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Studies on the Metabolic Products of a Strain of *Aspergillus fumigatus* (DH 413)[1]. IV.[2] Biosynthesis of Toluquinones and Chemical Structures of New Metabolites

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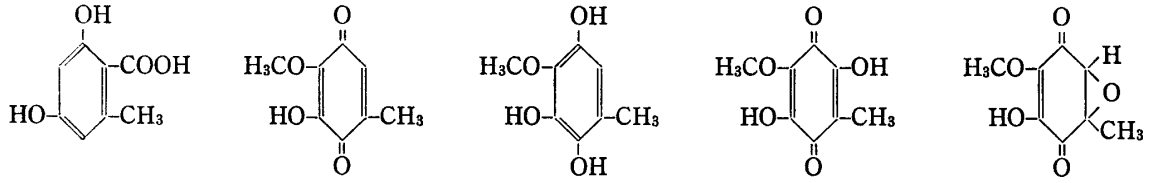
Four new compounds, spinulosin–hydrate (VI), spinulosin quinol-hydrate (II), dihydrospinulosin quinol (VII), and fumigatin chlorohydrin (VIII) were isolated from the culture medium of *Aspergillus fumigatus* DH 413 and Fersenius 4399 by ion exchange chromatography, and their chemical structures were determined. From the tracer experiments using ^{14}C-labeled compounds, the metabolic relationships among metabolites were proposed as shown in Chart 4. The key different point between spinulosin producing and unproducing strains in *Aspergillus fumigatus* were also discussed.

In the previous papers,[2] it was reported that several metabolites including orsellinic acid, fumigatin, fumigatin quinol, spinulosin, and fumigatin oxide (2-methyl-5-methoxy-6-hydroxy-2,3-epoxy-*p*-benzoquinone) (I) were isolated from the culture medium of *Aspergillus fumigatus* DH 413 by extraction with ethyl acetate. And the metabolic relationships among these compounds were discussed.



orsellinic acid fumigatin fumigatin quinol spinulosin fumigatin oxide (I)

Fig. 1

In the course of the tracer experiments with ^{14}C-labeled compounds, as reported in the Part III of this series, it was found that about half amount (41—57%) of the administered radioactivity still remained in the aqueous solution in spite of exhaustive extraction with ethyl acetate. This paper deals with the isolation and chemical structure of the new metabolites from the water soluble fraction, and the more detailed metabolic relationships are presented.

After the culture medium was extracted with ethyl acetate, the aqueous layer was concentrated to one tenth of initial volume below 40° under reduced pressure. The concentrated solution was passed through a column of Dowex-1X4 (formate type) and the column was washed with water to remove unmetabolized sugar, and then the adsorbed metabolites were eluted with 0.005—0.01 N hydrochloric acid. The effluent was divided into four portions by checking of ultraviolet (UV) absorption at 270 mμ and 310 mμ.

The first fraction having λ_{max} at 270 mμ which had been named Fraction 1 in Part I of this series was further divided into Fractions 1a and 1b by elaborate elution with very dilute

1) A part of this work was presented at the 89th annual meeting of the Pharmaceutical Society of Japan, Nagoya, April, 1969.
2) Part III: Y. Yamamoto, K. Nitta, and A. Jinbo, *Chem. Pharm. Bull.* (Tokyo), **15**, 427 (1967).
3) Location: *Takaramachi 13, Kanazawa, 920, Japan.*

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hydrochloric acid in this case. From the Fraction 1a, compound VII, mp 166° was isolated as colorless needles, and compound II, mp 191° was obtained as colorless prisms from the Fraction 1b.[4]

From the second fraction (Fraction 2) which had λ_{max} at 305 mμ, compound VI, mp 182° was isolated as described in the Part I.

The following fraction which showed λ_{max} at about 270 mμ was named Fraction 3. From this part, ethylene oxide α,β-dicarboxylic acid,[5] oxalic aicd, and slightly yellowish prisms (VIII), mp 170—171° were isolated. But the main component which showed λ_{max} at 270 mμ was not yet obtained in pure state.

Compound II, mp 191° was a monobasic acid (pK_a', 3.9) with equivalent 205 (calcd., 204) and the formula $C_8H_{12}O_6$ was assigned from elementary analysis and mass spectrum (M^+, *m/e*, 204). It was positive to ferric chloride (violet) and periodate reagent, and had one methoxy group (Zeisel), but showed no optical activity.

