Note

[Chem. Pharm. Bull. 13(8)1009~1011(1965)]

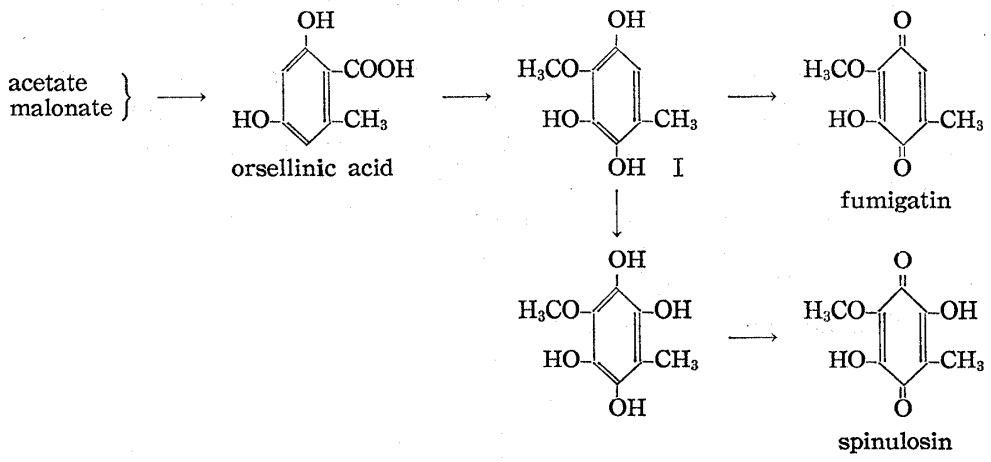
UDC 581.13 : 582.282.12 : 547.97

Yuzuru Yamamoto, Keiichi Nitta, Yoshie Terashima, Jitsukazu Ishikawa, and Nobuo Watanabe: Studies on the Metabolic Products of a Strain of *Aspergillus fumigatus* (DH 413). II.*1 Biosynthesis of Metabolites.

(*Faculty of Pharmacy, Kanazawa University**2)

In the preceding paper, the authors reported on the isolation methods and the chemical structures of metabolites of a strain of *Aspergillus fumigatus* (DH 413) (compounds, m.p. 74°, 182°, 204°, spinulosin, fumigatin and its quinol). These metabolites consisted of C_8-unit except compound, m.p. 204° and belonged to toluquinone pigments.

Biosynthesis of toluquinones has been studied by G. Pettersson[1~3] by using *Asp. fumigatus* Fresenius A 46 and A 49, and he proposed the biosynthetic route as shown



in the above scheme. In this route, several steps from orsellinic acid to quinol (I) are unknown. So it was particularly interested for the authors to study the biosynthetic situation of the compound, m.p. 74° (II) (2-methyl-5-methoxy-6-hydroxy-*p*-benzoquinone 2,3-epoxide) in toluquinone biosynthesis.

The fungus (DH 413) was cultivated on the same medium as described in Part I, and each 6 L. of 3 to 20 days' cultivation broth was used for determination of the yield of metabolites. The culture filtrate was extracted with ethyl acetate and the metabolites were isolated as shown in Fig. 2 of Part I. In this time, with careful treatment of smaller amount

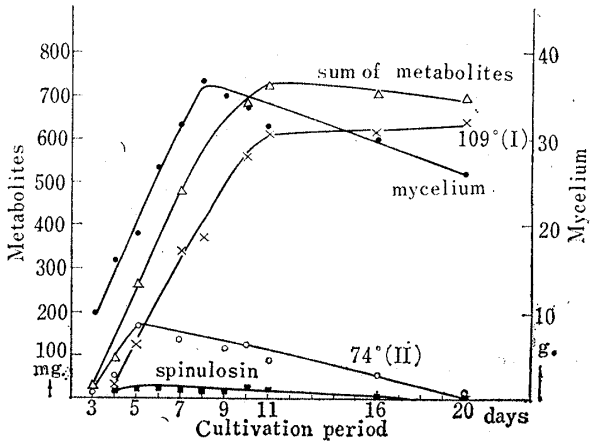


Fig. 1. Time Course Curve of the Variation of the Metabolites

*1 Part I: This Bulletin, **13**, 935 (1965).

*2 Takaramachi 13, Kanazawa (山本 譲, 新田啓一, 寺島佳江, 石川実計, 渡辺信郎).

