Chem. Pharm. Bull. 13(8) 935~941 (1965)

UDC 581.13 : 582.282.12 : 547.97

122. Yuzuru Yamamoto, Keiichi Nitta, Kimiko Tango, Taeko Saito, and Mikiko Tsuchimuro : Studies on the Metabolic Products of a Strain of *Aspergillus fumigatus* (DH 413). I. Isolation and Chemical Structures of Metabolites.

(*Faculty of Pharmacy, Kanazawa University*[*1])

Studying the relation between asthma and fungi, about one hundred strains of fungi were collected from the air and dust of asthmatic patients' rooms. One of them, *Oospora astringenes*, was found to produce some metabolites, and their chemical structures[1] and biological activities[2] were studied.

In this paper, another fungus (DH 413, a strain of *Aspergillus fumigatus*) is dealt with. This fungus as well as *Oospora astringenes* showed contractive activity to tracheal muscle in the preliminary experiments.

This microorganism was cultivated on a malt extract medium at 27° for 11 days. The incubation period was determined by examining the medium about pH, optical rotation (sugar) and ultraviolet absorption (see Fig. 1).

The culture broth of 11 days' cultivation was pale yellow or slightly reddish yellow and the λ_{max} in ultraviolet spectrum were at 215, 275, and 370 mμ, but the color turned to bright red on exposure to air or more rapidly by shaking.

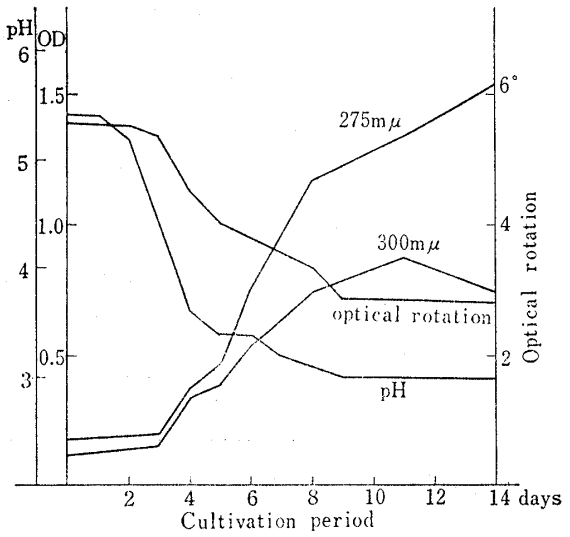


Fig. 1. Variation of pH, Optical Rotation and Optical Density

The culture broth was extracted with ethyl acetate to obtain red pigments (λ_{max} : 220, 275, 300, and 500 mμ), and the extract was treated with boiling benzene. The soluble part was distributed between ether and buffer solution (pH 7). From the ethereal layer slightly brownish prisms, m.p. 107.5~109° (I) were obtained. The buffer layer was acidified and extracted with ether. The ether extract was treated with benzene and deep purple crystals, m.p. 200~201° which were identified with spinulosin,[3~5] were isolated from sparing soluble fraction and slightly orange crystals, m.p. 73~74° (II), were obtained with a small amount of compound, m.p. 204°, from soluble fraction.

As the separation method described above (Fig. 2) seemed to destruct fairly large amounts of metabolites, the method was modified as shown in Fig. 3. The culture filtrate was extracted with chloroform, benzene and ethyl acetate, successively, and the solvents were evaporated. All operations were carried out below 45°. The chloroform extract was chromatographed on silica gel to obtain maroon-colored crystals,

*1 Takaramachi 13, Kanazawa (山本 譲, 新田啓一, 丹後紀美子, 斎藤妙子, 土室美紀子).

