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IDENTIFICATION OF 5-*n*-(2′-OXO)ALKYLRESORCINOLS FROM ETIOLATED RICE SEEDLINGS

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Key Word Index—*Oryza sativa*; Gramineae; rice; seedlings; structures; biosynthesis; collisionally activated dissociation; (2′-oxo)alkylresorcinol; alkylresorcinol.

Abstract—Six 5-*n*-(2′-oxo)alkylresorcinols were isolated from etiolated rice seedlings. The identification of these compounds was achieved by spectroscopy. From a comparison of the double bond position between 5-*n*-alkyl- and 5-*n*-(2′-oxo)alkylresorcinols in rice seedlings, the predicted biosynthetic pathway of 5-*n*-alkylresorcinols was supported. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Recently, Suzuki *et al.* [1] has reported the identification of 5-*n*-alkylresorcinols (ARs) as antifungal agents specific to etiolated rice seedlings (Fig. 1). The level of rice ARs is low under light illumination and exposure of the etiolated seedlings to light results in a marked reduction of the AR level [2]. Bouillant *et al.* [3] has shown that a high level of ARs is detected in the root exudates. These findings suggest that rice ARs protect the rice seedlings against microbial attack. Interestingly, accumulation of ARs can be detected in Indica-type cultivars, weedy- and wild-type rice [2], but not Japonica-type cultivars. Long-chain ARs, such as rice ARs, have been found in higher plants [4, 5] including gramineous cereals, brown algae [6], Streptomyces species [7] and bacteria [8], and the ARs are mostly localized in specific organs of these organisms. Since ARs exhibit inhibitory activity against a number of fungi, ARs are assumed to protect these organisms against microbial attack. In order to determine the actual roles of ARs in rice seedlings and the mechanism of AR accumulation, AR biosynthesis and/or metabolism must be established. To our knowledge, however, studies on AR biosynthesis and metabolism are few. It has been reported that β-hydroxybutyric acid is incorporated into bacterial ARs [9]. We investigated AR-related compounds in etiolated rice seedlings in an attempt to predict the biosynthetic pathway of rice ARs.

In this report, the isolation and identification of an AR-related compound, mixture A, in etiolated rice seedlings are described. In addition, the biosynthetic pathway of rice ARs is discussed based on the structure of mixture A.

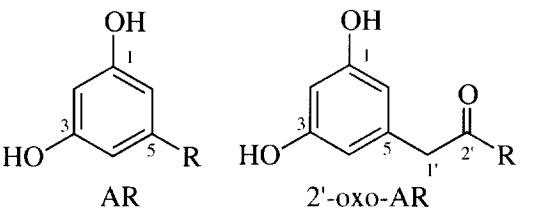

Fig. 1. Structure of mixture A: 5-*n*-(2′-oxo)alkylresorcinols R = -$(CH_2)_{12}CH_3$, R = -$(CH_2)_7CH$ = $CH(CH_2)_5CH_3$, R = $(CH_2)_{14}CH_3$, R = -$(CH_2)_7CH$ = $CH(CH_2)_7CH_3$, R = $(CH_2)_7$ CH = $CHCH_2CH$ = $CH(CH_2)_4CH_3$, R = -$(CH_2)_{16}CH_3$.

RESULTS AND DISCUSSION

The MeOH extract of 12-day-old etiolated rice seedlings (3.2 kg fr. wt) was partitioned between water and EtOAc. The AR-containing fraction was collected by Sephadex LH-20 column chromatography of the EtOAc extract. Silica-gel column chromatography of the AR-containing fraction followed by prep. TLC gave an AR-related compound, mixture A (6.3 mg), as a single spot on TLC. Analyses of mixture A indicated that it contains six closely related substances having different alkyl groups. The gross structure of mixture A was elucidated in the mixed state.

