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Identification of a Novel Casbane-Type Diterpene Phytoalexin, *ent*-10-Oxodepressin, from Rice Leaves

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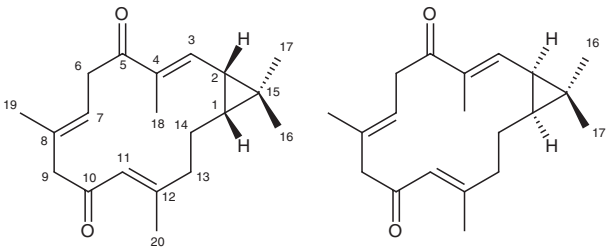
A 70% methanol extract of UV-irradiated rice leaves (400 g) was separated by chromatographic methods to give UV-induced compound 1 (2.1 mg) which showed a possible molecular ion at *m/z* 300 in the GC/MS analysis. Its structure was determined by NMR and MS methods. The ^{1}H- and ^{13}C-NMR spectra of 1 were identical to those of 10-oxodepressin (2), a casbane-type diterpene derived from the soft coral, *Sinularia depressa*. The specific rotation of 1 was positive, whereas that of 2 was negative. We therefore established 1 as *ent*-10-oxodepressin. The accumulation of 1 was also induced by an inoculation of the rice blast fungus. Compound 1 inhibited spore germination (IC_{50} 30 ppm) and germ tube growth (IC_{50} 10 ppm) of the rice blast fungus. We thus concluded that 1 was a novel rice phytoalexin.

Key words: phytoalexin; rice; blast fungus; diterpene; casbane

Phytoalexins are low-molecular-weight antimicrobial compounds which accumulate in plants invaded by pathogenic microorganisms.[1] The accumulation of phytoalexins has been recognized as one of the mechanisms for the induced resistance of higher plants against pathogens. Rice (*Oryza sativa*) is known to produce 15 phytoalexins, including 14 diterpenes and the flavanone, sakuranetin.[2] The diterpene phytoalexins are classified into four groups according to their structures; momilactones A and B,[3] oryzalexins A–F,[4–8] oryzalexin S,[9] and phytocassanes A–E.[10,11] The importance of those phytoalexins for resistance against the rice blast fungus, *Magnaporthe oryzae*, has been suggested in several studies.[2,12–14] We have recently shown that diterpene phytoalexins accumulated more quickly and to a greater extent in resistant rice than in susceptible rice, inducing severe restriction to fungal growth.[15] Moreover, the structural diversity of rice phytoalexins has been drawing attention as a model system for the biosynthesis of plant secondary metabolites.[16–18]

UV irradiation has been used to induce the accumulation of rice phytoalexins because of its ease and convenience.[2,7–9,19] We have been searching unidentified UV-induced substances as candidates for novel phytoalexins or the biosynthetic intermediates of phytoalexins. We report here the identification of the novel casbane-type diterpene phytoalexin, *ent*-10-oxodepressin (**1**, Fig. 1) from UV-irradiated rice leaves.



1 **2**

Fig. 1. Structures of *ent*-10-Oxodepressin (**1**) and 10-Oxodepressin (**2**).

Materials and Methods

General analytical methods. ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) and 2D-NMR spectra were measured 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 disk 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 spectrum was measured by a JMS-BU25 (GCmate II) mass spectrometer (Jeol) in the direct inlet EI mode (70 eV). GC/MS was performed by a JMS-BU25 mass spectrometer coupled with an HP6890 gas chromatograph (Agilent Technologies) under the following conditions: column, J&W Scientific HP-5 (0.32 mm i.d. × 30 m, 0.25 μm film thickness; Agilent Technologies); injection port temperature, 280 °C; carrier gas, He; flow rate, 1.0 mL min^{-1}; temperature program of column oven, 70 °C for 1 min before heating to 300 °C at 10 °C min^{-1}, and then maintained at 300 °C for 3 min; ionization mode, EI (70 eV); ion chamber temperature, 200 °C; scan range, *m/z* 61–627; scan rate, 1 scan s^{-1}.

Chemicals. Momilactones A and B were purified from rice husks according to the method reported by Kato *et al.*[20] with some modifications. Sakuranetin was chemically synthesized from naringenin (Aldrich) according to the method reported by Aida *et al.*[21] Oryzalexins A and S were purified from UV-irradiated rice leaves according to the methods previously described.[9,19]

Plant material. Rice plants (*Oryza sativa* cv. Koshihikari) were cultured in a phytotron (27 °C) for 1–3 months, and these leaves were detached for use in the subsequent experiments.

