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Studies on the Metabolic Products of a Strain of *Aspergillus fumigatus* DH 413. VI.[1] Metabolic Position of 3,4-Dihydroxytoluquinone and Fumigatin Chlorohydrin in Fumigatin Biosynthesis

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3,4-Dihydroxytoluquinone and fumigatin chlorohydrin were isolated from a strain of *Aspergillus fumigatus* DH 413 and the biosynthetic position of these metabolites were discussed.

As reported in the previous papers, several matabolites which are related to the biosynthesis of toluquinones had been isolated from the culture medium of *Aspergillus fumigatus* DH 413, and the biosynthetic route of these compounds had been presented.[3,4] In the pathway, several steps between orsellinic acid and fumigatin oxide including O-methylation have remained unclear. The effort to isolate reasonable intermediates by inhibiting methylation with ethionine was failed, and an orsellinic acid derivative, 4-carboxy-5,5′-dihydroxy-3,3′-dimethyldiphenylether was isolated.[1]

This time, a new isolation procedure mainly using column chromatography was adopted, and 3,4-dihydroxytoluquinone (I) and fumigatin chlorohydrin (II) were obtained together with other usual metabolites.

Fumigatin chlorohydrin (II) had been isolated by us[4] from ethyl acetate-unextracted fraction of the culture medium by the isolation procedure including hydrochloric acid treatment. Since the chlorohydrin was formed easily from fumigatin oxide (III) by treatment with this acid, we considered this compound (II) to be an artifact formed from fumigatin oxide (III) during the isolation procedure. However, it was obtained by the isolation procedure using no hydrochloric acid, therefore, it becomes necessary to study its biogenetic behavior.

3,4-Dihydroxytoluquinone (I) which could not be isolated by previous isolation procedure is also a pending metabolite in fumigatin biosynthesis. Pettersson[5] proposed this compound as a possible natural precursor of fumigatin by incorporation experiments, but the conversion ratio was unexpectedly low (1.6% in the best). Simonat[6] also supported the Pettersson's scheme from the results of resting cell experiments. On the other hand, Packter[7] observed no incorporation from labeled methylsalicylic acid, orcinol, or orsellinic acid to this compound, and concluded that this compound is not an intermediate in fumigatin biosynthesis.

The culture broth of *Aspergillus fumigatus* DH 413 was extracted with ethyl acetate and the extract was dissolved in dichloromethane. Previously, phosphate buffer solution (pH 7) was used for further fractionation, but, this time, the dichloromethane solution was chromatographed on a column of silicagel which was treated with oxalic acid (see Experimental) to avoid decomposition of metabolites.

1) Part V: Y. Yamamoto, K. Nitta, Y. Oohata, and T. Furukawa, *Chem. Pharm. Bull.* (Tokyo), **20**, 931 (1972).
2) Location: *Takaramachi 13, Kanazawa, 920, Japan.*
3) Y. Yamamoto, K. Nitta, and A. Jinbo, *Chem. Pharm. Bull.* (Tokyo), **15**, 427 (1967).
4) Y. Yamamoto, M. Shinya, and Y. Oohata, *Chem. Pharm. Bull.* (Tokyo), **18**, 561 (1970).
5) G. Pettersson, *Acta. Chem. Scand.*, **17**, 1771 (1963); *idem, ibid.*, **19**, 543 (1965).
6) P. Simonat and H. Verachtert, *Bull. Soc. Chim. Biol.*, **49**, 543 (1967).
7) N.M. Packter, *Biochem. J.*, **97**, 321 (1965); *idem, ibid.*, **98**, 353 (1966).

On the chromatography, red (fumigatin, IV), orange (fumigatin oxide, III), deep red, yellow, and pale yellow (fumigatin quinol, V) zones were eluted successively with dichloromethane. From the deep red zone, purple crystals were obtained and purified by sublimation *in vacuum* as deep purple prisms, mp 157—159° (I) (yield, 30 mg from 6 liter broth). This compound was identified with 3,4-dihydroxytoluquinone as described below. Fumigatin chlorohydrin (II) was isolated from the following yellow fraction and crystallized from chloroform as colorless needles, mp 170—171°.