The UV spectrum of II showed λ_{max} at 270 mμ in ethanol or acidic aqueous solution, which was reversibly shifted to 295 mμ in neutral or basic aqueous solution. In the infrared (IR) spectrum, II showed strong peaks at 3400 (OH), 1660 (C=O), 1620 (C=C), 1000 cm^{-1} (δ-OH), and so on. The nuclear magnetic resonance (NMR) spectrum of II showed signals at τ 8.92 (CH_3), 6.40(OCH_3), 5.60 (CH), and 5.34 (CH) (all singlet) in deuterium oxide. In dioxane, further two signals of hydroxyl groups were recognized at τ 2.03 and −0.85.

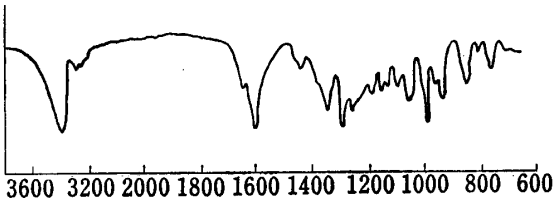

Fig. 2. IR Spectrum of Compound, mp 191° (II)

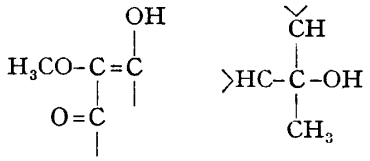

Fig. 3

II afforded both spinulosin quinol, mp 166° and spinulosin, mp 202° by boiling with ethanolic hydrochloric acid. Acetylation of II with acetic anhydride and sodium acetate in the presence of zinc powder afforded tetraacetate of spinulosin quinol, mp 192°. These results showed the close relation between II and spinulosin quinol. II was methylated with etherial diazomethane to give colorless prisms (III), mp 131.5—132.5°. Since III showed two signals of methoxy groups in NMR spectrum at τ 6.37 and 5.87, the presence of one enolic hydroxyl group in II was confirmed.

On treatment with ethanolic hydrochloric acid by the same method for II, III gave both spinulosin quinol and spinulosin instead of the corresponding methyl derivative. III was treated with acetic anhydride and sodium acetate, and the resulting acetate was deacetylated with sulfuric acid. The product was purified by chromatography to obtain 2,3-dimethoxy-5-hydroxy-6-methylbenzoquinone (IV), mp 108—109°.[6] On further methylation with diazomethane, IV gave spinulosin dimethyl ether (V), mp 80°,[7] which was identified with the specimen obtained from spinulosin. These results showed that II and III were easily suffered aromatization by dehydration, and the hydroxyl group which was eliminated as H_2O in aromatization must be located together with the aliphatic methyl group (τ 8.93[8]) on the same carbon atom. Acetate of IV showed UV absorption at 270 mμ (in ethanol) suggesting that the methoxy

4) Silicagel thin layer chromatography (solvent: acetic anhydride) was the most suitable to distinguish II (*Rf*, 0.6) from VII (*Rf*, 0.1).

5) K. Sakaguchi, S. Inoue and Y. Tada, *Nogei-Kagaku Kaishi*, **13**, 241 (1937); *idem*, *ibid.*, **14**, 362, 366 (1938); K. Sakaguchi and S. Inoul, *ibid.*, **16**, 1015 (1940). J.H. Birkinshaw and H. Raistrick, *Biochem. J.*, **39** 70(1945).

6) T.R. Seshadri and G.B. Venkatasubramanian, *J. Chem. Soc.*, **1959**, 1660.

7) W.K. Anslow and H. Raistrick, *J. Chem. Soc.*, **1939**, 1446.

8) H.W. Moore, *J. Org. Chem.*, **32**, 1996 (1967).

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group and enolic hydroxyl group in II were located in the adjacent position.[9] The fact that the methyl group introduced into II with diazomethane was easily hydrolized in acidic condition and that II had strong acidity (pK_a', 3.9) would be attributable to the partial structure of II shown in Fig. 3. The UV spectrum of II (λ_{max}, 270 mμ) showed no further extended conjugated system in II. The two methine groups in II were not in adjacent position, because the NMR signals of both methine groups at τ 5.80 and 5.40 were singlets.

From these results the chemical structure of the compound II, mp 191° was proposed as the keto form of spinulosin quinol-hydrate (5,6).

