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51. Yuzuru Yamamoto, Keiichi Nitta, and Akiko Jinbo : Studies on the Metabolic Products of a Strain of *Aspergillus fumigatus* (DH 413). III.*1 Biosynthesis of Toluquinones.

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The relationship among the metabolites of *Aspergillus fumigatus* DH 413 was studied by using ^{14}C-labelled compounds : ^{14}C-Labelled 2-methyl-5-methoxy-6-hydroxy-2,3-epoxy-*p*-benzoquinone (III) was well incorporated into fumigatin and fumigatin quinol, but the reverse reaction was not observed, and it is suggested that the epoxydation step should be involved in the biosynthesis of toluquinones.

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Biosynthesis of toluquinone pigments produced by *Aspergillus fumigatus* has been independently studied by various workers,[1~10] and it has become clear in the following points : 1) the toluquinone pigments are biosynthesized from acetate and malonate *via* orsellinic acid, 2) the carbon atom of methoxy group is originated from L-methionine, 3) conversion of fumigatin to spinulosin depends on the strain used, and 4) these metabolites exist in the reduced forms, and they change to the quinone forms by oxidation when culture becomes old or during extraction process artificially. It is summarized in Chart 1.

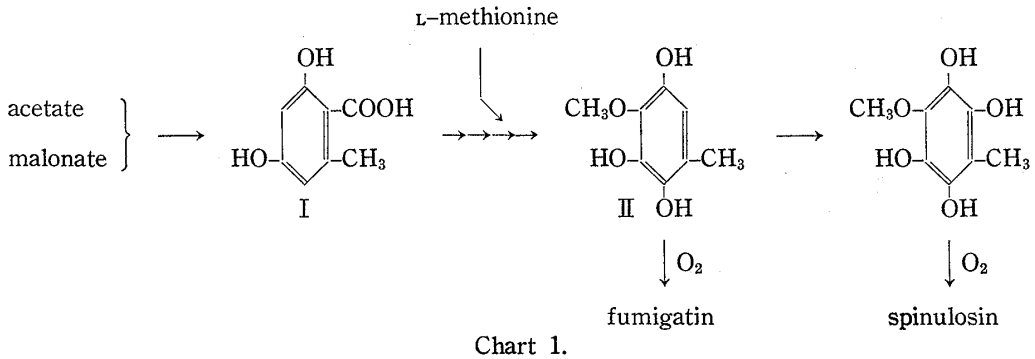

Chart 1.

However, the several steps from orsellinic acid (I) to fumigatin quinol (II) is still remained to be obscure.

Pettersson[6] isolated orcinol and toluquinone pigments from *Aspergillus fumigatus* A 46 and A 49, and presented the interpretation of the biosynthetic pathway by using ^{14}C-labelled metabolites. Although the incorporation of the labelled metabolites were

*1 Part II : This Bulletin, **13**, 1009 (1965)
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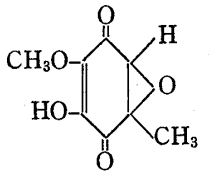
1) G. Pettersson : Acta Chem. Scand., **17**, 1323 (1963).
2) *Idem* : *Ibid.*, **17**, 1771 (1963).
3) *Idem* : *Ibid.*, **18**, 335 (1964).
4) *Idem* : *Ibid.*, **18**, 1202 (1964).
5) *Idem* : *Ibid.*, **18**, 1428 (1964).
6) *Idem* : *Ibid.*, **19**, 543 (1965).
7) Y. Yamamoto, *et al.* : This Bulletin, **13**, 935 (1965).
8) *Idem* : *Ibid.*, **13**, 1009 (1965).
9) N. M. Packter : Biochem. J., **97**, 321 (1965).
10) *Idem* : *Ibid.*, **98**, 353 (1966).

unexpectedly low, 3,4-dihydroxy-2,5-toluhydroquinone is suggested to be very possible intermediate between orsellinic acid and fumigatin quinol (or fumigatin), even though all the other biosynthetic routes cannot be excluded.

Recently Packter[9,10] also studied the biosynthesis of fumigatin in *Aspergillus fumigatus* I.M.I. 86353, and it was concluded that orcinol was very possible to be as an intermediate, but that 6-methylsalicylic acid or 3,4-dihydroxy-2,5-toluhydroquinone is not an actual intermediate.

