# A new sesquiterpenoid metabolite from Psilocybe samuiensis

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*Title*

**Two new sesquiterpenoids from culture broth of *Psilocybe samuiensis***

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*Abstract*

Two new *ent*-2,3-secoaromadendrane-type sesquiterpenoids, psilosamuiensin A and psilosamuiensin B, were isolated from the broth of *Psilocybe samuiensis*. Their structures were established by spectroscopic data and the configurations of psilosamuiensin A were confirmed by single crystal X-ray crystallographic analysis. Both compounds were inactive when tested for antimicrobial activity against *Bacillus subtilis* ATTC 6633, *Staphyllococcus aureus* ATTC 25923, *Escherichia coli* ATTC 25922, *Pseudomonas aeruginosa* ATTC 27853 and *Candida albicans* ATTC 10231 using disk diffusion assay at 200 μg/disc and for cytotoxic activity against HEP-G2 (hepatoma), SW620 (colon), Chago (lung), KATO-3 (gastric) and BT474 using MTT colorimetric method at concentration of 100 μg/ml.

ข้อคิดเห็น**[G1]:** 100 หรือ 10

**Keywords**

*Psilocybe samuiensis*; magic mushroom; hallucinogenic mushroom, secondary metabolite; sesquiterpene; secoaromadendrane.

## 1. Introduction

Magic mushroom or hallucinogenic mushroom is the name most commonly given to mushrooms that grow naturally and have hallucinogenic properties (Seivewright and Lagundoye, 2000). After the psychedelic effect of some species of genus *Psilocybe* was first reported by Wasson in 1957 (Wasson, 1957), Hofman and co-workers then isolated two important hallucinogenic compounds, psilocybin as the main psychotropic compound, and psilocin from *Psilocybe mexicana* (Hofmann et al., 1958; Hofmann et al., 1959). Later Leung and Pual found two more 4-phosphoryloxytryptamine derivatives, baeocystin and norbaeocystin, from *Psilocybe baeocystis* (Leung and Pual, 1968) and Koike and co-workers found psilocybin and psilocin together with ergosterol, ergosterol peroxide and α,α-trehalose from dried fruiting bodies of *Psilocybe argentipes* (Koike et al., 1981). Due to psychedelic effect of these mushrooms, they were investigated worldwide. It found that they belong to the genus *Panaeolus*, *Psilocybe*, *Conocybe*, *Gymnopilus*, *Inocybe* and *Pluteus* and mainly belong to the genus *Panaeolus* and *Psilocybe* (Stamets, 1996) and the psilocybin and psilocin content in various species was highly variable depended on species (Beug and Bigwood 1981; Beug and Bigwood 1982; Marcano et al. 1994; Gartz et al. 1994; Musshoff et al. 2000) and mainly contained in cap of the mushroom (Tsujikawa 2003).

In 1991 Allen reported the exploration of the hallucinogenic mushrooms in Thailand (Allen, 1991). He found that the mushrooms included the following species: *P. cubensis, P. subcubensis, Copelandia cyanescens* (Berkeley et Broome) Singer, and a new bluing *Psilocybe* species, *Psilocybe samuiensis* Guzman, Bandala and Allen (Allen and Merlin 1992; Gartz et al., 1994; Guzmán et al., 1993.). The alkaloid content of both naturally occurring and in vitro cultivated fruit bodies of *P. samuiensis* was analyzed by HPLC and the results revealed that high concentrations of psilocybin and psilocin and small amounts of baeocystin were detected (Allen, 1992; Guzmán et al., 1993; Gartz et al., 1994).

Recently we studied the metabolites of *P. samuiensis* cultured in malt extract broth (MEB) and we found two new *ent*-2,3-secoaromadendrane-type sesquiterpenoids, named psilosamuiensin A **1** and psilosamuiensin B **2** (Figure 1), from the ethyl acetate extract of culture broth. Herein we described the isolation and structural elucidation of those compounds and their antimicrobial activity and for cytotoxic activity.

