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Secondary Metabolites and Bioactivity of the Endophytic Fungus *Phomopsis theicola* from Taiwanese endemic plant

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Abstract: A new cytochalasan named as phomocytochalasin (**1**), together with five previously identified compounds, cytochalasin H, cytochalasin N, RKS-1778, dankasterone B, cyclo(L-Ile-L-Leu), were isolated from the solid fermentate of *Phomopsis theicola* BCRC 09F0213, an endophytic fungus isolated from the leaves of an endemic Formosan plant *Litsea hypophaea* Hayata. The structure of the new compound was established by spectroscopic methods, including UV, IR, HR-ESIMS, and extensive 1D- and 2D-NMR techniques. Among the isolates, cytochalasin N showed NO inhibitory activity with IC_{50} values of 77.8 μM. Cytochalasin H showed the progesterone receptor (PR) antagonism with the IC_{50} value of 1.42 μM.

Keywords: *Phomopsis theicola*; endophytic fungus; *Litsea hypophaea*; phomocytochalasin.

1. Introduction

The plant endophytes refer to a group of microorganisms, including fungi and bacteria, which live within plants' internal tissues or organs, but typically cause no apparent symptoms of disease in the host plant [1–3]. An endophytic fungus, *Phomopsis theicola* (Diaporthaceae), internal strain number BCRC 09F0213, was isolated from the leaves of an endemic plant *Litsea hypophaea* Hayata (Lauraceae). Previously, the Phomopsis genus have been investigated and identified various classes of chemical constituents [4–10], and the bioactivity of the isolates showed the antimicrotubule, antimycobacterial, anticandida and cytotoxic activity [11–14]. However, the chemical constituents and biological activities of the endophytic fungus, *P. theicola* BCRC 09F0213 have never been reported. In this paper, a new cytochalasan was isolated and identified from the active EtOAc layer of *P. theicola.*

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2. **Materials and Methods**

2.1. Microorganism Material

The leaves of *Litsea hypophaea* Hayata (Lauraceae) were collected on March 2010 in Mutan, Pingtung County, Taiwan, and identified by one of the authors Prof. I. -S. Chen. A voucher specimen (no. Chen 5595) was deposited in the Herbarium of the School of Pharmacy, College of Pharmacy, Kaohsiung Medical University. And the fungal strain was isolated from the leaves of *L. hypophaea*, and identified as *Phmompsis theicola* (Diaporthaceae) by one of the authors, S. -Y. Hsieh, based on cultural and anamorphic data. The identification was further confirmed by sequence analysis of the rDNA-ITS (internal transcribed spacer) region. By using BLAST (nucleotide sequence comparison program) to search the GenBank database, it was found to have a 100% similarity to *P. theicola* accession No. FJ441631.1.

2.2. Cultivation and Preparation of the Fungal Strain

Two-weeks-old colonies of the *P. theicola* strain on malt extract agar (MEA) medium in 9-cm Petri dish were cut into the bottle and blended for 30 s with 100 mL of dist. H_2O to prepare the fungal inoculum for solid fermentation. To each 500-mL flask containing 150 mL of liquid cultural media (ingredients: rice, 0.2% kombucha, 0.2% NH_4Cl, 0.2% yeast extract) were added 10 mL of fungal inocula and incubated at 25° for 28 days without illuminated in static state. The strain is preserved with the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI), under the ID No. 09F0213.

