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AN ANTIFUNGAL COMPOUND, 9,12,13-TRIHYDROXY-(*E*)-10-OCTADECENOIC ACID, FROM *COLOCASIA ANTIQUORUM* INOCULATED WITH *CERATOCYSTIS FIMBRIATA*

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Key Word Index—*Colocasia antiquorum*, Araceae, taro tubers; isolation, lipid peroxide, antifungal compound, 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid

Abstract—An antifungal compound has been isolated from tubers of taro (*Colocasia antiquorum*) inoculated with black rot fungus (*Ceratocystis fimbriata*) and identified as 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid.

INTRODUCTION

Various strains of *Ceratocystis fimbriata* have been isolated from black rot lesions on various plants such as taro and sweet potato; host plants differ in their response to them [1, 2]. When taro, *Colocasia antiquorum*, tubers are inoculated with an endoconidial spore suspension of the taro strain of *C. fimbriata*, the spores germinate and invade the inner tissue, resulting in the appearance of symptoms of black rot. In contrast, when taro tubers are inoculated with the sweet potato strain of *C. fimbriata*, which is incompatible with taro, the spores germinate but hyphal extension is inhibited. No black rot symptoms develop but the inoculated surface turns a reddish colour within 24 hr. These observations suggest that taro tubers show a hypersensitive reaction and may produce some antifungal compound(s) in response to inoculation with the sweet potato strain. As far as we know, there is no report of the isolation of any antifungal compounds from inoculated taro tubers. Therefore, we tried to isolate such a compound from taro tubers inoculated with the sweet potato strain of *C. fimbriata*.

RESULTS AND DISCUSSION

The acetone extracts from freshly prepared, wounded (incubated without inoculation) and inoculated taro tuber disks were applied to HPLC (ODS) and the resultant fractions were assayed for antifungal activity (Fig. 1). Fractions with antifungal activity were detected from all extracts. However, the highest antifungal activities were detected in fractions 14–15, all extracts from inoculated disks; no antifungal activity was detected in extracts from the fungus grown on potato–dextrose agar medium Thus, taro tuber produces antifungal compound(s) in response to inoculation with the sweet potato strain of *C. fimbriata*.

Rechromatography of the above antifungal fraction from inoculated disks indicated that several antifungal compounds were present. One of these was isolated (see Experimental) and its structure established as **1** through IR, NMR and mass spectral analysis The positive and

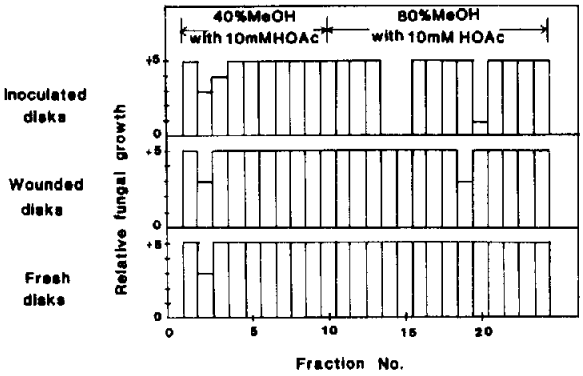
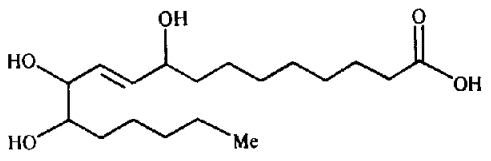


Fig. 1 Demonstration by HPLC of production of antifungal compounds in the inoculated taro tubers The acetone extracts from disks of fresh, wounded and inoculated taro tubers were subjected to HPLC and every 2 ml of eluate was fractionated and assayed for antifungal activity by the method described in Experimental.



1

negative ion FABMS gave [M + Na] and [M − H] peaks at *m/z* 353 and 329, respectively, indicating that the M_r of the compound is 330. The IR spectrum demonstrated the presence of OH (3300 cm^{-1}), CH_2 (2930 and 2850) and COOH (1710) groups. In the ^{13}C NMR spectrum, one double bound (δ131.9 and 137.4), three –CH(OH)–(73.8, 76.7 and 77.3) and 12 aliphatic carbons were identified. The data of ^1H–^1H COSY and the spin decoupling experiments showed the presence of a double bond in the *E*-configuration ($J_{10,11}$ = 15 Hz) and the spin system