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Abbreviations: CE, collision energy; CXP, collision cell exit potential; DP, declustering potential; SRM, selected reaction monitoring

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 spore suspension of the fungus was prepared by the method previously described.[22]

UV irradiation of the rice leaves. The detached rice leaves were UV-irradiated for 20 min with a germicidal lamp (15 W) at a distance of 20 cm.[19] The irradiated leaves were incubated at 26 °C in a moist box at high humidity under lighting. The leaves for the purification of **1** were incubated for 3 d, while the leaves for the quantification of **1** and phytoalexins were incubated for 0, 1, 2, 3, 4, and 5 d. The UV-irradiated leaves were stored at −20 °C before being used.

Purification of ent-*10-oxodepressin (**1**) from the UV-irradiated rice leaves.* The UV-irradiated rice leaves (400 g) were cut and homogenized in 70% (v/v) MeOH (6 L) with a Physcotron ultra-homogenizer (Microtec). The homogenate was shaken overnight with a shaker and then filtered to obtain an extract. The leaves were re-extracted with 70% MeOH (4 L) in the same manner. The combined extract was concentrated *in vacuo* to remove MeOH. The resulting aqueous concentrate was extracted with EtOAc (1 L × 4), and the organic layer was evaporated to dryness *in vacuo*. The EtOAc extract was subjected to silica gel column chromatography (Kanto Chemical silica gel 60N, spherical neutral, 63–210 μm particle size; 60 g) eluting with a stepwise gradient of *n*-hexane/EtOAc (95:5, 80:20, 60:40, 40:60, 20:80, and 0:100; 300 mL of each). Each fraction was analyzed by GC/MS to detect the UV-induced substances. A compound (**1**) exhibiting a plausible molecular ion at m/z 300 was detected in the fraction eluted with 60:40 *n*-hexane/EtOAc. Its MS and t_R did not correspond to those of known phytoalexins. The fractions containing **1** were determined by a GC/MS analysis during the subsequent procedure. The fraction eluted with 60:40 *n*-hexane/EtOAc was applied to preparative TLC (Merck silica gel 60F_{254} PLC plate, 20 × 20 cm, 0.5 mm thickness) developed with 3:1 benzene/EtOAc. An R_f 0.69–0.80 TLC region, which contained **1**, was scraped off and extracted with EtOAc. The EtOAc extract was evaporated *in vacuo* to dryness to give a TLC-purified fraction (53.4 mg). The subsequent purification was performed by preparative ODS-HPLC, using a Jasco PU-980 HPLC pump and a Jasco MD-910 photodiode array detector. A 50-μL amount of a 5% (w/v) MeOH solution of the TLC-purified fraction was repeatedly separated by ODS-HPLC under the following conditions: column, Inertsil ODS-3 (1 cm i.d. × 25 cm, 5-μm particle size; GL Science); solvent, MeOH/H_2O (3:1, v/v); flow rate, 2.0 mL min^{-1}; detection, UV 210–350 nm. A peak containing **1** (t_R 42–44 min) was collected and evaporated to dryness *in vacuo* to afford crude **1** (2.7 mg). A 50-μL amount of a 1% (w/v) MeOH solution of crude **1** was repeatedly separated by ODS-HPLC under the following conditions: column, Inertsil ODS-3 (1 cm i.d. × 25 cm, 5-μm particle size; GL Science); solvent, MeCN/H_2O (1:1, v/v); flow rate, 2.0 mL min^{-1}; detection, UV 210–350 nm. A peak containing **1** (t_R 95–100 min) was collected and evaporated to dryness *in vacuo* to afford purified **1** (2.1 mg). Compound **1** was identified as *ent*-10-oxodepressin by spectroscopic methods as described in the Results section. **1**. HRMS m/z (M^+): calcd. for $C_{20}H_{28}O_2$, 300.2089; found, 300.2087; ^{1}H- and ^{13}C-NMR (C_6D_6): see Table 1; EIMS m/z (rel. int.): 67 (28), 79 (21), 82 (27), 95 (50), 108 (100), 122 (25), 135 (38), 150 (75), 161 (32), 189 (22), 257 (57) 300 (M^+, 42); UV λ_{max} (MeOH) nm (log ε): 245 (4.3); IR $\tilde{\nu}_{max}$ (KBr) cm^{-1}: 1686, 1665, 1651, 1623; $[\alpha]_D^{20}$ +124.2 (*c* 0.17, $CHCl_3$).

Assay of the antifungal activity against the rice blast fungus. The germination rate and germ tube length of *M. oryzae* were measured under a microscope, after the spore suspension had been incubated at 26 °C for 16 h. The inhibitory activity against spore germination was determined according to the previously reported method.[23] In order to measure the germ tube length, spores were observed and their images were recorded as digital data by using a BZ-8100 microscope (Keyence). The germ tube lengths of the germinated spores were measured by using ImageJ software (http://rsbweb.nih.gov/ij/).