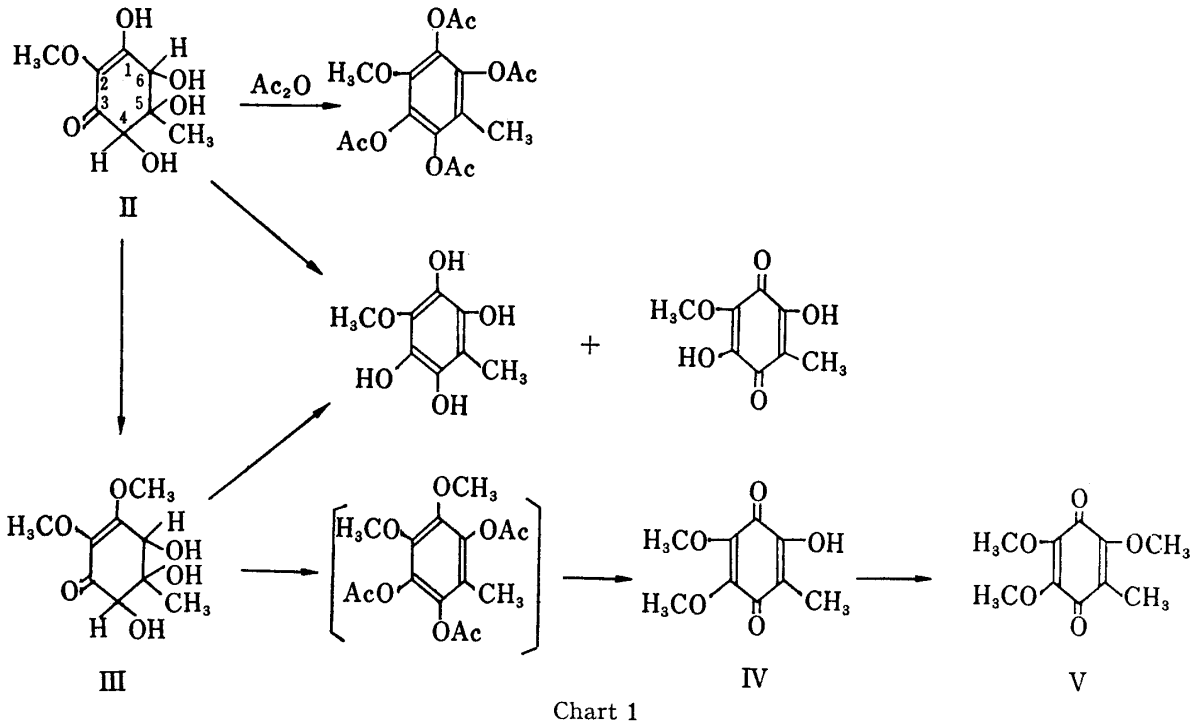

Chart 1

As mentioned above, II was optically inactive in spite of having three asymmetric carbons. It is easily recognized that II tautomerizes between IIa and IIb (Chart 2). And it will be necessary that they are antipodal each other to counterbalance the optical activity, and so, the hydroxyl groups at C-4 and C-6 might have the same conformation in II.

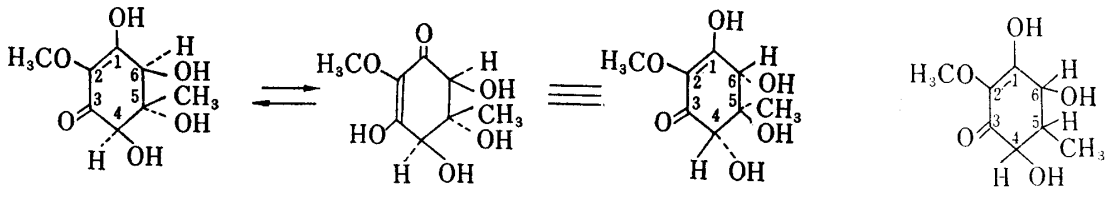

Chart 2

Fig. 4

Some properties of the slightly yellowish compound VI, mp 182° had been reported in the Part I, and supposed the existence of two hydroxyl groups instead of epoxy ring in fumigatin oxide (I). In NMR spectrum, VI showed signals at τ 8.80 (singlet, CH_3), 6.18 (OCH_3), 5.98 (doublet, CH), 6.30 (broad, 1H, OH), and 4.15 (broad, 2H, 2 OH) in dimethylsulfoxide, while in deuterium oxide the signals of the last three protons disappeared and the signal of CH (5.98) became singlet as reported in the Part I. As the presence of one enolic hydroxyl group

9) G. Pettersson, *Acta Chem. Scand.*, **17**, 177 (1963); R.A. Morton, U. Gloor, O. Schindler, G. M. Wilson, L. H. Chopard-dit-Jean, F. W. Hemming, O. Isler, W. M. F. Leat, J. F. Pennock, R. Rüegg, U. Schwieter, and O. Wiss, *Helv. Chim. Acta*, **41**, 2343 (1958); S. Natori, *Chem. Pharm. Bull.* (Tokyo), **12**, 236 (1964).

