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Five new compounds from the fungus *Ganoderma petchii*

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ABSTRACT

The fungal species of the genus *Ganoderma* attracted great interest in the last decades. Our recent investigation on *Ganoderma petchii* afforded five new compounds, (−)-petchioics A and B (**1** and **2**), petchiates A and B (**3** and **4**), petchine (**5**), and a known compound. The structures of the new compounds were elucidated on the basis of spectroscopic data. The absolute configurations of **1** and **2** were assigned by computational methods. Biological activities of these isolates towards human cancer cells, COX-1/2, and influenza virus were evaluated.

1. Introduction

Ganoderma is a genus of polypore mushrooms which grows on wood and distributed mainly in tropical regions [1]. Many *Ganoderma* species are known as Lingzhi mushrooms and of significant medical values in traditional Chinese medicines which attracted great interest in the last decades [2]. A literature search via SciFinder found that more than 15,000 documents dealing with investigations on *Ganoderma*, indicating its significance there. Previous chemical investigations revealed that triterpenoids and polysaccharides are major components of this genus [3]. We have reported a novel meroterpenoid with renoprotective effects from *G. lucidum* [4], since then, several structurally novel and biologically active meroterpenoids were isolated and characterized [5–10]. During our continuous research on *Ganoderma* species, *Ganoderma petchii* was investigated. This mushroom spreads over China, Sri Lanka, Malaysia, Singapore and Indonesia. It is sold in several markets of Chinese medical materials and used for the treatment of a wide range of diseases in Chinese folk. However, so far no chemical investigation on this species was documented, our effort on it afforded six metabolites including five new compounds. Herein, we report their isolation, structure characterization and biological evaluation.

2. Experimental

2.1. General

Optical rotations were recorded on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrometer. CD spectra were measured on a Chirascan instrument. NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer, with TMS as an internal standard. ESIMS and HRESIMS were measured on an API QSTAR Pulsar 1 spectrometer. C-18 silica gel (40–60 μm; Daiso Co., Japan), MCI gel CHP 20P (75–150 μm, Mitsubishi Chemical Industries, Tokyo, Japan) and Sephadex LH-20 (Amersham Pharmacia, Uppsala, Sweden) were used for column chromatography. Silica gel for thin-layer-chromatography (Qingdao Marine Chemical Inc., Qingdao, PR China) was used for preparative TLC. Semi-preparative HPLC was carried out using an Agilent 1200 liquid chromatograph with an YMC-Pack ODS-A column (250 × 10 mm, i.d., 5 μm).

2.2. Fungal material

The fruiting bodies of *G. petchii* were purchased from a market of Chinese medical materials located at Zhonghao-Luoshi-Wan of Kunming, PR China, in July 2014. The material was identified by Prof. Zhu-Liang Yang at Kunming Institute of Botany, Chinese Academy of Sciences, and a voucher specimen (CHYX-0588) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West

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2.3. Extraction and isolation

The powders of fruiting bodies of *G. petchii* (60 kg) were extracted by reflux with 70% EtOH (150 L × 2 h × 3) to give a crude extract (3.39 kg), which was suspended in water followed by partition with EtOAc to afford an EtOAc soluble extract. This extract (2.09 kg) was divided into eight parts (Fr.1–Fr.8) by using a MCI gel CHP 20P column eluted with gradient aqueous MeOH (10%–100%). Fr.3 (185 g) was further separated via MCI gel CHP 20P washed with gradient aqueous MeOH (20%–100%) to yield 7 fractions (Fr.3.1–Fr.3.7). Among them, fr.3.2 (10.1 g) was submitted to a RP-18 column eluted with gradient aqueous MeOH (10%–100%) to yield 6 subfractions (Fr.3.2.1–Fr.3.2.6). Fr. 3.2.3 (170 mg) was purified by preparative TLC (2 drops of formic acid in $CHCl_3$/MeOH, 9:1) followed by semipreparative HPLC (MeOH/H_2O, 40%) to yield compounds **1** (2.3 mg, t_R = 13.4 min) and **2** (2.5 mg, t_R = 12.3 min). Fr. 3.6 (49 g) was gel filtrated over Sephadex-LH20 (MeOH) to afford 5 parts (Fr.3.6.1–Fr.3.6.5), in which fr.3.6.3 (19 g) was further divided into 7 fractions by increasing amounts of MeOH in water (30%–100%). Fr.3.6.3.2 (210 mg) was purified by semipreparative HPLC (MeOH/H_2O, 47%) to produce compounds **3** (2.2 mg, t_R = 14.8 min) and **4** (2.0 mg, t_R = 9.2 min). Fr.3.4 (21 g) was submitted to a RP-18 column eluted with gradient aqueous MeOH (30%–100%) to yield 5 fractions (Fr.3.4.1–Fr.3.4.5). Fr.3.4.3 (2.8 g) was fractionated into 4 parts (Fr.3.4.3.1–Fr.3.4.3.4) by using Sephadex LH-20 (MeOH). Fr.3.4.3.1 (155 mg) was purified by preparative TLC ($CHCl_3$/MeOH 9:1) followed by semipreparative HPLC (MeOH/H_2O, 56%; MeOH/H_2O, 37%) to yield compounds **5** (3.0 mg, t_R = 19.2 min) and **6** (3.9 mg, t_R = 26.1 min), respectively.

