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Biosynthesis of Ipomeamarone

II. Synthetic Mechanism[1]

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Radioactive ipomeamarone synthesized by black rot sweet potato root tissues incubated with acetate-2-C^{14} and mevalonate-2-C^{14} was oxidatively degraded by potassium permanganate, and ipomeanic acid was proved to be a major degradation product. The determination of ipomeanic acid radioactivity compared with that of ipomeamarone gave figures close to the theoretically predicted ones based on the isoprenoid rule, thus verifying this as the biosynthetic route of ipomeamarone. The enzymatic mechanism of ipomeamarone biogenesis is discussed.

The ready incorporation of acetate-2-C^{14} into ipomeamarone has prompted us to postulate the isoprenoid rule of its synthetic mechanism; this was corroborated by an experiment showing a similar utilization of mevalonate-2-C^{14} in the system (1, 2). The final confirmation for this view would be possible either by an enzymatic synthesis starting from an appropriate isoprenoid precursor, or by the chemical degradation of the biosynthetically labeled compound isolated from the fungus-infected root tissues after feeding with radioactive precursors such as acetate-2-C^{14} and mevalonate-2-C^{14}. This paper reports the results of experiments employing the latter approach; it was found that the labeling pattern of the oxidized product conforms with the predicted distribution of C^{14} in the molecule formed by the isoprenoid rule. Accordingly, ipomeamarone, a postinfection furanoterpenoid of the diseased sweet potato root tissues, has been newly added to the isoprenoid family which is commonly synthesized from mevalonate as an active isoprene unit.

[1] Part 39 of the phytopathological chemistry of the black rot of the sweet potato.

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MATERIALS AND METHODS

Fungus inoculation of sweet potato root tissues and the subsequent handling were as reported previously (2, 3). Twenty four- and 48-hour fungus-infected root slices (1.2 mm. in diameter and 2.0 mm. in thickness) were used for incubation with either acetate-2-C^{14} or mevalonate-2-C^{14}. Previously, incubation was terminated at 6 hours, but in the present work slices were incubated for 6 and 15 hours to examine the effect of time. Na-acetate-2-C^{14} was purchased from the California Corporation for Biochemical Research, and mevalonate-2-C^{14} from the Nuclear Chicago Corporation. The latter compound was in benzene solution and was converted to the sodium salt after evaporation of the solvent. Five to ten μc of the labeled compound was added to 20–25 root tissue slices per Petri dish.

Furanoterpenoids were extracted by the standard method, and ipomeamarone was further purified by column chromatography. In order to determine the percentage incorporation of radioactive substrates, fractions containing ipomeamarone were pooled and an aliquot was analyzed for radioactivity by a Nuclear Chicago windowless gas flow counter [cf. Fig. 2 and Table I of ref. (2)]. Nonradioactive ipomeamarone was added as a carrier, and an aliquot was at first converted to the semicarbazone for the determination of specific activity; then the residual material was suspended in about 10 ml. of H_2O and was oxidized according to the method of Kubota and Matsuura (4). A 2% $KMnO_4$ solution was added dropwise to the water

suspension with vigorous stirring; a violet color that lasted a short time was taken as the indication of the end point. The temperature was maintained at 30°–50°C. throughout the process. After removing precipitated MnO_2, the supernatant was concentrated by a Rinco flash evaporator and acidified to pH 2.0 by adding conc. H_2SO_4. It was then continually extracted with ethyl-ether, and after solvent evaporation the ethereal fraction was applied to a silica gel column for organic acid separation by the method of Moyle *et al.* (5). Five ml. each of effluent was collected and aliquots were analyzed for radioactivity and alkaline titration by standardized 0.01 *N* methanolic KOH. The major radioactive fractions eluted by 20% *n*-butanol in $CHCl_3$ were combined, and after neutralization they were converted to *S*-benzylthiuronium salt by the method of Schriner (6). *S*-Benzylthiuronium chloride was previously synthesized by Dr. M. Miyano in this laboratory. The repeatedly crystallized thiuronium salt of ipomeamarone was accurately weighed (1–8 mg.) with a microbalance and placed in a glass vial. Ten ml. of scintillator (PPO, 5.0 g.; dimethyl-POPOP, 0.3 g.; and toluene, 1 liter) were added, and the radioactivity was measured by a Packard Tri-Carb liquid scintillation spectrometer. In a preliminary experiment it was confirmed that no quenching occurred under such conditions. All the experiments were duplicated, and an average value is presented.

