[Chem. Pharm. Bull. 20(5) 931—935 (1972)]

UDC 547.63.02 : 581.192

Studies on the Metabolic Products of a Strain of *Aspergillus fumigatus* DH 413.[1] V.[2] A New Metabolite produced by Ethionine Inhibition

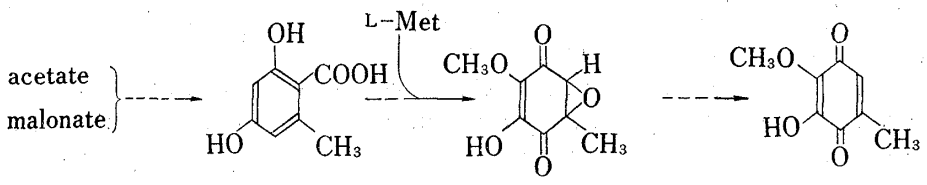
Yuzuru Yamamoto, Keiichi Nitta, Yasuharu Oohata, and Toshiko Furukawa

Faculty of Pharmaceutical Sciences, Kanazawa University[3]

(Received September 6, 1971)

A new metabolite (I) was isolated from the ethionine inhibited culture fluid of *Aspergillus fumigatus* DH 413, without accompanying the usual toluquinone derivatives such as fumigatin and spinulosin. The chemical structure of the new metabolite was established as 4-carboxy-5,5′-dihydroxy-3,3′-dimethyl-diphenylether based on the chemical degradations and physical properties.

In the previous papers, it was reported the biogenetic pathway to the toluquinone pigments produced by *Aspergillus fumigatus* DH 413 as shown in Chart 1. In the pathway, the step from orsellinic acid to fumigatin contains several chemical processes such as hydroxylation, decarboxylation, epoxidation, methylation of hydroxy group, and oxidation to quinone. However, there are still few experimental evidences on these processes.



orsellinic acid fumigatin epoxide fumigatin

Chart 1

The methyl moiety in the methoxy group of the toluquinone pigments, as elucidated by Pettersson,[4] derives from C_1-unit such as L-methionine. Therefore, if the methylation step is inhibited with methionine antagonist such as ethionine, it is possible to obtain the new intermediate in toluquinone biosynthesis.

When DL-ethionine was added to the culture medium, neither desired intermediates nor the usual toluquinone metabolites were obtained, but a new metabolite (I) was accumulated in the culture medium. In this paper, the studies on the new metabolite are described.

The cultural conditions were the same as described in the previous papers, and 150 mg per liter of ethionine was added to the culture. When ethionine was administered on the first day of cultivation, the growth of the fungus was depressed, and 9 days' cultivation was required until all the surface of the medium was covered with mycelium, and the yield of dried mycelium (3 g/liter on the 20th day) was decreased (normal case, 5—7 g/liter on the 14th day). The formation of green spores, which is a characteristic of this strain, was also inhibited, and the color of the mycelium was slightly brown even on the harvest day (20th day).

1) A part of this work was presented at 91st Annual Meeting of the Pharmaceutical Society of Japan, Fukuoka, April, 1971.
2) Part IV: Y. Yamamoto, M. Shinya, and Y. Oohata, *Chem. Pharm. Bull.* (Tokyo), **18**, 561 (1970).
3) Location: *Takaramachi 13, Kanazawa, 920, Japan.*
4) G. Pettersson, *Acta Chem. Scand.*, **17**, 1323 (1963).

The broth of the inhibited culture was extracted with ethyl acetate, and the extract was applied on a silica gel column, and eluted with benzene and ether successively. The new metabolite (I) was eluted as a slightly yellow solution with benzene, and red unknown pigments and orsellinic acid were eluted with ether. The yield of the compound (I) was 80—100 mg per liter of the culture, but no toluquinone pigments were isolated.

When ethionine was added on the 4th day of cultivation, the yield of the compound (I) was reduced to 40 mg per liter, and the growth of the mycelium was slightly recovered (3.6 g). When ethionine was added on the 11th day, the fungus grew almost normally as the intact culture. The compound (I) was not isolated, but 75 mg of fumigatin was isolated (100 mg from the intact culture).