(−)-Petchioic A (**1**): yellowish gum; $[\alpha]_D^{22} = -33.2$ (*c* 0.37, MeOH); UV (MeOH) λ_{max} (log ε): 278 (2.73), 203 (3.62); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (negative) *m/z* 227.0918 $[M-H]^-$ (calcd for $C_{11}H_{15}O_5$, 227.0925).

(−)-Petchioic B (**2**): yellowish gum; $[\alpha]_D^{22} = -22.7$ (*c* 0.28, MeOH); UV (MeOH) λ_{max} (log ε): 203 (3.26); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (negative) *m/z* 227.0920 $[M-H]^-$ (calcd for $C_{11}H_{15}O_5$, 227.0925).

Petchiate A (**3**): yellowish gum; UV (MeOH) λ_{max} (log ε): 326 (4.26), 208 (3.84); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 261.0732 $[M + Na]^+$ (calcd for $C_{12}H_{14}O_5Na$, 261.0733).

Petchiate B (**4**): yellowish gum; UV (MeOH) λ_{max} (log ε): 321 (4.29), 202 (3.98); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 261.0732 $[M + Na]^+$ (calcd for $C_{12}H_{14}O_5Na$, 261.0733).

Petchione (**5**): yellowish gum; UV (MeOH) λ_{max} (log ε): 345 (3.50), 307 (3.90), 287 (3.82), 204 (4.03); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS (positive) *m/z* 190.0499 $[M + H]^+$ (calcd for $C_{10}H_8NO_3$, 190.0499).

2.4. Cytotoxic assay

Cell lines, K562, MCF-7, A549, Huh-7, Hela, DU145, H1975, and A431 were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Cells were routinely grown and maintained in mediums RPMI or DMEM with 10% FBS and with 1% penicillin/streptomycin. All cell lines were incubated in a Thermo/Forma Scientific CO_2 Water Jacketed Incubator with 5% CO_2 in air at 37 °C. Cell viability assay was determined by the CCK8 (DojinDo, Japan) assay. Cells were seeded at a density of 400–800 cells/well in 384 well plates and treated with various concentration of compounds or solvent control. After 72 h incubation, CCK8 reagent was added, and absorbance was measured at 450 nm using Envision 2104 multi-label Reader (Perkin Elmer, USA).

2.5. Cyclooxygenase (COX) inhibitory assay

COX inhibitory activities of the compounds were evaluated as a previously described method [11].

2.6. MDCK cell based anti-influenza assay

Cell-based anti-influenza virus inhibitor screening was based on the principle of cytopathic effect (CPE) protection assay. Madin–Darby canine kidney (MDCK) cells cultured to approximately 90% confluence were detached with 0.25% Trypsin–EDTA (Invitrogen), washed and resuspended in complete EMEM, 2.5×10^4 MDCK cells were plated in triplicate in a 96-well plate and incubated overnight at 37 °C in a humidified 5% CO_2 incubator. The confluent MDCK monolayers cells were rinsed twice with Hanks' solution devoid of serum, and then the cells were treated with 50 μL medium with 1 mg/mL TPCK and 0.3% BSA and infected by different influenza virus strains at a multiplicity of infection (MOI) of 0.01 PFU/cell. After 2 h incubation, serially diluted compounds were added. After 3-day incubation, the medium was removed and 50 μL medium containing 5 μL CCK8 reagent was added into each well followed by additional 2 h incubation, the absorbance was measured at 450 nm using a UV-star-Microplates Synergy HT plate reader.

Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of compounds **1**–**5** (δ in ppm, *J* in Hz, CD_3OD).

