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Biosynthesis of Rice Phytoalexin: Enzymatic Conversion of 3β-Hydroxy-9β-pimara-7,15-dien-19,6β-olide to Momilactone A

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Momilactone A, a major rice diterpene phytoalexin, could be synthesized by dehydrogenation at the 3-position of 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide in rice leaves. The presence of 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide in UV-irradiated rice leaves was confirmed by comparing the mass spectra and retention times after a GC/MS analysis of the natural and synthetic compounds. The soluble protein fraction from UV-irradiated rice leaves showed dehydrogenase activity to convert 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide into momilactone A. The enzyme required NAD^+ or $NADP^+$ as a hydrogen acceptor. The optimum pH for the reaction was 8. The K_m value to 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide was 36 μM when NAD^+ was supplied as a cofactor at a concentration of 1 mM. 3β-Hydroxy-9β-pimara-7,15-dien-19,6β-olide and its dehydrogenase activity were induced in a time-dependent manner by UV irradiation.

Key words: momilactone A; dehydrogenase; phytoalexin; diterpene; 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide

The rice plant produces diterpene and flavanone phytoalexins which are involved in the defense mechanism of the plant.[1–4] Their production in the rice plant is triggered by fungal invasion, heavy metals such as copper chloride or UV irradiation. The biosynthetic pathway and enzyme of the flavanone phytoalexin, sakuranetin, have been studied in detail;[5,6] however, in the case of diterpene phytoalexins, information on their biosynthetic pathway is limited. It has been reported that geranylgeranyl diphosphate was cyclized to yield 9βH-pimara-7,15-diene as the precursor of momilactone A.[7] However, the remaining steps leading to the biosynthesis of momilactone A have not previously been elucidated. The work described in this paper was undertaken to provide information about the last step of momilactone A biosynthesis. We hypothesized that momilactone A could be synthesized by dehydrogenation at the 3-position of 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide (**1**) and that this reaction would be catalyzed by dehydrogenase (Fig. 1). Although compound **1** has been reported as a reduction product of momilactone A,[8] the natural occurrence of this compound has not previously been reported. The presence of **1** in rice plants was therefore confirmed by a GC/MS analysis, and the conversion of **1** to momilactone A by dehydrogenase was studied.

Materials and Methods

Chemicals. Momilactone A was purified from rice husks in our laboratory. 3β-Hydroxy-9β-pimara-7,15-dien-19,6β-olide (**1**) was prepared by the reduction of momilactone A with lithium aluminum hydride by the method of Kato *et al.*[8]

Plant material. Rice plants (*Oryza sativa* L. cv. Nipponbare) were cultivated in a greenhouse, and at

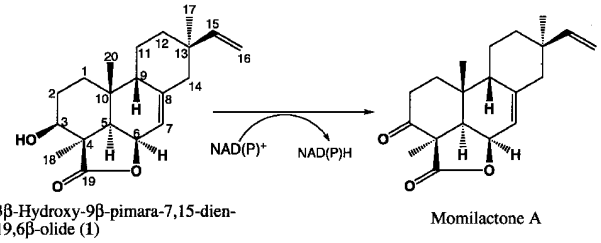

Fig. 1. Proposed Pathway for the Oxidation of 3β-Hydroxy-9β-pimara-7,15-dien-19,6β-olide (**1**) to Momilactone A by Dehydrogenase.

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Abbreviations: CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; MRM, multiple reaction monitoring

the sixth-leaf stage, the fourth- and fifth-stage leaves were detached for use as experimental material. These detached rice leaves were UV-irradiated and incubated as described previously.[4]

