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Biosynthetic correlation of various phytoalexins in sweet potato root tissue infected by *Ceratocystis fimbriata*[1]

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In sweet potato root tissue infected by *Ceratocystis fimbriata*, changes in a one hour-incorporation of radioactivities from 2-^{14}C-acetate into furano-terpene phytoalexins were examined during incubation. The data showed that radioactivities in the $CHCl_3$ fractions (containing lipid) were increased after 15 hr of incubation, and were found in furano-terpenes as visualized by the TLC radioautograms. Pulse and chase feeding of 2-^{14}C-acetate at low temperature was also performed using tissue discs incubated for 36 hr with the infected region. Changes in the radioactivities of the individual components were investigated with TLC. Several components such as OHDHMy were labeled rapidly, then followed a decrease in the label. These changes were accompanied by increases in the radioactivities of other components such as DHIp, Ip, IpOH and Component B_1. These results are further evidence for the hypothetical pathway that has been proposed previously, with some modifications.

Key words: *Ceratocystis fimbriata* — Ipomeamarone — *Ipomoea batatas* — Phytoalexin — Sesquiterpene.

In sweet potato root tissue, large amounts of sesquiterpenes containing the furan ring accumulate in response to infection of *C. fimbriata* or to treatment with toxic chemicals such as $HgCl_2$. They include ipomeamarone (Ip) (*6*), dehydroipomeamarone (DHIp) (*14*), ipomeamaronol (IpOH) (*10*, *20*), 4-hydroxymyoporone (OHMy) (*5*), 4-hydroxydehydromyoporone (OHDHMy) (*9*), 1-(3′-furyl)-6,7-dihydroxy-4,8-dimethylnonan-1-one (*3*) and other components of unknown structure. These sesquiterpenes serve as phytoalexins against some pathogenic fungi, e.g. *C. fimbriata* (*19*), and have been found in the toxicity of infected sweet potatoes to domestic animals (*2*).

The early stage of the biosynthetic pathway of these components has been determined by in vitro (*15–18*) experiments, but the later stage from farnesyl pyrophosphate to the furano-terpenes remains to be determined, although several in vivo (*4*, *11–13*) experiments have been performed. Oguni and Uritani reported that

Abbreviations: TLC, thin layer chromatography; MeOH, methanol; EtOAc, ethyl acetate; Ip, ipomeamarone; DHIp, dehydroipomeamarone; IpOH, ipomeamaronol; OHMy, 4-hydroxymyoporone; OHDHMy, 4-hydroxydehydromyoporone.

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2-^{14}C-farnesol was readily incorporated into Ip (*12*) and that DHIp was the closest precursor of Ip in the pathway (*13*). In addition, labeled Ip was incorporated into IpOH (*11*) and OHMy (*4*). Recently we observed that OHDHMy is easily converted in OHMy when ^{14}C-labeled OHDHMy is administered to the tissue inoculated and incubated for 36 hr (unpublished data). The hypothetical biosynthetic pathway, shown in Fig. 5, may be presented by such previous observations.

This report deals with the validity of the hypothetical biosynthetic pathway for terpenes, based on experimental data on 2-^{14}C-acetate incorporation, which is more efficient than ^{14}C-mevalonate incorporation (*1*).

Materials and methods

Materials

Sweet potato (*Ipomoea batatas* Lam. cv. Norin No. 1) roots were harvested at Kariya Farm in Aichi Prefecture in the autumn, then stored at 10 to 14°C until use. Roots were cut perpendicularly into slices 15 to 20 mm thick. The slices were inoculated on their cut surfaces with a spore suspension of *Ceratocystis fimbriata* Ell. et Halst. then incubated at 29°C under high humidity. 2-^{14}C-Sodium acetate was purchased from the Radiochemical Centre, Amersham.

Preparation of tissue discs and administration of 2-^{14}C-acetate

Two different experiments were performed in this study; time course labeling, and pulse and chase feeding.

In a prior experiment, 4 discs (20 mm in diameter and 2 mm thick, including the infected region) were prepared from inoculated slices at appropriate incubation times and left on a steel net in a petri dish, with the inoculated surface face down. 2-^{14}C-Acetate solution (3.8 μCi, 31 μmoles in a total volume of 160 μl) was applied to the upper cut surfaces of the 4 discs, after which the discs were incubated at 29°C for 1 hr.

