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Stereochemical Investigations of Isochromenones and Isobenzofuranones Isolated from *Leptosphaeria* sp. KTC 727

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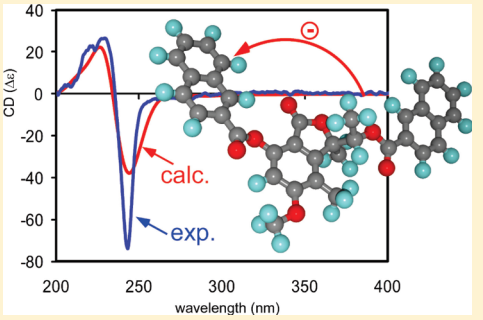
Supporting Information

ABSTRACT: Two new metabolites, (*R*)-3,4-dihydro-4,6,8-trihydroxy-4,5-dimethyl-3-methyleneisochromen-1-one (**1**) and (*R*)-7-hydroxy-3-((*S*)-1-hydroxyethyl)-5-methoxy-3,4-dimethylisobenzofuran-1(3*H*)-one (**2**), were isolated along with two structurally known related compounds (**3** and **4**) from the culture broth of *Leptosphaeria* sp. KTC 727 (JCM 13076 = MAFF 239586). These structures were disclosed mainly with ^{1}H and ^{13}C NMR spectroscopic analyses. The relative configuration of **2** was established by NOE studies. The absolute configuration of this molecule was determined by a combination of the modified Mosher's method and CD spectra after derivatizations. The theoretical CD profiles also supported these assignments. Structural correlations enabled us to establish the absolute configurations of metabolites **1**, **3**, and **4**, in which configurations of the latter two had not been established. Compound **2** exhibited the strongest antifungal activity among them, inhibiting the hyphal growth of *Cochliobolus miyabeanus* at about 0.5 μg/mL.

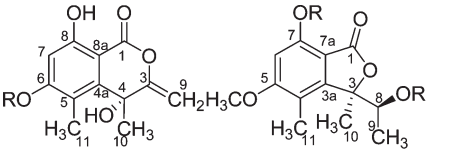


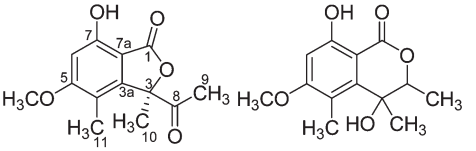
In the course of our investigations exploring novel biologically active metabolites from ecologically unique fungi,[1−5] the EtOAc extracts from the culture broth of *Leptosphaeria* sp. KTC 727 (JCM 13076[6] = MAFF 239586[7]) potently inhibited the growth of the fungus *Cochliobolus miyabeanus*. Further purification and investigation disclosed new isochromenone **1** and isobenzofuranone **2** from the aforementioned extracts. This study enabled us to establish the configurations of not only **1** and **2** but also structurally related **3** and **4**, which were also isolated from the extracts. The antifungal activities of **1**−**4** against *Cochliobolus miyabeanus* are also reported.

RESULTS AND DISCUSSION

When the extracts were fractionated by silica gel column chromatography, activity was found in the fraction that eluted with 20% EtOAc/hexane. Further purification afforded **1** (1.9 mg), **2** (91.3 mg), **3** (13.3 mg), and **4** (3.3 mg). Although **3** and **4** are known metabolites from *Halorosellinia oceanica*,[8] our investigation as described in this report disclosed **1** and **2** as new compounds.

The ^{1}H and ^{13}C NMR spectra of **1** strongly resembled those of **3** except that the C-6 methoxy group was missing. The C-6 resonance appeared at 166.2 ppm in the ^{13}C NMR spectrum to indicate a phenol function at this position, establishing the desmethyl form of **3**. The molecular formula of **1** was determined to be $C_{12}H_{12}O_5$ on the basis of the protonated molecular ion peak at *m*/*z* 237.0773. The provided molecular formula was "CH_2" smaller than that of **3**, which is consistent with the above





assumption. The HMBC correlations (Table 1) led us to confirm the suggested structure.

