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Note

Novel Antifungal Compounds Produced by Sterile Dark, an Unidentified Wheat Rhizosphere Fungus

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Two novel antifungal compounds were isolated from a culture broth of Sterile Dark, an unidentified fungus isolated from the rhizosphere of wheat grown in a continuous cropping field. These compounds were elucidated to be phthalide-based compounds by spectroscopic analyses.

Key words: phthalide; antifungal activity; Sterile Dark; take-all disease

The "take-all" disease is a major wheat disease caused by the fungus *Gaeumannomyces graminis* var. *tritici*.[1)] The disease occurs with continuous cropping of wheat and causes production to decline seriously.[2)] However, in some wheat fields continuously cropped over a long period of time, the take-all disease has never occurred. An unidentified fungus, provisionally named Sterile Dark, was originally isolated from the rhizosphere of wheat grown in such a field.[3)] Due to the lack of sporulation, this fungus has not yet been identified. When Sterile Dark was applied to wheat seeds, it colonized the roots and suppressed the growth of *Gaeumannomyces graminis* var. *tritici* on the roots.[3)] This result indicated that the compounds produced by Sterile Dark were probably suppressing the take-all disease. We report here two novel antifungal compounds produced by Sterile Dark that may be related with the suppression of take-all disease. This paper reports the production, isolation, structural elucidation, and antifungal activity of these two compounds.

A slant culture of Sterile Dark grown on potato dextrose agar was inoculated into two hundred 500-ml Erlenmeyer flasks containing 150 ml of a medium composed of malt extract (2.0%) and yeast extract (0.2%). These flasks were incubated statically at 22 °C for 50 days. The acetone extract of the mycelia of Sterile Dark (3.46 kg) was concentrated *in vacuo* to give an aqueous residue. This residue was extracted with EtOAc, and the soluble portion was evaporated to obtain a brown residue (10.5 g). The brown residue was subjected to silica gel column chromatography, and various mixtures of $CHCl_3$/MeOH were eluted to provide the fraction with antifungal activity. The obtained fraction (291 mg), which was eluted with a mixture of $CHCl_3$/MeOH (95:5), was subjected to Sephadex LH-20 column chromatography using a mixture of $CHCl_3$/MeOH (1:1), and then to silica gel column chromatography using a mixture of $CHCl_3$/MeOH (98:2). The fraction with antifungal activity (8.2 mg) was purified by reverse-phase HPLC (Inertsil ODS-2 column, 4.6 × 250 mm, GL Science) using a mixture of MeOH/H_2O/acetic acid (39.9:60:0.1) at a flow rate of 1 ml/minute, to yield compounds **1** (2.4 mg) and **2** (3.6 mg).

Compound **1** was obtained as a colorless powder. The IR spectrum of **1** indicated the presence of a hydroxyl group at 3400 cm^{-1}, a carbonyl group at 1720 cm^{-1}, and a phenyl group at 1600 cm^{-1}. The molecular formula of **1** was established as $C_{15}H_{16}O_6$[*m*/*z* 292.0937 (M^+), Δ1.0 mmu] on the basis of an HREI-MS analysis. A color reaction with the phenol reagent indicated the presence of a phenolic group in **1**. The ^{1}H-NMR spectrum of **1** exhibited signals for 14 protons including three methyl singlets, one methylene singlet, one methylene doublet, and one methine triplet. In addition, the ^{13}C-NMR and DEPT spectra of **1** revealed 15 carbons that could be classified into three methyl, two methylene, one methine and nine quaternary carbons including two carbonyl carbons. The direct connectivity of the protons and carbons was established from an HMQC spectrum. The HMBC correlations of **1** are summarized in Table 1. The HMBC spectrum showed long-range correlations from H-3 to C-1, C-4, C-4a and C-7a, from 4-Me to C-4, C-4a and C-5, and from 6-Me to C-5, C-6 and C-7. These NMR spectral data and a proton signal at δ5.22 (H-3), which is characteristic of the lactone in

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Table 1. NMR Spectral Data for Compounds **1** and **2** (500 MHz, $CDCl_3$)

Position	**1**			**2**		
	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC
1		172.9			173.0	
3	5.22[a] (2H[b], s[c])	70.1	1, 4, 4a, 7a	5.19 (2H, s)	70.0	1, 4, 4a, 7a
4		116.5			116.5	
4a		143.5			143.4	
5		162.3			162.6	
6		118.6			118.5	
7		158.3			153.7	
7a		106.5			106.1	
4-Me	2.16 (3H, s)	11.7	4, 4a, 5	2.12 (3H, s)	11.5	4, 4a, 5
6-Me	2.22 (3H, s)	8.9	5, 6, 7	2.17 (3H, s)	8.7	5, 6, 7
1′	4.58 (2H, d, 5.8[d])	69.6	5, 2′, 3′	3.85 (2H, t, 6.3)	70.4	5, 2′, 3′
2′	7.16 (1H, t, 5.8)	138.4	3′, 4′	2.26 (1H, m)	33.7	1′, 3′, 4′, 5′
				1.96 (1H, m)		1′, 3′, 4′, 5′
3′		129.0		2.88 (1H, m)	36.0	1′, 2′, 4′, 5′
4′		171.0			182.2	
5′	1.88 (3H, s)	12.7	2′, 3′, 4′	1.33 (3H, d, 7.1)	17.3	2′, 3′, 4′