1) Y. Yamamoto, *et al.* : Arg. Biol. Chem. Japan, **24**, 628 (1960); **25**, 400, 409 (1961); **26**, 486 (1962); **27**, 813, 817, 822 (1963).
2) S. Ōhashi, M. Yamaguchi, Y. Kobayashi : Proc. Japan Acad., **38**, 766 (1962).
3) J. Birkinshaw, H. Raistrick : Phil. Trans. Royal Soc. London, Series B, **220**, 245 (1931).
4) T. R. Seshadri, *et al.* : J. Chem. Soc., **1959**, 1660.
5) W. K. Anslow, H. Raistrick : Biochem. J., **32**, 803 (1938).

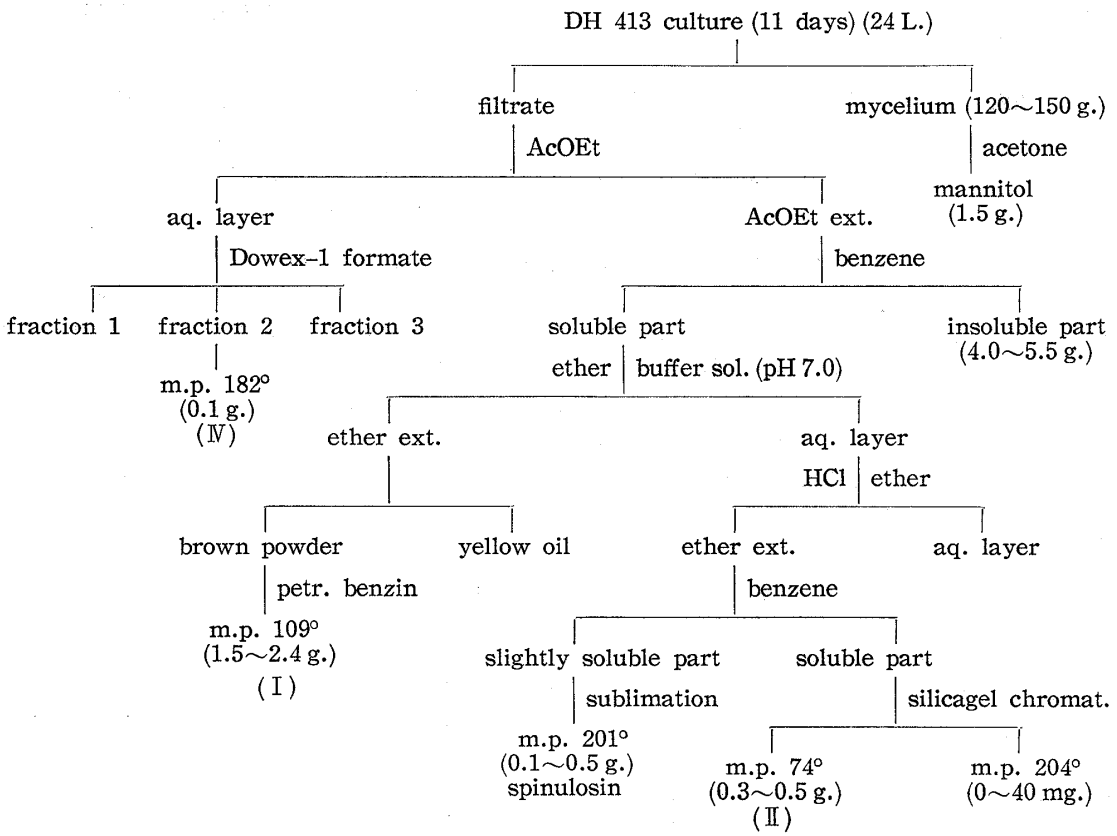


Fig. 2. Isolation Method of the Metabolites (1)

