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Note

Identification of a Degradation Intermediate of the Momilactone A Rice Phytoalexin by the Rice Blast Fungus

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We have already shown that major rice diterpene phytoalexin, momilactone A, was detoxified by *Magnaporthe oryzae*. We report here the identification by NMR, MS, and chemical synthesis of 3,6-dioxo-19-nor-9β-pimara-7,15-diene (1) as the degradation intermediate. Compound 1 exhibited similar antifungal activity to that of momilactone A, indicating 1 to be a precursor of possible detoxified compounds.

Key words: phytoalexin; rice; blast fungus; detoxification; momilactone

Phytoalexins are low-molecular-weight antimicrobial compounds which accumulate in plants invaded by pathogenic microorganisms.[1,2] The accumulation of phytoalexins is recognized as one of the mechanisms for the induced resistance of higher plants against pathogens. However, pathogenic microorganisms have the ability to avoid the resistance mechanisms of plants. The detoxification of phytoalexins is an effective solution to evade the growth inhibition of pathogenic microorganisms caused by phytoalexins.[3,4]

We have recently reported that momilactone A (Fig. 1), a major diterpene phytoalexin, was detoxified by the rice blast fungus, *Magnaporthe oryzae*, and that a possible metabolite of momilactone A was maximized after 4 h and then lost after 8 h in a fungal suspension culture containing momilactone A.[5] We therefore tried to purify the metabolite and elucidate its structure. A GC/MS analysis showed the metabolite to exhibit a plausible molecular ion at m/z 286, so we have designated it as M286. Fractionation guided by a GC/MS analysis, using solvent extraction and ODS-HPLC separation, gave 1.32 mg of M286 from two batches of a blast fungal suspension culture (80 mL) 4 h after adding 5 mg of momilactone A.

The high-resolution mass spectrum of M286 gave a molecular weight of 286.1909, agreeing with the molecular formula $C_{19}H_{26}O_2$ (calcd. as 286.1933). This formula indicated the loss of one carbon and one oxygen from momilactone A ($C_{20}H_{26}O_3$). ^{1}H- and ^{13}C-NMR, COSY, HSQC, HMBC and a comparison of ^{1}H- and ^{13}C-NMR data with those of momilactone A[6] suggested that the original carbon skeleton of momilactone A, except for γ-lactone, had been preserved in the structure of M286 (Table 1, Fig. 1). A methyl doublet signal at δ_H 1.21 ppm (H-18), a methine signal at δ_C 40.55 ppm, and the absence of a carbonyl carbon signal at around δ_C 170 ppm suggested a modification to the γ-lactone ring of momilactone A, the carbonyl carbon of which appeared at δ_C 174.25 ppm.[6] Two carbonyl signals (δ_C 198.81 and 212.26 ppm) were observed in the ^{13}C-NMR data of M286. The carbonyl signal at δ_C 212.26 ppm was assigned to a C-3 ketone by HMBC correlations from H-2/H-18 to C-3. The carbonyl signal at δ_C 198.81 ppm was reasonable for an α,β-unsaturated ketone, and an HMBC correlation with H-5 supported the assignment of this signal as a C-6 ketone. COSY correlation between H-5 and a methine signal at δ_H 2.69 ppm suggested that the methine proton was H-4. The large $J_{4,5}$ coupling (10.7 Hz) should represent an anti relationship between H-4 and H-5. NOESY cross-peaks between H-4 and H-20, and between H-5 and H-18 supported this configuration. M286 was thus identified as 3,6-dioxo-19-nor-9β-pimara-7,15-diene (Fig. 1, **1**).

Compound **1** was chemically synthesized from momilactone A to confirm the spectroscopic elucidation of the structure. 6β-Hydroxy-3-oxo-19-nor-9β-pimara-7,15-diene (**2**) has already been reported as a decarboxylation product of momilactone A which was designated as 19-nor-MA in the original report.[7] Compound **2** was converted to **1** by Dess-Martin oxidation. The identity of compound **1** obtained by the chemical synthesis and by fungal biotransformation was confirmed by a comparison of the NMR, MS, t_R in GC/MS, and specific rotation data.

