Illudane Sesquiterpenoids from Edible Mushroom Agrocybe salicacola and Their Bioactivities

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ABSTRACT: To comprehensively understand the chemical constituents of the edible mushroom Agrocybe salicacola and their biological functions, a phytochemical separation of the cultural broth of A. salicacola led to the isolation of four new illudane sesquiterpenoids, agrocybins H−K (1−4), along with 10 known analogues (5−14). Compounds 2−4 were racemates of which 2 and 3 were further separated into single enantiomers as 2a/2b and 3a/3b. All new structures with absolute configurations were elucidated on the basis of an extensive spectroscopic analysis and quantum chemistry calculations. Compound 1 possesses a new carbon skeleton that might be derived from the protoilludane backbone. Compounds 1, 5, 8, and 9 show a certain degree of cytotoxicity to five human cancer cell lines. Compound 1 shows a mild inhibitory effect on nitric oxide production with an IC50 value of 31.4 μM. It is concluded that A. salicacola is rich in illudin derivatives with potential bioactivity prospects, which would make A. salicacola a good material of medicine and food homology.

INTRODUCTION

Agrocybe is a genus of mushrooms in the family Strophariaceae. Approximately 100 species in the Agrocybe are widely distributed all over the world.1 Agrocybe salicacola is an edible mushroom similar to Agrocybe cylindrica. It is endemic to Yunnan Province, People’s Republic of China, and is usually found at the trunks of willows and poplars from September to October.2,3 Our previous chemical investigations on this fungus have revealed a number of illudin derivatives including agrocybone,3 agrocybins A−G,4 and illudin T.5 Among them, agrocybone was a novel illudane−illudane dimer with a highly combined six-ring system,3 while agrocybin A was also a illudin derivative having seven chiral carbons highly cyclized in a six-ring system.4 It is understood that illudins are a group of sesquiterpene metabolites available from fruiting bodies of many wood decay fungi.6 Previous pharmacological investigations have demonstrated that illudins have extensive cytotoxicity activities and antibacterial activities.6−8 For instance, hydroxymethylacylfulvene (HMAF), a semisynthetic antitumor agent based on the naturally occurring illudin S from mushroom Omphalotus olearius,8 has been advanced into human clinical trials for the treatment of cancers.9,9 In addition, there are many other kinds of sesquiterpenoids such as aromadendrane-type and fomannosane-type.10,11

Inspired by the novel structures and their significant biological activities, a comprehensive chemical investigation on the liquid fermentation of A. salicacola was accomplished, which resulted in the isolation of four new illudane derivatives, agrocybins H−K (1−4), together with 10 known analogues (5−14) (Chart 1). Structurally, compound 1 possesses an undescribed carbon backbone that might be derived from the protoilludane skeleton via a carbon−carbon bond cleavage, while compounds 2−4 are aromatized illudane racemates, of which compounds 2 and 3 are further separated into single enantiomers as 2a/2b and 3a/3b, respectively. All compounds were evaluated for their cytotoxicities to five human cancer cell lines and their bioactivities.
inhibitory activities against nitric oxide (NO) release. The isolation, structural elucidation, and biological activities of the isolates are reported herein.

