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Two New Biologically Active Illudane Sesquiterpenes from the Mycelial Cultures of *Panaeolus retirugis*

WENZHE MA[a], YICUN HUANG[b], LIDONG LIN[a], XIAOFENG ZHU[c], YIZHU CHEN[a], HANHONG XU[d] and XIAOYI WEI[a,*]

[a] South China Botanical Garden, Chinese Academy of Sciences, Leyiju, Guangzhou 510650, China
[b] Institute of Microbiology, Chinese Academy of Sciences, Zhongguancun, Beijing 10080, China
[c] Cancer Institute, Cancer Center, Sun Yat-sen University, 651 Dongfeng Road East, Guangzhou 510060, China
[d] Key Lab. of Pesticides and Chemical Biology, Ministry of Education, Wushan, Guangzhou 510642, China

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Two new illudane sesquiterpenes, paneolic acid and paneolilludinic acid, along with a known antibiotic diterpene, pleuromutilin, were isolated from the mycelial solid cultures of *Panaeolus retirugis*. Their structures were elucidated on the basis of spectroscopic analysis. Both compounds exhibited antibacterial activity against *Staphylococcus aureus*, and paneolic acid showed cytotoxicity to HL60 cell with an IC_{50} of 18.9 μg/ml.

Panaeolus retirugis (Fr.) Gill. (Coprinaceae) is a poisonous mushroom in China[1,2]. During an ongoing screening for new antibacterial metabolites produced by the fungi growing in south China, we isolated two new illudane sesquiterpenes, paneolic acid (**1**) and paneolilludinic acid (**2**), along with the known antibiotic diterpene, pleuromutilin[3,4], from the mycelial solid cultures of this Basidiomycete. Both **1** and **2** exhibited antibacterial activity against *Staphylococcus aureus* and slight cytotoxicities to some human tumor cells. In this paper, we report the isolation, structure elucidation, and biological activities of these new compounds.

Experimental

General

Optical rotations were obtained on a Perkin-Elmer 343 polarimeter with MeOH as solvent. The UV spectra were recorded in MeOH on a Perkin-Elmer Lambda 25 UV-vis spectrophotometer. The IR spectra were measured in KBr on a WQF-410 FT-IR spectrophotometer. The ^1H (400 MHz), ^{13}C (100 MHz), and 2D NMR spectra were recorded in $CDCl_3$ on a Bruker DRX-400 instrument using the signal of $CDCl_3$ as a reference (the singlet at δ 7.24 for the ^1H NMR data and a triplet centered at δ 77.0 for the ^{13}C NMR data). HRTOFMS data were obtained on an API QSTAR mass spectrometer in positive-ion mode. EIMS were collected on a Micromass Platform EI 200 GC/MS instrument at 70 eV by direct inlet. For column chromatography, silica gel 60 (200~300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Develosil ODS (10 μm, Nomura Chemical Co. Ltd., Japan), and Sephadex LH-20 were used. TLC was performed on precoated plates (Kieselgel $60GF_{254}$, Merck) with detection effected by exposure in I_2 vapor and spraying with H_2SO_4 (10%) in EtOH followed by heating.

Producing Fungus

Fruiting bodies of *Panaeolus retirugis* (Fr.) Gill. were collected in Dinghu Mountain Biosphere Reserve, Guangdong, China, in May 2002. The mycelia were isolated from tissue plugs of a young fruiting body. Voucher specimen (DHS305) and mycelial cultures (SC0641) are deposited in the culture collection of South China Botanical Garden, Chinese Academy of Sciences,

* Corresponding author: wxy@scbg.ac.cn

Guangzhou, China. For maintenance on agar slants and submerged cultures, the fungus was grown on MEA medium.

Fermentation

The mycelia of *P. retirugis* grown on MEA plates were used to inoculate ten 500 ml Erlenmeyer flask containing 100 ml of YMG medium (glucose 0.4%, malt extract 1.0%, yeast extract 0.4%, and pH 5.5). The flasks were incubated on a rotary shaker for 5 days at 28°C with shaking at 120 rpm. The cultures were transferred into ten 5000 ml flasks containing 1000 ml of YMG medium and 500 g of wheat grains, and the cultivation was carried out in the stationary phase at 28°C for 13 days.

Extraction and Isolation

The mycelial solid cultures were extracted with 95% EtOH three times at room temperature. The EtOH extract was suspended in H_2O, and the aqueous suspension was sequentially extracted four times each with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH. The combined $CHCl_3$ extract which showed the most potent antibacterial activity, upon evaporation, yielded a deep brown syrup (7.0 g). This syrup was subjected to silica gel column chromatography, eluted with petroleum ether - acetone mixtures of increasing polarities (9 : 1～1 : 1), to obtain six fractions (I～VI). Fraction IV, obtained on elution with petroleum ether - acetone (4 : 1), showed antibacterial activity, and thus was further separated by silica gel column chromatography eluted with benzene - acetone (9 : 1～7 : 3) to give two antibacterial subfractions, IV-A and IV-B. Subfraction IV-A was applied to a reversed phase C-18 column using MeOH - H_2O (6 : 4～9 : 1) as solvent system to afford **1** (30 mg) and **2** (20 mg). Subfraction IV-B was purified on a Sephadex LH-20 column, by elution with tetrahydrofuran, to afford pleuromutilin (120 mg).