The ^{1}H NMR spectrum of mixture A in $CDCl_3$ revealed signals at δ 6.25 (3H, *s*), 5.82 (2H, *s*, phenol), 3.57 (2H, *s*), 2.47 (2H, *t*) and 1.55 (2H, *m*) ppm (Table

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Table 1. ^1H and ^{13}C NMR data (δ ppm) of mixture A

Atom no.	Mixture A		ARs
	^1H NMR $CDCl_3$ (C_6D_6)	^{13}C NMR $CDCl_3$	^{13}C NMR $CDCl_3$
C-1	/	157.10*s*	156.4*s*
C-2	6.25*s* (6.11*t*)	101.83*d*	100.1*d*
C-3	/	157.10*s*	156.4*s*
C-4	6.25*s* (6.17*d*)	109.02*d*	108.0*d*
C-5	/	136.52*s*	146.1, 146.2*s*
C-6	6.25*s* (6.17*d*)	109.02*d*	108.0*d*
C-1′	3.57*s* (3.18*s*)	49.82*t*	
C-2′	/	210.23*s*	
C-3′	2.47*t* (2.07*t*)	42.22*t*	
C-4′	1.55*m* (1.44*m*)	23.74*t*	
OH	5.82*s* (5.15*s*)	/	

1). In addition, signals at δ 0.88 (3H, *t*), 2.00 (*ca.* 2.05H, *m*), 5.34 (*ca.* 1.20H, *m*), 1.30 (*ca.* 20H, *m*) and 2.78 (weak, *m*) were seen. The singlet at δ 6.25 ppm in $CDCl_3$, assigned to aromatic protons, split into two peaks at δ 6.17 (2H, broad *d*, J = 2 Hz) and 6.11 (1H, broad *t*, J = 2 Hz) ppm in C_6D_6. This splitting pattern in C_6D_6 suggested that the structural unit of mixture A was that of a 5-alkylated resorcinol. In the ^{13}C NMR spectrum ($CDCl_3$) of mixture A, chemical shifts of five aromatic carbons in the 5-alkylated resorcinol were near those of rice ARs (Table 1) [1]. This result also supported the presence of the 5-alkylated resorcinol structure in mixture A. IR and ^{13}C NMR spectra of mixture A indicated the presence of an oxo group (ν 1700 cm^{-1} and δ 210.23 ppm), the location of which was determined to be on C-2′ of the alkyl chain, since the proton signals at δ 3.57, 2.47 and 1.55 ppm could be assigned to H-1′, H-3′ and H-4′ methylenes, respectively. The results of FG/HMQC, FG/HMBC and ROESY correlations ($CDCl_3$) of mixture A confirmed the 5-(2′-oxo)alkylresorcinol structure, as illustrated in Fig. 2. Of the proton signals described above, those at δ 5.34, 2.00 and 2.78 ppm, which were assigned to olefinic and allylic protons in the alkyl groups, were nonintegrable. This suggests that mixture A is a mixture of closely related substances having different alkyl groups. This was confirmed by the detection of six quasi-molecular ions in the negative and positive FAB/MS spectra of mixture A (Table 2). The molecular formulae of these components were determined from high-resolution FAB/MS (see Experimental). The Z geometry of the double bonds in the alkyl groups was shown by the presence of methylene carbons at δ 27.16 and 27.19 ppm in the ^{13}C NMR spectrum of mixture A, being due to the allylic methylene carbons adjacent to a Z double-bond group [10]. Further characterization of the positions of the double bonds and branching in the alkyl groups was achieved by analysis of collisionally activated dissociation mass (CAD/MS) spectra using tandem mass spectrometry. CAD/MS spectra of compounds with a long-chain alkyl group and a localized charge, such as rice ARs, are very useful for determining the positions of double bonds, branchings and functional groups [11].

Mixture A, which consists of closely related substances having different alkyl groups, was purified by HPLC on an ODS column. Fractions corresponding to six of the baseline-separated peaks, peak/1 to peak/6, which were detected at the UV absorbance of 274 nm, were collected. Each peak represented a single component, judging from the FAB/MS spectrum. The CAD/MS spectrum for each component was taken, using the same method as that described for rice and rye ARs [1, 12].