Instead of the metabolites obtained by Pettersson from *Aspergillus fumigatus* A 46 and A 49, the authors[7,8] isolated 2-methyl-5-methoxy-6-hydroxy-2,3-epoxy-*p*-benzoquinone (Ⅲ) from *Aspergillus fumigatus* DH 413, and it was assumed to be a possible intermediate (in the reduced form) from the time course curve of the varieties of the metabolites (cf. Part Ⅱ of this series). In order to elucidate this assumption, the authors studied the incorporation of Ⅲ into fumigatin and fumigatin quinol by using ^{14}C-labelled compounds, and a reasonable pathway for the biosynthesis of toluquinone pigments is presented in the present paper.



Ⅲ

Experimental

Cultivation of *Aspergillus fumigatus* DH 413——The fungus was innoculated in 3L.-Fernbach flasks containing 800 ml. of malt extract medium (malt extract, 20 g.; anhyd. glucose, 20 g.; peptone, 1 g. and tap water, 1L.). It was cultivated stationarily at 27° for about two weeks. In every experiment with labelled compound, the cotton plug was changed to rubber stopper which carries two cotton-plugged glass tubes immediately after the addition of labelled compound. The gentle stream of CO_2-free air was introduced to one tube, and respiratory CO_2 was trapped in NaOH solution and subsequently collected as $BaCO_3$.

Preparation of Labelled Compounds——After 4-days' cultivation of the fungus about 100 μCi of CH_3-$^{14}COONa$ (purchased from Daiichi Chemical Co.) was added. Three biologically labelled metabolites were isolated after 10 days by the method described in Part I. Yields : the epoxy compound (Ⅲ), 25 mg.; fumigatin quinol (Ⅱ), 10 mg.; fumigatin, 40 mg. They were purified to constant specific radioactivity and were used for the incorporation experiments.

Incorporation Experiments of Labelled Compounds——Aqueous solutions of labelled Ⅲ, Ⅱ, and fumigatin were added to each of the 3~4 days' old culture. The cultures were harvested on the 11th day after the addition of labelled compounds, and the radioactivity of isolated metabolites and each fraction was assayed.

Assay of Radioactivity——The toluquinone or toluquinol pigments had a tendency to be sublimed on drying under the radiation of an infrared lamp, and so the pigments were placed evenly on stainless steel planchets with a few drops of dil. NaOH, dried and counted in a gas-flow counter (Kobe Kogyo Co., PR-123 series). The samples on the planchets were weighed and self-absorption was corrected to infinite thinness. The specific radioactivity and the amount of the metabolites were determined by the direct assay of the isolated compounds and/or by reverse dilution analysis (2~3 times). The crystalline compounds were purified to constant specific radioactivity. Radioactivity of the mycelium was measured in the form of $BaCO_3$ which was obtained by Van-Slyke-Folch oxidation.

Results and Discussion

^{14}C-Labelled epoxy compound (Ⅲ), fumigatin quinol (Ⅱ), and fumigatin which were obtained by feeding of $CH_3{}^{14}COONa$ to the fungus had nearly the same specific radioactivity (20,800, 20,600, and 21,700 c.p.m./mg., respectively) which showed that these metabolites might be originated in the common precursors (orsellinic acid, etc.).

In the three experiments administered, no radioactivity was incorporated into the respiratory carbon dioxide and mycelium, which meant these compounds added were converted to the other pigments as a unit without primary degradation. Fractionation methods are shown in Fig. 1, and the results are summarized in Table I.

The incorporation of epoxy compound (Ⅲ) to fumigatin quinol (Ⅱ) and fumigatin was found to be 4.7% and 10%, respectively (Experiment 1). In Experiment 2, fumigatin quinol (Ⅱ) added was converted to fumigatin in 26% extent, and 1.5% of the added

