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# Metabolites from the endophytic mitosporic Dothideomycete sp. LRUB20

Porntep Chomcheon [a,b], Suthep Wiyakrutta [c], Nongluksna Sriubolmas [d], Nattaya Ngamrojanavanich [b,e], Chulabhorn Mahidol [a,f], Somsak Ruchirawat [a,f], Prasat Kittakoop [a,*]

[a] Chulabhorn Research Institute and Chulabhorn Graduate Institute, Vipavadee-Rangsit Highway, Bangkok 10210, Thailand
[b] Program of Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[c] Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
[d] Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand
[e] Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[f] Chulabhorn Research Centre, Institute of Science and Technology for Research and Development, Mahidol University, Thailand

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ABSTRACT

The endophytic mitosporic Dothideomycete sp. LRUB20 was found to produce pyrone derivatives, dothideopyrones A–D (**1**, **3**, **4**, and **5**), together with seven known compounds, including questin (**9**), asteric acid (**10**), methyl asterrate (**11**), sulochrin (**12**), and eugenitin (**13**), 6-hydroxymethyleugenitin (**14**), and *cis*, *trans*-muconic acid (**15**). Dothideopyrone D (**5**) and its acetate derivative **6** exhibited moderate cytotoxic activity. This is the first report on a naturally occurring muconic acid, which is commonly known as a biomarker in environments after exposure to benzene and phenol (or derivatives). Interestingly, the LRUB20 fungus could produce muconic acid in relatively high yield (47.8 mg/L). The utility of endophytic fungi in the field of white biotechnology is discussed.



## 1. Introduction

Fungal endophytes normally colonize living internal tissues of plants without causing any obvious negative effects or external symptoms. Endophytic fungi may offer either significant benefit to their host plants by producing secondary metabolites that provide protection and survival advantage to the plants. Particular secondary metabolites produced by endophytic fungi are believed to benefit the host plants as they may be plant growth regulators, antimicrobials, antivirals, and insecticidals, or even mediate resistance to some types of abiotic stress (Stone et al., 2004). 3-Nitropropionic acid (3-NPA) is a good example of an endophytic metabolite having both ecological and biological impacts. 3-NPA is involved in nitrification processes in leguminous plants (Hipkin et al., 2004); however, 3-NPA accumulated in leguminous plants may be produced by associated endophytes (Chomcheon et al., 2005). Fungal 3-NPA therefore possibly participates in the nitrification process of the nitrogen cycle. 3-NPA (previously reported as 3-hydroxypropionic acid) exhibited potent nematicidal activity (Schwarz et al., 2004), which might benefit the hosts to fight against plant parasitic nematodes. Endophytic fungi are proving to be rich sources of biologically active compounds with potential applications in agrochemical and pharmaceutical industries (Gunatilaka, 2006; Pongcharoen et al., 2008; Rukachaisirikul et al., 2008; Schulz et al., 2002; Strobel, 2006; Tan and Zou, 2001; Wiyakrutta et al., 2004).

We recently isolated the endophytic fungus LRUB20 from a Thai medicinal plant, *Leea rubra* Blume ex Spreng (family Leeaceae). Based upon analysis of the DNA sequences of the 18S and ITS region of the ribosomal RNA gene, the fungus LRUB20 is potentially a new species (see Section 3). Interestingly, we have found that this fungus can produce a large amount (gram scale) of 2-hydroxymethyl-3-methyl-cyclopent-2-enone, useful scaffold for organic synthesis (Chomcheon et al., 2006). The term "white biotechnology" has been recently introduced for microbial production of building blocks, and various 2-oxocarboxylic acids and isoprenoids are examples of chemical scaffolds from microbial fermentation (Maury et al., 2005; Stottmeister et al., 2005). Moreover, the use of microbial metabolites in organic syntheses provides fundamental advantages in comparison to chemical methods. Because the LRUB20 fungus is potentially a new species, we have extended our study on secondary metabolites by varying the culture media. It was found that this fungus could produce various types of

* Corresponding author. Tel.: +66 86 9755777; fax: +66 2 5740622x1513.
*E-mail address:* prasatkittakoop@yahoo.com (P. Kittakoop).

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metabolites, and might be useful for the production of building blocks for organic syntheses. Twelve secondary metabolites were isolated from the fungus LRUB20, four of which (compounds **1**, **3**, **4**, and **5**) were new. In addition, *cis*, *trans*-muconic acid (**15**) was found for the first time as a natural product. This paper reports the isolation, characterization, and biological activity of the isolated compounds from the endophytic fungus LRUB20 (Figs. 1 and 2).

## 2. Results and discussion

### 2.1. Structural determination

A crude extract of the endophytic mitosporic Dothideomycete LRUB20 cultured in Czapek yeast autolysate medium was separated by Sephadex LH-20 and silica gel column chromatography, preparative TLC, and semi-preparative $C_{18}$ reversed-phase HPLC, furnishing four new pyrone derivatives, dothideopyrones A–D (**1**, **3**, **4**, and **5**), together with the known fungal metabolites questin (**9**), asterric acid (**10**), methyl asterrate (**11**), sulochrin (**12**), and eugenitin (**13**). However, the LRUB20 fungus cultured in the M1D medium (Strobel et al., 1996) supplemented with malt extract produced two known compounds, 6-hydroxymethyleugenitin (**14**) and *cis*, *trans*-muconic acid (**15**). Spectroscopic data of known compounds **9**–**15** were identical to those reported in the literature (Barba et al., 1992; Elvidge et al., 1950; Feng et al., 2002; Hargreaves et al., 2002; Huneck, 1972; Kalidhar, 1989; Shimada et al., 2003; Stermitz et al., 1973; Wijeratne et al., 2006).

