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FUNCTIONAL MOIETY FOR THE ANTIFUNGAL ACTIVITY OF PHYTOCASSANE E, A DITERPENE PHYTOALEXIN FROM RICE

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Key Word Index—*Oryza sativa*; Gramineae; rice; phytoalexins; elicitors; *Magnaporthe grisea*; *Phytophthora infestans*; diterpenes; phytocassane E.

Abstract—Large amounts of phytoalexins were produced in suspension-cultured rice cells by treatment with a mycelial extract of the potato pathogenic fungus *Phytophthora infestans*. Among them, a new cassane-type diterpene, designated phytocassane E, was isolated and identified as 1β-hydroxy-12,15-cassadien-3,11-dione. The ED_{50} values of phytocassane E in the prevention of spore germination and germ tube growth of the rice pathogenic fungus *Magnaporthe grisea* were 6 and 2 $\mu g\ ml^{-1}$, respectively. This result, together with earlier results, suggests that the hydroxyl group at the C-1 position is the main functional moiety for the high antifungal activity of phytocassane E. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

When plants interact with pathogens, some resistant plants protect themselves by accumulating antimicrobial compounds called phytoalexins. The production of such compounds is thought to be involved in plant defences against pathogenic fungi. Momilactones A and B [1], oryzalexins A–F [2–5] and S [6], and sakuranetin [7] have been isolated as phytoalexins from rice plants. To determine the main phytoalexins which are involved in defence mechanisms against pathogenic fungi, rice leaves that had been infected with *Magnaporthe grisea* were screened for phytoalexins possessing high antifungal activity. As a result, four novel phytoalexins with a cassane skeleton, designated phytocassanes A, B, C and D, were isolated [8]. Large amounts of phytocassanes A, B, C and D were produced in rice leaves and stems infected with *M. grisea* and *Rhizoctonia solani*, respectively. Although phytocassanes have high antifungal activity against the pathogenic fungi *M. grisea* and *R. solani*, it remained unclear which aspect of their chemical structures was responsible for the activity. In the present study, phytocassane E, a new cassane-type phytoalexin, was isolated from suspension-cultured rice cells and, from its structure, the functional moiety for the antifungal activity was deduced.

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RESULTS AND DISCUSSION

Phytoalexins are induced by molecules called elicitors that are produced by pathogenic microorganisms [9]. Elicitors have been studied extensively and, in most cases, have been shown to be oligo- or polysaccharides derived from the mycelia of pathogenic fungi [10, 11]. However, it was difficult to induce the production of large amounts of phytoalexins in suspension-cultured rice cells by treatment with an elicitor. Therefore, we have established a method for phytoalexin produced by using small aggregates of rice cells treated with a mycelial extract of the potato pathogenic fungus *Phytophthora infestans*, which have a potent elicitor activity among hydrophilic mycelial elicitors. As shown in Table 1, large amounts of phytoalexins, including a new phytoalexin, designated phytocassane E, were produced in suspension-cultured rice cells by treatment with the elicitor. Furthermore, the amounts of phytoalexin produced by using small aggregates of rice cells were larger than those obtained by using large aggregates of rice cells. The relative level of each phytoalexin produced in suspension-cultured rice cells differed from that in rice leaves and stems infected with *M. grisea* and *R. solani*, respectively (Table 2). In particular, there was a higher relative production of phytocassanes C and E (Fig. 1) and momilactone B [1] in suspension-cultured rice cells than in rice leaves and stems infected with them. Because of its high antifungal activity, the new phytoalexin, phytocassane E (**1**), was purified and its structure determined (Fig. 1).

The IR spectrum of phytocassane E (see Experimental) showed the presence of an $\alpha,\beta,\gamma,\delta$-unsaturated

Table 1. Amounts of phytoalexins in suspension-cultured rice cells obtained by treatment with *Phytophthora infestans* elicitor

	Phytocassane ($\mu g\ ml^{-1}$ culture medium)					Momilactone ($\mu g\ ml^{-1}$ culture medium)	
	A	B	C	D	E	A	B
Untreated	ND	ND	ND	ND	ND	ND	ND
Treated with elicitor*							
Large callus	0.6	0.1	2.4	0.1	1.2	0.3	1.1
Small callus	3.3	0.4	11.3	0.4	5.3	1.1	5.3

ND, not detectable.