1) G. Pettersson: Acta Chem. Scand., **17**, 1323 (1963).

2) *Idem*: *Ibid.*, **18**, 335 (1964).

3) *Idem*: *Ibid*,, **18**, 1202 (1964).

of the broth, the decomposition of the metabolites could be rendered less, and unstable fumigatin was obtained together with other metabolites. The results from each two experiments are shown in Fig. 1.

The amount of mycelium reached to peak on about 8th day of cultivation, and thereafter decreased gradually. The sum of metabolites began to increase remarkably after 8th day and maintained the constant amount after 11th day.

The absorption at 430 mμ to which fumigatin (λ_{max}, 270, 430 mμ at pH 3.0) was responsible did not appear in the broth until 19 days' cultivation. And when fumigatin was added to the 5, 6, 7 or 8th day's culture, the decoloration occurred within 24 hours. So fumigatin was absent in the broth, or at least, its yield was very little until 19th day. Pettersson[4,5] reported that reduced form (hydroquinone) was predominant during first 14 days in A. 46. So the majority of fumigatin isolated from DH 413 was supposed to be an artificial product which was produced on the isolation course.

Quinol of fumigatin, m.p. 109° (I) was appeared on the 4th day and the yield increased rapidly as far as 11th day and kept the amounts thereafter, which showed it was a final metabolite.

The yield of fumigatin became considerable amount from 8th day on, which seemed to suggest the relationships to I.

Compound, m.p. 74° (II) was appeared on the 3rd day and gave the maximum yield on the 5~6th day and thereafter it began to decrease gradually until it could not be isolated on the 20th day of cultivation. This phenomena meant this compound was an intermediate that was produced in the early stage.

Spinulosin was obtained from the culture of 4th to 11th day, but the yield was constantly small and seemed to depend on the amount of II. And it was not given in careful and mild conditions (Fig. 3 in Part I). Moreover, the ultraviolet-spectrum of the broth (215, 275, 370 mμ) had no absortion of spinulosin (225, 272, 535 mμ) and it was changed with ease from II, *i.e.* by alkali treatment (cf. Part I). From these results, it was probable that spinulosin did not exist in the broth, but it was derived from II artificially during isolation.

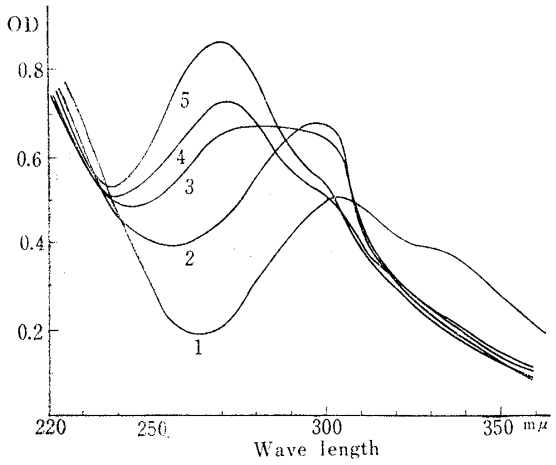


Fig. 2. Change of the Ultraviolet Spectrum of the Compound, m.p. 74° (II) by Incubation with the Washed Mycelium

curve 1: control, II (8 mg.) in buffer pH 3.2 (20 ml.)
curve 2: II (40 mg.) in the buffer (100 ml.) with washed mycelium. 0 hr.
curve 3: 24 hr. curve 4: 48 hr.
curve 5: 96 hr.

Another compound, m.p. 204° which was not C_8- but probably C_{18}-compound, was obtained only in small yield (usually 0~7 mg.), but whenever the yield of II was very small (17 mg.) or null, it could be isolated in fairly good yield (58 and 53 mg. from 6 L. broth of 7th and 10th day's culture, respectively). Hence, it might be an artifact which was produced from II as well as spinulosin.

Although II was obtained from early culture and was attractive as an intermediate of benzoquinone biosynthesis, it was very speculative to treat it as an intact metabolite: In the buffer solution (pH 3.2) suspended with the washed mycelium prepared from 5th day's culture (at which time II was most prosperous in the extraction

4) G. Pettersson: Acta Chem. Scand., **18**, 1428 (1964).
5) *Idem*: *Ibid.*, **18**, 1839 (1964).

experiment, and the pH of the culture medium was 3.2) added II was changed into an unknown compound (λ_{max} 270 mμ), while in the 5th day's culture broth without mycelium the ultraviolet spectrum of added II was not changed throughout 7 days.