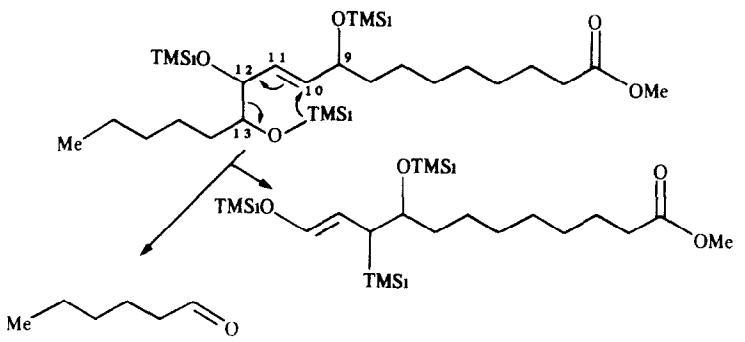


Fig 2 Mass-fragmentation of tris-TMSi of **2** at 70 eV

[$-CH_2-CH(OH)-CH=CH-CH(OH)-CH(OH)-CH_2-$]
In order to prepare a sample for EIMS, **1** was esterified with diazomethane to afford the methyl ester (**2**), followed by treatment with trimethylsilylimidazole to give the tris-TMSi derivative of **2** EIMS gave a parent peak at *m/z* 460 corresponding to [M − 100], which was caused by McLafferty-type rearrangement (Fig 2), strongly suggesting that the double bound is at C-10 Thus, **1** is 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid * This compound has already been isolated from *Oryza sativa* inoculated with *Pyricularia oryzae* [3], *Allium cepa* [4] and *Rudbeckia fulgida* [5].

Fifty ppm of 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid isolated from inoculated taro tubers completely inhibited the germination of spores of *C fimbriata* It is assumed that 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid is produced from linolenic acid and/or linoleic acid by a peroxidative reaction *in vivo* Antifungal activity was detected in assay mixtures containing lipoxygenase and linolenic acid with three double bonds or linoleic acid with two double bonds, while antifungal activity was not detected in the assay mixture containing the enzyme and oleic acid with one double bond or palmitic acid, a saturated fatty acid (Table 1) HPLC analysis showed that lipoxygenase produced not a single, but many antifungal oxidation products from linolenic acid and linoleic acid. Furthermore, the increase of lipoxygenase activity and accumulation of lipid peroxides take place in taro tubers in response to inoculation (H Masui, T Kondo and M Kojima, unpublished data) These results show that peroxidation of lipids is important in the defence reaction of taro tubers inoculated with *C. fimbriata*, as in other host–parasite interactions [3, 6].

Involvement of peroxidation of lipids in early host defence reaction seems to be general in the incompatible host–parasite interactions from the following considerations In most of incompatible host–parasite interactions, hypersensitive necrosis of host tissues occurs at an early stage of infection [7, 8]. In these tissues, the cellular membranes must be disorganized and the lipids in such membranes become susceptible to peroxidation [9, 10] The lipid peroxides thus formed show antifungal activity against the invading parasite

Table 1 Conversion by lipoxygenase of unsaturated fatty acids to antifungal compounds

Additions to mixture		
Fatty acid	Lipoxygenase	Relative fungal growth
None	active	+9
None	boiled	+9
Linolenic acid (18 3)	active	0
Linolenic acid	boiled	+9
Linoleic acid (18 2)	active	0
Linoleic acid	boiled	+8
Oleic acid (18 1)	active	+8
Oleic acid	boiled	+7
Palmitic acid (16 0)	active	+9
Palmitic acid	boiled	+8

Spores were incubated in the medium containing various fatty acids and lipoxygenase or boiled lipoxygenase at 25° After incubation for 17 hr, the growth of spores was examined under microscope

EXPERIMENTAL

Preparation of inoculum The sweet potato strain of *Ceratocystis fimbriata*, incompatible with taro, was used in all experiments in the present papers Inoculum (7×10^6 spores/ml) was prepared as described in our preceding paper [1]

Preparation of taro tuber disks and inoculation Disks (18 × 2 mm) of taro tubers (*Colocasia antiquorum*) were prepared and were rinsed in dist H_2O to remove the latex on the surfaces The rinsed disks were blotted with filter paper and divided into 3 groups consisting of 5 disks One group of disks was frozen immediately and stored at −30° for 24 hr (fresh disks), the second group of disks was dipped in the dist H_2O and incubated at 25° for 24 hr in moist chamber (wounded disks) and the third group of disks was inoculated by dipping in spore suspension and incubated at 25° for 24 hr in a moist chamber (inoculated disks) Each group of disks was extracted by shaking in 20 ml Me_2CO at 4° for 17 hr The extracts were applied to HPLC after being concd

Bioassay of antifungal activity Samples in soln were concd in a test tube (12 × 120 mm) and finally lyophilized The dried sample was dissolved in 10 μl of 200 mM of 12-(*N*-morpholino) ethanesulphonic acid(MES)–NaOH buffer, pH 6 5, including 10 μl of H_2O extract from sweet potato root (1 ml H_2O per 1 g

*^{1}H NMR data of **1** was not coincident with that for a sample of 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid provided by Dr T Kato (personal communication) This might be due to different configuration of the two compounds

fresh weight root) as a nutrient and 70 μl H_2O. Then 10 μl of spore suspension was added to the soln and incubated at 25° on a shaker. After incubation for 4 5 hr, the growth of the spores was examined under a microscope, and denoted by positive figures on an arbitrary scale Zero denotes no germination.