2.3. Extraction and Isolation

Solid fermentate of *P. theicola* (10.5 Kg) was extracted with MeOH to yield a MeOH extract (840 g) (approximately 95% inhibition of NO and IL-6 production at a concentration of 10 μg/mL), which was partitioned in EtOAc–H_2O (1:1; 2 L × 3) to produce an EtOAc-soluble fraction (60 g) (approximately 95% inhibition of NO and IL-6 production at a concentration of 10 μg/mL) and an H_2O-soluble fraction (700 g). The active EtOAc-soluble fraction (60 g) subjected to silica gel column chromatography (CC) using *n*-hexane with acetone (10:1) as the primary eluent and gradually increasing the eluent polarity with acetone to produce 13 fractions. *Fr. 5* (1.7 g) was subjected to silica gel CC using CH_2Cl_2–MeOH (50:1) as the eluent to yield 11 fractions (*Fr. 5.1–Fr. 5.11*). *Fr. 5.5* was a great amount of pure compound, dankasterone B (64.8 mg). *Fr.5.3* (30.9 mg) was subjected to MPLC (*RP-18*, H_2O/MeOH (1 : 5)) and gain RKS-1778 (12.3 mg). *Fr.5.7* (87 mg) was subjected to MPLC (*RP-18*, H_2O/MeOH (1 : 2)) to yield **1** (8.7 mg) and cytochalasin N (2.8 mg). *Fr. 7* (7.8 g) was subjected to silica gel CC using CH_2Cl_2–MeOH (10:1) as the eluent to produce 14 fractions (*Fr. 7.1–Fr. 7.14*). *Fr. 7.10* (335 mg) recrystallized from CH_2Cl_2 to gain cytochalasin H (300 mg). *Fr. 8* (1.9 g) was subjected to silica gel CC using CH_2Cl_2–MeOH (30:1) as the eluent to produce 11 fractions (*Fr. 8.1–Fr. 8.11*). *Fr. 8.7* (145 mg) divided into 5 fractions (*Fr. 8.7.1–Fr. 8.7.5*) by MPLC (SiO_2, CH_2Cl_2/MeOH (40:1)). *Fr. 8.7.3* recrystallized from CH_2Cl_2 to gain cyclo(L-Ile-L-Leu) (2.4 mg). 2.4*rel-(3S,4R,8R,9R,13E,16S,18R,19E,21R)-21-acetoxy-18-hydroxy-5,6,16,18-tetramethyl-l0-phenly [11]-cytochalasa-5,13,19-trien-l,7-dione* (**1**): whitish solid; $[\alpha]_D^{25}$: +104.3° (*c* 0.75, MeOH); UV λ_{max} (MeOH) (log ε) 207(3.82) and 345(1.57) nm; IR ν_{max} (KBr) 3370, 2919, 1739, 1695, 1669, 1644 cm^{-1};^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) spectral data see table 1; ESIMS *m/z* 514 $[M+Na]^+$; HRESIMS *m/z* 514.25637 $[M+Na]^+$ (calcd. for $C_{30}H_{37}NO_5Na$, 514.25639).

Table 1. ^{1}H and ^{13}C NMR (600/150 MHz) of phomocytochalasin (**1**) (δ in ppm).

phomocytochalasin (**1**)					
Position (H)	δ_H (J in Hz)	δ_C	**Position (H)**	δ_H (J in Hz)	δ_C
1	—	173.6	17	1.55 (1H, dd, J = 14.4, 3.6 Hz) 1.86 (1H, dd, J = 14.4, 6.6 Hz)	53.8
2-NH	5.89 (1H, br s, D_2O exchangeable)	—	18	—	74.4
3	3.42 (1H, tt, J = 7.8, 1.5 Hz)	59.6	19	5.55 (1H, dd, J = 16.8, 2.4 Hz)	138.7
4	2.81(1H, d, J = 1.5 Hz)	50.0	20	5.64 (1H, dd, J = 16.8, 2.4 Hz)	124.8
5	—	146.3	21	5.92 (1H, t, J = 2.4 Hz)	74.7
6	—	132.4	22	1.04 (3H, d, J = 6.6 Hz)	26.4
7	—	198.4	23	1.35 (3H, s)	30.5
8	3.55 (1H, d, J = 9.6 Hz)	51.7	24	—	170.2
9	—	51.2	25	2.32 (3H, s)	21.1
10	3.06 (1H, dd, J = 13.8, 7.8 Hz) 3.07 (1H, dd, J = 13.8, 7.8 Hz)	44.7	26	—	137.0
11	1.53 (3H, s)	18.5	27	7.23 (2H, d, J = 7.2, 1.2 Hz)	129.2
12	1.69 (3H, s)	12.1	28	7.35 (2H, td, J = 7.2, 1.2 Hz)	129.0
13	6.05 (1H, ddd, J = 16.2, 9.6, 1.2 Hz)	124.4	29	7.27 (1H, dd, J = 7.8, 1.2 Hz)	127.2
14	5.19 (1H, ddd, J = 16.2, 10.8, 4.8 Hz)	137.1	30	7.35 (2H, td, J = 7.2, 1.2 Hz)	129.0
15	1.90 (1H, br dd, J = 13.2, 1.8 Hz) 2.03 (1H, br dd, J = 13.2, 4.8 Hz)	43.0	31	7.23 (2H, d, J = 7.2, 1.2 Hz)	129.2
16	1.76 (1H, m)	28.1			

