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**Antimicrobial Activity of Bioactive Compounds from  
*Periconia siamensis* CMUGE015**

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**Abstract:** *Periconia siamensis* (strain CMUGE015) was isolated from leaves of the grass, *Thysanoleana latifolia* (Poaceae). It was antagonistic against the human pathogens *Bacillus cereus*, *Listeria monocytogenes*, MRSA (Methicillin Resistance *Staphylococcus aureus*) and *Pseudomonas aeruginosa* the causative agents of food borne disease, Listeriosis, skin infections and lung disease, respectively. Metabolites in both culture filtrates and crude extracts of the filtrates were inhibitory against the microbes tested. The major active ingredients from the culture filtrate were purified by silica gel column chromatography and identified to be Modiolide A, 5, 8-dihydroxy-10-methyl-5, 8, 9, 10-tetrahydro-2H-oxecin-2-one (Compound 1) and 4-Chromanone, 6-hydroxy-2-methyl- (5CI) (Compound 2) by IR, NMR and mass spectral (MS) data. Bioassays showed that both compounds had antibacterial activity against all the test bacteria. Minimum inhibitory concentrations were determined to be 50 µg mL<sup>-1</sup> for compound 1 and 100 µg mL<sup>-1</sup> for compound 2. This is the first report of the production of these two antibacterial metabolites a terrestrial endophytic fungus.

**Key words:** Antimicrobial activity, bioactive compound, fungi, grass

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## INTRODUCTION

The diversity of endophytic fungi and particularly those in grasses from temperate regions has been well documented (Wei *et al.*, 2007), but comparative studies on tropical species are few, especially from Thailand (Bacon and White, 2000). These groups of fungi are widely recognized as prolific sources of bioactive secondary metabolites that might represent useful leads for the development of new pharmaceutical bioagents (John *et al.*, 1999; Strobel *et al.*, 1999; Brady *et al.*, 2000; Singh *et al.*, 2000; Yamada *et al.*, 2002). Since more than 1.5×10<sup>6</sup> endophytic fungi are thought to thrive within the estimated 270,000 species of vascular plants, the prospects for additional discoveries of metabolites from these fungi are promising (Dreyfuss and Chapela, 1994). During recent investigation of the endophytic fungi of grasses in Thailand, we isolated many species of endophytic fungi including the novel endophytic species e.g., *Periconia siamensis* CMUGE015. In this research, we report on the extraction, isolation and structure elucidation, as well as the biological activities of the bioactive compounds, produced by this strain.

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## MATERIALS AND METHODS

### Fungal Isolation

*Periconia siamensis* CMUGE015 is a new species of an endophytic taxon isolated from leaf tissues of *Thysanoleana latifolia* collected from the Suthep-Pui National Park, Chiang Mai, Thailand. The isolation methods followed the previous study by Bussaban *et al.* (2001).

### Screening for Antimicrobial Metabolite Production

The screening and characterization of bioactive compounds were carried out during January 2006- January 2007. For initial screening various media (F1, F2, F3, F4 and F5) (Cheeptham *et al.*, 1999) were used for culture fermentations. *Periconia siamensis* CMUGE015 was subcultured on to PDA plate and incubated for 1 week. After incubation, 8 mm discs of mycelium were transferred, one per flask, to the five fermentation media. The cultures were incubated at room temperature  $28\pm 2^\circ\text{C}$  for 7 days using a reciprocal shaker at 130 rpm.

### Extraction and Concentration

At the end of the fermentation, the cultures were harvested by filtration. The culture broths were extracted twice with an equal volume of ethyl acetate (EtOAc). The extracts were pooled and dried in a rotary evaporator (BÜCHI Switzerland) at  $45^\circ\text{C}$  under reduced pressure. The extract residue was dissolved in dimethyl sulfoxide (DMSO) and stored at  $4^\circ\text{C}$  until the bioassays (described below) could be conducted.