Isolation of 9,12,13-*trihydroxy*-(E)-10-*octadecenoic acid from inoculated taro tuber slices* The taro tubers were cut into 2 mm thick slices, which were rinsed in the dist. H_2O to remove the latex on the surface and blotted with filter papers. The slices were kept in a moist chamber at 25° for 1 day and then inoculated with the spore suspension. After being incubated at 25° for 2 days, the slices were boiled in EtOH for 10 min to inactivate the enzymes and then extracted with EtOH. The extract was evapd to leave a H_2O soln which was extracted twice with EtOAc The EtOAc extract was taken to dryness The residue was dissolved in a small volume of 65% MeOH containing 10 mM HOAc and centrifuged to remove insoluble material The resultant supernatant was subjected to HPLC on a reversed-phase column (Develosil ODS-5, 0.4 × 25 cm) with 65% MeOH containing 10 mM HOAc; flow 0.5 ml/min and UV detector set at 270 nm The peak eluting at 15.5 ml was collected. Three mg of the purified compound was isolated from 3 kg of the inoculated slices.

9,12,13-*trihydroxy*-(E)-10-*octadecenoic acid* (**1**). ν_{max}^{KBr} cm^{-1}: 3300 (*br*, OH), 2930, 2850 (CH_2), 1710, 1410 (COOH) ^1H NMR (CD_3OD). 500 MHz, δ0.91 (3H, *t*, J = 6 5 Hz, 18-Me), 1 1 ~ 1.7 (20H, *m*, $-CH_2-$), 2.25 (2H, *br t*, J = 6 5 Hz, H-2), 3 14 (1H, *ddd*, J = 1 5, 6.5 and 9 0 Hz, H-13), 3 90 (1H, *t*, J = 6 5, H-12), 4.05 (1H, *q*, J = 6.5 Hz, H-9), 5 67 (1H, *dd*, J = 6.5 and 15 Hz, H-11), 5 71 (1H, *dd*, J = 6 5 and 15 Hz, H-10). ^{13}C NMR (CD_3OD) 125 MHz, δ15 2 (C-8), 24 5, 27 0, 27 2, 27 2, 27 4, 31 1, 31 2, 31 3, 34.4, 36.3, 39 2, 11C, ($-CH_2-$), 73 8, 76 7, 77 3 (>CH–OH), 131 9, 137.4 (–CH=CH–), 159 8 (–COOH)

Preparation of **2**. To a soln of **1** (2 mg) in MeOH (2 ml) was added excess CH_2N_2 in Et_2O. After 20 min, the soln was dried *in vacuo* to give **2** in quantitative yield.

Methyl ester of **1** (**2**). ^1H NMR ($CDCl_3$): 500 MHz, δ0.86 (3H, *t*, J = 6 5 Hz, 18-Me), 1.2 ~ 1 7 (20H, *m*, $-CH_2-$), 2.27 (2H, *t*, J = 6 5 Hz, H-2), 3.41 (1H, *m*, H-13), 3.64 (3H, *s*, COOMe), 3 89 (1H, *t*, J = 6.5 Hz, H-12), 4.08 (1H, *br q*, J = 6.5 Hz, H-9), 5.66 (1H, *ddd*, J = 1.5, 6 5 and 15 Hz, H-11), 5 76 (1H, *ddd*, J = 1.5, 6.5 and 15 Hz, H-10)

Silylation of **2** *for GC-MS* **2** was treated with Tri-Sil (1 5 M soln of trimethylsilylimidazole in pyridine, Pierce), and then the soln was directly loaded in MS on DV-1: *m/z* 545 [M − 15] (17.5), 460 (100), 439 (22), 387 [M − 173], 298 (40), 259 (95), 173 [M − 387] (100)

Conversion of unsaturated fatty acid by lipoxygenase to antifungal compounds. Spores (5×10^6) were incubated in 1 0 ml of 20 mM MES–NaOH buffer, pH 6 5, containing 18 μmol of various fatty acids, 2×10^4 units of lipoxygenase (from soybean, Biozyme Laboratories) and 50 μl of sweet potato root H_2O extract as a nutrient at 25°. In controls, boiled lipoxygenase was included instead of active lipoxygenase After incubation for 17 hr, spores were examined for germination under microscope

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