The ESIMS spectrum of **2** showed a protonated molecular ion signal at *m*/*z* 253.1071, indicating its molecular formula as $C_{13}H_{16}O_5$. The mass was two units greater than **3** or **4**. In the ^{1}H NMR spectrum, the doublet methyl signal (6.8 Hz) was at 0.93 ppm in place of the singlet (1.74 ppm) observed in **1**. These

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Table 1. NMR Spectroscopic Data (^{1}H, 500 MHz; ^{13}C, 125 MHz, $CDCl_3$) for 1, 2, and 3

	1			2			3	
position	δ_C, mult.	δ_H (J in Hz)	HMBC[a]	δ_C, mult.	δ_H (J in Hz)	HMBC[b]	δ_C, mult.	δ_H (J in Hz)
1	166.9, C			171.5, C			166.4, C	
3	160.5, C			91.3, C			160.6, C	
4	72.1, C			112.2, C			72.1, C	
5	114.2, C			165.4, C			116.4, C	
6	166.2, C			98.2, CH	6.43, s	1, 4, 5, 7, 7a	165.8, C	
7	102.7, CH	6.40, s	5, 8, 8a	156.5, C			98.6, CH	6.44, s
8	162.6, C			70.9, CH	4.22, m		163.2, C	
9	95.3, CH_2	5.10, d (1.7)	3	17.8, CH_3	0.93, d (6.8)	3, 8	95.4, CH_2	5.10, d (2.0)
		4.97, d (1.7)	3, 4					4.96, d (2.0)
10	29.1, CH_3	1.74, s	3, 4, 4a	21.3, CH_3	1.81, s	3, 3a, 8	29.0, CH_3	1.74, s
11	11.8, CH_3	2.44, s	4a, 5, 6	11.2, CH_3	2.11, s	3a, 4, 5	12.0, CH_3	2.39, s
3a				149.9, C				
4a	143.8, C						142.4, C	
7a				103.0, C				
8a	99.2, C						98.2, C	
C_5OCH_3				56.3	3.88, s	5		
C_6OCH_3							56.0	3.87, s
C-7 OH					7.88, s	6, 7, 7a		
C-8 OH		11.20, s	7, 8, 8a		1.97, d (10.3)			11.32, s

[a] HMBC correlations are from the proton(s) stated to the indicated carbon. [b] H-8 in **2** showed no HMBC correlations, which could eliminate **5** as the possible structure.

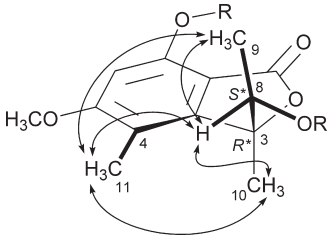

Figure 1. NOEs observed in **2**.

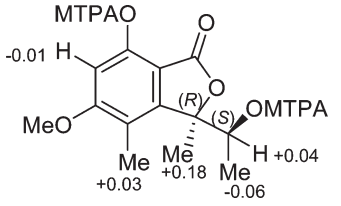

Figure 2. $\Delta\delta$ values ($\delta_S - \delta_R$) of MTPA esters **6** and **7** in $CDCl_3$.

analyses suggested structures **2** and **5** as the possible structures. Although HMBC and IR spectra did not distinguish between these two, irradiation of the arylmethyl signal (2.11 ppm, H_3-11 in structure **2**) in the ^{1}H NMR induced a considerable NOE at the doublet methyl (H_3-9 in structure **2**), which unambiguously excludes the possibility of **5**. This was confirmed by high-frequency shifts of H-8 by esterifications of the C-8 OH, giving **6**, **7**, and **8**, as will be described later.

Compound **2** has two asymmetric centers. Irradiation of H_3-11 of **2** induced NOEs not only at H_3-9 but at H_3-10, and H-8 also as shown in Figure 1. The NOE between H_3-11 and H-8 was most prominent. Similar NOE profiles were observed for diesters **6**, **7**, and **8**. These observations indicated that the C-9 and C-10 methyls are almost fixed conformationally by taking an *anti*-orientation in these compounds. Only this conformation provides enough room for the bulky C-8 ester groups in **6**−**8**. These data suggested a 3*R**,8*S**-isomer. This is the lone isomer that satisfied these NOEs.

