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Isolation, Identification, and Bioactivity of Monoterpenoids and Sesquiterpenoids from the Mycelia of Edible Mushroom *Pleurotus cornucopiae*

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Supporting Information

ABSTRACT: Edible mushroom is a profilic source of bioactive metabolites for the development of drugs and nutraceuticals. In this work, four new monoterpenoids (**1**−**4**) and one new sesquiterpenoid (**6**) were isolated from the mycelia of edible mushroom *Pleurotus cornucopiae* fermented on rice. Their structures were established by nuclear magnetic resonance, mass spectrometry, and circular dichroism (CD) data analysis. Compound **1** possesses an unusual spiro[benzofuran-3,2′-oxiran] skeleton. The absolute configuration of the 6,7-diol moieties in compounds **1**, **2**, and **6** was assigned using the *in situ* dimolybdenum CD method. Compounds **1**−**5**, **7**, and **8** showed moderate inhibitory activity against nitric oxide production in lipopolysaccaride-activated macrophages, with IC_{50} values in the range of 60−90 μM. Compounds **6** and **7** also exhibited slight cytotoxicity against HeLa and HepG2 cells.

KEYWORDS: *Edible mushroom, Pleurotus cornucopiae, terpenoids, nitric oxide inhibition, cytotoxicity*

INTRODUCTION

Edible mushrooms are featured by the edible macroscopic fruiting bodies. They have been consumed by humans for centuries because of their substantial nutritional value and their potential medicinal value. Edible mushrooms are a prolific source of bioactive secondary metabolites for the development of drugs and nutraceuticals.[1,2] Biologically active secondary metabolites produced by edible mushrooms contribute great to their supposed medicinal usage. In our searching for bioactive metabolites from edible mushrooms, the novel terpenoid derivatives with diverse structural skeleton and interesting bioactivities have been isolated from the mycelia of edible mushrooms *Flammulina velutipes* (Curt.: Fr.) Sing,[3−5] *Pleurotus eryngii*,[6] and *Pleurotus cornucopiae* (Paulet) Rolland.[7]

In mammals, including humans, nitric oxide (NO) is a significant cellular signaling molecule, participating in many physiological and pathological processes. It was evidenced that the chronic exposure to high levels of NO often led to the occurrence of inflammatory and cancer diseases.[8,9] The inhibitors of the overproduction of NO may be useful leading compounds for the development of drugs in the treatment of inflammatory diseases and cancers.[10] Various natural products with NO inhibitory activity have been isolated from plants and fungi.[7,11−14]

P. cornucopiae is an edible mushroom frequently consumed in China. In our early report, five new pleurospiroketals with NO inhibitory activity and cytotoxicity against HeLa cells were isolated from the mycelia of *P. cornucopiae*.[7] In addition to these metabolites, several minor secondary metabolites were also detected in the solid culture extract during the separation. To obtain new natural products with NO inhibitory activity, *P. cornucopiae* was refermented at a bigger scale with the same condition. Chemical investigation on the fractions containing the minor metabolites from the EtOAc-soluble part of the scale-up fermentation extract of *P. cornucopiae* led to the isolation of eight terpenoid derivatives, including four new monoterpenoids (**1**−**4**) and one new sesquiterpenoid (**6**) (Figure 1). The present work describes the isolation and structural elucidation of these compounds, as well as the evaluation of the NO inhibitory activity and growth inhibition against cancer cells of these isolated compounds.

MATERIALS AND METHODS

General Procedure. Silica gel (Qingdao Haiyang Chemicals, China), Sephadex LH-20 (Amersham Pharmacia Biotech AB, Sweden), and ODS (Merck) were used for column chromatography (CC). Semi-preparative high-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6AD instrument with an SPD-20A detector, using the YMC-Pack ODS column (9.4 mm inner diameter × 250 mm, 5 μm). Organic solvents used were from Beijing

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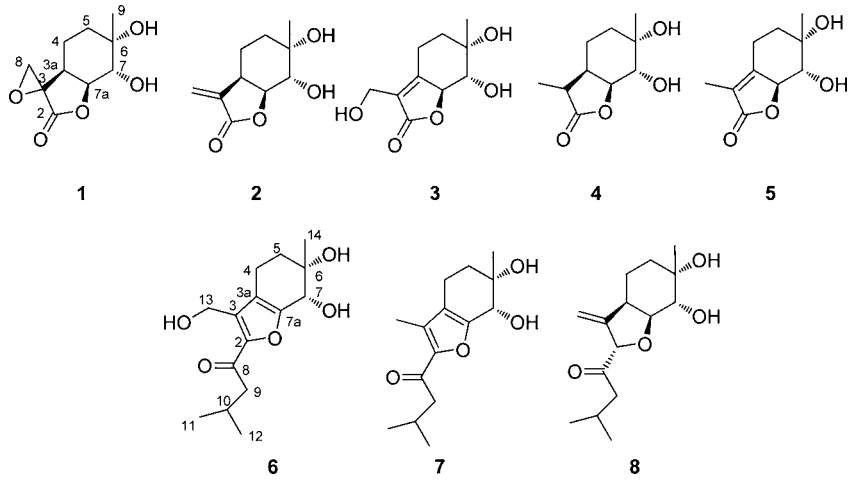

Figure 1. Structures of compounds **1**–**8**.

