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# Aromatase inhibitory, radical scavenging, and antioxidant activities of depsidones and diaryl ethers from the endophytic fungus *Corynespora cassiicola* L36

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ABSTRACT

Isolation of a broth extract of the endophytic fungus *Corynespora cassiicola* L36 afforded three compounds, corynesidones A (**1**) and B (**3**), and corynether A (**5**), together with a known diaryl ether **7**. Compounds **1**, **3**, **5**, and **7** were relatively non-toxic against cancer cells, and inactive toward normal cell line, MRC-5. Corynesidone B (**3**) exhibited potent radical scavenging activity in the DPPH assay, whose activity was comparable to ascorbic acid. Based on the ORAC assay, compounds **1**, **3**, **5**, and **7** showed potent antioxidant activity. However, the isolated natural substances and their methylated derivatives (**1**–**8**) neither inhibited superoxide anion radical formation in the XXO assay nor suppressed TPA-induced superoxide anion generation in HL-60 cell line. Corynesidone A (**1**) inhibited aromatase activity with an $IC_{50}$ value of 5.30 μM.



## 1. Introduction

One of eight American women will develop breast cancer in her lifetime (Brueggemeier et al., 2005), while one of nine women in the UK are diagnosed with breast cancer at some point in their life (Paoletta et al., 2008). In Thailand, it was estimated that the number of breast cancer were 5592 cases per 100,000 population in 1996 (Sriplung et al., 2005). Around 50–80% of breast cancer patients have estrogen-dependent breast cancer, the growth of the tumors being stimulated by circulating estrogen (Elledge and Osborne, 1997). In breast cancer tissues, high levels of the enzyme aromatase (also known as CYP19) are found either in or around tumor sites (Brueggemeier et al., 2005). Inhibition of aromatase activity significantly reduces the incidence of breast cancer, and examples of aromatase inhibitor drugs for the treatment of metastatic estrogen-dependent breast cancer are anastrozole, letrozole, and exemestane. It is known that reactive oxygen species (ROS) play an important role in tumor initiation, and that ROS levels, in a healthy organism, are controlled by endogenous mechanisms including glutathione and enzymes like catalase or superoxide dismutase (Gerhauser et al., 2003). Elevated ROS levels can initiate DNA damage, and might ultimately lead to carcinogenesis (Halliwell et al., 2000). Compounds capable of either scavenging free radicals or suppression of superoxide generation and antioxidant compounds show cancer chemopreventive effects (Lippman et al., 1994).

Endophytic fungi are rich sources of biologically active compounds, and several new compounds have been isolated from fungal endophytes (Gunatilaka, 2006; Pongcharoen et al., 2008; Rukachaisirikul et al., 2008; Schulz et al., 2002; Tan and Zou, 2001). Bioactive substances from endophytic fungi are of great interest to scientists because they have potential applications in agrochemical and pharmaceutical industries (Shrestha et al.,

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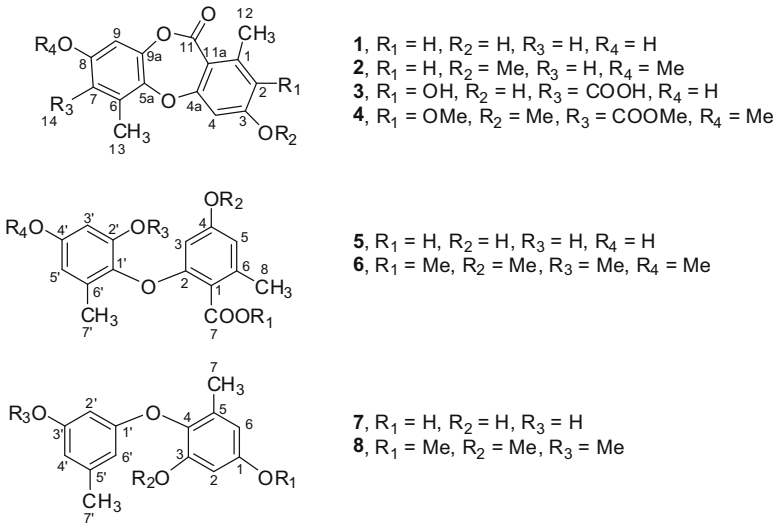


**Fig. 1.** Structure of the isolated compounds and their methylated derivatives.

2001; Stierle et al., 1993; Strobel, 2006; Wiyakrutta et al., 2004). Our continuing search on bioactive compounds from endophytic fungi led to the isolation and characterization of new depsidones and diaryl ethers from the endophytic fungus *Corynespora cassiicola* L36. Herein, we report the isolation and characterization of new depsidones **1** and **3** and a new diaryl ether **5**, as well as a known diaryl ether **7**, from the fungus *C. cassiicola* L36 (Fig. 1). Furthermore, radical scavenging, antioxidant, aromatase inhibitory, and cytotoxic activities of depsidones and diaryl ethers (**1**–**8**) are also reported in this paper.