RESULTS AND DISCUSSION

Utilization of Mevalonate-2-C^{14} in Ipomeamarone Biosynthesis

In a preceding paper, the less marked utilization of mevalonate-2-C^{14} as compared to acetate-2-C^{14} was reported (2). Although we assumed this to be due to differences in the permeability of root tissues to these two substances, the results weakened our original postulation. However, as shown in Table I, a greater utilization of mevalonate was observed on longer incubation, indicating the more efficient utilization of mevalonate in the ipomeamarone synthesis; considering the biosynthetic utilization of only one of the optical isomers of mevalonate the efficiency must be even greater.

Oxidation of Ipomeamarone

A primary objective of the oxidative degradation was to obtain *iso*-valeric acid from the terminal aliphatic side chain attached to the α-position of the tetrahydrofuran ring in the ipomeamarone molecule, as this portion should have been derived from one unit of isoprene. Since the radioactivity determination of effluents collected from a silica gel column by 20% *n*-butanol in $CHCl_3$ gave a proportional coincidence curve with the alkaline consumptions, the acid component of the fraction was assumed to be *iso*-valeric acid. However, this was denied by both paper chromatography with several solvent systems and spectral analyses. Also, the preparation of the *p*-bromophenacyl ester of the acid fraction was not successful, though this would have been easily accomplished with *iso*-valeric acid. Accordingly, the mechanism of oxidation by $KMnO_4$ was reinvestigated. Kubota's group had already reported that the major oxidation product of ipomeamarone under the same

TABLE I

Incorporation of Acetate-2-C^{14} and Mevalonate-2-C^{14} into Ipomeamarone[a]

Expt.	Compounds added (cpm)	Ipomeamarone		
		Yield mg.	Total cpm	% Incorporation[b]
1	Acetate-2-C^{14} (5.0×10^6)	3.9	5.6×10^4	1.1
	Mevalonate-2-C^{14} (5.0×10^6)	4.4	2.3×10^5	4.6 (9.2)
2	Acetate-2-C^{14} (2.5×10^6)	10.7	1.7×10^5	6.8
	Mevalonate-2-C^{14} (2.5×10^6)	13.3	9.5×10^4	3.8 (7.6)
3	Acetate-2-C^{14} (2.5×10^6)	19.2	2.9×10^5	11.6
	Mevalonate-2-C^{14} (2.5×10^6)	20.1	6.0×10^5	24.0 (48.0)

[a] In expt. 1, 25 root slices at the 24-hour infection stage were used. Twenty slices each of root tissues at the 48-hour infection stage were used in expts. 2 and 3. Incubation time was 15 hours in expts. 1 and 3 and 6 hours in expt. 2.

[b] Figures in parentheses represent the percentage incorporation of the biologically active form of mevalonate-2-C^{14}.

condition was not *iso*-valeric acid but an unknown oily liquid substance (4), and it was thought that the present main degradation product may correspond to this fraction. Consequently, the *S*-benzylthiuronium salt of the unknown acid was prepared, and the recrystallized salt gave the following analytical results; (Found: C, 61.13%; H, 7.24%; N, 7.21%; S, 8.09%. Calc. for $C_{20}H_{30}N_2SO_4$: C, 60.89%; H, 7.67%; N, 7.10%; S, 8.11%), ascribing the chemical formula of $C_{12}H_{20}O_4$ to the original acid. This formula is identical with that of ipomeanic acid, an ozonolyzed product as reported by Kubota's group (7). In accordance with this view, a titrimetric consumption of 2% $KMnO_4$ was roughly in agreement with the proposed oxidation mechanism of ipomeamarone (Fig. 1), that is, approximately eight equivalents of oxygen were consumed (Table II). Thus, the oxidized product is proven to be ipomeanic acid.