**RESULTS AND DISCUSSION**

Compound 1 was isolated as a colorless oil. The molecular formula was determined as C_{15}H_{24}O_{3} by the positive high-resolution electrospray ionization mass spectrometry (HREIMS) ion data at m/z 275.16159 [M + Na]^+ (calcd for C_{15}H_{24}O_{3}Na, 275.16177), implying four degrees of unsaturation. Inspection of the ^1H, ^13C nuclear magnetic resonance (NMR) (DEPT) and heteronuclear single quantum coherence (HSQC) spectra revealed the existence of 15 carbon signals, including four CH_{3}, four CH_{2}, two CH, and five quaternary carbons (Tables 1 and 2). Among them, two signals at δ_{C} 59.5 (t, C-4) and 74.1 (t, C-5) were assigned to two oxygenated methylene groups. One signal at δ_{C} 202.4 (s, C-8) was assigned to a carbonyl group, which should be conjugated to a double bond by their shifts at δ_{C} 153.4 (s, C-6) and 134.7 (s, C-7). This was supported by heteronuclear multiple bond correlations (HMBCs) from one methyl signal at δ_{H} 1.95 (3H, s, H-13) to C-6, C-7, and C-8 (Figure 1). The ^1H−^1H COSY spectrum revealed one fragment as given in Figure 1. The preliminary analysis of the HMBC spectrum indicated that 1 should be an illudane derivative. However, the HMBCs from δ_{H} 1.22 (3H, s, H-12) to δ_{C} 45.6 (d, C-2), 42.7 (s, C-3), C-6 (s, 153.4), and C-4 (59.5) suggested that one oxygenated methylene (C-4) was connected to C-3, while the other oxygenated methylene (C-5) was supposed to be connected to C-6 by the HMBCs from δ_{H} 3.60 and 3.52 (each 1H, d, J = 9.9 Hz, H-5) to C-3, C-6, and C-7. Therefore, compound 1 was elucidated to possess a new backbone, which might be derived from a protoilludane skeleton by the bond cleavage between C-4 and C-5 (Scheme 1). In the ROESY spectrum, the cross-peak of H-2 and H-9 indicated that they were on the same side. Then, the cross-peak between H-9 and H-4 indicated that hydroxymethylene of C-4 possessed the same orientation as
Analysis of its 1D NMR data (Tables 1 and 2) suggested that 2 should also be an illudane sesquiterpenoid with a structure related to that of dihydrogranuloinden (6). The 2D NMR data revealed that the carbonyl group at C-10 in dihydrogranuloinden was replaced by a hydroxymethine group in 2, as supported by the HMBCs from δH 4.67 (1H, s, H-10) to δC 141.8 (s, C-9) and 43.8 (s, C-11), as well as the ROESY correlation between H-10 and H-8 (δH 7.23, s). A detailed analysis of 2D NMR data revealed that other parts of 2 were the same as those of 6. After separation by a chiral column eluted with n-hexane-isopropanol (79:21, v:v, 1 mL/min), the single enantiomers 2a ([α]D26 +7.6) and 2b ([α]D26 −5.2) were obtained in an approximate ratio of 1:1. According to the ECD calculations, 2a showed a negative Cotton effect at 208 nm, suggesting the absolute configuration of C-10 to be R (Figure 3). Correspondingly, the configuration of C-10 in 2b was defined as S (Figure 3). Compound 2 was, therefore, identified as (+)-agrocybin I (2a) and (−)-agrocybin I (2b).

The colorless oil 3 possesses a molecular formula C17H24O4 on the basis of the positive HRESIMS data at m/z 293.17441 [M + H]+ (calcd for C17H25O4, 293.17474). The 1H and 13C NMR data (Tables 1 and 2), as well as the UV absorptions, revealed structural features similar to those of 2. One O-acetyl...
group should be placed at C-15 as supported by the HMBCs from δ_11 4.00 (2H, s, H-15) and 2.05 (3H, s, −OCOCH_3) to δ_8 171.5 (s, −OCOCOCH_3). In addition, one hydroxyl group was placed at C-12 as indicated by the HMBCs from δ_6 4.63 (2H, s, H-12) to δ_6 140.6 (s, C-2), 135.8 (s, C-3), and 133.9 (s, C-6). Compound 3 was also identified as a racemate, which was further separated into pure enantiomers 3a ([(α)26D +8.6] and 3b ([(α)26D −7.7). The ECD calculations established the absolute configurations as S for 3a and R for 3b (Figure 4).

Since illudins have been demonstrated to have good cytotoxicity properties, all compounds were, primarily, tested for their cytotoxicities against five human cancer cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW480) by the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as reported previously. As a result, compounds 1, 5, 6, 8, and 9 showed cytotoxicities to a certain extent. Particularly, compounds 1 and 8 showed significant cytotoxicities to SW480 and MCF-7, respectively, with IC_{50} values of less than 10 μM. The other compounds exhibited no cytotoxicities at the concentration of 40 μM (Table 3). In addition, all compounds were tested for their inhibitory activities against NO production in lipopolysaccharide (LPS)-activated RAW264.7 macrophages, and only compound 1 showed a mild inhibitory effect with an IC_{50} value of 31.4 μM.

In summary, a total of 14 illudin derivatives including four previously undescribed ones have been obtained from cultures of the mushroom A. salicaco. Compound 1 possesses a new backbone. Many compounds were found to possess promising cytotoxicities. This study further confirmed that A. salicaco is expected to be a good resource for bioactive illudin products.