## 2. Results and discussion

### 2.1. Structural determination

A crude broth extract of the endophytic fungus *C. cassiicola* L36 was separated by Sephadex LH-20 and preparative TLC to yield new depsidones **1** and **3** (named corynesidones A and B) and diaryl ether **5** (named corynether A), together with a known diaryl ether **7** (Fig. 1). Corynesidone A (**1**) had a molecular formula $C_{15}H_{12}O_5$, as indicated by its ESI–TOF–MS spectrum. The IR absorption band at 1693 $cm^{-1}$ and the $^{13}C$ resonance at $\delta_C$ 163.3 suggested the presence of a carbonyl of an aromatic ester (Table 1). The $^1H$ NMR spectrum (acetone-$d_6$) of **1** showed signals of two pairs of *meta*-coupling aromatic protons and two singlet methyls (Table 1), while the $^{13}C$ NMR spectrum revealed 15 lines attributable to two $sp^3$ methyls, four $sp^2$ methines, and nine non-protonated carbons (as indicated by DEPT techniques). The HMBC spectrum of **1** showed correlations from H-2 to C-3, C-4, C-11a, and C-12; H-4 to C-2, C-3, C-4a, and C-11a; H-7 to C-5a, C-8, and C-9; H-9 to C-5a, C-8, and C-9a; $H_3$-12 to C-1, C-2, and C-11a; and $H_3$-13 to C-5a, C-6, and C-7. However, these HMBC data could not place position of the C-11 ester carbonyl on the structure of corynesidone A (**1**). Corynesidone A (**1**) was methylated to give a di-*O*-methyl derivative **2**, confirming the presence of two hydroxyl groups in **1**. Interestingly, the HMBC spectrum of **2** showed a four-bond correlation

**Table 1**
$^1H$ and $^{13}C$ NMR data for compounds **14**.

| Position | **1**[a] | | **2**[b] | | **3**[a] | | **4**[b] | |
|---|---|---|---|---|---|---|---|---|
| | $\delta_H$ (*J* in Hz) | $\delta_C$ | $\delta_H$ (*J* in Hz) | $\delta_C$ | $\delta_H$ (*J* in Hz) | $\delta_C$ | $\delta_H$ (*J* in Hz) | $\delta_C$ |
| 1 | – | 145.1 | – | 145.5 | – | 128.1 | – | 136.5 |
| 2 | 6.66 (br s) | 115.5 | 6.60 (d, 2.4) | 114.0 | – | 141.6 | – | 145.2 |
| 3 | – | 162.4 | – | 163.0 | – | 149.3 | – | 156.8 |
| 4 | 6.66 (br s) | 104.7 | 6.60 (d, 2.4) | 103.5 | 6.78 (s) | 104.1 | 6.62 (s) | 101.7 |
| 4a | – | 161.5 | – | 163.1 | – | 155.0 | – | 158.7 |
| 5a | – | 142.1 | – | 142.8 | – | 142.8 | – | 142.9 |
| 6 | – | 131.3 | – | 131.3 | – | 133.5 | – | 129.2 |
| 7 | 6.53 (dd, 2.8, 0.7) | 113.5 | 6.53 (d, 2.9) | 112.7 | – | 110.0 | – | 120.9 |
| 8 | – | 154.5 | – | 156.5 | – | 160.3 | – | 153.7 |
| 9 | 6.52 (d, 2.8) | 104.9 | 6.62 (d, 2.9) | 103.7 | 6.64 (s) | 106.4 | 6.67 (s) | 102.0 |
| 9a | – | 144.9 | – | 144.8 | – | 149.4 | – | 145.6 |
| 11 | – | 163.3 | – | 163.3 | – | 161.6 | – | 162.3 |
| 11a | – | 112.8 | – | 109.5 | – | 112.9 | – | 113.7 |
| 12 | 2.39 (s) | 20.2 | 2.50 (s) | 21.5 | 2.31 (s) | 12.5 | 2.41 (s) | 13.3 |
| 13 | 2.37 (s) | 15.1 | 2.43 (s) | 16.2 | 2.70 (s) | 14.1 | 2.40 (s) | 13.6 |
| 14 | – | – | – | – | – | 172.0 | – | 167.3 |
| 2-OMe | – | – | – | – | – | – | 3.73 (s) | 60.3 |
| 3-OMe | – | – | 3.83 (s) | 55.6 | – | – | 3.90 (s) | 56.0 |
| 8-OMe | – | – | 3.74 (s) | 55.7 | – | – | 3.77 (s) | 56.3 |
| COO*Me* | – | – | – | – | – | – | 3.91 (s) | 52.4 |

[a] Acquired in acetone-$d_6$.
[b] Acquired in $CDCl_3$.