These results showed ethionine was very effective in the early stage, but not so effective in the later stage.

The compound (I) was crystallized from benzene as colorless prisms, mp 194° (decomp.). It was optically inactive, and the chemical formula, $C_{15}H_{14}O_5$ was assigned by elementary analysis and mass spectrometry (M^+, *m/e* 274). It showed positive reaction for phenol with ferric chloride (violet), Gibbs reagent (green), and Millon reagent (red). The infrared (IR) spectrum of the compound (I) showed the absorptions at 3400 (OH), 1620 (chelated C=O), and 2600—2800 cm^{-1} (COOH), and the ultraviolet (UV) spectrum was very similar to that of hydroxyphenoxybenzoic acids.[5] The nuclear magnetic resonance (NMR) spectrum showed the signals at τ 7.75 (3H), 7.48 (3H), 3.5—4.0 (6H), and 0.47 (2H), among which the signals at τ 4.0 (1H) and 0.47 (2H) disappeared on addition of deuterium oxide.

On methylation with diazomethane, the compound (I) gave a monomethyl ester (II), $C_{16}H_{16}O_5$ (M^+, *m/e* 288), as colorless prisms, mp 113.5—114°, and the methylation of the compound (I) with dimethyl sulfate gave a dimethyl ether (III), $C_{17}H_{18}O_5$, as colorless prisms, mp 126° (decomp.). The IR spectrum of III showed no absorption around 3400 cm^{-1} to show all the hydroxy groups were methylated, and the carbonyl absorption shifted to 1680 cm^{-1}. The shift meant that the carboxyl group of the compound (I) was chelating with an adjacent hydroxy group.

The NMR spectrum of the acetate of II also showed the presence of two hydroxy groups.

Potassium fusion of the compound (I) was attempted to identify the remained oxygen atoms, but the compound (I) was resistant to such a drastic condition. From this result, the absence of methoxy and ester groups[6] was determined, and the presence of a diphenyl ether linkage was suggested, although the compound (I) gave no xanthone derivative by treating with concentrated sulfuric acid[6,7] or hydroiodic acid.[8]

On treating with bromine water, the compound (I) gave a hexabromo compound (IV), $C_{14}H_8O_3Br_6$, as yellow prisms, mp 204—205°. As suggested by the chemical formula, the carboxyl group of the compound (I) was lost during the bromination and replaced by a bromine atom. The NMR spectrum of IV showed each sharp singlet due to two hydroxy and two methyl groups to suggest a symmetrical structure for IV.

The methyl ester (II) was brominated without decarboxylation to a pentabromide, $C_{16}H_{11}O_5Br_5$, mp 181°.

These results showed the compound (I) had five unsubstituted positions at *ortho*- or *para*-position to the two hydroxy groups. Hence, each of the two hydroxy groups was located in the different benzene ring separately. Further, it was also conceivable the two methyl groups were located in each benzene ring separately, occupying *meta*-positions to the hydroxy

5) H.E. Ungnade, E.E. Pickett, L. Rubin, and E. Youse, *J. Org. Chem.*, **16**, 1318 (1951); S. Natori and H. Nishikawa, *Chem. Pharm. Bull.* (Tokyo), **10**, 117 (1962).
6) H. Nishikawa, *Bull. Agr. Chem. Soc. Japan*, **18**, 13 (1942).
7) S. Archer, *J. Am. Chem. Soc.*, **76**, 588 (1954); A.A. Goldberg and H.A. Walker, *J. Chem. Soc.*, **1953**, 1348; C.H. Hassall and T.C. McMorris, *ibid.*, **1959**, 2831.
8) R.F. Curtis, C.H. Hassall, D.W. Jones, and W.W. Williams, *J. Chem. Sco.*, **1960**, 4838.

groups. The fact that the carboxyl group was easily removed by bromination also showed the carboxyl group was situated at the *ortho*- or *para*-position to one of the hydroxy groups.[9]

The compound (I) was easily decarboxylated on boiling with 85% phosphoric acid under the nitrogen atmosphere to a colorless oily compound (V), which afforded hexabromide (IV) by treating with bromine water.