*For culture of a small callus, 2 g of the rice callus were filtered through the stainless-steel mesh and the resultant small aggregates of rice cells were incubated with elicitor. For culture of a large callus, the large aggregates of rice cells were incubated with elicitor without filtering through the stainless-steel mesh.

Table 2. Amounts of phytoalexins in rice leaves and stems infected with *Magnaporthe grisea* and *Rhizoctonia solani*, respectively

	Phytocassane ($\mu g\ g^{-1}$ fresh weight)					Momilactone ($\mu g\ g^{-1}$ fresh weight)	
	A	B	C	D	E	A	B
Rice leaves (cv. Jukkoku)							
Uninfected	ND	ND	ND	ND	ND	ND	ND
Infected with *M. grisea*	37.7	10.9	4.4	15.8	3.1	40.2	12.7
Rice stems (cv. Koshihikari)							
Uninfected	ND	ND	ND	ND	ND	ND	ND
Infected with *R. solani*	58.2	20.7	2.6	15.9	6.5	49.9	19.3

ND, not detectable.

ketone at 1635 cm^{-1}, a carbonyl group at 1713 cm^{-1} and a hydroxyl group at 3408 cm^{-1}. The identification of 20 carbon atoms in the ^{13}C NMR (Table 3) and DEPT experiment, and the high-resolution mass spectrum, which showed a $[M]^+$ at m/z 316.2062, indicated a molecular formula of $C_{20}H_{28}O_3$ (see Experimental). The ^1H NMR spectrum ($CDCl_3$) of phytocassane E showed signals for four methyl groups (δ 0.88, 1.07, 1.11, 1.12), a proton geminal to a hydroxyl group (δ 4.16) and four olefinic protons (δ 5.57, 5.75, 5.88, 6.38). The connectivities between each of the ^{13}C signals and the related ^1H signals were established by

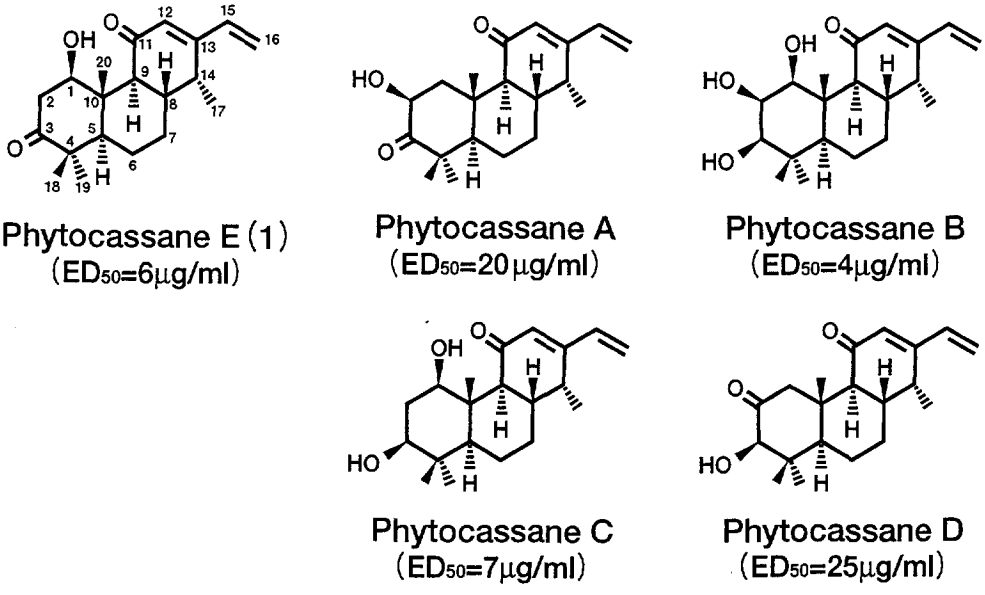

Fig. 1. Structures and antifungal activities of phytocassanes A, B, C, D and E. The ED_{50} values of phytocassanes in prevention of spare germination of *M. grisea* are shown.

