Hirsutane Sesquiterpenes from the Fungus *Lentinus connatus* BCC 8996

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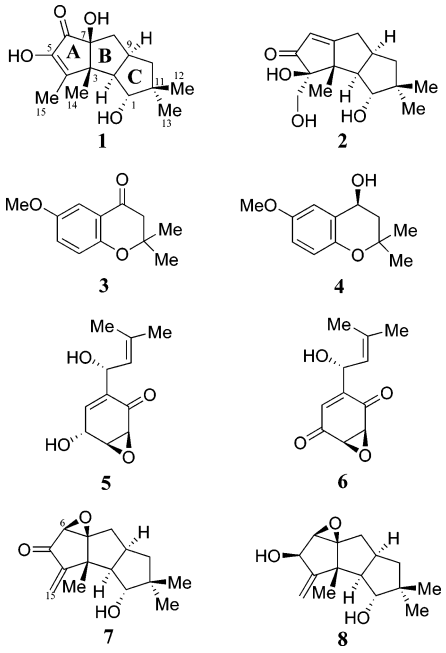
Two new hirsutane sesquiterpenes, connatusins A (**1**) and B (**2**), were isolated from the fungus *Lentinus connatus* BCC 8996. The structures, closely related to hypnophilin, were elucidated on the basis of the spectroscopic data. An X-ray analysis was performed to confirm the structure of **1**. Six known compounds were also obtained. Panepoxydone (**5**), panepoxydione (**6**), and dihydrohypnophilin (**8**) exhibited significant antimalarial and cytotoxic activities.

Many biologically active secondary metabolites have been isolated from the genus *Lentinus*, for example, an antitumor polysaccharide,[1] the antibiotic cortinellin,[2] and antimicrobial hirsutane sesquiterpenes.[3] However, chemical investigation of *L. connatus* has not been reported. The crude EtOAc extract of the culture broth of *L. connatus* BCC8996 showed significant cytotoxicity against human oral epidermoid carcinoma cells (KB), human breast cancer cells (BC), human lung cancer cells (NCI-H187), and Vero cells, as well as activity against the malarial parasite *Plasmodium falciparum* K1, with respective IC_{50} values of 3.0, 1.5, 0.18, 0.90, and 3.1 μg/mL. Investigation of the extracts of the fungal culture filtrate and mycelium led to the isolation and structural determination of two new hirsutane sesquiterpenes, connatusins A (**1**) and B (**2**), along with six known compounds, 2,2-dimethyl-6-methoxy-4-chromanone (**3**),[4] 2,2-dimethyl-3-hydroxy-6-methoxy-4-chromanone (**4**),[4] panepoxydone (**5**),[4,5] panepoxydione (**6**),[5] hypnophilin (**7**),[3,6] and the dihydro derivative of hypnophilin (**8**).[3,6] The known compounds were identified by comparison of the NMR data with those previously reported. All compounds were tested for antimalarial and cytotoxic activities.



Connatusin A (**1**) was isolated as colorless needles, from which the molecular formula was established as $C_{15}H_{22}O_4$ by HREIMS. The UV maximum at 268 nm and IR absorptions at 1704 and 1648 cm^{-1} indicated the presence of an α,β-unsaturated carbonyl group.[6] The ^1H NMR signals were similar to those of **7**,[3,6] except for the replacement of an oxymethine proton signal at δ 3.44 (H-6) and nonequivalent exomethylene signals at δ 5.46 and 6.14 (H_2-15) with a singlet signal of a vinylic methyl group at δ 1.99. Two additional singlets due to hydroxy protons (δ 5.32 and 5.99) were also observed. The ^{13}C NMR spectrum showed 15 carbon signals: one ketone carbonyl (δ 202.8); five quaternary (δ 43.2, 55.0, 85.5, 145.5, and 152.2); three methine (δ 34.6, 54.1, and 81.0); two methylene (δ 44.2 and 46.6); and four methyl (δ 10.3, 16.6, 22.6, and 28.5) carbons. The COSY and HMBC spectra (see Supporting Information) indicated that ring C of **1** was identical to that of **7**, with nonequivalent methylene protons attached at C-8 (δ 46.6). HMBC correlations between H_2-8 and the carbonyl carbon (C-6), an oxyquaternary carbon (δ 85.5, C-7), and a methylene carbon (δ 44.2, C-10), together with those between angular methyl protons (δ 1.31, Me-14) and a methine carbon (δ 54.1, C-2), a quaternary carbon (δ 55.0, C-3), an olefinic carbon (δ 152.2, C-4), and C-7, further constructed ring B with a hydroxyl group and a ketone moiety attached at C-7, as well as a methyl group and a vinyl moiety at C-3. The vinylic methyl protons (Me-15) gave HMBC cross-peaks with C-3, C-4, and an oxyquaternary sp^2 carbon (δ 145.5, C-5). These established a cyclopentenone for ring A, carrying a methyl substituent and a hydroxyl group at C-4 and C-5, respectively. The relative configuration of ring C was identical to that of **7** by NOEDIFF results (see Supporting Information). The coupling constant of 9.9 Hz between H-2 and H-9 indicated a *cis*-junction between rings A and B.[3,6] The angular methyl group (Me-14) was *cis* to H-1 and *trans* to H-2 since irradiation of H-1 enhanced only the signal intensity of Me-14. The orientation of the hydroxyl group at C-7 could not be determined by NOE experiments; however, it was proposed to have a *cis* relationship to Me-14, as two five-membered rings (A and B) would be fused in the more stable *cis* fashion. This was confirmed by X-ray crystallographic analysis (Figure 1).