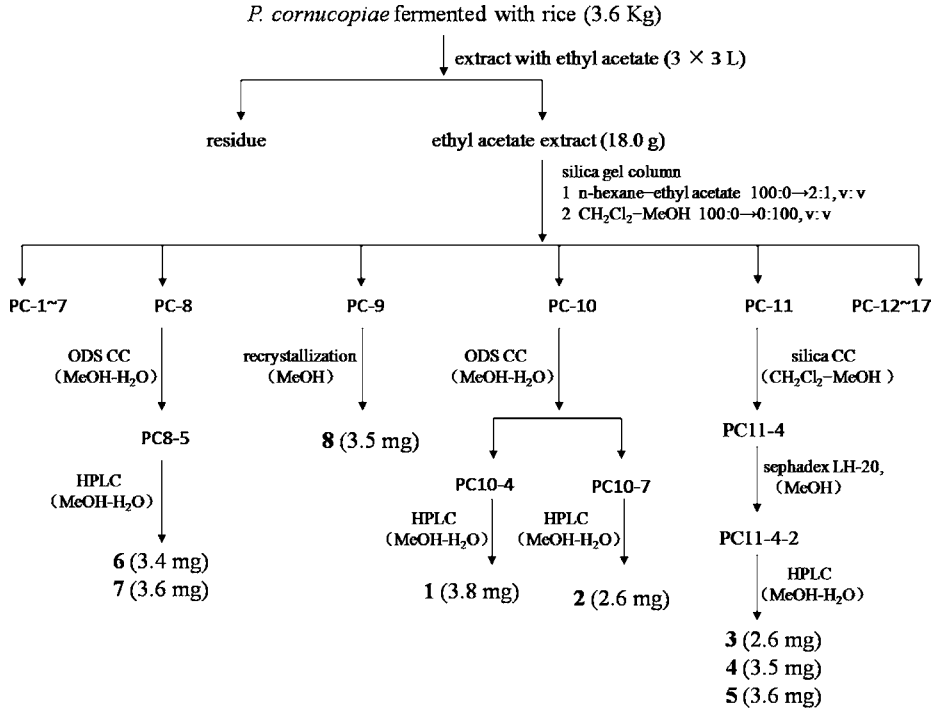

Figure 2. Extraction and isolation flow schematic.

Chemicals (China). Glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin G, lipopolysaccharide (LPS), Roswell Park Memorial Institute (RPMI) medium, streptomycin, and fetal calf serum (FCS) were purchased from Sigma Chemical Co. (St. Louis, MO). Specific rotations were measured on a Perkin-Elmer 341 polarimeter (Perkin Elmer, Waltham, MA). Ultraviolet (UV) spectra were recorded on a Shimadzu UV-2450 UV/vis spectrophotometer. Fourier transform infrared (FTIR) spectra were recorded on a Nicolet Magna-IR 750. Circular dichroism (CD) spectra were measured on JASCO J-815. High-resolution electrospray ionization mass spectrometry (HRESIMS) data were recorded on an Agilent 1200 HPLC/6520Q-TOF mass spectrometer. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker-III-500 spectrometer.

Fungus Material. The strain isolation and identification of *P. cornucopiae* CGMCC 5.366 used in this work was described in an earlier report.[7] *P. cornucopiae* was cultured on slants of potato dextrose agar at 25 °C for 10 days. Agar plugs were inoculated in a 500 mL Erlenmeyer flask containing 120 mL of media (0.4% glucose, 1% malt extract, and 0.4% yeast extract; the final pH of the media was adjusted to 6.5) before sterilization and incubated at 25 °C on a rotary shaker at 170 rpm for 1 week. The scale-up fermentation was carried out in 40 Fernbach culture flasks (500 mL) each containing 80 g of rice and 120 mL of distilled water. Each flask was inoculated with 5.0 mL of the culture broth and incubated at 25 °C for 40 days.

Extraction and Isolation. The fermented rice substrate in 40 flasks was combined and extracted with ethyl acetate by exhaustive maceration (3 × 3 L) at room temperature. The organic solvent was evaporated under reduced pressure to afford the EtOAc extract (18.0 g). The extract was subjected to silica gel (150–250 μm) CC eluted with a gradient of *n*-hexane/ethyl acetate (100:0 → 2:1, v/v), followed by CH_2Cl_2/MeOH (100:0 → 0:100, v/v). The collected solutions were combined by thin-layer chromatography (TLC) analysis into 17 fractions (PC1–PC17). Fractions (PC8–PC11) containing secondary metabolites not obtained in an early study were selected for separation.

Fraction PC10 (1.2 g), obtained by eluting with CH_2Cl_2/MeOH (20:1), was first isolated on ODS CC eluted with MeOH/H_2O (10:90 → 0:100, v/v) to give 27 subfractions (PC10-1–PC10-27).

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Table 1. ^{1}H (500 MHz) NMR Data of Compounds 1−4

position	**1** (MeOH-d_4) δ_H (J in Hz)	**2** (MeOH-d_4) δ_H (J in Hz)	**3** (MeOH-d_4) δ_H (J in Hz)	**4** (MeOH-d_4) δ_H (J in Hz)	**4** (DMSO-d_6) δ_H (J in Hz)
3				2.58, m	2.57, m
3a	3.08, m	3.33		2.38, m	2.26, m
4α	2.02, m	2.16, m	2.88, m	2.06, m	1.88, m
4β	1.61, m	1.90, m	2.60, m	1.61, m	1.40, m
5α	1.47, m	1.59, m	1.98, m	1.60, m	1.50, m
5β	1.33, m	1.46, m	1.50, m	1.59, m	1.40, m
7	3.27, d (8.5)	3.02, d (8.2)	3.13, d (9.0)	3.31 (o)[a]	3.15, dd (6.8, 5.8)
7a	4.73, t (8.5)	4.60, t (8.2)	4.91, d (9.0)	4.45, t (8.1)	4.25, t (6.8)
8	3.15, d (5.5)	6.18, d (3.0)	4.28, s	1.17, d (6.8)	1.02, d (6.8)
	3.06, d (5.5)	5.61, d (3.0)			
9	1.26, s	1.20, s	1.28, s	1.25, s	1.09, s
6-OH					4.17, s
7-OH					4.95, d (5.8)

[a]Signal overlapped with the solvent signal.