Table 3. ^{13}C NMR and HMBC data of phytocassane E in $CDCl_3$

C	^{13}C NMR (δ)	Correlated protons*
1	77.1	OH-1, H-2α, H-2β, H-9, H-20
2	42.9	OH-1
3	214.8	H-2α, H-2β, H-16, H-18, H-19
4	47.4	H-2α, H-18, H-19
5	51.0	H-6β, H-7β, H-18, H-19, H-20
6	22.2	H-5, H-7α
7	30.8	H-5
8	39.1	H-6α, H-6β, H-7β, H-9, H-14, H-17
9	57.5	H-1α, H-7β, H-8, H-12, H-14, H-20
10	43.4	H-2α, H-2β, H-5, H-6α, H-9, H-20
11	203.5	H-9
12	128.2	H-14, H-15
13	163.4	H-14, H-15, H-16, H-17
14	33.8	H-9, H-12, H-15, H-17
15	135.8	H-12, H-16
16	122.1	
17	13.4	
18	20.1	H-19
19	28.0	H-18
20	10.9	

*Correlations were observed using HMBC.

the ^1H–^{13}C COSY spectrum. The connectivities between ^{13}C signals were established by the ^1H–^1H COSY spectrum and by HMBC experiments, as shown in Table 3. The carbon signal (δ 77.1, Table 3) bearing a hydroxyl group, the chemical shifts (δ 4.16) of the proton at C-1 and the IR spectrum revealed that the hydroxyl group should be at C-1. The carbon signals (δ 203.5, 214.8) bearing carbonyl groups, the IR spectrum, the ^1H–^{13}C COSY spectrum and HMBC correlations revealed that the $\alpha,\beta,\gamma,\delta$-unsaturated ketone and the carbonyl group should be at C-11 and at C-3, respectively. The olefinic carbon signals at δ 122.1, 128.2, 135.8 and 163.4 and the chemical shifts (δ 5.57, 5.75, 5.88, 6.38) of the olefinic protons at C-12, C-15 and C-16 revealed that the olefinic carbon atoms should be at C-12, C-13, C-15 and C-16. The stereochemistry was confirmed by NOE experiments. The irradiation of H-1 showed effect with H-2α, H-5 and H-9; H-2α showed effect with H-1 and H-5; H-2β showed effect with Me-20; H-5 showed effect with H-1, H-2α and Me-18; H-8 showed effect with H-14 and Me-20; H-9 showed effect with H-1 and Me-17; H-14 showed effect with H–8, H-16 and Me-17; Me-17 showed effect with H-9, H-14 and H-16; Me-18 showed effect with H-5; Me-19 showed effect with Me-20; and Me-20 showed effect with H-2β, H-8 and Me-19. Consequently, phytocassane E was confirmed to be 1β-hydroxy-12, 15-cassadien-3,11-dione or its enantiomer (Fig. 1).

To the best of our knowledge, phytocassane E is a new diterpene phytoalexin. However, its overall spectral data are very similar to those of phytocassanes A, B, C and D, which were previously isolated as phytoalexins [8]. To compare the structures of these phytocassanes, the structures and antifungal activities of phytocassanes A, B, C and D are also with structure **1**. The IR band (1635 cm^{-1}) for $\alpha,\beta,\gamma,\delta$-unsaturated ketone of phytocassane E showed a similar value as those (1635 and 1637 cm^{-1}) of phytocassanes B and C, but showed different values from those (1637 and 1655 cm^{-1}) of phytocassanes A and D. The C-11 signal at δ 203.5 of phytocassane E was shifted to a slightly lower field as compared to those of phytocassanes A and D, which appeared at δ 200.3 and 200.6 [8], respectively. The OH-1 signal of phytocassane E was markedly shifted downfield (δ 6.21). Furthermore, in the stereostructure of phytocassane E, the proton of the OH-1 group was in close spatial proximity to the carbonyl group at C-11. These common features, observed also in phytocassanes B and C [8], suggest that the hydroxyl group at C-1 forms an intramolecular hydrogen bond with the carbonyl group at C-11.