Position	**1**		**2**		**3**		**4**		**5**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		46.1		46.2						
2	5.72, s	124.6	5.72, s	124.3		149.8		150.0		165.5
3		141.5		141.7	6.94, d, 3.5	121.6	6.84, d, 3.4	120.3	7.19, d, 2.4	111.6
4	1.99, m	26.2	1.99, m	26.2	6.51, d, 3.5	111.5	6.46, d, 3.4	111.3		182.2
5	1.76, m 1.68, m	20.4	1.71, m	20.4		161.3		160.9	6.44, dd, 5.6, 2.4	117.5
6	2.10, m 1.57, m	32.6	2.12, m 1.57, m	32.6		131.1		130.3	8.18, d, 5.6	157.8
7	2.69, d, 16.1 2.64, d, 16.1	44.4	2.68, d, 16.2 2.60, d, 16.2	45.6	7.45, s	128.3	7.35, s	126.9		140.4
8		173.3		176.0		169.4		165.9	7.90, d, 8.6	123.6
9		179.4		178.0		196.9		204.6	7.31, dd, 8.6, 2.5	123.8
10	3.92, s	67.1	3.93, s	67.0	4.53, s	57.4	4.50, s	57.4		158.0
11					2.37, s	26.0	2.46, s	26.0	8.25, d, 2.5	140.1
1′					4.37, q, 7.2	62.9	4.25, q, 7.2	62.5		
2′					1.35, t, 7.2	14.4	1.30, t, 7.2	14.4		
OCH_3	3.63, s	51.9	3.66, s	52.5						

3. Results and discussion

Compound **1** had a molecular formula $C_{11}H_{16}O_5$ on the basis of its negative HRESIMS at *m/z* 227.0918 $[M-H]^-$ (calcd for 227.0925, $C_{11}H_{15}O_5$), indicating four degrees of unsaturation (Fig. 1). The ^{13}C NMR and DEPT spectral data (Table 1) showed 11 carbon resonances ascribe to one methoxyl, five methylenes (including one hydroxymethyl), one methine, and four quaternary carbons (two carbonyls, one olefinic and one aliphatic). The HSQC spectrum allowed assignments of all the protons to their bonding carbons. Besides two carbonyls and one double bond, the remaining one unsaturation degree should be due to a ring. The structure construction of **1** was performed by 2D NMR experiments (Fig. 2). The ^1H–^1H COSY spectrum showed correlations of H-4/H-5/H-6. The HMBC correlations of H-2/C-4, C-6, H-4/C-2, C-3 and H-6/C-1, C-2 indicated the presence of a ring as shown. The HMBC correlations of H-7/C-1, C-2, C-6, C-9 and H-2/C-9 suggested that both C-7 and C-9 (δ_C 179.4) were attached to C-1. The presence of a fragment CH_3–O–C-8–C-7 was supported by the HMBC correlations of CH_3(δ_H 3.63) and H-7/C-8. The molecular composition and the chemical shift of C-9 (δ_C 179.4) indicated a carboxylic acid. Taken together, the planar structure of **1** was determined as shown. There is one chiral center in **1**, its absolute configuration was determined by computational methods (see Supporting Information) [12]. Based on the result of ECD calculations which were conducted at the B3LYP/6-31G//B3LYP/6-31G level, the absolute configuration of **1** was assigned as *S*-form (Fig. 4). Therefore, the structure of **1** was established as (*S*)-3-(hydroxymethyl)-1-(2-methoxy-2-oxoethyl)cyclohex-2-ene-1-carboxylic acid and named as (−)-petchioic A.

The molecular formula of compound **2** was assigned as $C_{11}H_{16}O_5$ by the negative HRESIMS at *m/z* 227.0918 $[M-H]^-$ (calcd for 227.0925), having four degrees of unsaturation. The ^1H and ^{13}C NMR data (Table 1) of **2** were very similar to those of **1**, indicating that they are analogues, only differing from the position of a methyl. The HMBC correlation of OCH_3/C-9 but not OCH_3/C-8 allowed the assignment of this methyl group. The *S*-form of **2** was determined using the above computational methods (Fig. 4). Therefore, the structure of **2** was established as (*S*)-2-(3-(hydroxymethyl)-1-(methoxycarbonyl)cyclohex-2-en-1-yl)acetic acid and named (−)-petchioic B.

