

カナメモチで発見された2種類のファイトアレキシン(オーキュパリン関連物質)について

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Isolation and Characterization of Two Aucuparin-Related Phytoalexins from *Photinia glabra* Maxim

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Abstract

Two kinds of biphenyl phytoalexins accumulated in leaves of *Photinia glabra* Maxim (*Rosaceae*) in response to inoculation with spores of *Entomosporium mespili*, a pathogen of *P. glabra*, or to treatment with 10^{-4} M HgCl_2 . The phytoalexins were identified as 2'-methoxyaucuparin and 4'-methoxyaucuparin. The contents of 2'-methoxyaucuparin and 4'-methoxyaucuparin were 3.578 and 1.233 $\mu\text{g/g}$ of leaf (fresh weight), respectively, 4 days after the inoculation with spores of *E. mespili*, whereas their contents in the uninoculated leaf were minute. The two phytoalexins exhibited toxicity to *Pestalotia photiniae*, a pathogenic fungus to *P. glabra*, and to *Botrytis cinerea* and *Cochliobolus miyabeanus*, the non-pathogens. The toxicity of 4'-methoxyaucuparin to *P. photiniae* and *Botrytis cinerea* was higher than that of 2'-methoxyaucuparin.

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Key words: *Rosaceae*, *Photinia glabra*, biphenyl phytoalexins, 2'-methoxyaucuparin, 4'-methoxyaucuparin.

INTRODUCTION

Historically, the name of aucuparin was first used in the 1960s to describe a biphenyl compound isolated from healthy heartwood of *Sorbus aucuparia* L. (mountain ash)²⁾. Twenty years later, the same compound was found to accumulate in the cortical layer of shoots of loquat (*Eriobotrya japonica* Lindl.) inoculated with *Colletotrichum lindemuthianum*¹⁰⁾. The compound was then proved to have an important contribution as a phytoalexin in the disease resistance of loquat⁸⁾.

Aucuparin and its related phytoalexins have been isolated and characterized in two more plant species, *Mallus pumila* Mill.⁵⁾ and *Rhaphiolepis umbellata* Makino¹¹⁾. This paper describes the isolation of 2'-methoxyaucuparin and 4'-methoxyaucuparin from the leaves of *Photinia glabra* Maxim. (the Japanese name is 'kanamemochi') and evaluation of their roles as phytoalexins of the plant. An abstract of this study was previously reported¹⁴⁾.

MATERIALS AND METHODS

Plant and fungal materials. The plant materials were prepared vegetatively by using healthy softwood cuttings of *P. glabra* (photinia). The cuttings were grown in greenhouse (average temperature 25°C, natural light) in a coarse sand until developing the roots and shoots. After developing small plants, they were transplanted to the pots containing a mixture of soil and organic fertilizer (1 : 1), grown for 3-6 months and then used for experiment.

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Pestalotia photinia Thumen, a pathogen of *P. glabra*, was isolated from a naturally occurring lesion on a leaf of *P. glabra*. *Botrytis cinerea* Persoon and *Cochliobolus miyabeanus* S. Ito et Kuribayashi which are nonpathogens of *P. glabra* were isolated from the lesions on leaves of *Rosa multiflora* Thunb. and *Oryza sativae* L., respectively. The procedures for culture and sporulation of fungi were previously described^{12,13}. The spores of *Entomosporium mespili* were prepared by collecting directly from leaf lesions on which *E. japonica* infected and sporulated.

Induction of phytoalexins. The induction of accumulation of phytoalexins in leaves of *P. glabra* was done as described previously¹². To elicit phytoalexins by fungal infection, the whole plants were inoculated with spore suspension (10^5 spores/ml) of *E. mespili* with a glass atomizer and kept under humid condition. The detached leaves of *P. glabra* were sprayed with 10^{-4} M HgCl_2 , an abiotic elicitor, and incubated in a moist chamber. Both of them were kept in the dark at 25°C for 4 days. After the incubation, the phytoalexin production was estimated.

Extraction of aucuparin-related phytoalexins. The extraction of aucuparin-related compounds from leaves of *P. glabra* was done as described previously¹². The samples were extracted with 80% ethanol [10 ml/g of sample (fresh weight)] for 24 hr at 25°C . The ethanolic extract was filtered through two layers of cheesecloth and then evaporated at 35°C under reduced pressure. The condensed extract was added with water and centrifuged ($9,000\times g$, 30 min). The supernatant was adjusted to pH 3.0 with HCl and extracted with an equal volume of petroleum ether (3 times). The petroleum ether phase was evaporated to dryness at 35°C . The residue was dissolved in ethanol, and the sample was applied to a silica gel TLC plate (Merk GF₂₅₄, 0.25 mm) developed with the solvent consisting of hexane : ethyl acetate : methanol (60 : 40 : 1, v/v/v) or a reversed phase silica gel TLC (RP=18) developed with acetonitrile : water (4 : 1, v/v). Aucuparin-related compounds were detected on TLC plate by illumination at 365 nm.