Paneolic Acid (**1**)

Colorless gummy solid, $[\alpha]_D^{25}$ +32.1° (*c* 2.776, MeOH); UV (MeOH) λ_{max} (log ε) 251 (3.98) nm; IR (KBr) ν_{max} 2968～2560, 1706, and 1652 cm^{-1}; ^1H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR, see Table 1; EIMS *m/z* 264 $[M]^+$ (33), 246 $[M-H_2O]^+$ (4), 231 $[M-H_2O-Me]^+$ (4), 221 $[M-Ac]^+$ (8), 189 (4), 175 (87), 161 (8), 147 (8), 135 (12), 121 (4), 105 (8), 91 (16), 43 (100); HRESIMS *m/z* 287.1245 $[M+Na]^+$ (calcd for $C_{15}H_{20}O_4Na$, 287.1259).

Paneolilludinic Acid (**2**)

Colorless gummy solid, $[\alpha]_D^{25}$ +53.1° (*c* 0.700, MeOH); UV (MeOH) λ_{max} (log ε) 203 (3.93) nm; IR (KBr) ν_{max} 3363, 2954～2560, and 1695 cm^{-1}; ^1H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR, see Table 1; EIMS *m/z* 250 $[M]^+$ (33), 232 $[M-H_2O]^+$ (4), 222 $[M-CO]^+$ (100), 205 $[M-CO-OH]^+$ (8), 189 (24), 175 (66), 161 (75), 149 (25), 136 (83), 121 (33), 107 (25), 91 (42); HRESIMS, *m/z* 273.1465 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_3Na$, 273.1466).

Antibacterial Activity

Antibacterial activity was evaluated by the agar diffusion method[5] with paper disks (Whatman No. 6 paper, 6 mm diameter). The test microorganisms were *Staphylococcus aureus* (strain GIM1.55) and *Escherichia coli* (strain GIM1.27) supplied by Guangdong Institute of Microbiology, Guangzhou, China. The paper disks with 5, 10, 25, 50, and 100 μg of **1**, **2**, and Ceftazidime (positive control) were placed on agar media inoculated test microorganisms in Petri dishes. The inhibition zones around the disks were measured after 48 hours at 30°C. The experiment involved the use of three plates for each test. At the dosages of 5, 10, 25, 50, and 100 μg, **1** exhibited the average inhibition zones to *S. aureus* of 3, 4, 7, 7, and 8 mm, respectively, **2** gave the zones of 0, 1, 1, 2, and 4 mm, and Ceftazidime showed those of 0, 1, 1, 3, and 10 mm. At the same dosages, both **1** and **2** showed no inhibition zones to *E. coli*.

Cytotoxicities

Cytotoxicities were determined by MTT method[6] using human colon cancer (SW620), human promyelocytic leukemia (HL60), human chronic myelogenous leukemia (K562), human hepatoma (Hep3B), and human lung carcinoma (A549) cells grown in RPMI-1640 medium plus 10% heat-inactivated fetal bovine serum. The assays were performed in 96-well microtiter plates. Compounds **1** and **2** were dissolved in DMSO and two-fold serially diluted to 7 different concentrations (62.5 to 400 μg/ml), then 5 μl of each serial solution was added to 195 μl (about 10,000 cells) culture medium in wells. After incubation at 37°C for 68 hours, 10 μl of MTT (5 mg/liter) was added to each well and incubated for four more hours, and then liquid in the wells was removed. DMSO (200 μl) was added to each well. The absorbance was recorded on a microplate reader (Bio-Rad model 550) at a wavelength of 570 nm. IC_{50} was defined as 50% reduction of absorbance in the control assay which was treated with 0.1% DMSO alone. The IC_{50} values for **1** were determined to be 43.5 (SW620), 18.9 (HL60), 23.8 (K562), 89.9 (Hep3B), and 64.9 (A549) μg/ml; the values for **2** were 31.7 (HL60) and more than 100 (other cells) μg/ml.

Results and Discussion

The fungus was isolated from tissue culture of the fruiting bodies of *Panaeolus retirugis* collected in Dinghu Mountain, Guangdong, China. The mycelia were grown on solid cultures for 13 days at 28°C. The antibacterial activity-guided fractionation and isolation of the EtOH extract of the mycelial cultures afforded **1** and **2** besides pleuromutilin which was identified by direct comparison of its spectral data with those published[7,8].

