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# Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15

Vatcharin Rukachaisirikul [a,*], Ubonta Sommart [a], Souwalak Phongpaichit [b], Jariya Sakayaroj [c], Kanyawim Kirtikara [c]

[a] *Department of Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*
[b] *Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*
[c] *National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Klong Luang, Pathumthani 12120, Thailand*

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**Abstract**

From the endophytic fungus *Phomopsis* sp. PSU-D15, three metabolites named as phomoenamide (**1**), phomonitroester (**2**) and deacetylphomoxanthone B (**3**), were isolated together with three known compounds, dicerandrol A (**4**), (1*S*,2*S*,4*S*)-*p*-menthane-1,2,4-triol (**5**) and uridine. Their structures were elucidated by spectroscopic methods. Phomoenamide (**1**) exhibited moderate in vitro antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra.
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*Keywords:* *Phomopsis* sp.; Endophytic fungus; Secondary metabolites; Antimycobacterial activity

## 1. Introduction

During our ongoing search for bioactive constituents from endophytic fungi, strong antimycobacterial activity against *Mycobacterium tuberculosis* (H37Ra strain) was shown by the extract of *Phomopsis* sp. PSU-D15, isolated from leaves of *Garcinia dulcis* (Roxb.) Kurz. The genus *Phomopsis* is a rich source of biologically active secondary metabolites including antimicrotubule phomopsidin (Kobayashi et al., 2003), antimalarial and antitubercular phomoxanthones (Isaka et al., 2001), antifungal phomoxanthone A (Elsaesser et al., 2005) and phomodiol (Horn et al., 1994), herbicidal biraryl ethers (Dai et al., 2005), algicidal phomosines (Krohn et al., 1995), cytokine production inhibitory phomalactone derivative (Wrigley et al., 1999), antimicrobial phomopsichalasin (Horn et al., 1995) and the plant growth regulator cytochalasin H (Wells et al., 1976). Chemical investigation of the ethyl acetate extracts from the culture broth and the cells of the endophytic fungus *Phomopsis* sp. PSU-D15 led to isolation of three new compounds, namely phomoenamide (**1**), phomonitroester (**2**) and deacetylphomoxanthone B (**3**), together with three known compounds, dicerandrol A (**4**) (Wagenaar and Clardy, 2001), (1*S*,2*S*,4*S*)-*p*-menthane-1,2,4-triol (**5**) (Thappa et al., 1976) and uridine (Rosemeyer et al., 1990). The known metabolites were identified by comparison of their spectroscopic data with those reported in the literature. We report herein the isolation, and structural elucidation of the metabolites as well as the antimycobacterial activity against *M. tuberculosis* H37Ra.

* Corresponding author. Tel.: +66 74 288 435; fax: +66 74 212 918.
*E-mail address:* vatcharin.r@psu.ac.th (V. Rukachaisirikul).

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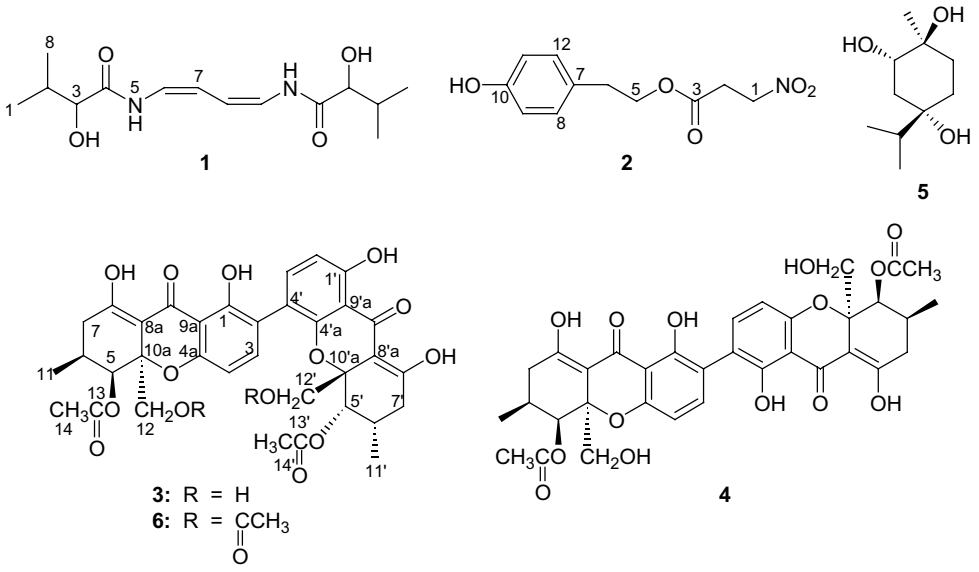