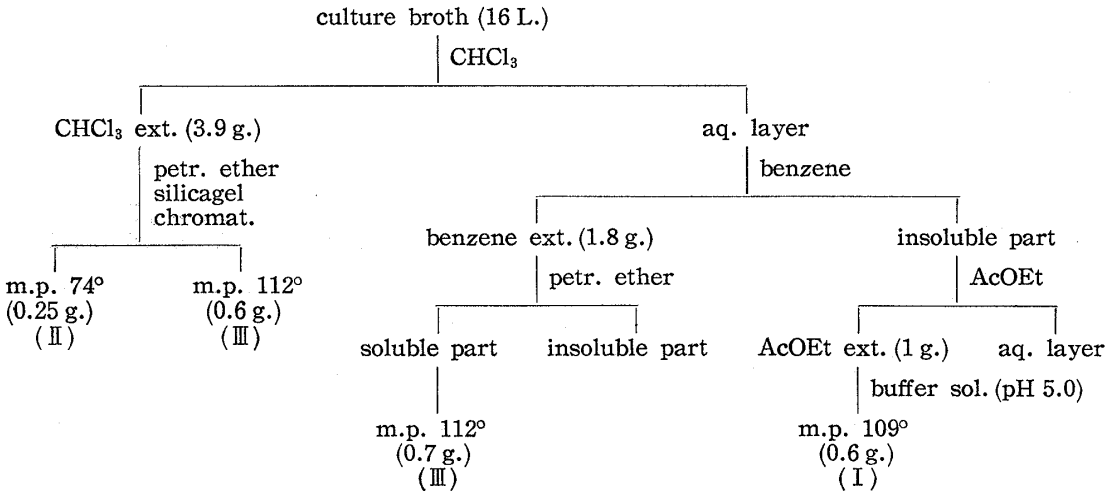


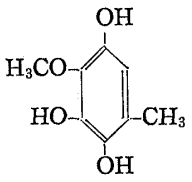
Fig. 3. Isolation Method of the Metabolites (2)

m.p. 112° together with Ⅱ. Ⅲ was also obtained from the benzene extract. I was isolated from ethyl acetate extract. But spinulosin and compound, m.p. 204° did not appear in this procedure.

The results of benzoylation (tribenzoate m.p. 148~149°), ultraviolet spectrum (λ_{max}^{EtOH} 284 mμ), infrared spectrum (3380, 3320, 2850 cm^{-1}) and nuclear magnetic resonance (τ values : 6.27 (OCH_3), 7.93 (CH_3) and 3.77 (-CH=) in D_2O) showed I to be a methoxy-trihydroxytoluene. It was oxidized with aqueous ferric chloride to a quinone, m.p. 112° which was identified with Ⅲ. Ⅲ was reduced to I with sodium hydrosulfite solution.

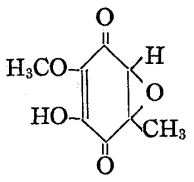
From these results, Ⅲ was expected to be fumigatin[6] or its isomer,[7] and the both compounds were synthesized[8] and Ⅲ was identified with fumigatin (3-hydroxy-4-methoxytoluquinone). Accordingly, I was confirmed to be the quinol of fumigatin. This compound had been reported by various workers[4,8,9], and all the melting points described were between 99° and 101°.

The aqueous solution which had extracted with ethyl acetate in Fig. 2 had still absorption at 270 mμ. It was chromatographed on Dowex-1 formate. By eluting with 0.1*N* hydrochloric acid it was divided into three fractions. From the second fraction a colorless needles, m.p. 181~182° (decomp.) (Ⅳ) were isolated. The first and the third fractions gave no crystalline compound, and under investigation.

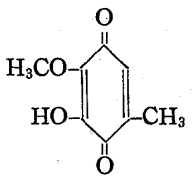


I

fumigatin quinol

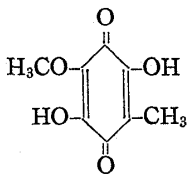


Ⅱ



Ⅲ

fumigatin



spinulosin

The compound, m.p. 73~74° (Ⅱ) was assigned as $C_8H_8O_5$ from the elementary analysis, determination of molecular weight (cryoscopic method) and methoxy determination. It had optical activity. It was fairly stable in acidic solution, but very sensitive to alkaline medium, especially to a medium of pH above 12.0. It produced reddish-brown color with conc. sulfuric acid, purple with 10% sodium hydroxide and greenish-pale brown in aqueous sodium bicarbonate. The yellow aqueous solution was changed in color into brown with ferric chloride with foaming. This compound was negative for magnesium acetate reagent and decolored with zinc powder in acetic acid or with sodium hydrosulfite solution without recovering the original compound in the