The ED_{50} values of phytocassane E in prevention of spore germination and germ tube growth of *M. grisea* were 6 and 2 $\mu g\,ml^{-1}$, respectively. Three of the five phytocassanes isolated, including phytocassane E, have ED_{50} values in the range 4–7 $\mu g\,ml^{-1}$, whereas the other two have ED_{50} values 4–5 times higher. The common structural feature of the three more effective forms is the presence of a 1β-hydroxyl bond to the carbonyl group at the C-11 position. This structural feature seems to enhance the antifungal activity. However, since phytocassanes A and D, which lack this feature, are active, albeit less so, it is possible that the other groups (e.g. 2β- and 3β-hydroxyl groups) are involved in the antifungal activity

EXPERIMENTAL

General procedures. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions (500.2 MHz for ^1H, 125.8 MHz for ^{13}C), using $SiMe_4$ and $CDCl_3$ as internal standards. Electron impact mass spectra (EI-

MS) were recorded on an Nippondenshi DX-303 mass spectrometer. For analytical HPLC, TSKgel ODS-120A and ODS-120T (4.6 mm i.d. × 30 cm; TOSOH Co. Ltd, Japan) were used as reverse phase HPLC columns at a flow rate of 1.2 ml min^{-1} at 50°. For preparative HPLC, TSKgel ODS-120A and ODS-120T (21.5 mm i.d. × 37.5 cm; TOSOH) were used as reverse phase HPLC columns at a flow rate of 10 ml min^{-1} at 40°.

Phytoalexin production in suspension-cultured rice cells by treatment with elicitor. Phytophthora infestans (Mont.) de Bary, race 0, was cultured in liquid medium containing rye-seed extract (60 g rye seeds), 20 g l^{-1} sucrose and 2 g l^{-1} yeast extract [12]. After incubation for 25 days at 18°, 100 g mycelia was harvested and suspended in 300 ml H_2O. The suspension of mycelia was homogenized for 10 min, sonicated for 30 min, and then autoclaved for 60 min at 121°. The resultant sample was centrifuged at 15 000 *g* for 1 hr and the supernatant used as elicitor.

Callus of *Oryza sativa* L. cv. Koshihikari, K13 was kindly supplied by Mr Osamu Kawakami. For suspension culture, approximately 2 g callus, which was filtered through a stainless-steel mesh (20 mesh) to generate small aggregates, was transferred to a 500 ml flask containing 90 ml liquid medium. The medium contained 30 g l^{-1} sucrose, 809 mg l^{-1} KNO_3, 66 mg l^{-1} $(NH_4)_2SO_4$, 312 mg l^{-1} $NaH_2PO_42H_2O$, 2.2 mg l^{-1} $MnSO_44H_2O$, 2.2 mg l^{-1} $ZnSO_47H_2O$, 0.2 mg l^{-1} $CuSO_45H_2O$, 0.13 mg l^{-1} $Na_2MoO_42H_2O$, 2.9 mg l^{-1} H_3BO_3, 148 mg l^{-1} $CaCl_22H_2O$, 246 mg l^{-1} $MgSO_47H_2O$, 20 mg l^{-1} Fe-EDTA, 1 mg l^{-1} nicotinic acid, 10 mg l^{-1} thiamine-HCl, 1 mg l^{-1} pyridoxine-HCl, 100 mg l^{-1} inositol, 700 mg l^{-1} aspartic acid, 700 mg l^{-1} glutamine and 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid, pH 5.8 (DKN medium [13]). The suspension culture was incubated on a rotary shaker at 25° with agitation at 80 rpm in the light (3000 lux). After incubation for 14 days, 2 g callus was again filtered through the stainless-steel mesh and the small aggregates of cells transferred to a new 500-ml Erlenmeyer flask containing 90 ml DKN medium plus 2 ml of a preparation of elicitor from *P. infestans*. After incubation for 2 days, 1 ml of the preparation of elicitor was again added to the culture. After further incubation for 5 days, the culture medium was extracted with ethyl acetate and the amounts of phytocassanes and momilacetones produced were quantitated by HPLC as described previously [8].

