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ISOLATION AND ANTIMICROBIAL ACTIVITY OF THE PHYTOALEXIN 6-METHOXYMELLEIN FROM CULTURED CARROT CELLS

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Key Word Index—*Daucus carota*; Umbelliferae; phytoalexin; 6-methoxymellein; suspension culture; antimicrobial activity.

Abstract—6-Methoxymellein was identified as a common phytoalexin produced by carrot roots irrespective of the species of challenging fungi. It was also shown that cultured carrot cells produced 6-methoxymellein when the culture was inoculated with fungi. This compound has a broad antimicrobial spectrum and inhibits the growth of several fungi, yeasts and bacteria.

INTRODUCTION

Phytoalexins are defined as the low MW antimicrobial compounds produced by plants in response to the infection by micro-organisms. These compounds are accumulated at the site of infection and are considered to be involved in the defense mechanism of plant to potential pathogens [1, 2]. Many different classes of compounds have been isolated as phytoalexins and their antibiotic activities studied [3, 4].

In carrot, Condon *et al.* have isolated 6-methoxymellein as an antifungal substance from the roots following inoculation with the non-pathogenic fungi, *Ceratocystis fimbriata* and *Thielaviopsis basicola* [5, 6]. Recently, Harding and Heale [7] reported that 6-methoxymellein, *p*-hydroxybenzoic acid and falcarinol accumulated as antimicrobial substances in carrot roots infected by *Botrytis cinerea*. In physiological studies on the phytoalexin production, the use of plant cell cultures may offer an advantage because a large number of cells grown under identical conditions can be brought into contact with fungal hyphae or the appropriate elicitor. Phytoalexin production in plant cultures has been studied with the use of biotic (fungal cell wall materials, cytoplasmic supernatants and extracellular metabolites, etc.) and abiotic (metal salts and sulfhydryl reagents) elicitors [8–11]; most work has been with isoflavonoids in legume cultures.

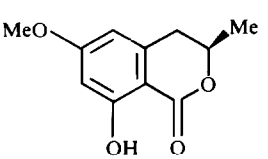
The present experiments were undertaken to identify the phytoalexin produced in carrot roots infected by different fungi, and study its toxicity to various micro-organisms. Special attention was focused on the possibility of observing phytoalexin production in cultured carrot cells.

RESULTS AND DISCUSSION

Isolation and identification of the phytoalexin of carrot

Chaetomium globosum was used as a micro-organism for challenge to carrots, and antimicrobial compounds in carrot extracts were recognized as inhibited zones on TLC bioassay. Four samples, described below, were prepared and tested for the purpose of the identification of the antibiotic substances produced only by the interaction between the fungus and live carrots. Killed by autoclaving and untreated live carrot slices were prepared and challenged by *C. globosum*, respectively, and the use of unchallenged controls allowed the comparison with response triggered by wounding alone. All four samples showed inhibition zones at R_f values 0.06, 0.16 and 0.69. However, the extract of untreated-live carrots gave one more characteristic inhibition zone at 0.84 when challenged by the fungus. It was obviously caused by the mutual interaction between *C. globosum* and live carrot tissues, and was, therefore, judged as being the phytoalexin of carrots.

This compound was soluble in *n*-hexane, and formed a water soluble salt with sodium hydroxide. On the basis of these properties, the phytoalexin of carrots was isolated by Si gel CC after the preparation of an acidic fraction from 2 kg infected carrot root slices. The antifungal activity of the eluate was tested with a TLC plate bioassay, and crystallization from *n*-hexane gave 75 mg colorless plates. The mp, IR, mass spectral and ^{1}H NMR data of the isolated phytoalexin were identical to those of 6-methoxymellein reported earlier [5, 7], and the elemental composition was also satisfied. (Found: C, 63.43; H, 5.87. Calc. for $C_{11}H_{12}O_4$: C, 63.46; H, 5.77%.) Stereochemistry of 3-position was determined by polarimetric analysis. 6-Methoxymellein isolated from carrot roots was levorotatory and, therefore, was assumed to have the 3*R* configuration based on comparison with other mellein derivatives [12, 13]. Thus, the structure of the phytoalexin of carrots is determined as 3,4-dihydro-8-hydroxy-6-methoxy-3*R*-methylisocoumarin (**1**).