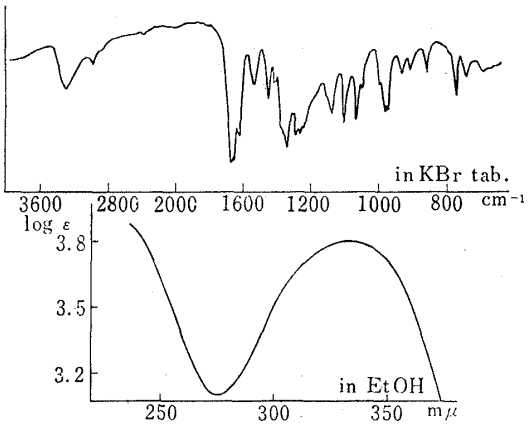


Fig. 4. Infrared and Ultraviolet Spectra of the Compound m.p. 74° (Ⅱ)

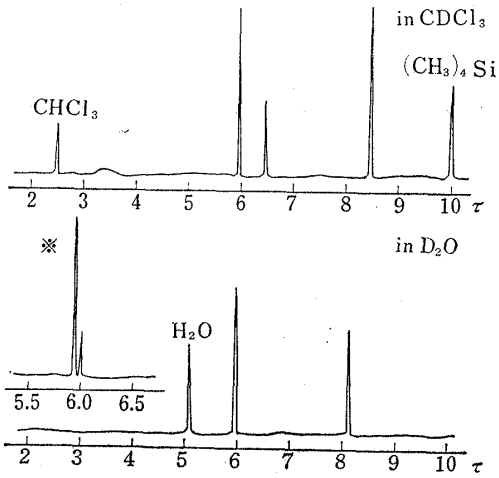


Fig. 5. Nuclear Magnetic Resonance Spectra of the Compound m.p. 74° (Ⅱ)

* magnified chart of 6 (τ) region in D_2O.

6) H. Raistrick, W. Baker : J. Chem. Soc., **1941**, 670.
7) G. Pettersson : Acta Chem. Scand., **17**, 1323 (1963) without m.p., UV- and IR-spectra.
8) The isomer was identified as ethyl ether, cf. W. K. Anslow, H. Raistrick : Biochem. J., **32**, 687 (1938).
9) G. Pettersson : Acta Chem. Scand., **18**, 1839 (1964).

both cases. The ultraviolet absorption had the λ_{max} at 216 and 334 mμ in ethanol, but this λ_{max} shifted to shorter region in strongly acidic media (324 mμ at pH 1.6; 315 mμ at pH 1.0). The infrared spectrum had the peaks at 3300, 2857, 1657, and 1628 cm^{-1}.

Nuclear magnetic resonance spectrum showed four peaks at 8.35 (s), 6.32 (s), 5.96 (s), and 3.40 (broad) (in τ values) at the ratio of 3:1:3:1 in deuterochloroform and three peaks at 8.32 (3), 5.98 (1), and 5.95 (3) in deuterium oxide (all singlet). So the broad peak at 3.40 in deuterochloroform which was disappeared in deuterium oxide was assigned for phenolic OH. From the above results all eight hydrogens became clear: namely, each one of CH_3, OCH_3, OH, and CH. II was changed to a quinone, m.p. 200°, by treating with *N* sodium hydroxide for 2 minutes at room temperature and this quinone was identified with spinulosin.

