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ISOLATION AND CHARACTERIZATION OF TWO PHYTOALEXINS FROM RICE AS MOMILACTONES A AND B

DAVID WILLIAM CARTWRIGHT,*‡ PETER LANGCAKE,† ROBERT JOHN PRYCE,† DAVID PAUL LEWORTHY† and JONATHAN PAUL RIDE*

* Department of Microbiology, The University, Birmingham, B15 2TT, U.K.; † Shell Research Ltd., Sittingbourne Research Centre, Kent, U.K.

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Abstract—Two phytoalexins were isolated as chromatographically homogeneous amorphous solids from UV-irradiated, dark-grown rice coleoptiles. From their mass and ^1H NMR spectra, the compounds were characterized as the known diterpenes, momilactones A and B. The same compounds were also produced in blast-infected, WL 28325-treated rice leaves. They appear to be the first clearly identified cereal phytoalexins.

INTRODUCTION

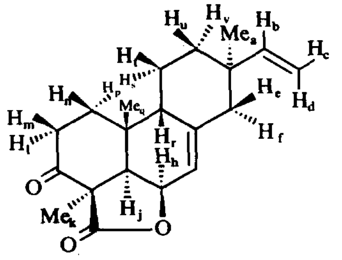
Two phytoalexin-like compounds are produced by rice in response to either infection by the blast fungus (*Pyricularia oryzae*) or irradiation with UV light [1, 2]. Enhanced accumulation of these highly antifungal compounds is a prominent feature of dichlorocyclopropane-induced blast resistance [3]. There are earlier reports of phytoalexin-like activity in blast- and *Helminthosporium oryzae*-infected rice [4, 5]. The present paper describes the isolation, characterization and chromatographic properties of the two phytoalexin-like compounds from rice as momilactones A and B.

RESULTS AND DISCUSSION

UV-Irradiated dark-grown coleoptiles were used as a convenient source of the two phytoalexin-like compounds. Since the activity of both compounds was greatly reduced by prolonged drying on TLC plates, emphasis was placed on the use of liquid chromatographic systems. The isolation procedure is described in the Experimental.

Examination of the ^1H NMR spectra of the two compounds indicated their identity with momilactones A (1) and B (2), diterpenes isolated previously from rice husks [6, 7]. The proton assignments for momilactone A (Table 1) were made on the basis of the selective decoupling experiments listed in Table 2. The resonance signals arising from the methyl groups (a and q) were assigned unambiguously by the results of irradiation 8 (i.e. proton e or f) which sharpened signal 17 (hence methyl a), and irradiation 10 (proton q) which sharpened signal 16 (hence methyl q).

The 100 MHz spectrum of momilactone B showed the expected differences when compared with the spectrum of momilactone A. These were: (a) the methyl resonance signal at δ 1.0 (assigned as Me_q in momilactone A) was not present, but an AB quartet was present at δ 4.1/3.5 (consistent with the replacement of methyl by —CH_2O moeity); (b) the methylene resonance signal in the region δ 2.58–2.69 (assigned as l, m in momilactone A) moved upfield in the spectrum of B (consistent with replacement of carbonyl with O—C—O); and (c) the methyl resonance signal at δ 1.52 (Me_k in momilactone A) moved upfield in the spectrum of B (again consistent with replacement of the carbonyl function). These were the major differences observed in the spectrum of momilactone B compared



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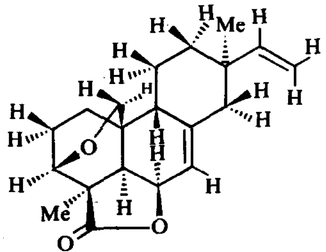

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‡ Current address: Department of Applied Biology, Chelsea College, University of London, London, U.K.

Table 1. 360 MHz ^{1}H NMR assignments for momilactone A

Signal	Shift	Multiplicity	Coupling (Hz)	Assignment
1	5.85	*dd*	$J_{13} = 13$ $J_{14} = 10.5$	b
2	5.71	*d*	$J_{25} = 5$	g
3	4.98	*dd*	$J_{34} = 1$	d
4	4.95	*dd*		c
5	4.84	*t*	$J_{57} = 5$	h
6	2.58–2.69	complex		l, m
7	2.32	*d*		j
8	2.21	*d*	$J_{89} = 12.5$	e or f
9	2.06	*d*		e or f
10	1.90	*dd*		p
11	1.80	*dd*		r
12	1.75	complex		s
13	1.50–1.65	complex		n, u, v
14	1.52	*s*		k
15	1.32	complex		t
16	1.00	*s*		q
17	0.90	*s*		a

Table 2. 360 MHz ^{1}H NMR decoupling experiments with momilactone A

	Signals affected	
Irradiation of signals	Major coupling removed	Minor coupling removed
1	3, 4	8, 9, 17
2	5	8, 9, 11
5	2, 7	
6	10, 13	
7	5	
8	9	17
9	8	13
10	6, 13	16
11/12	13, 15	2, 16
13/14	6, 10, 12, 15	9
15	11, 12, 13	

with that of momilactone A. Lack of material did not allow comprehensive spin decoupling experiments and the complete assignments of momilactone B.