The absolute configuration of **2** was then studied by taking advantage of the C-8 secondary alcohol. Conventional conditions for MTPA esters[9] afforded diastereomeric bis-MTPA esters **6** and **7**. In the ^{1}H NMR spectra of **6** and **7**, H-8 (5.74 and 5.70 ppm, respectively) shifted to a higher frequency from that of **2** (4.22 ppm), which confirmed the C-8 OH group. The chemical shifts of (*R*,*R*)-bis-MTPA ester **7** in the ^{1}H NMR spectrum were subtracted from the corresponding signals in the (*S*,*S*)-bis-MTPA ester **6** to give the $\Delta\delta$ values as shown in Figure 2. Based on Kusumi's modified Mosher's method,[9] the obtained $\Delta\delta$ values indicated an *S*-configuration for the C-8 stereogenic center. However, these results were not conclusive because (1) **2** is a sterically hindered alcohol (application for less hindered alcohols is recommended), (2) the other MTPA group at C-7 is spatially close and might induce undesirable effects on the $\Delta\delta$ values, and (3) only three signals were available for these analyses (Kusumi recommends consideration of as many signals as possible for a higher level of confidence in the assignment of configuration).

To reinforce the presumed absolute configuration assignment, CD spectroscopic analyses were employed. Naphthoyl groups were covalently introduced at the C-7 and C-8 hydroxy groups in **2** as exciton-coupling chromophores, yielding bisnaphthoate **8**.

As shown in Figure 3, the observed CD for **8** in CH_3CN exhibited a pair of strong exciton-split Cotton effects at 243 nm ($\Delta\varepsilon$ −74) and 227 nm ($\Delta\varepsilon$ +26). This result is qualitatively attributable to a negative chirality for the two naphthoyl groups as depicted, suggesting a 3*R*,8*S*-configuration.[10] Independently

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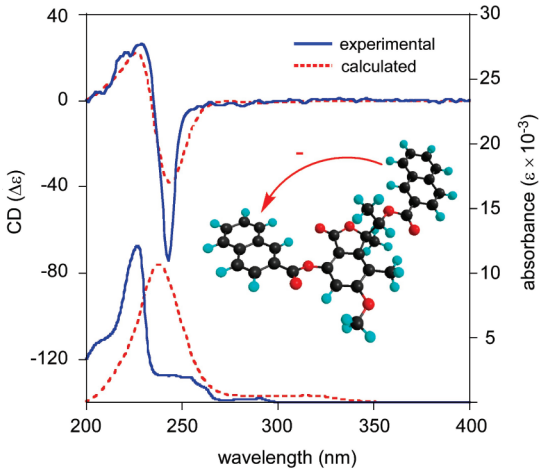


Figure 3. Experimental and calculated CD (upper) and UV (lower) spectra of dinaphthoate **8**.

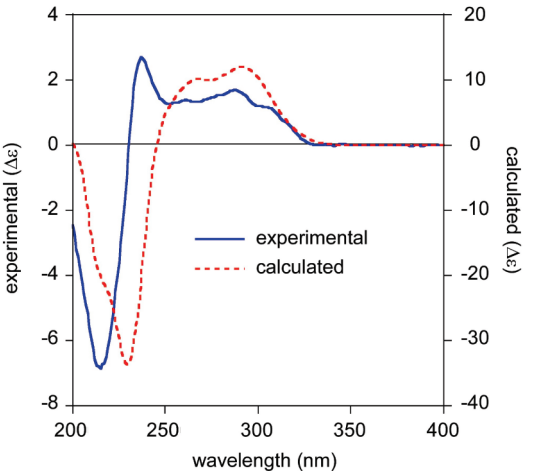
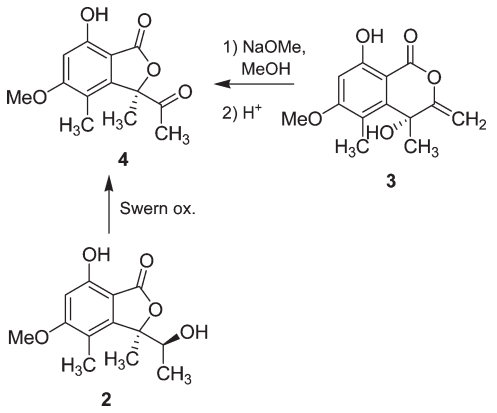