From these results it was presumed that the arrangement of the substituted groups was the same as that of spinulosin, but it had not quinoid form and had less conjugated system and had asymmetric center. Then it became necessary to suppose the compound carrying epoxy ring. So, thiosulfate test by Ross[10] for epoxy ring was tested to be found positive (red). Though study on infrared spectrum of epoxy ring[11~13] was insufficient, the peaks at 1420, 1260, and 1140 cm^{-1} (triangular ring), 930 cm^{-1} (ring vibration), 775 cm^{-1} (or 745 cm^{-1}) (trisubstituted epoxy ring) seemed to originate from epoxy ring. The above rather high τ value of CH (6.32) in nuclear magnetic resonance was also understood, thus this CH was related to epoxy ring. And the optical activity should be caused from this position.

Terreic acid,[14,15] a metabolite of *Aspergillus terreus*, had been known as the only example having epoxy ring in benzoquinone series. So these two compounds were

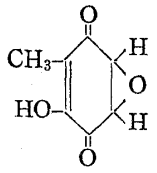
Table I. Comparison of Properties of the Compound m.p. 74° (II), Terreic Acid and the Compound m.p. 182° (IV)

	Color	$[\alpha]_D$	pKa	$FeCl_3$	Tollen's reagent	HIO_4	2,4-Dinitrophenyl hydrazone	Stability	IR cm^{-1}	UV mμ (log ε) in EtOH	UV mμ (log ε) in acid	NMR (τ) in $CDCl_3$	NMR (τ) in D_2O
m.p. 74° (II)	slightly orange leaflets	+28.5° (14°) (EtOH)	4.0	brown	+	+	+	stable in acid unstable in alkali	3300 1675 1657 1628	216 (4.05) 334 (3.78)	315 (pH 1.0)	8.35 (3) (CH_3) 6.32 (1) (epoxy H) 5.96 (3) (OCH_3) 3.40 (1) (OH)	8.32 (3) 5.98 (1) 5.95 (3)
terreic[15] acid m.p. 127°	pale-yellow needles	−12.6° (27°) (aq. MeOH)	4.5	red	+	+	+	do as above	3300 1690 1655 1629	213 (4.03) 316 (3.88)	304 (pH 1.0)	7.90 (3) (CH_3) 6.00 (2) (epoxy H) 2.90 (1) (OH)	/
m.p. 182° (IV)	colorless needles	−213° (14°) (EtOH)	/	brown	/	/	/	do as above	3420 3320 1659 1630	225 (3.78) 305 (4.06)	345 (pH 4.0) 303 (pH 3>)	/	8.68 (3) (CH_3) 6.08 (3) (OCH_3) 5.35 (1) (CH)

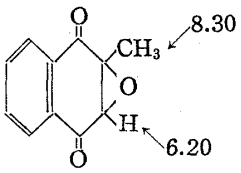
10) W. C. J. Ross: J. Chem. Soc., **1950**, 2257.
11) S. Tsutsumi: "Kagaku no Ryoiki," Special No. **37**, 85 (1959).
12) H. B. Heubert: J. Chem. Soc., **1957**, 1459.
13) J. Bromstein: Anal. Chem., **30**, 544 (1958).
14) M. A. Kaplan, *et al.*: Antibiotic and Chemotherapy, **4**, 746 (1954).
15) J. C. Sheehan, W. B. Lauson, R. J. Gaul: J. Am. Chem. Soc., **80**, 5536 (1958).

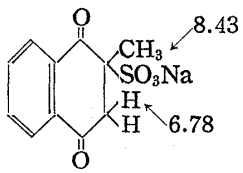
compared and found to be closely similar in infrared, ultraviolet, nuclear magnetic resonance and other properties (see Table I).

The infrared absorption of OCH_3 group in Ⅱ appeared at 2857 cm^{-1} and its methyl ether, m.p. 76~77°, had single peak at 2859 cm^{-1} which showed the presence of olefinic methoxy groups. In nuclear magnetic resonance spectrum the peak at 8.46 (s) was assignable to CH_3 which was not adjacent to unsaturated group. To make sure of this point by comparison with a model compound, 3-methylnaphthoquinone epoxide,[16] m.p. 81° was prepared. The nuclear magnetic resonance spectrum of the compound had the peaks at 8.30 (CH_3) and 6.20 (CH adjacent to epoxy oxygen) in deuterochloroform. Menadion-bisulfite was reported by Asahi[17] to have the peak of CH_3 at 8.43 in deuterium oxide (see the formula).



terreic acid





menadion-bisulfite

From these experimental results the chemical structure of Ⅱ was proposed as 2-methyl-5-methoxy-6-hydroxy-*p*-benzoquinone 2,3-epoxide.

