Farinomalein, a Maleimide-Bearing Compound from the Entomopathogenic Fungus *Paecilomyces farinosus*

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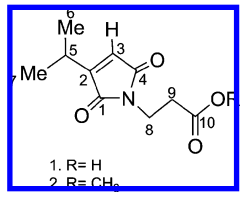
A new maleimide-bearing compound, farinomalein (**1**), was isolated from the entomopathogenic fungus *Paecilomyces farinosus* HF599. The structure was determined on the basis of spectroscopic analyses and chemical conversion. Compound **1** showed potent activity (5 μg/disk) against the plant pathogenic *Phytophthora sojae* P6497.

Entomopathogenic fungi are well known for their ability to produce various bioactive compounds during infection and proliferation in insects[1−3] and, thus, are considered as potential sources of novel bioactive compounds.[4−6] Those belonging to the genus *Paecilomyces* have been the source of a wide range of bioactive metabolites, including the antimalarial and antitumor cyclohexadepsipeptide paecilodepsipeptide A from *P. cinnamomeus*,[7] an antibiotic containing a tetramic acid moiety (paecilosetin from *P. farinosus*[8]), and neuritogenic pyridine alkaloids (farinosones A−C and militarinones A−D from *P. farinosus* and *P. militaris*, respectively[9,10]). During screening for an antioomycete compound against plant pathogenic oomycetes, the EtOAc extract of *P. farinosus* HF599 showed strong inhibitory activity against *Phytophthora sojae*.[11] Detailed investigation of the fungal extract led to the identification of the new maleimide compound 3-(3-isopropyl-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)propanoic acid, which we have named farinomalein (**1**). Farinomalein (**1**) showed potent inhibition of the plant pathogen *Phytophthora sojae,* with an MIC value of 5 μg/disk, whereas the MIC of the antifungal agent amphotericin B is 10 μg/disk. We herein describe the isolation, structure elucidation, and biological activity of farinomalein (**1**).

The producing strain was isolated from a lepidopteran larval cadaver collected on Mt. Tsukuba, Ibaraki, Japan. This fungus was identified as *Paecilomyces farinosus* on the basis of morphological criteria. Compound **1**, the major metabolite of *P. farinosus* HF599, was not detected in the extract of another active isolate of *P. farinosus*, HF656 (data not shown).



Farinomalein (**1**) was obtained as a white powder. High-resolution ESITOFMS revealed an $[M + Na]^+$ at *m/z* 234.0710, corresponding to the molecular formula $C_{10}H_{13}NO_4$ (calcd for $C_{10}H_{13}NO_4Na$, 234.0737). The ^1H and ^{13}C NMR spectroscopic data (Table 1) were in agreement with the assigned molecular formula. The ^{13}C NMR spectrum of **1** showed 10 signals, indicating five aliphatic carbons (one of them bonded to nitrogen), two carbons in the olefinic region, and two carbonyl carbons. The ^1H NMR spectrum in combination with the HSQC spectrum revealed proton signals for two methyl, two methylene, an aliphatic methine, and an olefinic methine group. The COSY spectrum demonstrated two fragments: H-6−H-5−H-7 and H-8−H-9 (Figure 1). HMBC correlations from H-3 to C-1, C-2, and C-4 and from H-5 to C-1, C-2, and C-3 established the maleimide core and the substitution of an isopropyl group at C-2. Furthermore, the HMBC correlation from H-8 to C-1 and C-4 suggested bonding of C-8 to the maleimide nitrogen. HMBC correlations from H-8 and H-9 to C-10, in consideration of the molecular formula, led to the suggestion of the presence of a carboxylic acid at the terminus. The location of the nitrogen was confirmed by the observation of $^1H-^{15}N$ long-range couplings between the protons H-3, H-8, and H-9 and the nitrogen (δ 147.1). For verification of the presence of carboxylic acid, **1** was treated with MeI and DBU to afford a methylated derivative (**2**). In the ^1H NMR of **2**, a singlet methyl was observed at δ 3.64, and it showed HMBC correlation to C-10, thereby confirming the location of carboxylic acid. The UV absorption at 223 nm is typical for a maleimide ring.[12] High-resolution ESITOFMS of **2** revealed an $[M + Na]^+$ at *m/z* 248.0910 corresponding to the molecular formula $C_{11}H_{15}NO_4$ (calcd for $C_{11}H_{15}NO_4Na$, 248.0893). The IR spectrum was consistent with the presence of the symmetric (1778 cm^{-1}) and asymmetric (1717 cm^{-1}) maleimide C═O stretches and the maleimide symmetric C−N−C stretch (∼1405 cm^{-1}),[13] as well as the presence of OH (3113 cm^{-1}) and carbonyl (1702 cm^{-1}) groups, thereby confirming the structure of **1** as indicated.