### Experimental Section

#### Instrumentation.

Optical rotations were measured on a Rudolph Autopol IV polarimeter (Hackettstown). Ultraviolet (UV) spectra were obtained using a double beam spectrophotometer (UH5300, Hitachi High-Technologies, Tokyo, Japan). Infrared (IR) spectra were obtained on a Shimadzu Fourier transform infrared spectrometer using KBr pellets. High-resolution electrospray ionization mass spectra (HRESIMS) were measured on an Exacta Orbitrap mass spectrometer with a HESI ion source (ThermoFisher Scientific, Bremen, Germany). Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker Avance III 600 MHz spectrometer (Bruker, Karlsruhe, Germany) with tetramethylsilane (TMS) as an internal standard. Circular dichroism (CD) spectra were measured with an Applied Photophysics spectrometer (Chirascan, New Haven). Silica gel (200–300 mesh), Sephadex LH-20 (GE Healthcare), and reverse phase (RP)-18 gel (20–45 μm, Fuji) were used for column chromatography. Preparative or semipreparative high-performance liquid chromatography (HPLC) was performed on an Agilent 1260 liquid chromatography system equipped with Zorbax SB-C18 columns (5 μm, 9.4 mm × 150 mm or 21.2 mm × 150 mm). Chiral separation was carried out on a Chiralpak AD-H chiral column (5 μm, 250 × 4.6 mm, Daicel).

#### Fungal Material and Cultivation Conditions.

The fungus A. salicaco was collected at the Botanic Garden of Kunming Institute of Botany, Chinese Academy of Sciences (CAS), China, in spring 2008, and identified by Prof. Mu Zang.
of Kunming Institute of Botany, CAS. The voucher specimen (HFC20170912-AS.3) has been deposited at the School of Pharmaceutical Sciences, South-Central University for Nationalities. The liquid culture medium included yeast powder 0.5%, saccharine 5%, peptone 0.15%, MgSO₄ 0.05%, and KH₂PO₄ 0.05%. Inoculums of *A. salicaca* were incubated in a 15 L fermentor for 6 days with an initial pH of 6.0, culture temperature 24 °C, inoculation volume of 10% (by volume), and aeration rate of 1.0vvm. Then, this was transferred into a 50 L fermentation tank under the same conditions for 25 days to afford a 21 L culture broth.

**Extraction and Isolation.** The culture broth of *A. salicaca* (21 L) was initially filtered, and the filtrate was extracted three times with EtOAc. The EtOAc layer was concentrated under reduced pressure to give a crude extract (10 g), which was then subjected to CC over silica gel using a petroleum ether–acetone gradient (1:0–0:1) to afford fractions A–K. Fraction E (900 mg) was first isolated by CC over silica gel (petroleum ether–acetone) and then purified by HPLC (MeCN/H₂O, from 25:75 to 40:60 in 20 min) to give 4 (3.3 mg, retention time (t<sub>R</sub>) = 11.3 min), 8 (1.8 mg, t<sub>R</sub> = 14.2 min), and 11 (6.4 mg, t<sub>R</sub> = 15.3 min). Fraction F (1.1 g) was subjected to CC over silica gel eluted with CHCl₃–MeOH (1:1) to yield compounds 10 (4.5 mg, t<sub>R</sub> = 17.7 min) and 12 (3.7 mg, t<sub>R</sub> = 14.4 min). Compounds 7 (2.9 mg, t<sub>R</sub> = 12.7 min), 9 (3.5 mg, t<sub>R</sub> = 17.7 min), and 12 (4.8 mg, t<sub>R</sub> = 16.6 min) were obtained from fraction F (400 mg) by HPLC (MeCN/H₂O 80:20 to 30:70 in 20 min). Fraction H (600 mg) was subjected to Sephadex LH-20 CC eluted with CHCl₃/MeOH (1:1) and then purified by HPLC (MeCN/H₂O, from 20:80 to 25:75 in 22 min) to yield compounds 5 (5.3 mg, t<sub>R</sub> = 13.2 min) and 6 (1.5 mg, t<sub>R</sub> = 15.2 min). Fraction H (580 mg) was isolated by HPLC (MeCN/H₂O, from 15:85 to 25:75 in 25 min) to give 13 (3.1 mg, t<sub>R</sub> = 14.8 min) and 14 (6.7 mg, t<sub>R</sub> = 16.6 min). Chiral separation of 3 and 4 was performed by semipreparative HPLC using a Chiralpak AD-H column (5 μm, 250 × 4.6 mm², Daicel, P. R. China), eluting with n-hexane–isopropanol in a ratio of 79:21 (v/v, 1 mL/min) and 87:13 (v/v, 1 mL/min), respectively. Finally, 3a (0.7 mg, t<sub>R</sub> = 10.0 min), 2b (0.5 mg, t<sub>R</sub> = 21.2 min), 3a (0.6 mg, t<sub>R</sub> = 22.3 min), and 3b (0.4 mg, t<sub>R</sub> = 7.5 min) were obtained.