## 2. Results and Discussion

On the basis of 1H NMR analysis, the hexane, ethyl acetate and methanol extracts of *Psilocybe samuiensis* mycelia were mainly fatty acid. The culture broth of *Psilocybe samuiensis* was concentrated and then extracted with hexane, ethyl acetate and methanol, respectively. The hexane extract of the

culture broth was obtained only in a small amount. The ethyl acetate extract of the culture broth was isolated by column chromatography in stepwise fashion. Compound **1** was isolated as a major metabolite (67 mg/litre of broth). The molecular formula of **1** was established as $C_{15}H_{26}O_4$ on the basis of HRESIMS which indicated three degrees of unsaturation. IR spectrum was consistent with the presence of hydroxyl functional group (3425-3352 $cm^{-1}$). $^1$H-NMR spectrum of **1** showed three signals of methyl groups at $\delta_H$ 0.83, 1.13 and 1.34 and five signals of the protons attached to carbons bearing oxygen atom at $\delta_H$ 3.27, 3.55, 3.42, 3.87 and 5.30. The $^{13}$C-NMR and HSQC experiments identified 15 carbon signals consisting of three methyl carbons at $\delta_C$ 11.4, 14.5 and 31.6, four methylene carbons at $\delta_C$ 18.4, 38.6, 61.8 and 73.4, six methine carbons at $\delta_C$ 21.5, 24.2, 30.4, 35.6, 56.6 and 92.9 and two quaternary $sp^3$-carbons at $\delta_C$ 26.5 and 73.3. Additionally the carbon signals at $\delta_C$ 92.9, 73.4, 73.4 and 61.8 indicated that these carbons attached with oxygen atom and the carbon signal at $\delta_C$ 92.9 was an anomeric carbon. Since no $sp^2$- or *sp*-carbon showed in the $^{13}$C-NMR data and the three unsaturations were accounted for, it was implied that **1** should contain three rings. A series of COSY and HSQC experiments established partial connectivities as showed in Fig. 2 in bold. The assignment of **1** was made from HMBC and NOESY experimental data (Table 1) and the crucial HMBC and NOESY correlations were shown in Figure 2. The relative stereochemistry of **1** was determined by a combination of coupling constant

(*J*) and NOESY experiment (Fig. 2). The large coupling constant of $H_{ax}$-3 with H-4 (*J* = 11.6 Hz) and the observed NOEs between $H_{ax}$-3 and H-6 and between $H_{ax}$-3 and methyl protons of C-15 in the NOESY experiment suggested that H-3, H-4 and the methine carbon (C-6) were axially oriented. The small coupling constant of H-2 with H-1 and the observed NOEs between H-2 and the methyl protons of C-14 in the NOESY experiment suggested that H-2 and the methyl protons of C-14 were equatorially orientated and H-1 was axially oriented. The observed NOEs between H-5 and the methyl protons of C-13 and between the methyl protons of C-13 and $H_a$-8 in the NOESY experiment suggested that those protons were on the same face. The observed NOEs in the NOESY experiment for H-6:$H_b$-9, $H_b$-9:H-7, $H_a$-12:H-6, $H_b$-12:H-6 and $H_b$-12:H-7 suggested that the bridge-head protons (H-6 and H-7) and the hydroxymethylene group were on the same face of the cyclopropane ring and $H_b$-9 was axially oriented. A single crystal X-ray analysis of **1** (Fig. 3) was also carried out and the relative stereochemistry was found to be completely consistent with the assignments based on NMR data as described above.

Compound **2** was determined as $C_{16}H_{28}O_4$ on the basis of HRESIMS which indicated three degrees of unsaturation. The spectroscopic data of **2**, compared with those of **1**, suggested that compound **2** was a derivative of **1**, named psilosamuiensin B (Figure 1), and the methoxy group of **2** was axially oriented which assigned by the NOESY data (Table 1). Due to a

presence of the hemiacetal, compound **1** may undergo the reaction with methanol during isolation to provide **2**. To prove this doubt compound **1** was treated with excess methanol in the presence of a catalytic amount of *p*-toluenesulfonic acid and without *p*-toluenesulfonic acid and the reactions were monitored by TLC. It found that the reaction under acid condition was complete within 3 hours while the reaction under non-acid condition was undergone very slowly. After evaporation of the solvent and purification by silica gel column chromatography, $^{1}H$ and $^{13}C$ NMR spectra indicated that the product from the reaction was mainly compound **2**. This result suggested that compound **2** may be arisen during the process of extraction and isolation and crystallization of **1**.

Furthermore, compound **1** and **2** were tested for antimicrobial activity against *Bacillus subtilis* ATTC 6633, *Staphyllococcus aureus* ATTC 25923, *Escherichia coli* ATTC 25922, *Pseudomonas aeruginosa* ATTC 27853 and *Candida albicans* ATTC 10231 using disk diffusion assay and for cytotoxic activity against HEP-G2 (hepatoma), SW620 (colon), Chago (lung), KATO-3 (gastric) and BT474 using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. Both were inactive against those five standard microorganisms at 200 μg/disc and those five cell lines at concentration of 100 μg/ml.

