Chemical Constituents from the Fungus *Phomopsis* sp.

องค์ประกอบทางเคมีจากรา *Phomopsis* sp.

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ABSTRACT

The chromatographic separation of crude extracts from the fungus *Phomopsis* sp. led to the isolation of seven compounds. They were three new sesquiterpenes (**1**-**3**), two known compounds, kampanol A (**4**) and *R*-mevalonolactone (**5**), along with two known sterols, ergosterol (**6**) and 24(*R*)-5α,8α-epidioxyergosta-6,22-diene-3β-ol (**7**). Their structures were characterized by extensive spectroscopic analyses. Compounds **1** and **2** showed cytotoxicity against the KB cell line with IC_{50} values of 42.07 and 9.44 μg/mL. Compound **2** also exhibited antimalarial activity against *Plasmodium falciparum* with IC_{50} value of 0.789 μg/mL.

บทคัดย่อ

จากการแยกส่วนสกัดหยาบของรา *Phomopsis* sp. ด้วยวิธีทางโครมาโทกราฟีได้สาร 7 สาร ได้แก่สารใหม่ในกลุ่มเซควิสเทอร์ปีน 3 สาร (**1**-**3**) สารที่ทราบโครงสร้างแล้ว 4 สาร คือ kampanol A (**4**) *R*-mevalonolactone (**5**) ergosterol (**6**) และ 24(*R*)-5α,8α-epidioxyergosta-6,22-diene-3β-ol (**7**) การพิสูจน์โครงสร้างของสารเหล่านี้อาศัยเทคนิคทางสเปกโทรสโกปี จากการทดสอบฤทธิ์ทางชีวภาพ พบว่า สาร **1** และ **2** มีความเป็นพิษต่อเซลล์มะเร็งชนิด KB ด้วยค่า IC_{50} 42.07 และ 9.44 μg/mL และยังพบว่าสาร **2** มีฤทธิ์ยับยั้งเชื้อ *Plasmodium falciparum* สาเหตุของไข้มาลาเรีย โดยมีค่า IC_{50} เท่ากับ 0.789 μg/mL

Key Words: Sesquiterpenes, Endophytic fungus, *Phomopsis* sp.

คำสำคัญ: เซสควิสเทอปีน เชื้อราเอนโดไฟท์ ราสกุลโฟม๊อบซิส

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Introduction

Endophytic fungi in the genus phomopsis are rich source of bioactive metabolites.[1] Previous investigations of secondary metabolites from *Phomopsis* species resulted in the isolation of compounds such as phenochalasins,[2] phomopsins,[3] phomoenamide,[4] phomoeuphorbins,[5] and oblongolides.[6] However, no phytochemical investigation of *Phomopsis archeri* has been reported. We report herein the isolation, characterization and bioactivities of three new sesquiterpenes (**1**-**3**) and four known compounds (**4**-**7**).

Materials and methods

The fungus, *P. archeri*, was collected from cortex stem of *Vanilla albidia* at Pathumthani province Thailand, in 2008 and was identified by Assoc. Prof. K. Soytong. A voucher specimen (no. Pac01) was deposited at the Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Air dried fungal biomass (225 g) was ground and extracted successively at room temperature with hexane, EtOAc and MeOH to give three crude extracts hexane 16.3 g (7.2%), EtOAc 16.4 g (7.3%), and MeOH 13.2 g (5.9%), respectively. CH_2Cl_2 (300 mL) was added to the hexane extract (6.8 g) and a solid (95 mg) was precipitated, which was then recrystallized from EtOAc-hexane to give a white solid of **6** (34.0 mg). The EtOAc extract was subjected to flash column chromatography, eluted with a gradient system of hexane-EtOAc and EtOAc-MeOH. On the basis of their TLC characteristic, the fractions which contained the same major compounds were combined to give ten fractions P_1-P_{10}. Fraction P_5 was purified by silica gel FCC, eluted with a gradient system of hexane-EtOAc to give six subfractions, $P_{5.1}$-$P_{5.6}$. Subfraction $P_{5.4}$ was chromatographed on FCC, eluted with CH_2Cl_2 to give a white solid of **4** (10.2 mg,) and **3** (7.5 mg). Fraction P_7 was further subjected to flash CC, eluted with a gradient system of hexane-EtOAc to give ten subfractions, $P_{7.1}$-$P_{7.10}$. Subfraction $P_{7.6}$ was purified by recrystallization from EtOAc to give a white solid of **1** (700 mg). Fraction P_8 was chromatographed on silica gel FCC, eluted with a gradient system of hexane-EtOAc to give ten subfractions, $P_{8.1}$-$P_{8.10}$. Recrystallization of subfraction $P_{8.5}$ with 2% MeOH-CH_2Cl_2 gave colorless needles of **2** (600 mg). Fraction P_9 was chromatographed on silica gel FCC, eluted with a gradient system of CH_2Cl_2-EtOAc to give ten subfractions, $P_{9.1}$-$P_{9.10}$. Subfraction $P_{9.7}$ was further subjected to silica gel FCC, eluted with an isocratic system of 50% EtOAc-hexane to yield a yellow-brown viscous of **5** (32.6 mg). The MeOH extract was fractionated by silica gel FCC, eluted with a gradient system of hexane-CH_2Cl_2 and CH_2Cl_2-MeOH to provided eight fractions, PM_1-PM_8. Fraction PM_6 was chromatographed on a silica gel CC, eluted with a gradient system of hexane-EtOAc to give an additional amount of **6** (300.1 mg) and 24(*R*)-5α,8α-epidioxyergosta-6,22-diene-3β-ol (36 mg). Three isolated compounds were sent to evaluate for their bioactivities at BIOTECH, Pathumthani, Thailand.