Identification of 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide (1). Rice leaves (1.2 g), which had been incubated for 2 d after UV irradiation, were boiled in 6 ml of 70% aqueous MeOH for 20 min. After 3 ml of brine had been added to the extract, the mixture was partitioned against 9 ml of EtOAc. The EtOAc layer was concentrated to dryness and adsorbed to a Sep-Pak Light Silica cartridge (Waters) with *n*-hexane. After the cartridge had been washed with 20% EtOAc in *n*-hexane, the fraction eluted with 50% EtOAc in *n*-hexane was collected and evaporated to dryness. The concentrate was dissolved in 10 μl of acetone. A 1-μl aliquot of the synthetic compound **1** solution (10 ppm in acetone) and 1 μl of the extract obtained from UV-irradiated rice leaves were each directly injected into the GC/MS instrument (Saturn 2000R, Varian) which was equipped with an ion-trap mass spectrometer. Separation was carried out with a CP-Sil 8 CB low-bleed/MS column (0.25 mm × 30 m, 0.25 μm film thickness, Varian). Helium was used as the carrier gas at a flow rate of 1.5 ml/min, the injector temperature was 250°C, and the temperature program of a column oven was 60°C for 1 min, before heating to 300°C at 10°C/min. The conditions used for the mass spectrometer were as follows: ionization mode, EI (70 eV); ion trap temperature, 220°C; scan range, *m/z* 70-600; scan rate, 1 s/scan.

Protein assay. The protein concentration was measured by the method of Bradford *et al.*,[9] using bovine serum albumin as a standard.

Enzyme preparation. Rice leaves were collected at 2 d after UV irradiation or at the times indicated in the time-course experiment. The leaves were cut into small pieces and then homogenized with a mortar and pestle on ice in 5 ml per gram fresh tissue weight of a buffer consisting of 0.2 M Tris-HCl at pH 8.5, 10 mM 2-mercaptoethanol, 1 mM of phenylmethylsulfonyl fluoride, and 1% (w/v) polyvinylpolypyrrolidone. The subsequent extraction was performed at 4°C. The resulting homogenate was filtered through four layers of cheesecloth, and the filtrate centrifuged at 10,000 × *g* for 40 min to remove the dense membranes and other cellular debris. The resulting supernatant, which is referred to as the crude extract, was subjected to ultracentrifugation at 100,000 × *g* for 60 min to provide a supernatant (termed the soluble protein fraction) and pellet (microsome fraction). This pellet was dissolved in the small amount of the extraction buffer. The microsome fraction and crude extract were dialyzed with a 0.2 M Tris-HCl buffer at pH 8.0 before the assay. The soluble protein fraction was concentrated by ultrafiltration with a Centricon YM-30 membrane (Millipore). This fraction was desalted in a PD-10 or NAP-10 column (Amersham Pharmacia Biotech) that had been equilibrated with the assay buffer or by dialysis with the 0.2 M Tris-HCl buffer at pH 8.0 before the assay. The protein concentrations of the crude extract, soluble protein fraction and microsome fraction were 100–200, 60–500 and 430 μg/ml, respectively, in the assay.

Enzyme assay for dehydrogenase. The reaction mixture containing 150 μl of the enzyme solution and 150 μl of the assay buffer consisting of 0.2 M Tris-HCl at pH 8.0 and 10 mM 2-mercaptoethanol was pre-incubated for 5 min at 30°C. To determine the optimum pH value, 0.2 M Hepes-NaOH, Tris-HCl and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)-NaOH were used as the assay buffer for the pH ranges of 6–8, 7–9 and 9–10, respectively. The reaction was initiated by adding 83 μl of 600 μM compound **1** in the assay buffer containing 0.5% DMSO, 100 μl of 5 mM NAD^+ in the assay buffer and 17 μl of distilled water. After incubating at 30°C for 40 min, the reaction was stopped by the addition of 0.5 ml of MeOH, before the solution was centrifuged (10,000 × *g* for 10 min). A 10-μl aliquot obtained from the assay was subjected to LC/MS/MS (Perkin-Elmer SCIEX API-300, Applied Biosystems), the instrument being equipped with an APCI inlet system, to determine the amount of momilactone A by the method described previously.[10] Momilactone A was detected at a combination of *m/z* 315/271 in the multiple reaction monitoring (MRM) mode.