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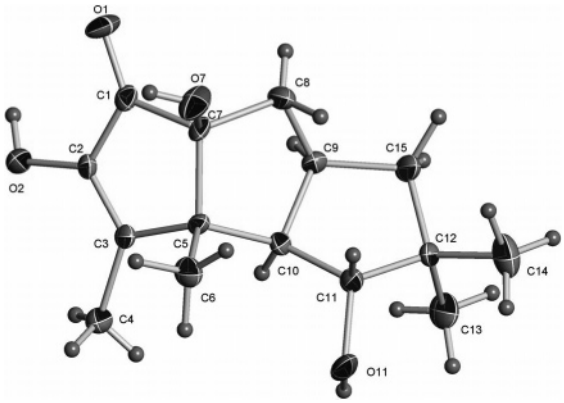

Figure 1. ORTEP view of **1**.

Table 1. Antimalarial and Cytotoxic Activities of Compounds **1**–**8**

	P. falciparum K1	cytotoxicity (IC_{50} (μg/mL)			
compound	IC_{50} (μg/mL)	KB	BC	NCI-H187	Vero
EtOAc extract from broth	3.1	3.0	1.5	0.18	0.90
1	>20	>20	>20	>20	>50
2	>20	>20	>20	>20	>50
3	>20	>20	>20	>20	>50
4	>20	15	16	6.1	8.5
5	3.4	1.9	2.9	0.66	0.90
6	2.1	0.80	0.80	0.22	0.20
7	>20	>20	>20	>20	>50
8	3.1	>20	>20	0.67	1.1
dihydroartimisinin[a]	0.0018	*c*	*c*	*c*	*c*
ellipticine[b]	*c*	0.21	0.27	0.32	0.60

[a] Standard antimalarial drug. [b] Standard compound for cytotoxicity assay. [c] Not tested.

Connatusin B (**2**), a colorless solid, had the same molecular formula as **1** by HREIMS. The UV and IR spectra were similar to those of **7**. The ^{1}H NMR, COSY, and HMBC data (see Supporting Information) of **2** indicated that it had ring C identical to **7** with a methylene group and a quaternary carbon carrying a methyl group attached at C-9 (δ 38.9) and C-2 (δ 51.1), respectively. In addition, signals of the oxymethine proton (H-6) and nonequivalent exomethylene protons (H_2-15) in **7** were replaced by signals for an olefinic proton at δ 5.78 and nonequivalent oxymethylene protons at δ 3.49 and 3.67, respectively, in **2**. The olefinic proton (H-6) and nonequivalent oxymethylene protons (H_2-15) gave HMBC correlations with the same carbons: a quaternary carbon (δ 54.5, C-3); an oxyquaternary carbon (δ 84.6, C-4); and a carbonyl carbon (δ 209.0, C-5). These data established a cyclopentenone structure for ring A having a hydroxyl group and a hydroxymethyl substituent at C-4. The HMBC cross-peaks of Me-14 (δ 1.18, s)/C-2, C-4, and C-7 (δ 194.2) and those of H_2-8/C-6 (δ 119.2) and C-7 connected rings A and C. The relative configuration of rings B and C was identical to that of **7** by NOEDIFF results (see Supporting Information). In addition, the NOE enhancement of H_2-15 was observed upon irradiation of H-2 (δ 2.51, dd, J = 7.8, 12.0 Hz), suggesting that they were in a *cis* relationship.

The antimalarial and cytotoxic activities of compounds **1**–**8** are presented in Table 1. Compounds **5** and **6** exhibited strong cytotoxicity against all cell lines and strong activity against the malarial parasite *P. falciparum*. Compound **8** was cytotoxic against most of the cell lines and also showed strong activity against *P. falciparum*. Compound **6** inhibited all cell lines, with IC_{50} values of 0.80 (KB), 0.80 (BC), 0.22 (NCI-H187), and 0.20 (Vero) μg/mL. It was also active against *P. falciparum*, with IC_{50} 2.1 μg/mL. Compound **8**, the alcohol derivative of the ketone **7**, was much more active than **7** in all tests, indicating the important role of a C-5 hydroxyl group.