Figure 3 shows the CAD/MS spectra for three components that have a C-19 side chain, as a typical example. In the negative CAD/MS spectrum (1) of peak/6 component having a saturated alkyl group, a sequence of odd-mass series that was spaced by 14 amu after an initial loss of methane was observed. Such fragment ion series is a characteristic of the linear saturated alkyl structure. The linear saturated alkyl group was also detected in peak/1 and peak/4 homologues (data not shown). In the negative CAD/MS spectrum (2) of peak/5 component having a double bond in the alkyl group, a typical "window" of 54 amu formed by the peaks at *m/z* 233 and 287, being due to allylic cleavage *via* a 1,4-elimination on H_2 on either side of the double bond, was observed. This result indicates that the double bond is found on C-10′. Similarly, the position of the double bond for peak/2 homologue was on C-10′ (data not shown). Peak/3 component has two double bonds in the alkyl group. These double bonds are present as a homoconjugated diene system, based on the ^1H NMR data of mixture A in $CDCl_3$; the weak multiplet at δ 2.78 ppm, being assigned to methylene protons characteristic of the homoconjugated diene system, was observed. To determine the position of such a homoconjugated diene system, CAD/MS spec-

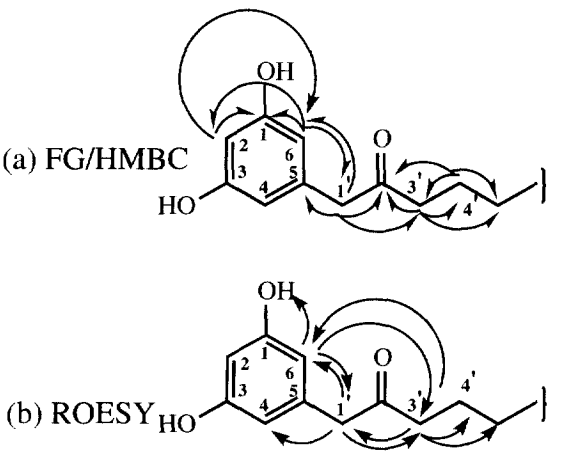


Fig. 2. Structural subunit of mixture A, assigned on the basis of NMR data.

Table 2. Separation of the components by HPLC

Peak no.	Rt/min[a]	Rel. area %[b]	$[M+H]^{+}$[c]	$[M-H]^{-}$[c]	Rel. int. %[d]
1	13.4	51	*m/z* 335	*m/z* 333	28
2	14.7	8	*m/z* 361	*m/z* 359	10
4	21.0	73	*m/z* 363	*m/z* 361	68
3	17.1	10	*m/z* 387	*m/z* 385	11
5	23.0	100	*m/z* 389	*m/z* 387	100
6	35.0	8	*m/z* 391	*m/z* 389	11

[a] ODS (ϕ10 × 250 mm)/90% MeOH (0.2% AcOH): 3 ml/min.
[b] A_{274}.
[c] Mixture A and the isolated components.
[d] The quasi-molecular ions in negative FAB/MS of mixture A.

tra using lithium adduct ions as precursor ions are useful, as shown previously in rye ARs having a homoconjugated diene group [12]. In the positive CAD/MS spectrum (3) of peak/3 component, a typical pair of windows of 40 amu formed by the peaks at *m/z* 301 and 341 and of 54 amu formed by the peaks at *m/z* 247 and 301, being due to allylic cleavage *via* an elimination of the illustrated side of the double bonds, was observed. This result indicates that the double bonds are found on C-10′ and C-13′. Thus, the structures of all six components of mixture A were established to be 5-*n*-(2′-oxo)alkylresorcinols (2′-oxo-ARs), as shown in Fig. 1.

