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METABOLISM OF IPOMEAMARONE IN SWEET POTATO ROOT SLICES BEFORE AND AFTER TREATMENT WITH MERCURIC CHLORIDE OR INFECTION WITH *CERATOCYSTIS FIMBRIATA*

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Key Word Index—*Ipomoea batatas*; Convolvulaceae; sweet potato; *Ceratocystis fimbriata*; metabolism; phytoalexin; furanoterpene; ipomeamarone; ipomeamaronol.

Abstract—Furanoterpenes produced in sweet potato (*Ipomoea batatas*) root tissue in response to fungal infection or injury with mercuric chloride were metabolized to compounds negative to Ehrlich's reagent. The metabolism of ipomeamarone in sweet potato root tissue was enhanced in response to cut injury. The enhanced metabolism of ipomeamarone in the cut tissue was partly prevented by $HgCl_2$ treatment. The initial metabolite of ipomeamarone was identified as ipomeamaronol, 15-hydroxyipomeamarone. A time-course analysis of the metabolism of [^{14}C]ipomeamarone by cut tissue indicated that ipomeamarone was converted to ipomeamaronol, which was further metabolized to unknown compounds.

INTRODUCTION

Furanoterpenes are accumulated in sweet potato root tissue infected with *Ceratocystis fimbriata* or injured by application of toxic chemicals to cut tissue. Researches on the mechanism of accumulation of furanoterpenes have been focused on the biosynthesis ever since ipomeamarone (the main furanoterpene) was isolated from sweet potato root tissue infected with *C. fimbriata* and the chemical structure was determined [1–3]. These studies have demonstrated that the activities of the enzymes involved in the biosynthesis are enhanced in response to fungal infection or injury with toxic chemicals and the enhancement is followed by the production of furanoterpenes. However, the extent of accumulation of furanoterpenes could be regulated by the relative rates of biosynthesis and biodegradation. We recently observed that the furanoterpenes produced in $HgCl_2$-treated tissue are metabolized to compounds negative to Ehrlich's reagent [4]. The fact that the amount of [^{14}C]ipomeamarone produced from [2-^{14}C]acetate in the fungal-infected tissue decreased in the later stage of incubation suggested that furanoterpenes are also metabolized in tissue infected with *C. fimbriata* [5].

In the present paper, we show that: (1) ipomeamarone is metabolized in cut, $HgCl_2$-treated and fungus-infected tissues, (2) induction of ipomeamarone metabolism in cut tissue is dependent upon the *de novo* synthesis of some of the enzymes involved in the degradation, (3) ipomeamarone is converted to ipomeamaronol in the cut tissue for the first 1 hr after feeding of [^{14}C]ipomeamarone, and ipomeamaronol is further metabolized to unknown compounds.

RESULTS

Changes in furanoterpene content after $HgCl_2$ *treatment*

When cut tissue was treated with $HgCl_2$ on the cut surface, furanoterpenes were rapidly accumulated in the injured region. A time-course experiment demonstrated that fluctuation of the amounts of furanoterpenes accumulated in the injured region differed depending on the incubation periods before $HgCl_2$ treatment (Fig. 1): a 16- or 24-hr incubation before $HgCl_2$ treatment resulted in larger accumulation of furanoterpenes following $HgCl_2$ treatment than an 8-hr incubation before $HgCl_2$ treatment. When the 16- or 24-hr incubated cut tissue was treated with $HgCl_2$, furanoterpenes were accumulated 3 hr after $HgCl_2$ treatment and the content reached a maximum in 6–9 hr and then decreased gradually. The results suggest that metabolism of furanoterpenes is induced in response to cut injury or $HgCl_2$ treatment.