Final purification of phytoalexins was conducted by using high performance liquid chromatography (HPLC). A Waters 6,000 A pump system (Waters Associates) equipped with a Waters U6K universal injector (Waters Associates) was used for analytical and preparative HPLC. Samples dissolved in ethanol were filtered through an $0.45\text{ }\mu\text{m}$ filter (chromatodisc 4A filters, Biofield INC.). A $20\text{ }\mu\text{l}$ portion of each sample was applied to an ODSpak F-411A column ($150\times 4.6\text{ mm}$, $5\text{ }\mu\text{m}$ particle size, Shoko Co. LTD.) equipped to the HPLC system and eluted with methanol : water (65 : 35, v/v, 0.5 ml/min). Chromatographic and spectral data of the eluate were obtained with a UVDEC-100-II detector (Jasco, LTD.).

For quantification of phytoalexins, $10\text{ }\mu\text{l}$ of authentic sample ($20\text{ }\mu\text{g/ml}$) was subjected to HPLC analysis under the same condition as mentioned above, and the chromatographic profile was used as a standard.

Characterization of the phytoalexins. Ultraviolet (UV) spectra of phytoalexins were measured in ethanol by using Shimadzu 265 spectrophotometer (Shimadzu). The $^1\text{H-NMR}$ spectra were recorded at 90 MHz in CDCl_3 with Hitachi R-90H (Hitachi). The mass spectra were obtained by direct inlet method at 70 eV with Hitachi M-80 (Hitachi).

Bioassay of the phytoalexins. The activity of purified phytoalexins was assayed by measuring their effects on the spore germination of *P. photinia*, a pathogenic fungus, and two other nonpathogenic fungi, *B. cinerea* and *C. miyabeanus*, by following the method of Király et al.⁶⁾ An ethanolic solution of each phytoalexin was added to water to give an ethanol concentration at 2% (v/v). A drop of the solution was added to water agar block containing spores (10^5 spores/ml) and then incubated at 25°C . When more than 80% of control spores germinated, the germination rate of the treated spores was measured by using a light microscope.

RESULTS

Characterization of aucuparin-related compounds

During isolation of aucuparin-related compounds from leaves of *P. glabra* inoculated with spores of *E. mespili*, we detected two compounds (A and B) as candidates of the phytoalexins. Purification of the

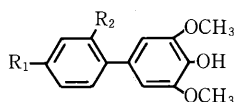


Fig. 1. Structures of two phytoalexins from *Photinia glabra*. A: 4'-methoxyaucuparin, $R_1 = \text{OCH}_3$, $R_2 = \text{H}$, B: 2'-methoxyaucuparin, $R_1 = \text{H}$, $R_2 = \text{OCH}_3$.

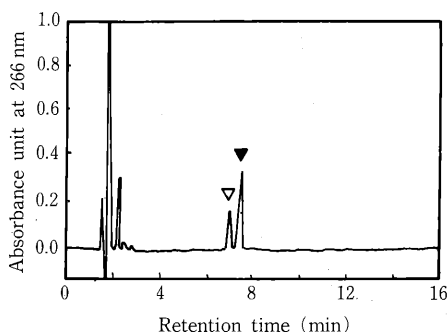


Fig. 2. Chromatogram at 266 nm of 2'-methoxyaucuparin (▽) and 4'-methoxyaucuparin (▼). Column: ODSpak F-411A, Solvent: 60% methanol.

Table 1. The accumulation of 2'-methoxyaucuparin and 4'-methoxyaucuparin in *Photinia glabra* leaves inoculated with spores of *Entomosporium mespili* or treated with 10^{-4} M HgCl_2

Elicitor	Phytoalexin ($\mu\text{g/g}$ fresh weight) ^{a)}	
	2'-methoxyaucuparin	4'-methoxyaucuparin
Water ^{b)}	0.010	0.014
<i>Entomosporium mespili</i> ^{b)}	3.578	1.233
Water ^{c)}	0.020	ND ^{d)}
10^{-4} M HgCl_2 ^{c)}	2.166	0.747

a) Accumulation of each compound was measured 4 days after treatments as described in Materials and Methods. Each value represents an average of three experiments with triplicate samples.

b) The whole plants were used for the inoculation, and leaves attaching to the plants were assayed for phytoalexin accumulation.

c) The detached leaves were used for the treatment and assayed for phytoalexin accumulation.

d) Not detected.

two compounds was carried out by HPLC with a reverse phase column. The purified compounds were subjected to chemical characterization by measuring the UV spectra, $^1\text{H-NMR}$ spectra and Mass spectra.