## 2. Results and discussion

The broth extract was subjected to successive column and thin layer chromatography to yield two new compounds (**1** and **2**) as well as one known compound (**5**). Upon chromatographic separation, the mycelial extract gave one new compound (**3**) and two known metabolites (**4** and uridine). Compound **4** was the known metabolite isolated from the fungus *Phomopsis longicolla* (Wagenaar and Clardy, 2001), whereas **5** was obtained for the first time from a fungal source.

Phomoenamide (**1**), with the molecular formula $C_{14}H_{24}N_2O_4$ from HREIMS, was obtained as a colorless gum with $[\alpha]_D^{26} + 32.64$. It exhibited an UV absorption band at 260 nm. Hydroxyl and amino absorption bands were found at 3374 $cm^{-1}$ while a carbonyl absorption band was observed at 1665 $cm^{-1}$ in the IR spectrum. The $^1H$ NMR spectrum (Table 1) consisted of characteristic signals of a 1-hydroxy-2-methylpropyl group [$\delta$ 4.07 (1H, *d*, $J = 3.3$ Hz), 2.13 (1H, *m*), 0.99 (3H, *d*, $J = 6.9$ Hz) and 0.82 (3H, *d*, $J = 6.9$ Hz)], one amino proton ($\delta$ 10.97, *d*, $J = 10.8$ Hz), two *cis*-olefinic protons [$\delta$ 7.50 (1H, *dd*, $J = 10.8$ and 8.7 Hz) and 5.15 (1H, *d*, $J = 8.7$ Hz)] and one hydroxy proton ($\delta$ 6.49, *brs*). Apart from four carbon resonances of the 1-hydroxy-2-methylpropyl group at $\delta$ 76.2, 32.2, 18.9 and 15.7, the $^{13}C$ NMR spectrum (Table 1) showed one amide carbonyl carbon ($\delta$ 172.4) and two olefinic carbons ($\delta$ 138.6 and 96.8). The lower-field olefinic proton, H-6 ($\delta$ 7.50), gave a $^1H$–$^1H$ COSY cross peak with the amino proton, 5-NH ($\delta$ 10.97), suggesting the presence of a *cis*-aminovinyl moiety. HMBC correlations of both H-6 of the aminovinyl group and H-3 ($\delta$ 4.07) of the 1-hydroxy-2-methylpropyl group with the amide carbonyl carbon (Table 1) linked both units with the carbonyl carbon to form an enamide moiety. These data together with the molecular formula $C_{14}H_{24}N_2O_4$ suggested that **1** was a symmetrical molecule. Therefore, phomoenamide was identified as a new enamide dimer (**1**).

Table 1
The $^1H$, $^{13}C$ NMR and selected HMBC data for compound **1**

| Position | $^1H$ ($\delta$) | $^{13}C$ ($\delta$) | | HMBC |
|---|---|---|---|---|
| 1 | 0.82 *d* (6.9) | 15.7 | $CH_3$ | C-2, C-3, C-8 |
| 2 | 2.13 *m* | 32.2 | CH | C-1, C-3, C-8 |
| 3 | 4.07 *d* (3.3) | 76.2 | CH | C-1, C-2, C-4, C-8 |
| 3-OH | 6.49 *brs* | | | |
| 4 | | 172.4 | C | |
| 5-NH | 10.97 *d* (10.8) | | | |
| 6 | 7.50 *dd* (10.8, 8.7) | 138.6 | CH | C-4, C-7 |
| 7 | 5.15 *d* (8.7 ) | 96.8 | CH | C-4, C-6 |
| 8 | 0.99 *d* (6.9) | 18.9 | $CH_3$ | C-1, C-2, C-3 |