[a]δ in ppm, [b]integral, [c]multiplicity, [d]coupling constant (Hz).

phthalide,[4,5] indicated the presence of phthalide in **1**. The presence of a 3-carboxy-2-butenyloxy moiety was indicated by HMBC correlations from H-1′ to C-2′ and C-3′, and from H-5′ to C-2′, C-3′ and C-4′. Moreover, HMBC correlation between H-1′ and C-5 showed that the 3-carboxy-2-butenyloxy moiety was linked to C-5. To confirm the configuration of the double bond in the 3-carboxy-2-butenyloxy side chain, NOE difference spectroscopy was carried out. NOE was observed between H-5′ and H-1′, and therefore the double bond configuration was determined to be (*E*). Consequently, the structure of **1** was elucidated to be (*E*)-5-(3-carboxy-2-butenyloxy)-7-hydroxy-4,6-dimethylphthalide (Fig. 1).

Compound **2** was also obtained as a colorless powder. An HREI-MS analysis enabeled the molecular formula of **2** to be determined as $C_{15}H_{18}O_6$ [*m/z* 294.1104 (M^+), Δ0.1 mmu], differing by two protons from **1**. The specific rotation value of **2** was +6.2° (*c* 0.36, MeOH). The IR spectrum of **2** indicated the presence of a hydroxyl group at 3420 cm^{-1}, a carbonyl group at 1740 cm^{-1}, and a phenyl group at 1600 cm^{-1}. The presence of a phenolic group in **2** was indicated by the phenol reagent. The ^{1}H-NMR spectrum of **2** was similar to that of **1**. Proton signals of **2** at δ2.17 (3H, s), δ2.12 (3H, s) and δ5.19 (2H, s) suggested the presence of a phthalide skeleton. A comparison of the ^{1}H-NMR spectra of **2** and **1** revealed the double bond proton signal at δ7.16 that was present in **1** to be missing in **2**. Instead, proton signals at δ2.88 (1H, m), δ2.26 (1H, m) and δ1.96 (1H, m) were identified in **2**. Through an analysis of the 2D NMR data, the structure 3-carboxy-butoxy moiety was identified by the COSY connectivity from H-1′ to H_2-2′, from H_2-2′ to H-3′, and from H-3′ to H-5′, and the long-range coupling between H-3′ and C-4′. The HMBC correlation between H-1′ and C-5 revealed connectivity between C-5 and C-1′ through an oxygen. Accordingly, the structure of **2** was determined to be (+)-5-(3-carboxy-butoxy)-7-hydroxy-4,6-dimethylphthalide.

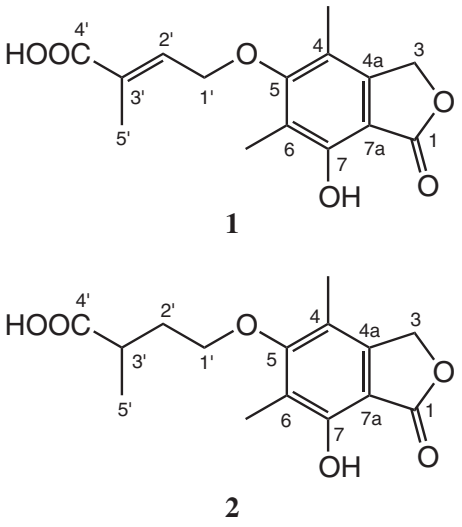

Fig. 1. Structures of Compounds **1** and **2**.

The antifungal activities of these two compounds were evaluated by TLC bioautography, using *Gaeumannomyces graminis* var. *tritici* and *Cladosporium herbarum*. Compound **1** inhibited spore germination of *G. graminis* var. *tritici* at 50 μg/spot, and of *C. herbarum* at 5 μg/spot. Compound **2** inhibited the assay of *C. herbarum* at 50 μg/spot, but, showed no inhibitory activity in the assay with *G. graminis* var. *tritici* at 50 μg/spot. These results suggest that the double bond in the side chain of **1** contributed to antifungal activity.

Experimental

Instrumentation. IR spectra were measured with a Hitachi 285 infrared spectrophotometer, mass spectra were obtained with a Jeol JMS-DX300 mass spectrometer, specific rotation value was measured with a Jasco DIP-4 digital polarimeter, and NMR spectra were

recorded on a Brucker AM-500 FT-NMR spectrometer.

Antifungal activity. Antifungal activity was evaluated by spotting a $CHCl_3$ solution of each isolated compound on silica gel TLC plates (0.25 mm thick, Merck). After removing $CHCl_3$ under reduced pressure, spore suspensions of *Gaeumannomyces graminis* var. *tritici* and *Cladsporium herbarum* were respectively sprayed on the plates, before the plates were incubated at 22 °C under high humidity for 3 days.

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