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A Novel Phytoalexin Formed in Mulberry Leaves at the Infection of *Phloeospora maculans*, Causal Agent of Leaf Spot

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A novel terpenoid-type phytoalexin was isolated from mulberry leaves infected with *Phloeospora maculans*, causal agent of leaf spot. The molecular formula and weight of this substance were decided as $C_2H_4O_4$ (MWt. 340) by HR/(+)FAB mass spectrometry. This substance is detectable at Rf 0.56 on TLC chromatogram developed with chloroform-methanol(9:1, v/v) as a reddish-purple spot by spraying of 1% vanillin-sulfuric acid and heating at 110°C. This substance appeared at Rt 13.6 min on the GLC chromatogram (OV–101, 200°C, N_2 50 ml/min) and at Rt 4.5 min on HPLC chromatogram with reversed-phase column (C18, 1 ml/min, 80% aqueous methanol). Other kind of substances which showed antifungal activity were also detected on the TLC chromatogram. These substances were absent in the extract from healthy check.

INTRODUCTION

The phytoalexin production at the site of infection has been well–documented since the first report of Müller and Börger in 1940. The close association of these phytoalexins with the defence to the parasites has also been indicated (Bailey *et al.*, 1982).

The production of various kind of phytoalexins such as 26 kinds of Moracin (A~Z), Oxyresveratrol, 4'–prenyloxyresveratrol and Chalcomoracin has been well noted (Shirata, 1978, 1981; Shirata *et al.*, 1979, 1982a, 1982b; Takasugi *et al.*, 1978a, 1978b, 1978c, 1979, 1980). The reason why the existence of such numerous kind of phytoalexins in mulberry plant has been attributed to the tissue–specific production of phytoalexin. Moracin M, however, was isolated from diseased epidermal tissues and xylem of twigs. Furthermore, Chalcomoracin was isolated not only from diseased leaf and also from diseased epidermal tissues and xylem of twigs (Takasugi *et al.*, 1980).

Since 1991, mulberry plants in Goto islands (Nagasaki prefecture) have been severely attacked every year by *Phloeospora maculans*, causal agent of leaf spot (Negi *et al.*, 1992) and silk–worm industry has been heavily depressed. According to our survey, there was a resistant variety to this pathogen. To examine if the phytoalexin will associate with the resistance, the extraction of phytoalexin in mulberry leaves was conducted.

The abstract was reported elsewhere (Kim *et al.*, 1992) and the detail will be contributed in this report.

MATERIALS AND METHODS

Extraction and purification of phytoalexin

Twenty gram of diseased leaves of mulberry plant, cultivar "Minamisakari" was

scissored and dipped in 200 ml of 70% aqueous methanol. After keeping for 3 min in boiling water bath, the leaf sample was homogenized with the Physcotron homogenizer. The extract was concentrated to ca. 50 ml and shaked with twice volume of petroleum ether. The ether extract was concentrated completely and dissolved in 3 ml of methanol.

The extract was applied on TLC plate of silica gel 60 (Merck Co.) and developed with chloroform–methanol (9:1, v/v). The TLC plate was sprayed with 1% vanillin–sulfuric acid and heated at 110°C for detection of terpenoid-type phytoalexin. UV irradiation to TLC plate was also conducted to detect other types of phytoalexin.

Preparative TLC was conducted and the corresponding area where phytoalexin-like substance was detected at preliminary tests was scraped. The silica-gel powder was suspended in chloroform–methanol (2:1, v/v) and the substance was extracted. This sample was dissolved in 80% aqueous methanol and was subjected to HPLC with reversed-phase column (M&S pack C18, 20×250 mm) and eluted with 80% aqueous methanol monitoring at 236 nm.

Analytical GLC and HPLC

Analytical GLC and HPLC were conducted. GLC was performed by Shimadzu GLC 7AG equipped with FID and OV-101 column (1% loading on Gas Chrom Q, 100/200 mesh in silanized glass column 0.3×150 cm). Analysis was conducted at 200°C and 50 ml/min N_2 flow rate. HPLC was performed by Gilson 303 LC system equipped with the reversed-phase column (M&S pack C18, 4.6×150 mm). Elution was carried out with 80% aqueous methanol at a flow rate of 1 ml/min monitoring at 236 nm.

Mass spectrometry

The purified substance was analyzed by a high resolution HR/(+)FAB mass spectrometer at the Faculty of Pharmaceutical Sciences, Kyushu University.

Antifungal activity

Antifungal activity of the substances against *P. maculans* was tested by suspending spores which were formed on PSA medium in a drop of each sample solution on a glass slide keeping under humid condition.