LC/MS/MS analysis of ent-*10-oxodepressin (**1**) and phytoalexins.* 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 binary gradient of 0.1% (v/v) aqueous formic acid (solvent A) and MeOH containing 0.1% (v/v) formic acid (solvent B) at a flow rate of 0.2 mL min^{-1} and 40 °C. The graded solvent was programmed as follows: initial, 20% B; 0–5 min, a linear gradient from 20% B to 100% B; 5–9 min, isocratic elution by 100% B. The equilibration time between two runs was 2 min. The parameters of the mass spectrometer were optimized for detecting **1** and the phytoalexins 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 source parameters were set as follows: curtain gas, 10 psi; temperature, 400 °C; spray gas (GS1), 30 psi; dry gas, (GS2) 30 psi; ion spray voltage, 5500 V. The following SRM transitions (t_R) were monitored: **1**, m/z 301→177 (6.5 min); momilactone A, m/z 315→271 (6.3 min); sakuranetin, m/z 287→167 (5.8 min); oryzalexin A, m/z 303→145 (6.5 min); oryzalexin S, m/z 287→105 (6.8 min). The optimized parameters of the mass spectrometer for each compound were as follows: **1**, 15 V collision energy (CE), 4 V collision cell exit potential (CXP), 41 V declustering potential (DP); momilactone A, 17 V CE, 6 V CXP, 41 V DP; sakuranetin, 31 V CE, 4 V CXP, 41 V DP; oryzalexin A, 21 V CE, 4 V CXP, 46 V DP; oryzalexin S, 43 V CE, 4 V CXP, 46 V DP. Calibration curves were obtained from the SRM peak areas of the standards for **1** and phytoalexins in a concentration range of 1–1,000 ng mL^{-1}. The concentrations of the analytes were calculated from the SRM peak area of each analyte based on the calibration curves.

Quantification of ent-*10-oxodepressin (**1**) and the phytoalexins from UV-irradiated rice leaves.* UV-irradiated rice leaves (*ca.* 0.3 g) were cut and homogenized in MeOH (10 mL) with a mortar and pestle. The homogenate was filtered through a cotton-plugged funnel. The residue was re-extracted with MeOH (5 mL) and then filtered. The volume of the combined extract was adjusted to 20 mL with MeOH, and distilled water (5 mL) was then added to the extract. The extract was passed through a Bond Elut C18 cartridge (500 mg/3 mL; Agilent Technologies). A 2-μL amount of the solution was subjected to the aforementioned LC/MS/MS analysis to quantify **1** and the phytoalexins.

Quantification of ent-*10-oxodepressin (**1**) and the phytoalexins from* M. oryzae-*inoculated rice leaves.* A spore suspension of *M. oryzae* (4×10^5 spores mL^{-1}) was applied to press-injured spots of 1 mm in diameter on the rice leaves. The leaves were maintained in a highly humidified box and incubated at 26 °C in the light. Leaf tissues around the injured spots were excised with a cork borer (4 mm in diameter). Approximately 25 pieces of the excised leaf tissues (*ca.* 10 mg) were collected and homogenized in MeOH (3 mL) with a mortar and pestle. The homogenate was filtered through a cotton-plugged funnel. The residue was re-extracted with MeOH (1 mL) and filtered. The volume of the combined filtrate was adjusted to 5 mL with MeOH, and distilled water (1.25 mL) was added to the extract. The extract was then passed through a Bond Elut C18 cartridge. A 2-μL amount of the solution was subjected to the aforementioned LC/MS/MS analysis to quantify **1** and the phytoalexins.

Results

Purification and structural elucidation of ent-*10-oxodepressin (**1**) from UV-irradiated rice leaves*

We found a UV-induced compound (**1**) in the silica gel column chromatography-purified fraction from UV-irradiated rice leaves by a GC/MS analysis. Compound **1** was detected at t_R 18.3 min. Its mass spectrum showed a plausible molecular ion at m/z 300. Compound **1** was not detected in healthy rice leaves which had not been UV-irradiated (data not shown). Its t_R and mass spectrum did not correspond to those of known phytoalexins or their biosynthetic precursors, authentic samples of which we compared with (data not shown). We therefore attempted to isolate **1** to determine its structure. Compound **1** (2.1 mg, a colorless oil) was

