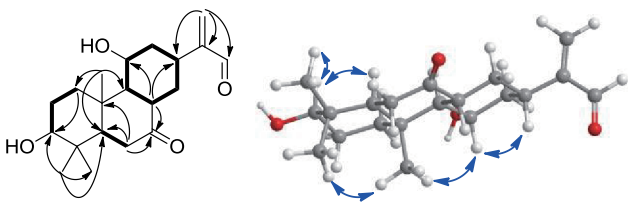

Figure 3 ^{1}H-^{1}H COSY (bold), selected HMBC (arrow) and key ROESY correlations of compound **3** (color online).

Table 3 Cytotoxic activities of compounds **3** and **4** against five tumor cell lines [a] (IC_{50}, μM)

Compound	Tumor cell lines				
	HL-60	SMMC-7721	A-549	MCF-7	SW-480
3	4.1	4.6	15.1	4.2	5.1
4	7.1	16.3	29.8	4.6	4.9
cis-Platin	3.2	5.8	7.1	15.0	11.3

a) Compounds **1**, **2**, and **5** were inactive (IC_{50}>40 μM) for all cell lines.

and similar structure. The ^{1}H-^{1}H COSY revealed three spin systems, H-1/H_2-2/H-3, H-5/H-6 and H-8/H-9/H_2-11/H_2-12/H-13/H_2-14. The HMBC correlations from H-9 and H_3-20 to C-1, from H_2-2, H_3-18 and H_3-19 to C-3, and from H-5 to C-6, implied an OH group at C-1, C-3, and C-6, respectively. The ROESY correlations of H-1/H-3/H_3-18β and H-6/H_3-20α, demonstrated that H-1, H-3, and H-6 were β,β,α-oriented, respectively. Accordingly, the structure of compound **4** was identified as 1α,3α,6β-trihydroxy-7-oxo-*ent*-abiet-15-en-17-al, and given the trivial name phyllostachysin L.

Compounds **1**–**5** were tested for their cytotoxic activity against five human cancer cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW-480). As the results, compounds **3** and **4** exhibited significant cytotoxic activity (Table 3).

Nitric oxide (NO), which is produced by the inducible NO synthase (iNOS) isoform, is an essential component of the host innate immune and inflammatory response to a variety of pathogens [14]. Compounds **1**–**5** were tested for their inhibitory activity against NO production in LPS-stimulated RAW264.7 cells using the MTT assay. Compounds **3** and **4** exhibited significant inhibitory effects, with IC_{50} values 1.34 and 2.09 μM respectively.

4 Conclusions

This is the first time that compounds **1**–**5** have been obtained from *I. phyllostachys*, and compounds **1**–**4** are four new diterpenoids. Compounds **3** and **4** exhibited significant cytotoxic activity and inhibitory activity against NO production. The motif (α,β-unsaturated aldehyde function) is structurally required for biological activities. This result is consistent with the conclusion of previous structure-activity relationship [15].

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Conflict of interest The authors declare that they have no conflict of interest.

Supporting information The supporting information is available online at http://chem.scichina.com and http://link.springer.com/journal/11426. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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