Compound **1** and momilactone A were extracted after different incubation times from a rice blast fungus suspension culture containing 300 µM (*ca.* 100 ppm) momilactone A, and were quantified by LC/MS/MS. The results are summarized in Fig. 2. The level of momilactone A rapidly decreased up to 8 h after the addition of momilactone A, and then gradually decreased up to 24 h. The level of **1** reached a maximum 4 h after the addition of momilactone A. The concentration of **1** at the maximum level was approximately 70 µM, this being approximately 1/4 of the initial concentration of momilactone A. However, the level of momilactone A

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Abbreviations: SRM, selected reaction monitoring; PDB, potato dextrose broth

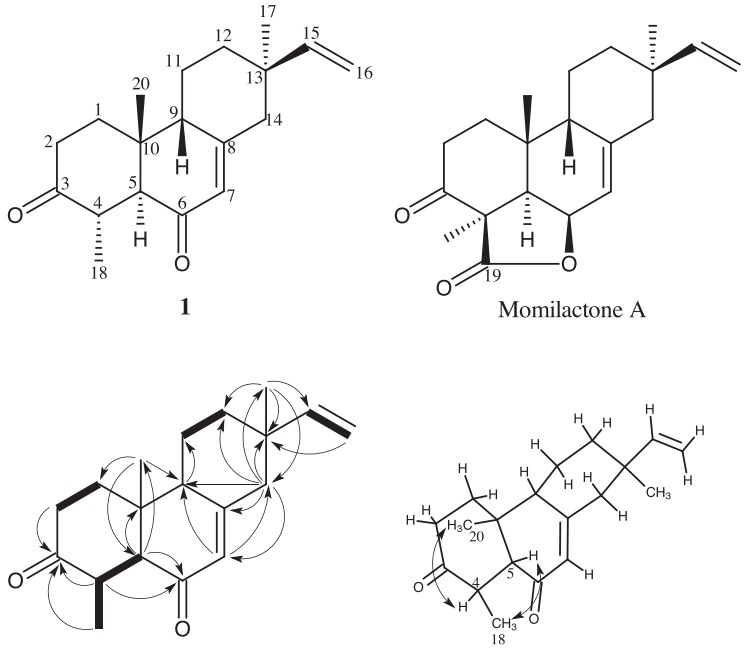

Fig. 1. Structures of 3,6-Dioxo-19-nor-9β-pimara-7,15-diene (**1**) and Momilactone A, and COSY, Selected HMBC, and Selected NOESY Correlations for **1**.

COSY correlations are represented by bold lines, HMBC correlations are represented by single-headed arrows from H to C, and NOESY correlations are represented by double-headed arrows. COSY correlations between H-9 and H-11 were not clearly observed because of the overlapping signals.

Table 1. ^{13}C–^{1}H-NMR Data for 3,6-Dioxo-19-nor-9β-pimara-7,15-diene (**1**)

Position	δ_C	δ_H (multiplicity, J in Hz)
1	35.69	1.63 (1H, m, overlapped)
		2.01 (1H, m, overlapped)
2	36.95	2.40 (1H, ddd, 14.8, 4.3, 2.8)
		2.55 (1H, dddd, 14.8, 14.8, 5.4, 0.9)
3	212.26	—
4	40.55	2.69 (1H, dqd, 10.7, 6.4, 0.9)
5	55.44	2.45 (1H, d, 10.7)
6	198.81	—
7	124.25	5.82 (1H, br s)
8	160.42	—
9	51.61	1.97 (1H, m, overlapped)
10	38.19	—
11	23.76	1.61 (1H, m, overlapped)
		1.88 (1H, m)
12	36.83	1.63 (2H, m, overlapped)
13	40.94	—
14	47.58	2.13 (1H, dd, 12.0, 2.2)
		2.28 (1H, br d, 12.0)
15	148.49	5.86 (1H, dd, 17.5, 10.7)
16	110.51	4.97 (1H, dd, 10.7, 1.0)
		5.01 (1H, dd, 17.5, 1.0)
17	22.05	0.95 (3H, s)
18	14.32	1.21 (3H, d, 6.4)
20	20.52	1.15 (3H, s)

δ_H values for the overlapped signals were estimated by an HSQC experiment.

decreased to approximately 2/3 of its initial level after 4 h of incubation. Compound **1** could not be detected in a suspension culture without momilactone A, in a suspension culture heated in boiling water, or in a culture without the fungus (data not shown). These results suggest that **1** would have been one of the major intermediates during the fungal degradation of momilactone A. The accumulation of **1** remained at the maximal level for 1 h, and then decreased to almost the zero level 8 h after the addition of momilactone A. This result indicates that **1** must have been rapidly converted to other compounds by the rice blast fungus. The identification of the converted products is one objective of our future research.

The inhibitory activity against spore germination of the rice blast fungus was almost the same as that of momilactone A. IC_{50} values for **1** and momilactone A were 0.50 mM and 0.54 mM, respectively. We had concluded in our previous report[5] that the rice blast fungus could detoxify the phytoalexin, momilactone A, because the antifungal activity of a rice blast fungus suspension culture became lower after 8 h of incubation with momilactone A. This result suggested that **1** could be an intermediate in the detoxification pathway for momilactone A, and that further conversion of **1** would be necessary for detoxification.