The biosynthetic pathway of long-chain ARs is predicted, as shown in the upper scheme of Fig. 4, from analogy with 6-methylresorcylic acid (orsellinic acid) biogenesis [13] and from the co-occurrence of ARs, the corresponding 6-alkylresorcylates, 6-alkylphloroglucinol or 4-hydroxy-6-(2′-oxo)alkyl-2-pyrones in *Ginkgo biloba* [14], brown algae [6, 15] and bacteria [8]. The addition of three acetate units to a fatty acid leads to triketone (1), which by ring closure through aldol condensation gives the 6-alkylresorcylic acid derivative (2). Decarboxylation of (2) produces ARs. Of the AR biosynthesis-related compounds, the 4-hydroxy-6-(2′-oxo)alkyl-2-pyrones in bacteria [8] and 6-alkylresorcylates [6, 8, 14, 15] are structurally comparable to triketone (1) and 6-alkylresorcylic acid derivative (2), respectively. In this study, we identified six 2′-oxo-ARs. There are two possible routes for 2′-oxo-AR biosynthesis; are ARs involved as intermediates or not? A comparison of the double-bond positions between ARs and 2′-oxo-ARs could answer this question. The double bond is positioned at C-8′ in the ARs [1]. If 2′-oxo-ARs were formed by oxidation of the 2′-methylene carbon of ARs, the double bonds in 2′-oxo-ARs would be found on C-8′. However, all the double bonds in 2′-oxo-ARs were found on C-10′. This implies that ARs are not the intermediates for 2′-oxo-ARs biosynthesis, and that 2′-oxo-ARs are biosynthesized from an intermediate prior to the cyclization process from (1) to (2) in the upper scheme of Fig. 4. It is reasonable to consider that tetraketone (3), which is derived by the addition of an acetate unit to triketone (1), produces 2′-oxo-ARs, through the same reactions as those predicted in the AR biosynthetic route. If the AR biosynthetic pathway in rice is the same as that in other plants and bacteria, the identification of 2′-oxo-ARs in this study supports the predicted biosynthetic pathway of long-chain ARs.

Mixture A inhibited spore germination of the fungus *Pyricularia oryzae* at an $[ED]_{50}$ of 40 μg/ml. Considering the $[ED]_{50}$ of 45 μg/ml for the corresponding rice AR mixture [1], the oxo function at C-2′ in the alkyl group did not reduce the biological activity of ARs.