Phomonitroester (**2**), with the molecular formula $C_{11}H_{13}NO_5$ from HREIMS, was isolated as a colorless gum. It exhibited UV absorption bands at 221 and 277 nm while hydroxyl, carbonyl and nitro absorption bands were found at 3409, 1730 and 1556 $cm^{-1}$ in the IR spectrum, respectively. The $^1H$ NMR spectrum indicated the presence of a 1,4-disubstituted benzene [$\delta$ 7.00 (2H, *d*, $J = 8.4$ Hz) and 6.70 (2H, *d*, $J = 8.4$ Hz)], as well as four sets of methylene protons [$\delta$ 4.55 (2H, *t*, $J = 6.3$ Hz), 4.25 (2H, *t*, $J = 7.2$ Hz), 2.89 (2H, *t*, $J = 6.3$ Hz, 2H) and 2.80 (2H, *t*, $J = 7.2$ Hz, 2H)]. The $^{13}C$ NMR spectrum showed one carbonyl carbon ($\delta$ 169.0), one oxyquaternary carbon ($\delta$ 153.9), one quaternary carbon ($\delta$ 130.0), two carbon resonances for four aromatic carbons ($\delta$ 115.5 and 129.9) and four methylene carbons ($\delta$ 31.1, 34.1, 66.1 and 69.7). These data established that one of the substituents in the 1,4-disubstituted benzene was a hydroxyl group. In the $^1H$–$^1H$ COSY spectrum, two sets of coupled methylene protons were observed: H-1 ($\delta$ 4.55) with H-2 ($\delta$ 2.89) and H-5 ($\delta$ 4.25) with H-6 ($\delta$ 2.80). HMBC correlations of H-5 with the carbonyl carbon, C-3 ($\delta$ 169.0), and C-7

($\delta$ 130.0) of the 4-hydroxyphenyl ring and that of H-2 with C-3 established the ester linkage between C-2 and C-5 with an oxygen atom attached to C-5 and further linked C-6 with C-7 of the 4-hydroxyphenyl ring. The chemical shifts of C-2, C-5 and C-7 supported the assigned linkage. These results together with the molecular formula $C_{11}H_{13}NO_5$ suggested the substituent at C-1 to be a nitro group. Therefore, phomonitroester (**2**) was an ester derivative of 3-nitropropionic acid. It is worth to note that this acid was previously isolated from a *Phomopsis* sp. (Elsaesser et al., 2005).

Deacetylphomoxanthone B (**3**), with the molecular formula $C_{34}H_{34}O_{14}$ from HREIMS, was isolated as a yellow gum. The UV and IR spectrum absorption bands were similar to those of dicerandrol A (**4**). These results suggested the presence of a tetrahydroxanthone dimer. The $^{13}C$ NMR spectrum (Table 2) contained 32 carbon resonances for 34 carbons, thus indicating that **3** was an unsymmetrical dimer. The $^{1}H$ NMR spectrum (Table 2) confirmed the above conclusion by the presence of two sets of proton resonances for two different tetrahydroxanthones. Analyses of the $^{1}H$, $^{13}C$ and HMBC data suggested that **3** consisted of one monomer, identical to that of **4**, and the other one, differing from that of **4** in the substitution pattern of an aromatic ring. $^{3}J$ HMBC correlations of H-3 ($\delta$ 7.06)/C-4′ ($\delta$ 115.9) and H-3′ ($\delta$ 7.21)/C-2 ($\delta$ 117.3) (Table 2) established a C-2-C-4′ linkage between two tetrahydroxanthones. Therefore, **3** was assigned as a new unsymmetrical tetrahydroxanthone dimer, which was the deacetyl derivative of phomoxanthone B (**6**) (Isaka et al., 2001). Their $^{1}H$ and $^{13}C$ NMR spectroscopic data were similar except that $H_{a,b}$-12 and $H_{a,b}$-12′ in **3** resonated at higher field than those in **6**. These results supported that the substituents at C-12 and C-12′ in **3** were hydroxyl groups. Due to the close chemical shifts of H-6, 6′ and $H_{a,b}$-7, 7′, the relative configuration in **3** was determined by comparison of multiplicity of H-5 and H-5′ together with $J$ value with those of **6**. Since they displayed identical data, compound **3** was proposed to possess the same relative configuration as **6**.