($^4J_{CH}$ coupling) from $H_3$-12 to C-11, which confirmed the position of the C-11 ester carbonyl in **2**. It is known that H–H couplings substantially reduce intensities of the C–H correlation, therefore, $^nJ_{CH}$ correlations for $n > 3$ are very weak or non-observable (Griesinger et al., 1994). The observation of longer range $^nJ_{CH}$ couplings could be possible for compounds with either few or no H–H couplings, for example, the observation of $^5J_{CH}$ and $^7J_{CH}$ couplings in excelsione, a depsidone from an unidentified endophytic fungus (Lang et al., 2007). The NOESY spectrum of **2** demonstrated correlations from 3-OMe protons to H-2 and H-4; and from 8-OMe protons to H-7 and H-9, readily placing the methoxy groups in **2** (the hydroxyl groups in **1**). The above NOESY correlations also implied that the two aromatic parts were linked either at C-5a/C-4a and C-9a/C-11 or at C-5a/C-11 and C-9a/C-4a. Fortunately, the key NOESY correlation from $H_3$-13 to H-4 was observed for both **1** and **2**, which indicated the linkage at C-5a/C-4a and C-9a/C-11 in **1** and **2**. On the basis of these spectroscopic data, the structure of corynesidone A (**1**) was secured as shown. Protons and carbons in **1** were assigned by analysis of $^1H$–$^1H$ COSY and HMBC spectra (Table 1). It should be noted that di-*O*-methyl derivative **2** was synthetically known (Sala and Sargent, 1981), however, comparison of the 60 MHz $^1H$ NMR spectroscopic data ambiguously confirmed the structural identity of compound **2**. Analysis of $^1H$–$^1H$ COSY and HMBC spectra assisted the assignment of proton and carbon signals in **2** (Table 1). It is worth noting that structural elucidation of hydroxylated depsidones, i.e. corynesidone A (**1**), needs analysis of HMBC and NOESY spectral data of its corresponding methylation product, in order to locate individual substituents (particularly on the hydroxyl positions) and the linkage between the two aromatic parts.

Analysis of the ESI–TOF–MS spectrum resulted in deduction of molecular formula, $C_{16}H_{12}O_8$, for corynesidone B (**3**). The $^1H$ NMR spectrum (acetone-$d_6$) of corynesidone B (**3**) showed only four signals (two $sp^2$ singlet methines and two singlet methyls; Table 1), while the $^{13}C$ NMR spectrum contained sixteen lines, twelve of which were non-protonated carbons (as indicated by HMQC and DEPT techniques; Table 1). The $^{13}C$ NMR resonance at $\delta_C$ 172.0 indicated the presence of a carboxylic acid functionality in **3**. The IR spectrum of **3** showed absorption bands for a carboxylic acid (1703 $cm^{-1}$) and an aromatic ester carbonyl (1687 $cm^{-1}$). HMBC correlations were observed from H-4 to C-2, C-3, C-4a, and C-11a; H-9 to C-5a, C-7, C-8, and C-9a; $H_3$-12 to C-1, C-2, and C-11a; and $H_3$-13 to C-5a, C-6, and C-7. Similar to the depsidone, excelsione (Lang et al., 2007), there were no H–H couplings for corynesidone B (**3**); therefore, it was expected that longer range $^nJ_{CH}$ couplings could be observable in the HMBC spectrum. Indeed, there were $^4J_{CH}$ couplings, e.g., from both H-4 and $H_3$-12 to the C-11 ester carbonyl and from H-9 and $H_3$-13 to C-14 carboxylic acid carbonyl, placing positions of ester and carboxylic acid in each aromatic ring. Methylation of corynesidone B (**3**) gave a tetra-*O*-methyl derivative **4**. The NOESY spectrum of **4** showed correlations between 3-OMe and H-4, between 8-OMe and H-9, and between $H_3$-13 and H-4. The HMBC correlations in **4** were observed from 2-OMe to C-2; 3-OMe to C-3; H-4 to C-3, C-4a, C-11a, and C-11 ($^4J_{CH}$); 8-OMe to C-8; H-9 to C-5a, C-7, C-8, C-9a, and C-14 ($^4J_{CH}$); $H_3$-12 to C-1, C-2, C-11a, and C-11 ($^4J_{CH}$); and $H_3$-13 to C-5a, C-6, C-7, and C-14 ($^4J_{CH}$). Based upon the spectroscopic data, the structure of corynesidone B (**3**) was established. Assignments of proton and carbon signals for compounds **3** and **4** are shown in Table 1.

Corynether A (**5**) demonstrated a molecular formula $C_{15}H_{14}O_6$, as indicated by ESI–TOF–MS. The $^1H$ NMR spectrum (DMSO-$d_6$) of corynether A (**5**) revealed the presence of two pairs of *meta*-coupling aromatic protons and two singlet methyls (Table 2). The $^{13}C$ NMR and DEPT spectra of **5** had 15 lines attributable to two $sp^3$ methyls, four $sp^2$ methines, and nine non-protonated carbons. The $^{13}C$ NMR resonance at $\delta_C$ 173.5 indicated the presence of carboxylic acid in **5**. The HMBC spectrum of **5** showed correlations from H-3 to C-1, C-2, C-4, and C-5; H-5 to C-1, C-3, C-4, C-6, and