Table 1. ^{13}C–^{1}H-NMR Data for *ent*-10-Oxodepressin (**1**) in C_6D_6

Position	δ_C	δ_H (multiplicity, J in Hz)
1	34.18	0.68 (1H, m, overlapped)
2	27.74	1.29 (1H, m, overlapped)
3	140.12	6.16 (1H, dq, 10.5, 1.3)
4	138.32	—
5	197.68	—
6	39.57	2.92 (1H, m)
		3.50 (1H, dd, 13.8, 9.8)
7	124.29	5.16 (1H, m)
8	133.09	—
9	56.10	2.70 (1H, d, 13.9)
		2.77 (m)
10	198.29	—
11	123.32	5.98 (1H, br s)
12	156.82	—
13	41.17	1.65 (1H, m, overlapped)
		1.88 (1H, m)
14	26.88	0.66 (1H, m, overlapped)
		1.72 (1H, m, overlapped)
15	25.33	—
16	28.87	0.93 (3H, s)
17	15.67	0.84 (3H, s)
18	12.21	2.00 (3H, d, 1.3)
19	16.28	1.32 (3H, m, overlapped)
20	17.98	2.05 (3H, dd, 1.3, 0.4)

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

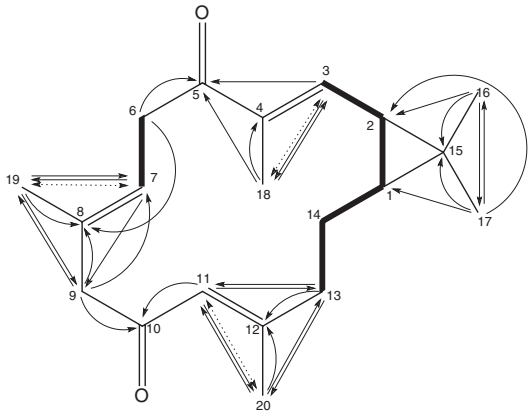

Fig. 2. 2D NMR Correlations for *ent*-10-Oxodepressin (**1**).
The COSY and TOCSY correlations are represented by bold lines, HMBC correlations are represented by single-headed arrows from H to C, and long-range COSY correlations are represented by double-headed dotted arrows.

successfully purified from UV-irradiated rice leaves (400 g) by chromatographic methods.

The high-resolution mass spectrum of **1** gave a molecular weight of 300.2089, agreeing with the molecular formula $C_{20}H_{28}O_2$ (calcd. as 300.2087) and seven degrees of unsaturation. The numbers of carbons and hydrogens agreed with the ^{1}H- and ^{13}C-NMR data (Table 1). The DEPT and HSQC data revealed that all hydrogens were directly connected to carbons. ^{13}C-NMR and IR data indicated that two oxygens belonged to two carbonyl groups. ^{13}C-NMR and DEPT data indicated the presence of three olefinic methines and three olefinic quaternary carbons, suggesting that **1** had three C–C double bonds. Seven degrees of unsaturation and the presence of three C–C double bonds and two carbonyl groups indicated that **1** had two rings. COSY, TOCSY, and HSQC correlations suggested C-13/C-14/C-1/C-2/C-3 and C-6/C-7 connectivity. The typical chemical shifts of two methine protons at δ_H 0.68 (H-1) and 1.29 (H-2) suggested the presence of a cyclopropane ring. HMBC correlations indicated that the cyclopropane ring included a quaternary carbon (C-15) which had two geminal methyls (C-16 and C-17). The HMBC and long-range COSY correlations were used to connect those fragment structures and the remaining fractions such as two carbonyls (C-5 and C-10), three olefinic quaternary carbons (C-4, C-8, and C-12), a methylene (C-9), an olefinic methine (C-11), and three methyls (C-18, C-19, and C-20). The deduced structure showed a casbane-type diterpene skeleton which had a 14-membered ring fused with a cyclopropane ring (Fig. 2).

Based on this structure, we surveyed the literature reporting casbane-type diterpenes, and found one casbane-type compound identical to the deduced structure. This compound, 10-oxodepressin (**2**, Fig. 1), had been reported as a Dess-Martin oxidation product of the casbane-type diterpene, 10-hydroxydepressin, which had been isolated from the Hainan soft coral, *Sinularia depressa*.[24] The ^{1}H- and ^{13}C-NMR spectra of **1** and **2** are similar, although the solvents used for the NMR analysis were different. We therefore redissolved **1** in $CDCl_3$, which was used in the literature, and subjected it to an NMR analysis. The obtained ^{1}H- and ^{13}C-NMR spectra were identical to those of **2** in the reference (data not shown).[24] The sign for the specific rotation of **1** was positive (+124.2), whereas that for **2** was negative (−76) according to the reference.[24] The structure of **1** was therefore determined as *ent*-10-oxodepressin (Fig. 1).