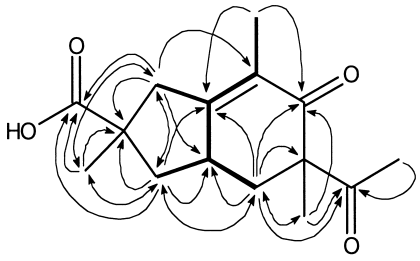
Paneolic acid (**1**) was found to have the molecular formula $C_{15}H_{20}O_4$ by combined analysis of its HRTOFMS, ^{13}C NMR, and DEPT data. The IR spectrum of **1** exhibited two strong absorptions at 1706 and 1652 cm^{-1} for carbonyl groups. The UV spectrum showed an absorbance at 251 nm for a conjugated system. The ^1H NMR spectrum (Table 1) exhibited signals for 19 nonexchangeable protons, including two singlets at δ 1.28 (H-13) and 1.38 (H-15) for two tertiary methyl groups, a singlet at δ 2.05 (H-5) for an acetyl methyl group, and a broad singlet at δ 1.70 (H-12) for a methyl group on an olefinic quaternary carbon. The ^{13}C NMR (Table 1) and DEPT spectra indicated four methyl groups (δ 26.3, C-5; 25.5, C-15; 23.1, C-13; 11.8, C-12), three methylenes (δ 44.8, C-10; 42.2, C-1; 39.4, C-8), and a methine (δ 38.6, C-9), as well as seven quaternary carbons, of which two were ketone carbonyls (δ 207.4, C-4; 198.3, C-6), one carboxylic (δ 182.6, C-14), two olefinic (δ 165.9, C-2; 127.9, C-3), and two aliphatic [δ 59.0, C-7; 48.0, C-11). The protonated carbons were assigned by the ^{13}C-^1H COSY spectrum. The connectivity of the above carbons and groups was deduced from the ^1H-^1H COSY and HMBC spectra. In the ^1H-^1H COSY spectrum, the cross peaks between H_3-12 and H-9 as well as the methylene protons at δ 3.22 (H-1α) and 2.29 (H-1β) due to homoallylic long-range couplings, and between H-9 and the protons of two methylenes at δ 1.45 (H-8α), 2.59 (H-8β), 1.20 (H-10α), and 2.56 (H-10β) were observed, suggesting a partial structure shown by bold lines in Figure 1. The HMBC correlations (Figure 1) from both H_2-1 and H_2-10 to C-11, C-15, and C-14 and from H_3-15 to C-1, C-10 and C-14 indicated connectivity of C-11 to C-1 and C-10, with C-15 and C-14 attached to C-11. The correlations from H_3-12 to C-6, from H_2-8 to C-6 and C-7, from H_3-13 to C-6, C-7, C-4, and C-8, and from H_3-5 to C-4 indicated a six-membered ring formed by C-2, C-3, C-6, C-7, C-8, and C-9, with C-13 and C-4 attached to C-7. Thus, the gross structure of **1** was derived as shown in Figure 1.

The relative stereochemistry of **1** was determined from the NOE interactions in the NOESY spectrum (Table 1) and the proton coupling constants in the ^1H NMR spectrum. The presence of NOE correlations between H-1β/H_3-15, H-10β/H_3-15, H-9/H_3-15, H-9/H-8β, H-9/H-10β, H-9/H_3-5, H_3-13/H-8α, and H_3-13/H-8β, and the absence of the correlations between H_3-15/H-1α, H_3-15/H-10α, H-9/H-10α, H-9/H-8α, and H-8α/H_3-5 indicated that H-9, 7-Ac, and 11-Me were at the same side and in β-orientations, while 7-Me and 11-COOH were in α-orientations. The above evidence in combination of the ^1H NMR large coupling constants between H-9 and H-8α (11.6 Hz) and H-10α (12.4 Hz) indicated that the five-membered ring was in E_{10} form, and the six-membered ring was in a half chair form with C-2, C-3, C-6, and C-9 held in the same plane, while C-7 was oriented up and C-8 down the plane. In conclusion, the whole structure of **1** was established as depicted.

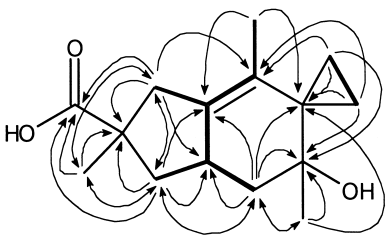
The structure of **1** is interesting because a cyclopropane ring in illudane skeleton is absent, instead, an acetyl group attaches to C-7. It is presumably derived by rearrangement from illudane or protoilludane skeleton. This is the first example to be assigned with such a novel illudane skeleton.