### Characterization of Bioactive Compounds from *Periconia siamensis*

#### Culture Conditions and Extraction

Strain CMUGE015 was grown on 100 plates of F1 medium for 2 weeks at  $28^\circ\text{C}$  or until it completely felled each plate. The F1 colonies formed on each plate were cut into small pieces ( $0.5\times 0.5$  cm) using a sterilized cutter. The agar pieces were placed in a 3 L Erlenmeyer flask and ethyl acetate (2 L) was used to extract the metabolites produced. The flasks were shaken on a reciprocal checker at 180 rpm for 30 min. The process was repeated 5 times. The combined extracts (10 L) were dried by flash evaporation at  $40^\circ\text{C}$  and the recovery yield of dry material was about 2.8 g.

#### Fractionation and Purification of the Compounds

The dried extract was mixed with silica gel 60 (size 0.015-0.040 mm, Merck) and packed into a chromatography column. Fractionation using increased concentrations of Hexane:EtOAc:MeOH (100:0:0, 80:20:0, 60:40:0, 40:60:0, 20:80:0, 0:100:0, 0:99:1, 0:98:2, 0:96:4, 0:92:2, 0:90:10, 0:80:20, 0:60:40, 0:40:60, 0:20:80 and 0:0:100) produced fifty five 25 mL fractions. These were evaporated to dryness, weighed and fractionated again by column chromatography (silica gel 60 size 0.063-0.200 mm, Merck). A white crystalline material (compound 1, Fig. 1a) was recovered in fraction 7 and a yellow powder (compound 2, Fig. 1b) found in fraction 5 were active in the initial antibacterial assay.

The white crystals were washed in cold EtOAc for purification (120 mg). The material from Fraction 5 (194 mg dry weight) was subjected to repeated fractionation by column chromatography, using, Hexane:EtOAc (60:40) as eluant, the product (105 mg) was recovered in fractions 12-16. These were combined and checked by TLC result. The two compounds were screened again for their antimicrobial activity against the test organisms as described below, using the paper disk method (Venugopal and Venugopal, 1994).

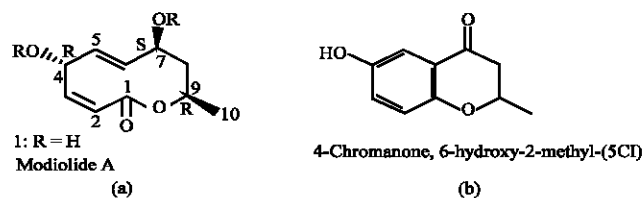


Fig. 1: Structural of antimicrobial compounds from *Periconia siamensis* CMUGE015. (a) Modiolide A; (b) 4-Chromanone, 6-hydroxy-2-methyl- (5CI)

### Structure Elucidation of Compounds

<sup>1</sup>H NMR (300 MHz) and <sup>13</sup>C NMR (75 MHz) spectra were analysed at the Department of Chemistry, Faculty of Science, Sanamchun Palace Campus, Silpakorn University, Thailand using a Bruker DPX300 spectrometer, with TMS ( $\delta$ H 0 ppm) and CDCl<sub>3</sub> ( $\delta$ C 77.0 ppm) as internal references. Melting points were determined using an electrothermal melting point apparatus. Optical rotations were measured with a JASCO J-810 spectropolarimeter. IR spectra were recorded on an FT-IR system 2000 (Perkin-Elmer) spectrometer. Mass spectra were recorded on a JEOL JMS- X505WA mass spectrometer.

### Bioassays

#### Screening for Antibacterial Metabolite Production

Ethyl acetate extracts of culture filtrates and column chromatography eluants of similar extracts of agar plates, were assayed for the presence of metabolites and with antibacterial activity using a paper disk diffusion assay method. Sterile paper disks (8 mm diam., Advantec, Toyo Roshi Kaisa, Ltd., Japan) were soaked in the extracts and allowed to air dry and placed on seeded assay plates. Controls were disks impregnated with solvents, used for extraction of culture filtrates and in column chromatography, with had been allowed to air dry. All assay plates were then incubated at 37°C for 24 h for all bacterial strains. The bacterial strains used were *Bacillus cereus*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*, were provided by Department of Biology, Faculty of Science, Chiang Mai University and MRSA (Methicillin Resistance *Staphylococcus aureus*) was kindly provided by Department of Associated Medical Science, Chiang Mai University. Nutrient broth cultures of these organisms were used to produce inocula for the bioassay plates. Screening for antibacterial metabolite production, was conducted in triplicate and data generated was analyzed using the SPSS v.10 package for one-way analysis of variance (ANOVA).