Accumulation of ent*-10-oxodepressin (**1**) in UV-irradiated and* M. oryzae*-inoculated rice leaves*

We established the quantification method for **1** by using LC/MS/MS. The extract of the leaves was subjected to an LC/MS/MS analysis, and **1** could be detected in the SRM mode by using positive ESI. The change in the accumulated level of **1** after UV irradiation was analyzed by this method (Fig. 3A). An increase of the level of **1** was clearly detected after 48 h, the level of **1** reaching a maximum 72–96 h after UV irradiation. The leaves which had not been UV-irradiated did not clearly accumulate **1**. The accumulation of **1** in *M. oryzae*-inoculated leaves was also quantified by LC/MS/MS (Fig. 3B). The increase in the level of **1** was clearly detected after 72 h, and the level of **1** continued to increase to 120 h after inoculation. The mock-inoculated leaves did not clearly accumulate **1**.

The contents of **1** and phytoalexins in the UV-irradiated and *M. oryzae*-inoculated leaves are summarized in Table 2. Momilactone A and sakuranetin accumulated at the highest level in the UV-irradiated leaves. The level of **1** in the UV-irradiated leaves was lower than of momilactone A and sakuranetin, but higher than of oryzalexins A and S. Sakuranetin accumulated at the highest level in the *M. oryzae*-inoculated leaves. The accumulation of **1** in the *M. oryzae*-inoculate leaves was at the lowest level among the quantified compounds, and at almost the same level as oryzalexin A.

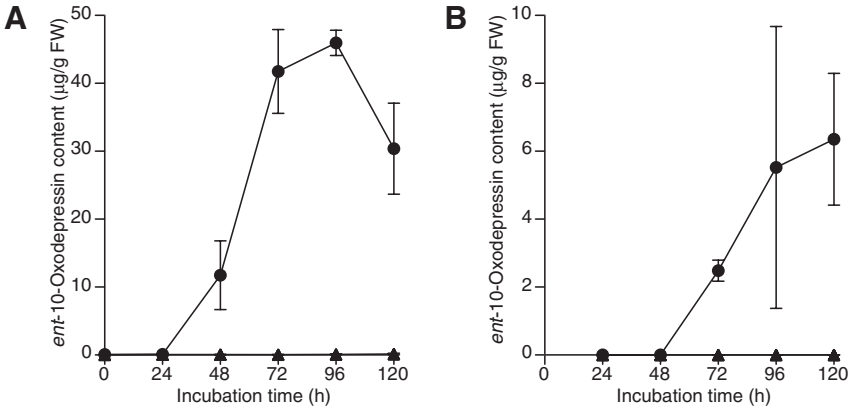

Fig. 3. Time-Dependent Accumulation of *ent*-10-Oxodepressin (**1**) in UV-Irradiated (A) and Rice Blast Fungus-Inoculated (B) Rice Leaves.

Compound **1** was extracted from rice leaves at different times after UV irradiation (A) and rice blast fungus inoculation (B). The concentration was quantified by using LC/MS/MS. Values are presented as the mean ± SD ($n = 3$ for the UV-irradiated, rice blast fungus-inoculated, and control leaves; $n = 2$ for the mock-inoculated leaves). The concentrations of **1** in UV-irradiated (A) and rice blast fungus-inoculated (B) leaves are represented by circles. The concentrations of **1** in the control (A) and mock-inoculated (B) leaves are represented by triangles.

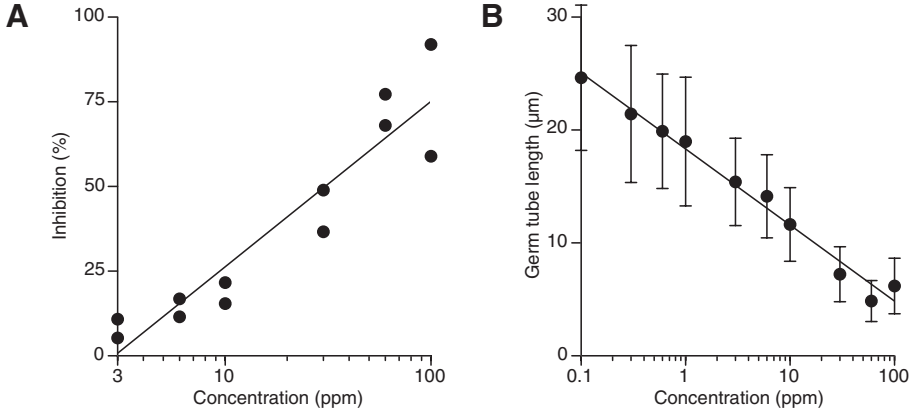

Fig. 4. Antifungal Activity of *ent*-10-Oxodepressin (**1**) against the Spore Germination (A) and Germ Tube Growth (B) of Rice Blast Fungus.

Spore germination was obsereved under a microscope after incubating with **1** for 16 h at 26 °C. (A) The inhibitory activity rate was calculated as follows: inhibition (%) = (1 − b/a) × 100, where a is the germination rate of the control group and b is the germination rate of the sample group. Duplicate data are represented by circles. The line shows logarithmic regression. (B) The germ tube length of germinated spores was measured as described in the Materials and Methods section. Values are presented as the mean ± SD ($n = 90$–103). The germ tube length of the control experiment was 23.4 ± 7.8 μm ($n = 91$). The line shows logarithmic regression.