The molecular formula of **3** was deduced as $C_{12}H_{14}O_5$ by the combination of its HRESIMS and NMR data. The ^1H NMR spectrum of **3** (Table 1) revealed the presence of two methyls [δ_H 2.37 (3H, s); δ_H 1.35 (3H, t, J = 7.2 Hz)], and two olefinic protons [δ_H 6.94, 6.51 (each 1H, d, J = 3.5 Hz)] characteristic of a furan ring. The ^{13}C NMR and DEPT spectra of **3** (Table 1) displayed 12 carbon signals including two methyls, two oxygenated methylenes, three olefinic methines, and five quaternary carbons (one ketone, one ester carbonyl, three olefinic including two oxygenated). The structure of **3** was mainly constructed by aid of an HMBC experiment which showed correlations of H_2-10 (δ_H 4.53)/C-4, C-5, indicating that a hydroxymethyl was attached to C-5. The HMBC correlations of H-11, H-7/C-9, C-6, H-7, H-1′/C-8 supported the presence of the fragment C-11—C-9—C-6—C-7—C-8—O—C-1′—C-2′. This unit was positioned at C-2 supported by the HMBC cross peaks of H-3/C-6 and H-7/C-2. Thus far, the planar structure of **3** was constructed. The geometry of the double bond between C-6 and C-7 was *trans*-form seen from the ROESY correlation of H-7/H_3-11. Therefore, the structure of **3** was deduced as ethyl (*E*)-3-(5-(hydroxymethyl)furan-2-yl)-4-oxopent-2-enoate and named petchiate A.

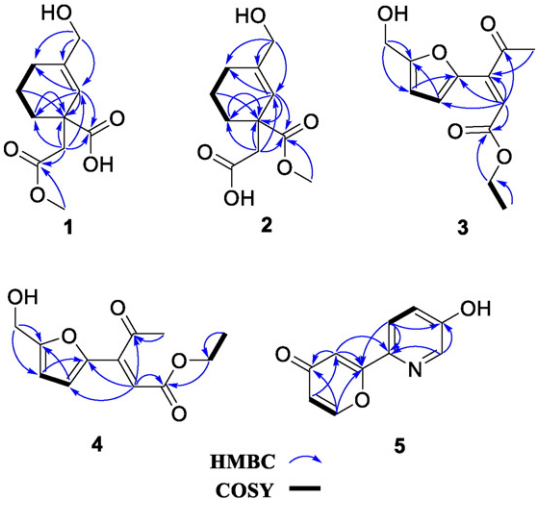

Fig. 2. Key HMBC and ^1H–^1H COSY correlations of compounds **1**–**5**.

Compound **4** has the same molecular formula as that of **3**. The extremely similar NMR data of **3** and **4** suggested that they might be isomers. Careful interpretation of 2D NMR data of **4** implied that **4** is a *Z*-isomer of **3**, which could be readily confirmed by the ROESY correlation of H-3/H-7 instead of H-11/H-7 (Fig. 3). The structure of **4** was therefore identified as ethyl (*Z*)-3-(5-(hydroxymethyl)furan-2-yl)-4-oxopent-2-enoate, named as petchiate B.

Of note, the presence of a methyloxy group in **1** and **2** and an ethoxyl functionality in **3** and **4** allowed us to speculate whether they are artificial products during extraction and isolation. Extraction of material by $CHCl_3$ followed by a careful analysis by LC–MS found that both **1** and **2** are natural products. In this study, **3** and **4** were not detectable, in consideration of the extraction solvent, we tentatively concluded that these two compounds might be artifacts.

Compound **5** had a molecular formula $C_{10}H_7NO_3$ as deduced from its positive HRESIMS and NMR data, indicating eight degrees of unsaturation. The ^{13}C NMR and DEPT spectra showed 10 carbons attributive to six olefinic methines (two oxygenated), four quaternary carbons (one ketone, three olefinic, two of which are oxygenated). In addition to one ketone and five double bonds accounting for six degrees of unsaturation, there should be two rings in the structure occupied the remaining two degrees of unsaturaion. The ^1H–^1H COSY spectrum gave cross peaks of H-5/H-6 and H-8/H-9; the ^1H NMR exhibited two ABX-like coupling systems [δ_H 8.25 (1H, d, J = 2.5 Hz, H-11), δ_H 7.90 (1H, d, J = 8.6 Hz, H-8), δ_H 7.31 (1H, dd, J = 8.6, 2.5 Hz, H-9); δ_H 8.18 (1H, d, J = 5.6 Hz, H-6), δ_H 7.19 (1H, d, J = 2.4 Hz, H-3), δ_H 6.44 (1H, dd, J = 5.6, 2.4 Hz, H-5)], these data