#### Minimum Inhibitory Concentration (MICs)

Purified compounds 1 and 2 were assayed to determine the MICs of these compounds against *Bacillus cereus*, *Listeria monocytogenes*, MRSA and *Pseudomonas aeruginosa*. Antibacterial activity was determined by the 2-fold microtiter broth dilution method (Kim and Oh, 2002). Dilutions of the test compound dissolved in dimethyl sulfoxide (DMSO) were added to each well of a 96 well microtiter plate containing a fixed volume of standard methods broth (SM broth, Difco) (final 0.5% DMSO). Each well was inoculated overnight with bacteria ( $10^5$  CFU mL<sup>-1</sup>) and incubated at 37°C for 24 h. The MIC was calculated as the concentration at which no growth of bacteria was observed.

## RESULTS

Preliminary tests for antibacterial activity showed that *Periconia siamensis* CMUGE015 produced metabolites that are inhibitory against the growth of *Bacillus cereus*, *Listeria monocytogenes*, MRSA and *Pseudomonas aeruginosa* on all media.

Table 1: The Inhibition zone (diameter, mm) of crude extract from *Periconia siamensis* against pathogenic bacteria in fermentation media F1, F2, F3, F4 and F5

Pathogenic bacteria	Inhibition zone (mm) <sup>a</sup>				
	F1	F2	F3	F4	F5
<i>Bacillus cereus</i>	15.0±0.15	12.9±0.15	8.9±0.10	9.2±0.10	8.5±0.11
<i>Listeria monocytogenes</i>	13.1±0.11	10.7±0.05	7.1±0.05	8.3±0.10	6.4±0.05
<i>Staphylococcus aureus</i>	14.3±0.05	10.8±0.11	7.4±0.17	8.0±0.05	7.5±0.05
<i>Pseudomonas aeruginosa</i>	10.1±0.11	8.4±0.05	6.8±0.05	8.1±0.10	6.8±0.05

<sup>a</sup>Means of inhibition clear zones surrounding each application point, three replicates

The result shows that F1 medium containing a combination of carbon source (fructose) and soybean meal (as N-source) was identified as the most effective for the production of antibiotics active against the test bacteria (Table 1). Data analysis using SPSS v.10 package for one-way analysis of variance (ANOVA) showed that F1 medium was significantly better than the other media used (results not shown).

A crude extract (1500 mg) of *P. siamensis* CMUGE015 was subjected to silica gel-column chromatography and 16 fractions, fraction 1 to fraction 16, were obtained. Each fraction was tested for the presence of antibacterial activity. Fractions 5 and 7 were markedly effective in inhibiting the growth of the test microorganism in this experiment indicating that they contained bioactive compounds. Fraction 7 (hexane:ethyl acetate = 99:1) was precipitated with cold ethyl acetate and designated as the hyaline white, pin-shaped crystal, Compound 1 (120 mg). Fraction 5 was then subjected to repeated chromatography in a mixture of hexane and ethyl acetate (6:4) and then designated Compound 2 (105 mg). Purification of fraction 5 also resulted in other compounds (ii) (43 mg), (iii) (32 mg) and (iv) (14 mg).