Table 2. Accumulation of Phytoalexins and *ent*-10-Oxodepressin (**1**) in UV-Irradiated and *M. oryzae*-Inoculated Rice Leaves after 72 h of Incubation

Compound	Accumulation (μg/g FW)	
	UV irradiation	*M. oryzae* inoculation
ent-10-Oxodepressin (**1**)	41.7 ± 6.2	2.48 ± 0.31
Momilactone A	139 ± 3	17.7 ± 2.2
Oryzalexin A	4.23 ± 0.95	2.11 ± 1.60
Oryzalexin S	31.9 ± 5.4	11.5 ± 2.2
Sakuranetin	135 ± 10	50.6 ± 13.1

Compound **1** was extracted from rice leaves 72 h after UV irradiation and rice blast fungus inoculation. The concentration was quantified by using LC/MS/MS. Values are presented as the mean ± SD ($n = 3$). The control and mock-inoculated leaves contained less than 1/30 of the quoted compounds respectively compared to UV-irradiated and *M. oryzae*-inoculated leaves.

Antifungal activity of ent*-10-oxodepressin (**1**)*

The antifungal activity of **1** against *M. oryzae* was determined by spore germination and a germ tube growth assay (Fig. 4). The respective IC_{50} values were calculated as *ca.* 30 ppm (100 μM) and *ca.* 10 ppm (33 μM) for germination and germ tube growth. This inhibitory activity against spore germination was stronger than that of momilactone A (IC_{50} 540 μM),[25] but weaker than that of sakuranetin (IC_{50} 52 μM).[2] These results support the proposal of **1** being a novel rice phytoalexin.

Discussion

UV irradiation is a useful method to accumulate phytoalexins in rice for their identification.[2,7–9,19] We have identified in this study the novel casbane-type diterpenoid, *ent*-10-oxodepressin (**1**), from the UV-irradiated rice leaves. Compound **1** was induced by both UV irradiation and *M. oryzae* infection, and showed strong inhibitory activity against the spore germination and germ tube growth of *M. oryzae*. We thus concluded **1** to be a novel rice phytoalexin. All other known rice diterpene phytoalexins belong to the labdane-related diterpenoid super-family,[26] whose basal hydrocarbon skeletons are biosynthesized from geranylgeranyl diphosphate *via* two-step enzymatic cyclization.[17,18] However, casbene, a hydrocarbon of the casbane-type diterpene, has been reported to be biosynthesized *via* single-step cyclization catalyzed by casbene synthase.[27]

The biological origin of known casbane-type diterpenes has been very limited. The first-reported casbane-type diterpene, casbene, was identified as a conversion product from mevalonic acid, a common precursor of terpenoids, by cell-free extraction of castor bean seedlings (*Ricinus communis*; Euphorbiaceae).[28] Since then, casbane-type diterpenes have been isolated from Euphorbiaceae plants[29–45] and soft corals of the genus *Sinularia*.[24,46] To our knowledge, this is the first report of a casbane-type diterpene originating from an organism other than a Euphorbiaceae plant or *Sinularia* soft coral. Casbene has been recognized as a phytoalexin of the castor bean, because it showed antifungal activity and its biosynthetic activity was induced by fungal infection.[47] Other casbane-type diterpenoids have been reported to have cytotoxicity against animal cells[24,31,32,36,37,40,43,46] and antimicrobial activity.[24,36,42,45]

Rice phytoalexins are known to have extraordinary structural diversity.[16] Our finding of *ent*-10-oxodepressin (**1**) revealed the further structural diversity of rice phytoalexins. We had already found that the rice diterpene phytoalexin, momilactone A, was detoxified by *M. oryzae*.[15,25] In respect of pathogens, detoxifying phytoalexins is an effective solution to evade the growth inhibition caused by them.[48] We speculate that the rich structural diversity of rice phytoalexins may be a powerful competitive strategy to overcome the microbial detoxification of phytoalexins.

In addition to phytoalexins, rice plants have been reported to produce various self-defensive substances against pathogenic microorganisms. Oryzalides are recognized as kaurane-type diterpene phytoanticipins to inhibit the growth of *Xanthomonas oryzae*.[49] Oxygenated fatty acids have been reported as antifungal substances against *M. oryzae*,[50] and serotonin has been reported to be involved in the defense response against *Bipolaris oryzae*.[51] We have found that such volatile organic compounds as sesquiterpenes and monoterpenes were emitted from *M. oryzae*-inoculated rice leaves, although their role in disease resistance is still unclear.[22] Concerning other plants, it has recently been reported that maize could produce novel diterpene and sesquiterpene phytoalexins to protect itself from fungal pathogens,[52,53] although benzoxazinoid phytoanticipins have only been recognized as secondary metabolites that were involved in the resistance of maize against fungal pathogens and herbivores.[54] We speculate that this diversity of plant antimicrobial secondary metabolites, including the novel rice casbane-type phytoalexin described here, may have resulted from the competitive evolution of plants against pathogens.