6-Methoxymellein

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To examine whether the carrot produces 6-methoxymellein as the common phytoalexin regardless of the species of challenging fungi, we selected three fungi, *Botrytis cinerea*, *Helminthosporium oryzae* and *Fusarium moniliforme*, and prepared extracts of carrots for TLC bioassay as described above. In every case, carrot extracts showed upon bioassay an identical pattern to that obtained in the case of *C. globosum*. GC/MS analysis was carried out on acidic fractions from respective infected carrots, and the existence of 6-methoxymellein was confirmed in every sample. These observations suggest that 6-methoxymellein production is not a specific response to an attacking fungus but the same compound is formed irrespective of the species of challenging fungi.

Production of phytoalexin by cultured carrot cells

A spore suspension (1×10^8 spores/ml) of *C. globosum* in 10 ml water was inoculated to the 110 ml culture of carrot cells. The culture medium for carrot cells was supplemented with 200 mg malt extract and 400 mg glucose to support germination of the fungal spores and mycelial growth. To a control culture was added only water. It was confirmed microscopically that *ca* 70% spores germinated after 6 hr incubation in this modified medium. 6-Methoxymellein in infected cultured carrot cells was identified by GC/MS and this compound was not detected in unchallenged cultured carrot cells. Figure 1 shows the time course of the production of 6-methoxymellein by carrot cells in suspension culture. Intracellular level of 6-methoxymellein increased with time after a lag period of *ca* 12 hr. Maximum accumulation of 6-methoxymellein generally occurred after 1–2 days but this was always followed by a rapid decrease in its concentration during the next 24 hr. The extracellular concentration of 6-methoxymellein was very low indicating that the compound is not released from the cell. These results suggest that 6-methoxymellein is metabolically unstable. In a suspension culture of *Phaseolus vulgaris*, it was reported that phaseollin accumulated in growing culture was rapidly converted to phaseollinisoflavan [14]. Inoculation with the fungus resulted in a general brownish appearance of the carrot cells after *ca* 24 hr. However, the culture still contained uninjured cells and it is not certain whether cell damage or senescence is associated with 6-methoxymellein production in carrot cells.

In leguminous plants and their cell cultures, phytoalexin production is induced by a culture filtrate of fungi or a hydrolysate of fungal cell walls [8–10]. Certain metal ions, such as Hg^{2+} and Cu^{2+}, also stimulate the synthesis of phytoalexin in some plants including sweet potato tissues [15] and white clover [11]. To examine the elicitor activity of these substances on carrot cells, a partial hydrolysate of fungal cell wall [8], cytoplasmic supernatant and extracellular metabolite [9] of *C. globosum* and *B. cinerea* were prepared and added to our culture in the concentration range of 0.1–50 μg glucose equivalents/ml. However, 6-methoxymellein was not detectable after 2 days incubation. Metal salts (mercuric and cupric chlorides) were also ineffective in the range of tested concentrations (0.01 μM–100 mM).

Antimicrobial activities of 6-methoxymellein

The ability of 6-methoxymellein to inhibit the growth of several fungi, yeasts and bacteria was examined. 6-Methoxymellein was effective in inhibiting the growth of all fungi tested (Table 1). In particular, *Alternaria alternata* was the most sensitive fungus and 90% inhibition was observed with 0.05 mM 6-methoxymellein. The inhibitory effect of 6-methoxymellein on *Saccharomyces cerevisiae* was rather weak, but it appreciably inhibited *Candida albicans* (Table 2). The antibacterial activities of 6-methoxymellein are summarized in Table 3. 6-Methoxymellein inhibited the growth of four Gram-positive and seven Gram-negative bacteria tested, except for *Pseudomonas aeruginosa*. In general, Gram-negative bacteria were less sensitive. Most phytoalexins that have been studied show either no effect on bacteria or are selectively toxic to Gram-positive bacteria. However, glycinol, recently isolated from soybean, was reported to inhibit Gram-negative as well as Gram-positive bacteria [16]. Our results show that 6-methoxymellein is an antibiotic substance effective against diverse micro-organisms.