Experimental

General analytical methods. ^{1}H-NMR (400.13 MHz), ^{13}C-NMR (100.61 MHz) and 2D-NMR spectra were measured in $CDCl_3$ with TMS as an internal standard by an AVANCE III FT-NMR spectrometer (Bruker BioSpin) equipped with a 5-mm BBFO probe. The IR spectrum was measured as a KBr pellet by an FT/IR-4100 spectrometer (Jasco) and the UV spectrum was measured in MeOH by a V-550 spectrometer (Jasco). The specific rotation was measured in $CHCl_3$ by a P-2100 polarimeter equipped with a halogen lamp and a 589 nm filter (Jasco). The mass spectra were measured by a JMS-BU25 (GCmate II) mass spectrometer (Jeol) in the direct inlet EI mode (70 eV).

Chemical. Momilactone A was purified from rice husks according to the method reported by Kato *et al.*[8] with some modifications.

Fungal material. The rice blast fungus (*Magnaporthe oryzae* strain P-2) had been maintained on potato dextrose agar (Nissui Pharmaceutical) as a stock culture in our laboratory. A small piece of this stock culture was inoculated and grown on potato dextrose agar or oatmeal agar in a Petri dish (9 mm in diameter) at 26 °C in the dark before inoculating into the suspension culture.

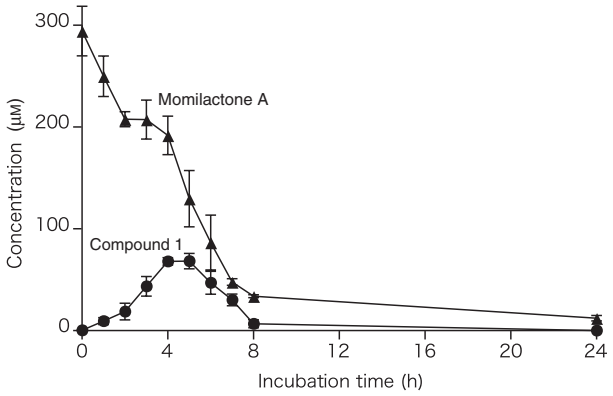

Fig. 2. Time-Dependent Accumulation of 3,6-Dioxo-19-nor-9β-pimara-7,15-diene (**1**) and Degradation of Momilactone A in a Rice Blast Fungus Suspension Culture Containing Momilactone A.

Compound **1** and momilactone A were extracted from the rice blast fungus suspension culture at different times after adding momilactone A. Their concentrations were quantified by using LC/MS/MS. Values are presented as the mean $\pm$ SD ($n = 3$). The concentrations of **1** and momilactone A are respectively represented by circles and triangles.

*Purification of 3,6-dioxo-19-nor-9β-pimara-7,15-diene (**1**) from the rice blast fungus suspension culture containing momilactone A.* The mycelia of rice blast fungus grown on potato dextrose agar were inoculated into 80 mL of a potato dextrose broth medium (PDB; Sigma-Aldrich). After the suspension culture had been incubated at 27 °C for 2 months in the dark while shaking at 150 rpm, 5 mg of momilactone A (1 mL of an MeOH solution) was added to the culture. After a further 4 h of incubation, 373 mL of MeOH was added to the culture. The aqueous MeOH suspension was heated in a boiling water bath for 5 min. The suspension was filtered through cotton to remove the mycelia, and the resulting filtrate was evaporated *in vacuo*. After adding 100 mL of distilled water to the residue, the aqueous solution was extracted with EtOAc (100 mL × 3). The EtOAc extract was evaporated *in vacuo* to dryness to give a crude extract (*ca.* 25 mg). A 170 μL amount of a 10,000 ppm MeOH solution of the crude extract was repeatedly separated by ODS-HPLC under the following conditions: column, Cosmosil 5C18AR (1 cm i.d. × 25 cm, 5 μm particle size; Nacalai Tesque); solvent, 50% (v/v) aq. MeCN; flow rate, 2.0 mL min^{-1}; column oven temperature, 40 °C; detection, UV 210 nm. Compound **1** was eluted immediately after momilactone A (t_R 44.0 min) as an isolated peak at t_R 46.9 min. The elution of compound **1** was confirmed by a GC/MS analysis under the previously described conditions.[5] The foregoing procedure was conducted twice to purify compound **1** (1.32 mg). **1**. HRMS m/z (M^+): calcd. for $C_{19}H_{26}O_2$, 286.1933; found, 286.1909; NMR: see Table 1; EIMS m/z (rel. int.): 81 (72), 91 (59), 105 (55), 119 (53), 147 (71), 203 (74), 215 (39), 229 (64), 243 (37), 258 (23), 271 (45), 286 (M^+, 100); $[\alpha]_D^{25}$ −256° (*c* 0.095, $CHCl_3$).