Dothideopyrone A (**1**) was isolated as colorless oil, and APCI–TOF MS suggested the molecular formula $C_{14}H_{22}O_5$. The IR spectrum of **1** showed absorption bands for hydroxyl group (3373 $cm^{-1}$) and a conjugated carbonyl (1682 $cm^{-1}$). The UV spectrum exhibited a maximum absorption at 299 nm, characteristic of a pyrone. The $^1H$ NMR spectrum of **1** showed signals for an isolated $sp^2$ methine ($\delta_H$ 6.44), an oxygenated $sp^3$ methine ($\delta_H$ 4.40), a methoxy group ($\delta_H$ 3.92), an oxygenated methylene ($\delta_H$ 4.48), and a hexyl moiety ($\delta_H$ 0.86–1.82). The $^{13}C$ NMR spectrum of **1** contained 14 lines, and the DEPT data established the presence of two methyls, six methylenes, two methines, and four non-protonated carbons. The $^1H$–$^1H$ COSY spectrum of dothideopyrone A (**1**) assisted the assembling of a partial structure from C-1′ to C-7′. The HMBC spectrum showed correlations from H-5 to C-1′, C-4, and C-6; from the 4-OMe protons to C-4; from H-1′ to C-5 and C-6; and from $H_2$-1″ to C-2, C-3 and C-4. These correlations established the positions of the substituents on the pyrone ring. The side chain in **1**, 1-heptanol, was linked to the pyrone unit at C-6 as evident from the HMBC correlations of H-1′ and $H_2$-2′ to C-6. Acetylation of dothideopyrone A (**1**) yielded a diacetate derivative **2**, confirm-

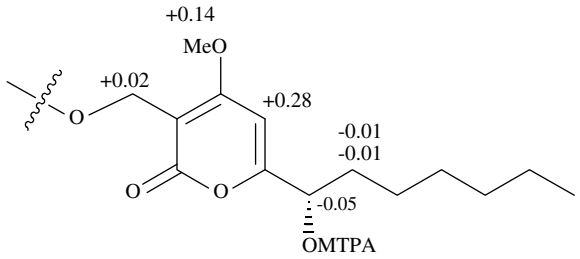


**Fig. 2.** $\Delta\delta$ Values [$\delta_{(S)}$–$\delta_{(R)}$] for the MTPA esters **7** and **8**.

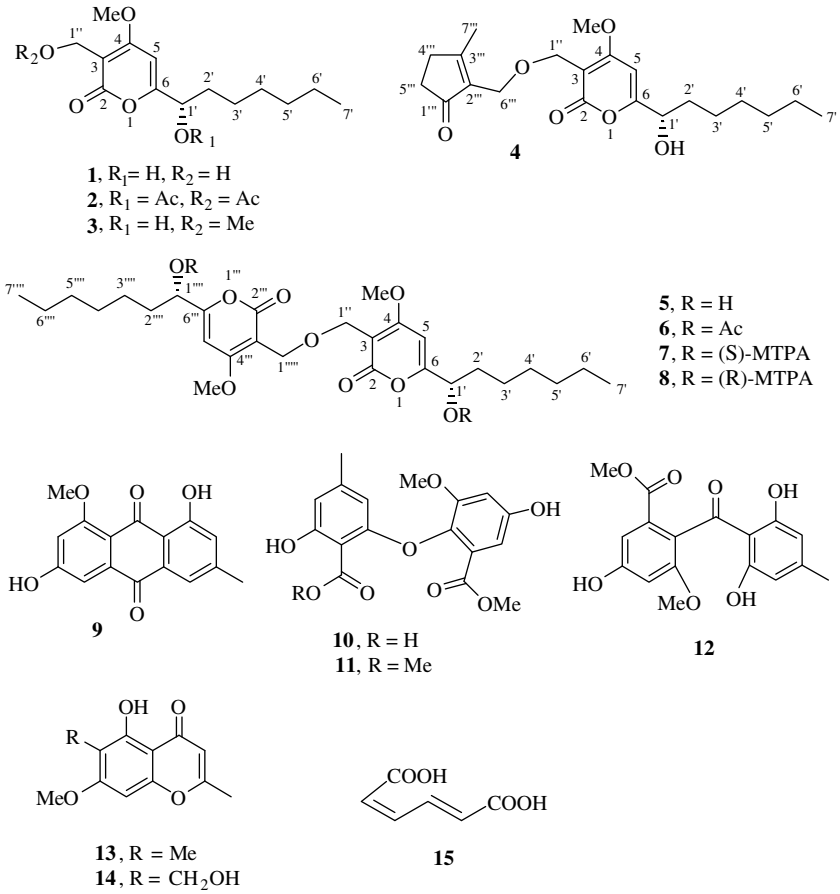


**Fig. 1.** Structures of the isolated compounds and their derivatives.

ing the presence of two hydroxyl groups in **1**. On the basis of these data, the gross structure of dothideopyrone A (**1**) was established. Assignments of all $^{1}H$ and $^{13}C$ NMR spectroscopic signals for **1** were made by analysis of $^{1}H$–$^{1}H$ COSY and HMBC spectra (Table 1).