Compound, m.p. 182° (Ⅳ) was soluble in water (pH of the solution, 3.0) and fairly unstable. From the results of elementary analysis, determination of methoxyl group, titration with alkali and nuclear magnetic resonance spectrum an empirical formula $C_8H_{10}O_6$ was assigned. It had a fairly high value of optical rotation and positive to Fehling and ferric chloride solutions. Absorption peak in ultraviolet spectrum was in 305 mμ at pH 3.0, and 345 mμ at pH 4.0. The peaks of nuclear magnetic resonance spectrum in deuterium oxide were at 8.68 (CH_3), 6.08 (olefinic OCH_3) and 5.35 (CH), and the ratio of their strength was 3:3:1 (cf. Table I). The difference between Ⅱ and Ⅳ in formula was H_2O, and it was shown also in the infrared spectra of these two compounds, especially in the following points : The compound (Ⅳ) had absorption peaks of hydroxyl groups at 3420 and 3320 cm^{-1}, whereas Ⅱ had only one peak at about 3300 cm^{-1}, and the absorption peak originated from epoxy group, 1480 cm^{-1} in Ⅱ was not recognized in Ⅳ. In nuclear magnetic resonance spectrum the peak at 6.34 (epoxy H) in Ⅱ also disappeared in Ⅳ.

These results showed that Ⅳ had the closely similar structure to Ⅱ. Thus, Ⅳ might have two more hydroxyl groups than Ⅱ, which might derive from epoxy ring of Ⅱ. The direct confirmation of the structure of Ⅳ is in progress.

Compound, m.p. 204° was obtained as red needles or red rods from benzene or chloroform. The color test with conc. sulfuric acid (cherry-red → violet), ammonia (orange-red) and zinc in acetic acid showed to keep the hydroxy-quinone moiety in the

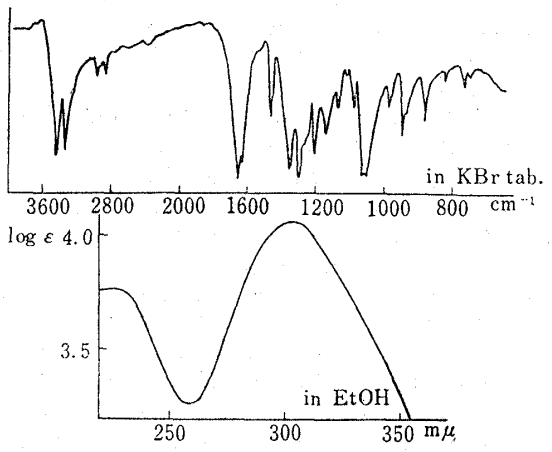


Fig. 6. Infrared and Ultraviolet Spectra of the Compound m.p. 182°(Ⅳ)

16) L. F. Fieser : J. Biol. Chem., **133**, 391 (1940).
17) Y. Asahi : This Bulletin, **11**, 815 (1963).

molecule. It had no optical activity. Molecular weight (Rast) and nuclear magnetic resonance spectrum in which the absorptions of two CH_3 groups (8.33 and 7.69) and two OCH_3 groups (6.20 and 6.02) were appeared, suggested that this compound was not belonged to C_8-unit compound different from others.