The empirical formula of the compound, mp 157—159° (I) was determined as $C_7H_6O_4$ from the results of elementary analysis and mass spectroscopy. The infrared (IR) spectrum showed the compound was a three substituted hydroxybenzoquinone derivative (3350, 1695, 1625, and 880 cm^{-1}). It was positive for the Craven test (blue), and methoxy group was absent. It was unstable in aqueous solution of pH above 7. When measured immediately after dissolving in dilute sodium hydroxide solution, this compound exhibited absorption at 440 mμ which was different from absorptions of 3,6-, mp 179° (536 mμ)[8] and 4,6-dihydroxytoluquinone, mp 160° (583 mμ).[9] In ethanol solution the compound (I) showed maximum absorptions at 264 mμ (log ε, 4.13) and 480 mμ (log ε, 3.10). These absorptions were almost coincident with the data described by Packter[8] (262 mμ, log ε, 4.04; 475 mμ, log ε, 2.81), but not with Pettersson's report[10] (279 and 385 mμ). Final confirmation was performed by comparison with the sample derived from fumigatin quinol (V) by demethylation.

Packter[7] and Pettersson[10] obtained 3,4-dihydroxytoluquinone (I) by treating fumigatin quinol with hydriodic acid and red phosphorus in the yield of 5 and 10—30%, respectively, but in our own experiment it was obtained only a trace amount. As it is necessary to prepare some amount of this compound for tracer experiments, more efficient methods were investigated. When hydrobromic acid was used instead of hydriodic acid, the yield increased to 15% (30 mg from 200 mg of V). It was further advanced to 45% (81 mg from 200 mg of V) by refluxing the quinol with anhydrous aluminum bromide in benzene, and ^{14}C-labeled 3,4-dihydroxytoluquinone was prepared by this procedure. Fumigatin (IV) itself was not converted to this quinone by this method.

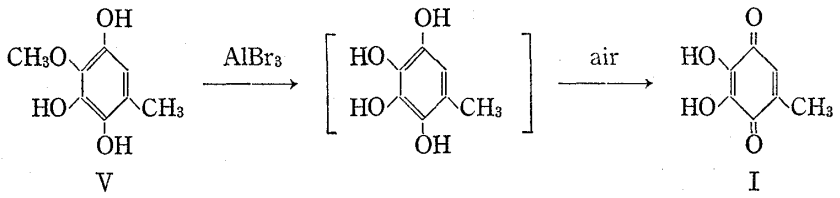

Chart 1

Using the labeled compound, the metabolism of 3,4-dihydroxytoluquinone was examined. The labeled compound was added to the medium of *Aspergillus fumigatus* DH 413 on the 4th day of cultivation and growth was continued for further 10 days. The culture broth was extracted with ethyl acetate and its dichloromethane soluble pigments were chromatographed on oxalic acid treated silicagel column. The unextracted aqueous fraction was divided into three fractions by ion exchange chromatography as described in Part IV. The radioactivity of administered 3,4-dihydroxytoluquinone (346×10^4 dpm, 20 mg) was incorporated into fumigatin oxide (III) (3.5%), fumigatin (IV) and its quinol (V) (total, 7.0%), 191° compound (VI) (26.6%), 182° compound (VII) (1.9%), and the fraction 3 (19.3%) together with recovered 3,4-dihydroxytoluquinone (1.3%) and decomposed parts (23%). This is normal incorporation pattern in this fungus (*cf.* Part IV), so it is certain that 3,4-dihydroxytoluquinone (I) is an intermediate in fumigatin (toluquinone) biosynthesis.

8) J.F. Corbett and A.G. Fooks, *J. Chem. Soc.* (*C*), **1967**, 1909.
9) J.F. Corbett, *J. Chem. Soc.* (*C*), **1967**, 2408.
10) G. Pettersson, *Acta Chem. Scand.*, **17**, 1323 (1963).

Then, biosynthetic position of 3,4-dihydroxytoluquinone was examined. When ^{14}C-labeled fumigatin oxide was added to the fungus, it was little converted to 3,4-dihydroxytoluquinone (0.1—0.2%). This value was very small and not out of experimental error. To make sure this point, washed cell method was also used.

When 3,4-dihydroxytoluquinone was added to the washed cell, fumigatin oxide (III) and fumigatin quinol (V) were detected on thin-layer chromatogram (TLC) after 2 and 4 days' incubation at 27°. On the other hand, 3,4-dihydroxytoluquinone (I) was not detected in the case when fumigatin oxide (III) or fumigatin quinol (V) was added.