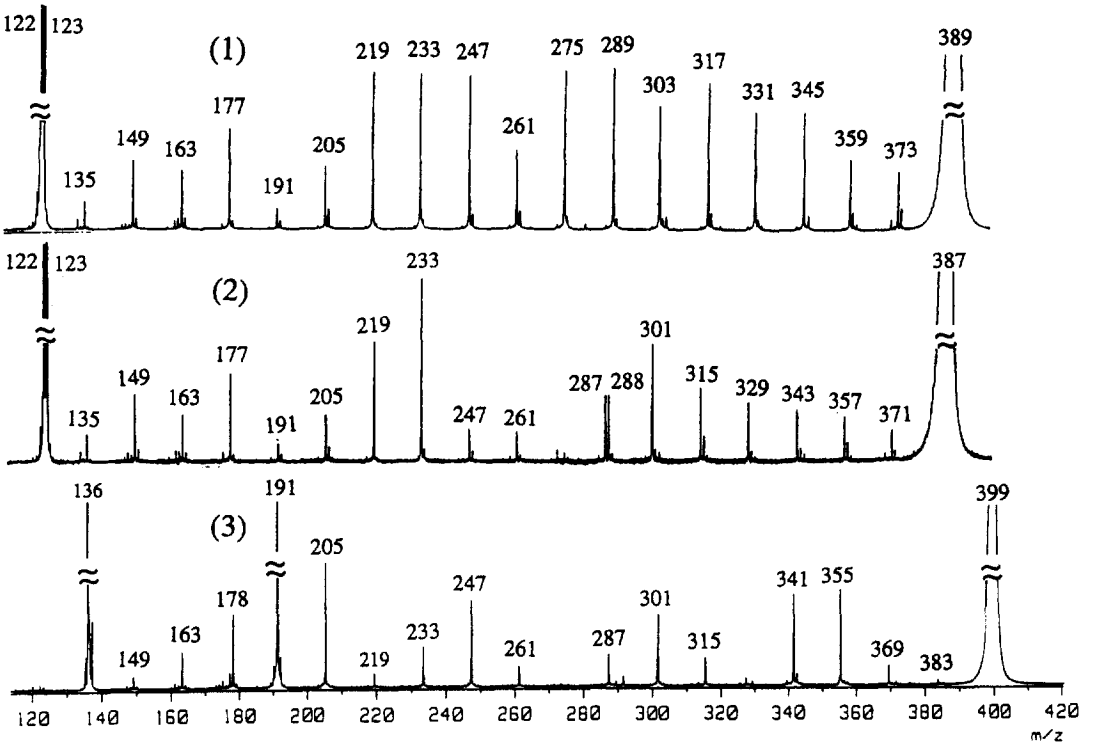
EXPERIMENTAL

General. NMR spectra were recorded with TMS as an internal standard. IR spectra were measured as films.

Rice seedlings. Seeds (*Oryza sativa* L. indica-type, *cv.* RD-25) were sterilized with 70% EtOH for 3 min, planted in pots filled with vermiculite and grown in a growth chamber (28° to 30°).

Isolation of mixture A. Whole 9-day-old etiolated rice seedlings (*ca.* 740 g fr. wt) were homogenized with MeOH (5 L) and the homogenate was allowed to stand for one week. The filtrate was concentrated *in vacuo* and the resulting material was extracted with EtOAc. The EtOAc fraction (8.11 g) was subjected to Sephadex LH-20 C.C. with MeOH as eluent. The fraction containing AR (1.28 g) was collected, as judged from TLC. The resulting AR-containing fraction was subjected to silica-gel C.C. with EtOAc-CH_2Cl_2 (10:90 and then 20:80) as eluent, from which the crude AR (561 mg) and mixture A (27.5 mg) fractions were obtained. Each fraction was purified by prep. TLC on silica gel to give AR (318 mg) and mixture A (6.3 mg) as a single spot on TLC, respectively.

Mixture A, IR ν_{max} (film) cm^{-1}: 3400, 2920, 2850, 1700, 1640, 1590, 1150, 1000, 850, 910; ^1H NMR (400 MHz, $CDCl_3$): δ 6.25 (3H, *s*, 2, 4, 6-H), 5.82 (2H, *s*, phenol), 3.57 (2H, *s*, 1′-H), 2.47 (2H, *t*, J = 7.6 Hz, 3′-H), 1.55 (2H, *m*, 4′-H), 2.00 (*ca.* 2.05H, *m*,