Subfraction PC10-4 (50 mg) was further separated by reversed-phase (RP)-HPLC using 10% MeOH in H_2O to give compound **1** (3.8 mg, 32.0 min). Compound **2** (2.6 mg, 23.0 min) was obtained from the subfraction PC10-7 (30.3 mg) by RP-HPLC using 18% methanol in water.

Fraction PC11 (1.5 g) eluted with CH_2Cl_2/MeOH (15:1) was separated on silica gel CC eluted with CH_2Cl_2/MeOH (100:0 → 0:100, v/v) to give 11 subfractions (PC11-1−PC11-10). Subfraction PC11-4 (400.0 mg) was subjected to Sephadex LH-20 separation by MeOH elution to give four subfractions (PC11-4-1−PC11-4-4). Subfraction PC11-4-2 (50.0 mg) was further purified by RP-HPLC using 18% methanol in water to afford compounds **3** (2.6 mg, 7.0 min), **4** (3.5 mg, 31.0 min), and **5** (3.6 mg, 25.0 min).

Fraction PC8 (160.0 mg), obtained by eluting with CH_2Cl_2/MeOH (50:1), was separated on ODS CC (40−63 μm) eluted with MeOH/H_2O (10:90 → 0:100, v/v) to give 10 subfractions (PC8-1−PC8-10). Compounds **6** (3.4 mg, 27.0 min) and **7** (3.6 mg, 43.0 min) were purified from the subfraction PC8-5 by RP-HPLC using 51% methanol in water. Compound **8** (3.5 mg) was recrystallized in methanol from the fraction PC9 (25.0 mg) eluted with CH_2Cl_2/MeOH (30:1). The extraction and isolation schematic is shown in Figure 2. The physical properties and spectroscopic and high-resolution time of flight mass spectrometry (HR-TOF-MS) data of the new compounds are as follows.

Compound 1. White needles. $[\alpha]_D^{25}$ +52.9 (c 2.2 × 10^{-3}, methanol). CD (c 2.3 × 10^{-4} M, methanol). λ_{max} ($\Delta\varepsilon$): 216 (−2.2). UV (methanol) λ_{max}, nm (log ε): 202 (2.6). IR (neat) ν_{max}: 3406, 2932, 1766, 1054, 906 cm^{-1}. Positive ESI−MS m/z: 215 $[M + H]^+$. Positive HR-TOF-MS m/z: $[M + H]^+$ 215.0918 (calcd for $C_{10}H_{15}O_5$, 215.0914). For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

Compound 2. White needles. $[\alpha]_D^{25}$ +56.9 (c 1.3 × 10^{-3}, methanol). CD (c 2.5 × 10^{-4} M, methanol). λ_{max} ($\Delta\varepsilon$): 240 (−2.4). UV (methanol) λ_{max}, nm (log ε): 215 (3.3). IR (neat) ν_{max}: 3424, 2932, 1759, 1675, 1453, 1141, 970 cm^{-1}. Positive ESI−MS m/z: 221 $[M + Na]^+$. Positive HR-TOF-MS m/z: $[M + Na]^+$ 221.0789 (calcd for $C_{10}H_{14}O_4Na$, 221.0784). For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

Compound 3. White needles. $[\alpha]_D^{25}$ +27.4 (c 2.7 × 10^{-3}, methanol). CD (c 4.7 × 10^{-4} M, methanol). λ_{max} ($\Delta\varepsilon$): 240 (−2.8). UV (methanol) λ_{max}, nm (log ε): 220 (2.8). IR (neat) ν_{max}: 3402, 1739, 1681, 1011, 917 cm^{-1}. Positive ESI−MS m/z: 215 $[M + H]^+$. Positive HR-TOF-MS m/z: $[M + H]^+$ 215.0914 (calcd for $C_{10}H_{15}O_5$, 215.0914). For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

Compound 4. Colorless solid. $[\alpha]_D^{25}$ +42 (c 1.2, methanol). CD (c 0.45 × 10^{-3} M, methanol). λ_{max} ($\Delta\varepsilon$): 216 (−0.34). UV (methanol) λ_{max}, nm (log ε): 205 (2.5). IR (neat) ν_{max}: 3399, 2971, 2934, 1764, 1679, 1382, 1302, 1204, 1176, 1090, 1005, 978 cm^{-1}. Positive ESI−MS m/z: 223 $[M + Na]^+$. Positive HR-TOF-MS m/z: $[M + Na]^+$ 223.0947 (calcd for $C_{10}H_{16}O_4Na$, 223.0941). For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

Compound 6. Oil. $[\alpha]_D^{25}$ +24.1 (c 0.8, methanol). UV (methanol) λ_{max}, nm (log ε): 290 (2.8). IR (neat) ν_{max}: 3366, 2960, 1679, 1537, 1204, 1021, 953 cm^{-1}. Positive ESI−MS m/z: 283 $[M + Na]^+$. Positive HR-TOF-MS m/z: $[M + Na]^+$ 305.1356 (calcd for $C_{15}H_{22}O_5Na$, 305.1359). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 3.