Table 2
The $^{1}H$, $^{13}C$ NMR and selected HMBC data for compound **3**

| Position | $^{1}H$ ($\delta$) | $^{13}C$ ($\delta$) | | HMBC |
|---|---|---|---|---|
| 1-OH | 11.95 *s* | 158.4 | C | C-1, C-2, C-9a |
| 2 | | 117.3 | C | |
| 3 | 7.06 *d* (8.5) | 137.4 | CH | C-2, C-4a, C-4′ |
| 4 | 6.41 *d* (8.5) | 107.2 | CH | C-2, C-4a, C-9a |
| 4a | | 156.6 | C | |
| 5 | 5.46 *d* (1.5) | 68.3 | CH | C-7, C-8a, C-10a, C-11, C-13 |
| 6 | 2.31 *m* | 26.7 | CH | C-5, C-11 |
| 7 | a: 2.31 *m* | 32.1 | $CH_2$ | C-5, C-6, C-8a |
| | b: 2.39 *m* | | | |
| 8-OH | 13.85 *s* | 177.3 | C | C-7, C-8, C-8a |
| 8a | | 100.0 | C | |
| 9 | | 187.0 | C | |
| 9a | | 105.6 | C | |
| 10a | | 81.2 | C | |
| 11 | 0.92 *d* (6.5) | 16.3 | $CH_3$ | C-5, C-6, C-7 |
| 12 | a: 3.33 *d* (12.0) | 64.0 | $CH_2$ | C-5, C-10a |
| | b: 3.92 *d* (12.0) | | | |
| 13 | | 169.4 | C | |
| 14 | 1.90 *s* | 19.7 | $CH_3$ | C-13 |
| 1′-OH | 11.36 *s* | 160.9 | C | C-1′, C-2′, C-9′a |
| 2′ | 6.52 *d* (8.5) | 109.2 | CH | C-4′, C-9′a |
| 3′ | 7.21 *d* (8.5) | 138.1 | CH | C-2, C-1′, C-4′a |
| 4′ | | 115.9 | C | |
| 4′a | | 153.3 | C | |
| 5′ | 5.59 (*s*) | 70.1 | CH | C-6, C-7, C-8′a, C-10′a, C-11′, C-13′ |
| 6′ | 2.38 *m* | 26.6 | CH | C-5′, C-11′ |
| 7′ | a: 2.20 *m* | 32.5 | $CH_2$ | C-6′ |
| | b: 2.37 *m* | | | |
| 8′-OH | 13.82 *s* | 176.6 | C | C-7′, C-8′, C-8′a |
| 8′a | | 100.1 | C | |
| 9′ | | 187.0 | C | |
| 9′a | | 105.6 | C | |
| 10′a | | 81.7 | C | |
| 11′ | 1.01 *d* (6.0) | 16.5 | $CH_3$ | C-5′, C-6′, C-7′ |
| 12′ | 3.58 *d* (10.0) | 64.7 | $CH_2$ | C-5′, C-10′a |
| | 3.95 *d* (10.0) | | | |
| 13′ | | 169.7 | C | |
| 14′ | 2.07 *s* | 19.9 | $CH_3$ | C-13′ |

The crude ethyl acetate extract of the broth showed interesting antimycobacterial activity against *M. tuberculosis* H37Ra with a minimum inhibitory concentration (MIC) value of 0.195 μg/ml. Only compound **1** was tested for the activity as it was obtained in sufficient amount. It exhibited the MIC value of 6.25 μg/ml. Phomoxanthone B (**6**), the diacetate analogue of **3**, has been reported to show this activity with the same MIC value as **1** (Isaka et al., 2001). In addition, deacetylphomoxanthone A has been noted to be inactive while phomoxanthone A gave a MIC value of 0.50 μg/ml (Isaka et al., 2001). These data suggest that **3** might display much weaker antimycobacterial activity than **6**. Further work is required to explain the activity of the crude extract.