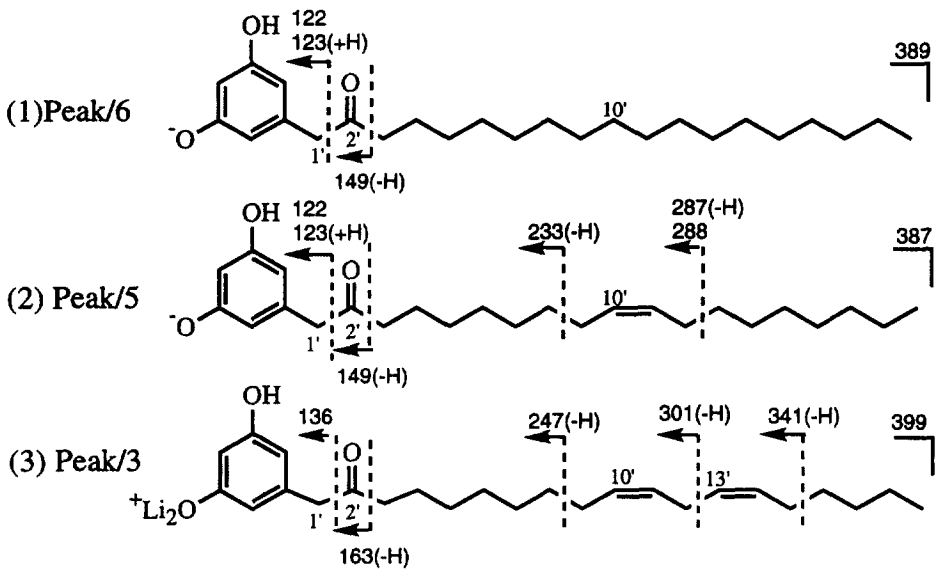


Fig. 3. CAD/MS spectra of mixture A components: (1) Peak/6, $[M-H]^-$, (2) Peak/5, $[M-H]^-$, Peak/3, $[M+2Li\text{-}H]^+$.

—CH_2—CH=CH—), 5.34 (*ca.* 1.20H, *m*, —CH_2—CH=CH—), 1.30 (*ca.* 20H, *m*), 0.88 (3H, *t*, J = 6.8 Hz, —CH_2CH_3), 2.78 (weak, *m*, =CH—CH_2CH=); ^{1}H NMR (400 MHz, C_6D_6): δ 6.17 (2H, *d*, J = 2.0 Hz, 4, 6-H), 6.11 (1H, *t*, J = 2.0 Hz, 2-H), 5.15 (2H, *s*, phenol), 3.18 (2H, *s*, 1′-H), 2.07 (2H, *t*, J = 7.3 Hz, 3′-H), 1.44 (2H, *m*, 4′-H), 2.07 (2.38H, *m*, —CH_2—CH=CH—), 5.50 (*ca.* 1.16H, *m*, —CH_2—CH=CH—), 1.30 (*ca.* 22H, *m*), 0.90, 0.91 (each *ca.* 1.5H, *t*, J = 6.7 Hz, —CH_2CH_3), 2.90 (weak, *m*, =CH—CH_2CH=); ^{13}C NMR (100 MHz, $CDCl_3$): δ 200.23 (*s*, C-2′), 157.10 (*s*, C-1, 3), 136.52 (*s*, C-5), 109.02 (*s*, C-4, 6), 101.83 (*s*, C-2), 49.82 (*t*, C-1′), 42.22 (*t*, C-3′), 23.74 (*t*, C-4′), 129.75, 129.98 (each, *d*, —CH_2CH=CH—), 27.16, 27.19 (*t*, —CH_2CH=CH—), 31.90 (*t*), 14.10 (*q*, —CH_3), 29.04, 29.09, 29.25, 29.30, 29.35, 29.45, 29.51, 29.60, 29.61, 29.74 (each, *t*), 22.67 (*t*), 23.74 (*t*); FAB/MS *m/z* $[M+H]^+$ (Rel. int.): 391 (22), 389 (63), 387 (7), 363 (100), 361 (13), 335 (45), *m/z* $[M-H]^-$ (Rel. int.):