Quantification of momilactone A and 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide (1). UV-irradiated rice leaves were collected at the time indicated. These leaves were cut into small pieces and then boiled in 70% aqueous MeOH (5 ml per gram fresh tissue weight) for 20 min. Ten μl of the extract was subjected to an LC/MS/MS analysis by the method described previously.[10] Compound **1** was detected at a combination of *m/z* 317/299 in the MRM mode.

Results and Discussion

Identification of 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide in UV-irradiated rice leaves

We had speculated that momilactone A would be biosynthesized from **1** by dehydrogenation at the 3-position. Since **1** had not been previously reported as a natural product prior to this investigation, it was necessary to clarify whether this compound was present in rice plants. The retention time and mass spectrum of synthetic **1** was determined by GC/MS with a capillary column. The peak at the same retention time had an identical mass spectrum when the

extract obtained from UV-irradiated rice leaves was analyzed by GC/MS (Table 1). This result indicates that **1** had been biosynthesized in the UV-irradiated rice leaves and may play a role as the precursor for momilactone A biosynthesis. This is the first report on **1** from a natural source. The antifungal activity of **1** was measured by the spore germination of the rice blast fungus, *Magnaporthe grisea*. Its activity was almost the same as that of momilactone A (data not shown).

Demonstration of 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide dehydrogenase

The last step for momilactone A biosynthesis had been speculated to be oxidation at the 3-position of **1** by dehydrogenase. In the 10,000×*g* UV-irradiated rice leaf supernatant, **1** was converted into momilactone A in the presence of $NAD(P)^+$, while the conversion of **1** into momilactone A in the presence of NAD^+ showed higher activity. Moreover, 70% of activity was also found in the presence of $NADP^+$. The boiled control and assay without a cofactor or **1** showed no activity for producing momilactone A (Fig. 2). These results demonstrate that momilactone A was enzymatically synthesized from **1** by $NAD(P)^+$-dependent dehydrogenase in the UV-irradiated rice leaves.

To study the enzymes involved in the biosynthesis of secondary metabolites, a GC/MS analysis or radioisotope tracing is usually used.[11–13] However, the GC/MS analysis usually requires some purification of the samples and takes more than 20 min for each analysis. Although radioisotope tracers are very useful, their availability is limited. We used in this study an LC/MS/MS analysis for the enzyme assay. The assay mixture could be directly analyzed, and the time for the analysis of each sample was less than 5 min. We propose that the LC/MS/MS technique can substitute for a GC/MS analysis and radioisotope tracing when investigating the biochemistry of secondary metabolites.

3β-Hydroxy-9β-pimara-7,15-dien-19,6β-olide dehydrogenase activity was detected in a soluble protein fraction

To determine whether the dehydrogenase activity existed in the soluble or microsome fraction, subcellular fractionation preparations were examined for their dehydrogenase activity to convert **1** to momilactone A. The result is shown in Table 2. After ultracentrifugation at 100,000×*g*, the dehydrogenase activity was retained in the supernatant, showing that it originated from the soluble enzyme. Only 1% of dehydrogenase activity was found in the microsome fraction. These results demonstrate that the dehydrogenase which synthesized momilactone A from **1** was a soluble protein.

Table 1. Comparison of the Retention Time and Relative Intensity of the Characteristic Ion between the Extract Obtained from UV-Irradiated Rice Leaves and Synthesized 3β-Hydroxy-9β-pimara-7,15-dien-19,6β-olide

	Retention time (min)	Diagnostic ion (*m*/*z*) with percentage abundance						
		183	199	239	255	273	288	316
Synthesized	22.116	43	45	100	38	46	26	7
Natural	22.109	52	55	100	38	49	26	10