Metabolism of [^{14}C]*ipomeamarone in fresh, cut or* $HgCl_2$*-treated tissue*

To investigate the metabolism of furanoterpenes in the tissue, [^{14}C]ipomeamarone was fed to fresh, cut or $HgCl_2$-treated tissue, which was incubated at 30° for 0.5, 3 and 6 hr. As shown in Table 1, the radioactivity in the $CHCl_3$-soluble fraction containing ipomeamarone and other furanoterpenes rapidly decreased during the first 30 min and then fell gradu-

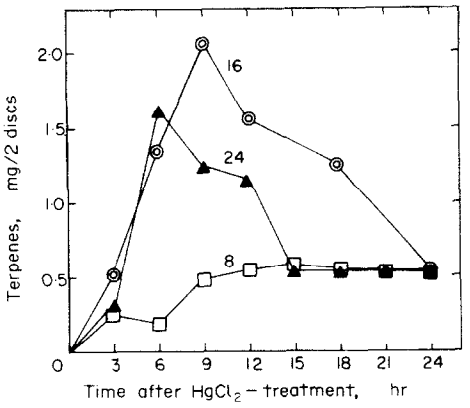

Fig. 1. Time course of furanoterpene accumulation in the injured region of $HgCl_2$-treated tissue. Discs [16 mm (diam.) × 4 mm] were incubated for 8 (□–□), 16 (◎–◎) and 24 hr (▲–▲) at 30°. They were then treated with $HgCl_2$.

ally thereafter in the three tissues. In contrast, the radioactivity recovered in the MeOH–H_2O-soluble fraction and in the 20% KOH fraction increased, although the total amount recovered in these two fractions was less than that lost from the $CHCl_3$-soluble fraction. Time-course studies showed that the radioactivity associated with the ipomeamarone zone recovered from ITLC (see Experimental), decreased with incubation time in fresh, cut and $HgCl_2$-treated tissues when [^{14}C]ipomeamarone was applied on the cut surface of the tissues, although the metabolism by fresh tissue was small as compared with that by cut or $HgCl_2$-treated tissue.

Change in the metabolism of ipomeamarone in tissue in response to cut injury, $HgCl_2$ treatment or fungal inoculation

As shown in Fig. 2, the rate of metabolism of ipomeamarone did not change for the first 4 hr after cutting, it then began to increase and reached a maximum at about 18 hr after cutting, This increase in the rate of metabolism following cutting was almost completely inhibited by the administration of cycloheximide (5 μg/ml).

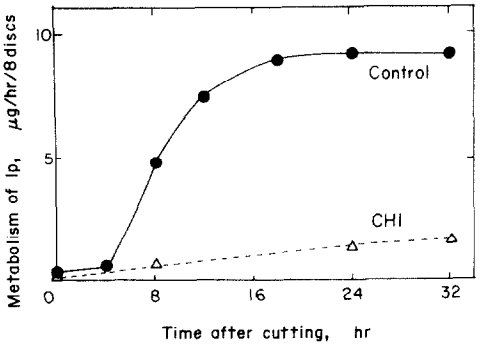

Fig. 2. Induction of metabolism of ipomeamarone in cut tissue and effect of cycloheximide on the induction. Distilled water (control, ●–●) and cycloheximide solution (5 μg/ml) (CHI, △–△) was administered to discs by vacuum infiltration (40 mm Hg, for 1 min) immediately after disc preparation. [^{14}C]Ipomeamarone (8900 dpm, 48 μg/8 discs) was placed on the freshly cut surface of the discs, and the rate of metabolism of ipomeamarone (μg/hr per 8 discs) was calculated as described in the Experimental.

Table 1. Time course of metabolism of ipomeamarone in fresh, cut and $HgCl_2$-treated tissues

Tissue		Radioactivity (dpm) 0.5 hr	3.0 hr	6.0 hr
Fresh	$CHCl_3$ fraction*	6470	5630	5030
	MeOH–H_2O fraction	2630	1540	2110
	$^{14}CO_2$(20% KOH)	143	130	100
	Recovery (%)	96.7	76.4	75.7
Cut	$CHCl_3$ fraction	6510	4110	2740
	MeOH–H_2O fraction	1710	3580	4170
	$^{14}CO_2$ (20% KOH)	110	140	180
	Recovery (%)	87.2	82.1	74.2
$HgCl_2$-treated	$CHCl_3$ fraction	5110	4030	3020
	MeOH–H_2O fraction	890	1120	1270
	$^{14}CO_2$ (20% KOH)	160	270	190
	Recovery (%)	64.5	56.7	46.9

[^{14}C]Ipomeamarone (9600 dpm) in 480 μl 66 mM KPi buffer (pH 6.0) containing Me_2CO in a final concentration of 5% was placed on the freshly cut surfaces (see Experimental) of eight discs of fresh, cut and $HgCl_2$-treated tissues, and incubated for 0.5, 3.0 and 6.0 hr at 30°.