**Table 2**
$^1H$ and $^{13}C$ NMR data for compounds **5–8**.

| Position | **5**[a] | | **6**[b] | | **7**[c] | | **8**[b] | |
|---|---|---|---|---|---|---|---|---|
| | $\delta_H$ (*J* in Hz) | $\delta_C$ | $\delta_H$ (*J* in Hz) | $\delta_C$ | $\delta_H$ (*J* in Hz) | $\delta_C$ | $\delta_H$ (*J* in Hz) | $\delta_C$ |
| 1 | – | 123.8 | – | 115.9 | – | 154.8 | – | 157.0 |
| 2 | – | 156.7 | – | 161.2 | 6.35 (d, 2.9) | 101.4 | 6.41 (d, 2.8) | 97.9 |
| 3 | 5.91 (br s) | 101.9 | 5.85 (d, 2.1) | 97.6 | – | 150.6 | – | 153.0 |
| 4 | – | 157.1 | – | 157.2 | – | 133.1 | – | 135.3 |
| 5 | 6.22 (br s) | 111.6 | 6.33 (d, 2.1) | 107.7 | – | 132.4 | – | 133.1 |
| 6 | – | 136.8 | – | 138.5 | 6.25 (d, 2.9) | 108.1 | 6.36 (d, 2.8) | 106.2 |
| 7 | – | 173.5 | – | 168.4 | 1.98 (s) | 15.4 | 2.13 (s) | 16.4 |
| 8 | 2.24 (s) | 20.5 | 2.35 (s) | 20.0 | – | – | – | – |
| COO*Me* | – | – | 3.91 (s) | 51.8 | – | – | – | – |
| 1-OH | – | – | – | – | 8.17 (br s) | – | – | – |
| 3-OH | – | – | – | – | 8.01 (br s) | – | – | – |
| 4-OH | 12.75 (br s) | – | – | – | – | – | – | – |
| 1-OMe | – | – | – | – | – | – | 3.81 (s) | 55.5 |
| 3-OMe | – | – | – | – | – | – | 3.74 (s) | 56.0 |
| 4-OMe | – | – | 3.66 (s) | 55.2 | – | – | – | – |
| 1′ | – | 137.3 | – | 135.4 | – | 159.7 | – | 159.5 |
| 2′ | – | 152.1 | – | 153.2 | 6.03 (dd, 2.1, 2.1) | 99.1 | 6.20 (dd, 2.2, 2.2) | 98.1 |
| 3′ | 5.96 (br s) | 102.4 | 6.39 (d, 2.1) | 98.3 | – | 158.4 | – | 160.6 |
| 4′ | – | 154.8 | – | 157.3 | 6.28 (m) | 109.2 | 6.34 (m) | 107.6 |
| 5′ | 6.03 (br s) | 106.3 | 6.34 (d, 2.1) | 106.7 | – | 139.8 | – | 140.2 |
| 6′ | – | 131.4 | – | 133.1 | 6.13 (m) | 106.8 | 6.22 (m) | 107.7 |
| 7′ | 2.16 (s) | 16.7 | 2.13 (s) | 16.2 | 2.17 (s) | 20.6 | 2.25 (s) | 21.8 |
| 2′-OH | 9.04 (br s) | – | – | – | – | | – | |
| 3′-OH | – | – | – | – | 8.23 (br s) | – | – | – |
| 4′-OH | 9.37 (br s) | – | – | – | – | – | – | – |
| 2′-OMe | – | – | 3.72 (s) | 56.1 | – | – | – | – |
| 3′-OMe | – | – | – | – | – | – | 3.73 (s) | 55.2 |
| 4′-OMe | – | – | 3.80 (s) | 55.5 | – | – | – | – |

[a] Acquired in DMSO-$d_6$.
[b] Acquired in $CDCl_3$.
[c] Acquired in acetone-$d_6$.

C-8; H-3′ to C-1′, C-2′, C-4′, and C-5′; H-5′ to C-1′, C-3′, C-4′, C-6′, and C-7′; and $H_3$-7′ to C-1′, C-5′, and C-6′. Although the $^{13}C$ NMR resonance at $\delta_C$ 123.8 of C-1 implied that the carboxylic acid may be situated at C-1, the available NMR spectroscopic data could not conclusively establish the structure of corynether A (**5**), particularly on the positions of ether linkage and free hydroxyl groups. Therefore, corynether A (**5**) was subjected to methylation to yield a tetra-*O*-methyl derivative **6**. The NOESY correlations from 4-OMe to H-3 and H-5; from 2′-OMe to H-3′; and from 4′-OMe to H-3′ and H-5′ readily indicated the 1′/2 ether linkage in **6**. The HMBC correlations from $H_3$-8 to C-1, C-5, C-6, and C-7 ($^4J_{CH}$) and from COO$CH_3$ to C-7 and C-1 ($^4J_{CH}$) unambiguously assigned the position of ester carbonyl (C-7) in **6**, and thus establishing the carboxylic acid position in corynether A (**5**). The evidence from these spectroscopic data supported the structure of corynether A (**5**) as shown; proton and carbon signals in **5** and **6** were assigned by analysis of 2D NMR spectroscopic data (Table 2). It should be noted that analysis of NOESY and HMBC spectra of the corresponding methylated product is crucially necessary for structure elucidation of hydroxylated diaryl ether.

Compound **7** was a demethyl derivative of 2-hydroxy-4-methoxy-6-methylphenyl 3-hydroxy-5-methylphenyl ether (Cannon et al., 1972; Sargent et al., 1971), also known as LL-V125α, a fungal metabolite of the order Sphaeropsidales (McGahren et al., 1970). However, LL-V125α was incorrectly identified as 2-hydroxy-6-methoxy-4-methylphenyl 3-hydroxy-5-methylphenyl ether (McGahren et al., 1970); it was subsequently revised, by chemical synthesis, to 2-hydroxy-4-methoxy-6-methylphenyl 3-hydroxy-5-methylphenyl ether (Cannon et al., 1972; Sargent et al., 1971). Compound **7** was synthetically prepared during the synthesis of LL-V125α, unfortunately, only the 60 MHz $^1H$ NMR spectroscopic data of compound **7** was available in the literature (Cannon et al., 1972; Sargent et al., 1971). Furthermore, comparison of such NMR data was not sufficient to confirm the structural identity of compound **7**, particularly on the assignment of aromatic substituents. In order to prove the structure of **7**, the corresponding methylated product **8** was prepared and subjected to NOESY analysis. The NOESY correlations from 1-OMe to H-2 and H-6; from 3-OMe to H-2; and from 3′-OMe to H-2′ and H-4′ readily indicated 1′/4 ether linkage in **8**, and thus confirming the structure of diaryl ether **7**. Assignments of $^1H$ and $^{13}C$ NMR signals for **7** and **8** are in Table 2.