In the second experiment, plugs (10 mm in diameter) were prepared with a cork borer from the slices incubated for 36 hr. The outer tissue (0.5 mm thick) including the infected region was removed from each plug. One disc (2 mm thick) was prepared from the tissue of each plug adjacent to the cut surface from which the infected region had been removed. The discs were washed with distilled water and incubated at 6.5°C for 20 min. Seven discs were placed on a petri dish containing 4 ml of 10 mM potassium phosphate buffer, pH 7.5, with 2-^{14}C-acetate (264 μCi, 4.5 μmoles); these were incubated at 6.5°C for 2 min. The discs then were washed for 30 sec three times with 200 ml of ice cold potassium acetate solution (50 mM, pH 7.5). The washed discs were placed on a steel net in a large petri dish containing 150 ml of 50 mM potassium acetate solution, pH 7.5, in which the discs were immersed to about half their thickness. The discs were incubated at 6.5°C, and removed at appropriate times for assay. In both experiments, the control samples were prepared by the same procedures except that fungal inocultaion was omitted.

Extraction of furano-terpenes

In the first experiment, the 4 discs each were washed twice with 50 ml of ice cold acetate solution (50 mM, pH 5.5) at the end of incubation, then they were homo-

genized with 20 ml $CHCl_3$-MeOH (1:1, v/v) in a glass homogenizer. In the second experiment, each disc was homogenized in the above solvent (20 ml) without washing. The homogenate was filtered through a glass filter. A half volume of water was added to the filtrate and the mixture was shaken. The $CHCl_3$ layer was separated from the MeOH-H_2O layer (MeOH-H_2O fraction) by centrifugation and evaporated in vacuo to an oily residue. This was dissolved in $CHCl_3$, and the solution ($CHCl_3$ fraction) was used to measure radioactivity and for the TLC assay.

Measurement of radioactivities

Radioactivities were counted with a Packerd Tri-Carb liquid scintillation spectrometer, Model 3320, with 5 ml of 33 vol % Nonion (Nissan Yushi Co., Tokyo) in toluene containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-*bis*-2-(5-phenyloxazole) benzene (POPOP).

Quantitative assay of furano-terpenes

A sample of the $CHCl_3$ fraction was concentrated in vacuo to an oily residue, which then was dissolved in ethanol and assayed for the amount of furano-terpenes according to the method of Hyodo et al. (*7*).

Results and discussion

The time course labeling

A one hour incorporation of the label from 2-^{14}C-acetate into furano-terpenes was examined during incubation of discs prepared from slices with or without fungal inoculation. As shown in Fig. 1-a, the incorporation rate (1 hr) of the label of 2-^{14}C-acetate into the $CHCl_3$ fraction was rather small at the initial time (0 time). However, in both the inoculated or unioculated slices, the incorporation rate had increased by 3 hr and reached a plateau that was maintained up to the 12 hr of incubation. The primary increase in the rate probably is due to the response to the cut injury in both slices. The substances related to this increase mainly may be phospholipids, as reported by Imaseki et al. (*8*).

The incorporation rate into the $CHCl_3$ fractions of inoculated discs again began to increase 15 hr after incubation (Fig. 1-a); accumulation of furano-terpenes in these discs was observed about 3 hr later (Fig. 1-b). In the radioautogram (Fig. 2-a), several spots that did not correspond to furano-terpenes, were present from the early (3 hr-incubated) stage to the end of the experimental period. These spots also may be due to the response to cut-injury. By contrast, labeled spots corresponding to furano-terpenes were present beginning at the 18th hour; the number of labeled spots did not change thereafter throughout the experimental periods. However, positive Ehrlich's reagent spots appeared first at the 18th hour and increased up to the end of incubation (27 hr) (Fig. 2-b). The data on the radioautogram suggest that the enzymes that participate in the biosynthesis of these furano-terpenes are activated or induced over a short period.

The above data indicate that the secondary increase in incorporation may be due to furano-terpene accumulation.