From these results, it was determined 3,4-dihydroxytoluquinone (I) is an intermediate (probably as quinol form) and situated before fumigatin oxide (III) in fumigatin biosynthesis.

Next, the metabolic position of fumigatin chlorohydrin (II) was investigated. This compound was appeared in the medium on the 8th day of cultivation when the yield of fumigatin oxide (III) reached maximum amount, and the yield of the chlorohydrin increased rapidly until the 14th day and then gradually as shown in Fig. 1. It suggested this compound (II) is closely related to fumigatin oxide (III). When this fungus was cultivated on a medium replaced sodium chloride by sodium bromide, the corresponding bromohydrin, mp 154° (VIII) was isolated instead of the chlorohydrin.

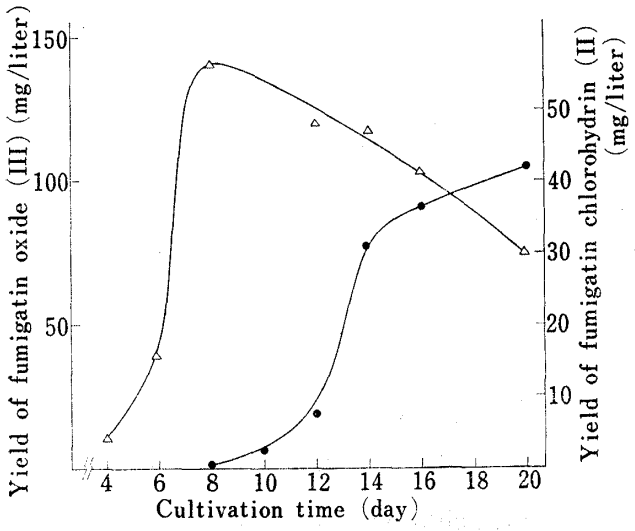

Fig. 1.

—●—: yield of fumigatin chlorohydrin (II)
—△—: yield of fumigatin oxide (III)

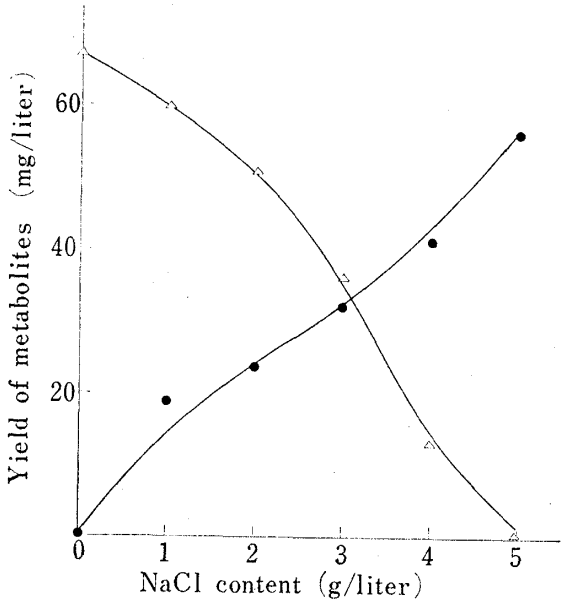

Fig. 2.

—●—: yield of fumigatin chlorohydrin (II)
—△—: yield of fumigatin oxide (III)

The yield of fumigatin oxide (III) and fumigatin chlorohydrin (II) were compared at various sodium chloride concentration in the culture medium. As shown in Fig. 2 the yield of fumigatin oxide (III) was the highest when sodium chloride was absent, and decreased linearly to zero at the concentration of 5 g per liter. The yield of the chlorohydrin (II) was zero at no addition of sodium chloride, and increased paralleling with the sodium chloride concentration.

The radioactivity of fumigatin oxide (III) was incorporated to the chlorohydrin to the extent of 6.2% when 3 g per liter of sodium chloride was present in the culture medium. The washed cell experiments also supported this results. Fumigatin oxide (III) changed to the chlorohydrin (II) in existence of sodium chloride, but did not in sodium chloride free medium. Otherwise, the chlorohydrin (II) was changed to fumigatin oxide (III) in sodium chloride free medium but not in the containing medium.