Experimental*2

Cultivation of the Strain of *Asp. fumigatus* (DH 413)——The culture medium used was the solution of following composition: Difco's malt extract, 20 g.; anhydrous glucose, 20 g.; peptone, 1 g. in 1000 ml. of tap water, and the pH was adjusted to 7.2 with Na_2CO_3. This medium was distributed in 200 ml. portions into Roux flasks, sterilized, inoculated with spore and cultivated at 27° for 11 days. The culture broth (pH 2.8~3.0) was separated by filtration from the mycelium which was green in color with sporing patches and with occasional pink spot on reverse side. The culture filtrate was yellowish- or reddish-brown in color. The fungus mats were dried and ground; yield, 5~7 g. per 1 litre broth. Any compound except mannitol was not isolated from the mycelium.

Treatment of Culture Filtrate——The culture filtrate was extracted with AcOEt by shaking at least 5 times (total about 20 L.). The aqueous fraction was slightly brown in color and treated with ion-exchange resin (see later).

The AcOEt solution was evaporated and the red syrupy residue was refluxed with benzene (2.5 L.) for several hours. The dark brown insoluble part (4.0~5.5 g.) was removed by filtration and the filtrate was concentrated under reduced pressure. The benzene extract was dissolved in ether (750 ml.) and buffer solution (500 ml., pH 7.0; 1*M* KH_2PO_4, 50 ml.+*N* NaOH, 29.6 ml. and add water to 100 ml.) and shaken. The yellowish orange ethereal solution was concentrated and the brown residue was treated with cold petr. benzin to remove the contaminating yellow oil and then extracted with benzene or petr. benzin (1 L.) under reflux. The slightly brownish prisms, m.p. 105~107°, were separated by concentration, which were purified by sublimation under reduced pressure to give almost colorless micro crystals, m.p. 107.5~109° (I), yield 2.4 g. *Anal.* Calcd. for $C_8H_{10}O_4$: C, 56.46; H, 5.92; OCH_3, 18.23. Found: C, 56.79, 56.17; H, 5.86, 6.11; OCH_3, 17.82.

The purple buffer solution was acidified with conc. HCl and extracted with ether. The red ethereal solution was concentrated and the residue was dissolved in hot benzene. It was concentrated and kept at room temperature. The separated deep violet prisms were purified by sublimaion at 140~160° in vacuum to give spinulosin, m.p. 200~201°, yield 0.1~0.5 g.

The benzene easily soluble part (orange-red) was chromatographed on a silicagel column (200 mesh) and eluted with benzene. The orange eluate was concentrated to dryness and the residue was crystallized from petr. benzin as slightly orange needles, m.p. 73~74° (II), yield 0.3 g.

Then the column was eluted with $CHCl_3$ and red prisms, m.p. 204° was obtained (from $CHCl_3$), yield 0~40 mg.

Isolation of III (fumigatin)——As shown in Fig. 3 the metabolic broth (16 L.) was extracted with $CHCl_3$, benzene and AcOEt, successively. $CHCl_3$ extract (3.9 g.) was washed with petr. ether and the residue was dissolved in benzene and chromatographed on a silicagel column. From the benzene eluate II was obtained (0.25 g.) and by eluting with acetone, evaporation and recrystallization from CCl_4 gave fumigatin (III), m.p. 112°.

The benzene extract (1.8 g.) was extracted with hot petr. benzin and III (0.7 g.) was obtained.

Isolation of the Compound, m.p. 74° (IV)——After the culture broth was extracted with AcOEt, the aqueous layer was adsorbed on Dowex-1×8 formate (3×40 cm.) in a 3 L. portions, and eluted with 0.1*N* HCl after washing the column with H_2O. The eluate was collected in 20 ml. portions, and divided into three fractions by checking with OD at 270 and 310 mμ.

The second fraction (tube No. 46~57) was concentrated under reduced pressure and the residue was dissolved in EtOH and concentrated. The separated crystals were collected and recrystallized from EtOH-benzene mixture as colorless needles, m.p. 181~182° (decomp.). This compound is soluble in aqueous $NaHCO_3$ (with foaming) and NaOH (yellow) and conc. H_2SO_4 (yellow→orange→brown). *Anal.* Calcd. for $C_8H_{10}O_6$: C, 47.53; H, 4.99; OCH_3, 15.35; mol. wt., 202.2. Found: C, 47.89; H, 5.11; OCH_3, 15.22, 15.42; mol. wt., 195.0 (titration). $[\alpha]_D^{14}$ −213° (c=1, EtOH).