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was confirmed by titration, other two hydroxyl groups must be alcoholic ones. VI had a molecular formula $C_8H_{10}O_6$, and it was confirmed by mass spectrum (M^+, *m/e*, 202) and the fragmentation pattern contained all the peaks observed in fumigatin oxide (I) (*m/e*, 184, 156, 142, 127, 113, 99, 85 *etc.*) with a few additional peaks (*m/e*, 173, 159). Therefore, VI is probably changed to fumigatin oxide (I) (*m/e*, 184) by elimination of water under the condition of mass measurement.

When VI was treated with ethanolic hydrochloric acid, spinulosin was obtained, while spinulosin quinol was not formed on the contrary to the case of II. From these results the chemical structure of the compound VI, mp 182° was determined to be spinulosin–hydrate.

Although the production of the compound VII, mp 166° by *Aspergillus fumigatus* DH 413 was rather poor, Fresenius 4399 produced considerable amount of VII (*ca.* 100 mg from 1 liter culture). The molecular formula $C_8H_{12}O_5$ was given to VII by elementary analysis combining with mass spectrum (M^+, 188). It had optical activity ($[\alpha]_D=-93°$ in H_2O) on the contrary to II, but other properties such as UV and IR spectra suggested close resemblance of VII to II. It had one enolic hydroxyl group (pK_a', 4.05) and reacted with etherial diazomethane under evolution of nitrogen, but the product was too unstable to be purified.

NMR spectrum gave the most effective informations for the chemical structure of VII, especially by means of decoupling. It showed signals at τ 8.89 (doublet, $J=6.5$ cps, CH_3), 6.38 (OCH_3), 7.68 (center, multiplet, CH), 5.76 (doublet, $J=8.6$ cps, CH), and 5.56 (doublet, $J=4.0$ cps, CH) in deuterium oxide. When irradiated at the signal of τ 8.89, the multiplet signal at τ 7.68 changed to quartet, while two other signals of CH did not change. When the multiplet signal at τ 7.68 was irradiated, the signal of CH_3 became singlet, and two other doublet signals of CH turned to singlet. These results showed the three methine groups and one methyl group were arranged as shown in Fig. 6, and the multiplet signal at τ 7.68 was properly assigned to the centered methine group. Whereas it was not certain which signal 5.56 or 5.76 should be assigned to methine group at position 4 (or 6). The coupling constant among protons of the three methine groups gave informations about conformation. The value $J=8.6$ cps would be assigned to adjacent two axial hydrogens, and $J=4.0$ cps to axial–equatorial or equatorial–equatorial displacement of hydrogens. If the hydrogen at C-5 was axial and that of C-4 and C-6 were opposite conformation each other, the above coupling constants were the most agreeable. In this case, two alcoholic hydroxyl groups became opposite conformation each other, so on the contrary to II, VII gave no enantiopode by tautomery, and this would be the reason VII had optical activity in contrast to II.