RESULTS AND DISCUSSION

Extraction and characterization of phytoalexins formed in the leaves of mulberry by the infection with *P. maculans* were conducted. As can be seen in Fig. 1-1, bluish, purplish and reddish spots were detected on TLC plate at Rf 0.47, 0.43 and 0.33 when UV (254 nm) light was irradiated. These spots were not detected on the chromatogram of the healthy check leaves. At the visualization with 1% vanillin–sulfuric acid followed by heating at 110°C, reddish-purple spot was observed at Rf 0.56 on the chromatogram of the infected leaves (Fig. 1-2).

Antifungal activity as phytoalexin was tested for eluates from corresponding areas at Rf 0.56, 0.47, 0.43 and 0.33, respectively. Every fraction showed antifungal activity and relatively strong inhibition was observed for the fractions from Rf 0.56, 0.47 and 0.33. However, further analyses for these fractions with other kind of solvent-systems of TLC

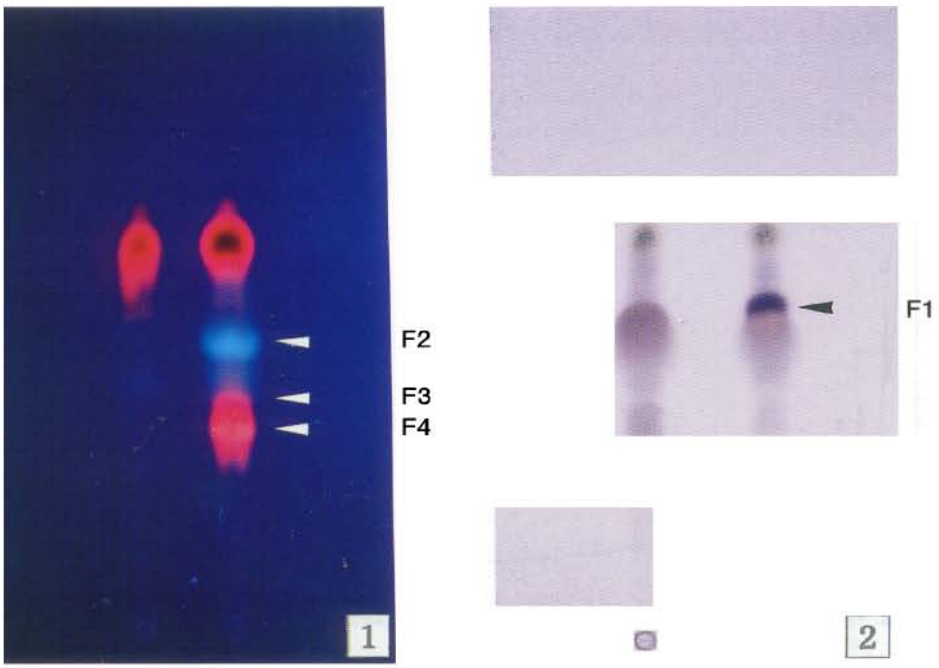

Fig. 1. TLC chromatograms of petroleum ether extracts from healthy (left of each figure) and infected leaves with *P. maculans* (right of each figure).
1. At UV irradiation (254 nm)
2. At spraying 1% vanillin–sulfuric acid and heating
F1–F4 : Fractions detected at Rf 0.56, 0.47, 0.43 and 0.33
Solvent : chloroform–methanol (9:1, v/v)
Plate : Silica gel 60

such as ethyl acetate–carbon tetrachloride (6:4, v/v) and cyclohexane–dioxane (7:3, v/v) indicated that each fraction is a complex of weakly antifungal substances.

Only the substance at Rf 0.56 which is detectable with 1% vanillin–sulfuric acid seemed to be single substance and showed strong antifungal activity. This substance was detected at Rt 13.6 min on GLC chromatogram with OV–101 column and appeared at Rt 4.5 min on HPLC chromatogram with reversed–phase column (Fig. 2). This fraction was subjected to preparative HPLC and the fraction at Rt 32.5 min was collected. After the tests for purity, the substance was subjected to a high resolution mass spectrometry (HR/(+)FABMS). The molecular formula and weight of this purified substance were decided as $C_{21}H_{24}O_4$ (MWt. 340)(Fig. 3). Since this substance was also detectable with 1% vanillin–sulfuric acid and 25% antimonate–chloroform, it was imagened that this novel substance will be a terpenoid–type phytoalexin.