Reports of natural compounds bearing maleimide rings are very limited. Examples include showdomycin from *Streptomyces showdoensis*, pencolide from *Penicillium multicolor*, and turrapubesin from the twigs and leaves of *Turraea pubescens.*[14−16] The isopropyl moiety attached at C-2 on the maleimide ring is, to our best knowledge, a unique structural feature that has not been reported previously in natural compounds.

Recently, maleimides such as *N*-methylmaleimide, *N*-ethylmaleimide, and phenylmaleimide PM-20 have attracted the interest of many researchers due to their cytotoxicity toward tumor cell lines through the inhibition of human topoisomerase II[17] or the inhibition of Cdc25A.[18] These facts suggested that, in addition to its potent antioomycete activity, farinomalein (**1**) could also possess other biological functions, such as cytotoxicity toward tumor cell lines.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. IR spectra were recorded on a

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Table 1. NMR Spectroscopic Data for Farinomalein (**1**) and Its Methyl Ester (**2**) in CD_3OD

	farinomalein (**1**)			farinomalein-Me (**2**)		
position	δ_H (mult, J in Hz)[b]	δ_C[c]	HMBC[b]	δ_H	δ_C	HMBC
1		172.6[a]			170.8[a]	
2		157.3			156.0	
3	6.37 (d, 1.6)	126.0	1, 2, 4, 5, 6, (7)	6.37 (d, 1.6)	126.0	
4		172.3[a]			170.8[a]	
5	2.80 (dh, 1.6, 6.9)	27.2	1, 2, 3, 6, (7)	2.80 (dh, 1.6, 6.9)	27.2	
6, 7	1.21 (d, 6.9)	21.3	2, 5, 6, 7	1.21 (d, 6.9)	21.3	2, 5, 6, 7
8	3.74 (t, 7.2)	34.9	1, 4, 9, 10	3.75 (t, 7.0)	34.9	1, 9
9	2.58 (t, 7.2)	34.0	8, 10	2.60 (t, 7.0)	34.0	8, 10
10		175.0			172.0	
11				3.64 (s)	52.5	10

[a] Exchangeable. [b] Recorded at 500 MHz. [c] Recorded at 100 MHz.

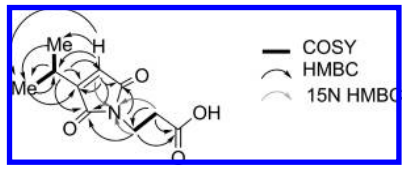

Figure 1. COSY, HMBC, and ^{15}N HMBC correlations of **1**.

Perkin-Elmer Spectrum 100 spectrophotometer. NMR spectra were obtained on a Bruker AV400 M or on a Bruker AVANCE 500 spectrometer. The ^1H and ^{13}C chemical shifts were referenced to the solvent signals (δ_H 3.3 and δ_C 49.1 in CD_3OD). The ^{15}N chemical shift was referenced to the external signal of formamide. HRESITOFMS were recorded on a Bruker microTOF focus spectrometer. HPLC analyses were carried out on an Agilent HP1100 system using a Cosmosil 5C18-AR-II column (4.6 × 100 mm; Nacalai Tesque Inc.). The solvent used to dissolve **1** was methanol unless stated otherwise.

Microorganism. The entomopathogenic fungus *Paecilomyces farinosus* HF599 was isolated from a Lepidopteran larval cadaver collected on Mt. Tsukuba, Ibaraki, Japan. Fungal conidia that developed on the surface of the dead larva were transferred to SMY and incubated at 25 °C for several days.[20] After the conidia had developed on SMY slants, the strain was kept at −30 °C as a stock culture. The isolated strain was identified as *P. farinosus* according to its morphology by one of the authors (F.I.). The fungus is deposited at the culture collection of National Institute of Fruit Tree Science.