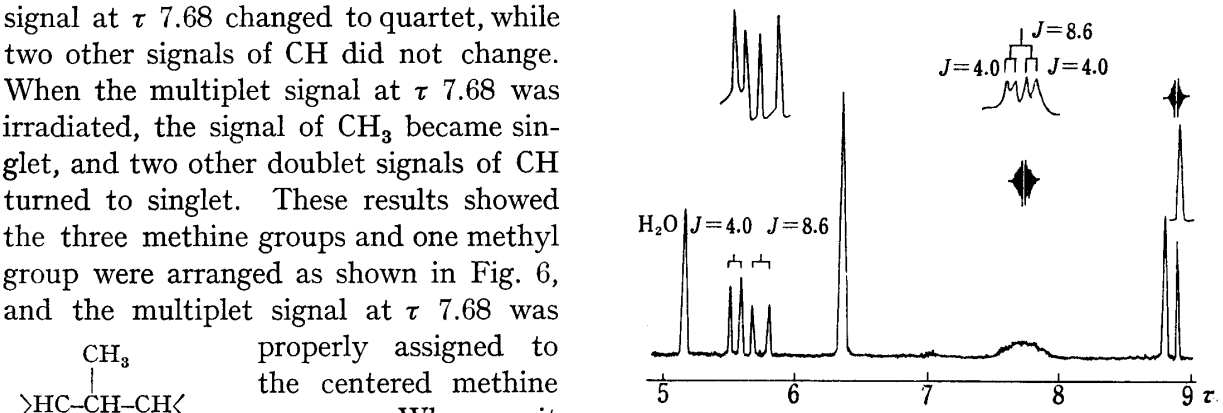

Fig. 5. NMR Spectrum of Compound, mp 166° (VII) in D_2O

Fig. 6

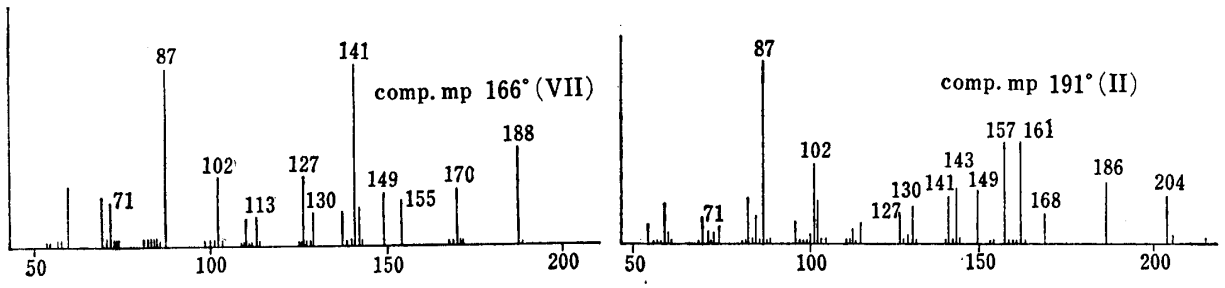

Fig. 7. Mass Spectra of Compound, mp 166° (VII) and Compound, mp 191° (II)

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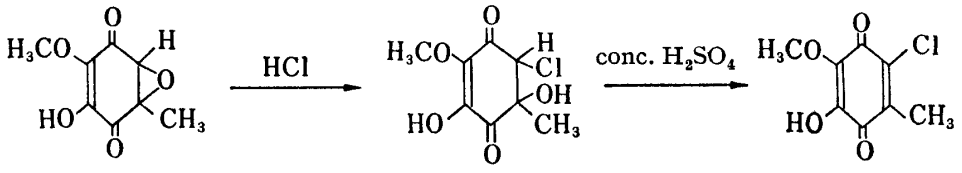
In mass spectrum, one molecule of water was eliminated from VII, whereas two molecules of water from II. It might be caused from the difference of substitution at C-5 (OH and H in II and VII, respectively).

From the results above, the chemical structure of VII was supposed to be the keto form of dihydrospinulosin quinol, though the chemical informations were not enough (Fig. 4).

Slightly yellowish compound VIII, mp 170—171° from the Fraction 3 was given the formula $C_8H_9O_3Cl$ by mass spectrum (M^+, 220.012; Calcd. for $C_8H_9O_3{}^{35}Cl$: 220.014). It showed NMR signals at τ 8.54 (singlet, CH_3), 6.05 (OCH_3), 4.70 (singlet, CH) in deuterium oxide, and IR absorptions at 3400, 3300 (OH), 2820 (OCH_3), and 1660, 1615 cm^{-1} (quinone). The fragmentation pattern of mass spectrum very closely resembled to that of VI. VIII had UV absorptions at 250 and 354 mμ (in ethanol) and was optically active ($[\alpha]_D = -160°$ in H_2O).

VIII was dehydrated with cold concentrated sulfuric acid to give purple-red prisms (IX), mp 92°. IX had the molecular formula $C_8H_7O_4Cl$, and its fragmentation pattern in mass spectrum was well corresponding to that of fumigatin or spinulosin. These results suggested that VIII was fumigatin chlorohydrin and it was confirmed by its synthesis from fumigatin oxide (I) and dry etherial hydrogen chloride. It was presumably an artifact produced in the course of isolation.



I VIII IX

Chart 3

Next, the metabolic relationships among the above metabolites were discussed. In the Part II and III of this series, it had been reported about the biosynthetic pathway to toluquinones and the intermediate role of fumigatin oxide (I), and presented the following metabolic sequence:orsellinic acid→fumigatin oxide (I)→fumigatin quinol→water soluble fraction (Fractions 1,2, and 3).