*Ipomeamarone and other furanoterpenes were recovered in the $CHCl_3$ fraction. At 0 hr all counts were recovered in the $CHCl_3$ fraction.

When $HgCl_2$ (0.1%) was applied to cut tissue which had been incubated for 16 hr, the enhanced rate of metabolism of ipomeamarone in cut tissue was decreased gradually by $HgCl_2$ treatment until 6 hr and reached a constant rate thereafter (Fig. 3). The prevention of metabolism of ipomeamarone by $HgCl_2$ treatment was not affected by administration of cycloheximide.

The changes in the rate of metabolism of ipomeamarone in the tissue infected by *C. fimbriata* are shown in Fig. 4. The rate of metabolism began to increase after a short lag period and reached a maximum at about 18 hr of incubation after inoculation of *C. fimbriata*, then decreased by 48 hr. The amounts of furanoterpenes accumulated in the injured browning region began to increase at the time when the rate of metabolism of ipomeamarone began to decrease in both $HgCl_2$-treated and fungus-infected tissues (Figs. 3 and 4).

Identification of a metabolite of ipomeamarone as ipomeamaronol

ITLC (Fig. 5) demonstrated that there were three radioactive peaks whose R_f values coincided with those of ipomeamarone, 4-hydroxymyoporone and ipomeamaronol. Sometimes the radioactivity in the peak corresponding to 4-hydroxymyoporone was comparable with that of ipomeamaronol. To identify the compound which ran as ipomeamaronol, a $CHCl_3$-soluble fraction was subjected to TLC and the EtOAc extract of the area corresponding to ipomeamaronol run on HPLC. The radioactive peak was coincident with the mass peak of ipomeamaronol (detected by monitoring at A_{230}). The MS of the compound in the fraction corresponding to ipomeamaronol gave the same M^+ peak (*m/z* 266) as ipomeamaronol ($C_{15}H_{22}O_4$).

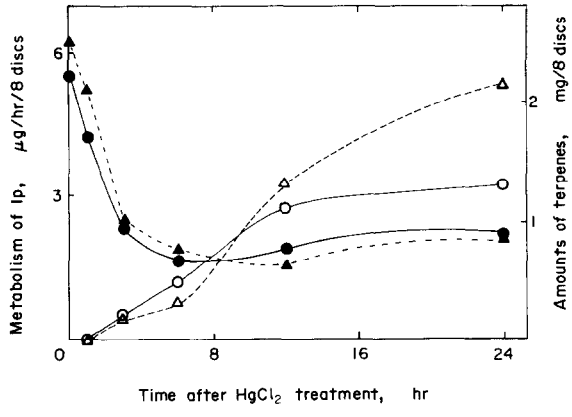

Fig. 3. Changes in the rate of metabolism of ipomeamarone and furanoterpene content after $HgCl_2$ treatment and effect of cycloheximide on the changes. $HgCl_2$ (0.1%) solution with (△, ▲) or without (○, ●) cycloheximide (5 μg/ml) was placed on the surface of cut tissue incubated for 16 hr, and the tissues further incubated for the periods shown. The injured browning region was separated from the non-infected tissue. The former and the latter tissues were used for the assays of furanoterpene content (○, △) and the rate of metabolism of ipomeamarone (●, ▲), respectively.