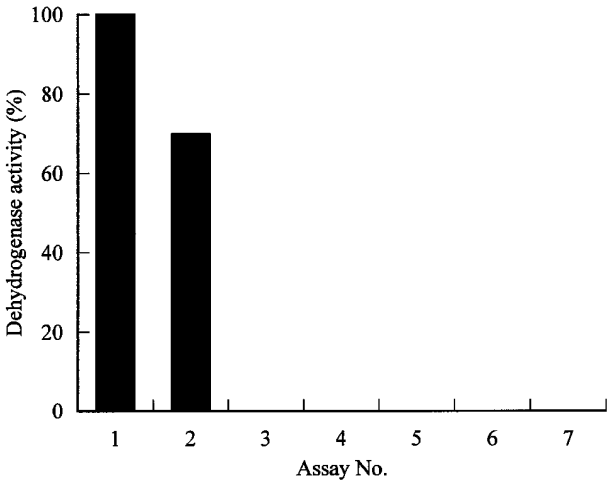

Fig. 2. Demonstration of Dehydrogenase in UV-Irradiated Rice Leaves.

The complete assay mixture contained the crude extract, a cofactor such as 1 mM NAD^+ (1) or 1 mM $NADP^+$ (2) and 100 μM compound **1**. The control was this assay mixture containing the denatured enzyme which had been prepared by dipping the crude extract into boiling water for 5 min before the assay. (3) and (4) are the control assay mixture respectively containing NAD^+ and $NADP^+$. An incomplete assay mixture containing the crude extract and compound **1** without any cofactor (5) was also prepared. Assay mixtures containing the enzyme and cofactors NAD^+ (6) and $NADP^+$ (7) without compound **1** were also prepared. The maximum activity corresponds to a specific activity of 120 fkat/mg of protein.

Table 2. Fractionation of the UV-Irradiated Rice Leaf Extract and Subcellular Localization of the Dehydrogenase

Fraction	Dehydrogenase activity in one gram of fresh leaf (% of crude extract)	Specific activity (fkat/mg of protein)
Crude extract	100	183
Microsome	1.1	21.8
Soluble protein	77	240

The crude extract was prepared by centrifuging the rice leaf extract at 10,000×*g*. After ultracentrifuging this crude extract at 100,000×*g*, the supernatant was obtained and is termed the soluble protein fraction. The microsome fraction was obtained from the resulting pellet. All of the fractions were dialyzed with 0.2 M Tris-HCl at pH 8 before the assay. The reaction mixtures are described in the Materials and Methods section. The activity corresponding to the 100% value was 248 fkat/g fresh weight equivalent of leaves.

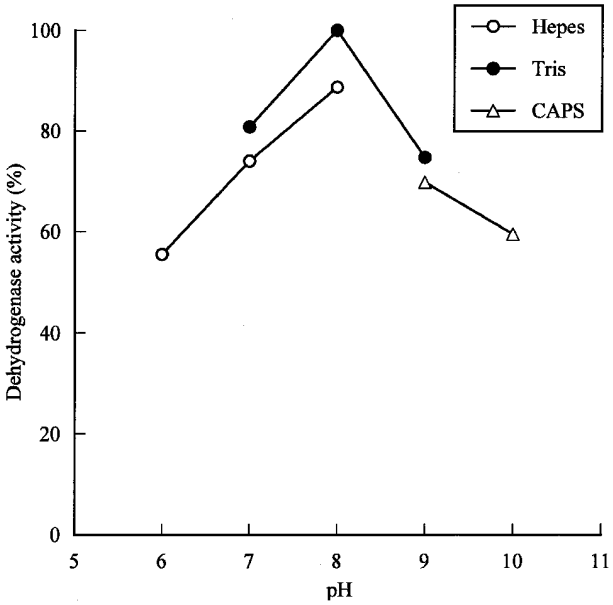

Fig. 3. Activity-pH Profile of Dehydrogenase Assayed for the Optimal pH for Activity with Different Buffers in the Range pH 6–10.

The experiment was performed with the soluble protein fraction used as the enzyme. Dehydrogenase activity is presented as a percentage of the maximum activity. The maximum activity corresponds to a specific activity of 41 fkat/mg of protein.