Radioactivity Measurement

Results of the radioactivity measurements of ipomeamarone-semicarbazone and the *S*-benzylthiuronium salt of ipomeanic acid are recorded in Table III. Comparisons of the specific radioactivities (cpm/μmole) of ipomeamarone and ipomeanic acid are important to speculate the mechanism of ipomeamarone biogenesis. The data show that when acetate-2-C^{14} was fed, the specific activity of ipomeanic acid decreased compared with that of ipomeamarone. It will be shown also that the ratio of the specific activity of two compounds is rather constant under different incubation conditions. However, when mevalonate-2-C^{14} was used as a substrate, the specific activity of ipomeanic acid was much the same as that of ipomeamarone in experiments 1 and 2; thus the ratio was more or less uniform. But in experiment 3, in which the root tissues of the 48-hour infection stage were incubated with the substrate for 15 hours, the ratio was rather declined. The reason for the latter case is not immediately known from this experiment only; however, the over-all results could be understandable in the light of the isoprenoid rule, because the C^{14}-labeling pattern of the two molecules based on this mechanism would be as illustrated in Fig. 1. Retention of C^{14} in ipomeanic acid was well in agreement with the experimental results, when mevalonate-2-C^{14} was used as a substrate; the loss of two C^{14} in ipomeanic acid, when acetate-2-C^{14} was fed, also gave theoretically close values.

Biosynthetic Mechanism of Ipomeamarone

A partial oxidative degradation, only giving rise to ipomeanic acid, may not be sufficient to reach a firm conclusion for the synthetic mechanism of ipomeamarone.

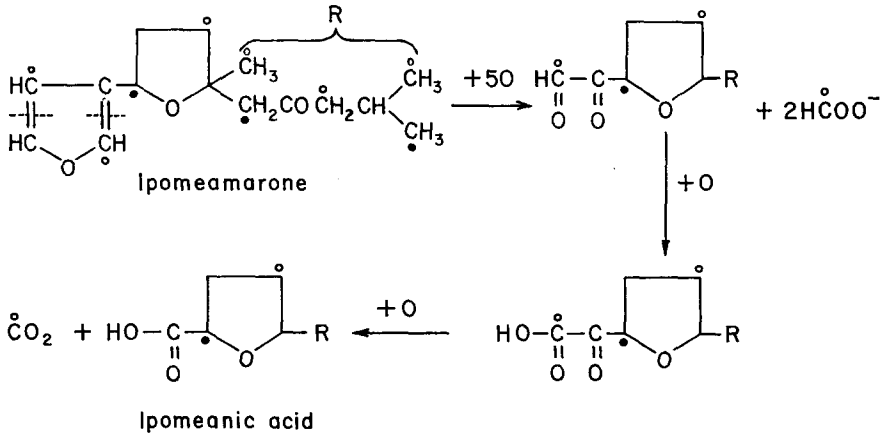


Fig. 1. Oxidative degradation of ipomeamarone to ipomeanic acid and their labelling pattern. Symbol (○) denotes the carbon atoms originated from acetate-2-C^{14} and (●) those from mevalonate-2-C^{14}; (◉) also shares those from acetate-2-C^{14}.

However, attempts to obtain any other small fragments like *iso*-valeric acid have been unsuccessful, and neither Kubota's group (4, 7) nor other workers (8, 9) who have undertaken the structural study of ngaione, an optical isomer of ipomeamarone, have succeeded along this line. Thus, based on the present experimental results, the biosynthetic mechanism of ipomeamarone is that illustrated in Fig. 2. As in the synthesis of other natural terpenoids (10–13), a stepwise condensation of acetyl-CoA to mevalonate (C_5), and the succeeding synthesis of farnesyl-pyrophosphate (C_{15}), via geranyl-pyrophosphate (C_{10}), will be presumed. As can be seen from the comparison of chemical structures between farnesyl-pyrophosphate (farnesol $C_{15}H_{26}O$) and ipomeamarone ($C_{15}H_{22}O_3$), at the final step of the formation of the latter compound

TABLE II

OXIDATION OF IPOMEAMARONE

Expt.	Ipomeamarone		$KMnO_4$[a]		
	Amount added		Consumed	Oxygen	
	mg.	μmole	ml.	matom	equivalent
1	91	0.36	15.0	2.85	7.9
2	101	0.40	15.0	2.85	7.1
3	142	0.57	25.0	4.75	8.3

[a] Two % $KMnO_4$ solution was used.

TABLE III

RADIOACTIVITY MEASUREMENT OF IPOMEAMARONE AND IPOMEANIC ACID

Expt.[a]	Compounds added	Ipomeamarone[b]		Ipomeanic acid[c]		Ratio
		cpm/mg.	cpm/μmole [A]	cpm/mg.	cpm/μmole [B]	[B]/[A]
1	Acetate-2-C^{14}	293	90	163	64	0.72
	Mevalonate-2-C^{14}	560	172	493	194	1.13
2	Acetate-2-C^{14}	2743	842	1462	577	0.69
	Mevalonate-2-C^{14}	762	234	546	216	0.92
3	Acetate-2-C^{14}	2437	748	1454	574	0.77
	Mevalonate-2-C^{14}	6445	1979	4218	1666	0.84

[a] Expts. 1, 2 and 3 as explained in Table I.