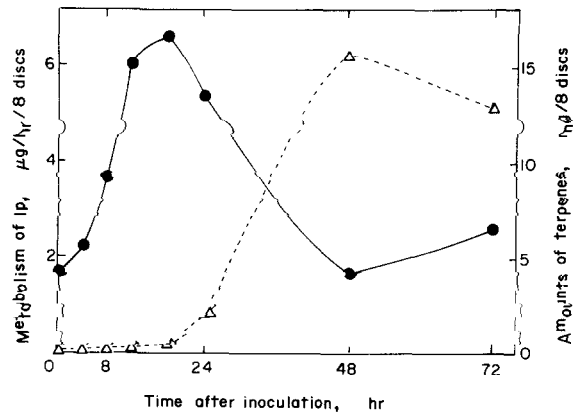

Fig. 4. Changes in the rate of metabolism of ipomeamarone and furanoterpene content in tissue infected by *C. fimbriata*. Infected browning region and the non-infected tissue adjacent to the infected region were used for the assays of furanoterpene content (△–△) and the rate of metabolism of ipomeamarone (●–●), respectively.

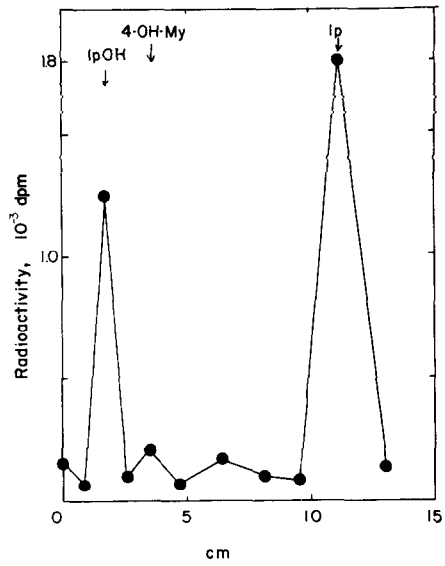

Fig. 5. ITLC of the metabolites of [^{14}C]ipomeamarone. Chloroform-soluble materials extracted from the cut tissue incubated for 1 hr after feeding [^{14}C]ipomeamarone (7450 dpm, 48 μg/8 discs) were applied to an ITLC plate. The plate was first developed for 7 cm with *n*-hexane–EtOAc (7:3) and then for 15 cm with *n*-hexane–EtOAc (9:1). Ip, 4-OH–My and IpOH: ipomeamarone, 4-hydroxymyoporone and ipomeamaronol. The positions of Ip; 4-OH–My and IpOH were visualized by spraying with Ehrlich's reagent.

A spot on the radioautogram of an ITLC coincided exactly with that of ipomeamaronol visualized by spraying with Ehrlich's reagent.

Figure 6 shows the time-course pattern of [^{14}C]-label following the feeding of [^{14}C]ipomeamarone to cut tissue. The data indicate that ipomeamarone was

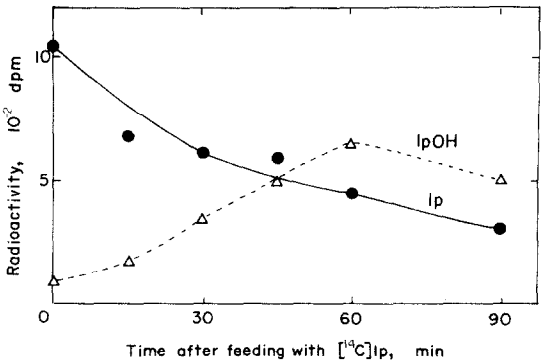

Fig. 6. Time course of decrease in ipomeamarone and increase in ipomeamaronol after feeding cut tissue with [^{14}C]ipomeamarone. Radioactivities in the respective zones of ipomeamarone and ipomeamaronol on an ITLC plate were assayed separately. Ip and IpOH: ipomeamarone and ipomeamaronol.

converted stoichiometrically to ipomeamaronol during the first 60 min. The ipomeamoronol was then metabolized to other compound(s).

DISCUSSION

Time course patterns of furanoterpene content after $HgCl_2$ treatment (Fig. 1) reconfirmed the previous observation that furanoterpenes produced in $HgCl_2$-treated tissue are metabolized to a compound negative to Ehrlich's reagent at the later stage of incubation [4]. It should be emphasized that the maximum amount of furanoterpenes accumulated in the injured region after $HgCl_2$ treatment differs among the tissues incubated for different periods before $HgCl_2$ treatment. Therefore, mechanical wounding may play some role in the production of furanoterpenes after $HgCl_2$ treatment.