*Concluding remarks*: The isolation of a derivative of 3-nitropropionic acid (**2**) and phomoxanthone derivatives (**3** and **4**) from the endophytic fungus PSU-D15 confirmed the identification of this fungus as *Phomopsis* sp. The related metabolites were observed for the fungi *P. longicolla* (Wagenaar and Clardy, 2001) and *Phomopsis* sp. (Isaka et al., 2001; Elsaesser et al., 2005; Chomchoen et al., 2005).

## 3. Experimental

### 3.1. General experimental procedures

Infrared spectra (IR) were recorded neat on a Perkin Elmer 783 FTS165 FT-IR spectrometer. Ultraviolet (UV) absorption spectra were measured in MeOH on a SHIMADZU UV-160A spectrophotometer. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a 300 MHz Bruker FTNMR Ultra Shield™ spectrometer in $CDCl_3$. Mass spectra were obtained on a MAT 95 XL Mass Spectrometer (Thermofinnigan). Optical rotations were measured in MeOH on a JASCO P-1020 polarimeter. Thin-layer chromatography (TLC) and precoated TLC were performed on silica gel $GF_{254}$ (Merck). Column chromatography (CC) was carried out on Sephadex LH-20 or silica gel (Merck) type 100 (70–230 Mesh ASTM).

### 3.2. Fungal material

The endophytic fungus *Phomopsis* PSU-D15 was isolated from the leaves of *Garcinia dulcis* (Roxb.) Kurz, collected in Songkhla Province, Thailand, in 2005. This fungus was deposited as PSU-D15 (GenBank accession number DQ480353) at the Department of Microbiology, Faculty of Science, Prince of Songkla University and the Culture Collection Laboratory, the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand (BCC number 26568). The fungus was identified based on the analysis of the DNA sequences of the ITS1-5.8S-ITS2, ITS regions of their ribosomal RNA gene. Its ITS sequence (DQ480353) matched with four fungal sequences from GenBank, comprising *Diaporthe phaseolorum* AY577815, *Diaporthe* sp. AY148440, *Phomopsis sojae* AY050627 and *Phomopsis* sp. AY050628, but with sequence identity less than 99%. These results indicated that the endophyte PSU-D15 might be a *Diaporthe*/*Phomopsis* species complex (Zhang et al., 1998). Due to the lack of a sexual structure in the culture, this endophytic fungus was then identified as *Phomopsis* sp.

### 3.3. Fermentation and isolation

The endophytic fungus *Phomopsis* PSU-D15 was grown on potato dextrose agar (PDA) at 25 °C for 5 days. Three pieces ($0.5 \times 0.5$ cm$^2$) of mycelial agar plugs were inoculated into 500 ml Erlenmeyer flasks containing 300 ml potato dextrose broth (PDB) at room temperature for 4 weeks. The culture (5 l) was filtered to give the filtrate and mycelia. The filtrate was extracted with EtOAc ($3 \times 800$ ml) to afford a broth extract (750.0 mg) as a brown gum. The crude EtOAc extract was separated by CC over Sephadex LH-20 using MeOH to yield five fractions (A–E). Fraction B (59.3 mg) was further purified by silica gel CC using a gradient of MeOH–$CH_2Cl_2$ to give **5** (2.5 mg). Fraction C (147.5 mg) was subjected to Sephadex LH-20 CC using 50% MeOH in $CH_2Cl_2$ to yield **1** (5.5 mg). Fraction E (6.2 mg) was subjected to precoated TLC using 5% MeOH in $CH_2Cl_2$ (5 runs) to afford **2** (1.3 mg). Wet mycelia were extracted with MeOH ($2 \times 500$ ml). After concentration of the MeOH solution to ~200 ml, $H_2O$ (100 ml) was added with the mixture washed with hexane (500 ml). The aqueous MeOH layer was concentrated under reduced pressure. The residue was dissolved in EtOAc (1 l) and washed with $H_2O$ (200 ml), dried ($Na_2SO_4$), and concentrated under reduced pressure to obtain a brown gum (190.0 mg). The crude mycelial extract was fractionated on Sephadex LH-20 column, eluted with MeOH to yield 3 fractions. The last fraction (31.8 mg) was purified using the same method as fraction C to afford **3** (1.5 mg), **4** (1.2 mg) and uridine (3.5 mg).