**Table 3**
Radical scavenging, antioxidant, and aromatase inhibitory activities of depsidones and diaryl ethers.

| | Radical scavenging and antioxidant activities ($IC_{50}$, μM) | | | | | Aromatase inhibition |
|---|---|---|---|---|---|---|
| | DPPH | XXO | IXO | HL-60 | ORAC (unit)[a] | ($IC_{50}$, μM) |
| 1 | >250 | ND | 19.1 | >100 | 5.9 | 5.30 |
| 3 | 22.4 | ND | 226.5 | >100 | 5.9 | >25 |
| 4 | >250 | ND | >500 | >100 | 0.1 | >25 |
| 5 | 182.4 | >500 | >500 | >100 | 4.3 | >25 |
| 7 | >250 | >500 | 421.4 | >100 | 5.8 | >25 |
| 8 | >250 | >500 | >500 | >100 | 0.0 | ND |

[a] Results were expressed as ORAC units, where one ORAC unit equals the net protection of β-phycoerythrin produced by 1 μM of Trolox.

### 2.2. Radical scavenging, antioxidant, aromatase inhibitory, and cytotoxic activities

The isolated natural substances and their methylated derivatives (**1**–**8**) were evaluated for cancer chemopreventive properties, i.e. measuring radical scavenging, antioxidant, aromatase (CYP19) inhibitory activities. Among the compounds tested, corynesidone B (**3**) showed the best activity for the scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals with an $IC_{50}$ value of 22.4 μM (Table 3), comparable to ascorbic acid ($IC_{50}$ 21.2 μM). Compounds **1**–**8** did not inhibit superoxide anion radical formation in the xanthine/xanthine oxidase (XXO) assay, while corynesidone A (**1**) inhibited xanthine oxidase (IXO) with an $IC_{50}$ value of 19.1 μM (Table 3). None of the tested compounds could suppress superoxide anion generation (at 100 μM), induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), in differentiated HL-60 human promyelocytic leukemia cells (Table 3). Interestingly, natural products, corynesidones A (**1**) and B (**3**), corynether A (**5**), and the diaryl ether **7** showed potent antioxidant activity, exhibiting oxygen radical absorbance capacity (ORAC) 4.3–5.9 units, while the methylated derivatives **4** and **8** did not showed this activity (Table 3). It is clear that hydroxyl groups in depsidones (**1** and **3**) and diaryl ethers (**5** and **7**) are required for antioxidant property. Corynesidone A (**1**) inhibited aromatase activity with an $IC_{50}$ value of 5.30 μM (Table 3), comparable to that of ketoconazole standard ($IC_{50}$ 2.4 μM). Compounds **1**–**8** exhibited only weak cytotoxic activity or were inactive (at 50 μg/mL) towards some cell lines (Table 4). However, a derivative **8** exhibited moderate cytotoxicity with $IC_{50}$ values of 1.4–9.0 μg/mL (Table 4).

In general, natural compounds, corynesidones A (**1**) and B (**3**), corynether A (**5**), and diaryl ether **7** were relatively non-toxic, but, exhibited antioxidant activities. Corynesidone A (**1**) exhibited aromatase inhibitory activity with an $IC_{50}$ value of 5.30 μM; this activity magnitude is comparable to the first generation aromatase inhibitor drug, aminoglutethimide. Both anti-aromatase and antioxidant activities of corynesidone A (**1**) are interesting functions because this dual biological activity may be useful for cancer

**Table 4**
Cytotoxic activity of compounds **1**–**8**.

| Compound | Cytotoxic activity[a] ($IC_{50}$, μg/mL); mean (±s.d.), $n$ = 3 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HeLa | HuCCA-1 | HepG2 | T47D | MDA-MB231 | S102 | A549 | HL-60 | MOLT-3 | MRC-5 |
| 1 | 22.5 (±2.2) | >50 | 17.5 (±3.5) | >50 | >50 | >50 | >50 | 14.7 (±1.7) | 12.2 (±0.06) | >50 |
| 2 | 32.7 (±2.0) | >50 | 35.5 (±3.5) | >50 | 47.6 (±2.5) | 44.0 (±5.6) | >50 | 19.3 (±1.5) | 14.7 (±0.5) | >50 |
| 3 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| 4 | 23.3 (±3.0) | 38.0 (±1.4) | 35.0 (±0.0) | 25.0 (±0.0) | >50 | 45.0 (±0.0) | 35.0 (±0.6) | 17.8 (±0.3) | 12.4 (±0.3) | >50 |
| 5 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| 6 | 29.5 (±0.7) | >50 | 23.5 (±2.1) | 41.0 (±5.5) | 31.0 (±1.4) | >50 | 46.3 (±4.7) | 17.5 (±1.9) | 9.6 (±0.2) | >50 |
| 7 | 35.0 (±3.0) | >50 | >50 | >50 | >50 | >50 | >50 | 43.6 (±1.6) | 34.6 (±1.2) | >50 |
| 8 | 2.7 (±1.1) | >50 | 4.0 (±1.4) | >50 | 9.0 (±0.0) | 25.0 (±0.0) | >50 | 2.2 (±0.1) | 1.4 (±0.04) | >50 |
| Doxorubicin | 0.12 (±0.04) | 0.40 (±0.11) | 0.23 (±0.00) | 0.04 (±0.01) | 0.28 (±0.00) | 1.40 (±0.00) | 0.39 (±0.03) | ND | ND | >50 |
| Etoposide | ND | ND | ND | ND | ND | ND | ND | 0.61 (±0.05) | 0.02 (±0.00) | ND |