Paneolilludinic acid (**2**) was established as having a molecular formula of $C_{15}H_{22}O_3$ by its HRTOFMS, EIMS, and NMR (^1H, ^{13}C, and DEPT) data. The IR spectrum exhibited absorptions for hydroxyl (3363 cm^{-1}) and

Fig. 1. ^1H-^1H and long ^{13}C-^1H correlations of **1** and **2**.



1

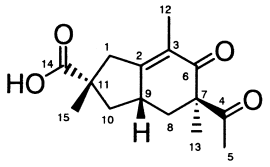


2

Table 1. ^{1}H and ^{13}C NMR data and NOESY correlations of compounds **1** and **2** (in $CDCl_3$)[a].

position	**1**			**2**		
	^{1}H	NOESY	^{13}C	^{1}H	NOESY	^{13}C
1α	3.22 *br d* (18.8)	1β, 12	42.2	2.87 *br d* (17.4)	1β, 12	41.0
1β	2.29 *br d* (18.8)	1α, 12, 15		2.26 *br d* (17.4)	1α, 12, 15	
2			165.9			138.1
3			127.9			127.7
4α			207.4	1.23 *td* (10.0, 2.0)	4β, 5α	21.9
4β				1.48 *ddd* (10.0, 8.8, 2.0)	4α, 5β, 12	
5α	2.05 *s*	8β, 9, 13	26.3	2.11 *ddd* (10.8, 10.0, 2.0)	4α, 5β, 13	34.3
5β				1.99 *ddd* (10.8, 8.8, 2.0)	4β, 5α, 12	
6			198.3			43.7
7			59.0			73.3
8α	1.45 *dd* (12.8, 11.6)	8β, 10α, 13	39.4	0.88 *dd* (12.8, 11.2)	8β, 10α, 13	34.6
8β	2.59 *dd* (12.8, 4.8)	5, 8α, 9, 13		1.59 *dd* (12.8, 6.0)	8α, 10β, 9, 13	
9	2.80 *m*	5, 8β, 10β, 15	38.6	2.55 *m*	8β, 10β, 15	36.7
10α	1.20 *t* (12.4)	8α, 10β	44.8	1.68 *t* (12.4)	8α, 10β	44.0
10β	2.56 *dd* (12.4, 7.2)	9, 10α, 15		1.87 *dd* (12.4, 6.8)	9, 10α, 15	
11			48.0			46.7
12	1.70 *br s*	1α, 1β	11.8	1.64 *br s*	1α, 1β, 4β, 5β	12.8
13	1.28 *s*	5, 8α, 8β	23.1	1.20 *s*	5α, 8α, 8β	23.4
14			182.6			183.2
15	1.38 *s*	1β, 9, 10β	25.5	1.36 *s*	1β, 9, 10β	25.1

[a] Coupling constants (parentheses) are given in Hz.



1 **2**

carbonyl (1695 cm^{-1}) groups. Its ^{1}H, ^{13}C NMR (Table 1), and DEPT spectra indicated three methyl groups, five methylenes, a methine, and six quaternary carbons, with one of them oxygenated, two olefinic, and one carboxylic. Interpretation of the ^{1}H-^{1}H COSY spectrum suggested a $-CH_2CH_2-$ residue besides the same partial structure as that in **1** (Figure 1). The further connectivity was deduced from the HMBC spectrum (Figure 1). Thus, the HMBC correlations were observed of H_2-4 and H_2-5 with C-3, C-6, and C-7, and of H_3-13 with C-8, C-7, and C-6, besides the same correlations of H_2-1, H_2-10, H_3-15, H_2-8, and H_3-12 as those in **1**. Its relative stereochemistry was also assigned from the NOESY data and the ^{1}H NMR coupling constants. As in **1**, the NOE cross peaks in the NOESY spectrum

(Table 1) were observed between H-1β/H_3-15, H-10β/H_3-15, H-9/H_3-15, H-9/H-8β, H-9/H-10β, H_3-13/H-8α, and H_3-13/H-8β. These together with the $J_{8\alpha,9}$ (11.2 Hz) and $J_{9,10\alpha}$ (12.4 Hz) values indicated that the five-membered ring was also in E_{10} form, and the six-membered ring in a half chair ($^7H_8$) form, with H-9, 7-OH, and 11-Me in β-orientations, while 7-Me and 11-COOH in α-orientations. Compound **2** was thus elucidated as shown. A similar compound, illudinic acid, has been previously reported[9].

In an assessment of antibacterial activity by the agar diffusion method, both **1** and **2** were found to possess potency against *Staphylococcus aureus* but no activity against *Escherichia coli*. The activity to *S. aureus* of **1** was much more potent than that of **2** (see Experimental section). In an evaluation of cytotoxicities by the MTT method, **1** and **2** were slightly cytotoxic to some human tumor cells as shown in Experimental section.

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