Figure 4. Experimental and calculated CD spectra for **4**.

Scheme 1. Chemical Correlations among 2, 3, and 4



from this empirical assignment, theoretical calculations were also performed to account for the CD appearance.[11] The CD curve of (3*R*,8*S*)-**8** was obtained by the time-dependent density functional theory (TDDFT) using the B3LYP functional[12] at a triple-ζ quality with a TZVP basis set.[13] These calculations provided the CD spectra for each conformation. The final CD spectrum was obtained by considering their Boltzmann distributions. The resultant CD curve from the calculations reproduced the experimental CD profile well enough to confirm the absolute configuration.

Chemical correlations enabled us to establish as well the chiralities of **3** and **4**, which have not yet been reported. Swern oxidation of **2** smoothly produced **4** as shown in Scheme 1. It was also found that basic methanolysis of **3** followed by neutralization gave rise to a ring contraction, providing **4** in good yield. The NMR data and CD spectra of synthetic **4** prepared from **2** and **3** were identical to those of natural **4**, disclosing its 3*R*-configuration. The absolute configuration of **1** was also proposed to be *R*, by observing the same sign of the optical rotations for both **1** (+161, *c* 0.19) and **3** (+96, *c* 0.11). The configurations of the oxygen-bearing quaternary centers (C4 in **1** and **3**, C3 in **2** and **4**) thus revealed the same chirality, which is plausible considering their biogenesis.

Additionally, although the intensity could not be exactly reproduced, the calculated CD profile of (*R*)-**4** accorded well with that of the experimentally obtained spectrum of natural **4** (Figure 4). This further supports the *R*-configuration of **4** as discussed above. The calculations were performed in a similar manner as described for **8**. The difference observed in CD intensities between theoretical and experimental spectra may be attributed to overestimation of the UV absorbance in the calculations.

As described, novel isochromenone and isobenzofuranone derivatives were isolated from the culture broth of *Leptosphaeria* sp. KTC 727. NOE experiments revealed the relative configuration of **2**. The absolute configuration of **2** was established through the modified Mosher's method, which was further confirmed with CD studies involving both experimental data and theoretical calculations. This study also established the configurations of **1**, **3**, and **4** based on chemical correlations and comparison of their CD/$[\alpha]_D$ data.

Finally, **1**−**4** were subjected to an antifungal assay against *Cochliobolus miyabeanus* to disclose the IC_{50} values at ca. 10, 500, 0.5, and 10 μg/mL, respectively. Minor structural differences of metabolites lead to significant differences in the ability to inhibit fungal hyphal growth.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation values were measured on a Horiba SEP-700 spectrometer. UV spectra were obtained with a Hitachi U-2010 spectrophotometer. CD spectra were recorded on a Jasco J-725 spectropolarimeter. Measurements of IR spectra were performed with a Horiba FT-720 spectrometer on a KBr cell. The ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a JEOL JNM-ECA500 spectrometer. In $CDCl_3$, the signal due to tetramethylsilane (0 ppm) was used as the standard, while the CHD_2COCD_3 peak at 2.04 ppm was employed for acetone-d_6. Electrospray ionization (ESI) MS spectra were obtained from a Hitachi NanoFrontier LD spectrometer.

Fungal Material. *Leptosphaeria* sp. KTC 727 (JCM 13076[6] = MAFF 239586[7]) was collected on woody debris from Akaiwa, Rebun Island in Hokkaido, Japan, on August 30, 2001. The fungal isolate was deposited at the Japan Collection of Microorganisms (JCM) and the Ministry of Agriculture, Forestry and Fisheries, Japan (MAFF).