Then, the cleavage of the diphenyl ether linkage with sodium in liquid ammonia[10] was attempted. Although the compound (I) and its methyl ester (II) were not affected, the decarboxylated compound (V) was smoothly cleaved. The resulted phenol fraction was purified on thin-layer chromatography (TLC), and orcinol was obtained as colorless prisms, mp 103°. The phenol fraction was also treated with excess bromine water, and the resulted yellow precipitates were fractionated with petroleum benzin. Pentabromorcinol was obtained as yellow prisms, mp 126° from the soluble part, and 2,4,6-tribromo-*m*-cresol was isolated as yellow needles from the less soluble part.

From these results, V was confirmed to be a derivative of diphenyl ether which was composed of *m*-cresol and orcinol.

The oxidative cleavage of the compound (I) with hydrogen peroxide was unsuccessful.

On permanganate oxidation, dimethyl ether (III) gave tricarboxylic acid (VI), $C_{17}H_{14}O_9$ as colorless prisms, mp 215—218° (solidified after melting and melted again at 235°). When the acid (VI) was heated at 250°, it gave an anhydride (VII), $C_{17}H_{12}O_8$ as colorless prisms, mp 251—253°. The IR spectrum of VII showed the absorptions at 1837 and 1775 cm^{-1} which were the characteristic of phthalic anhydride type compounds. From this results combined with the IR spectrum of I and that the compound (I) gave no xanthone derivative, it was determined that the carboxyl group of the compound (I) was located in the position 4, not 2, surrounded by hydroxy and methyl groups.

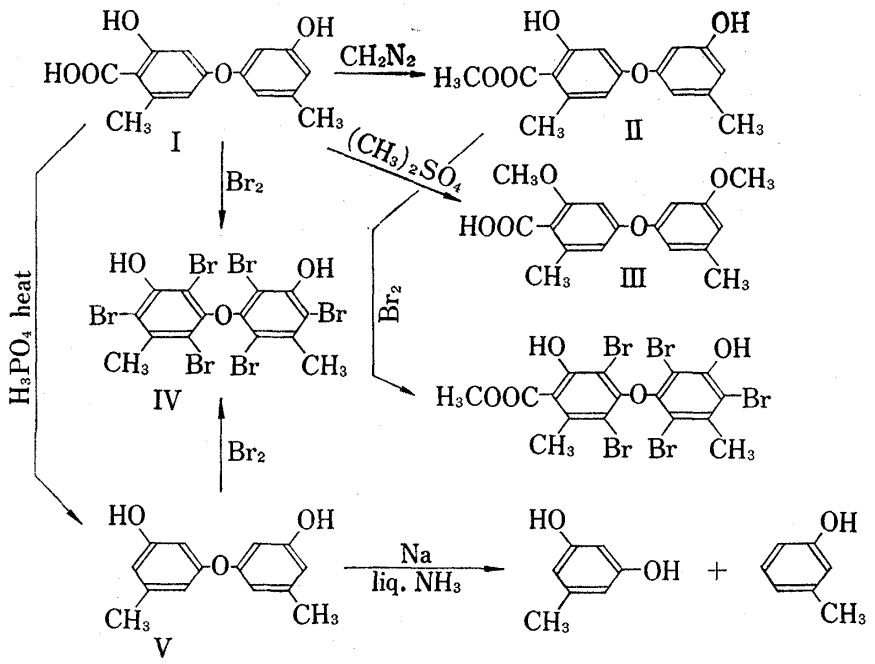


Chart 2

From the foregoing experimental evidences, summarized in Chart 2, the new metabolite was determined to have the structure I which was biosynthesized from two molecules of orsellinic acid.

9) W. Robertson, *J. Chem. Soc.*, **81**, 1480 (1902); H. Hlasiwetz and L. Barth, *Ann. Chem.*, **134**, 276 (1865).
10) M. Tomita, "Jikkenkagaku-Koza," Vol. 22, ed. by the Chemical Soc. of Japan, Maruzen Co. Tokyo, p. 436.

Experimental[11]

Cultural Conditions——*Aspergillus fumigatus* DH 413 was cultivated on the malt extract medium (malt extract, 20 g; glucose, 20 g; peptone, 1 g; tap water, 1 liter) containing 150 mg of DL-ethionine per liter at 27°. The cultivation period was extended to 20 days.