Compound A exhibited the following spectroscopic properties: MS m/z 260 [M^+], 245 [$\text{M}^+ - \text{CH}_3$] and 149 (base peak); UV $\lambda_{\text{max}}^{\text{EtOH}}$ 268 nm (ϵ , 13,700)¹²⁾; $^1\text{H-NMR}$ $\delta_{\text{TMS}}^{\text{CDCl}_3}$ 7.43 (2H, d, J 9Hz), 6.95 (2H, d, J 9Hz), 6.76 (2H, s), 3.98 (3Hx2, s, OCH_3) and 3.85 (3H, s, OCH_3). These data were identical to those of 4'-methoxyaucuparin which has been previously isolated from *R. umbellata* and characterized as its phytoalexin (Fig. 1A)¹¹⁾. Final identification was conducted by direct comparison of the spectrum with that of the authentic sample of 4'-methoxyaucuparin.

Compound B showed the following physicochemical data: mp. 120–121°C; MS m/z 260 [M^+] and 105 (base peak); UV $\lambda_{\text{max}}^{\text{EtOH}}$ 265 nm (ϵ , 12,500) and 286 nm (ϵ , 9,600); $^1\text{H-NMR}$ $\delta_{\text{TMS}}^{\text{CDCl}_3}$ 7.05–7.40 (4H, m, aromatic protons), 6.77 (2H, s), 3.93 (3Hx2, s, OCH_3) and 3.81 (3H, s, OCH_3). Its mass spectrum indicated that compound B is one of isomers of 4'-methoxyaucuparin (Fig. 1B). The $^1\text{H-NMR}$ signal patterns of four

Table 2. Effect of 2'-methoxyaucuparin on the spore germination of three plant pathogenic fungi

Fungi (Host plant)	Concentration of 2'-methoxyaucuparin ($\mu\text{g/ml}$)	Germination rate (%) ^{a)}
<i>Pestalotia photiniae</i> (photinia)	0 ^{b)}	83 (100.0) ^{c)}
	40	30 (36.1)
	80	6 (7.2)
<i>Botrytis cinerea</i> (rose)	0	92 (100.0)
	40	46 (50.0)
	80	10 (10.8)
<i>Cochliobolus miyabeanus</i> (rice)	0	94 (100.0)
	40	54 (57.4)
	80	35 (37.2)

a) Each value represents an average of three experiments. In each experiment, 100 spores were observed.

b) Control (treated with 2% ethanol).

c) Number in parenthesis represents an exponent against the value of the control (100).

Table 3. Effect of 4'-methoxyaucuparin on the spore germination of three plant pathogenic fungi

Fungi (Host plant)	Concentration of 4'-methoxyaucuparin ($\mu\text{g/ml}$)	Germination rate (%) ^{a)}
<i>Pestalotia photiniae</i> (photinia)	0 ^{b)}	83 (100.0) ^{c)}
	10	68 (81.9)
	40	24 (28.9)
<i>Botrytis cinerea</i> (rose)	0	92 (100.0)
	10	26 (28.3)
	40	3 (3.3)
<i>Cochliobolus miyabeanus</i> (rice)	0	94 (100.0)
	10	78 (82.9)
	40	49 (52.1)

a) Each value represents an average of three experiments. In each experiment, 100 spores were observed.

b) Control (treated with 2% ethanol).

c) Number in parenthesis represents an exponent against the value of the control (100).

aromatic protons as well as other singlet signals suggested that compound B should be 2'-methoxyaucuparin which has already been isolated from the heartwood of mountain ash²⁾. By direct comparison of the spectrum with that of the authentic specimen, it was identified as 2'-methoxyaucuparin.

HPLC analysis showed that the retention times for 4'-methoxyaucuparin and 2'-methoxyaucuparin were 7.6 ± 0.1 min and 7.0 ± 0.1 min, respectively (Fig. 2).