**Agrocybin H (1).** Colorless oil; [α]<sub>D</sub><sup>26</sup> +24.0 (c 1.0, MeOH); UV (CH₃OH) λ<sub>max</sub> (log ε) 250 (3.42), 209 (3.14) nm; IR (KBr) ν<sub>max</sub> 3344, 2945, 1701, 1448, 1116, 1028 cm<sup>−1</sup>; 13C NMR data, see Table 1; 1H NMR data, see Table 2; HRESIMS m/z 293.17441 [M + H]<sup>+</sup> (calcld for C₁₇H₂₂O₄Na<sup>+</sup>, 293.17474).

**Agrocybin K (4).** Colorless oil; [α]<sub>D</sub><sup>26</sup> 0 (c 1.0, MeOH); UV (CH₃OH) λ<sub>max</sub> (log ε) 285 (1.83), 230 (2.16), 205 (2.74) nm; IR (KBr) ν<sub>max</sub> 3390, 2947, 1653, 1114, 1031 cm<sup>−1</sup>; 13C NMR data, see Table 1; 1H NMR data, see Table 2; HRESIMS m/z 315.15646 [M + Na]<sup>+</sup> (calcld for C₁₇H₂₂O₄Na<sup>+</sup>, 315.15668).

**Equivalent Circulating Density (ECD) Calculation.** The ECD calculation was performed by the Gaussian 16 software package. Systematic conformational analyses were carried out via SYBYL-X 2.0 with the MMFF94s molecular mechanics force field calculation, with 10 kcal/mol cutoff energy. The obtained conformers were optimized at the B3LYP/6-31G(d) level of theory with an IEF-PCM solvent model (MeOH), followed by frequency calculation. The Boltzmann distribution was used to calculate the population of each conformer by Gibbs free energy. The ECD (TDDFT) was calculated at the B3LYP/6-31+G(d) level of theory with IEF-PCM solvent model (MeOH). The calculated ECD curves and weighted ECD were used by SpecDis V1.71 with σ = 0.30 eV with a UV shift of −6 nm for 2 and σ = 0.30 eV with a UV shift of 6 nm for 3.

**Specific Optical Rotation Calculation.** A conformation search based on molecular mechanics with MMFF force fields was performed for 3a and 3b, giving 2 and 2 stable conformers with populations higher than 1%, respectively. All of these conformers were further optimized by the density functional theory method at the B3LYP/6-31G(d) level by the Gaussian 16 program package and then subjected to specific optical rotation calculations at the B3LYP/6-311++G(2d,p) level in MeOH with a PCM model. The calculated specific optical rotation data of these conformers were averaged according to the Boltzmann distribution theory and their relative Gibbs free energy.

**Cytotoxic Assays.** All compounds were assessed for their cytotoxicity against five human cancer cell lines including colon cancer SW480, breast adenocarcinoma MCF-7, lung cancer A549, hepatocellular carcinoma SMMC-7721, and human myeloid leukemia HL-60 cells. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) or Roswell Park Memorial Institute (RPMI)-1640 (HyClone), supplemented with 10% fetal bovine serum (FBS, HyClone) in 5% CO₂ at 37 °C. The assays were performed according to the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method in 96-well microplates. Briefly, 100 μL of adherent cells was seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before addition of test compounds, while suspended cells were seeded just before drug addition with an initial density of 1 × 10<sup>5</sup> cells/mL. Each tumor cell line was exposed to the test compound at concentrations of 0.0625, 0.32, 1.6, 8, and 40 μM in triplicate for 48 h, with taxol as a positive control. After compound preincubation, the cells were treated with serial dilutions of the test compounds, up to a maximum concentration of 25 μM. The culture plates were incubated for 48 h, and 10 μL of MTT solution was added to each well. After 4 h of incubation, 100 μL of DMSO was added to each well to dissolve the MTT formazan crystals, and the absorbance was measured at 570 nm with a microplate reader.
μM, in the presence of 1 μg/mL LPS for 18 h. The compounds were dissolved in dimethyl sulfoxide (DMSO) and further diluted in the medium to produce different concentrations. NO production in each well was assessed by adding 100 μL of Griess reagent (reagent A and reagent B, Sigma) to 100 μL of each supernatant from the lipopolysaccharide (LPS, Sigma)-treated or LPS- and compound-treated cells in triplicate. After incubation for 5 min, the absorbance of samples was measured at 570 nm with a 2104 EnVision multilabel plate reader (PerkinElmer Life Sciences). MG-132 was used as a positive control (IC$_{50}$ = 0.2 μM).

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at [https://pubs.acs.org/doi/10.1021/acsomega.0c03314](https://pubs.acs.org/doi/10.1021/acsomega.0c03314).

Spectroscopic data including 1D and 2D NMR, HRMS, CD, and calculation details of compounds 1–4 (PDF)

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### Notes

The authors declare no competing financial interest.

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