[a] Cytotoxicity was tested against the following cell lines: HeLa, cervical adenocarcinoma cell line; HuCCA-1, human lung cholangiocarcinoma cancer cells; HepG2, human hepatocellular liver carcinoma cell line; T47D, human mammary adenocarcinoma cell line; MDA-MB231, human breast cell line; S102, human liver cancer cell line; A549, human lung carcinoma cell line; HL-60, human promyelocytic leukemia cell line; and MOLT-3, T-lymphoblast (acute lymphoblastic leukemia) cell line.
MRC-5 was normal embryonic lung cell. ND, not determined.

chemoprevention, particularly for breast cancer. A recent study showed that depsidones and depsides from lichens could prevent UV light and nitric oxide-mediated plasmid DNA damage and induce apoptosis in human melanoma cells, whose mechanism of action was partly involved in the inhibition of reactive oxygen species and reactive nitrogen species (Russo et al., 2008). Some fungal depsidones showed better superoxide anion scavenging activity than quercetin (Lohezic-Le Devehat et al., 2007); however, a few depsidones showed moderate antioxidant activity (Hidalgo et al., 1994). Previous reports also demonstrated that fungal depsidones exhibited cytotoxicity (Bezivin et al., 2004; Pittayakhajonwut et al., 2006) and antibacterial activity against methicillin- and multidrug-resistant *Staphylococcus aureus* (Kokubun et al., 2007). Several natural products are known to be aromatase inhibitors (Balunas et al., 2008; Paoletta et al., 2008); however, to our knowledge, aromatase inhibitory activity of depsidones has never been reported to date. This is, therefore, the first report on anti-aromatase activity of natural depsidones.

### 2.3. Concluding remarks

Three new compounds, corynesidones A (**1**) and B (**3**), and cornether A (**5**), together with a known diaryl ether **7** were isolated from the fungal endophyte *C. cassiicola* L36. Corynesidone B (**3**) could scavenge DPPH free radicals at the same activity as that of ascorbic acid. All natural products isolated (**1**, **3**, **5**, and **7**) showed potent antioxidant activity, as revealed by ORAC assay. However, compounds **1**–**8** neither inhibited superoxide anion radical formation in the XXO assay nor suppressed TPA-induced superoxide anion generation in HL-60 cell line. Corynesidone A (**1**) acted as an aromatase inhibitor, showing comparable activity to the first generation aromatase inhibitor drug, aminoglutethimide.

## 3. Experimental

### 3.1. General experimental procedures

Melting points were measured on a digital Electrothermal 9100 Melting Point Apparatus and reported without correction. UV–Vis spectra were obtained from Shimadzu UV-1700 PharmaSpec Spectrophotometer. FTIR data were obtained using a universal attenuated total reflectance (UATR) attachment on a Perkin–Elmer Spectrum One spectrometer. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker AM 400 NMR instrument (operating at 400 MHz for $^{1}H$ and 100 MHz for $^{13}C$) and a Bruker AVANCE 600 NMR spectrometer (operating at 600 MHz for $^{1}H$ and 150 MHz for $^{13}C$). ESI–TOF–MS were determined using a Bruker MicroTOF$_{LC}$ spectrometer.

### 3.2. Fungal material and identification

Apparently healthy leaves of a Thai medicinal plant, *Lindenbergia philippensis* (Cham.) Benth. [H]. (family Scrophulariaceae), were collected from Kanchanaburi Province, Thailand. The samples were cleaned under running tap $H_2O$ and air-dried. The cleaned leaves were surface-sterilized according to the previously described method (Chomcheon et al., 2006). The surface-sterilized leaves were cut into small pieces using a sterile blade and placed on sterile water agar plates for further incubation at 30 °C. The hyphal tip of the endophytic fungus growing out from the plant tissue was cut by a sterile Pasteur pipette and transferred onto a sterile potato dextrose agar (PDA) plate. After incubation at 30 °C for 7 days, culture purity was determined from colony morphology.

