A New Antifungal Metabolite from *Penicillium expansum*

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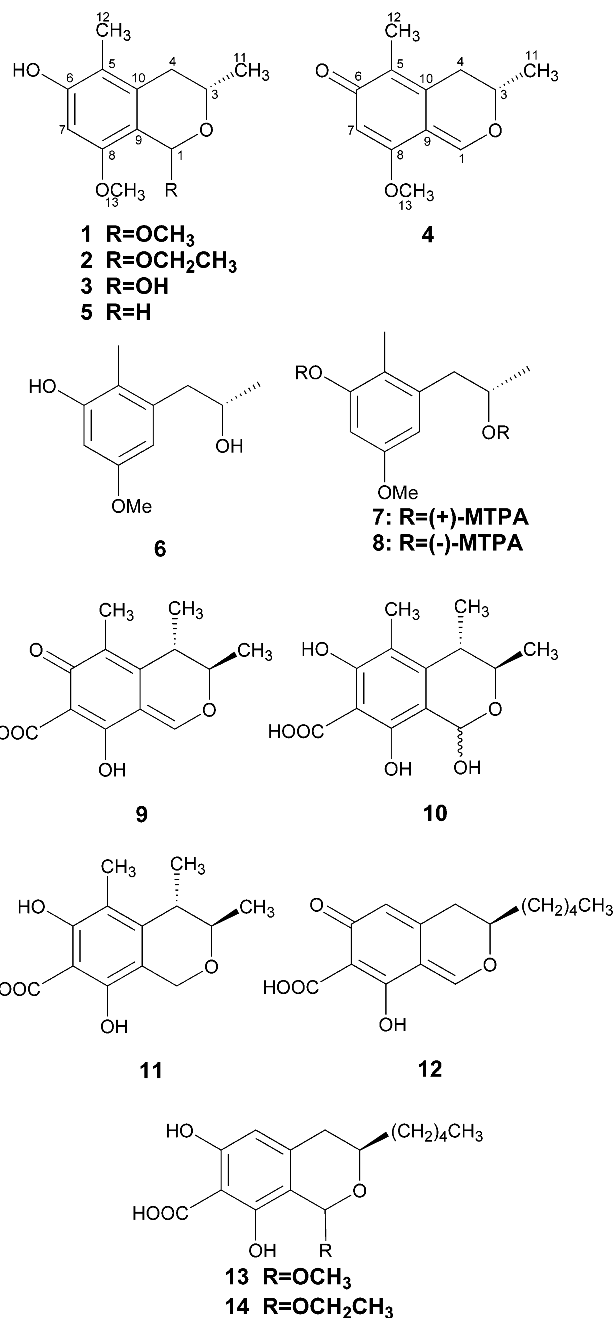
A new antifungal compound, (3*S*)-4,6-dihydro-8-methoxy-3,5-dimethyl-6-oxo-3*H*-2-benzopyran (**4**), was isolated from *Penicillium expansum.* During the isolation procedure **4** was determined to be unstable and readily reacted with methanol, ethanol, and water, forming three new isochromans, (1*S*,3*S*)-6-hydroxy-1,8-dimethoxy-3,5-dimethylisochroman (**1**), 1-ethoxy-6-hydroxy-8-methoxy-3,5-dimethylisochroman (**2**), and 1,6-dihydroxy-8-methoxy-3,5-dimethylisochroman (**3**), respectively. (3*S*)-6-Hydroxy-8-methoxy-3,5-dimethylisochroman (**5**) was reisolated from *P. expansum*. In fungicide disk assays, compounds **1**, **2**, and **4** inhibited the mycelial growth of *Lasiodiplodia theobromae* at 100 μg/mL by 76%, 74%, and 69%, respectively.