Experimental Section

General Experimental Procedures. Melting points were determined on an Electrothermal 9100 melting point apparatus and are uncorrected. Ultraviolet spectra (UV) were measured with a UV-160A spectrophotometer (Shimadzu) in MeOH. Infrared spectra (IR) were obtained on a FTS 165 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a FTNMR, Bruker Avance 300 MHz spectrometer using tetramethylsilane (TMS) as the internal standard. HPLC was performed on a preparative HPLC apparatus (C_{18}, 5 μm, 9.4 × 250 mm, Agilent-1100). Optical rotations were measured in $CHCl_3$ at 589 nm on an automatic polarimeter (JASCO P-1020). EI and HREI mass spectra were measured on a Thermofinnigan MAT95XL spectrometer. Precoated thin-layer chromatography (TLC) was performed on silica gel 60 GF_{254} (Merck). Column chromatography was performed on silica gel (Merck) type 100 (70–230 mesh ASTM) eluted with a gradient system of MeOH/CH_2Cl_2, or as otherwise stated.

Fungal Material. Fruiting bodies of *Lentinus connatus* BCC 8996 were obtained at an open market in Khanchanaburi Province on June 5, 2000. A single spore was isolated, and a culture was maintained on PDA (potato dextrose agar), by Mr. Chainarong Boonkhemthong. The culture was deposited at the Thailand BIOTEC Culture Collection as BCC 8996 on December 6, 2000.

Fermentation and Isolation. *L. connatus* BCC 8996 was maintained on potato dextrose agar at 25 °C for 10 days, which was cut into pieces (1 × 1 cm) and inoculated into 4 × 250 mL Erlenmeyer flasks containing 25 mL of Difco potato dextrose broth (PDB; composition, potato starch 4.0 g, dextrose 20.0 g, per liter) (15 pieces for each flasks). After incubation at 25 °C for 7 days on a rotary shaker (200 rpm), each primary culture was transferred into a 1000 mL Erlenmeyer flask containing 250 mL of PDB and incubated at 25 °C for additional 7 days on a rotary shaker (200 rpm). Each 25 mL portion of the secondary cultures (in 4 flasks) was transferred into 40 × 1000 mL Erlenmeyer flasks, each containing 250 mL of bacto-malt extract broth (MEB: composition; malt extract 6.0 g, maltose 1.8 g, dextrose 6.0 g, yeast extract 1.2 g, per liter), and static fermentation was carried out at 25 °C for 14 days. The cultures were separated by filtration into mycelia and filtrate. The filtrate (ca. 10 L) was extracted with an equal volume of EtOAc to obtain a dark brown oil (1.4 g). The crude extract was fractionated on silica gel column chromatography (CC) to afford six fractions. Compound **3** (130.4 g) was present in fraction 1. Fraction 2 (12.4 mg) was subjected to silica gel CC, eluted with 0.5% MeOH/CH_2Cl_2, to yield **6** (3.1 mg). Fraction 3 (240.9 mg) was separated by flash silica gel CC to afford two subfractions. The first subfraction, eluted with 1% MeOH/CH_2Cl_2, contained **4** (32.5 mg). The second subfraction (38.0 mg) was further purified by silica gel CC, eluted with 1% EtOAc/CH_2Cl_2, to yield **7** (1.6 mg). Fraction 4 (121.7 mg) was submitted to silica gel CC, eluted with 1% MeOH/CH_2Cl_2, to afford **5** (75.4 mg). Fraction 5 (43.1 mg) was purified on silica gel CC to yield three subfractions. Subfraction 3 was further recrystallized from a mixture of MeOH/CH_2Cl_2 to afford **1** (6.7 mg). Fraction 6, eluted with 4% MeOH/CH_2Cl_2, contained **2** (2.6 mg). Wet mycelia were extracted twice with 1 L of MeOH. After concentration of the MeOH solution to 1 L, H_2O (50 mL) was added and the mixture washed with hexane (1 L). The aqueous MeOH layer was concentrated under reduced pressure. The residue was dissolved in EtOAc (1 L), washed with H_2O (200 mL), dried over $MgSO_4$, and concentrated under reduced pressure to obtain a brown oil (391.3 mg). The crude mycelial extract was fractionated on a Sephadex LH-20 column, eluted with MeOH, to yield three fractions. Compound **8** (4.6 mg) was obtained from fraction 1.