Endophytic fungus isolate L36 was identified based on both morphology on PDA and analysis of the DNA sequences of the ITS1-5.8S-ITS2 ribosomal RNA gene region. Total DNA was extracted from fungal mycelia grown in potato dextrose broth using FTA® Plant Kit (Whatman®, USA) according to the manufacturer's instruction. Primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990) were used to amplify the ITS1-5.8S-ITS2 region from total cellular DNA as previously described (Prachya et al., 2007). The amplified DNA was purified and directly subjected to sequencing reactions using primers ITS5 and ITS4. BLASTN 2.2.18+ (Zhang et al., 2000) was used to search for similar sequences in GenBank. DNA sequence similarity was determined by the ClustalW (Thompson et al., 1994) in BioEdit version 7.0.1 (Hall, 1999). Endophytic fungus isolate L36 grew on PDA as brown velvety colony. Conidiophores were pale brown and formed singly with successive cylindrical proliferations. Conidia (52–124 × 7–10 μm) were in chains, obclavate to subcylindrical with 4–10 pseudosepta. These correspond well with the characteristics of *C. cassiicola* (Ellis, 1971). A GenBank search for DNA sequence similarity revealed that ITS1-5.8S-ITS2 of the L36 was 100% homology to those of *C. cassiicola* reference strains (CBS1, GenBank Accession No. EU364555; ATCC64204, GenBank Accession No. AY238606). Based on microscopic morphological characteristics and DNA sequence of the ribosomal RNA gene region, this endophytic fungus is identified as *C. cassiicola* L36. The ITS1-5.8S-ITS2 DNA sequence of the L36 fungus has been submitted to GenBank with the Accession number of FJ225970. The culture of *C. cassiicola* L36 has been deposited at the MIM Laboratory, Department of Microbiology, Mahidol University, Thailand.

### 3.3. Extraction and isolation

The endophytic fungus isolate L36 was cultured in a malt extract medium (MEB) for 21 days at 25 °C (stationary condition). Fungal cells and broth (5 L) were separated by filtered, and the filtrate was extracted with equal volumes of EtOAc (3×) to obtain a crude broth extract (4.5 g). Fungal cells were macerated in MeOH (500 mL) for 2 days, followed by $CH_2Cl_2$ (500 mL) for 2 days. Mycelial crude extract was found to be, as shown by $^{1}H$ NMR spectroscopic analysis, a mixture of triglycerides and fatty acids. A crude broth extract was subjected to Sephadex LH-20 CC (3 × 90 cm), eluted with MeOH, to yield 14 fractions (A1–A14). Fractions A8–A11 were combined and further purified by Sephadex LH-20 CC (3 × 90 cm), eluted with acetone:MeOH (1:1), and twelve fractions (B1–B12) were obtained. Fractions B5 and B6 gave cornether A (**5**, 460 mg), while fractions B8, B9 and B10 contained corynesidone B (**3**, 1.2 g). Fractions A5 and A6 were combined and subjected to Sephadex LH-20 CC (3 × 48 cm), eluted with 100% MeOH, yielding eight fractions (C1–C8). Fractions C2 and C3 were combined and purified by Sephadex LH-20 CC (2 × 100.5 cm) using MeOH as a mobile phase to obtain eight fractions (D1–D8). Fraction D3 was further purified on preparative TLC eluted with hexane:$CH_2Cl_2$:acetone (2:2:1), to yield 40 mg of diaryl ether **7**. Fraction C5 was subjected to Sephadex LH-20 CC (2 × 100.5 cm), eluted with MeOH, to provide nine fractions (E1–E9). Fractions E5 and E6 were combined and separated on preparative TLC using hexane:$CH_2Cl_2$:acetone (2:2:1) as eluent, yielding 89 mg of corynesidone A (**1**).

### 3.4. Spectroscopic data of compounds

#### 3.4.1. Corynesidone A (*1*)

White solid; m.p. 235–237 °C; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 222 (4.12), 267 (3.80) nm; IR (UATR) $\nu_{max}$ 3373, 3253, 2924, 2854, 1693, 1615, 1459, 1380, 1354, 1343, 1294, 1252, 1218, 1146, 1092, 853, 845, 732 cm$^{-1}$; ESI–TOF–MS (negative ion mode): *m/z* 271.0616 $[M-H]^-$ (calcd. for $C_{15}H_{11}O_5$, 271.0607); for $^{1}H$ and $^{13}C$ NMR spectroscopic data, see Table 1.

*3.4.2. Corynesidone B (**3**)*

Pale brown solid; m.p. 212–214 °C; UV (MeOH) $\lambda_{max}$ (log ε) 223 (4.38), 304 (3.80) nm; IR (UATR) $\nu_{max}$ 3241, 2924, 2853, 1703, 1687, 1616, 1460, 1375, 1306, 1243, 1214, 1154, 1067, 846, 730 $cm^{-1}$; ESI–TOF–MS (negative ion mode): *m/z* 331.0458 $[M-H]^-$ (calcd. for $C_{16}H_{11}O_8$, 331.0454); for $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 1.

*3.4.3. Corynether A (**5**)*

Brown solid; m.p. 145–147 °C; UV (MeOH) $\lambda_{max}$ (log ε) 221 (3.87), 282 (3.18) nm; IR (UATR) $\nu_{max}$ 3275, 2926, 2860, 1691, 1606, 1460, 1380, 1318, 1267, 1206, 1153, 1051, 979, 840 $cm^{-1}$; ESI–TOF–MS (negative ion mode): *m/z* 289.0720 $[M-H]^-$ (calcd. for $C_{15}H_{13}O_6$, 289.0712); for $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 2.