When [^{14}C]ipomeamarone is placed on the tissues, it is metabolized in fresh, cut and $HgCl_2$-treated tissues, although ipomeamarone is not synthesized in fresh and cut tissues, and the rate of metabolism of ipomeamarone in fresh tissue is small as compared with that in cut or $HgCl_2$-treated tissue. [^{14}C]Ipomeamarone should be metabolized to either water-soluble components or volatile components although they have not been detected, as judged by the observation that radioactivity in the MeOH–H_2O-soluble fraction increases with increase in time of incubation whereas the total recovery of radioactivity decreases (Table 1).

It has been reported that rishitin in potato tuber tissue [6], capsidiol in pepper tissue [7] and glyceollin in soybean hypocotyls [8] are metabolized by these tissues, so degradative metabolism of phytoalexins in plants seems to be a general phenomenon.

The metabolic activity of ipomeamarone in sweet potato root tissue is induced in response to cut injury and the enhanced rate of metabolism of ipomeamarone in the cut tissue is prevented by $HgCl_2$ treatment (Fig. 3). As the induction of the metabolism of ipomeamarone in response to cut injury is inhibited by the administration of cycloheximide but the prevention after $HgCl_2$ treatment is not inhibited by cycloheximide (Figs. 3 and 4), induction of the metabolism of ipomeamarone by cutting seems to be dependent upon *de novo* protein synthesis and the prevention of the metabolism by $HgCl_2$ treatment seems to be due to the death of cells or due to inactivation of the enzymes involved in the metabolism of ipomeamarone. Decrease in the metabolism of ipomeamarone in $HgCl_2$-treated tissue or fungus-infected tissue (Figs. 3 and 4) may play some role in the accumulation of furanoterpenes in the sweet potato root tissue injured with toxic chemicals or fungus. However, the formation or activation of the enzymes involved in the biosynthesis of furanoterpenes must play an important role in the accumulation of furanoterpenes, because the activity of HMG–CoA reductase, the key enzyme in the biosynthetic pathway, is enhanced and followed by the accumulation of furanoterpenes in response to $HgCl_2$ treatment or the fungus-infection, but neither activation of HMG–CoA reductase nor accumulation of furanoterpenes occurs in cut tissue [1, 9].

In the present research, the initial metabolite of ipomeamarone produced in sweet potato root tissue was isolated and identified as ipomeamaronol, 15-hydroxyipomeamarone. Ipomeamaronol was first isolated from *C. fimbriata*-infected sweet potato root tissue and its chemical structure was elucidated by Kato *et al.* [10]. The present study also shows that ipomeamarone is metabolized to ipomeamaronol and then to unknown compounds (Fig. 6), but does not reveal whether the conversion of ipomeamarone into ipomeamaronol is a rate-limiting step of its metabolism. Burka and Kuhnert [11] proved that ipomeamarone is oxidized to 4-hydroxymyoporone by $HgCl_2$-treated sweet potato root tissue, using [^{14}C]ipomeamarone. Usually, we detected only small amounts of radioactivity in the spot of 4-hydroxymyoporone on an ITLC plate (Fig. 5), although on occasions we observed comparable levels of radioactivity in the spots of 4-hydroxymyoporone and ipomeamaronol. Therefore, there seem to be at least two pathways for the metabolism of ipomeamarone in sweet potato root tissue.

Heuvel *et al.* [12] demonstrated that *Fusarium solani* f. sp. *phaseoli* metabolizes phaseollin, a main phytoalexin of *Phaseolus vulgaris*, to 1a-hydroxy phaseollone which is less toxic to the fungi than phaseollin. Ishiguri *et al.* [6] demonstrated that rishitin, a main phytoalexin of potato tuber, is hydroxylated to rishitin M-1 and rishitin M-2, which have less antifungal activity than rishitin, by cut potato tuber tissue. Ipomeamarone is toxic to sweet potato root [13] and ipomeamaronol is similarly inhibitory to spore germination of *C. fimbriata* to the same extent of ipomeamarone [14, 15], and hence ipomeamaronol formation cannot be considered to be a detoxification process *per se*. However, ipomeamaronol disappears with increase in time of incubation and it may be an intermediate in an over all degradation process. The same situation holds for the metabolism of phaseollin to 6a-hydroxyphaseollin and to 6a,7-dihydroxyphaseollin by *Colletotrichum lindemuthianum*, the cause of anthracnose disease of *P. vulgaris* [16].