Connatusin A (1): colorless needles (MeOH/CH_2Cl_2); mp 84.1–85.0 °C; $[\alpha]^{26}_D$ −36.0° (*c* 0.47, $CHCl_3$); UV (MeOH) λ_{max}

(log ϵ) 268 (3.94); IR (neat) ν_{max} 3399, 2935, 1704, 1648 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.99 (1H, brs, 5-OH), 5.32 (1H, brs, 7-OH), 3.90 (1H, d, J = 10.2 Hz, H-1), 2.37 (1H, dd, J = 13.8 and 8.7 Hz, H-8α), 2.28 (1H, dd, J = 10.2 and 9.9 Hz, H-2), 2.13 (1H, m, H-9), 1.99 (3H, s, H-15), 1.80 (1H, dd, J = 13.8 and 8.7 Hz, H-10α), 1.59 (1H, dd, J = 13.8 and 9.9 Hz, H-8β), 1.31 (3H, s, H-14), 1.29 (1H, m, H-10β), 1.11 (3H, s, H-12), 0.91 (3H, s, H-13); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 202.8 (C, C-6), 152.2 (C, C-4), 145.5 (C, C-5), 85.5 (C, C-7), 81.0 (CH, C-1), 55.0 (C, C-3), 54.1 (CH, C-2), 46.6 (CH_2, C-8), 44.2 (CH_2, C-10), 43.2 (C, C-11), 34.6 (CH, C-9), 28.5 (CH_3, C-12), 22.6 (CH_3, C-13), 16.6 (CH_3, C-14), 10.3 (CH_3, C-15); LREIMS m/z 266 [M]$^+$ (35), 238 (14), 210 (16), 140 (100), 138 (53), 109 (30), 94 (13), 67 (9); HREIMS m/z 266.1512 [M]$^+$ (calcd for $C_{15}H_{22}O_4$, 266.1518).

X-ray Structure Determination of 1.[7] X-ray diffraction data collection was carried out on a Bruker APEX CCD diffractometer. Crystal data of **1**: $C_{15}H_{22}O_4$, MW 266.33, monoclinic, $P2_1$(No. 4), a = 9.716(4) Å, b = 5.813(2) Å, c = 12.107(4) Å, β = 94.668(6)°, V = 681.5(4) Å^3, D_x = 1.298 g/cm^3, Z = 2. A total of 4836 reflections and 2378 observed reflections ($I \geq 2\sigma(I)$) were measured at room temperature from a 0.242 × 0.077 × 0.068 mm^3 colorless crystal using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). The crystal structure was solved by direct methods, and all atoms except hydrogen atoms were refined anisotropically by full-matrix least-squares method on F^2 using SHELXTL-NT to give a final R-value of 0.074, wR(gt) = 0.1141.

Connatusin B (2): colorless solid; mp 85.3−85.6 °C; $[\alpha]^{26}_D$ +14.0° (c 0.44, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 232 (4.00) nm; IR (neat) ν_{max} 3365, 2927, 1705, 1629 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.78 (1H, d, J = 2.1 Hz, H-6), 3.80 (1H, d, J = 7.8 Hz, H-1), 3.67 (1H, d, J = 12.0 Hz, H-15), 3.49 (1H, d, J = 12.0 Hz, H-15), 2.83 (1H, dd, J = 15.6 and 7.2 Hz, H-8α), 2.72 (1H, m, H-9), 2.51 (1H, dd, J = 12.0 and 7.8 Hz, H-2), 2.30 (1H, ddd, J = 15.6, 7.8, and 2.1 Hz, H-8β), 1.90 (1H, dd, J = 12.6 and 7.5 Hz, H-10α), 1.90 (1H, m, H-10β), 1.18 (3H, s, H-14), 1.09 (3H, s, H-12), 0.98 (3H, s, H-13); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 209.0 (C, C-5), 194.2 (C, C-7), 119.2 (CH, C-6), 84.6 (C, C-4), 79.5 (CH, C-1), 65.8 (CH_2, C-15), 54.5 (C, C-3), 51.1 (CH, C-2), 47.2 (CH_2, C-10), 45.8 (C, C-11), 38.9 (CH, C-9), 33.1 (CH_2, C-8), 26.8 (CH_3, C-12), 22.6 (CH_3, C-14), 20.3 (CH_3, C-13); LREIMS m/z 266 [M]$^+$ (4), 206 (31), 176 (57), 163 (41), 149 (100), 133 (76), 93 (27), 71 (27), 57 (39); HREIMS m/z 266.1509 [M]$^+$ (calcd for $C_{15}H_{22}O_4$, 266.1518).

Biological Assays. Antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain), using the microculture radioisotope technique based on the method described by Desjardins et al.[8] The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. The standard compound was dihydroartimisinin. Cytotoxic assays against human epidermoid carcinoma (KB), human breast cancer (BC), and human small cell lung cancer (NCI-H187) cell lines and African green monkey kidney fibroblast (Vero) cells were performed employing the calorimetric method as described by Skehan and co-workers.[9] The reference substance was ellipticine.

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Supporting Information Available: Table of HMBC and NOE correlations for compounds **1** and **2** is available free of charge via the Internet at http://pubs.acs.org.

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