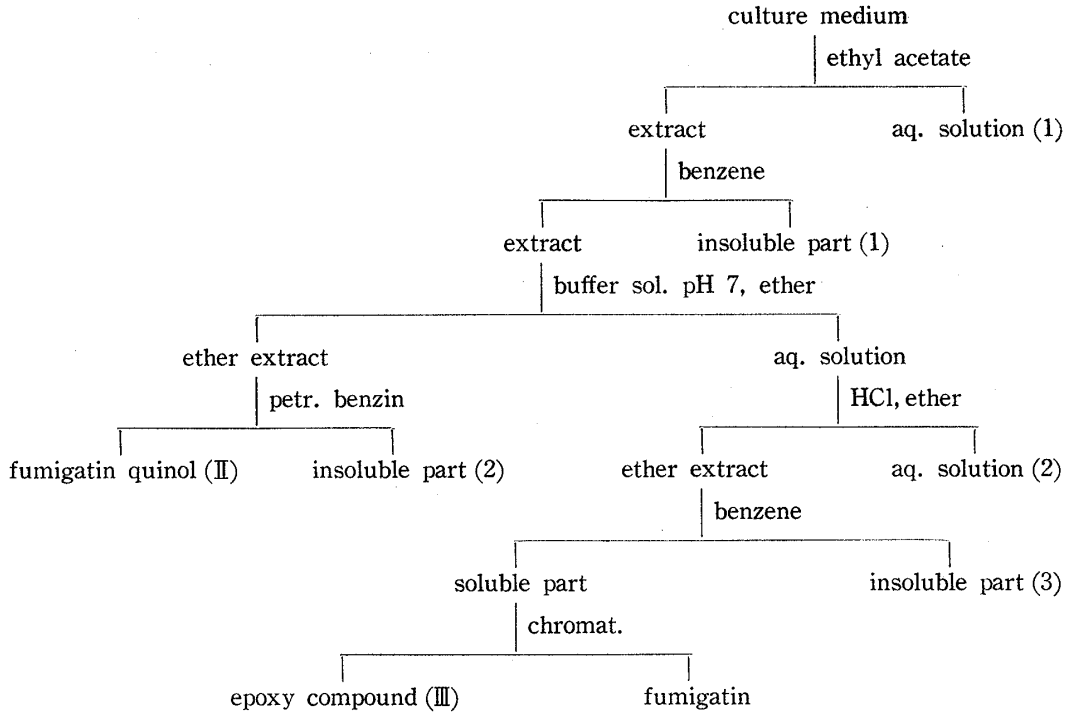


Fig. 1. Isolation of the Metabolites

TABLE I.

	Experiment 1 with epoxy compound (III)		Experiment 2 with fumigatin quinol (II)		Experiment 3 with fumigatin	
	Radioactivity (counts/min.)	Amount (mg.)	Radioactivity (counts/min.)	Amount (mg.)	Radioactivity (counts/min.)	Amount (mg.)
Amount added	30.0×10^4(100%)	14.4	14.4×10^4(100%)	7.0	15.0×10^4(100%)	6.9
Aq. solution (1)	11.0×10^4		6.6×10^4		7.5×10^4	
〃 (2)	1.3×10^4		0.7×10^4		1.0×10^4	
Aq. solutions (1)+(2)	12.3×10^4 (41%)		7.3×10^4 (51%)		8.5×10^4 (57%)	
Insoluble part (1)	5100		5900		2900	
〃 (2)	2500		11000		3000	
〃 (3)	26000		8000		6400	
Insoluble parts (1)+(2)+(3)	33600 (11%)		24900 (17%)		12300 (8.2%)	
Epoxy compound (III)	2300 (0.8%)	9.5	66 (0%)	2.4	90 (0%)	1.7
Fumigatin quinol (II)	14200 (4.7%)	41.5	2200 (1.5%)	10.0	3700 (2.5%)	50.0
Fumigatin	29900 (10%)	46.4	36800 (26%)	93.4	17500 (12%)	27.2

quinol was recovered. In Experiment 3 it is shown that fumigatin was converted to its quinol in 2.5% extent, and 12% of the added fumigatin was recovered unchanged. At least, a part of fumigatin obtained here might be produced from fumigatin quinol (II) by air oxidation during extraction, and so the incorporation ratios were similarly expressed to be the total sum of the two compounds. It was recognized that much radioactivity is rather present in the aqueous layer (see below) and also in the decomposed parts in all experiments. From these considerations the incorporation ratio of the epoxy compound (III) to fumigatin (plus fumigatin quinol (II) and further metabolites) was at a high level.

Experiments 2 and 3 showed that both fumigatin quinol (II) and fumigatin were not converted to the epoxy compound (III), whereas in Experiment 1 the epoxy compound

was well incorporated into fumigatin quinol and fumigatin. It means that the reaction between Ⅲ and others is irreversible, and that Ⅲ is a precursor of the two, but the reverse reaction is not possible.