Dothideopyrone B (**3**) possessed the molecular formula $C_{15}H_{24}O_5$, as deduced from APCI–TOF MS data. The $^{1}H$ and $^{13}C$ NMR spectroscopic data of **3** were similar to those of **1**, except for the presence of additional methoxy signals in **3**. In the HMBC spectrum, 1″-OMe protons ($\delta_H$ 3.33) showed correlation to C-1″, indicating that **3** was a 4-*O*-methyl derivative of **1**. Protons and carbons in **3** were assigned by analysis of $^{1}H$–$^{1}H$ COSY and HMBC spectra (Table 1).

Dothideopyrone C (**4**) has the molecular formula $C_{21}H_{30}O_6$, as indicated by APCI–TOF MS data. The $^{1}H$ and $^{13}C$ NMR spectroscopic data of **4** were again similar to those of **1**, suggesting that **4** was a derivative of **1**. Analysis of $^{1}H$ and $^{13}C$ NMR spectra of **4** indicated additional signal characteristic of a 2-hydroxymethyl-3-methyl-cyclopent-2-enone unit (Chomcheon et al., 2006). The HMBC spectrum of **4** showed correlations of $H_2$-1″ to C-6‴, and $H_2$-6‴ to C-1″, indicating the presence of C-1″/C-6″ ether linkage between the 2-hydroxymethyl-3-methyl-cyclopent-2-enone unit and a dothideopyrone A (**1**) unit. The $^{1}H$–$^{1}H$ COSY and HMBC spectra assisted the assignment of all proton and carbon signals for **4** (Table 2).

Dothideopyrone D (**5**) possessed $^{1}H$ and $^{13}C$ NMR spectra similar to those of **1**. The $^{13}C$ NMR spectrum of **5** showed 14 lines. However, the APCI–TOF MS data indicated the molecular formula $C_{28}H_{42}O_9$, indicating that **5** had C2 symmetry. The HMBC spectrum readily established the ether linkage of the two identical units at the hydroxyl methylene, showing correlations from $H_2$-1″ to C-1⁗ and from $H_2$-1⁗ to C-1″. Upon acetylation of **5**, a diacetate derivative **6** was obtained; only the hydroxyl groups at C-1′ and C-1⁗ were acetylated, which further confirmed the structure of **5**. Proton and carbon signals for **5** were assigned by analysis of $^{1}H$–$^{1}H$ COSY and HMBC spectra (Table 2). Pyrone dimers are very rare in nature. To our knowledge, multiforisin D is the only natural bis-pyrone dimer, previously isolated from the fungus *Gelasinospora multiforis* (Fujimoto et al., 1995).

Dothideopyrones A–D (**1**, **3**, **4**, and **5**) all process a chiral secondary alcohol unit, and all exhibited similar negative specific rotations. The absolute configuration of dothideopyrones A–D was assigned by application of the modified Mosher's method (Dale and Mosher, 1973), and dothideopyrone D (**5**) was selected as a model compound. (*S*)-MTPA (**7**) and (*R*)-MTPA (**8**) esters of **5** were prepared. The $\Delta\delta$ values [$\delta_{(S)}$–$\delta_{(R)}$] of the MTPA esters indicated the *S*-configuration at C-1′ (or C-1⁗) in **5** (Fig. 2). Based on the similar negative specific rotations, the absolute configuration of C-1′ in dothideopyrones A–D (**1**, **3**, **4**, and **5**) was also assumed to be *S*.

**Table 1**
$^{1}H$ and $^{13}C$ NMR spectroscopic data ($CDCl_3$) for compounds **1** and **3**.

| Position | **1** | | **3** | |
|---|---|---|---|---|
| | $\delta_H$ (*J* in Hz) | $\delta_C$ | $\delta_H$ (*J* in Hz) | $\delta_C$ |
| 2 | – | 165.2 | – | 164.6 |
| 3 | – | 103.6 | – | 101.3 |
| 4 | – | 167.7 | – | 169.1 |
| 5 | 6.44 (s) | 92.8 | 6.34 (s) | 92.4 |
| 6 | – | 169.1 | – | 169.2 |
| 1′ | 4.40 (dd, 4.0, 7.9) | 70.5 | 4.36 (dd, 4.1, 8.1) | 70.8 |
| 2′ | 1.82 (m); 1.64 (m) | 35.3 | 1.79 (m); 1.58 (m) | 35.5 |
| 3′ | 1.40 (m) | 24.9 | 1.35 (m) | 25.0 |
| 4′ | 1.25 (m) | 28.9 | 1.21 (m) | 28.9 |
| 5′ | 1.25 (m) | 31.6 | 1.21 (m) | 31.6 |
| 6′ | 1.25 (m) | 22.5 | 1.21 (m) | 22.5 |
| 7′ | 0.86 (t, 6.7) | 13.9 | 0.81 (t, 6.7) | 14.0 |
| 1″ | 4.48 (s) | 54.3 | 4.25 (s) | 63.2 |
| 4-OMe | 3.92 (s) | 56.7 | 3.87 (s) | 56.8 |
| 1″-OMe | – | – | 3.33 (s) | 58.4 |