Compound, m.p. 74° (II)——It must be kept in a cool, dry and dark place to avoid decomposition. *Anal.* Calcd. for $C_8H_8O_5$: C, 52.18; H, 4.38; OCH_3, 16.85; mol. wt., 184.1. Found: C, 52.00, 52.13; H, 4.60, 4.52; OCH_3, 16.71, 16.50; mol. wt., 184.1, 179.0 (cryoscopic method in benzene). $[\alpha]_D^{14}$ +28.5° (c=1.3, EtOH).

*2 All melting points are not corrected.

Reaction of II with Diazomethane——II (100 mg.) was added to the large excess of ethereal CH_2N_2. The reaction occurred vigorously and slightly yellow needles were obtained by evaporation. It was crystallized from petr. benzin as colorless needles (100 mg.), m.p. 76~77°. From the elementary analysis $C_{10}H_{12}O_5$ was assigned to the product, but the increase of methoxyl group was only one. *Anal.* Calcd. for $C_{10}H_{12}O_5$: C, 56.60; H, 5.70; 2 OCH_3, 28.94. Found: C, 56.64; H, 5.71; OCH_3, 28.77, 28.57. NMR in D_2O (τ, 8.45 (CH_3), 6.31 (OCH_3), 6.01 (OCH_3), 6.94 (one H) (all singlet) and 6.9~6.4 (quartet, CH_2)) might show the ring expansion of epoxy ring to 4-membered ring containing $-O-CH_2-$ group. The attempts to obtain simple methyl ether was not successful.

Decomposition of II with Sodium Hydroxide——II (80 mg.) was added to 2 ml. of 2*N* NaOH and allowed to stand for 2 min. at room temperature (color turned to violet). The reaction mixture was neutralized with 10% HCl and extracted with AcOEt. The orange solution was dried with Na_2SO_4 and evaporated. The residue was crystallized from hot petr. ether as dark violet prisms, m.p. 200° (20 mg.). It was identified with spinulosin by mixed melting point and IR spectra.

Compound, m.p. 204°——It began to sublime from 190° and melted at 204~205° under decomposition. UV λ_{max}^{EtOH} mμ: 294, 380. *Anal.* Found: C, 54.64, 54.67; H, 4.89, 5.24; OCH_3, 15.40, 15.32; mol. wt., 382, 396 (Rast).

The authors express their gratitude to Dr. I. Yamamoto (Kobe Municipal Hygienic Lab.) who supplied this fungus, Mr. Y. Itatani (this Faculty) for his elementary analyses, Prof. Y. Bando (Pharmaceutical Department, Mukogawa University) for his help for preliminary pharmacological test, and Dr. Y. Tsuda (Department of Pharmaceutical Sciences, Osaka University) for his discussion. NMR spectra were measured by Takeda Pharmaceutical Industry Co. and Kyoto University (Dr. T. Shingu), and a part of the expences for this work was defrayed by the Grant-in-Aid by the Ministry of Education, to which their thanks are also due.

Summary

Several fungal pigments were isolated by cultivation of a strain of *Aspergillus fumigatus* which was isolated from the dust of asthmatic patients' rooms. Spinulosin, fumigatin and its quinol (m.p. 107.5~109°) were determined, though the melting point of the quinol was rather much different from the literature (99~101°). The chemical structure of a new metabolite, m.p. 74° (II) was determined as 2-methyl-5-methoxy-6-hydroxy-*p*-benzoquinone 2,3-epoxide. Another compound, m.p. 182° (IV) was found to have a closely related structure to II. Compound, m.p. 204° had *p*-quinone moiety in the structure, but not the C_8 compound different from all other metabolites.

(Received March 17, 1965)