Determination of the Absolute Configuration in Compounds 1, 2, and 6 Using the *in Situ* Dimolybdenum CD

Table 2. ^{13}C (125 MHz) NMR Data of Compounds 1−4 in MeOH-d_4

position	**1**	**2**	**3**	**4**
2	176.2	173.0	176.1	182.4
3	58.0	139.3	124.6	37.5
3a	37.3	41.4	166.7	44.6
4	17.9	20.2	22.6	20.1
5	34.8	33.8	38.4	33.3
6	73.1	72.9	73.5	73.3
7	78.8	78.6	81.3	77.2
7a	84.0	84.4	86.0	84.5
8	52.0	120.0	54.4	13.9
9	26.4	26.5	25.9	26.6

Table 3. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of Compound 6

	6 (MeOH-d_4)	
position	δ_C	δ_H (J in Hz)
2	150.0	
3	134.2	
3a	123.5	
4	24.8	2.69, m; 2.47, m
5	33.7	2.02, m; 1.71, m
6	72.7	
7	70.5	4.33, s
7a	155.6	
8	193.9	
9	49.0	2.76, dd (7.2, 1.1)
10	26.4	2.24, m
11	23.1	0.98, d (6.6)
12	23.2	0.98, d (6.6)
13	56.2	4.79, d (13.7); 4.74, d (13.7)
14	19.1	1.25, s

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Method.[15,16] HPLC-grade dimethyl sulfoxide (DMSO) was dried with 4 Å molecular sieves. According to the published procedure, mixtures of 1:1.3 diol/$Mo_2(OAc)_4$ for compounds **1**, **2**, and **6** were subjected to CD measurements at concentrations of 1.0 mg/mL, respectively. The first CD spectrum was recorded immediately after mixing, and its time evolution was monitored until stationary (about 10 min after mixing). The inherent CD was subtracted. The observed signs of the diagnostic bands at around 310 and 400 nm in the induced CD spectrum were correlated to the absolute configuration of the 6,7-diol moiety.

NO Inhibition Assay.[7] RAW 264.7 cells were maintained in RPMI 1640 medium supplemented with penicillin (100 units/mL), streptomycin (100 mg/mL), and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified incubator with 5% CO_2 and 95% air. The medium was routinely changed every 2 days. RAW 264.7 cells were passaged by trypsinization until they attained confluence and were used for assays during the exponential growth phase. Compounds **1**−**8** were dissolved in DMSO and were further diluted with the culture medium to give a final DMSO concentration of 0.2% in the assay. This concentration of DMSO had no significant effect on the growth of the cell line tested. The cell concentration was adjusted to 5 × 10^5 cells/mL. A total of 200 μL of RAW cells were seeded and incubated for 1 h in a 96-well plate. After incubation, the cells were added with lipopolysaccharides (LPS, 1 μg/mL) and test compounds (10−200 μM), and then the plate was incubated for another 24 h. Control groups received an equal amount of DMSO. As a parameter of NO release, the nitrite concentration was measured in the supernatant of RAW 264.7 cells by the Griess reaction. Briefly, 100 μL of culture medium in each well was transferred to another plate, and the level of NO was assessed by measuring the accumulation of nitrite (NO_2^-) using 100 μL of Griess agent (mixture of 0.1% *N*-[naphthalen-1-yl]ethylenediamine in 5% phosphoric acid and 1% sulfanilamide). The concentration of NO_2^- was calculated by a standard curve from 0, 1, 2, 5, 10, 20, 50, and 100 μM $NaNO_2$ solutions. The inhibitory rate of the compounds on NO production induced by LPS was calculated by the NO_2^- levels. IC_{50} values were calculated using linear interpolation of inhibition curves for three independent experiments, each carried out in triplicate. The positive control of hydrocortisone showed NO inhibitory activity, with an IC_{50} value of 53.8 μM.

Growth Inhibition against HeLa and HepG2 Cells.[7] Cytotoxic activity for cancer cell lines HeLa and HepG2 were evaluated with the MTT assay. After the cells were treated with the compounds tested (DMSO as the solvent) for 72 h, 50 μL of MTT/medium solution (0.5 mg/mL) was added to each well and the tumor cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 air for 4 h. Upon removal of MTT/medium, 100 μL of DMSO was added to each well and the plate was agitated at 60 revolutions per minute (rpm) for 5 min to dissolve the precipitate. The assay plate was read at 540 nm using a microplate reader. IC_{50} values were calculated using linear interpolation of inhibition curves for three independent experiments, each carried out in triplicate. Cisplatin was used as the positive control.

RESULTS AND DISCUSSION

Identification of New Secondary Metabolites. The fermented rice substrate of *P. cornucopiae* was extracted repeatedly with EtOAc. The EtOAc-soluble extract was subjected to chromatographic purification on normal-phase silica gel and reverse-phase C18 CC, followed by preparative HPLC, to provide metabolites **1**−**8**. The known compounds **5**, **7**, and **8** were identified as 1,2-dihydroxymintlactone,[17] (6*S*,7*S*)-6,7-dihydroxy-3,6-dimethyl-2-isovaleroyl-4,5,6,7-tetrahydrobenzofuran,[18] and cheimonophyllon E,[19] respectively, by comparison of their NMR and MS data to those published.