Post-harvest storage rot in fruits causes serious economic losses worldwide and are primarily associated with fungal infection, which can occur before, during, or after harvest.[1] Among the post-harvest diseases, stem end rot is considered one of the major problems for fruit production in many areas of the world.[2] *Lasiodiplodia theobromae* is the causative organism of stem end rot in many fruits and causes decay that spreads rapidly from the stem end destroying the entire fruit.[3] The possibility of controlling the fruit rot caused by *L. theobromae* has been reported using other microorganisms.[4] One of the important mechanisms that contributed to their biocontrol activities has been proposed to produce antibiotic compounds.[5] Numerous examples of biocontrol studies revealed that one or more antibiotics produced by biocontrol agents were involved in the suppression of plant pathogens.[6−9] In screening for bioactive compounds from *Penicillium expansum,* an antagonistic microorganism against *L. theobromae* (unpublished data), we isolated a new compound, (3*S*)-4,6-dihydro-8-methoxy-3,5-dimethyl-6-oxo-3*H*-2-benzopyran (**4**), and its adducts **1**−**3**. Compounds **1**, **2**, and **4** inhibited the mycelial growth of *L. theobromae* at 100 μg/mL by 76%, 74%, and 69%, respectively, in fungicide disk assays.



Results and Discussion

The ethyl acetate extract of the culture filtrate (20 L) of *P. expansum* was chromatographed on a silica gel column eluted using $CH_3OH-CHCl_3$ (1:99, v/v) to yield a yellow residue. The antifungal activity against *L. theobromae* was monitored by the paper disk agar diffusion method. Crystallization of the yellow residue from solvents other than CH_3OH or EtOH could not be achieved, whereas after recrystallization from CH_3OH and EtOH, white crystals **1** (1.4 g) and **2** (53 mg) were obtained, respectively.

Compound **1** was shown to have the molecular formula $C_{13}H_{18}O_4$ on the basis of HREIMS data and was a phenolic compound as determined by its green color upon reaction with ferric chloride. Analysis of ^{1}H, ^{13}C NMR, DEPT, and HMQC spectra revealed the presence of two methyl, one methylene, and two methoxy groups, one methine adjacent to an oxygen, one acetal methine, one aromatic methine, and five aromatic carbons. The $^{1}H-^{1}H$ COSY spectrum of **1** revealed cross-peaks (from H-4 to H-3 and from H-3 to

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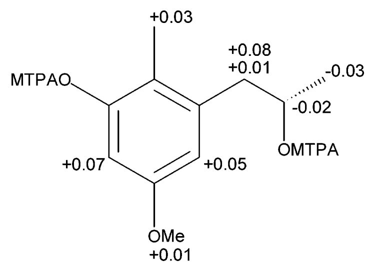

Figure 1. $\Delta\delta$ values [$\delta(-) - \delta(+)$] for (+)- and (−)-MTPA esters of compound **6**.

H-11) confirming the presence of the partial structure $-CH_2-CH(-O-)-CH_3$. The H-7 aromatic proton signal (δ 6.26) showed HMBC correlations to C-6 (δ 157.0), C-8 (δ 157.1), C-5 (δ 114.3), and C-9 (δ 114.9), suggesting the presence of a benzene ring. The unequivalent methylene proton signals of H-4 (δ 2.57 and 2.20) showed HMBC cross-peaks with C-3 (δ 63.8), C-10 (δ 136.0), C-11 (δ 21.6), C-9 (δ 114.9), and C-5 (δ 114.3). In addition, an acetal proton H-1 (δ 5.41) showed HMBC correlations to C-14 (δ 55.1), C-3 (δ 63.8), C-10 (δ 136.0), C-9 (δ 114.9), and C-8 (δ 157.1). Carbons C-1 and C-3 were determined to be linked through an oxygen, due to the HMBC cross-peak from H-1 (δ 5.41) to C-3 (δ 63.8). These results were consistent with an isochroman structure for **1**. The methoxy protons at δ 3.40 and 3.68 showed HMBC correlations to C-1 (δ 97.3) and C-8 (δ 157.1), respectively, implying that methoxy groups were attached at the C-1 and C-8 positions. A methyl proton resonating at δ 1.93 showed HMBC correlations with C-5 (δ 114.3), C-6 (δ 157.0), and C-10 (δ 136.0), suggesting this methyl group was on C-5. The phenolic hydroxy group had to be located on C-6 according to the ^{13}C chemical shift of C-6.