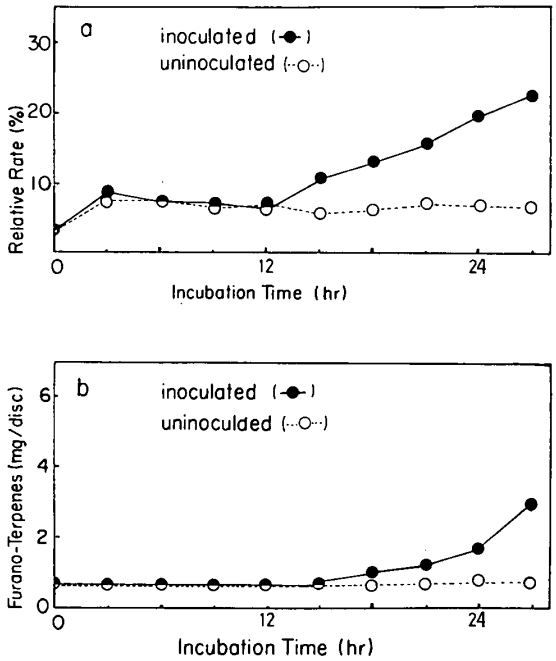

Fig. 1. *Changes in relative rates of radioactivities(a) and amounts of furano-terpenes(b) in $CHCl_3$ fractions.* Inoculated (diseased, -●-) and uninoculated (sliced only, --○--) slices (15–20 mm thick) were incubated for various periods as shown in the figure. At the end of each incubation, 4 discs (2×20 mm) were incubated with 2-^{14}C-acetate for 1 hr by the procedure described in **Materials and methods**. The $CHCl_3$ fraction was prepared from these 4 discs; then the radioactivity was measured (a), and the amount of furano-terpene accumulated in the fraction was assayed (b). The relative rates of the radioactivities are expressed as the percent (%) of the dpm of the assayed sample to that of the labeled acetate absorbed by the discs. The radioactivity (dpm) of the latter was calculated by subtracting the dpm of the remaining unabsorbed 2-^{14}C-acetate from that of the applied labeled acetate.

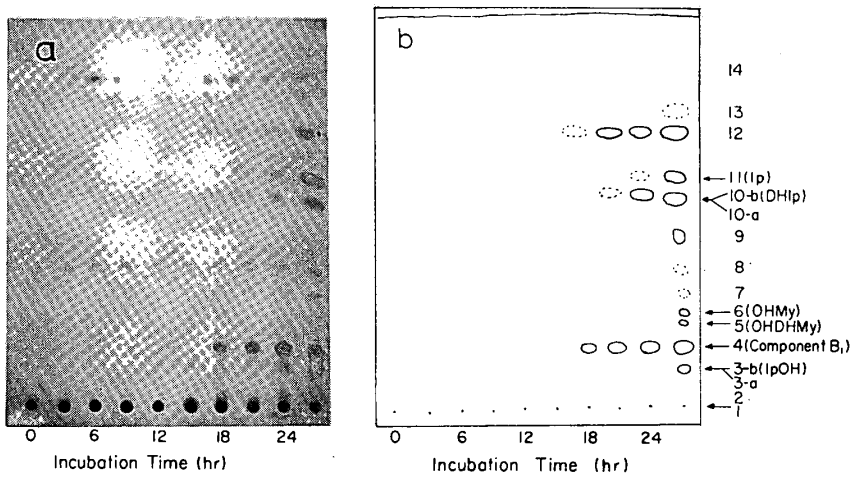

Fig. 2. *TLC radioautogram and furano-terpene pattern of the $CHCl_3$ fractions.* $CHCl_3$ fractions were prepared by the procedure described in **Materials and methods**. The fractions were applied to a TLC plate, that was developed with *n*-hexane-EtOAc (8 : 2, v/v). This plate was subjected to radioautography (a), then sprayed by Ehrlich's reagent (b). The definition of the numbers (1 to 14): see the legend to Fig. 4.