#### 3.3.1. Phomoenamde (*1*)

Colorless gum; $[\alpha]_D^{26} + 32.64 (c = 0.13, CHCl_3)$; UV $\lambda_{max}^{MeOH}$ nm ($\log \varepsilon$): 260 (3.97); FT-IR $\nu_{max}^{neat}$ cm$^{-1}$: 3374, 1665, 1622, 1023; For $^{1}H$ NMR and $^{13}C$ NMR ($CDCl_3$) spectra, see Table 1; EIMS *m*/*z* (% relative intensity): 284 (1), 96 (49), 87 (100), 73 (74); HREIMS *m*/*z* 284.1711 $[M]^+$ (calcd for $C_{14}H_{24}N_2O_4$ 284.1736).

#### 3.3.2. Phomonitroester (*2*)

Colorless gum; UV $\lambda_{max}^{MeOH}$ nm ($\log \varepsilon$): 221 (3.83), 277 (2.96); FT-IR $\nu_{max}^{neat}$ cm$^{-1}$: 3409, 1730, 1613, 1556, 1377; $^{1}H$ NMR (300 MHz): $\delta$ 7.00 (2H, *d*, $J = 8.4$ Hz, H-8, H-12), 6.70 (2H, *d*, $J = 8.4$ Hz, H-9, H-11), 4.55 (2H, *t*, $J = 6.3$ Hz, H-1), 4.25 (2H, *t*, $J = 7.2$ Hz, H-5), 2.89 (2H, *t*, $J = 6.3$ Hz, H-2), 2.80 (2H, *t*, $J = 7.2$ Hz, H-6); $^{13}C$ NMR (75 MHz): $\delta$ 169.0 (C, C-3), 153.9 (C, C-10), 130.0 (C, C-7), 129.9 (CH, C-8, C-12), 115.5 (CH, C-9, C-11), 69.7 ($CH_2$, C-1), 66.1 ($CH_2$, C-5), 34.1 ($CH_2$, C-6), 31.1 ($CH_2$, C-2); EIMS *m*/*z* (% relative intensity): 239 (5), 121

(97), 107 (100), 77 (40); HREIMS *m/z* 239.0748 $[M]^+$ (calcd for $C_{11}H_{13}NO_5$ 239.0794).

### 3.3.3. Deacetylphomoxanthone B (*3*)

Colorless gum; $[\alpha]_D^{26} + 106.80 (c = 0.01, CHCl_3)$; UV $\lambda_{max}^{MeOH}$ nm (log ε): 212 (4.45), 243 (3.87), 345 (4.30); FT-IR $\nu_{max}^{neat}$ $cm^{-1}$: 3450, 1735, 1606; For $^1H$ NMR and $^{13}C$ NMR ($CDCl_3$) spectra, see Table 2; EIMS *m/z* (% relative intensity): 666 (7), 665 (26), 635 (31), 634 (100); HREIMS *m/z* 666.1946 $[M]^+$ (calcd for $C_{34}H_{34}O_{14}$ 666.1949).

### 3.4. Antimycobacterial assay

Antimycobacterial activity was performed against *M. tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). Standard drugs, rifampicin, kanamycin sulfate and isoniazid, exhibited MIC values of 0.047, 1.25 and 0.25 μg/ml, respectively.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.09.006.

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