EXPERIMENTAL

Plant material. Sweet potato (*I. batatas* Lam. cv Norin 1) roots were harvested at Kariya Farm, Aichi, in the autumn and stored at 13° until use.

Preparation of fresh, cut, $HgCl_2$-treated or fungus-infected tissue. Roots were dipped in a soln of 0.1% $NaClO_4$ for 20 min for sterilization, washed with H_2O for 20 min, then cut transversely into blocks about 3 cm thick. Tissue cylinders, 16 mm in diam., were obtained from parenchymatous tissue with a cork borer. Discs 4 mm thick were prepared from the cylinder by cutting with a razor blade, washed with a large vol. of distilled H_2O, and lightly blotted. The discs were placed on a wire net in a plastic box under high humidity and incubated at 30° in the dark. Freshly cut discs and discs incubated for 22 hr were called fresh tissue and cut tissue, respectively. Sometimes, 60 μl 0.1% $HgCl_2$ soln/disc was added to filter paper (14 mm diam.) on one side of 16-hr incubated cut tissue. The tissue was then incubated for another 6 hr under the same condition, and called $HgCl_2$-treated tissue. The discs inoculated on the cut surface with a spore suspension (*ca* 1×10^7/ml) of *C. fimbriata* Ell. and Halst, and incubated at 30° for definite periods were called fungus-infected tissue.

Determination of furanoterpene content. This was determined by the method of Hyodo *et al.* [18].

Assay for metabolism of [^{14}C]ipomeamarone. [^{14}C]Ipomeamarone (1 μCi/μmol) was biosynthesized from [2-^{14}C]acetate (58 μCi/μmol) in $HgCl_2$-treated tissue and isolated as a pure sample by the method previously reported [17]. The upper surfaces (*ca* 0.5 mm thick) of cut, $HgCl_2$-treated and fungus-infected tissues were cut to remove injured browning regions. A mixture (30 μl/disc) of [^{14}C]ipomeamarone in Me_2CO and KPi buffer (66 mM, pH 6.0) (5:95) was placed on a freshly cut surface of the respective tissues (3.5 mm thick, 20 mm diam.). These tissues were incubated at 30° for 1 hr in a plastic box with a dish containing 1.0 ml 20% KOH for trapping $^{14}CO_2$ and then put into 20 ml $CHCl_3$–MeOH (1:1) containing crude furanoterpenes (2.5 μg/20 ml) and homogenized with a glass homogenizer. The homogenate was filtered through a glass filter (porosity No. 3 medium) into a centrifuge tube and the residue was washed with the same solvent mixture until the filtrate reached 35 ml. The soln was mixed with 14 ml H_2O, and the suspension was shaken vigorously, then the $CHCl_3$ fraction was separated from the MeOH–H_2O fraction. After evapn of the $CHCl_3$ fraction under red. pres. below 40°, the residue was dissolved in 0.3 ml EtOAc and a 0.1 ml aliquot subjected to chromatography on polysilisic acid gel-impregnated glass fiber sheet (ITLC SAF, German Industrial Company) with *n*-hexane–EtOAc (9:1). The ipomeamarone zone, detected under UV (2536 Å), was cut out and put in 5 ml Bray's soln for determination of radioactivity in a liquid scintillation spectrometer. The rate of metabolism of ipomeamarone was estimated from the decreases in radioactivity of [^{14}C]-label in the ipomeamarone zones as the experiments progressed. Ipomeamarone prepared by the method of Akazawa [19] was added as a carrier to obtain a linear decrease over the incubation time for 1 hr in the case of cut, $HgCl_2$-treated or fungus-infected tissue. No degradation of [^{14}C]ipomeamarone was detected either in incubations without tissue or in the course of the extraction procedure. HPLC: μ Bondapack C-18; 1 ml/min (110 kg/cm^2) MeOH–H_2O (1:1); UV (254 nm) and RI.

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