To determine the absolute configuration at C-3, cleavage of a nonaromatic ring of compound **1** was conducted under acid conditions to produce compound **6**.[10] The structure and assignments of **6** were established by ^{1}H and ^{13}C NMR spectra, and the advanced Mosher method[11] was subsequently applied to determine absolute configuration. Esterification of **6** with (+)- or (−)-MTPA by *N*,*N*-dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine in CH_2Cl_2 yielded **7** and **8**, respectively. The assignments of **7** and **8** were achieved by ^{1}H NMR and ^{1}H−^{1}H COSY spectra, and the chemical shift differences between **7** and **8** (Figure 1) indicated an *S* configuration at C-3. The absolute configuration at C-1 was deduced from the relative configuration of C-1 with C-3 by NOE experiments. Irradiation at the resonance of H-1 showed enhancement of the resonance of H-3. Thus, the structure of **1** was established as (1*S*,3*S*)-6-hydroxy-1,8-dimethoxy-3,5-dimethylisochroman.

The molecular formula of compound **2** was suggested as $C_{14}H_{20}O_4$ on the basis of HREIMS data. The ^{1}H and ^{13}C NMR data of **2** were almost identical to those of compound **1**, with the exception of signals due to an ethoxy unit rather than a methoxy group. Compound **2** was therefore 1-ethoxy-6-hydroxy-8-methoxy-3,5-dimethylisochroman.

P. expansum was reported to produce citrinin (**9**), a characteristic metabolite of several species of *Penicillium* and *Aspergillus*.[12,13] Citrinin has a wide variety of physiological activities including antifungal and antibiotic activities.[14] However, **9** was not detected in the strain used in this investigation. Furthermore, **9** was determined to form an adduct (**10**) with water at pH 7.4.[15] Pulvilloric acid (**12**) from *Penicillium pulvillorum* also had a quinone methide structure and readily formed adducts, such as CH_3OH adduct (**13**)[16] or EtOH adduct (**14**).[17] Herein, **1** and **2** were thought to be the addition products of CH_3OH and EtOH from the same compound. This prompted the investigation of the compound from which **1** and **2** were formed.

In an effort to examine the existence of such a compound, an isolation experiment using nonalcohol solvent systems was conducted. Fresh culture filtrate (2 L) was extracted with $CHCl_3$ and ether, independently. The $CHCl_3$ extract was then subjected to silica gel column chromatography, eluted with $CHCl_3$, to yield compounds **2** (354 mg) and **5** (110 mg) as the major components. Compound **2** was again obtained from the extract using $CHCl_3$ because a very small amount of EtOH (0.3−1%) was used to stabilize the $CHCl_3$. On the other hand, the extract using ether was subjected to silica gel column chromatography, eluted with *n*-hexane−acetone (3:1, v/v), to afford **5** (120 mg) and a mixture (136 mg) containing compounds **3** and **4**. Compounds **1** and **2**, however, could not be detected. Upon addition of a small amount of hot acetone−water to the mixture, bright yellow crystals of **4** (2 mg) were obtained.

Compound **4** was determined to have the molecular formula $C_{12}H_{14}O_3$ on the basis of the HREIMS data. Analysis of ^{1}H, ^{13}C NMR, DEPT, and HMQC spectra revealed the presence of two methyl, one methylene, and one methoxy group, one methine adjacent to an oxygen, two olefinic methines, four olefinic carbons, and one carbonyl carbon. The ^{1}H−^{1}H COSY spectrum of **4** revealed cross-peaks (from H-4 to H-3 and from H-3 to H-11) confirming the presence of the partial structure $-CH_2-CH(-O-)-CH_3$. A methine proton signal due to H-7 (δ 5.73) showed HMBC correlations to C-6 (δ 186.9), C-8 (δ 164.0), C-5 (δ 126.4), and C-9 (δ 109.3). The unequivalent methylene proton signals of H-4 (δ 2.90 and 2.53) showed HMBC cross-peaks with C-3 (δ 75.1), C-10 (δ 132.4), C-11 (δ 20.3), C-9 (δ 109.3), and C-5 (δ 126.4). In addition, an olefinic proton due to H-1 (δ 7.70) showed HMBC correlations with C-3 (δ 75.1), C-10 (δ 132.4), C-9 (δ 109.3), and C-8 (δ 164.0). Carbons C-1 and C-3 were determined to be linked through an oxygen due to the HMBC cross-peak from H-1 (δ 7.70) to C-3 (δ 75.1). The spectral data revealed that a partial structure of 4,6-dihydro-6-oxo-3*H*-2-benzopyran was present in compound **4**. A methyl proton signal (H-12, δ 1.92) showed HMBC correlations with C-5 (δ 126.4), C-6 (δ 186.9), and C-10 (δ 132.4), which suggested that this methyl group was on C-5. The remaining methoxy group was placed at C-8 due to the HMBC cross-peak from $-OCH_3$ (δ_H 3.75) to C-8 (δ 164.0). Thus, **4** was concluded to be 4,6-dihydro-8-methoxy-3,5-dimethyl-6-oxo-3*H*-2-benzopyran.