Fermentation. All chemicals, media, and reagents were purchased from Wako, Japan, unless stated otherwise. The seed culture was prepared as follows. The mycelium of *P. farinosus* HF599 grown on a slant culture was inoculated into 100 mL flasks each containing 30 mL of the seed medium, SMY [maltose 4%, yeast extract 1%, peptone 1%], and cultivated at 25 °C for 3 days. The seed culture (5 mL) was transferred into 500 mL baffled flasks containing 250 mL of the production medium [SMY supplemented with Diaion HP-20 (Mitsubishi Chemical Co., Japan) 1%], which were cultured at 25 °C for 21 days under static conditions.

Extraction and Isolation. The mixture of 21-day-old mycelium and fermentation broth of strain HF599 (250 mL × 8 flasks) was extracted with ethyl acetate (without prior saturation with water, 250 mL per flask) by stirring for 1 h. The mixture was separated by filtration using Miracloth (Calbiochem, San Diego, CA), and the filtrate was again extracted with 1 L of ethyl acetate. The organic layer was separated from the aqueous layer in an extraction funnel and dried with anhydrous Na_2SO_4. Evaporation of the solvent provided approximately 1 g of extract per 2 L of culture. A portion of the crude extract (500 mg) was subjected to reversed-phase column chromatography using a Sep-Pak Vac 35 cm^3 (10 g) C_{18} cartridge (Waters, Millford, MA) with a step gradient of CH_3CN-H_2O (0:1, 1:9, 2:8, and 1:0 v/v). Final purification was conducted by reversed-phase HPLC on a Shiseido Capcell-Pak C_{18} column (5 μm; 250 × 10 mm i.d.) with 20% CH_3CN + 0.1% TFA to yield **1** (45.6 mg/g extract).

Farinomalein (1): white powder; UV (MeOH) λ_{max} (log ε) 223 (4.27), 288 (2.26); IR 3113, 1778, 1717, 1702, 1405; ^1H, ^{13}C NMR, HMBC, see Table 1; HRESITOFMS m/z $[M + Na]^+$ 234.0710 (calcd for $C_{10}H_{13}NO_4$ Na 234.0737).

Methylation of Farinomalein (1). A solution of **1** (500 μg), methyl iodide (few drops), and 1,8-diazabicyclo[5.4.0]-7-undecene (trace amount) in acetone (50 μL) and CH_3CN (50 μL) was heated at 50 °C for 1 h in darkness. The reaction mixture was diluted with water and extracted with EtOAc. The organic layer was successively washed with diluted aqueous HCl and brine and dried over anhydrous Na_2SO_4. The organic layer was concentrated *in vacuo* to obtain methyl ester **2**. The obtained residue (200 μg) containing the methyl ester as the major constituent (>90% purity as shown by HPLC) was directly used for analysis.

Methyl Ester of Farinomalein (2): ^1H, ^{13}C NMR,[22] HMBC, see Table 1; HRESITOFMS m/z $[M + Na]^+$ 248.0910 (calcd for $C_{11}H_{15}NO_4Na$, 248.0893).

Antioomycete Assay. *In vitro* antioomycete activity was determined by the disk diffusion susceptibility test following the method described previously[21] with several modifications. Loaded paper disks (1, 5, 10, 50, 100 μg/disk) (8.0 mm; Advantec, Japan) were air-dried on a clean bench for 30 min prior to placing on the Petri dish. The minimal inhibitory concentration was defined as the lowest concentration resulting in inhibition of mycelial growth after 48−96 h of incubation at 25 °C. The indicator strain, *P. sojae* P6497, was grown at 25 °C on V8-juice agar [Vvegetable juice without NaCl (COOP, Japan) 20% v/v, $CaCO_3$ 0.2%, agar 1.8%]. The reference compound used in this assay, amphotericin B, was dissolved in DMSO. No growth inhibition was observed in the control disks (loaded with 50 μL of MeOH or DMSO).

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Supporting Information Available: ^1H NMR, ^{13}C NMR, and HMBC spectra of farinomalein (**1**) and ^1H NMR, HSQC, and HMBC spectra of its methyl ester (**2**). This information is available free of charge via the Internet at http://pubs.acs.org.

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(22) Due to the limited amount of the sample, ^{13}C signals were not fully detected and the chemical shifts were determined indirectly by analyzing HSQC and HMBC spectra.

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