Isolation of Metabolites——The culture broth (6 liter) was extracted exhaustively with AcOEt, and the solvent was evaporated under reduced pressure. The extract was dissolved in a small volume of ether, applied on a column of silica gel (Kanto Chemical Co., for chromatography, 3×30 cm), and eluted successively with benzene and ether. The effluent with benzene was evaporated to dryness, and the residue was crystallized from benzene as colorless prisms, mp 193—194°. They were easily soluble in AcOEt and ether, slightly soluble in $CHCl_3$ and benzene, and insoluble in H_2O. *Anal.* Calcd. for $C_{15}H_{14}O_5$: C, 65.69; H, 5.15. Found: C, 65.55, 65.58; H, 5.13, 5.22. UV λ_{max}^{EtOH} mμ (log ε): 218 (4.69), 262 (4.20), 282 (sh, 3.83), 304(3.78). The effluent with ether gave reddish syrup on evaporation, in which orsellinic acid was detected on TLC using several solvents.

Methyl Ester (II)——To the etherial solution of diazomethane (large excess) 175 mg of I in ether (20 ml) was added. After standing overnight the solvent was removed and the residue was crystallized from benzene mixed with small amount of petr. benzin as colorless prisms, mp 113.5—114°, yield 170 mg. *Anal.* Calcd. for $C_{16}H_{16}O_5$: C, 66.66; H, 5.59. Found: C, 66.71; H, 5.62. NMR (in $CDCl_3$)τ: 7.74 (CH_3), 7.52 (CH_3), 6.07 ($COOCH_3$), 4.5 (b, OH), 3.66—3.55 (6H), and −1.70 (OH).

Dimethyl Ether (III)——Dimethyl sulfate (0.32 ml) was added to the solution of I (314 mg) in 10% NaOH (6 ml) and warmed on a water bath. Every 3 hrs, dimethyl sulfate (0.3 ml) and 10% NaOH (3 ml) were added. After 10 hrs, the reaction mixture was acidified with H_2SO_4 and extracted with ether. The solvent was removed and the resulted syrupy material was crystallized from petr. benzin as colorless prisms, mp 126°, yield 200 mg. *Anal.* Calcd. for $C_{17}H_{18}O_5$: C, 67.54; H, 6.00. Found: C, 67.63; H, 6.13. NMR (in $CDCl_3$) τ: 7.75 (CH_3), 7.52 (CH_3), 6.20 (OCH_3), 6.11 (OCH_3), 3.54 (b, 5H), 0.1 (b, COOH, disappeared with D_2O).

Acetylation of II——Methyl ester (II) (83 mg) was refluxed with Ac_2O (2 ml) and AcONa (200 mg) for 1 hr, and poured into ice water. The product was extracted with ether after neutralization and the solvent was removed. The resulted oil was tried to crystallize without success, but NMR showed signals due to two acetyl groups. NMR (in $CDCl_3$)τ: 7.80 (AcO), 7.73 (AcO), 7.70 (CH_3), 7.66 (CH_3), 6.13 (OCH_3), 3.38—3.25 (5H).

Bromination of I——Bromine water (0.4 ml in 30 ml of H_2O) was added dropwise to the solution of 110 mg of I in EtOH (20 ml) until the decoloration was ceased, and finally warmed on a water bath. The resulted yellow precipitates were collected and thinlayer chromatographed (solvent: benzene). The main band at *Rf* 0.6 was extracted with ether and crystallized from the mixture of ether–petr. benzin as slightly yellow prisms, mp 204—205°, yield 150 mg. *Anal.* Calcd. for $C_{14}H_8O_3Br_6$: C, 23.89; H, 1.17; molecular weight, 703.7. Found: C, 24.36; 24.37; H, 1.28, 1.32; Mass Spectrum *m/e* 696 (1), 698 (6), 700 (15), 702 (20), 704 (15), 706 (6), 708 (1). NMR (in $CDCl_3$) τ: 7.36 (CH_3), 3.90 (b, OH, disappeared with D_2O).