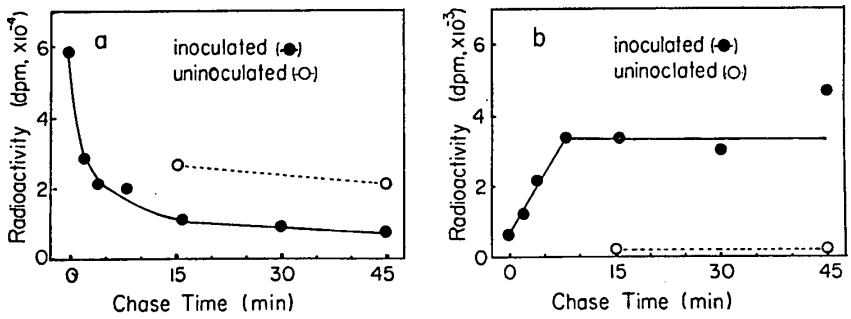

Fig. 3. *Radioactivities in the MeOH-H_2O (a) and $CHCl_3$ (b) fractions.* At each incubation time an MeOH-H_2O fraction and $CHCl_3$ fraction were prepared and assayed for radioactivity by the procedure described in **Materials and methods.**

Pulse and chase feeding

To verify the hypothetical pathway, pulse and chase feeding experiments were performed. Twenty seven hours after fungal inoculation, labeled acetate was actively utilized for the biosynthesis of furano-terpenes (Fig. 1-a). Therefore, labeled acetate was administered for 2 min at 6.5°C to discs prepared from the inoculated fungal and 36 hr-incubated slices (see **Materials and methods**).

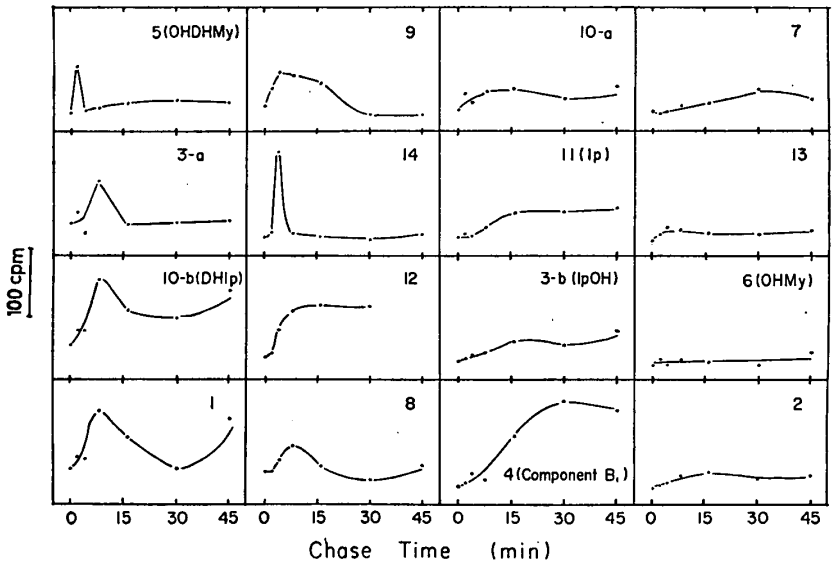

Fig. 4. *Changes in the radioactivities of each fraction by TLC.* At an appropriate time, each radioactive $CHCl_3$ fraction was mixed with the cold standard of furano-terpene prepared from inoculated and 36 hr-incubated tissue, then applied to a TLC plate (silica gel HF_{254}), and developed with *n*-hexane-EtOAc (8 : 2, v/v). Fourteen zones, corresponding to Ehrlich's reagent positive and UV absorbed spots, were individually scraped off and extracted with EtOAc. These were called spots 1 to 14 from the lower position. The solutions were concentrated in vacuo to oily residues, which then were dissolved in EtOAc. Spot 3 and spot 10 were further separated on TLC with *n*-hexane-EtOAc (7 : 3, v/v) and *n*-hexane-EtOAc (9 : 1, v/v), respectively. These were called spot 3-a and 3-b, and spot 10-a and 10-b. The individual spots were named 1, 2, 3-a, 3-b (IpOH), 4 (Component B_1), 5 (OHDHMy), 6(OHMy), 7, 8, 9, 10-a, 10-b (DHIp), 11(Ip), 12, 13 and 14; all were assayed for radioactivity. The time course patterns shown in the figure are in the probable order of earliest incorporated to latest incorporated spot.