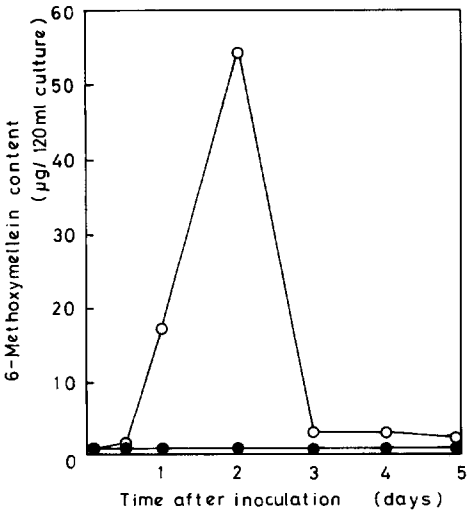

Fig. 1. Time course of 6-methoxymellein contents in infected carrot cells in suspension culture. (○) in cells (●) in medium.

Table 1. Toxicity of 6-methoxymellein to fungi

Fungi	Incubation time (hr)	Inhibition (%) Concentration (mM) 0.05	0.1	0.5
Curvularia intermedia	72	27	21	91
C. clavata	48	22	28	87
C. ovoidea	48	54	70	100
C. lunata	96	N.I.	N.I.	85
C. inaequalis	96	N.I.	43	94
Alternaria alternata	96	90	94	99
A. padwickii	96	N.I.	22	98
Rhizopus chinensis	48	N.I.	N.I.	33
Fusarium moniliforme	72	N.I.	N.I.	72
Botrytis cinerea	72	N.I.	73	92
Helminthosporium oryzae	48	N.I.	N.I.	72
Chaetomium globosum	48	N.I.	N.I.	48
Aspergillus fumigatus	48	N.I.	N.I.	85

N.I., not inhibited.

Table 2. Toxicity of 6-methoxymellein to yeasts

Yeasts	Incubation time (hr)	Inhibition (%) Concentration (mM) 0.05	0.1	0.5
Saccharomyces cerevisiae	24	N.I.	N.I.	39
	30	N.I.	N.I.	18
Candida albicans	48	54	66	93
	60	N.I.	27	76

N.I., not inhibited.

Table 3. Toxicity of 6-methoxymellein to bacteria

Bacteria	Incubation time (hr)	Inhibition (%) Concentration (mM) 0.05	0.1	0.5
Gram-positive				
Staphylococcus aureus	30	42	92	93
	48	20	26	31
S. epidermidis	12	22	53	59
	24	N.I.	13	33
Streptococcus pyogenes	12	53	58	58
	24	12	18	22
Bacillus subtilis	12	N.I.	N.I.	73
	24	N.I.	N.I.	65
Gram-negative				
Salmonella typhimurium	12	N.I.	11	20
	24	N.I.	N.I.	15
Shigella sonnei	12	N.I.	14	22
	24	N.I.	18	22
Escherichia coli	24	N.I.	N.I.	21
Klebsiella pneumoniae	10	N.I.	N.I.	19
	12	N.I.	N.I.	19
Enterobacter aerogenes	12	N.I.	N.I.	29
Serratia marcescens	12	N.I.	20	18
	24	N.I.	12	21
Proteus vulgaris	12	26	32	65
	24	12	21	37
Pseudomonas aeruginosa	12	N.I.	N.I.	N.I.
	24	N.I.	N.I.	N.I.

N.I., not inhibited.

The data presented here indicate that 6-methoxymellein is accumulated in carrot roots as a phytoalexin after fungal infection. This compound was first reported by Sondheimer as an unusual metabolite of carrot, responsible for the bitter taste in carrots kept in a cold store [17]. Coxon *et al.* [18] showed that 6-methoxymellein was produced in carrot roots in the presence of low concentrations of ethylene. These findings suggest that 6-methoxymellein is accumulated in response to physiological imbalance and support the hypothesis that phytoalexins are also stress compounds [19].

EXPERIMENTAL

Micro-organisms. Curvularia intermedia, C. clavata, C. ovoidea, C. lunata, C. inaequalis, Alternaria alternata, A. padwickii, Rhizopus chinensis, Fusarium moniliforme and *Helminthosporium oryzae* were obtained from Toyama Agricultural Experimental Station, Toyama, Japan. *Botrytis cinerea* and *Chaetomium globosum* were obtained from Dr. K. Tsubaki, Institute of Biological Sciences, Tsukuba University, Ibaraki, Japan. *Aspergillus fumigatus* and *Candida albicans* were obtained from Dr. K. Tanaka, Faculty of Medicine, Nagoya University, Nagoya, Japan. These fungi and yeasts were maintained on agar slants of yeast–malt medium (yeast extract 0.4%, malt extract 1% and glucose 0.4%). *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), *Streptococcus pyogenes* (ATCC 10389), *Salmonella typhimurium* (ATCC 13311), *Shigella sonnei* (ATCC 11060), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 27736), *Enterobacter aerogenes* (ATCC 23355), *Serratia marcescens* (ATCC 8100), *Proteus vulgaris* (ATCC 13315) and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from Dr. K. Konishi, Faculty of Medicine of this university and maintained on heart infusion broth.