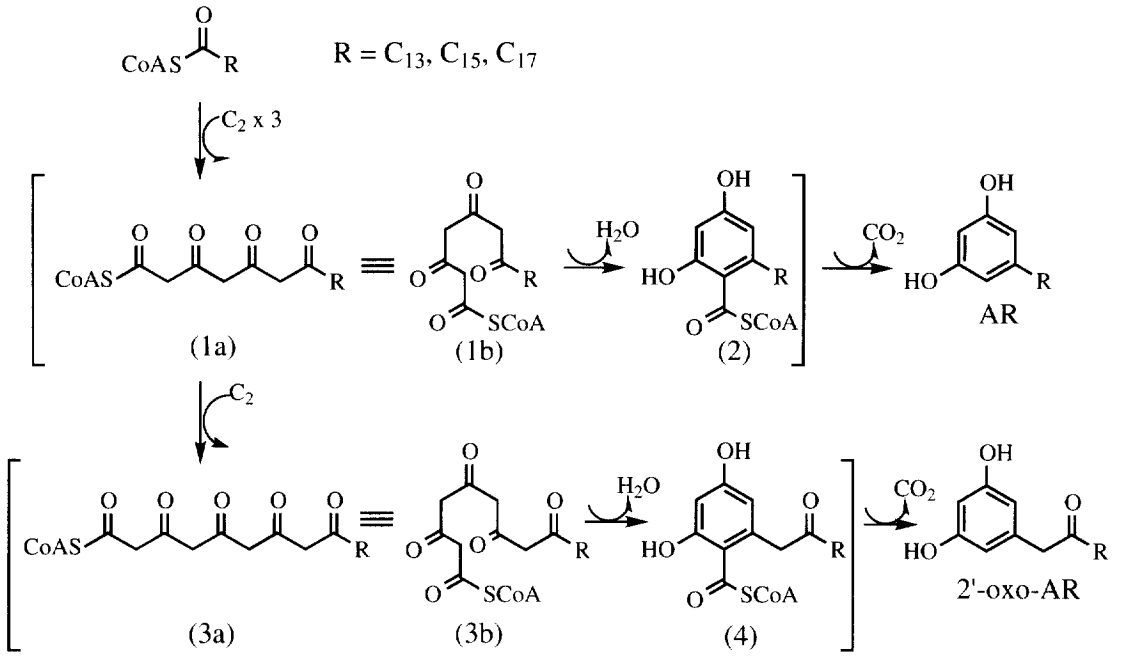


Fig. 4. Possible biosynthetic pathway of ARs and 2′-oxo-ARs in etiolated rice seedlings.

Table 2; high-resolution FAB/MS *m/z*: found, 391.3191 $[M+H]^+$, calcd. 391.3212 ($C_{25}H_{43}O_3$); found, 389.3040 $[M+H]^+$, calcd. 389.3055 ($C_{25}H_{41}O_3$); found, 387.2881 $[M+H]^+$, calcd. 387.2899 ($C_{25}H_{39}O_3$); found, 363.2895 $[M+H]^+$, calcd. 363.2899 ($C_{23}H_{39}O_3$); found, 361.2744 $[M+H]^+$, calcd. 361.2742 ($C_{23}H_{37}O_3$); found, 335.2570 $[M+H]^+$, calcd. 335.2586 ($C_{21}H_{35}O_3$); found, 333.2431 $[M+H]^+$, calcd. 333.2430 ($C_{21}H_{33}O_3$).

Separation of each component of mixture A by prep. HPLC. Prep. HPLC was performed on a Senshu Pak PEGASIL ODS column (5 μm particle size, φ 10 mm × 250 mm). The column was developed at a flow rate of 3 ml/min. with 90% MeOH containing 0.2% AcOH. Six peaks that were monitored with a UV detector (274 nm) were collected, concentrated and analyzed by FAB/MS. Each peak gave the corresponding single quasi-molecular ion shown in Table 2. Peak/3, FAB/MS *m/z* (rel. int): 393 (81, $M+Li)^+$, 399 (100, $M+2Li-H)^+$, 405 (35, $M+3Li-2H)^+$.

FAB/MS and FAB/MS/MS. A JMS HX-110/110A tandem mass spectrometer was used. Ions that were produced by bombarding the sample with 6 keV Xe atoms were accelerated through a potential of 10 kV. CAD/MS experiments were conducted by mass selection of ions with MS-I, the mass resolution for the mass-selected ions being approximately 1000. The 10 keV mass-selected ions were then activated by collision with helium in a collision chamber floated at 8 kV. Helium gas pressure was adjusted in order to attenuate the primary ion beam by 70%. Fragment ions were detected with a JEOL ADS 11S variable-dispersion array detector equipped with MS-II. Nitrobenzyl alcohol (3-NBA) for negative ion MS and magic bullet (dithioerythritol/dithiothritol = 1/3) saturated with lithium hydroxide for positive-ion MS were used as matrices. All CAD/MS spectra were obtained by using quasi-molecular anions and/or lithium-adduct cations produced by FAB as precursor ions.

Spore germination assay using Pyricularia oryzae. The spore germination test was conducted using the rice blast fungus, *Pyricularia oryzae*, according to a previously described procedure [1].

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