Radioactivities in the MeOH-H_2O fractions decreased hyperbolically with the time of chase (Fig. 3-a) with inoculated tissue, but radioactivities in the $CHCl_3$ fractions increased in the early stage and reached a plateau (Fig. 3-b). These changes in radioactivity suggest that water soluble precursors, mainly 2-^{14}C-acetate, disappeared rapidly from the discs and were exchanged with cold acetate during the chase period.

Furano-terpenes were separated by TLC into their various components as shown in Fig. 2-b; thus, the radioactivities of the individual components were measured at 7 different points during a 45 min incubation.

As shown in Fig. 4, OHDHMy was rapidly labeled followed by a rapid decrease of the label. The change was accompanied by an increase in the radioactivities of the other components including DHIp, Ip, IpOH and Component B_1 (*10*). These results show that OHDHMy was a precursor of the above 4 components. The scheme shown in Fig. 5 indicates that OHDHMy was also a precursor of OHMy, but its incorporation into OHMy was low according to the data in Fig. 4. These results suggest that the conversion from OHDHMy to OHMy was not active under these incubation conditions when compared with that from OHDHMy to DHIp or other components. The fact that the label was incorporated into DHIp earlier than Ip was consistent with a previous observation in which DHIp was regarded as the closest precursor of Ip (*13*). The hypothetical pathway involves reaction steps from both Ip to IpOH and Ip to OHMy. But in Fig. 4, the label from 2-^{14}C-acetate was clearly incorporated into Ip or IpOH in similar activity, but less was incorporated into OHMy. This indicates that conversion from Ip to IpOH was greater

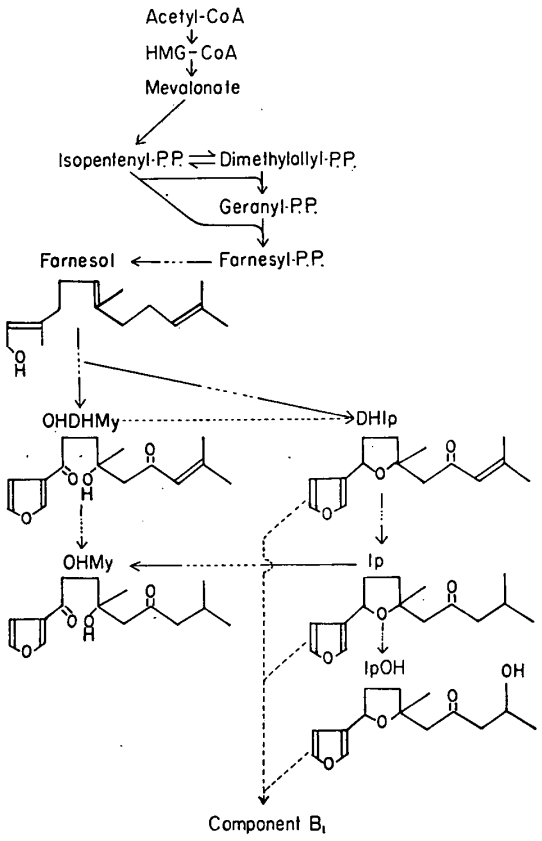

Fig. 5. *The hypothetical biosynthetic pathway of furano-terpenes.* →: The reaction step previously validated in in vivo and in vitro experiments. ⟶: The reaction step previously validated or suggested by in vivo experiments. -→: The reaction step assumed to take place based on the results of this study. pp: pyrophosphate. According to recent unpublished data from this laboratory, spot 12 is composed of two Components, A_1 and A_2; the latter is regarded as a precursor of DHIp or Ip.

than that from Ip to OHMy under these incubation conditions. The fact that no label at the position of OHMy was found up to 27 hr after inoculation (see Fig. 2-a) may mean that the rate of biosynthesis of OHMy, whether OHMy is produced from OHDHMy or Ip, is low in the early period of terpene biosynthesis. The radioactivity of Component B_1 gradually increased up to the 30th minute. Thus, it appears that Component B_1 is positioned at a step that comes later in the pathway than for DHIp, Ip or IpOH (Fig. 5). The results reported here lend further reliable support to the hypothetical pathway shown in Fig. 5.

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