[b] Counted and expressed as per ipomeamarone-semicarbazone, and calculated 0.307 mg. of the semicarbazone as 1 μmole.

[c] Counted and expressed as per *S*-benzylthiuronium-ipomeanic acid, and calculated 0.395 mg. of the thiuronium salt as 1 μmole.

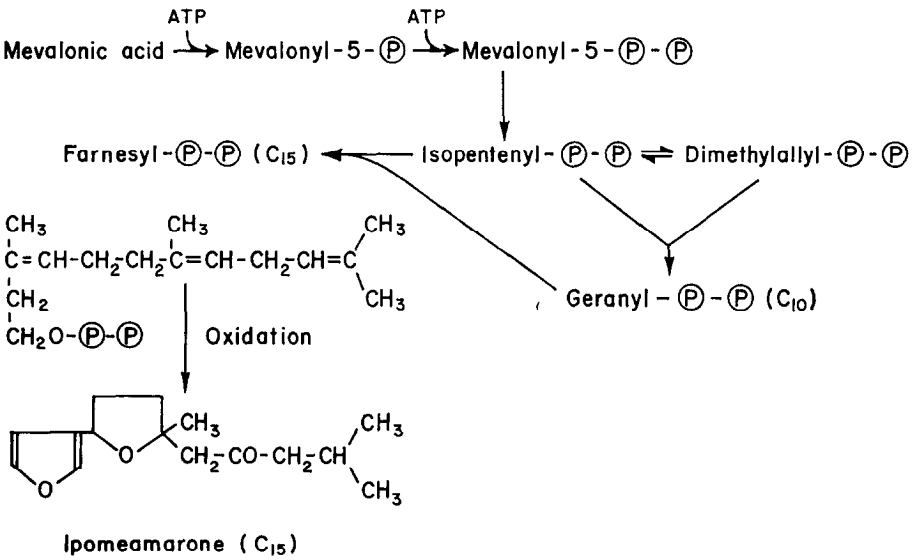
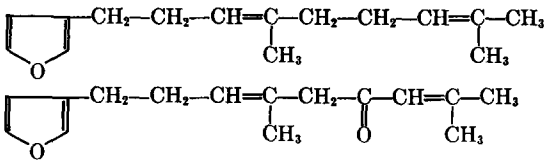


FIG. 2. Proposed scheme for the enzymatic mechanism of ipomeamarone biosynthesis.



(I)

(II)

some enzymatic oxidation reaction would occur. To confirm the role of geraniol or farnesol as precursors in the system, it would be worthwhile to examine the conversion of such C^{14}-labeled compounds to ipomeamarone, as has been undertaken by Varma and Chichester (14) in the biosynthesis of β-carotenoid. Another way of confirming this view is to investigate the occurrence of polyterpenoid precursors in the infected sweet potato root tissues. In an elegant work on the enzymatic synthesis of polyterpenoid compounds with yeast enzyme, Lynen and his associates (13) were able to detect the active form of terpenyl alcohol (presumably pyrophosphate) synthesized from isopentenyl pyrophosphate-C^{14}. Varma and Chichester (15) have also shown the incorporation of isopentenyl pyrophosphate-C^{14} into lycopene with cell-free homogenates of tomato. Thus, it is not unreasonable to predict that the black rot sweet potato root tissues, forming 20–30 mg. of ipomeamarone per gram fresh weight, may contain detectable amounts of such precursors.

Quilico *et al.* (16) have determined the chemical structure of dendrolasin (I), a furanoterpenoid pheromone of the ant, *Lasius fuliginosus*, and have discussed the isoprenoidal synthetic mechanism. The structures of ipomeamarone and dendrolasin are very similar, the former existing in a more oxidized form; hence, it is interesting to recall that Ogawa and Hirose (17) have isolated dendrolasin and 2,6-dimethyl-8-β-furfuryl-octanone-4 (II) from fusel oil of sweet potato root tissues, but so far we have not been able to detect these compounds[3] in such tissues infected with black rot.

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[3] Gifts of Dr. Y. Hirose.

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