Results and discussion

Chromatographic separation of the endophytic fungus *Phomopsis archeri* led to the isolation of seven compounds (**1**-**7**). The structures of these compounds were determined on the basis of ^1H and ^{13}C NMR, IR,

and MS spectral data, including 2D NMR techniques (COSY, HSQC, HMBC, and NOESY).

Compound 4 was obtained as a white powder and was assigned the molecular formula $C_{25}H_{32}O_6$, from HRESITOFMS (found m/z 429.2283, $[M+H]^+$), indicating ten degrees of unsaturation. The UV spectrum exhibited maximum absorptions at 263 and 310 nm. The IR spectrum showed the absorption bands for a hydroxy group (3360 cm^{-1}), lactone/ester groups (1736 cm^{-1}) and an aromatic ring (1619 cm^{-1}). The ^{13}C NMR and DEPT spectra displayed 25 carbon resonances of five methyl, six methylene, and four methine (an aromatic, an oxymethine and two aliphatic), and ten quaternary carbons. The ^{1}H NMR spectrum (Table 1) exhibited resonances for four methyl singlet signals in the upfield region of the spectrum (δ_H 0.69, H_3-15; 0.86, H_3-14; 0.91, H_3-13; and 1.19, H_3-12) and one in a lower field at δ_H 2.06 (H_3-2″). Apart from a number of resonances in the upfield region of the spectrum, the ^{1}H NMR spectrum displayed downfield signals readily assignable to an oxymethine at δ_H 4.49 (dd, J = 4.8, 11.8 Hz, H-3), an oxymethylene at δ_H 5.17 (ABq, J = 18.8 Hz, H_2-8′) and an aromatic methine at δ_H 6.94 (s, H-3′). The COSY spectrum showed correlations of H-1/H-2, H-6/H-7, and H-9/H-11, indicating three partial units of a sesquiterpene. The HMBC spectrum (Figure 1) demonstrated correlations of H-3 to C-4, C-13, C-14 and C-1″; H-5 to C-4, C-6, C-9, and C-15; H_3-15 to C-5 and C-10; H-9 to C-11, C-18, and C-1′; H_3-12 to C-7, C-8, and C-9; H_2-11 to C-8, C-9, C-10, C-1′, C-2′, and C-6′; and H_2-8′ to C-4′, C-5′, C-6′, and C-7′, establishing the pentacyclic skeleton (ABCDE ring system) of **4**. The NOESY spectrum (Figure 2) exhibited correlation of H_3-15/H_3-14/H-6; H-3/H-5/H_{ax}-1; H-5/H-9; H_3-13/H-6, indicating a *trans*-fused decalin ring. These data and its optical rotation are compatible with those reported, as kampanol A.[7]

Table 1 ^{1}H and ^{13}C NMR spectral data (δ values) of **4** in $CDCl_3$

position	δ_H (J in Hz)	δ_C, mult.
1_{eq}	1.91, d (13.2)	37.6 t
1_{ax}	1.25, m	
2	1.59, m	23.4 t
3	4.49, dd (8.4, 11.8)	80.9 t
4	-	38.7 s
5	1.02, d (11.6)	54.3 d
6	1.54, m	17.8 t
7_{eq}	2.19, d (10.4)	40.1 t
7_{ax}	1.65, m	
8	-	76.3 s
9	1.45, d (8.0)	48.4 d
10	-	38.0 s
11_{eq}	2.85, d (18.8)	18.3 t
11_{ax}	2.73, dd (11.2, 18.8)	
12	1.19, s	27.0 q
13	0.91, s	28.4 q
14	0.86, s	16.9 q
15	0.69, s	14.2 q
1′	-	117.1 s
2′	-	155.4 s
3′	6.94, s	101.6 d
4′	-	124.4 s
5′	-	126.9 s
6′	-	150.1 s
7′	-	172.3 s
8′	5.17, ABq (18.8)	68.3 t
1″	-	171.4 s