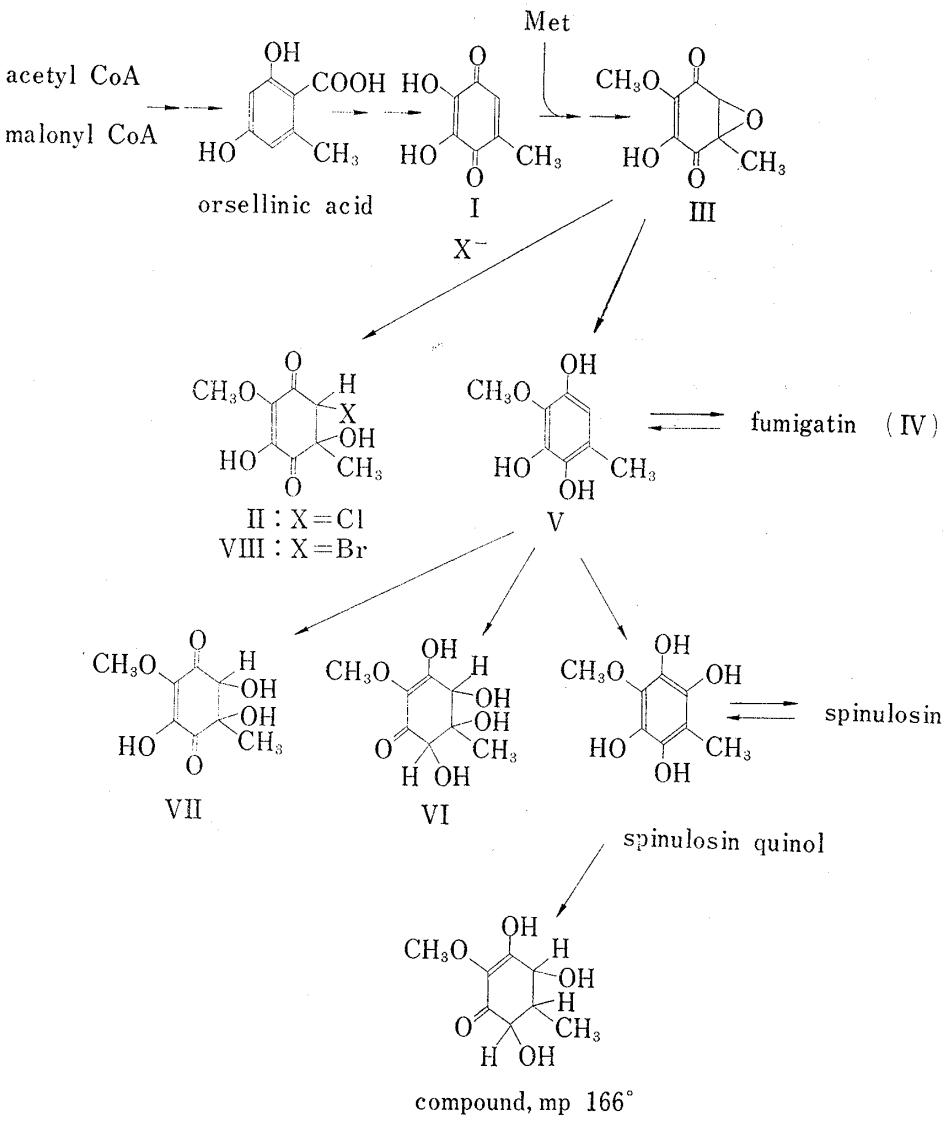

Chart 2. Biosynthetic Map around Toluquinones in *Aspergillus fumigatus*

From these results, it was concluded that fumigatin chlorohydrin (II) was a special kind of metabolite derived from fumigatin oxide (III) only when sodium chloride was present in the culture medium.

Finally, the biosynthetic pathway of toluquinones was reinforced as shown in Chart 2.

Experimental[11]

Cultivation of the Fungus——*Aspergillus fumigatus* DH 413 was cultivated in Roux flasks at 27° for 14 days using the following medium: malt extract, 20 g; glucose, 20 g; polypeptone, 1 g; NaCl, 1 g in 1000 ml of tap water.