**Table 2**
$^{1}H$ and $^{13}C$ NMR spectroscopic data ($CDCl_3$) for compounds **4** and **5**.

| Position | **4** | | **5**[a] | |
|---|---|---|---|---|
| | $\delta_H$ (*J* in Hz) | $\delta_C$ | $\delta_H$ (*J* in Hz) | $\delta_C$ |
| 2 | – | 164.6 | – | 164.9 |
| 3 | – | 101.3 | – | 101.3 |
| 4 | – | 169.2 | – | 169.4 |
| 5 | 6.40 (s) | 92.6 | 6.35 (s) | 92.9 |
| 6 | – | 169.2 | – | 169.4 |
| 1′ | 4.41 (dd, 4.0, 8.1) | 70.8 | 4.32 (dd, 4.0, 8.1) | 70.6 |
| 2′ | 1.85 (m); 1.64 (m) | 35.5 | 1.74 (m); 1.57 (m) | 35.3 |
| 3′ | 1.40 (m) | 25.1 | 1.33 (m) | 25.1 |
| 4′ | 1.28 (m) | 29.0 | 1.20 (m) | 29.0 |
| 5′ | 1.28 (m) | 32.0 | 1.20 (m) | 31.7 |
| 6′ | 1.28 (m) | 22.6 | 1.20 (m) | 22.5 |
| 7′ | 0.88 (t, 6.6) | 14.1 | 0.80 (t, 6.7) | 14.0 |
| 1″ | 4.37 (s) | 61.6 | 4.32 (s) | 61.3 |
| 4-OMe | 3.92 (s) | 56.8 | 3.85 (s) | 56.9 |
| 1‴ | – | 208.6 | – | – |
| 2‴ | – | 136.9 | – | – |
| 3‴ | – | 177.5 | – | – |
| 4‴ | 2.53–2.55 (m) | 31.7 | – | – |
| 5‴ | 2.38–2.40 (m) | 34.5 | – | – |
| 6‴ | 4.20 (s) | 60.9 | – | – |
| 7‴ | 2.20 (s) | 17.7 | – | – |

[a] Compound **5** has C2 symmetry, so NMR data for the two halves are identical.

### *2.2. Biological activity and prospects on fungal metabolites in white biotechnology*

The isolated compounds and derivatives were evaluated for cytotoxicity toward nine cancer cell lines (Table 3). Compounds **1**–**4** and **9**–**15** were either inactive (at 50 μg/mL) or showed only weak activity (14.8–45.0 μg/mL) towards some cell lines (Table 3). Dothideopyrone D (**5**) and its acetate derivative **6** exhibited moderate cytotoxic activity (8.6–25.0 μg/mL). Previous reports have demonstrated that many pyrones are not cytotoxic (Zhan et al., 2007). However, members of this class show phytotoxic activity (Pedras and Chumala, 2005), inhibitory effects on NF-kappaB (Folmer et al., 2008), and interactions with the GABA-A receptor (Boonen and Haberlein, 1998). Questin (**9**) was recently found to be an inhibitor of Cdc25B phosphatase (Choi et al., 2007).

Interestingly, the LRUB20 fungus cultured in M1D medium (supplemented with 0.5 g/L malt extract) produced large quantities of *cis*, *trans*-muconic acid (**15**), up to 47.8 mg/L. Generally, *cis*, *cis*-muconic acid and *trans*, *trans*-muconic acid are known metabolites from the metabolism of benzene and phenol (or derivatives) in living organisms, including humans (Tsai et al., 2005; Xiao et al., 2007; Yoshikawa et al., 1993), and thus are employed as biomarkers for benzene contamination in living organisms (Melikian et al., 2002; Navasumrit et al., 2008, 2005; Yardley-Jones et al., 1991). To date, only a monomethyl ester of *cis*, *cis*-muconic acid isolated from a marine sponge *Plakortis simplex* has been reported as a natural product (Shen et al., 2001). To our knowledge, the present work is the first report on naturally occurring muconic acid. Interestingly, a prior report showed that genetically engineered *Escherichia coli* can catalytically convert D-glucose to *cis*, *cis*-muconic acid (Draths and Frost, 1994). Consequently, such muconic acid production initiated an environmentally compatible synthesis of adipic acid, an important ingredient for the production of nylon 6.6, gelatin, jams, polyamides, polyurethanes, and lubricants (Niu et al., 2002; Thomas et al., 2003). The current industrial production of adipic acid involves nitric acid oxidation of intermediates, generating $N_2O$ as a byproduct, which contributes to both ozone depletion and global warming. Overproduction of *cis*, *trans*-muconic acid (**15**) through a genetic manipulation of the LRUB20 fungus, by analogy to that for the genetically engineered *E. coli* (Draths and Frost, 1994), is a challenging research approach