Acknowledgments

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References

1) Ahuja I, Kissen R, and Bones AM, *Trends Plant Sci.*, **17**, 73–90 (2012).
2) Kodama O, Miyakawa J, Akatsuka T, and Kiyosawa S, *Phytochemistry*, **31**, 3807–3809 (1992).
3) Cartwright D, Langcake P, Pryce RJ, and Leworthy DP, *Nature*, **267**, 511–513 (1977).
4) Akatsuka T, Kodama O, Kato H, Kono Y, and Takeuchi S, *Agric. Biol. Chem.*, **47**, 445–447 (1983).
5) Kono Y, Takeuchi S, Kodama O, and Akatsuka T, *Agric. Biol. Chem.*, **48**, 253–255 (1984).
6) Sekido H, Endo T, Suga R, Kodama O, Akatsuka T, Kono Y, and Takeuchi S, *J. Pestic. Sci.*, **11**, 369–372 (1986).
7) Kato H, Kodama O, and Akatsuka T, *Phytochemistry*, **33**, 79–81 (1993).
8) Kato H, Kodama O, and Akatsuka T, *Phytochemistry*, **36**, 299–301 (1994).
9) Kodama O, Li WX, Tamogami S, and Akatsuka T, *Biosci. Biotechnol. Biochem.*, **56**, 1002–1003 (1992).
10) Koga J, Shimura M, Oshima K, Ogawa N, Yamauchi T, and Ogasawara N, *Tetrahedron*, **51**, 7907–7918 (1995).
11) Koga J, Ogawa N, Yamauchi T, Kikuchi M, Ogasawara N, and Shimura M, *Phytochemistry*, **44**, 249–253 (1997).
12) Dillon VM, Overton J, Grayer RJ, and Harborne JB, *Phytochemistry*, **44**, 599–603 (1997).
13) Umemura K, Ogawa N, Shimura M, Koga J, Usami H, and Kono T, *Biosci. Biotechnol. Biochem.*, **67**, 899–902 (2003).
14) Bagnaresi P, Biselli C, Orrù L, Urso S, Crispino L, Abbruscato P, Piffanelli P, Lupotto E, Cattivelli L, and Valè G, *PLoS ONE*, **7**, e51609 (2012).
15) Hasegawa M, Mitsuhara I, Seo S, Imai T, Koga J, Okada K, Yamane H, and Ohashi Y, *Mol. Plant Microbe Interact.*, **23**, 1000–1011 (2010).
16) Harborne JB, *Biochem. Syst. Ecol.*, **27**, 335–367 (1999).
17) Peters RJ, *Phytochemistry*, **67**, 2307–2317 (2006).
18) Toyomasu T, *Biosci. Biotechnol. Biochem.*, **72**, 1168–1175 (2008).
19) Kodama O, Suzuki T, Miyakawa J, and Akatsuka T, *Agric. Biol. Chem.*, **52**, 2469–2473 (1988).
20) Kato T, Tsunakawa M, Sasaki N, Aizawa H, Fujita K, Kitahara Y, and Takahashi N, *Phytochemistry*, **16**, 45–48 (1977).
21) Aida Y, Tamogami S, Kodama O, and Tsukiboshi T, *Biosci. Biotechnol. Biochem.*, **60**, 1495–1496 (1996).
22) Obara N, Hasegawa M, and Kodama O, *Biosci. Biotechnol. Biochem.*, **66**, 2549–2559 (2002).
23) Lin F, Hasegawa M, and Kodama O, *Biosci. Biotechnol. Biochem.*, **67**, 2154–2159 (2003).
24) Li Y, Carbone M, Vitale RM, Amodeo P, Castelluccio F, Sicilia G, Mollo E, Nappo M, Cimino G, Guo Y-W, and Gavagnin M, *J. Nat. Prod.*, **73**, 133–138 (2010).
25) Imai T, Ohashi Y, Mitsuhara I, Seo S, Toshima H, and Hasegawa M, *Biosci. Biotechnol. Biochem.*, **76**, 414–416 (2012).
26) Peters RJ, *Nat. Prod. Rep.*, **27**, 1521–1530 (2010).
27) Mau CJD and West CA, *Proc. Natl. Acad. Sci. USA*, **91**, 8497–8501 (1994).
28) Robinson DR and West CA, *Biochemistry*, **9**, 70–79 (1970).
29) Burke BA, Chan WR, Pascoe KO, Blount JF, and Manchand PS, *J. Chem. Soc., Perkin Trans. 1*, 2666–2669 (1981).
30) Ghisalberti EL, Jefferies PR, Mori TA, Skelton BW, and White AH, *Tetrahedron*, **41**, 2517–2526 (1985).
31) Choi Y-H, Kim J, Pezzuto JM, Kinghorn AD, Farnsworth NR, Lotter H, and Wagner H, *Tetrahedron Lett.*, **27**, 5795–5798 (1986).
32) Choi Y-H, Pezzuto JM, Kinghorn AD, and Farnsworth NR, *J. Nat. Prod.*, **51**, 110–116 (1988).
33) Moura VLA, Monte FJO, and Braz Filho R, *J. Nat. Prod.*, **53**, 1566–1571 (1990).
34) Kashman Y, Bernart MW, Tischler M, Cardellina II JH, and Boyd MR, *J. Nat. Prod.*, **57**, 426–430 (1994).
35) Chen H and Jia ZJ, *Indian J. Chem.*, **35B**, 1308–1310 (1996).
36) Xu Z-H, Sun J, Xu R-S, and Qin G-W, *Phytochemistry*, **49**, 149–151 (1998).
37) Kong L-Y, Li Y, Wu X-L, and Min Z-D, *Planta Med.*, **68**, 249–252 (2002).
38) Bai Y, Yang Y, and Ye Y, *Tetrahedron Lett.*, **47**, 6637–6640 (2006).