Phytoalexin accumulation

2'-methoxyaucuparin and 4'-methoxyaucuparin were accumulated in leaves of *P. glabra* by the inoculation with spores of *E. mespili* and by the treatment with HgCl_2 , whereas without the inoculation and the treatment, the leaves contained only minute amounts of these compounds. Inoculation with the spores on attached leaves induced 2'-methoxyaucuparin and 4'-methoxyaucuparin accumulation of $3.578 \mu\text{g}$ and $1.233 \mu\text{g}$ per 1 g of leaf (fresh weight), respectively, within 4 days (Table 1). Detached leaves treated with 10^{-4} M HgCl_2 for 4 days contained $2.166 \mu\text{g}$ of 2'-methoxyaucuparin and $0.747 \mu\text{g}$ of 4'-methoxyaucuparin per 1 g leaf (Table 1).

In vitro antifungal activity of the phytoalexins

2'-methoxyaucuparin and 4'-methoxyaucuparin significantly prevented spore germination of three fungi tested at the concentration of $40 \mu\text{g/ml}$ (Tables 2 and 3). However, the antifungal activity of

2'-methoxyaucuparin was lower than 4'-methoxyaucuparin. The two phytoalexins were more toxic to *B. cinerea* and *P. photiniae* than *C. miyabeanus*.

DISCUSSION

Plants have various mechanisms for resistance to infection by pathogens. One of the dynamic resistance mechanisms is the production of antimicrobial compounds called as phytoalexins, that kill the pathogens or restrict their intracellular development. In this study, the two biphenyl compounds accumulated in the leaves of *P. glabra* (photinia) by the inoculation with fungal spores and the treatment with 10^{-4} M HgCl_2 were chemically identified as 2'-methoxyaucuparin and 4'-methoxyaucuparin and evaluated as phytoalexins of the plant. In the previous report¹¹⁾, 4'-methoxyaucuparin was identified as one of the phytoalexins of *R. umbellata*. Thus, *P. glabra* is the second plant that has been found to accumulate 4'-methoxyaucuparin in the response to microbial attack. When compared the accumulation of 4'-methoxyaucuparin between the two plants, *R. umbellata* seemed to produce more amount of the phytoalexin than *P. glabra*.

While 2'-methoxyaucuparin has been known as a secondary metabolite, but not as a phytoalexin, in *Sorbus aucuparia* L. (mountain ash)²⁾, we observed that the accumulation of 2'-methoxyaucuparin was induced in leaves of *P. glabra* by fungal inoculation and HgCl_2 treatments.

These results seemed to show that demarcation between prohibitin and phytoalexin in plants sometime is not clear⁹⁾. In *S. aucuparia*, for example, aucuparin and 2'-methoxyaucuparin are not phytoalexins but can be evaluated as prohibitin because the healthy wood also contains detectable amounts of these compounds²⁾. On the other hand, aucuparin of *E. japonica* (loquat) can be classified as a phytoalexin because this compound accumulates in living tissues as a specific consequence of contact with microorganism or treatment with abiotic elicitors¹⁰⁾. Further more, gossypol and congeners were found as phytoalexins in xylem tissue of cotton, but they appeared to occur as a major constituent of its pigment glands⁹⁾.

The similarity between the phytoalexins of *R. umbellata* and *P. glabra* was in agreement with previous reports that the phytoalexins isolated from the Rosaceous plants belong to biphenyl type compounds or dibenzofuran^{1,3,4,7,10,11)}.

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和 文 摘 要

Siti Muslimah WIDYASTUTI・野中福次・渡辺敬介・佐古宣道・田中欽二：カナメモチで発見された2種類のファイトアレキシシン（オーキユパリン関連物質）について

カナメモチの葉に病原菌であるビワごま色斑点病菌の胞子を接種すると、2種類のファイトアレキシシンが生成された。このファイトアレキシシンはいずれも biphenyl 化合物で、2'-methoxyaucuparin と 4'-methoxyaucuparin と同定された。両物質はエリシターとして 10^{-4} M HgCl_2 を噴霧した葉でも誘導生成された。ビワごま色斑点病菌の胞子を接種して4日目における 2'-methoxyaucuparin と 4'-methoxyaucuparin の生成量は、それぞれ $3.578 \mu\text{g/g}$ と $1.233 \mu\text{g/g}$ (生重葉)であり、無接種葉のそれぞれに対して 358 倍および 88 倍量であった。両ファイトアレキシシンの病原菌に対する発芽阻害活性をカナメモチのペスタロチア病菌と灰色かび病菌について比較した結果、4'-methoxyaucuparin の方が 2'-methoxyaucuparin より優っていた。