Characterization of 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide dehydrogenase

Some properties of the enzyme involved in the oxidation of **1** to form momilactone A have been established in this work. The optimum pH for the dehydrogenase was determined to be 8.0, with reduction of activity to 74% and 80% of the maximum at 1 pH unit above and below the optimum (Fig. 3). A substrate saturation experiment was performed under the optimum assay conditions with the soluble protein fraction, using **1** as a substrate at various concentrations. The K_m value for substrate **1** was estimated to be 36 μM from Lineweaver-Burk plots when NAD^+ was supplied as a cofactor at the concentration of 1 mM.

Time-dependent induction of momilactone A, 3β-hydroxy-9β-pimara-7,15-dien-19,6β-olide (1) and dehydrogenase activity in UV-irradiated rice leaves

Momilactone A and **1** were extracted after different incubation times from UV-irradiated rice leaves and quantified by LC/MS/MS. The level of momilactone A increased rapidly up to 48 hr and then slightly increased up to 72 hr after UV irradiation. The level of **1** reached a maximum 36 hr after irradiation (Fig. 4(A)). These results strongly support the notion that **1** was a precursor of momilactone A. The amount of **1** at the maximum level was about 20 times less than that of momilactone A, this being similar to the relationship between sakuranetin and its precursor, naringenin. The maximum level of

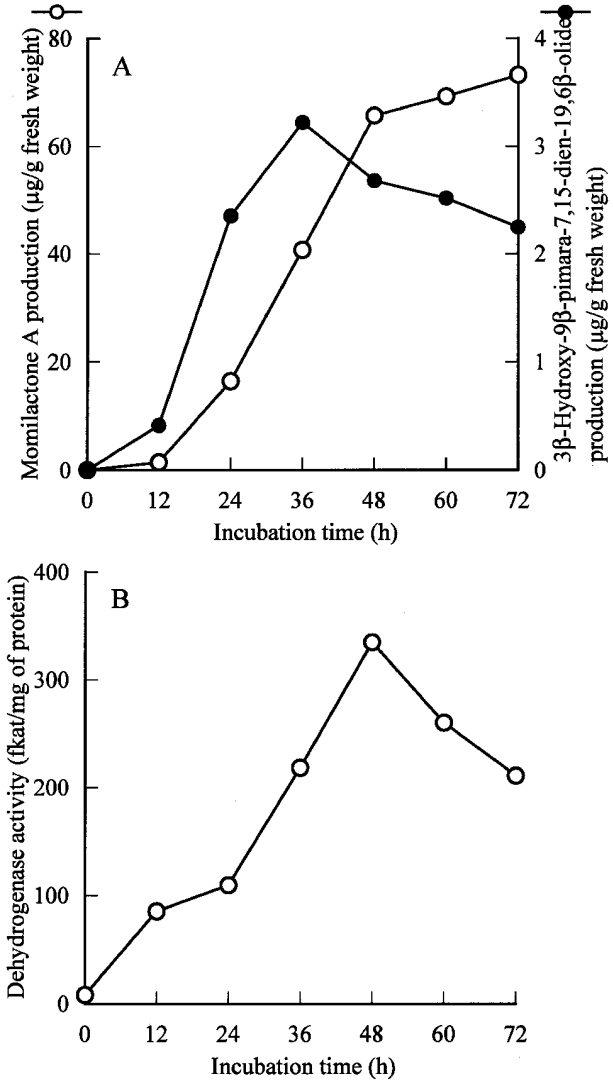

Fig. 4. Time-dependent Induction of Momilactone A (A, ○), 3β-Hydroxy-9β-pimara-7,15-dien-19,6β-olide (A, ●) and Dehydrogenase Activity (B) in UV-Irradiated Rice Leaves.

Following irradiation by UV light, the compounds were extracted with 70% MeOH, and the soluble protein fraction was prepared from the leaves at different time intervals. Details of the analysis are provided in the Materials and Methods section.

naringenin was 60 μg/g fresh weight of UV-irradiated rice leaves, while that of sakuranetin was 1.15 mg/g fresh weight (M. Hasegawa, unpublished results). These results suggest that **1** and naringenin were effectively converted to momilactone A and sakuranetin in UV-irradiated rice leaves.