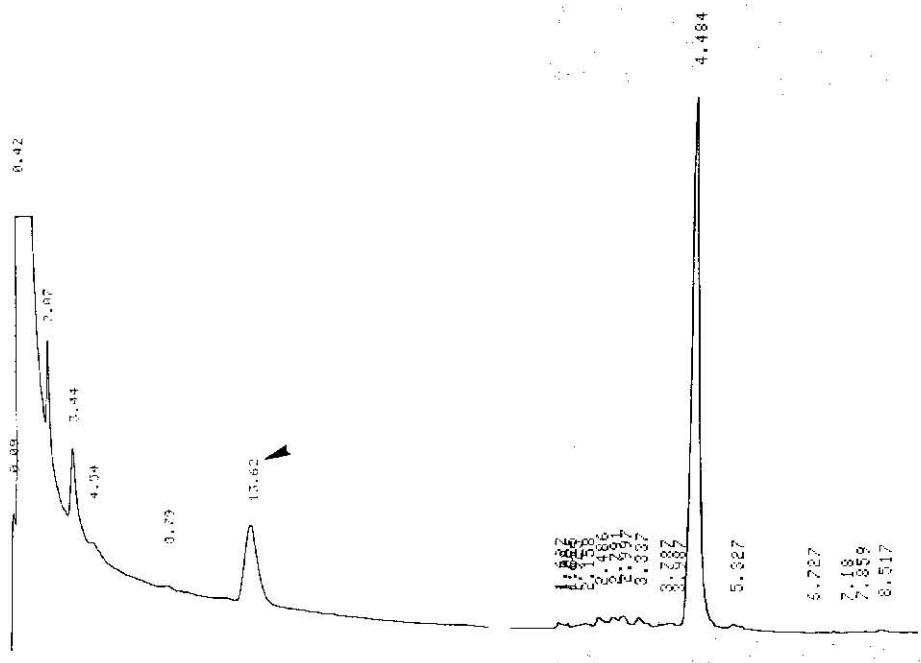

Fig. 2. Gas liquid chromatography analysis (left) and high performance liquid chromatography analysis (right) of the substance at Rf 0.56.
GLC : OV–101, 200 °C column temperature, N_2 50 ml/min
HPLC : Reversed–phase column C18, 1 ml/min, 80% aqueous methanol solution

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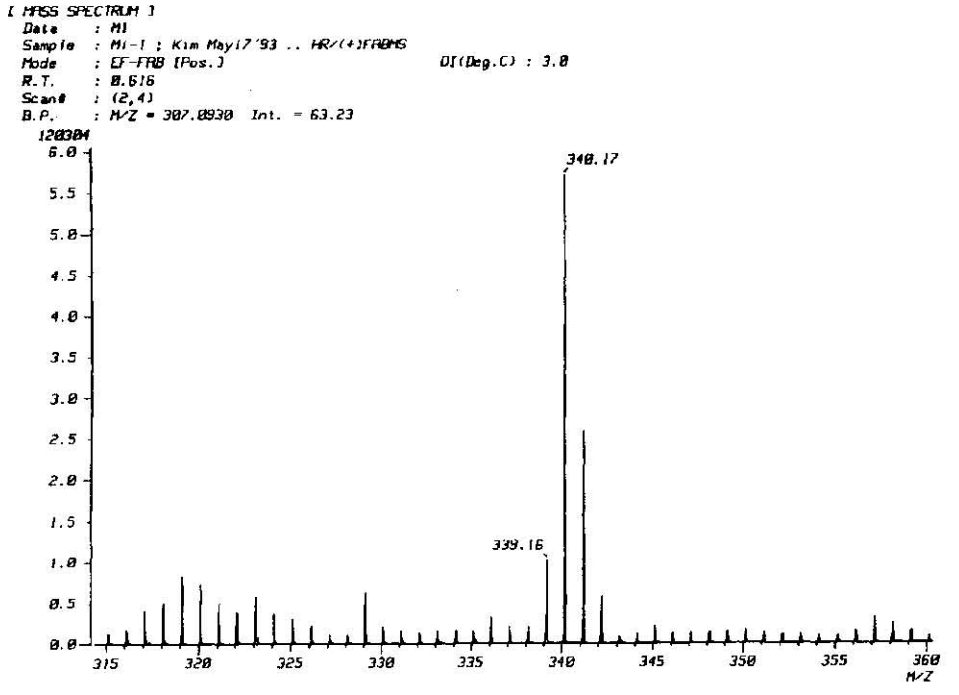

Fig. 3. HR/(+)FAB mass spectrum of a novel phytoalexin.

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