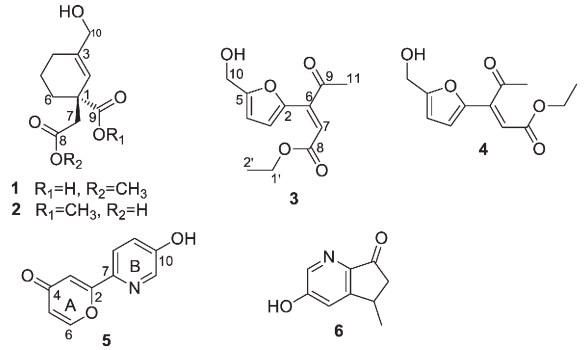

Fig. 1. Chemical structures of compounds **1**–**6**.

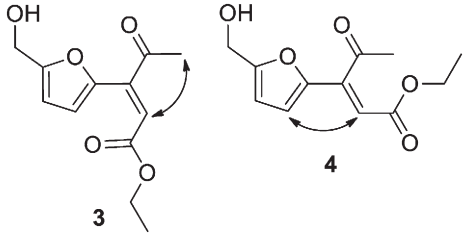

Fig. 3. Key ROESY (↷) correlations of compounds **3** and **4**.

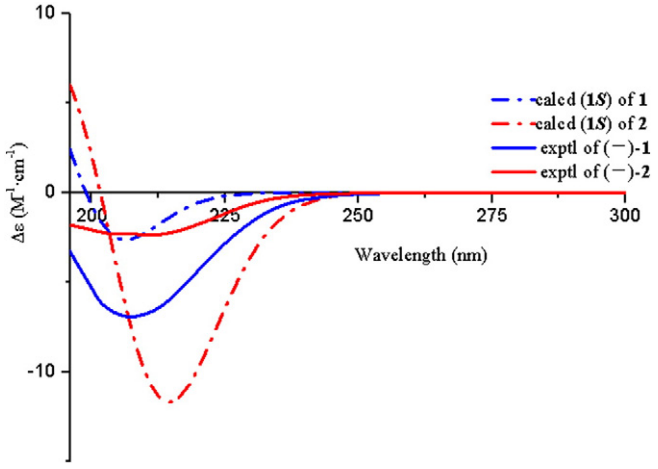

Fig. 4. Calculated and experimental ECD spectra of **1** and **2**.

suggested the presence of two six-membered rings. The architecture of **5** was finally constructed by an HMBC experiment, which showed correlations of H-3, H-5, H-6/C-4, H-3, H-6/C-2, in consideration of the chemical shifts of C-2 and C-6, suggesting the presence of ring A. This conclusion was further confirmed by comparison its NMR data with 2-(4-hydroxyphenyl)-4*H*-pyran-4-one [13]. The coupling pattern of H-8, H-9 and H-11, the HMBC correlation of H-11/C-7, and the downfield shifts of C-7 and C-11 indicated the presence of a typical pyridine. The position of the hydroxyl group in the pyridine was readily assigned due to the chemical shift of C-10. In addition, similar NMR data between 5-hydroxypicolinic acid [14] and a substituted pyridine-3-ol moiety further confirmed the presence of substructure B in **5**. The two rings was connected via C-2 and C-7 supported by HMBC correlations of H-3/C-7 and H-8/C-2. Thus far, the structure of **5** was identified as 2-(5-hydroxypyridin-2-yl)-4*H*-pyran-4-one and named as petchine.

Known compound **6** was identified as 3-hydroxy-5-methyl-5,6-dihydro-7*H*-cyclopenta[*b*]pyridin-7-one by comparison of its NMR data with literature data [15].

In general, triterpenoids and polysaccharides are known as characteristics of *Ganoderma* species. However, the isolation of more and more aromatic meroterpenoids in the recent years adds new facets for *Ganoderma* chemistry. In this study, the findings of compounds **1**–**6**, which are either intermediates of biosynthetic routes or end products in fungi, will further diversify the chemical constituents in *Ganoderma*.

All the isolates were evaluated for their cytotoxicity, cyclooxygenase (COX-1/2) inhibitory activities and anti-influenza activity. Unfortunately, none of them was found to be active towards these assays. Since *Ganoderma* species are used for a variety of diseases, whether these isolates have other significant biological activities needs further investigation.

Conflict of interest

We declare that there is no conflict of interest.

Acknowledgments

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