**Table 3**
Cytotoxic activity of compounds **1–6** and **9–15** against nine cancer cell lines.

| Cell line[a] | Cytotoxic activity ($IC_{50}$, μg/mL); mean (±s.d.), $n = 3$ | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | Etoposide[c]/ Doxorubicin[d] |
| HeLa | >50 | >50 | >50 | ND[b] | 23.0 (±2.1) | 23.0 (±0.7) | >50 | >50 | >50 | >50 | 18.0 (±4.2) | >50 | >50 | 0.04 (±0.01)[d] |
| HuCCA-1 | >50 | >50 | >50 | ND | 19.0 (±8.4) | 22.0 (±0.7) | >50 | >50 | >50 | >50 | >50 | >50 | >50 | 0.42 (±0.11)[d] |
| HepG2 | >50 | >50 | >50 | ND | 21.0 (±2.6) | 19.5 (±0.7) | 44.5 (±6.3) | >50 | 45.0 (±4.3) | >50 | 20.8 (±8.4) | >50 | >50 | 0.18 (±0.00)[d] |
| T47D | >50 | >50 | >50 | ND | 21.0 (±1.4) | 20.0 (±1.4) | >50 | >50 | >50 | >50 | 44.0 (±1.4) | >50 | >50 | 0.04 (±0.01)[d] |
| MDA-MB231 | >50 | >50 | >50 | ND | 20.0 (±0.0) | 18.0 (±2.8) | >50 | >50 | >50 | 35.0 (±0.0) | >50 | >50 | >50 | 0.20 (±0.00)[d] |
| S102 | >50 | >50 | >50 | ND | 24.0 (±1.4) | 22.5 (±4.9) | >50 | >50 | >50 | >50 | >50 | >50 | >50 | 1.20 (±0.00)[d] |
| A549 | >50 | >50 | >50 | ND | 25.0 (±1.5) | 19.0 (±1.4) | >50 | >50 | >50 | >50 | >50 | >50 | >50 | 0.31 (±0.01)[d] |
| HL-60 | >50 | 41.2 (±3.4) | >50 | ND | 16.0 (±1.1) | 18.0 (±0.7) | 23.4 (±3.3) | >50 | 38.9 (±4.3) | 32.9 (±4.4) | >50 | >50 | >50 | 0.82 (±0.04)[c] |
| MOLT-3 | >50 | 18.9 (±0.3) | 26.3 (±0.9) | ND | 13.8 (±0.9) | 8.6 (±0.4) | 11.8 (±0.3) | >50 | 30.2 (±1.3) | 16.4 (±0.2) | 14.8 (±1.1) | 31.5 (±1.0) | >50 | 0.02 (±0.00)[c] |

[a] Cell lines were: HeLa = human cervical adenocarcinoma cell line; HuCCA-1 = human lung cholangiocarcinoma cancer cells; HepG2 = human hepatocellular liver carcinoma cell line; T47D = hormone-dependent breast cancer cell line; MDA-MB231 = hormone-independent breast cancer cell line; S102 = human liver cancer cell line; A549 = human lung carcinoma cell line; HL-60 = human promyelocytic leukemia cell line; MOLT-3 = T-lymphoblast (acute lymphoblastic leukemia) cell line.
[b] ND = Not determined.
[c] Etoposide and doxorubicin were standard drugs.
[d] Etoposide and doxorubicin were standard drugs.

to be pursued. Based upon this investigation, endophytic fungi could prove to be potential sources of industrial chemicals, and may therefore offer potential utility in the field of white biotechnology.

### 2.3. Concluding remarks

Four new pyrone derivatives, dothideopyrones A–D (**1**, **3**, **4**, and **5**), and seven known compounds, questin (**9**), asterric acid (**10**), methyl asterrate (**11**), sulochrin (**12**), and eugenitin (**13**), 6-hydroxymethyleugenitin (**14**), and *cis*, *trans*-muconic acid (**15**) were isolated from the endophytic mitosporic Dothideomycete sp. LRUB20. This is the first report on a naturally occurring muconic acid, a biomarker in environments after exposure to benzene and phenol. Although the cytotoxic properties of the isolated metabolites are not promising, the LRUB20 fungus can produce muconic acid (**15**) (47.8 mg/L). The latter is an important reference compound in environmental studies. The fungus LRUB20 could also produce a gram scale of 2-hydroxymethyl-3-methyl-cyclopent-2-enone, a useful scaffold for organic synthesis (Chomcheon et al., 2006). The LRUB20 fungus is a potential fungal candidate for the use in the field of white biotechnology.

## 3. Experimental

### 3.1. General experimental procedures

$^1$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). FTIR data were obtained using a universal attenuated total reflectance (UATR) attachment on a Perkin–Elmer Spectrum One spectrometer. APCI–TOF MS were determined using a Bruker MicroTOF$_{LC}$ spectrometer. Optical rotations were measured with sodium D line (590 nm) on JASCO DIP-370 digital polarimeter.

### 3.2. Fungal material

The LRUB20 fungus used in this study was isolated as an endophyte from a Thai medicinal plant, *Leea rubra* Blume ex Spreng. (family Leeaceae) collected from a forest area in Pitsanulok Province, Thailand. DNA sequence analysis of the 18*S* ribosomal RNA gene demonstrated that this fungus was closely related (96% homology) to *Acrospermum compressum* UME 31704 and *A. gramineum* UME 31190 which are currently classified in the Dothideomycetes incertae sedis, subphylum Pezizomycotina (Eriksson, 2000; Lumbsch and Huhndorf, 2007). However, microscopic morphology of the LRUB20 differs from that of *Dactylaria* and *Virgariella*, the known anamorphs of *A. compressum* and *A. gramineum*, respectively (Tubaki, 1958; Webster, 1956). Additionally, the ITS1–5.8S–ITS2 DNA sequence was found to be unique, with the highest homology of only 78% to *Mycoleptodiscus terrestris*, a member of the mitosporic Magnaporthaceae, Sordariomycetes incertae sedis, subphylum Pezizomycotina. Accordingly, the LRUB20 fungus may represent a new species and it was characterized as mitosporic Dothideomycete LRUB20 (Chomcheon et al., 2006). The 18S rRNA gene sequence and ITS sequence of LRUB20 have been submitted to GenBank with accession number DQ381536 and DQ384608, respectively.