ข้อคิดเห็น**[G2]:** 100 หรือ 10

## 3. Experimental

### *3.1 General experimental procedures*

$^{1}H$ and $^{13}C$ NMR spectra were recorded on a Varian Mercury +400 MHz NMR spectrometer ($^{1}H$ at 400 MHz and $^{13}C$ at 100 MHz). Chloroform-*d* ($CDCl_3$) was used in NMR experiments and chemical shifts (δ) were referenced the signals of residual solvents at δ 7.26 ppm ($^{1}H$) and 77.0 ppm ($^{13}C$). HRESIMS spectra were recorded on Micromass LCT (LC/MS). Optical rotations were measured on a Perkin Elmer 341 polarimeter, using a sodium lamp at wavelength 589 nm. FT-IR spectra were recorded on a Perkin-Elmer Model 1760X Fourier Transform Infrared Spectrophotometer. Melting points were examined using a Fisher-John melting point apparatus. All solvents used for column chromatography were commercial grade and were distilled prior to use. TLC were carried out on precoated silica gel 60 (Merck's TLC aluminium sheet, silica gel 60 $F_{254}$ Art. 1.05554.0001) and spots were detected under UV (254 and 365 nm) before and after spraying with a vanillin/sulphuric acid solution followed by heating the plate. Isolations were carried out using column chromatography (CC) [silica gel 60 (Merck Art. 1.09385.9025, 0.040-0.063 mm)]. Malt extract for culture of the fungus was purchased from Himedia.

*3.2 Psilocybe samuiensis*

Spores print dry specimens of *Psilocybe samuiensis* from Koh Samui, Surat Thani Province, Thailand were received from John W. Allen in July 2004. The spores print was used for cultivation.

*3.3 Cultivation, extraction and isolation*

The spores of *Psilocybe samuiensis* from spore print were streaked in Petri dishes containing Potato Dextrose Agar (PDA). The Petri dishes were incubated at room temperature (25-30$^{o}$C) and examined for fungal mycelium from spores. Outgrowing mycelia were purified and transferred into others Petri dishes containing PDA. Stock culture of *P. samuiensis* was grown on MEA at room temperature (25-30$^{o}$C) for 2 weeks. The agar was then cut with a flamed 8 mm diameter cork borer. Five pieces of agar cultures were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of MEB (x 110), and then statically cultured at ambient temperature for 11 weeks. The culture was filtered through filter paper (Whatman No. 93) and the broth was then concentrated under reduced pressure at 35$^{o}$C into 500 ml. The concentrated broth was extracted with 200 ml of hexane (x 5), with 200 ml of ethyl acetate (x 5) and with 200 ml of methanol (x 5), respectively. The hexane extract of broth was obtained in a small amount. The combined ethyl acetate layers were dried over anhydrous sodium sulphate and then evaporated under reduced pressure at 35$^{o}$C to yield the ethyl acetate extract

(5.88 g) as yellowish brown viscous liquid. The ethyl acetate extract (4.97 g) was chromatographed on silica gel column eluted with $CH_2Cl_2$-MeOH gradient in a stepwise fashion (3% MeOH to 100% MeOH). Fractions with similar components were combined together according to the TLC profile.

Compound **1** was obtained from elution of 3% methanol in dichloromethane. The solvent was removed by rotary evaporation and the residue was obtained as white solid mixed with yellow viscous liquid (960 mg). The mixture was purified by crystallization from dichloromethane-methanol to give compound **1** as colorless crystals. The filtrate was further purified by silica gel column chromatography (Merck's silica gel 60 Art. 1.09385.9025), and crystallization from dichloromethane-methanol to give compound **1** as colorless crystals. Both crystals of **1** were combined and total amount of **1** was 736 mg (67 mg/litre of broth).

After evaporation of the filtrate from purification of compound **1**, a yellow viscous liquid (179 mg) was obtained. The residue was purified by TLC to give compound **2** as colorless oil (19 mg).

3.3.1 Psilosamuiensin A **1**

Colorless crystals; m.p. 91-92 $^{o}$C; $[\alpha]_D^{20}$ -48 (MeOH, *c* 0.25); $\nu_{max}$ (KBr) 3425-3352, 2960, 2927, 2872, 1140, 1102, 1043 and 1013 $cm^{-1}$; $\delta_H$ ($CDCl_3$, 400 MHz) 0.83 (3H, d, *J* = 7.2 Hz, H-15), 0.91 (1H, m, H-7), 0.93 (1H, m, H-6), 1.13 (3H, s, H-13), 1.34 (3H, s, H-14), 1.53 (1H, m, $H_a$-8),