Compound **3** could not be obtained by crystallization, and its structure was therefore determined indirectly. FDMS of the mixture obtained from the extract using ether showed two molecular ion peaks, indicating that it consisted of two compounds. The molecular formulas of peaks at *m*/*z* 206 and 224 were determined to be $C_{12}H_{14}O_3$ and $C_{12}H_{16}O_4$, respectively, by HREIMS. The formula of **3** corresponded to the loss of one CH_2 group from compound **1**. The ^{13}C NMR spectrum of this mixture exhibited 24 carbon signals, 12 of which were in good accordance with those of **4**, while the remaining signals were in good agreement with those from compound **1**, with the exception of the absence of signal due to one methoxy group. Therefore, the components of the mixture were determined to be 1,6-dihydroxy-8-methoxy-3,5-dimethylisochroman (**3**) and **4**. Compound **4** could not be isolated by reverse-phase HPLC. This was thought to be because **3** existed in the collected fractions, indicating that **4** was unstable and readily formed **3** in H_2O. Furthermore, it became clear that

1 and **2** were formed from the addition of CH_3OH and EtOH to **4**, respectively. According to the stereochemistry of **1**, the absolute configuration at position C-3 in **2**−**4** was also determined to be *S*.

The ^1H and ^{13}C NMR spectral data and the optical rotation of compound **5** suggested that the structure, including absolute configuration, was identical to that of (3*S*)-6-hydroxy-8-methoxy-3,5-dimethylisochroman.[18,19] Compound **5** was originally reported as a metabolite from a hybrid strain derived from *Penicillium citreo-viride*.[18] Later, **5**, having anticoccidial activity, was determined to be produced in *Penicillium* sp. FO-2295.[19] In this investigation, it was isolated as one of the major metabolites of *P. expansum*.

Compounds **1** and **2** inhibited the mycelial growth of *L. theobromae* in fungicide disk assays at 100 μg/mL by 76% and 74%, respectively. Compound **4**, which contained a mixture **3**, due to its poor stability in media containing H_2O, had a similar activity at the same concentration. However, compound **5** was characterized as inactive due to mycelial growth inhibition of <10% at the same concentration.

The fermentation yield of **2** reached about 180 mg/L of the broth, indicating that **4** was a major metabolite and the key antifungal substance from the culture filtrate of *P. expansum*. The yellow color of **4** was ascribed to its quinone methide structure, which readily formed an adduct with one molecule of alcohol to give a colorless, aromatic structure that was characteristic of quinone methides in general.[20,21]

It was interesting that **5** was much less active than adducts **1** and **2**. It is well known that citrinin (**9**) is transformed into dihydrocitrinin (**11**) while developing its antifungal activity.[22] In the same mechanism, the development of the antifungal activity of **4** may also involve its reduction to dihydro-derivative **5**. The derivatives of **4** with different antifungal activity may be useful to investigate the relationship between the structure and activity of **4** and citrinin-like substances. The structure of **4** resembles citrinin (**9**), suggesting that **4** and **9** may be produced via the same biogenetic pathway.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanaco micro melting point apparatus. Optical rotations were measured on a JASCO DIP-4 polarimeter. NMR spectra were recorded on a Bruker AM-500FT-NMR spectrometer or JEOL JNM-EX 270 FT-NMR system. FDMS and EIMS were taken on JEOL JMSOISG-2 and JMS-DX-300 mass spectrometers, respectively.