Comparisons of the mass (electron impact and field desorption ionization), IR and UV spectra, and the chromatographic properties (TLC and GLC, Table 3) of the phytoalexins from rice with authentic samples of momilactones A and B further confirmed their identities. Their identity with the two antifungal compounds produced in blast-infected, WL 28325-treated rice leaves, as opposed to UV-irradiated coleoptiles, was also confirmed by GC–MS. Momilactones A and B appear to be the first phytoalexins characterized from any member of the Gramineae.

EXPERIMENTAL

Production of UV-irradiated material. Seeds of the blast susceptible rice variety, Sasashigure, were sown on water-soaked vermiculite and grown at 25° in complete darkness. After 8 days the etiolated coleoptiles were irradiated for 15 min with a broad spectrum UV lamp (incident energy 140 W/m^2), returned to the dark for a further 48 hr, and then harvested for extraction.

Table 3. TLC and GLC characteristics of momilactones A and B

	R_f values in TLC solvent systems*				Retention times (min) on GLC phases†	
	DCM	CD	CE	AP	OV-17	OV-101
Momilactone A	0.13	0.22	0.55	0.77	5.5	8.4
Momilactone B	0.08	0.21	0.47	0.72	4.8‡	7.8‡

* Compounds applied in $CHCl_3$ and Si gel plates and developed in: DCM, CH_2Cl_2; CD, cyclohexane–dioxane (7:3); CE, $CHCl_3$–EtOH (97:3); AP, Me_2CO–petrol (17:3).

† Compounds applied in EtOAc to 1.5 m silanized glass columns, 1 % phase loadings, column temperatures 213° (OV-17) and 188° (OV-101); FID, N_2 carrier gas flow rate—45 ml/min.

‡ Retention time of TMSi derivative.

Isolation of phytoalexins. Irradiated coleoptiles (1.2 kg) were extracted by mincing twice into 3 l. EtOH and homogenizing the resultant suspension in an omnimixer. The solid residue was filtered, re-extracted twice overnight at 4° using 2 l. portions of fresh EtOH, and the three filtrates were finally combined. This extract was then dried (35°, *in vacuo*), the residue dissolved in H_2O (400 ml) and extracted ×4 with equal vols of Et_2O. The organic phases were combined, reduced to 200 ml and washed ×3 with equal vols of Pi buffer (1.4 M, pH 6.3) to remove strong acids. The Et_2O was removed (25°, *in vacuo*), the residue taken into EtOH (10 ml, 90%) and applied to a Sephadex LH-20 column (76 × 3 cm). Fractions were eluted with EtOH (90%) and tested for antifungal activity by the *Cladosporium cucumerinum* TLC bioassay of ref. [8], using extract loadings equivalent to 2 g fr. wt tissue and $CHCl_3$–EtOH (97:3) as developing solvent. Active fractions (all of which contained both phytoalexins) were combined, reduced to 5 ml and dried onto Celite 545 (2.5 g). This was applied to a column (32 × 2.5 cm) of Si gel in hexane, and fractions eluted with a gradient of 20, 50, 70 and 85% $CHCl_3$ in hexane (200 ml each solvent), and finally 90% $CHCl_3$ in hexane (500 ml). Both phytoalexins, detected by TLC bioassay, were eluted in the final fraction which was taken to dryness (30°, *in vacuo*) and redissolved in $CHCl_3$ (400 μl). The sample was fractionated further by HPLC on Partisil 10 [eluant, $CHCl_3$–EtOH–hexane (50:1:49), operating pressure, 1000 psi]. Fractions were taken according to a trace of UV absorbance at 225 nm. Since the two phytoalexins remained unresolved, all active fractions were combined, reduced to dryness (30°, *in vacuo*) and redissolved in $CHCl_3$ (200 μl). This was then chromatographed on Si gel TLC using cyclohexane–dioxane (7:3) as developing solvent. The area between R_f 0.20 and 0.24 corresponding with both phytoalexins was eluted with $CHCl_3$–EtOH (9:1, 3 × 1.5 ml), the eluates were combined, reduced to 150 μl, and re-chromatographed on Si gel TLC in $CHCl_3$–EtOH (97:3). To enable accurate location of the two phytoalexins, a separate 1 μl sample of the eluate was chromatographed simultaneously and then sprayed with 1% vanillin in conc H_2SO_4. On the basis of the purple colour reaction of momilactone A (R_f 0.55), and the light blue reaction of momilactone B (R_f 0.47), the compounds were eluted separately from the preparative chromatogram using $CHCl_3$–EtOH (9:1, 3 × 1.5 ml). Each phytoalexin was finally purified by elution in EtOH (90% v/v) from a Sephadex LH-20 column (32 × 2 cm), fractions being taken according to UV absorbance at 225 nm. Following solvent evapn (30°, *in vacuo*), the compounds were obtained as white amorphous solids.

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