3. Results and Discussion

3.1 Structure Elucidation

The EtOAc soluble fraction was subjected to series of silica gel column chromatography and preparative TLC to afford six compounds. Besides new compound (**1**), the other five compounds were known as by comparison of their spectra and physical data with the available literature [15–18].

All the ^{1}H and ^{13}C signals were asssigned as shown in Table 1. Compound **1** was obtained as an optically active white powder with $[\alpha]_D^{25} = +104.3°$ (c 0.75, MeOH). The ESI-MS and HR-ESIMS were used to establish the molecular formula of **1** as $C_{30}H_{37}NO_5$, which implies thirteen degrees of unsaturation. Its IR spectrum exhibited peaks at 3370 (OH group), 1739, 1695, 1669 (carbonyl group), and 1644 (amide group). The UV spectrum showed maxium absorption at 207 and 345 nm. Accroding to the IR and UV spectral data, it suggested a skeleton of 10-phenylcytochalasin [15]. ^{1}H-NMR (600 MHz, $CDCl_3$) showed the presence of four singlet methyl groups at δ 1.35 (3H, s, H-23), 1.53 (3H, s, H-11), 1.69 (3H, s, H-12), and 2.32 (3H, s, H-25), three unequivalent methylene at δ 1.55 (1H, dd, J = 14.4, 3.6 Hz, H-17b), 1.86 (1H, dd, J = 14.4, 6.6 Hz, H-17a), 1.90 (1H, br dd, J = 13.2, 1.8 Hz, H-15b), 2.03 (1H, br dd, J = 13.2, 4.8 Hz, H-15a), 3.06 (1H, dd, J = 13.8, 7.8 Hz, H-10b), 3.07 (1H, dd, J = 13.8, 7.8 Hz, H-10a), fourteen methine protons, including δ 1.76 (1H, m, H-16), 2.81(1H, d, J = 1.5 Hz, H-4), 3.42 (H, tt, J = 7.8, 1.5 Hz, H-3), 3.55 (H, d, J = 9.6 Hz, H-8), one oxymethine at δ 5.92 (1H, t, J = 2.4 Hz, H-21), two pairs of *trans*-form olefinic protons at δ 5.19 (1H, ddd, J = 16.2, 10.8, 4.8 Hz, H-14), 6.05 (1H, ddd, J = 16.2, 9.6, 1.2 Hz, H-13), 5.55 (1H, dd, J = 16.8, 2.4 Hz, H-19), 5.64 (1H, dd, J = 16.8, 2.4 Hz, H-20) and one monosubstituted benzene ring at δ 7.23 (2H, d, J = 7.2, 1.2

Hz, H-27, 31), 7.27 (1H, dd, *J* = 7.8, 1.2 Hz, H-29), 7.35 (2H, td, *J* = 7.2, 1.2 Hz, H-28, 30). The ^{13}C-NMR spectrum of **1** indicated the presence of an amide carbonyl δ 173.6 (C-1), an ester carbonyl *δ* 170.2 (C-24), α,β-unsaturated carbonyl *δ* 198.4 (C-7), and five methyl groups *δ* 18.5 (C-11), 12.1 (C-12), 26.4 (C-22), 30.5 (C-23), and 21.1 (C-25) together with nine sp^2 and five sp^3 methines, two sp^2 and three sp^3 quaternary carbons, and three methylene groups. Detailed analyses of the ^{1}H- and ^{13}C-NMR spectra of **1** with the aid of COSY and HMQC spectroscopy, confirmed the partial structure of **1** (Figure 1). The geometry of both the 13 and 19 positions was the *E* configuration from the large coupling constant (*J* = 16.2 and 16.8 Hz, respectively). The HMBC spectrum was measured to determine the total structure of **1**. The correlation from CH_3-25 and H-21 to C-24 revealed that an acetoxyl group was connected to the 21 position. Connectivities of the partial structures and other units were confirmed by the correlations from CH_3-11 to C-5, CH_3-12 to C-6, CH_3-23 to C-17, 18, 19, H-13 to C-7, H-3 to C-26, and H-10 to C-26, 27, 31. Thus, the planar structure of **1** was deduced as shown in Figure 1.