*Preparation of 3,6-dioxo-19-nor-9β-pimara-7,15-diene (**1**) from momilactone A.* An aqueous KOH solution (1 M, 1.2 mL) was added to an MeOH solution of momilactone A (40 mg in 2.0 mL). The mixture was stirred for 2 h at 80 °C, and then water (10 mL) was added to stop the reaction. After extracting with EtOAc (10 mL × 3), the EtOAc extract was evaporated to give a crude product. This crude product was separated by preparative TLC developed with *n*-hexane/EtOAc (1:1, v/v). An R_f 0.70–0.81 TLC region was scraped off and extracted with EtOAc. The resulting extract was evaporated to dryness *in vacuo* to give 6β-hydroxy-3-oxo-19-nor-9β-pimara-7,15-diene (**2**, 32.6 mg, 89%). Dess-Martin periodinane (96 mg, 2.0 eq.) was added to a CH_2Cl_2 (1.2 mL) solution of **2** (32.6 mg). The mixture was stirred for 1 d at room temperature and, after adding a sat. aqueous $NaHCO_3$ solution (3 mL) and water (7 mL), the mixture was extracted with EtOAc (10 mL × 3). The EtOAc extract was evaporated to give a crude product which was separated by preparative TLC developed with benzene/EtOAc (2:1, v/v). An R_f 0.75–0.81 TLC region was scraped off and extracted with EtOAc. The extract was evaporated to dryness *in vacuo* to give 3,6-dioxo-19-nor-9β-pimara-7,15-diene (**1**, 26.5 mg, 82%). NMR and MS data were identical to those of **1** which had been purified from an *M. oryzae* culture containing momilactone A. **1**. UV λ_{max} (MeOH) nm (ε): 243 (14,300); IR $\tilde{\nu}_{max}$ (KBr) cm^{-1}: 1712, 1673; $[\alpha]_D^{26}$ −254° (*c* 0.41, $CHCl_3$).

*Quantification of 3,6-dioxo-19-nor-9β-pimara-7,15-diene (**1**) in a rice blast fungus culture containing momilactone A.* The fungal layers on an oatmeal medium were cut into squares of approximately 1 mm^2. The small pieces were suspended in a PDB liquid medium (150 mL) and then incubated for 6 d in the dark at 27 °C with rotary shaking at 150 rpm. A portion of the culture (30 mL) was transferred to a fresh PDB medium (120 mL) and further incubated for 1 d under the same conditions. Approximately ten fungal clusters were collected from the culture and transferred to a fresh PDB medium (1 mL), to which 20 mM momilactone A in MeOH (15 μL) was added. The medium was incubated for 1–24 h at 27 °C with reciprocal shaking at 200 strokes per min. MeOH (2.3 mL) was added to the culture after this incubation. A portion of the supernatant (30 μL) was diluted with MeOH (0.87 mL), and 5 μL of the solution was subjected to an LC/MS/MS analysis to quantify **1** and momilactone A. The LC/MS/MS analysis was performed with an AB SCIEX 3200 QTRAP LC/MS/MS system coupled with a Shimadzu Prominence UFLC system. Liquid chromatographic separation of the analytes was achieved in a Shim-pack XR-ODS column (2.0 mm i.d. × 30 mm, 2.2 μm particle size; Shimadzu) with a linear binary gradient of 0.1% (v/v) aqueous acetic acid containing 10 mM ammonium acetate (solvent A) and MeOH (solvent B) at a flow rate of 0.2 mL min^{-1} at 40 °C. The graded solvent was programmed as follows: initial, 20% B; 0–5 min, a linear gradient from 20% B to 100% B; 5–7 min, isocratic elution by 100% B. The equilibration time between two runs was 3 min. The parameters of the mass spectrometer were optimized for detecting **1** and momilactone A by using Analyst 1.5.1 software (AB SCIEX) for selective reaction monitoring (SRM). The ion source (Turbo V) was operated in the positive ESI mode. The following SRM transitions were monitored: **1**, 287→175; momilactone A, 315→271. Calibration curves were obtained from the SRM peak areas of standards for **1** and momilactone A in respective concentration ranges of 10–10000 ng mL^{-1} and 1–1000 ng mL^{-1}.

Assay of the inhibitory activity against spore germination of the rice blast fungus. A spore suspension of the rice blast fungus (strain P-2) was prepared by the method described previously.[9] The antifungal activity was evaluated by using the spore suspension according to the previously reported method.[10]

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