TLC bioassay. Mycelial suspensions of a 2-day-old culture of fungi in a modified Murashige–Skoog's medium [20] (containing Murashige and Skoog's mineral salts, 0.2% malt extract and 0.4% glucose) were sown on the 50 g carrot root slices (2 mm thick) with sterilized pipettes. Only the medium was sown on control treatments. After 24 hr incubation at 27°, slices were homogenized in Me_2CO with a Waring blendor and extracted with a total of 100 ml Me_2CO for 7 days at room temp. The extract was filtered and the filtrate evaporated. The concd soln was re-extracted with EtOAc and the extract was evaporated to dryness. The dried material was redissolved in 0.2 ml Me_2CO, and 20 μl aliquots of the soln were applied on to Si gel plates (Si gel 60 F-254, Merck). The plates were developed in C_6H_6–MeOH (100:3) and air dried. Then the suspension of sonicated fragments of fungal mycelia cultured in yeast–malt medium was sprayed on the TLC plates. Clear inhibitory zones were observed in all fungi used here after the plates were incubated at 27° in a moist chamber for 2–5 days.

Isolation of 6-methoxymellein. Carrot roots (2 kg) were sliced *ca* 2 mm thick and incubated with *C. globosum* as mentioned above for 24 hr at 27°, and extracted with Me_2CO for 3 days at room temp. (2 × 6 l.). The extract was concd to 500 ml under red. pres. at 40° then mixed with several portions of *n*-hexane and extracted with 2N NaOH (2 × 500 ml). The aq. phase was neutralized with 6N HCl in an ice bath, and re-extracted with *n*-hexane (2 × 1 l.). The *n*-hexane soln (i.e. acidic fraction) was dried and evaporated to *ca* 5 ml at 40°. The concd extract was subjected to Si gel CC in C_6H_6–MeOH (100:3).

Chemical characterization. ^{1}H NMR spectrum was determined in $CDCl_3$ with TMS as int. standard at 200 MHz. Electron impact mass spectrometry used a heated direct inlet probe, and GC/MS analysis was carried out using a 1 m × 3 mm glass column packed with 3% OV-1 at column temp. 170°.

Determination of 6-methoxymellein in carrot suspension culture. Carrot cells were cultured in the synthetic liquid medium of Murashige and Skoog on a reciprocal shaker at 27°. The details of culture conditions were described previously [21]. A 7-day-old culture of carrot cells (late logarithmic phase) was inoculated with the spore suspension of *C. globosum*. Mixed cells of carrot and *C. globosum* were harvested by filtration and homogenized in Me_2CO with quartz sand. The acidic fraction was prepared as described above, and evaporated to dryness. The remaining material was dissolved in 0.1 ml Me_2CO and 20 μl of the soln was applied to Si gel plates. Acidic substances in medium were directly extracted with *n*-hexane after the addition of 10 ml 6N HCl. Then, the plates were developed in C_6H_6–MeOH (100:3) and analysed on a dual wavelength TLC scanner at λ_S 265 nm, λ_R 400 nm.

Inhibition bioassay. EtOH soln of 6-methoxymellein was added to each medium to give the concns of 0.05, 0.1 and 0.5 mM. The final concn of EtOH was adjusted to 1% in every expt. Fungal growth was determined by increase of dry wt. Equal amounts of mycelia were inoculated to a 5 ml yeast–malt medium in 22 mm test tubes and incubated at 27° until the control tube formed a mat on the surface of the medium. The mycelia were washed on a 0.45 μm millipore filter and transferred to previously weighed dishes and dried overnight at 80°. The growth of yeasts was followed by measuring turbidity in 14 mm test tubes at 540 nm. The tubes contained 5 ml yeast–malt medium and were incubated at 27°. Bacteria were grown in a heart infusion broth (5 ml) at 37° and the growth was followed likewise by the turbidity method.

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