1.55 (1H, dd, *J* = 6.8, 14.4 Hz, $H_a$-9), 1.69 (1H, m, $H_b$-8), 1.94 (1H, br s, H-1), 2.01 (1H, m, H-4), 2.09 (1H, m, H-5), 2.11 (1H, dd, *J* = 12.8, 14 Hz, $H_b$-9), 3.27 (1H, d, *J* = 10.8 Hz, $H_b$-12), 3.42 (1H, dd, *J* = 11.6, 4.4 Hz, $H_{eq}$-3), 3.55 (1H, d, *J* = 10.4, $H_a$-12), 3.87 (1H, t, *J* = 11.6 Hz, $H_{ax}$-3), and 5.30 (1H, br s, H-2) ppm; $\delta_C$ ($CDCl_3$, 100 MHz) 11.4 (C-13), 14.5 (C-15), 18.4 (C-8), 21.5 (C-6), 24.2 (C-7), 26.5 (C-11), 30.4 (C-5), 31.6 (C-14), 35.6 (C-4), 38.6 (C-9), 56.6 (C-1), 61.8 (C-3), 73.3 (C-10), 73.4 (C-12), and 92.9 (C-2) ppm; ESI-TOF MS *m/z* 293.1732 $[M+Na]^+$; $C_{15}H_{26}O_4Na$ *calc* 293.1729.

3.3.2 Psilosamuiensin B **2**

$[\alpha]_D^{20}$ -51 ($CHCl_3$, *c* 0.07,); $\nu_{max}$ (film) 3397, 2967, 2931, 2878, 1129, 1103 and 1045 $cm^{-1}$; $\delta_H$ ($CDCl_3$, 400 MHz) 0.77 (3H, d, *J* = 6.8 Hz, H-15), 0.82 (1H, m, H-7), 0.84 (1H, m, H-6), 1.08 (3H, s, H-13), 1.24 (3H, s, H-14), 1.47 (1H, m, $H_a$-8), 1.47 (1H, m, $H_a$-9), 1.64 (1H, m, $H_b$-8), 1.89 (1H, br s, H-1), 1.96 (1H, m, H-4), 2.00 (1H, m, H-5), 2.03 (1H, m, $H_b$-9), 3.21 (1H, d, *J*=11.2 Hz, $H_b$-12), 3.32 (1H, dd, *J*=11.6, 4.8 Hz, $H_{eq}$-3), 3.34 (3H, s, OMe), 3.51 (1H, d, *J*=11.2 Hz, $H_a$-12), 3.64 (1H, t, *J*=11.6 Hz, $H_{ax}$-3) and 4.70 (1H, d, *J*=2.4 Hz, H-2) ppm; $\delta_C$ ($CDCl_3$, 100 MHz) 11.5 (C-13), 14.4 (C-15), 18.5 (C-8), 21.9 (C-6), 24.3 (C-7), 26.3 (C-11), 30.8 (C-5), 31.7 (C-14), 35.6 (C-4), 38.6 (C-9), 55.3 (OMe), 56.6 (C-1), 61.8 (C-3), 73.4 (C-10), 73.9 (C-12) and 99.6 (C-2) ppm; ESI-TOF MS *m/z* 307.1890 $[M+Na]^+$; $C_{16}H_{28}O_4Na$ *calc* 307.1885.

*4. X-ray crystallography of* **1**

Crystal data of **1** were obtained by a BRUKER SMART CCD diffractometer, $M_o$ $K_\alpha$ radiation ($\lambda$ = 0.71073 Å), graphite monochromator, $C_8H_7ClO_3$, triclinic, space group *P(-1)*, unit cell dimensions *a* = 7.3122(9) Å, *b* = 7.3839(9) Å, *c* = 8.5878(10) Å, *alpha* = 112.135(2)°, *beta* = 97.002(2)°, *gamma* = 103.759(2)°, $V$ = 405.42(8) Å$^3$, $D_{calc}$ = 1.528 g/cm$^3$, *Z* = 2, *F*(000) = 192, $\mu$ = 0.430 mm$^{-1}$. Data was collected at 293 (2) K using $\omega$-2$\theta$ scans in the ranges $\theta$ = 2.63-28.32°. A total of 4793 reflections were collected, 1913 were unique ($R_{int}$ = 0.0212). The structure was refined by full-matrix least-squares on $F^2$. The non hydrogen atoms were refined anisotropically. Hydrogen atoms were located in difference Fourier maps and refined isotropically. The final refinement [$I > 2\sigma(I)$] gave $R_1$ = 0.0498, $wR_2$ = 0.1174.

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