*3.4.4. Diaryl ether **7***

Pale brown viscous oil; UV (MeOH) $\lambda_{max}$ (log ε) 223 (4.14), 282 (3.51) nm; IR (UATR) $\nu_{max}$ 3336, 2923, 1692, 1596, 1469, 1319, 1264, 1206, 1147, 1122, 1051, 1027, 967, 834, 800, 737, 682 $cm^{-1}$; ESI–TOF–MS (negative ion mode): *m/z* 281.0590 $[M+Cl]^-$ (calcd. for $C_{14}H_{14}Cl_1O_4$, 281.0581); for $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 2.

*3.5. Methylation of corynesidones A (**1**) and B (**3**), corynether A (**5**), and diaryl ether **7***

To a solution of corynesidone A (**1**) (9 mg) in DMF (1 mL) were added $K_2CO_3$ (20 mg) and MeI (0.3 mL), and the mixture was left stirring at room temperature for 20 h. The mixture was dried under vacuum, then dissolved in EtOAc (8 mL) and subsequently washed with $H_2O$ (5 × 8 mL), to afford a methylated derivative **2** (6.9 mg). Methylation of corynesidone B (**3**) (20 mg), corynether A (**5**) (10 mg) and diaryl ether **7** (16 mg) were performed in a similar manner as that of **1**, yielding methylated derivatives **4** (17.5 mg), **6** (8.2 mg), and **8** (13.5 mg), respectively.

*3.5.1. Methylated derivative (**2**)*

Pale brown viscous oil; ESI–TOF–MS (positive ion mode): *m/z* 301.1064 $[M+H]^+$ (calcd. for $C_{17}H_{17}O_5$, 301.1076); for $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 1.

*3.5.2. Methylated derivative (**4**)*

White solid (from $CH_2Cl_2$/MeOH, 1:1); m.p. 159–162 °C; ESI–TOF–MS (positive ion mode): *m/z* 389.1236 $[M+H]^+$ (calcd. for $C_{20}H_{21}O_8$, 389.1236); for $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 1.

*3.5.3. Methylated derivative (**6**)*

Yellow viscous oil; ESI–TOF–MS (positive ion mode): *m/z* 369.1309 $[M+Na]^+$ (calcd. for $C_{19}H_{22}Na_1O_6$, 369.1314); for $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 2.

*3.5.4. Methylated derivative (**8**)*

Pale yellow solid (from $CH_2Cl_2$/MeOH, 1:1); m.p. 94–96 °C; ESI–TOF–MS (positive ion mode): *m/z* 311.1254 $[M+Na]^+$ (calcd. for $C_{17}H_{20}Na_1O_4$, 311.1259); for $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 2.

*3.6. Bioassays*

*3.6.1. Scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals*

Scavenging DPPH free radicals was determined photometrically as described by Gerhauser et al. (2003). Ascorbic acid was used as the reference compound, exhibiting an $IC_{50}$ value of 21.2 μM.

*3.6.2. Inhibition of superoxide anion radical formation by xanthine/xanthine oxidase (XXO assay)*

The XXO assay was performed following the method essentially described by Gerhauser et al. (2003). Superoxide dismutase was used as a control. Allopurinol, the reference compound, inhibited xanthine oxidase (IXO) with the $IC_{50}$ value of 3.0 μM. Inhibition of superoxide anion radical formation was measured only when the tested compounds did not inhibit xanthine oxidase.

*3.6.3. Inhibition of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide anion radical generation in differentiated HL-60 cells (HL-60 assay)*

TPA-induced superoxide anion radical formation was detected in differentiated HL-60 human promyelocytic leukemia cells by photometric determination of cytochrome c reduction, following the method previously described by Gerhauser et al. (2003). Superoxide dismutase was used as a positive control. Only the test samples with cell viability more than 50% were considered for the calculation of scavenging potential.

*3.6.4. Measurement of oxygen radical absorbance capacity (ORAC)*

Peroxyl radical absorbance capacity of compounds was tested in a modified ORAC assay previously described by Gerhauser et al. (2003). Antioxidant potential of the test compounds (1 μM) was compared with that of 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), a water soluble vitamin E analog. Results were expressed as ORAC units, where 1 ORAC unit equals the net protection of β-phycoerythrin produced by 1 μM of Trolox. Scavenging capacities >1 ORAC unit were considered as positive.

*3.6.5. Inhibition of aromatase (CYP19)*

Aromatase inhibitory assay was performed according to the method reported by Stresser et al. (2000), using CYP19/methoxy-4-trifluoromethyl-coumarin (MFC) high throughput inhibition screening kit (BD Biosciences, Woburn MA, USA). The reference compound, ketoconazole, typically exhibits the $IC_{50}$ value of 2.4 μM.

*3.6.6. Cytotoxicity*

Cytotoxic activity for adhesive cell lines including HeLa, HuCCA-1, HepG2, T47D, MDA-MB231, S102, A549, and MRC-5 cancer cell lines was evaluated with the MTT assay (Carmichael et al., 1987; Doyle and Griffiths, 1997; Mosmann, 1983; Tominaga et al., 1999). For non-adhesive cells, HL-60 and MOLT-3 cell lines, the cytotoxicity was assessed using the XTT assay (Doyle and Griffiths, 1997). Etoposide and doxorubicin were used as the reference drugs (Table 4).

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