Compound **1** had a molecular formula of $C_{10}H_{14}O_5$ (4 degrees of unsaturation) as deduced from the HR-TOF-ESIMS at *m*/*z* 215.0918 and NMR data. The IR absorption bands for hydroxyl (3406 cm^{-1}) and carbonyl (1766 cm^{-1}) groups were observed in its IR spectrum. Analysis of the ^1H and ^{13}C NMR data of compound **1** aided by the heteronuclear single-quantum coherence (HSQC) technique revealed resonances for one methyl (δ_H 1.26, 3H, s; δ_C 26.4), three methylenes, including one oxygenated methylene [δ_H 3.06 (d, *J* = 5.5 Hz), 3.15 (d, *J* = 5.5 Hz), δ_C 52.0], three methines, including two oxymethines [δ_H 3.27 (d, *J* = 8.5 Hz), δ_C 78.8; δ_H 4.73 (t, *J* = 8.5 Hz), δ_C 84.0], three quaternary carbons, including two oxygenated carbons (δ_C 73.1 and 58.0), and a carbonyl carbon (δ_C 176.2) (Table 1). These data accounted for all of the ^1H and ^{13}C resonances and required compound **1** to be tricyclic. The $^1H-^1H$ correlation spectroscopy (COSY) spectrum of compound **1** showed the spin system of H-7-H-7a-H-3a-H_2-4-H_2-5. Heteronuclear multiple-bond correlations (HMBCs) from H_3-9 to C-5, C-6, and C-7, from H_2-8 to C-3 and C-3a, from H-7a to C-2, C-3, and C-4, from H_2-4 to C-3 and C-5, and from H_2-5 to C-7 and C-9 completed the structure of the tetrahydrobenzofuran skeleton (Figure 3). Considering the oxygenated nature of C-3 and C-8, as well as the unsaturation requirement for compound **1**, the formation of a three-membered oxygen ring at C-3 was confirmed by default. On the basis of these data, the gross structure of compound **1** was established, as shown in Figure 1. It possesses an unusual spiro[benzofuran-3,2′-oxiran] skeleton.

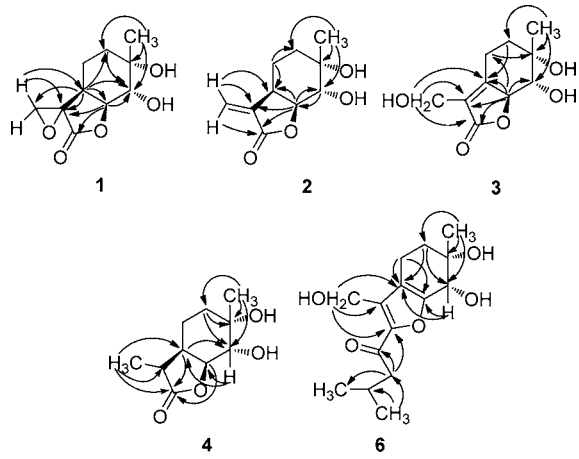

Figure 3. Selected HMBC (H → C) correlations of compounds **1**−**4** and **6**.

The nuclear Overhauser effect spectrometry (NOESY) correlations of H_2-8 with H-4α, H_3-9 with H-7, and H-3a with H-7a were observed in the NOESY spectrum of compound **1**, which in combination with the larger coupling constant of 8.5 Hz between H-7 and H-7a supported the relative configuration of compound **2** (Figure 4). The absolute configuration of C-6 and C-7 in compound **1** was assigned using the *in situ* dimolybdenum CD method.[15,16] As an auxiliary chromophore, a metal complex of compound **1** was produced by the addition of dimolybdenumtetraacetate [$Mo_2(OAc)_4$] to a solution of compound **1** in DMSO. The induced CD of the complex was obtained through subtracting the inherent CD of compound **1**. The sign of the Cotton effect observed in the induced spectrum comes from the chirality of the vic-diol moiety, as indicated by the sign of the O−C−C−O torsion angle. The negative Cotton effects observed at 310 and 400 nm in the induced CD spectrum (Figure 5) allowed for assignment of the 6*S* and 7*R* configurations according to the empirical rule (Figure 5).[15,16] On the basis of current analysis, the absolute configuration of the spiro carbon at C-3 was left unsolved.

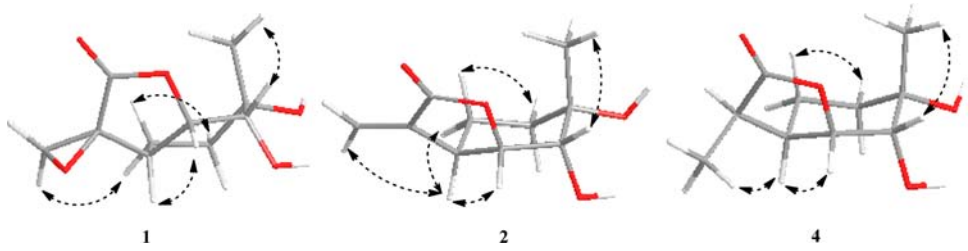

Figure 4. Key NOESY correlations of compounds **1**, **2**, and **4**.