Fungal Material. The seed fungus *P. expansum* was collected from Okinawa Prefectural Agricultural Experiment Station, Okinawa, Japan, and identified by S.K. The culture was deposited in the Department of Agriculture, Hokkaido University. The fungus was grown in a potato-glucose medium at 25 °C for 3 weeks.

Extraction and Isolation of 1 and 2. The culture filtrate (20 L) was concentrated in vacuo to one-fifth of its volume. The concentrated culture filtrate was extracted with EtOAc (× 3), and the EtOAc extract was evaporated to dryness in vacuo to afford a yellow residue (11 g). The residue was chromatographed on a silica gel column eluted with MeOH−$CHCl_3$ (1:99, v/v) to give five fractions. The antifungal activity was monitored by the paper disk diffusion method, and fraction 3 showed activity. Fraction 3 was further chromatographed on a silica gel column eluted with *n*-hexane−EtOAc (1:1) to give an active yellow oil. After recrystallization from MeOH−H_2O, **1** (1.4 g) was obtained as white crystals. From the remaining mother residue, **2** (53 mg) was recrystallized from EtOH−H_2O to give white crystals.

Extraction and Isolation of 3, 4, and 5. *P. expansum* was again cultured in potato-glucose medium at 25 °C for 2 weeks. A portion of the filtrate (2 L) was extracted with $CHCl_3$, and another portion (2 L) was extracted with ether. The residue (450 mg) from the $CHCl_3$ extract was subjected to chromatography (Si) and eluted with $CHCl_3$ to give **5**, which was crystallized from benzene to give fine white crystals (110 mg), and **2** (354 mg).

The residue (632 mg) from ether extract was separated twice using silica column chromatography (*n*-hexane−acetone, 3:1) to afford **5** (120 mg) and an active mixture (136 mg). After crystallization of the mixture from acetone−H_2O, **4** (2 mg) was obtained as yellow crystals.

(1*S*,3*S*)-1,8-Dimethoxy-3,5-dimethyl-6-hydroxyisochroman (1): white crystals; mp 60−62 °C; $[\alpha]^{25}_D$ +8.7° (*c* 0.30, MeOH); ^1H NMR (CD_3OD, 270 MHz) δ 6.26 (1H, s, H-7), 5.41 (1H, s, H-1), 4.15 (1H, m, H-3), 3.68 (3H, s, H-13), 3.40 (3H, s, H-14), 2.57 (1H, dd, *J* = 17.0, 3.5 Hz, H-4a), 2.20 (1H, dd, *J* = 16.7, 11.6 Hz, H-4b), 1.93 (3H, s, H-12), 1.27 (3H, d, *J* = 6.1 Hz, H-11); ^{13}C NMR (CD_3OD, 67.5 MHz) δ 157.1 (C, C-8), 157.0 (C, C-6), 136.0 (C, C-10), 114.9 (C, C-9), 114.3 (C, C-5), 97.6 (CH, C-7), 97.3 (CH, C-1), 63.8 (CH, C-3), 55.9 (OCH_3, C-13), 55.1 (OCH_3, C-14), 34.6 (CH_2, C-4), 21.6 (CH_3, C-11), 10.2 (CH_3, C-12); FDMS *m*/*z* 238 $[M]^+$; HREIMS *m*/*z* 238.1206 (calcd for $C_{13}H_{18}O_4$, 238.1205).