39) Santos HS, Mesquita FMR, Lemos TLG, Monte FJQ, and Braz-Filho R, *Quim. Nova*, **31**, 601–604 (2008).
40) Liang Q-L, Dai C-C, Jiang J-H, Tang Y-P, and Duan J-A, *Fitoterapia*, **80**, 514–516 (2009).
41) Tan Q-G, Cai X-H, Du Z-Z, and Luo X-D, *Helv. Chim. Acta*, **92**, 2762–2768 (2009).
42) Carneiro VA, dos Santos HS, Arruda FVS, Bandeira PN, Albuquerque MRJR, Pereira MO, Henriques M, Cavada BS, and Teixeira EH, *Molecules*, **16**, 190–201 (2011).
43) Shao F-G, Bu R, Zhang C, Chen C-J, Huang J, and Wang J-H, *J. Asian Nat. Prod. Res.*, **13**, 805–810 (2011).
44) Silva-Filho FA, Braz-Filho R, Silveira ER, and Lima MAS, *Magn. Reson. Chem.*, **49**, 370–373 (2011).
45) Sá NC, Cavalcante TTA, Araújo AX, dos Santos HS, Albuquerque MRJR, Bandeira PN, da Cunha RMS, Cavada BS, and Teixeira EH, *Arch. Oral Biol.*, **57**, 550–555 (2012).
46) Zhang C-X, Yan S-J, Zhang G-W, Lu W-G, Su J-Y, Zeng L-M, Gu L-Q, Yang X-P, and Lian Y-J, *J. Nat. Prod.*, **68**, 1087–1089 (2005).
47) Sitton D and West CA, *Phytochemistry*, **14**, 1921–1925 (1975).
48) Pedras MSC and Ahiahonu PWK, *Phytochemistry*, **66**, 391–411 (2005).
49) Kono Y, Kojima A, Nagai R, Watanabe M, Kawashima T, Onizawa T, Teraoka T, Watanab M, Koshino H, Uzawa J, Suzuki Y, and Sakurai A, *Phytochemistry*, **65**, 1291–1298 (2004).
50) Kato T, Yamaguchi Y, Uyehara T, Yokoyama T, Namai T, and Yamanaka S, *Naturwissenschaften*, **70**, 200–201 (1983).
51) Ishihara A, Nakao T, Mashimo Y, Murai M, Ichimaru N, Tanaka C, Nakajima H, Wakasa K, and Miyagawa H, *Phytochemistry*, **72**, 7–13 (2011).
52) Schmelz EA, Kaplan F, Huffaker A, Dafoe NJ, Vaughan MM, Ni X, Rocca JR, Alborn HT, and Teal PE, *Proc. Natl. Acad. Sci. USA*, **108**, 5455–5460 (2011).
53) Huffaker A, Kaplan F, Vaughan MM, Dafoe NJ, Ni X, Rocca JR, Alborn HT, Teal PEA, and Schmelz EA, *Plant Physiol.*, **156**, 2082–2097 (2011).
54) Ahmad S, Veyrat N, Gordon-Weeks R, Zhang Y, Martin J, Smart L, Glauser G, Erb M, Flors V, Frey M, and Ton J, *Plant Physiol.*, **157**, 317–327 (2011).