#### Characterization of Bioactive Compounds from *Periconia siamensis* CMUGE015

Compound 1 (Fig. 1a) was fractionated from fraction-7 and crystallized from solvent system EtOAc:MeOH (99:1), identified by IR, NMR and mass spectra data as 5,8-dihydroxy-10-methyl-5,8,9,10-tetrahydro-2H-oxecin-2-one (Modiolide A). Data from compound 1: Pin-shaped, translucence and hyaline-white crystal; m.p. = 188.85-190.85°C; MW 198.21 (EtOAc: MeOH [99:1]) and the molecular formula was shown to be C<sub>10</sub>H<sub>14</sub>O<sub>4</sub> by HREIMS. IR absorption bands at 3294 and 1717 cm<sup>-1</sup> were attributed to OH and carbonyl group- (s), respectively. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) data, (δ<sub>H</sub>: 5.85, 5.83, 4.68, 5.61, 5.56, 4.12, 1.71, 1.87, 5.25 and 1.22 ) and <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) data, (δ<sub>C</sub>:170.9, 123.7, 138.7, 73.0, 131.8, 139.4, 73.6, 44.7, 70.9 and 22.4) disclosed the existence of an ester carbonyl (δC 170.9), four sp<sup>2</sup> methines (δC 139.4, 138.7, 131.8 and 123.7), three oxymethines (δC 73.6, 73.0 and 70.9), one sp<sup>3</sup> methylene (δC 44.7) and one methyl group (δC 22.4). Since three out of four unsaturations were accounted for, Compound 1 was inferred to contain one ring. The 1H-1H COSY and HMQC spectra revealed connectivities from C-2 to C-10 (Fig. 1a). The HMBC correlation from H-2 to C-1 suggested that the ester carbonyl group was attached to C-2. The relatively lower-field resonance of H-9 (δH 5.25) suggested that C-9 was involved in an ester linkage to C-1. The existence of two hydroxyl groups at C-4 and C-7 was determined by a lower-field shift of H-4 and H-7 (δH 5.44 2H, m) by esterification with p-methoxycinnamoyl chloride (vide infra). This observation supported the presumption that the compound was a 10-membered macrolide. Geometries of two disubstituted olefins at C-2- C-3 and C-5-C-6 were assigned as Z and E, respectively, by 1H-1H coupling constants [J(H-2/H-3), 12.3 Hz; J(H-5/ H-6), 15.8 Hz] and the NOESY correlation for H-2/H-3 this result are similar with the previous report of Modiolide A compound. Modiolide A D +42° (c 0.25, MeOH); UV λ<sub>max</sub><sup>MeOH</sup> 203 nm (6400); IR (KBr) μmax 3294 and 1717 cm<sup>-1</sup>; 1H and 13C NMR; EIMS m/z 180 (M - H<sub>2</sub>O)<sup>+</sup> and 198 (M<sup>+</sup>); HREIMS m/z 198.0892 (M<sup>+</sup>, calcd for C<sub>10</sub>H<sub>14</sub>O<sub>4</sub> 198.21 (Tsuda *et al.*, 2003; Fun *et al.*, 2006).

Table 2: Antibacterial activity (MIC  $\mu\text{g mL}^{-1}$ ) of Modiolide A and 4-Chromanone, 6-hydroxy- 2-methyl- (5CI)

Microorganism	MIC ( $\mu\text{g mL}^{-1}$ )		
	Modiolide A	4-Chromanone, 6-hydroxy- 2-methyl- (5CI)	Penicillin G
<i>Bacillus cereus</i>	3.12	6.25	6.25
<i>Listeria monocytogenes</i>	6.25	12.50	6.25
<i>Staphylococcus aureus</i> (MRSA)	25.00	50.00	25.00
<i>Pseudomonas aeruginosa</i>	12.50	12.50	12.50
<i>Escherichia coli</i>	50.00	100.00	50.00