3,5-Dimethyl-1-ethoxy-6-hydroxy-8-methoxyisochroman (2): white crystals; mp 48−50 °C; $[\alpha]^{25}_D$ −13.5° (*c* 0.17, EtOH); ^1H NMR (CD_3OD, 270 MHz) δ 6.32 (1H, s, H-7), 5.59 (1H, s, H-1), 4.28 (1H, m, H-3), 3.85 (2H, m, H-14), 3.74 (3H, s, H-13), 2.65 (1H, dd, *J* = 16.8, 3.0 Hz, H-4a), 2.27 (1H, dd, *J* = 16.0, 11.9 Hz, H-4b), 1.99 (3H, s, H-12), 1.32 (3H, d, *J* = 6.3 Hz, H-11), 1.23 (3H, t, *J* = 7.1, 6.9 Hz, H-15); ^{13}C NMR (CD_3OD, 67.5 MHz) δ 156.9 (C, C-8), 156.8 (C, C-6), 135.9 (C, C-10), 114.7 (C, C-9), 114.1 (C, C-5), 97.5 (CH, C-7), 97.1 (CH, C-1), 63.8 (CH, C-3), 55.9 (OCH_3, C-13), 58.3 (OCH_2, C-14), 34.6 (CH_2, C-4), 21.7 (CH_3, C-11), 18.4 (CH_3, C-15), 10.3 (CH_3, C-12); FDMS *m*/*z* 252 $[M]^+$; HREIMS *m*/*z* 252.1377 (calcd for $C_{14}H_{20}O_4$, 252.1362).

1,6-Dihydroxy-3,5-dimethyl-8-methoxyisochroman (3): ^{13}C NMR ($CDCl_3$, 67.5 MHz) δ 155.0 (C, C-8), 154.2 (C, C-6), 135.2 (C, C-10), 114.5 (C, C-9), 112.8 (C, C-5), 96.9 (CH, C-7), 88.5 (CH, C-1), 62.6 (CH, C-3), 54.8 (OCH_3, C-13), 30.9 (CH_2, C-4), 21.6 (CH_3, C-11), 10.1 (CH_3, C-12); FDMS *m*/*z* 224 $[M]^+$; HREIMS *m*/*z* 224.1072 (calcd for $C_{12}H_{16}O_4$, 224.1049).

(3*S*)-4,6-Dihydro-3,5-dimethyl-8-methoxy-6-oxo-3*H*-2-benzopyran (4): yellow crystals; $[\alpha]^{25}_D$ −32.9° (*c* 0.08, $CHCl_3$); ^1H NMR ($CDCl_3$, 270 MHz) δ 7.70 (1H, s, H-1), 5.73 (1H, s, H-7), 4.35 (1H, m, H-3), 3.75 (3H, s, H-13), 2.90 (1H, dd, *J* = 16.7, 3.2 Hz, H-4a), 2.53 (1H, dd, *J* = 16.3, 12.3 Hz, H-4b), 1.92 (3H, s, H-12), 1.50 (3H, d, *J* = 6.1 Hz, H-11); ^{13}C NMR ($CDCl_3$, 67.5 MHz) δ 186.9 (C, C-6), 164.0 (C, C-8), 155.6 (CH, C-1), 132.4 (C, C-10), 126.4 (C, C-5), 109.3 (C, C-9), 101.4 (CH, C-7), 75.1 (CH, C-3), 55.2 (OCH_3, C-13), 32.4 (CH_2, C-4), 20.3 (CH_3, C-11), 10.3 (CH_3, C-12); FDMS *m*/*z* 206 $[M]^+$; HREIMS *m*/*z* 206.0941 (calcd for $C_{12}H_{14}O_3$, 206.0943).

(3*S*)-6-Hydroxy-8-methoxy-3,5-dimethylisochroman (5): white crystals (lit.[18,19] white powder); mp 121−123 °C; $[\alpha]^{25}_D$ +116.4° (*c* 0.1, MeOH); FDMS *m*/*z* 208 $[M]^+$.