The relative stereochemistry of **1** was established from the ROESY spectrum (Figure 2). The presence of ROESY correlations H-3↔H-21↔H-20↔H-23 indicated the α-orientation of these protons. The ROESY cross-peaks between H-4↔H-8↔H-14↔H-16 established the β-orientation of H-4, H-8, and H-16 (Figure 2). Compared to the known cytochalasin N, the major difference of ^{1}H- and ^{13}C-NMR spectra of **1** (Table 1) was the presence of a carbonyl group δ_C 198.4 at C-7, instead of a hydroxy group in cytochalasin N. Although there is different functional group at C-7 between **1** and cytochalasin N, it will not effect the relative configuration. So, the compound **1** displayed the same relative configuration with cytochalasin N [15]. On the basis of the above spectral evidence, the structure of **1** was elucidated as *rel*-(3*S*,4*R*,8*R*,9*R*,13*E*,16*S*,18*R*,19*E*,21*R*)-21-acetoxy-18-hydroxy-5,6,16,18-tetramethyl-10-phenyl[11]-cytochalasa-5,13,19-trien-l,7-dione, named phomocytochalasin.

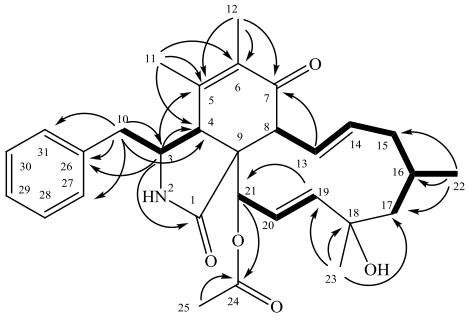

Figure 1. Key COSY (bold line) and HMBCs (H→C) correlations for compound **1**

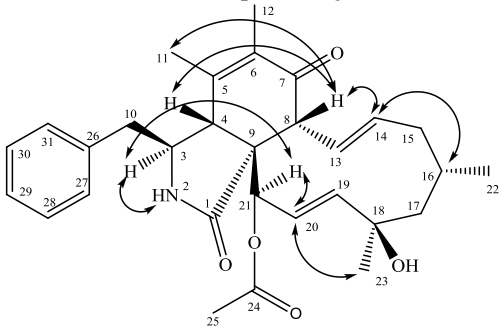

Figure 2. Key ROESY correlations for compound **1**

3.2. Anti-inflammatory Activity and Progesterone Receptor(PR) Antagonism

The isolates in sufficient amounts, were evaluated by examining their inhibitory effects on LPS-induced inducible nitric oxide synthase (iNOS)-dependent NO and IL-6 production in the murine macrophage cell line RAW 264.7 (Table 2) and progesterone receptor antagonism. The inhibitory activity data of the isolated compounds on NO and IL-6 generation by macrophages are shown in Table 2. From the results of above tests, the following conclusions can be drawn: Compared to quercetin (IC_{50} value 36.8±1.3 μM), which was used as positive control in this study, cytochalasin N exhibited NO inhibitory activity with IC_{50} values of 77.8 μM. And the cytochalasin H show progesterone receptor antagonism with IC_{50} values of 1.42 μM compare with positive control RU486 (IC_{50} values 0.063 nM). Among the cytochalasan analogs, cytochalasin N with a OH group (C-7) exhibited higher inhibition than its analog **1** with a carbonyl group (C-7).

Table 2. Inhibitory effects of the isolates on NO and IL-6 generations by RAW 264.7 macrophages in respones to lipopolysaccharide (LPS)

Compounds	IC_{50} (μM)	
	NO	IL-6
phomocytochalasin (**1**)	>100	>100
cytochalasin H	>100	>100
cytochalasin N	77.8	>100
dankasterone B	>100	>100
quercetin[a]	36.8	31.3

[a] Positive control

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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