2″	2.06, s	21.3 q

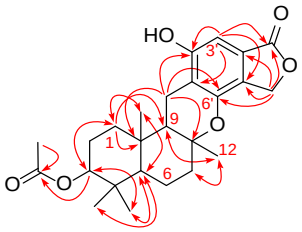

Figure 1 Selected HMBC correlations of compound **4**

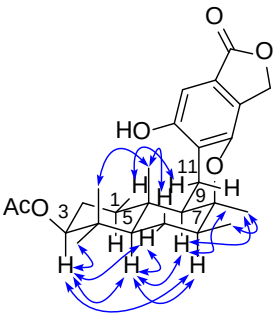

Figure 2 Selected NOESY correlations of compound **4**

Compound 5 was obtained as colorless oil. The IR spectrum displayed absorption bands of hydroxyl (3417 cm^{-1}) and δlactone (1715 cm^{-1}). The ^{13}C NMR and DEPT experiment (Table 2) revealed the presence of six carbons, attributable to one methyl, three methylenes and two quaternary (including a carbonyl group) carbons. The ^{1}H NMR (Table 2) showed the resonance of three set of methylene proton at δ_H 2.52 (ABq, J = 17.6 Hz, H-3), 1.84 (ddd, J = 4.8, 8.4, 14.4 Hz, H-5) and 4.53 (ddd, J = 2.4, 8.4, 14.8 Hz, H_b-6), 4.27 (ddd, J = 2.0, 9.2, 11.2 Hz, H_a-6), together with singlet signal of methyl group at δ_H 1.32 (s, 4-CH_3) were also observed. The HMBC correlations of H_2-3 to C-2, C-4, C-5, 4-CH_3; H_2-5 to C-2, C-3, C-4, C-6, H_2-6 to C-2, C-4, C-5; and methyl proton (4-CH_3) to C-2, C-3, C-4, C-5 confirmed the connectivity of these groups (Figure 3). On the basis of the above data, and comparison of the specific rotation [-29.0 (*c* 0.2, EtOH)] with the literature reported[8] [-23.0 (*c* 6.0, EtOH)], the structure of **5** was defined as a known *R*-mevalonolactone.

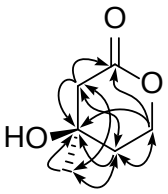

Figure 3 HMBC correlations of compound **5**

Table 2 ^{1}H and ^{13}C NMR spectral data (δ values) of **5** in $CDCl_3$

position	δ_H (J in Hz)	δ_C, mult.
2	-	171.1 s
3	2.52, ABq (17.6)	44.6 t
4	-	67.9 s
4-CH_3	1.32, s	29.6 q
5	1.84, ddd (4.8, 8.4, 14.4)	35.7 t
6	H_b 4.53, ddd (2.4, 8.4, 14.8)	66.2 t
	H_a 4.27, ddd (2.0, 9.2, 11.2)	

Compound 6 was obtained as a white solid. The IR spectrum showed a broad absorption band at 3429 cm^{-1} indicated the O-H stretching of a hydroxyl group. Weak absorption bands at 3044 and 1655 cm^{-1} due to C-H and C=C stretching of alkenes, respectively. A set of absorption bands at 2953, 2870 and 1458, 1382, 1368 cm^{-1} was characterized as saturated C-H stretching and bending, respectively. A medium absorption band at 1032 cm^{-1} was assigned to C-O stretching. The ^{1}H NMR and ^{13}C NMR spectra showed the characteristic signals of a steroid unit. The ^{1}H NMR spectral data showed the resonance signals of four olefinic protons at δ 5.58 (dd, J = 2.4, 5.6 Hz), 5.38 (dd, J = 2.4, 5.6 Hz), 5.22 (dd, J = 7.0, 15.6 Hz), and 5.18 (dd, J = 7.0, 15.6 Hz). An oxymethine proton displayed at δ 3.66 (m), while

signals between δ 2.50-1.10 were assigned to methine and methylene protons. The resonance signals of the methyl protons were displayed at δ 1.03 (d, J = 6.4 Hz), 0.94 (s), 0.91 (d, J = 6.8 Hz), 0.84 (d, J = 6.4 Hz), 0.82 (d, J = 6.4 Hz), and 0.63 (s). Comparison of the NMR spectral data[9], and mixed-TLC with the authentic sample indicated that compound **6** was ergosterol.