Fermentation and Isolation. *Leptosphaeria* sp. KTC 727 was cultured in 200 mL potato-sucrose medium [prepared from a potato extract (40 g potato), 4 g of sucrose, and H_2O] in 500 mL Erlenmeyer

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flasks (×5) at 25 °C for 40 days on a rotary shaker (100 rpm). After vacuum filtration, the filtrate was extracted with EtOAc (1.0 L × 2) and concentrated *in vacuo* to give an extract (350 mg), which was divided into several fractions by silica gel column chromatography. The activity was found in the fractions eluted by 20% EtOAc/hexane. Further silica gel column chromatography using a hexane/EtOAc system gave **3** (13.3 mg), **1** (1.9 mg), **4** (3.3 mg), and **2** (91.3 mg), respectively, as oils.

(*R*)-3,4-Dihydro-4,6,8-trihydroxy-4,5-dimethyl-3-methyleneisochromen-1-one (1):. colorless oil; $[\alpha]^{27}_{D}$ +161 (*c* 0.19, $CHCl_3$), +64 (*c* 0.12, EtOH); UV (CH_3CN) λ_{max} (log ε) 214 (4.00), 270 (3.58), 318 (3.39); CD (0.00025 M, CH_3CN) λ_{max} ($\Delta\varepsilon$) 226 (−2.2), 207 (+5.2) nm; IR (film) ν_{max} 3328, 2923, 1681, 1650, 1025 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 1; ESIMS *m*/*z* 237.0773 $[M + H]^+$ (calcd for $C_{12}H_{13}O_5$, 237.0758); R_f = 0.6 (silica gel, EtOAc/hexane, 1:1).

(*R*)-7-Hydroxy-3-((*S*)-1-hydroxyethyl)-5-methoxy-3,4-dimethylisobenzofuran-1(3*H*)-one (2):. colorless oil; $[\alpha]^{27}_{D}$ −27 (*c* 0.20, $CHCl_3$), −27 (*c* 0.37, EtOH); UV (CH_3CN) λ_{max} (log ε) 212 (4.71), 257 (4.15), 296 (3.86); CD (0.000030 M, CH_3CN) λ_{max} ($\Delta\varepsilon$) 258 (+2.7), 215 (−7.7) nm; IR (film) ν_{max} 3401, 2981, 1731, 1054 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$), see Table 1; ESIMS *m*/*z* 253.1071 $[M + H]^+$ (calcd for $C_{13}H_{17}O_5$, 253.1070); R_f = 0.3 (silica gel, EtOAc/hexane, 1:1).

(*R*)-3,4-Dihydro-4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochromen-1-one (3) (ref 8):. colorless oil; $[\alpha]^{27}_{D}$ +150 (*c* 0.05, EtOH), +96 (*c* 0.11, $CHCl_3$) [lit. +93 (*c* 0.05, EtOH)]; CD (0.000052 M, CH_3CN) λ_{max} ($\Delta\varepsilon$) 225 (−7.4), 207 (+12.3) nm. The ^1H and ^{13}C NMR data in acetone-d_6 showed good accordance with those in the literature.

(*R*)-3-Acetyl-7-hydroxy-5-methoxy-3,4-dimethylisobenzofuran-1(3*H*)-one (4) (ref 8):. colorless oil; $[\alpha]^{27}_{D}$ +20 (*c* 0.14, EtOH), +100 (*c* 0.09, $CHCl_3$) [(lit. +200 (*c* 0.05, EtOH)]; CD (0.00024 M, CH_3CN) λ_{max} ($\Delta\varepsilon$) 236 (+1.6), 213 (−4.0) nm. The ^1H and ^{13}C NMR data in acetone-d_6 showed good accordance with those in the literature.