### 3.3. Extraction and isolation

#### 3.3.1. Metabolites obtained from Czapek yeast autolysate medium

The fungus LRUB20 was cultured in Czapek yeast autolysate medium (5 L) for 21 days at 25 °C. Fungal cells and broth were

separated by filtration, and the filtrate was extracted with an equal volume of EtOAc three times to obtain a crude broth extract (1.4 g). Fungal cells were extracted sequentially with MeOH and $CH_2Cl_2$, yielding a crude extract (900 mg). The extract was subjected to Sephadex LH-20 column chromatography (CC) (3 × 85 cm), eluted with MeOH, to yield 14 fractions (A1–A14). Fraction A9 was re-crystallized from MeOH to afford asterric acid (**10**; 147 mg). Fraction A6 was further purified by Sephadex LH-20 CC (3 × 48 cm), eluted with MeOH, and ten fractions (B1–B10) were obtained. Fraction B6 was again subjected to chromatography purification on Sephadex LH-20 CC (2.5 × 52 cm) using MeOH as a mobile phase to provide fourteen fractions ($B_6$1–$B_6$14). Fraction $B_6$6 was subjected to semi-preparative HPLC ($C_{18}$ reversed-phase column; MeCN:$H_2O$ (1:1, v/v) as eluent; flow rate of 8.0 mL/min) to furnish dothideopyrone C (**4**; 28.8 mg; $t_R$ 8 min). Fraction B4 and B5 were combined and subjected to further CC over silica gel (2.5 × 40 cm), eluting with a mixture of EtOAc/$CH_2Cl_2$ (2:8, v/v) to yield ten fractions (C1–C9). Fraction C4 contained dothideopyrone A (**1**) (126 mg), while fraction C6 gave dothideopyrone B (**3**) (12 mg). Fraction C8 was further purified by preparative TLC using hexane:acetone (2:1, v/v) as eluent to furnish dothideopyrone D (**5**) (48 mg). Fraction A11 was re-crystallized from MeOH, and subjected to preparative TLC eluted with a hexane:$CH_2Cl_2$:acetone (1:1:1, v/v), yielding sulochrin (**12**) (10 mg). Fraction A13 was purified by preparative TLC, using acetone:hexane (1:1, v/v) as eluent, to yield questin (**9**) (25 mg). Crude cell extract was separated by Sephadex LH-20 CC (3 × 85 cm), eluted with MeOH, yielding twelve fractions (D1–D12). Fractions D7 and D8 were combined and subjected to further CC over silica gel (2 × 30 cm), eluting with EtOAc/hexane (2:3, v/v), to yield eight fractions (E1–E8). Fraction E3 gave methyl asterrate (**11**) (98 mg). Fraction D4 was subjected to Sephadex LH-20 CC (2 × 100.5 cm) to give nine fractions (F1–F9). Fraction F7 was separated on a preparative TLC using hexane:acetone (7:3, v/v) as eluent, yielding eugenitin (**13**) (12 mg).

### 3.3.2. Metabolites obtained from M1D medium (supplemented with malt extract)

The fungus LRUB20 was grown in M1D medium (supplemented with 0.5 g/L malt extract; 3.2 L) for 21 days at 25 °C. Mycelia were separated from the culture broth by filtration, and the broth was subsequently extracted three times with an equal volume of EtOAc, yielding a crude extract (630 mg). The extract was purified by MPLC ($C_{18}$ reversed-phase column, 3.6 × 46 cm), with a stepwise elution starting with a mixture of MeOH/$H_2O$ (1:1, v/v) to MeOH, to afford *cis*, *trans*-muconic acid (**15**; 153 mg, $t_R$ 22 min) and 6-hydroxymethyleugenitin (**14**; 72.3 mg, $t_R$ 66 min).

## 3.4. Spectroscopic data of compounds

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

Colorless oil; $[\alpha]_D^{25}$ − 77 (*c* 0.22, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 248 (3.2), 299 (4.1) nm; IR $\upsilon_{max}$ 3373, 2926, 1682, 1600, 1466, 1392, 1252, 1138, 999, 795 $cm^{-1}$; ESI–TOF–MS: *m*/*z* 271.1540 $[M + H]^+$ (calcd. for $C_{14}H_{23}O_5$, 271.1546); for $^1H$ and $^{13}C$ NMR spectroscopic data (see Table 1).

### 3.4.2. Dothideopyrone B (*3*)

Yellow viscous oil; $[\alpha]_D^{25}$ − 45 (*c* 0.15, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 233 (3.0), 299 (3.7) nm; IR $\upsilon_{max}$ 3400, 2925, 1687, 1561, 1466, 1395, 1230, 1083, 800 $cm^{-1}$; ESI–TOF–MS: *m*/*z* 307.1516 $[M + Na]^+$ (calcd. for $C_{15}H_{24}NaO_5$, 307.1521); for $^1H$ and $^{13}C$ NMR spectroscopic data (see Table 1).

