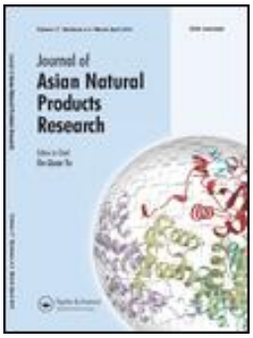

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Two new illudin type sesquiterpenoids from cultures of *Phellinus tuberculosus* and *Laetiporus sulphureus*

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Chemical investigation on the cultures of *Phellinus tuberculosus* and *Laetiporus sulphureus* lead to the isolation of two new illudin-type sesquiterpenoids (phellinuin J and sulphureuine A). Their structures were elucidated by 1D, 2D NMR and MS spectroscopic data. These compounds were purposely evaluated for their cytotoxicity against HL-60, SMMC-7721, A549, MCF-7, and SW480 cell lines.

Keywords: Hymenochaetaceae; *Phellinus tuberculosus*; *Laetiporus sulphureus*; sesquiterpenoids; phellinuin J; sulphureuine A

1. Introduction

Mushrooms have proved to be a rich source of bioactive metabolites with attractive structures. The genus *Phellinus* belonging to the family Hymenochaetaceae, was widely distributed in Yunnan province of China [1]. The crude extract of mushroom *Phellinus tuberculosus* was reported to possess antioxidant activity, which exhibited potent radical scavenging activity [2]. The higher fungus *Laetiporus sulphureus* is also well known for its rich chemical constituents including sesquiterpenoids, triterpenes, and alkaloids, as well as their bioactive diversity [3,4]. Our previous investigation on the cultures of *P. tuberculosus* revealed drimane-type sesquiterpenoids [5]. As part of our ongoing investigation to discover structurally novel and bioactive natural compounds, we isolated two new illudin-type sesquiterpenoids phellinuin J (**1**) and sulphureuine A (**2**) from cultures of *P. tuberculosus* and *L. sulphureus*, respectively (Figure 1). Compounds **1** and **2** were tested for their cytotoxic activities against human tumor cell lines HL-60, SMMC-7721, A549, MCF-7, and SW480 *in vitro*. Herein, we report on the isolation, structural determination, and cytotoxic activity of new compounds.

2. Results and discussion

Compound **1** was obtained as white amorphous powder. The molecular formula of **1** was determined as $C_{15}H_{22}O_3$ by its HR-EI-MS at *m/z* 250.1571 $[M]^+$, indicating five degrees of unsaturation. The absorption bands in the IR spectrum at 3434 and 1712 cm^{-1} indicated the presence of hydroxy and carbonyl groups. ^{13}C NMR and DEPT spectral data showed 15 carbons, which were classified to three methyl groups, five methylene, two methine, and five quaternary carbons (including two oxygenated carbons and one carbonyl carbon) (Table 1). The three

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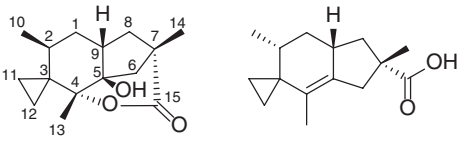

1 **2**

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

characteristic signals at δ_C 24.2 (s, C-3), 6.7 (t, C-11), 13.3 (t, C-12) revealed the existence of a three-membered ring carbon skeleton [6,7]. These characteristic signals suggested that compound **1** should be an illudin-type sesquiterpenoid possessing a 3/6/5 carbon skeleton. According to the HMBC correlations from H-10 to C-1, C-2, C-3, suggested that Me-10 at C-2. Me-13 at C-4 was deduced by the HMBC correlations from H-13 to C-3, C-4, and C-5. Me-14 was linked with C-7, which was also established by HMBC correlations of H-14 to C-6, C-7, C-8, and C-15. In the NMR spectra measured in DMSO-d_6, the hydroxyl group at δ_H 5.13 showed strong HMBC correlations with C-5 (δ_C 76.3) and C-9 (δ_C 41.3) (Figure 2), which indicated that the lactone ring should be constructed between C-4 and C-7, as well as supported by analysis of MS data. Therefore, the planar structure of compound **1** was established, as shown in Figure 1. The relative configuration was confirmed by ROESY spectrum measured in DMSO-d_6. The correlation between H-9 and 5-OH indicated that both H-9 and 5-OH were in the same side (assigned as the β orientation), while the correlations of Me-10/H-9 and 5-OH/Me-13 indicated that Me-10 and Me-13 were also β-oriented (Figure 2). All these data, as well as analyses of a molecular model, suggested that the lactone ring could only be α-oriented. Finally, the structure of compound **1** was established and named phellinuin J.

Compound **2** gave a molecular formula $C_{15}H_{22}O_2$ based on HR-EI-MS at *m/z* 234.1602 $[M]^+$, corresponding to five

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data of **1** and **2** (δ in ppm, *J* in Hz).