Compound 2 (Fig. 1b) was fractionated from fraction-5, identified by IR, NMR and mass spectra data as 4-Chromanone, 6-hydroxy-2-methyl- (5CI) the molecular formula was revealed to be  $\text{C}_{10}\text{H}_{10}\text{O}_3$  (Fig. 1b) by HREIMS. MW 178. Compound 2 was a yellow, amorphous powder: melting point 148-149°C (MeOH); UV  $\lambda_{\text{max}}^{\text{MeOH}}$  nm (log  $\text{\AA}$ ): 205 (4.04), 324 (4.22); UV  $\lambda_{\text{max}}^{\text{CH}_3\text{ONa}}$  nm: 210, 254;  $^1\text{H}$  NMR ( $\text{CD}_3\text{Cl}_2$ ):  $\delta$ 8.50 (1H, s, exchangeable  $\text{D}_2\text{O}$ , OH-4'), 7.14 (2H, d,  $J = 8.5$  Hz, H-2', H-6'), 6.82 (2H, d,  $J = 8.5$  Hz, H-3', H-5'), 6.51 (1H, d,  $J = 2.5$  Hz, H-8), 6.37 (1H, d,  $J = 2.5$  Hz, H-6), 5.80 (1H, s, H-3), 3.91 (3H, s, OMe-7), 3.53 (3H, s, OMe-5);  $\Delta\delta = \delta\text{C}_3\text{D}_5\text{N}_2 - \delta\text{CD}_3\text{COCD}_3 = \text{H-2}' + \text{H-6}' (+0.19)$ ,  $\text{H-3}' + \text{H-5}' (+0.32)$ ,  $\text{H-8} (+0.11)$ ,  $\text{H-6} (+0.01)$ ,  $\text{H-3} (+0.38)$  OMe-7 (20.16), OMe-5 (20.23); IR  $\nu_{\text{max}}$  (KBr)  $\text{cm}^{-1}$ : 3124, 2968, 1644, 1582, 1572, 1484 and 1372; MS  $m/z$  (relative intensities): 178  $[\text{M}]^+$  (100), 270  $[\text{M}-\text{CO}]^+$  (82), 255  $[\text{M}-\text{MeCO}]^+$  (29), 227  $[\text{M}-43-\text{CO}]^+$  (15).

#### Minimum Inhibitory Concentration (MICs)

Modiolide A and 4-Chromanone-6-hydroxy-2-methyl-(5I C) showed antibacterial activity against the five test bacteria used. Modiolide A was generally the most effective of the two (Table 2), however, the high MIC values with *S. aureus* and *E. coli* rule out further investigation. Both compounds were compared with standard antibiotic, Penicillin G with Modiolide A being the most similarly effective (Table 2).

## DISCUSSION

Fungi produce a diverse range of secondary metabolites which have no effect on the growth of the producer strain but can be strongly inhibitory against other microorganisms and many of these have important chemotherapeutic and other uses. In 1995 six of the top twenty best selling drugs were of fungal origin and there are numerous other potential drugs being developed from fungi (Concepcion *et al.*, 2001). Endophytic fungi are widely recognized as prolific sources of bioactive secondary metabolites that might represent useful leads in the development of new pharmaceutical agents (John *et al.*, 1999; Strobel *et al.*, 1999; Brady *et al.*, 2000; Singh *et al.*, 2000; Yamada *et al.*, 2002).

We are researching new biologically active metabolites from fungi and in this paper have characterized two antibacterial compounds from *P. siamensis* CMUGE015, endophytic fungi from the grass, *Thysanoleana latifolia*. Two antibacterial compounds were identified as Modiolide A ( $\text{C}_{10}\text{H}_{14}\text{O}_4$ ) and 4-Chromanone-6-hydroxy-2-methyl-(5IC) were identified.

Modiolide A was recently reported as a novel compound from the marine fungus, *Paraphaeosphaeria* sp., however, no antimicrobial activity was reported (Tsuda *et al.*, 2003). Recently, the crystallized structure of this compound was reported using x-ray crystallization (Fun *et al.*, 2006). In this study, it has been demonstrated that Modiolide A has potent activity against *B. cereus* and *L. monocytogenes*. MRSA (Methicillin Resistance *Staphylococcus aureus*) and *Pseudomonas aeruginosa* the causative agents of human pathogen, food borne disease, listeriosis, skin infections and lung disease, respectively. The compound was compared with standard antibiotic, Penicillin G, the results shows that Modiolide A inhibits gram positive bacteria rather than gram negatives (Table 2).

Compound 2 (Fig. 1b) or 4-Chromanone-6-hydroxy-2-methyl-(5IC) is similar with Tuckolide (C<sub>10</sub>H<sub>16</sub>O<sub>5</sub>) that has been reported as an inhibitor of cholesterol synthesis in liver cells (Andrus and Smith, 1996). However, it has never been reported as having antibacterial activities. It showed a similar spectrum of activity as Modiolide A, however, the latter was more potent and could have potential as a lead compound for the development of antibacterial agents for many bacterial strains.

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