Phytoalexin accumulation in rice plants infected with Magnaporthe grisea *and* Rhizoctonia solani. Rice plants (*Oryza sativa*, L. cv. Jukkoku) were cultivated in a phytotron and, at the fifth-leaf stage, the leaves were sprayed with the spore suspension of *M. grisea* (incompatible race 031), prepared as described previously [14]. After the rice plants had been kept in a moist chamber (100% humidity) at 24° for 24 hr, they were removed and cultivated in the phytotron at 23°, as described previously [14]. Seven days after inoculation, the lesions on the fourth and fifth leaves were collected. Rice stems (*Oryza sativa* L. cv. Koshihikari) infected with *R. solani* (2 kg) were collected in a paddy field of Konokan High School, Niigata-ken, Japan, in September 1993. The amounts of phytocassanes and momilactones produced were quantitated by HPLC, as described previously [8].

Isolation procedure of phytocassane E. A suspension culture that had been treated with the *P. infestans* elicitor was centrifuged at 15 000 *g* for 1 hr and the supernatant adjusted to pH 11 with Na_2CO_3 and partitioned with EtOAc. The EtOAc phase was evaporated and dissolved in 45% EtOH. The sample was subjected to HPLC on a TSKgel ODS-120A column (21.5 mm i.d. × 37.5 cm) that was eluted with 55% $MeCN_3$. The eluate was monitored using a UV detector (absorbance 280 nm). The fraction corresponding to the peak of phytocassane E was collected and evaporated. The residue was dissolved in 42% EtOH and subjected to HPLC on a TSKgel ODS-120T column (21.5 mm i.d. × 37.5 cm) that was eluted with 50% $MeCN_3$. The purified sample was obtained from the fraction corresponding to the peak of phytocassane E.

Phytocassane E (**1**). Gum. CD (λ_{max} nm), EtOH: 348 ($\Delta\varepsilon = -4.47$), 317 ($\Delta\varepsilon = -2.25$), 298 ($\Delta\varepsilon = -4.00$), 269 ($\Delta\varepsilon = +4.18$), 229 ($\Delta\varepsilon = -5.02$). UV ($\lambda_{max}$ nm), EtOH: 273. IR (ν_{max} cm^{-1}): 3408, 2980, 2939, 2872, 1713, 1635, 1622, 1591. ^1H NMR ($CDCl_3$) δ: 0.88 (3H, *s*, Me-20), 1.07 (3H, *s*, Me-18), 1.11 (3H, *s*, Me-19), 1.12 (3H, *d*, $J = 7.0$ Hz, Me-17), 1.54 (1H, *m*, H-6β), 1.56 (1H, *m*, H-5), 1.56 (1H, *m*, H-7α), 1.77 (1H, *m*, H-6α), 1.79 (1H, *m*, H-7β), 2.20 (1H, *dddd*, $J = 13.0$, 12.0, 4.0, 4.0 Hz, H-8), 2.27 (1H, *d*, $J = 13.0$ Hz, H-9), 2.47 (1H, *dd*, $J = 14.0$, 4.3 Hz, H-2β), 2.70 (1H, *dq*, $J = 7.0$, 4.0 Hz, H-14), 2.96 (1H, *dd*, $J = 14.0$, 7.6 Hz, H-2α), 4.16 (1H, *ddd*, $J = 7.6$, 4.3, 1.5 Hz, H-1β), 5.57 (1H, *d*, $J = 11.0$ Hz, H-16), 5.75 (1H, *d*, $J = 17.7$ Hz, H-16), 5.88 (1H, *s*, H-12), 6.21 (1OH, *d*, $J = 1.5$ Hz, OH-1), 6.38 (1H, *dd*, $J = 17.7$, 11.0 Hz, H-15). EIMS *m/z* (%): $[M - 18]^+$ 298 (24), 283 (5), 270 (4), 265 (5), 255 (7), 241 (3), 227 (5), 213 (27), 201 (7), 185 (13), 173 (9), 161 (9), 147 (32), 135 (12), 121 (100), 108 (35), 93 (43), 77 (42), 65 (17), 56 (21), 41 (44); HREIMS $[M]^+$ at *m/z* 316.2062 (calc. for $C_{20}H_{28}O_3$, 316.2086).

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