### 3.4.3. Dothideopyrone C (*4*)

Yellow viscous oil; $[\alpha]_D^{25}$ − 64 (*c* 0.10, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 207 (4.4), 228 (4.0), 299 (3.9) nm; IR $\upsilon_{max}$ 3403, 2927, 1694, 1643, 1563, 1466, 1390, 1269, 1227, 1049, 823 $cm^{-1}$; ESI–TOF–MS: *m*/*z* 401.1936 $[M + Na]^+$ (calcd. for $C_{15}H_{24}NaO_5$, 401.1940); for $^1H$ and $^{13}C$ NMR spectroscopic data (see Table 2).

### 3.4.4. Dothideopyrone D (*5*)

White amorphous solid; $[\alpha]_D^{25}$ − 72 (*c* 0.22, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 248 (3.0), 301 (5.0) nm; IR $\upsilon_{max}$ 3398, 2927, 1687, 1640, 1561, 1466, 1393, 1270, 1229, 1033, 823 $cm^{-1}$; ESI–TOF–MS: *m*/*z* 545.2715 $[M+Na]^+$ (calcd. for $C_{28}H_{42}NaO_9$, 545.2727); for $^1H$ and $^{13}C$ NMR spectroscopic data (see Table 2).

## 3.5. Acetylation of dothideopyrone A (*1*) and dothideopyrone D (*5*)

A solution containing dothideopyrone A (**1**; 22 mg), pyridine (1 mL), and $Ac_2O$ (3 mL) was stirred at room temperature for 2 h. $H_2O$ (6 mL) was added to the reaction mixture, which was subsequently extracted with $CHCl_3$. The organic layer was evaporated to give an oily mixture (33 mg), which was purified by preparative TLC eluted with a mixture of $CH_2Cl_2$:EtOAc (9:1), to afford the acetate **2** (17.8 mg). Acetylation of dothideopyrone D (**5**; 19.6 mg) was performed in a similar manner, yielding acetate derivative **6** (9.1 mg).

Compound **2**: colorless oil; $^1H$ NMR ($CDCl_3$, 400 MHz) $\delta$ 6.16 (1H, s, H-5), 5.37 (H, t, *J* = 6.7, H-1′), 4.90 (2H, s, H-1″), 3.88 (3H, s, 4-OMe), 2.07 (3H, s, 1′-$CO_2$Me), 1.99 (3H, s, 1″-$CO_2$Me), 1.83 (2H, m, H-2′), 1.21 (8H, m, H-3′, H-4′, H-5′ and H-6′), 0.81 (3H, t, *J* = 6.7, H-7′); $^{13}C$ NMR ($CDCl_3$, 100 MHz) $\delta$ 171.0 (C, 1″-$CO_2$Me), 169.9 (C, 1′-$CO_2$Me), 168.4 (C, C-6), 163.8 (C, C-4), 162.7 (C, C-2), 100.5 (C, C-3), 94.4 (CH, C-5), 72.3 (CH, C-1′), 56.8 ($CH_3$, 4-OMe or 4‴-OMe), 56.1 ($CH_2$, C-1″), 32.1 ($CH_2$, C-2′), 31.5 ($CH_2$, C-5′), 28.7 ($CH_2$, C-4′), 24.9 ($CH_2$, C-3′), 22.5 ($CH_2$, C-6′), 20.9 ($CH_3$, 1″-$CO_2$Me), 20.8 ($CH_3$, 1′-$CO_2$Me), 13.9 ($CH_3$, C-7′); ESI–TOF–MS: *m*/*z* 389.1382 $[M + Cl]^-$ (calcd. for $C_{18}H_{26}ClO_7$, 389.1367).

Compound **6**: colorless oil; $^1H$ NMR ($CDCl_3$, 400 MHz) $\delta$ 6.20 (2H, s, H-5 or H-5‴), 5.42 (2H, t, *J* = 6.7, H-1′ or H-1‴), 4.44 (4H, s, H-1″ or H-1⁗), 3.92 (6H, s, 4-OMe or 4‴-OMe), 2.12 (6H, s, 1′-$CO_2$Me or 1‴-$CO_2$Me), 1.87 (4H, m, H-2′ or H-2‴), 1.28 (16H, m, H-3′ or H-3‴, H-4′ or H-4‴, H-5′ or H-5‴, and H-6′ or H-6‴), 0.88 (6H, t, *J* = 6.7, H-7′ or H-7‴); $^{13}C$ NMR ($CDCl_3$, 100 MHz) $\delta$ 169.9 (C, 1′-$CO_2$Me or 1‴-$CO_2$Me), 168.3 (C, C-6 or C-6‴), 163.9 (C, C-4 or C-4‴), 162.8 (C, C-2 or C-2‴), 102.9 (C, C-3 or C-3‴), 94.9 (CH, C-5 or C-5‴), 72.3 (CH, C-1′ or C-1‴), 61.5 ($CH_2$, C-1″ or C-1⁗), 56.7 ($CH_3$, 4-OMe or 4‴-OMe), 32.1 ($CH_2$, C-2′ or C-2‴), 31.5 ($CH_2$, C-5′ or C-5‴), 28.8 ($CH_2$, C-4′ or C-4‴), 24.9 ($CH_2$, C-3′ or C-3‴), 22.5 ($CH_2$, C-6′ or C-6‴), 20.8 ($CH_3$, 1′-$CO_2$Me or 1‴-$CO_2$Me), 13.9 ($CH_3$, C-7′ or C-7‴); ESI–TOF–MS: *m*/*z* 641.2723 $[M + Cl]^-$ (calcd. for $C_{32}H_{46}ClO_{11}$, 641.2729).