Conversion of 1 to 3-(3-Hydroxy-5-methoxy-2-methylphenyl)propan-2-ol (6). To a solution of **1** (30 mg, 0.13 mmol) in THF (2 mL) was added water (100 μL) and 5 N HCl (0.3 mL). The solution was stirred at room temperature for 2 h and then poured into saturated aqueous $NaHCO_3$ (4 mL) and extracted with diethyl ether (3 × 1mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by preparative TLC (Merck, MeOH−$CHCl_3$, 1:9) to afford **6** (6.6 mg, 26%): $[\alpha]^{25}_D$ +31.3° (*c* 0.3, $CHCl_3$); ^1H NMR ($CDCl_3$, 270 MHz) δ 6.35 (1H, d, *J* = 2.1 Hz), 6.29 (1H, d, *J* = 2.1 Hz), 4.00 (1H, m), 3.75 (3H, s), 2.74 (2H, m), 2.13 (3H, s), 1.27 (3H, d, *J* = 6.3 Hz); ^{13}C NMR ($CDCl_3$, 67.5 MHz) δ 157.6 (C, C-5′), 155.1 (C, C-3′), 138.6 (C, C-1′), 115.2 (C, C-2′), 107.7 (C, C-6′), 99.8 (CH, C-4′), 68.2 (CH, C-2),

55.1 (OCH_3, C-8′), 43.4 (CH_2, C-3), 22.8 (CH_3, C-1), 11.1 (CH_3,C-7′); FDMS *m*/*z* 196 $[M]^+$; HREIMS *m*/*z* 196.1088 (calcd for $C_{11}H_{16}O_3$, 196.1100).

Preparation of (+)-MTPA Ester (7) of 6. 3-(3-Hydroxy-5-methoxy-2-methylphenyl)propan-2-ol (**6**) (10 mg), (+)-MTPA (75 mg), 4-(dimethylamino)pyridine (57 mg), and *N*,*N*′-dicyclohexylcarbodiimide (90 mg) were dissolved in dry CH_2Cl_2 under Ar atmosphere at room temperature, and the mixture was stirred for 24 h. The volatile components were removed, and the residue was purified by preparative TLC (hexane−EtOAc, 3:1) to give **7** (3 mg): ^{1}H NMR ($CDCl_3$, 270 MHz) δ 6.55 (1H, d, $J = 2.5$ Hz), 6.45 (1H, d, $J = 2.5$ Hz), 5.31 (1H, m), 3.46 (3H, s), 2.98 (1H, m), 2.72 (1H, m), 1.88 (3H, s), 1.35 (3H, d, $J = 6.3$ Hz); FDMS *m*/*z* 628 $[M]^+$.

Preparation of (−)-MTPA Ester (8) of 6. (−)-MTPA ester (**8**) (3 mg) from 3-(3-hydroxy-5-methoxy-2-methylphenyl)propan-2-ol (**6**) (10 mg) was prepared in the same manner as that used for (+)-MTPA ester (**7**). ^{1}H NMR ($CDCl_3$, 270 MHz) δ 6.62 (1H, s), 6.50 (1H, d, $J = 2.1$ Hz), 5.29 (1H, m), 3.47 (3H, s), 2.99 (1H, m), 2.80 (1H, m), 1.91 (3H, s), 1.32 (3H, d, $J = 5.9$ Hz); FDMS *m*/*z* 628 $[M]^+$.

Paper Disk Diffusion Method. The test fungus, *Ladiodiplodia theobromae* OCS-71, was subcultured in potato dextrose medium incubated at 25 °C. Sterile liquid potato dextrose agar media, mixed with freshly homogenized fungus at 40 °C, was poured into plastic dishes and allowed to solidify. A paper disk of 8 mm diameter was soaked with the solution of EtOH (30 μL) containing the test sample, and the paper disk without test sample but with EtOH was used as control. The soaked disks were allowed to dry at room temperature and placed at the center of the medium. Plates were then incubated at 25 °C for 3 days to obtain inhibition zones. The tests were conducted in triplicate.

Fungicide Disk Assays. Compounds in EtOH or acetone were added to 10 mL of PDA, in a concentration of 100 μg/mL. PDA plates containing only the solvents were used as controls. Seven-day-old cultures of test fungus, *L. theobromae* OCS-71, grown on PDA plates were used as an inoculum put onto the control and test plates. After 4 days of incubation at 25 °C, the mycelial growth diameter was measured, and the percentage of inhibition with respect to the control was calculated according to the following formula described by Shin et al.[23] as follows:

$$\text{inhibition (\%)} = [(\text{growth diameter in untreated control} - \text{growth diameter in treatment}) \times 100]/[\text{growth diameter in untreated control}]$$

The antifungal activity of each sample was determined in triplicate.

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