Bromination of Methyl Ester (II)——To the solution of II (113 mg) in EtOH (20 ml), bromine water was added dropwise. The calculated amount of bromine was consumed. After slight warming, the precipitates were collected, yield 254 mg (calcd. 270 mg). The bromide was recrystallized from aqueous MeOH as slightly yellow prisms, mp 180—181°. *Anal.* Calcd. for $C_{16}H_{11}O_5Br_5$: C, 28.15; H, 1.62. Found: C, 28.50, 28.42; H, 1.69, 1.65.

Decarboxylation of I——I (140 mg) was suspended in 85% H_3PO_4 (25 ml) and boiled for 1.5 hr under the stream of N_2. The liberated CO_2 was trapped in a $Ba(OH)_2$ solution and weighed (90% yield). The reaction mixture was diluted with H_2O and extracted with ether. After washing with $NaHCO_3$ solution, the solvent was evaporated to obtain a colorless oil. NMR (in $CDCl_3$) τ: 7.64 ($2CH_3$), 5.66 (2OH), 3.08 (b, 6H).

Cleavage of IV with Na in Liquid NH_3——To the solution of Na (0.5 g) in liquid NH_3 (150 ml), 200 mg of IV in ether (30 ml) was added dropwise from a separatory funnel under cooling with dry ice-acetone. When the blue color faded out, Na was supplemented until the color persisted (total 1.2 g of Na was consumed). After standing under a hood to evaporate the NH_3, H_2O was added. The reddish brown solution was acidified with H_2SO_4 and extracted with ether. The extract was preparatively thin layer chromatographed (solvent: benzene: MeOH=5:1). The band at *Rf* 0.25 corresponding to orcinol was extracted with ether, and rechromatographed on a TLC plate (solvent: $CHCl_3$: ether=10:1). The upper band of the chromatogram was extracted with ether, and recrystallized from $CHCl_3$ as colorless prisms, mp 103—105°, yield 10 mg. It was identified with orcinol by mixed mp and comparison of IR. The isolation of *m*-cresol was unsuccessful. The ether extract form another lot of reaction mixture (from 270 mg of IV) was dissolved in H_2O, and brominated as described above. The resulted yellow precipitates were extracted with hot petr.

11) All the melting points were not corrected. All the thin layer chromatography were carried out on Silica gel G (Merck).

benzin (5 ml×3). The combined benzin solution was concentrated to 3 ml and cooled, the precipitated crystals (105 mg) were recrystallized from petr. benzin as yellow prisms, mp 123—125°, and was identified with pentabromorcinol by comparison with the synthetic sample. The residue on petr. benzin extraction above was subjected to preparative TLC (solvent: benzene: MeOH=9:1), and the band at *Rf* 0.85 was extracted with EtOH and recrystallized from petr. benzin as yellow needles, mp 82° (10 mg). It was identified with 2,4,6-tribromo-*m*-cresol by comparison with the synthetic sample.

$KMnO_4$-Oxidation of III——To the suspension of III in H_2O (120 mg in 15 ml), powdered $KMnO_4$ was added until the color of permanganate persisted under gentle warming (1.8 g of $KMnO_4$ was required within 20 hr). The reaction mixture was acidified with H_2SO_4, MnO_2 was dissolved with $NaHSO_3$, and extracted with ether. The ether extract was crystallized from CH_2Cl_2–MeOH mixture as colorless prisms, mp 215—218°, yield 40 mg. *Anal.* Calcd. for $C_{17}H_{14}O_9$: C, 56.36; H, 3.90; molecular weight, 362.3. Found: C, 56.77; H, 4.22; Mass Spectrum, *m/e* 362.

Acid Anhydride (VII)——The above acid (50 mg) was heated at 250° in a flask equipped with an air condenser for 30 min, and the product was extracted with boiling benzene. It was crystallized from benzene with charcoal as slightly yellow prisms, mp 251—253°, yield 20 mg. *Anal.* Calcd. for $C_{17}H_{12}O_8$: C, 59.31; H, 3.51. Found: C, 59.54; H, 3.55. IR (KBr) cm^{-1}: 1837, 1775, 1695.

Acknowledgement The authors are very grateful to Mr. Y. Itatani and Miss T. Tsuji of this Faculty for elementary analyses and spectral measurements. This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, which is gratefully acknowledged.