Bis-(*S*)-MTPA Ester 6. Compound **2** (2.0 mg, 8.0 μmol) was treated with *R*-(−)-MTPACl (10 mg, 40 μmol) in pyridine (200 μL) at room temperature for 2 days. The mixture was diluted with EtOAc (20 mL) and washed successively with H_2O (20 mL) and 1.0 M aqueous $NaHCO_3$ (20 mL). After the organic layer was dried over $MgSO_4$, the filtrate was concentrated *in vacuo* to give the crude product, which was purified by preparative TLC (EtOAc/hexane, 1:9) to give bis-(*S*)-MTPA ester **6** (0.90 mg) as a colorless oil: ^1H NMR ($CDCl_3$) δ 7.26−7.80 (10H, aromatic protons), 6.56 (1H, s, H-6), 5.74 (1H, q, *J* = 6.3 Hz, H-8), 3.58, 3.76 (each 3H, s, OCH_3 × 2), 2.31 (3H, s, H_3-11), 1.69 (3H, s, H_3-10), 0.98 (3H, d, *J* = 6.3 Hz, H_3-9); ESIMS *m*/*z* 685.1833 $[M + H]^+$ (calcd for $C_{33}H_{31}F_6O_9$, 685.1867); R_f 0.5 (silica gel, EtOAc/hexane, 1:9).

Bis-(*R*)-MTPA Ester 7. In the same manner as described above, **2** (2.5 mg, 10.0 μmol) was treated with *S*-(+)-MTPACl (10 mg, 40 μmol) to give bis-(*S*)-MTPA ester **7** (1.5 mg) as a colorless oil: ^1H NMR ($CDCl_3$) δ 7.41−7.80 (10H, aromatic protons), 6.57 (1H, s, H-6), 5.70 (1H, q, *J* = 6.3 Hz, H-8), 3.90 (3H, s, C5OCH_3), 3.61, 3.74 (each 3H, s, OCH_3 × 2), 2.28 (3H, s, H_3-11), 1.51 (3H, s, H_3-10), 1.04 (3H, d, *J* = 6.3 Hz, H_3-9); ESIMS *m*/*z* 685.1876 $[M + H]^+$ (calcd for $C_{33}H_{31}F_6O_9$, 685.1867); R_f 0.5 (silica gel, EtOAc/hexane, 1:9).

Dinaphthoate 8. Compound **2** (8.0 mg, 32 μmol) was stirred with 2-naphthoyl chloride (47.7 mg, 251 μmol) and 4-*N*,*N*-dimethylaminopyridine (3.1 mg, 25 μmol) in pyridine (300 μL) at 65 °C for 8 h. MeOH (30 μL) was added, and the resulting mixture was stirred for 2 h. The mixture was diluted with EtOAc (20 mL), washed successively with H_2O (20 mL) and brine (20 mL), and then dried over $MgSO_4$. After filtration, the filtrate was concentrated *in vacuo* to give the crude product. Preparative silica gel TLC (EtOAc/hexane, 1:3) gave dinaphthoate **8** (7.7 mg, 14 μmol) as a colorless oil: $[\alpha]^{21}_{D}$ −72 (*c* 0.11, EtOH); UV (CH_3CN) λ_{max} (log ε) 239 (5.08); CD (0.0000035 M, CH_3CN) λ_{max} ($\Delta\varepsilon$) 243 (−73.9), 227 (+26.2) nm; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.56−8.91 (14H, aromatic naphthoate protons), 7.01 (1H, s, C6H), 5.96 (1H, q, *J* = 6.3 Hz, C8*H*), 3.98 (3H, s, C6OCH_3), 2.41 (3H, s, C4CH_3), 1.81 (3H, s, C3CH_3), 1.16 (3H, d, *J* = 6.3 Hz, C9H_3); ESIMS *m*/*z* 561.1889 $[M + H]^+$ (calcd for $C_{35}H_{29}O_7$, 561.1908); R_f 0.5 (silica gel, EtOAc/hexane, 3:7).