From these experiments II was not able to exist in culture medium owing to the reducing system in this fungus and might exist in the reduced form which changed to II. The investigation on this intact compound is under proceeding.

In order to research earlier metabolites, 2.5 days' cultivation (when a slight peak at 260 mμ appeared for the first time) was examined : From the culture broth colorless needles, m.p. 177° were obtained which was identified with orsellinic acid by mixed melting point and infrared spectra. But 6-methylsalicylic acid was not obtained.

In conclusion, the biosynthetic pathway of toluquinones in *Asp. fumigatus* DH 413 is likely to proceed on the route shown in the scheme, but it has no ability to form spinulosin.

Epoxy compound (II) which was newly isolated, seemes to be a significant intermediate (in the reduced form : 270 mμ compound) between orcellinic acid and quinol (I), so the study of this point will be reported in the future.

Spinulosin and compound, m.p. 204° are artificial products from II.

Experimental

Isolation of Metabolites——The isolation was carried out as described in Fig. 2 in Part I with some modifications. The AcOEt extract was dissolved in ether and extracted with buffer solution (pH 7.0). The buffer layer containing compound, m.p. 74° (II), fumigatin, and compound, m.p. 204° was acidified and extracted with ether. Ether extract was treated with benzene and soluble part was chromatographed on silicagel. Fumigatin and II were eluted with benzene and evaporated, then the residue was treated with ether and buffer solution (pH 3.8). Fumigatin was obtained from orange-red ether layer and II was isolated from buffer solution.

Treatment of Compound 74° (II) with Washed Mycelium——The fungus was cultivated on 100 ml. of the described medium in 200 ml.-Erlenmyer flasks for 5 days at 27°. The culture broth was decanted out and the remained mycelium was washed with buffer solution (pH 3.2, 0.1*M* H_3PO_4, 400 ml. +0.1*M* NaOH, 275 ml.), then 100 ml. of the same buffer solution and 40 mg. of II were added. Into the decanted broth, II was also added. Both were stood at 27°, and checked by UV-spectra and paper chromatography (acetone-petr. benzin-H_2O=2:2:1, upper layer) in every 24 hr. (see Fig. 2).

After removing mycelium, the reacted buffer solution was extracted with 200 ml. of AcOEt and evaporated. The reddish residue was treated with hot petr. benzin and obtained orange-red crystals which were identified with II (yield, 5 mg.). But main part of the 270 mμ compound still remained in aqueous solution.

Isolation of Orsellinic Acid——The culture filtrate (6 L.) of 2.5 days' cultivation was extracted with AcOEt and evaporated. The residue was treated with ether and the extract was refluxed with petr. benzin. The insoluble part was dissolved in ether and shaken with 10% $NaHCO_3$. The aqueous solution (slightly violet) was acidified with H_2SO_4 and extracted with ether and fractionated by silicagel chromatography, or more conveniently by thin-layer chromatography (silicagel, acetone-petr. benzin-H_2O=2:2:1, upper layer). There were several bands of which yellow band had the fluorescence under UV light. The yellow band was collected and extracted with ether. The solvent was evaporated and the residue was crystallized from benzene as slightly red needles and recrystallized from benzene as colorless needles, m.p. 176~177°, yield, 10 mg. *Anal.* Calcd. for $C_8H_8O_4$: C, 57.14; H, 4.80; mol. wt. 168.1. Found : C, 57.18; H, 4.98; mol. wt. (Rast), 168.1, 156.3. UV λ_{max} mμ 262, 298 (in EtOH).

The authors thank Mr. Y. Itatani for his elementary analyses and help for molecular weight determination. A part of the experiments was performed by Misses K. Sekimoto, E. Hayashi and A. Nagabayashi who were students in this Faculty. This work had been supported in part by the Grant-in-Aid from the Ministry of Education to which they express their gratitudes.

Summary

The relationships among the metabolites from a strain of *Asp. fumigatus* (DH 413) were discussed. Spinulosin and compound, m.p. 204° were not metabolites, but secondary compounds converted from epoxy compound (II) which existed in its reduced form. In the biosynthetic pathway of toluquinone the reduced form of II seems to be an intermediate placed between orsellinic acid and quinol of fumigatin (I).

(Received March 17, 1965)