The profile of dehydrogenase induction was also examined. Figure 4(B) shows that the dehydrogenase activity reached a maximum 48 hr after UV-irradiation. This result provides good correlation between the increase in enzyme activity and the accumulation of momilactone A. Neither the production of the compounds nor any dehydrogenase activity was induced in detached rice leaves which had not been UV-irradiated (data not shown).

The results reported here provided the information

about the involvement of dehydrogenase in momilactone A biosynthesis. Although there have been some reports on the involvement of dehydrogenase in terpenoid biosynthesis in plants,[11–13] this is the first report on a dehydrogenase being involved in a stress-inducible terpenoid compound.

References

1) Cartwright, D., Langcake, P., Pryce, R. J., Leworthy, D. P., and Ride, J. P., Chemical activation of host defence mechanisms as a basis for crop protection. *Nature*, **267**, 511–513 (1977).
2) Kato, H., Kodama, O., and Akatsuka, T., Oryzalexin F, a diterpene phytoalexin from UV-irradiated rice leaves. *Phytochemistry*, **36**, 299–301 (1994).
3) Koga, J., Ogawa, N., Yamauchi, T., Kikuchi, M., Ogasawara, N., and Shimura, M., Functional moiety for the antifungal activity of phytocassane E, a diterpene phytoalexin from rice. *Phytochemistry*, **44**, 249–253 (1997).
4) Kodama, O., Miyakawa, J., Akatsuka, T., and Kiyosawa, S., Sakuranetin, a flavanone phytoalexin from ultraviolet-irradiated rice leaves. *Phytochemistry*, **31**, 3807–3809 (1992).
5) Rakwal, R., Hasegawa, M., and Kodama, O., A methyltransferase for synthesis of the flavanone phytoalexin sakuranetin in rice leaves. *Biochem. Biophys. Res. Commun.*, **222**, 732–735 (1996).
6) Rakwal, R., Agrawal, G. K., Yonekura, M., and Kodama, O., Naringenin 7-*O*-methyltransferase involved in the biosynthesis of the flavanone phytoalexin sakuranetin from rice (*Oryza sativa* L.). *Plant Sci.*, **155**, 213–221 (2000).
7) Wickham, K. A. and West, C. A., Biosynthesis of rice phytoalexins: Identification of putative diterpene hydrocarbon precursors. *Arch. Biochem. Biophys.*, **293**, 320–332 (1992).
8) Kato, T., Aizawa, H., Tsunakawa, M., Sasaki, N., Kitahara, Y., and Takahashi, N., Chemical transformations of the diterpene lactones momilactones A and B. *J. Chem. Soc. Perkin I.*, 250–254 (1977).
9) Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
10) Nakazato, Y., Tamogami, S., Kawai, H., Hasegawa, M., and Kodama, O., Methionine-induced phytoalexin production in rice leaves. *Biosci. Biotechnol. Biochem.*, **64**, 577–583 (2000).
11) Bouwmeester, H. J., Gershenzon, J., Konings, M. C. J. M., and Croteau, R., Biosynthesis of the monoterpenes limonene and carvone in the fruit of caraway. I. Demonstration of enzyme activities and their changes with development. *Plant Physiol.*, **117**, 901–912 (1998).
12) Funk, C. and Croteau, R., Diterpenoid resin acid biosynthesis in conifers: Characterization of two cytochrome P450 dependent monooxygenases and an aldehyde dehydrogenase involved in abietic acid biosynthesis. *Arch. Biochem. Biophys.*, **308**, 258–266 (1994).
13) De Kraker, J.-W., Franssen, M. C. R., Dalm, M. C. F., and De Groot, A., Biosynthesis of germacrene A carboxylic acid in chicory roots. Demonstration of a cytochrome P450 (+)-germacrene A hydroxylase and $NADP^+$-dependent sesquiterpenoid dehydrogenase(s) involved in sesquiterpene lactone biosynthesis. *Plant Physiol.*, **125**, 1930–1940 (2001).