	1[a]		**1**[b]		**2**[c]	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	2.03–2.07 m 1.62 t (12.5, 3.0)	28.9	2.06 overlap 1.37 dt (13.8, 4.2)	29.2	1.05–1.11 m 1.85–1.89 m	37.3
2	1.15–1.19 m	37.1	1.90–1.21 m	35.7	1.97–2.03 m	33.8
3		24.2		24.2		24.2
4		88.8		88.7		127.5
5-OH		77.8	5.13 s	76.3		135.9
6	2.27 d (11.4) 1.79 d (11.4)	43.8	2.19 d (11.4) 1.64 d (11.4)	43.6	2.25 dd (17.4, 1.2) 2.90 brd (17.4)	41.7
7		45.1		44.2		47.0
8	2.03 ddd (11.5, 5.5, 1.0)	41.8	1.68 ddd (13.8, 6.0, 1.8)	41.2	1.69 t (12.0) 1.85–1.89 m	43.9
9	2.45–2.50 m	42.5	2.35–2.39 m	41.3	2.64–2.70 m	40.3
10	1.07 d (6.5)	20.3	0.91 d (7.8)	19.7	0.71 d (6.6)	16.9
11	0.85 dt (8.0, 4.0) 0.37 dt (6.5, 4.0)	6.7	0.60–0.64 m 0.20–0.24 m	5.8	0.70–0.74 m 0.53–0.58 m	7.2
12	0.65 dt (8.0, 4.0) 0.36 dt (6.5, 4.0)	13.3	0.58–0.62 m 0.26–0.30 m	13.4	0.61–0.65 m 0.48–0.51 m	6.2
13	1.13 s	18.5	1.00 s	18.2	1.27 s	29.7
14	1.27 s	21.7	1.10 s	21.4	1.36 s	24.9
15		176.0		175.2		184.1

[a] Measured in methanol-d_4.
[b] Measured in DMSO-d_6.
[c] Measured in $CDCl_3$.

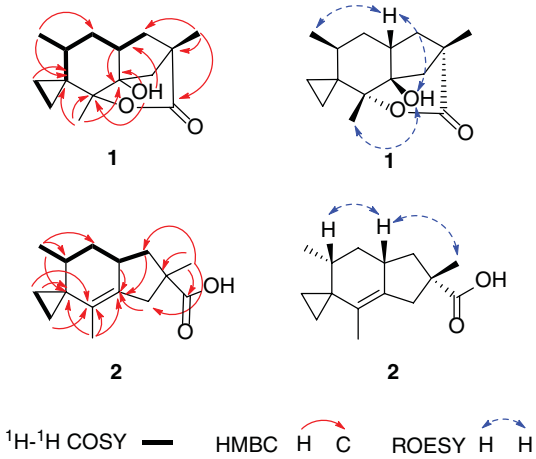

Figure 2. Key 2D NMR correlations of compounds **1** and **2**.

degrees of unsaturation. IR spectrum revealed the existence of hydroxy and carbonyl groups due to absorption bands at 3439 and 1702 cm^{-1}, respectively. The ^{1}H and ^{13}C NMR spectra (Table 1) showed similarities with those of compound **1**, revealing that compound **2** also shared an illudin-type skeleton. The HMBC correlations from H-10 to C-1, C-2, and C-3 demonstrated the linkage of Me-10 at C-2 (Figure 2). There was a fully substituted double bond (δ_C 127.5 and 135.9) between C-4 and C-5, as indicated by HMBC correlations from H-9 at δ_H 2.64–2.70 (1H, m) and H-8a at δ_H 1.85–1.89 (1H, m) to C-5, as well as from H-13 at δ_H 1.27 (3H, s) to C-3, C-4, and C-5. The HMBC correlations from H-11 and H-12 to C-4 also indicated the position of the double bond (Figure 2). Further, HMBC correlations from H-8a at δ_H 1.85–1.89 (1H, m), H-8b at δ_H 1.69 (1H, t, $J = 12.0$ Hz), and H-14 at δ_H 1.36 (3H, s) to C-15 at δ_C 184.1 (s) suggested that C-15 should be a carboxyl group (Figure 2). So far, the planar structure of compound **2** was determined as shown in Figure 1. The relative stereochemistry was established by ROESY experiments. The correlations from H-2/H-9/H-14 suggested the same orientation of H-2, H-9, and H-14 (Figure 2). Therefore, compound **2** was established and named sulphureuine A.

The antitumor activities of the two compounds were tested against HL-60, SMMC-7721, A549, MCF-7, and SW480 cell lines. Unfortunately, no significant inhibitory activity was found ($IC_{50} > 40$ μM).

3. Experimental

3.1. *General experimental procedures*

Optical rotations were obtained on a JASCO P-1020 digital polarimeter (Horiba, Kyoto, Japan). IR spectra were taken on a Bruker Tensor 27 FT-IR spectrometer (Bruker, Karlsruher, Germany) with KBr pellets. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 600 spectrometer (Bruker). HR-EI-MS and HR-ESI-MS were measured on a Waters AutoSpec Primier P776 instrument (Waters, Milford, MA, USA) and a Bruker HCT/Esquire (Bruker) instrument, respectively. Column chromatography (CC) was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), RP-18 (40–75 μm, Fuji Silysia Chemical Ltd, Kasugai, Japan), and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden). Fractions were monitored by TLC and spots were visualized by

heating silica gel plates immersed in vanillin-H_2SO_4 in EtOH.