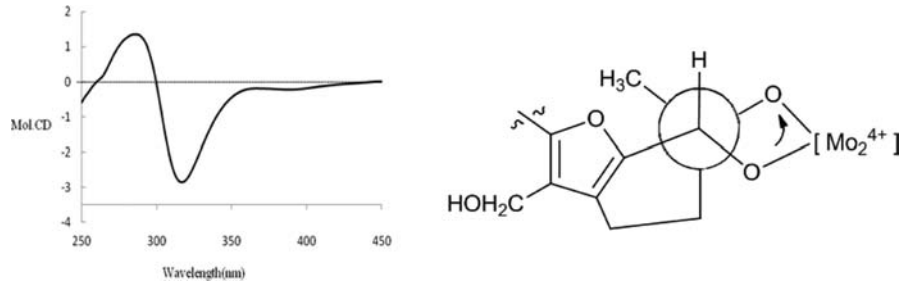

Figure 5. CD spectrum of compound **1** in DMSO containing $Mo_2(OAc)_4$ with the inherent CD spectrum subtracted and conformation of the Mo_2^{4+} complex of compound **1**.

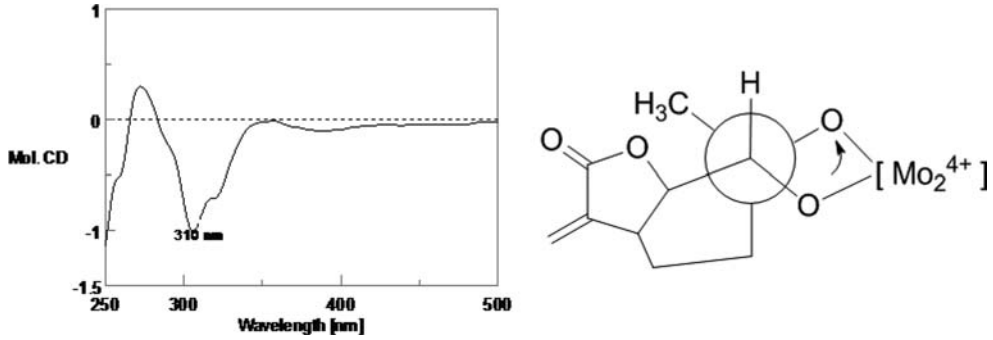

Figure 6. CD spectrum of compound **2** in DMSO containing $Mo_2(OAc)_4$ with the inherent CD spectrum subtracted and conformation of the Mo_2^{4+} complex of compound **2**.

Accordingly, the structure of compound **1** was assigned as (3a*S*,6*S*,7*R*,7a*S*)-6,7-dihydroxy-6-methylhexahydro-2*H*-spiro-[benzofuran-3,2′-oxiran]-2-one.

Compound **2** had a molecular formula of $C_{10}H_{14}O_4$ (4 degrees of unsaturation) as deduced from the HR-TOF-ESIMS at *m*/*z* 221.0789 and NMR data. The IR spectrum revealed the presence of hydroxyl (3424 cm^{-1}) and carbonyl (1759 cm^{-1}) groups. The NMR data of compound **2** (Table 1) displayed signals for one methyl [δ_H 1.20 (3H, s, H_3-9); δ_C 25.0], two methylenes, three methines, including two oxygenated methines [δ_H 3.02 (d, *J* = 8.2 Hz, H-7), 4.60 (t, *J* = 8.2 Hz, H-7a); δ_C 78.6 (C-7); 84.4 (C-7a)], and one α,β-unsaturated carbonyl moiety [δ_H 5.61 (d, *J* = 3.0 Hz, H-8), 6.18 (d, *J* = 3.0 Hz, H-8); δ_C 118.5 (C-8), 137.8 (C-3), and 171.1 (C-2)]. The HMBCs from H_3-9 to C-7/C-6/C-5, from H-4 to C-5/C-3, from H-7a to C-3a/C-7/C-2/C-3, and from H-8 to C-2/C-3/C-3a were observed in its HMBC spectrum (Figure 3), which in combination with $^1H-^1H$ COSY correlations of H-7H-7a-H-3a-H-4-H-5 supported the planar structure of compound **2**.

The larger coupling constant of 8.2 Hz between H-7 and H-7a together with the NOESY correlations of H_3-9 with H-7 and H-5β and H-3a with H-7a indicated the α configurations for H-3a and H-7a and β configurations for H_3-9 and H-7, as described in Figure 4. The absolute configuration of 6,7-diol moieties in compound **2** was determined as described in compound **1**. In the $Mo_2(AcO)_4$-induced CD spectrum of compound **2**, negative Cotton effects at 310 and 400 nm supported the 6*S* and 7*R* configuration for compound **2** on the basis of the empirical rule (Figure 6). Therefore, the structure of compound **2** was determined as (3a*R*,6*S*,7*R*,7a*S*)-6,7-dihydroxy-6-methyl-3-methylenehexahydrobenzofuran-2(3*H*)-one.