Swern Oxidation of 2 Providing 4. To a solution of DMSO (13.7 mg, 176 μmol) in CH_2Cl_2 (400 μL) was added oxalylchloride (20.3 mg, 160 μmol) at −78 °C. After stirring for 20 min, a solution of **2** (2.1 mg, 8.4 μmol) in CH_2Cl_2 (500 μL) was added at the same temperature. After 40 min, triethylamine (32.3 mg, 320 μmol) was added, and the solution was allowed to warm to 0 °C. The mixture was poured into H_2O (20 mL) and extracted with EtOAc (20 mL). The organic solution was washed with brine (20 mL), dried over $MgSO_4$, and then concentrated *in vacuo*. Preparative silica gel column chromatography of the residue (EtOAc/hexane, 3:7) gave **4** (1.8 mg, 7.0 μmol, 83%). CD spectra, ^1H NMR, and chromatographic behavior of this sample were identical with those of natural **4**. Colorless oil: $[\alpha]^{21}_{D}$ +24 (*c* 0.10, EtOH); UV (CH_3CN) λ_{max} (log ε) 213 (3.64), 259 (3.25), 299 (2.95); CD (0.00027 M, CH_3CN) λ_{max} ($\Delta\varepsilon$) 235 (+1.3), 213 (−4.6) nm (see Supporting Information); ESIMS *m*/*z* 251.0907 $[M + H]^+$ (calcd for $C_{13}H_{15}O_5$, 251.0914); R_f = 0.4 (silica gel, EtOAc/hexane, 1:1).

Chemical Correlation of 3 with 4. A solution of **3** (1.0 mg, 4.0 μmol) in MeOH (200 μL) was stirred with sodium methoxide (1.1 mg, 20 μmol) for 1 h at room temperature. The mixture was poured into 0.2 M aqueous HCl solution and extracted with EtOAc (20 mL). The organic solution was washed with brine (20 mL), dried over $MgSO_4$, and then concentrated *in vacuo*. Silica gel column chromatography of the residue (EtOAc/hexane, 1:4) gave **4** (0.9 mg, 90%). The ^1H NMR, CD spectra, and chromatographic behaviors were identical to those of natural **4**.

Biological Assay. Solutions of *Cochliobolus miyabeanus* spores provided by Mitsubishi Chemical Corporation were prepared containing 500, 100, 50, 10, 5.0 1.0, and 0.5 μg/mL in two replicates for each metabolite (**1**−**4**) with 2% sucrose in DMSO. After 36 h at 25 °C, germination and the shapes of the spores were observed under a microscope. The IC_{50} values were determined by the concentration that showed 50% inhibition of germination.

CD Calculation for (3*R*,8*S*)-8. A standard conformational search by CONFLEX6[14] using MMFF94S as the force field gave more than 200 stable conformers. Selected conformers (26) with distributions higher than 1% were further optimized by the density functional theory method at the B3LYP/6-31G(d) level by Gaussian 09,[15] leading to 22 representative conformers. Nine stable conformers, which represent more than 73.3% of the distribution, were subjected to the TDDFT calculation in Gaussian 09 for 48 excited states at the B3LYP/TZVP level. To guarantee the quality of all calculations, it was verified that the computed 48 transition states contain virtual orbitals with negative eigenvalues and have energies (3.9−5.9 eV) reasonably below the computed ionization potential (6.3 eV). The resultant rotational strengths for each conformer were converted to a Gaussian-type curve with a half-bandwidth of 2800 cm^{-1} and summed to give a CD curve. Those CD curves were weighted based on the Boltzmann distribution and added to afford the final CD calculation curve. Although the curve is not derived from all possible conformers, the adopted CD curve does not change much even with the rest of the conformers of distribution below 5%.

CD Calculation for 4. Calculations were performed in a similar manner to that described above. A conformational search provided only two stable conformers in this case.

■ ASSOCIATED CONTENT

Supporting Information. The NMR spectra of all compounds and other relevant information associated with this article are available free of charge via the Internet at http://pubs.acs.org.

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■ DEDICATION

Dedicated to Dr. Koji Nakanishi of Columbia University for his pioneering work on bioactive natural products.

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