3.2. Fungi material

Fruiting bodies of *P. tuberculosus* were collected at Jingdong, Yunnan Province, China in 2003 and identified by Prof. Zhu-Liang Yang of Kunming Institute of Botany, CAS. The voucher specimen (No. CGBWSHF00118) was deposited at herbarium of Kunming Institute of Botany. Fruiting bodies of *L. sulphureus* were collected at FenYi, JiangXi Province, China in 2012 and identified by Prof. Yu-Cheng Dai (Beijing Forestry University). The voucher specimen (No. CGBWSHF01013) was deposited at herbarium of Kunming Institute of Botany. Culture medium consisted of glucose (5%), pork peptone (0.15%), yeast (0.5%), KH_2PO_4 (0.05%), $MgSO_4$ (0.05%); the initial pH was adjusted to 6.0; and the fermentation was first carried out on an Erlenmeyer flask for 6 days till the mycelium biomass reached to the maximum. Later it was transferred to a fermentation tank (20 l) at 24°C and 250 rpm for 20 days, while ventilation was set to 1.0 vvm (vvm: air volume/culture volume/min).

3.3. Extraction and isolation

The *P. tuberculosus* culture broth (20 l) was concentrated under vacuum, and extracted three times with EtOAc. The organic layer was evaporated *in vacuum* to give a crude extract (3.1 g), which was separated by Sephadex LH-20 (MeOH) CC to afford fractions A–C. Fraction A (1.2 g) was separated by reversed-phase C18 column (MeOH–H_2O, 20% → 100%) to give sub-fractions A1 and A2. The sub-fraction A1 (20.0 mg) was further purified by silica gel ($CHCl_3$–MeOH, 10:1) to yield phellinuin J (4.3 mg).

The *L. sulphureus* culture broth (20 l) was concentrated under vacuum, and extracted three times with EtOAc. The organic layer was evaporated *in vacuum*, and yielded a crude extract (5.8 g), which was separated by Sephadex LH-20 (MeOH) CC to afford fractions A–C. Fraction A (1.8 g) was subjected to a silica gel column eluted with petroleum ether–acetone (1:0 → 0:1) to give sub-fractions A1 and A3. The sub-fraction A2 (50 mg) was purified further by Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) and silica gel column (petroleum ether–acetone, 20:1) to yield sulphureuine A (6.5 mg).

3.3.1. Phellinuin J (*1*)

White amorphous powder, $[\alpha]_D^{20.4}$ −22.7 (*c* 0.23, MeOH); IR (KBr) ν_{max} 3434, 2938, 2877, 1712, 1633, 1460, 1077 cm^{-1}; ^1H NMR spectral data (see Table 1); ^{13}C NMR spectral data (see Table 1); ESI-MS (pos.): *m/z* 273 $[M + Na]^+$; HR-EI-MS: *m/z* 250.1571 $[M]^+$ (calcd for $C_{15}H_{22}O_3$, 250.1569).

3.3.2. Sulphureuine A (*2*)

Colorless oil, $[\alpha]_D^{20.4}$ −16.9 (*c* 0.12, MeOH); IR (KBr) ν_{max} 3439, 2960, 2927, 2855, 1702, 1463 cm^{-1}; ^1H NMR spectral data (see Table 1); ^{13}C NMR spectral data (see Table 1); EI-MS: *m/z* 234 $[M]^+$; HR-EI-MS: *m/z* 234. 1620 $[M]^+$ (calcd for $C_{15}H_{22}O_2$, 234.1620).

3.4. Cytotoxic bioassay

The following human tumor cell lines were used: HL-60, SMMC-7721, A-549, MCF-7, and SW-480. All cells were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640) or DMEM (Dulbecco's Modified Eagle Medium) (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) at 37°C in a humidified atmosphere with 5% CO_2. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) (Sigma,

St Louis, MO, USA). Briefly, 100 μl of adherent cells were seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition, both with an initial density of 1×10^5 cells/ml in 100 μl medium. Each tumor cell line was exposed to the test compound at various concentrations in triplicate for 48 h, with cisplatin (Sigma) as the positive control. After the incubation, MTS (100 μg) was added to each well, and the incubation continued for 4 h at 37°C. The cells were lysed with 100 μl of 20% sodium dodecyl sulfate (SDS)–50% *N*,*N*-dimethylformamide (DMF) after removal of 100 μl medium. The optical density of the lysate was measured at 490 nm in a 96-well microtitre plate reader (Bio-Rad 680, Bio-Rad Laboratories Co., Ltd, Hercules, CA, USA). The IC_{50} value of each compound was calculated by the Reed and Muench's method.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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