The molecular formula of compound **3** was determined to be $C_{10}H_{14}O_5$ from the HR-TOF-ESIMS at *m*/*z* 215.0914 and NMR data analysis (Table 1), requiring 4 degrees of unsaturation. The IR spectrum of compound **3** revealed the presence of hydroxy (3402 cm^{-1}) and carbonyl (1739 cm^{-1}) groups. The NMR data of compound **3** were very similar to those of 1,2-dihydroxymintlactone (**5**), except for the absence of a methyl group and presence of an oxygenated methylene [δ_H: 4.28 (2H, s, H_2-8); δ_C: 54.4 (C-8)] in compound **2**. The HMBCs from H-7a to C-2, C-3, C-3a, C-4, and C-7, from H_2-8 to C-2, C-3, and C-3a, and from H_3-9 to C-5, C-6, and C-7 confirmed the planar structure of compound **3**. The larger coupling constant of 9.0 Hz between H-7 and H-7a together with the NOESY correlations of H_3-9 with H-7 indicated the *cis* configuration between C-6 and C-7 and the *trans* configuration between C-7 and C-7a. The absolute configuration of 1,2-

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dihydroxymintlactone (**5**) has been assigned by application of the modified Mosher's method in an earlier report.[19] The CD spectrum of compound **3** showed the similar negative Cotton effect at 240 nm to those of compound **5**, confirming the 7a*S* configuration in compound **3**. Thus, the structure of compound **3** was assigned as (6*S*,7*R*,7a*S*)-6,7-dihydroxy-3-(hydroxymethyl)-6-methyl-5,6,7,7a-tetrahydrobenzofuran-2(4*H*)-one.

The molecular formula of compound **4** was established as $C_{10}H_{16}O_4$ (3 degrees of unsaturation) by HR-TOF-ESIMS and NMR data (Table 4). Analysis of the ^{1}H and ^{13}C NMR and HMQC spectra of compound **4** indicated 10 carbon signals, including two methyls [δ_H 1.17 (3H, d, J = 6.8 Hz); 1.25 (3H, s); δ_C 13.9, 26.6], four methines, including two oxymethines [δ_H 3.31 (overlapped with solvent signal); δ_C 77.2 and δ_H 4.45 (t, J = 8.1 Hz); δ_C 84.5], two methylenes, and one carbonyl group (δ_C 182.4). The HMBC spectrum of compound **4** showed correlations from H_3-9 to C-5, C-6, and C-7, from H_3-8 to C-2, C-3, and C-3a, and from H-7a to C-2, C-3, C-3a, and C-7 (Figure 3), which in combination with the ^{1}H−^{1}H COSY correlations of H-7-H-7a-H-3a-H-4-H-5 and H_3-8-H-3-H-3a established the planar structure of compound **4**.

Table 4. NO Inhibitory Activity and Growth Inhibition against HeLa and HepG2 Cells of Compounds 1−8

	NO inhibition		growth inhibition (IC_{50}, μM)	
compounds	IC_{50} (μM)	cell viability (%)[a]	HeLa	HepG2
1	81.8	99.2 ± 1.6	inactive	inactive
2	88.8	97.8 ± 1.8	inactive	inactive
3	80.4	98.6 ± 2.0	inactive	inactive
4	65.6	99.6 ± 2.2	inactive	inactive
5	72.8	98.3 ± 1.3	inactive	inactive
6	>100	101.3 ± 1.8	70.6	76.8
7	76.5	98.8 ± 2.3	36.0	68.6
8	72.4	102.2 ± 2.4	inactive	inactive
hydrocortisone	53.8	100.8 ± 2.6		
cisplatin			16.8	18.6

[a]The percentage of viable cells was determined by comparing to control groups for tested compounds at the concentration of 100 μM.

Because the ^{1}H NMR signal of H-7 recorded in the solvent of MeOH-d_4 was overlapped with the solvent signal, the NOESY spectrum of compound **4** obtained in MeOH-d_4 is too poor to determine its relative stereochemistry. The ^{1}H NMR and NOESY spectra of compound **4** in DMSO-d_6 were recorded to assign its relative stereochemistry. Signals because of two hydroxyl groups were observed at δ_H 4.17 (s, OH-6) and 4.95 (d, J = 5.8 Hz, OH-7). In the NOESY spectrum recorded in DMSO-d_6, the NOESY correlations of H-3a with H-7a, H_3-8 with H-3a and H_3-9 with H-7 were observed, which indicated the relative stereochemistry of compound **4**, as described in Figure 4. In the CD spectrum of compound **4**, the negative Cotton effect at 216 nm (the exciton coupling of the $n-\pi^*$ transition in γ-lactone) confirmed the 3a*R* configuration in compound **4** according to the octane rule for γ-lactones.[20] Considering the relative configuration determined by NOESY data, an absolute configuration of 3*R*, 3a*R*, 6*S*, 7*R*, and 7a*S* was assigned to compound **4**.

The molecular formula of compound **6** was assigned as $C_{15}H_{22}O_5$ (5 degrees of unsaturation) from HR-TOF-ESIMS and NMR data (Table 3). Inspection of ^{1}H and ^{13}C NMR and HMQC data of compound **6** revealed a conjugated ketone carbon (δ_C 193.9), four sp^2 quaternary carbons (δ_C 155.6, 150.0, 134.2, and 123.5), an oxygenated quaternary carbon (δ_C 72.7), two methins, including one oxymethine [δ_H 4.33 (1H, s); δ_C 70.5], four methylenes, including one oxygenated methylene [δ_H 4.74 (d, J = 13.7 Hz), 4.79, (d, J = 13.7 Hz); δ_C 56.2), and three methyl groups [δ_H 1.25 (s), 0.98 (6H, d, J = 6.6 Hz); δ_C 19.1, 23.1, 23.2]. The methylene protons at δ_H 2.76 (H_2-9) showed HMBC to C-8, C-10, and C-11(12), which together with HMBCs from H_3-11(12) to C-9 and C-10 indicated an isovaleroyl group (C-8−C-12). HMBCs from H-7 to C-3a, C-5, C-6, C-7a, and C-14, from H_2-5 to C-3a, C-4, C-6, C-7, and C-14, from H_2-4 to C-3a, C-5, C-6, and C-7a, and from H_3-14 to C-5, C-6, and C-7 indicated the cyclohexene structural moiety. HMBCs from H_2-13 to C-2, C-3, and C-3a confirmed the linkage of C-2-C-3-C-3a. In addition, the HMBC from H_2-9 to C-2 confirmed the connection of the isovaleroyl group at C-2. Considering the chemical shifts of four sp^2 quaternary carbons (C-2, C-3, C-3a, and C-7a), as well as the unsaturation requirement for compound **6**, the formation of the furan ring was deduced. The relative configuration in compound **6** was determined by a one-dimensional (1D) nuclear Overhauser effect (NOE) experiment. Irradiation of the methyl signal at δ_H 1.27 (H_3-14) led to the NOE enhancement of the signal at δ_H 4.36 (H-7), which supported the *cis* orientation between C-6 and C-7. The absolute configuration of compound **7** has been assigned as 6*S* and 7*S* by application of the modified Mosher's method in an earlier report.[19] To determine the absolute configuration of 6,7-diol moieties in compound **6**, the *in situ* dimolybdenum CD method was applied. The $Mo_2(AcO)_4$-induced CD spectrum of compound **6** showed negative Cotton effects at 310 and 400 nm, indicating the 6*S* and 7*S* configuration for compound **6** (Figure 7).