Isolation of Metabolites——The culture broth was extracted with ethyl acetate and the solvent was evaporated under reduced pressure to dryness. The extract was dissolved in CH_2Cl_2 and was chromatographed on oxalic acid treated silicagel[12] column (3×30 cm) and eluted with the same solvent. It was separated in the order of fumigatin (IV), fumigatin oxide (III), 3,4-dihydroxytoluquinone (I), fumigatin chlorohydrin (II), and fumigatin quinol (V). 3,4-Dihydroxytoluquinone was crystallized from the mixture of benzene and cyclohexane and purified by sublimation as deep purple prisms, mp 157—159°. *Anal.* Calcd.

11) All mp were not corrected.

12) Oxalic acid treated silicagel: Silicagel (for chromatography, Kanto Chemical Co.) was suspended in 0.1M oxalic acid overnight, filtered, and washed with H_2O and dried in a oven at 100°.

for $C_7H_6O_4$: C, 54.55; H, 3.92. Found: C, 54.69; 55.00, H, 4.04, 4.02. Mass Spectrum *m/e*: 154 (M^+), 126, 98, 80. Fumigatin chlorohydrin was crystallized from $CHCl_3$ as colorless needles, mp 170—171°. *Anal.* Calcd. for $C_8H_9O_5Cl$: C, 43.55; H, 4.11. Found: C, 43.46; H, 4.02. NMR (in $CDCl_3$) τ: 8.67 (CH_3), 6.20 (OCH_3), 5.15 (CH), 3.55 (OH), −0.78 (OH).

Synthesis of 3,4-Dihydroxytoluquinone (I)——The mixture of fumigatin quinol (200 mg) and anhydrous $AlBr_3$ (1.0 g) in 10 ml of dried benzene was refluxed for 5 hr under leading off evolved bromomethane. The reaction mixture was treated with dilute HCl and extracted with ether. To the ether solution 0.1M phosphate buffer solution (pH 6.5) was added and shaken for oxidation. The mixture was acidified and extracted with ether several times. The red ether solution was concentrated and chromatographed on a oxalic acid treated silicagel column with CH_2Cl_2 as the eluting solvent. The red fraction of 3,4-dihydroxytoluquinone was concentrated and purified by sublimation as deep purple prisms, mp 157—159°. It was identified with the isolated metabolite (I) by mixed mp, TLC, and comparison of IR.

Administration of ^{14}C-Compound and Determination of Radioactivity——Several millions dpm of each labeled metabolite was added to the medium on the 4th day and further cultivated for 10 days. Radioactivity of isolated metabolites was determined in a liquid scintillation spectrometer (Packard, Model 3320) using dioxane scintillator. The color of quinones was decolorized with ascorbic acid before measurement.

Washed Cell Experiments——The cultivated mycelium of 7 days old was washed several times with 0.1M phosphate buffer solution (pH 3.5) and kept at 27° for several days with the testing substance (40 mg) in 200 ml of the same buffer solution in Roux flasks. The solution was extracted with ethyl acetate and concentrated. The extract was examined on TLC (Silicagel G treated with oxalic acid,[13] ethyl acetate–benzene, 2: 1 as the solvent). The spots were detected by treatment with ammonia vapor.

Fumigatin Bromohydrin——The fungus was cultivated on the medium of the following composition: malt extract, 30 g; glucose, 30 g; K_2HPO_4, 1 g; NaBr, 1 g; $MgSO_4 \cdot 7H_2O$, 0.01 g in 1 liter of distilled water, for 14 days at 27°. The culture broth was treated by the usual method and separated on chromatography. The bromohydrin (VIII) was crystallized from the mixture of acetone–CH_2Cl_2 as colorless needles, mp 154° (yield, 20 mg from 6 liter broth). *Anal.* Calcd. for $C_8H_9O_5Br$: C, 36.25; H, 3.42. Found: C, 36.73; H, 3.51. Mass Spectrum *m/e*: (M^+), 264, 266. UV λ_{max}^{EtOH} mμ: 240, 315. $[\alpha]_D^{20°} = -95°$ (c=0.78, H_2O). IR (KBr) cm^{-1}: 3425, 3225, 1695, 1665, 1620.

13) The TLC plates were prepared with Silicagel G (Merck) and 0.1M oxalic acid instead of H_2O.