Compound 7 was obtained as a white solid. The IR spectrum showed a O-H stretching of a hydroxyl group at 3301 cm^{-1}. The absorption bands due to C-H and C=C stretching of alkenes displayed at 3080 and 1655 cm^{-1}, respectively. A set of absorption bands at 2955, 2871 and 1458, 1377 cm^{-1} was characterized as saturated C-H stretching and bending, respectively. An absorption band at 1044 cm^{-1} was assigned to C-O stretching. Comparison of the NMR spectral data with those of known sterol[10] and mixed-TLC with the authentic sample indicated that compound **7** was 24(*R*)-5α,8α-epidioxyergosta-6-22-diene-3β-ol.

Compounds **1-4** showed cytotoxicity against four cholangiocarcinoma cell lines (0.1-19.6 μg/mL) while, **1** and **2** exhibited weak cytotoxicity against KB cell line with IC_{50} values of 42.1 and 9.4 μg/mL. In addition, compound **2** showed strong antimalarial activity against *Plasmodium falciparum* with IC_{50} value of 0.79 μg/mL (Table 3).

Table 3 Bioactivities activities of isolated compounds **1** and **2**

compound	antimalarial IC_{50} (μg/mL)	anti-TB MIC (μg/mL)	cytotoxicity, KB[a] IC_{50} (μg/mL)
1	>20	>200	42.07
2	0.79	50.0	9.4
artmisinin	0.0001		
isoniazid		0.05	
kanamycin sulfate		2.5	
ellipticine			0.36

[a]Human epidermoid carcinoma in the mouth

Conclusions

Separation of hexane, EtOAc and MeOH extracts of a fungus *Phomopsis* sp. gave three new compounds (**1-3**), and four known compounds (**4-7**). Their structures were identified by physical and spectroscopic data (IR, NMR, 2D NMR, and MS).

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References

Leslie Gunatilaka, AA. 2006. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. J. Nat. Prod. 69:509-526.

Tomoda, H., Namatame, I., Si, S., Kawaguchi, K., Masuma, R., Namikoshi, M., Omura, S. 1999. Phenochalasins, inhibitors of lipid droplet formation in mouse macrophages, produced by *Phomopsis* sp. FT-0211. J. Antibiot. 52:851-856.

Huang, Z., Cai, X., Shao, C., She, Z., Xia, X., Chen, Y., Yang, J., Zhou, S., Lin, Y. 2008.

Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. Phytochemistry. 69:1604-1608.

Rukachaisirikul, V., Sommart, U., Phongpaichit, S., Sakayaroj, J., Kirtikara, K. 2008. Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. Phytochemistry, 69:783-787.

Yu, BZ., Zhang, GH., Du, ZZ., Zheng, YT., Xu, JC., Luo, XD. 2008. Phomoeuphorbins A–D, azaphilones from the fungus *Phomopsis euphorbiae*. Phytochemistry. 69:2523-2526.

Bunyapaiboonsri, T., Yoiprommarat, S., Srikitikulchai, P., Srichomthong, K., Lumyong, S. 2010. Oblongolides from the Endophytic Fungus *Phomopsis* sp. BCC 9789. J. Nat. Prod. 73:55-59.

Singh, SB., Zink, DL., Williams, M., Polishook, JD., Sanchez, M., Silverman, KC., Lingham, RB. 1998. Kampanols: novel Ras Faenesyl-protein transferase inhibitors from *Stachybotrys kampalensis.* Bioorg. Med. Chem. Lett. 8:2071-2076.

Mash, EA., Arterburn JB. 1991. Enantiomerically pure acetals in organic synthesis. 3. A synthesis of (*R*)-mevalonic acid lactone. J. Org. Chem. 56(2):885-888.

Bok, JW., Lermer, L., Chilton, J., Klingeman, HG., Towers, GH. 1999. Antitumor sterols from the mycelia of *Cordyceps sinensis*. Phytochemistry. 51:891-898.

Rösecke, J., König, WA. 2000. Constituent of the fungi *Daedalea quercina* and *Daedaleopsis confragosa var. tricolor.* Phytochemistry. 54:757-762.