Bioactivity Evaluation. Compounds **1**−**8** were evaluated for their inhibitory activities of NO production from macrophage RAW 264.7 and growth inhibition against HeLa and

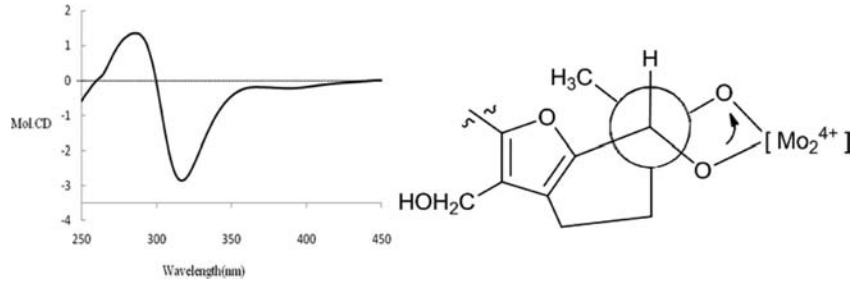

Figure 7. CD spectrum of compound **6** in DMSO containing $Mo_2(OAc)_4$ with the inherent CD spectrum subtracted and conformation of the Mo_2^{4+} complex of compound **6**.

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HepG2 cell lines. Compounds **1**–**8** showed no cytotoxicity to macrophage cells at the concentration of 100 μM by the MTT method. As shown in Table 4, compounds **1**–**5**, **7**, and **8** showed moderate inhibition against nitric oxide production in lipopolysaccaride-activated macrophages, with an IC_{50} value of 81.8, 88.8, 80.4, 65.6, 72.8, 76.5, and 72.4 μM, respectively. Hydrocortisone was used as the positive control, with the IC_{50} value of 53.8 μM. Terpenoid derivatives with various carbon skeletons have been reported to possess NO inhibitory activity. Examples included meroterpenes with IC_{50} values in the range of 7.71–27.63 μM from *Psoralea corylifolia* fruits,[21] one new drimane-type sesquiterpenoid ether of isocitric acid with an IC_{50} value of 86.4 μM from the fruiting bodies of *Cryptoporus sinensis*,[12] a novel sesquiterpene named as souliene A (IC_{50} value of 24.5 μM) from the roots of *Vladimiria souliei*,[22] β-dihydroagarofuran sesquiterpenes with IC_{50} values ranging from 14.7 to 24.7 μM from the fruits of *Celastrus orbicultus* Thunb.,[23] one new lathyrane diterpenes with an IC_{50} value of 14.56 μM from *Euphorbia prolifera*,[13] two new *neo*-clerodane diterpenes with IC_{50} values of 64.6 and 25.3 μM from *Ajuga decumbens*,[24] and two new cyathane-type diterpenoids with strong NO inhibitory activity (IC_{50} values of 2.57 and 1.45 μM) from the mycelia of the medicinal mushroom *Cyathus africanus*.[25]

In the growth inhibition assay against HeLa and HepG2 cell lines (Table 4), compounds **6** and **7** exhibited slight growth inhibition against HeLa cells (IC_{50} values of 70.6 and 36.0 μM) and HepG2 (IC_{50} values of 76.8 and 68.6 μM). Compounds **6** and **7** showed different effects on RAW 264.7, HeLa, and HepG2 cells. The reason for such difference is unknown, which deserves further investigation.

Metabolites with medicinal properties have been isolated from the fruiting body and mycelia of this fungus.[7,26−29] In this work, four new monoterpenoids (**1**–**4**) and one new sesquiterpenoid (**6**) were obtained from the solid culture of edible mushroom *P. cornucopiae* fermented on rice. Compound **1** possesses an unusual spiro[benzofuran-3,2′-oxiran] skeleton. Compounds **1**–**5**, **7**, and **8** showed moderate inhibitory activity against nitric oxide production in lipopolysaccaride-activated macrophages. Compounds **6** and **7** exhibited slight cytotoxicity against HeLa and HepG2 cancer cells. Compounds **1**–**8** were isolated for the first time from edible mushroom *P. cornucopiae*, which advances our understanding of the secondary metabolism in this fungus.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra data of the new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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