Much radioactivity is observed to be remained in the aqueous layer in every experiment (41%, 51%, and 57% in Experiments 1, 2, and 3, respectively) in spite of the repeated extractions with ethyl acetate. This portion corresponds to Fractions I~Ⅲ which were reported in Part I. As the conversion of the epoxy compound to fumigatin quinol and/or fumigatin is irreversible, incorporation of radioactivity to the water soluble fractions in these three experiments suggests that these fractions are not produced from the epoxy compound (Ⅲ) directly, but yielded *via* either fumigatin quinol (Ⅱ) or fumigatin. Consequently, the metabolic sequence of [epoxy compound → fumigatin quinol and/or fumigatin → water soluble fractions] is most conceivable (cf. reference 9), p. 326).

The decomposed portions (insoluble portions) produced from the metabolites, during the isolation and purification processes, owing to the unstability, also indicated considerably higher radioactivity (11%, 17%, and 8.2% in each experiment).

Particularly the epoxy compound (Ⅲ) is so unstable in alkaline medium that it is decomposed to several pigments, one of which was identified to be spinulosin (cf. Part Ⅱ). On chromatography using basic solvent such as butanol-propanol-2*M* ammonium hydroxide (6:1:3 by volume, according to the method used by Pettersson), natural metabolites are not seemed to be obtained as stated below, so the authors carried out the isolation with other than alkaline medium to avoid decomposition.

When the pigment mixture obtained according to our isolation procedure was developed by paper-chromatography (Toyo filter paper, No. 51A, acetone-petr. benzin-water (2:2:1, upper layer) as the solvent), it was separated into several spots: Rf values, 0.66 (fumigatin), 0.53 (epoxy compound (Ⅲ)), 0.28 (fumigatin quinol (Ⅱ)), 0.16 (orange-yellow), 0.09 (orange-yellow), and 0.0 (orange-yellow), however, the pigments isolated by Pettersson were not recognized.

On the other hand, when the same pigment mixture was paper-chromatographed with the basic solvent, it gave the following spots: Rf values, 0.74 (red-violet), 0.68 (red-violet), 0.62 (brown), 0.60 (yellow), 0.53 (violet), 0.44 (red-violet), 0.30 (red-violet), 0.21 (red-violet), and 0.14 (violet). By thin-layer chromatography (on silicagel (Wakogel B-5)) of the above pigment mixture with the basic solvent, several spots were recognized: 0.75 (violet), 0.71 (yellow), 0.67 (red-violet), 0.52 (orange), 0.41 (violet), 0.20 (orange-violet), 0.11 (red-violet), and 0.0 (blue-violet) in Rf values (cf. foot note*[3]).

When the paper-chromatogram on which the pigment mixture had developed once in neutral solvent was subjected again to the separation by chromatography with the basic solvent, additional three more spots were newly appeared from the spot of epoxy compound (Ⅲ) and at least three different spots were appeared from the 0.16, 0.09, and 0.0 spots. The pure epoxy compound (Ⅲ) on paper-chromatogram with the basic solvent gave the following spots: 0.76 (red-violet), 0.68 (yellow), 0.53 (red-violet), 0.37 (spinulosin). As shown in the above chromatographic experiments, the pigments produced by *A. fumigatus* DH 413 were so sensitive to alkaline medium that it seemed impossible to obtain the natural metabolites with basic solvent. If *A. fumigatus* A 46 and A 49 produce the same metabolites as DH 413, it is considered that some of the metabolites isolated by Pettersson might be the degradation products formed during chromatographic separation.

*[3] The Rf values obtained by Pettersson[2] were 0.65 (violet), 0.45 (violet), 0.30 (violet), 0.25 (blue-violet), 0.19 (red-violet) on Whatmann No. 1 paper with the same basic solvent; 0.60 (violet), 0.52 (orange), 0.49 (violet), 0.30 (violet), 0.10 (red-violet), 0.0 (blue-violet) on thin-layer chromatography (silicagel) with the same solvent.

Now, it has become clear that the epoxy compound (Ⅲ) is incorporated into fumigatin quinol. As reported in Part Ⅱ, the epoxy compound is present in the reduced form in the culture, therefore, it is proper to assume that the reduced form is the natural intermediate from which fumigatin quinol is formed. In any case, it seems to be possible that the process of epoxydation is essential in the several steps between orsellinic acid and fumigatin quinol, and the step of epoxydation is considered to be taken place prior to the hydroxylation and O-methylation.

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