## 3.6. Preparation of (R)- and (S)-MTPA esters of *5*

The reaction mixture containing dothideopyrone D (**5**; 3 mg), (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA; 13.9 mg), *N*-[3-(dimethyllaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (12.6 mg), and a catalytic amount of *N*,*N*-(dimethylamino)pyridine was dissolved in 4 mL of $(CH_2)_2Cl_2$, and heated until reflux began, this being maintained for 4 h. The reaction mixture was added to $H_2O$ (5 mL) and extracted with $CHCl_3$ (5 mL). The mixture was purified by preparative TLC, using EtOAc–$CH_2Cl_2$ (1:4, v/v) as an eluent, to give the (*R*)-MTPA ester (4.5 mg). The (*S*)-MTPA ester was prepared in the same manner; dothideopyrone D (**5**) was reacted with (*S*)-(−)-α-methoxy-α-(trifluoromethyl)-phenylacetic acid (MTPA), to give the (*S*)-MTPA ester (5.0 mg). (*S*)-MTPA ester (Data for half of a molecule); $^1H$ NMR ($CDCl_3$, 400 MHz) $\delta$ 7.53 (2H, m, aromatic signals of MTPA), 7.43 (3H, m, aromatic signals of MTPA), 6.23 (1H, s, H-5), 5.67 (1H, t, *J* = 6.4, H-1′), 4.54 (2H, s, H-1″), 3.83 (3H, s, 4-OMe), 3.52 (3H, br d, OMe

of MTPA), 1.94 (1H, m, H-2′a), 1.60 (1H, m, H-2′b), 1.25 (4H, m, overlapping signals of H-3′ and H-4′), 1.23 (2H, m, H-4′), 1.23 (2H, m, H-5′), 1.23 (2H, m, H-6′), 0.86 (3H, t, $J$ = 6.9, H-7′); ESI–TOF–MS: $m/z$ 955.3620 $[M + H]^+$(calcd. for $C_{48}H_{57}F_6O_{13}$, 955.3703). (*R*)-MTPA ester (Data for half of a molecule); $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 7.51 (2H, m, aromatic signals of MTPA), 7.44 (3H, m, aromatic signals of MTPA), 5.95 (1H, s, H-5), 5.72 (1H, t, $J$ = 6.1, H-1′), 4.52 (2H, s, H-1″), 3.69 (3H, s, 4-OMe), 3.58 (3H, br d, OMe of MTPA), 1.95 (1H, m, H-2′a), 1.60 (1H, m, H-2′b), 1.29 (4H, m, overlapping signals of H-3′ and H-4′), 1.25 (2H, m, H-4′), 1.25 (2H, m, H-5′), 1.25 (2H, m, H-6′), 0.88 (3H, t, $J$ = 6.7, H-7′); ESI–TOF–MS: $m/z$ 955.3610 $[M + H]^+$ (calcd. for $C_{48}H_{57}F_6O_{13}$, 955.3703).

### 3.7. Assay of cytotoxic activity

The MTT assay (Carmichael et al., 1987; Doyle and Griffiths, 1997; Mosmann, 1983; Tominaga et al., 1999) was applied for the evaluation of cytotoxicity against HeLa, HuCCA-1, HepG2, T47D, MDA–MB231, S102, and A549 cancer cell lines. Cells were plated in a 96-well microplate (Costar No. 3599, USA, 100 μL/well at a density of $5 \times 10^3$–$2 \times 10^4$ cells/well), and incubated for 24 h at 37 °C under 5% $CO_2$ and 95% humidity. The tested compounds at various concentrations were added to cell lines, which were incubated further for 48 h. Cell viability was determined by staining with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The MTT stock solution (5 mg/mL) was prepared in phosphate buffered saline (PBS), which was diluted (1:10) with a culture medium prior to use. After removing the culture medium, the diluted MTT solution (50 μL) was added to the adhesive cells, and plates were incubated at 37 °C under 5% $CO_2$ and 95% humidity for 2–4 h. Subsequently, DMSO (100 μL) was added to dissolve the resulting formazan by sonication. The plates were read on a microplate reader (Molecular Devices, CA, USA), using a test wavelength of 550 nm and a reference wavelength of 650 nm.

The cytotoxic activity against non-adhesive cells, HL-60 and MOLT-3 cell lines, was evaluated using the XTT assay (Doyle and Griffiths, 1997). Cells were plated in a 96-well microplate as mentioned above, and the tested compounds at various concentrations were added to cell lines, which were incubated further for 48 h. A dye solution was prepared by mixing 5 mL of XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) solution (1 mg/mL) with 100 μL of phenazine methosulfate solution (0.383 mg/mL). The dye solution (50 μL) was added to cells, which were further incubated for 4 h. The plates were read on a microplate reader (Molecular Devices, CA, USA) at the wavelengths of 492 and 690 nm. Etoposide and doxorubicin were used as the reference drugs (Table 3).

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