Aspergillus fumigatus DH 413 did not produce enough amount of spinulosin nor dihydrospinulosin quinol (VII) for tracer experiments, so the metabolism related to them was studied by using *Aspergillus fumigatus* Fresenius 4399 which was regarded to be a "spinulosin producing strain". It produced spinulosin (10—20 mg on the 7th day of cultivation, and none on the 14th day, from 1 liter culture) together with all the metabolites isolated from DH 413.

When sodium acetate (1- ^{14}C) was administered to the strain DH 413 and cultivated for 14 days, about 15% of radioactivity was incorporated into the isolated metabolites. Radioactivity was also incorporated into respiratory carbon dioxide (15%) and mycelium (30%). Each labeled metabolite was isolated, purified to constant radioactivity, and administered to the culture of DH 413 or Fresenius 4399. In the usual administration experiments each labeled compound was added on the 4—5th day of cultivation, and harvested on the 14th day. In all the tracer experiments, radioactivity was recovered in the metabolites including administered labeled compounds at the extent over 70%, and no radioactivity was detected in the respiratory carbon dioxide and mycelium. Therefore, it was probable that the labeled compounds administered did not suffer primary degradation prior to incorporation as reported in the previous paper.

The distribution of radioactivity in the tracer experiments with each labeled metabolite is summarized in Table I.

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Table I

Compound amount added (cpm)	Strain used	AcOEt extract				AcOEt unextracted aqueous part			
		Fumigatin-oxide (I) (%)	Fumigatin-quinol[a] (%)	Spinu-losin (%)	Decomp. part (%)	Comp. 191° (II) (%)	Comp. 182° (VI) (%)	Comp. 166° (VII) (%)	Fr. 3
Fumigatin oxide (I) 3.20×10^6	DH 413	1.1	15	*	11	33	4.1	*	10
Fumigatin quinol 2.37×10^6	DH 413	0.2 (30 mg)	17	*	17	30	3.8	*	4.2
Comp. 191° (II) 5.68×10^6	DH 413	~0 (25 mg)	~0 (150 mg)	*	0.5	84	0.1 (60 mg)	*	1.4
Comp. 191° (II) 9.50×10^6	Fres. 4399 (14 days)	~0 (85 mg)	~0 (15 mg)	~0 (6 mg)	—	91	0.1 (21 mg)	~0 (65 mg)	6.5
Comp. 182° (VI) 3.15×10^6	DH 413	~0 (12 mg)	~0 (6.2 mg)	*	—	1.0 (520 mg)	66 (102 mg)	*	7.3
Comp. 166° (VII) 3.56×10^6	Fres. 4399	~0 (10 mg)	~0 (176 mg)	~0 (18 mg)	—	~0 (170 mg)	~0 (26 mg)	74	1.7
Spinulosin 7.95×10^6	Fres. 4399	~0 (15 mg)	~0 (110 mg)	4.6	0.8	~0 (58 mg)	0.2 (4 mg)	69	6.0
Spinulosin 5.68×10^6	DH 413	~0 (9.5 mg)	~0 (50 mg)	4.0	17	0.2 (750 mg)	0.1 (50 mg)	45	7.8
Fumigatin quinol 4.40×10^6	Fres. 4399 (14 days)	~0 (10 mg)	8.1 (107 mg)	2.5 (27 mg)	30	10	0.9	11	7.7
Fumigatin quinol 2.05×10^6	Fres. 4399 (7 days)	*	11	4.5	25	20	2.3	3.3	—

a) including fumigatin * undetectable

In DH 413, administered labeled fumigatin oxide (I) was incorporated into fumigatin quinol (15%), spinulosin quinol-hydrate (II) (33%), spinulosin-hydrate (VI) (4.1%), and Fraction 3 (10%). Labeled fumigatin quinol was incorporated into II (30%), VI (3.8%), and Fraction 3 (4.2%). In the both experiments, the ratio of radioactivity of II to VI was about 8:1, and dihydrospinulosin quinol (VII) could not be detected. These results showed that the pathway to II from I or fumigatin quinol was the main metabolic route and the route to VI was the minor route. While the metabolic route to VII was almost negligible in the strain DH 413.

Radioactivity of II administered to DH 413 and Fresenius 4399 was not incorporated into other metabolites except a little incorporation into VI and Fraction 3 (recovered 84% and 91% in II, respectively). With spinulosin-hydrate (VI), similar results were obtained in DH 413, thus, 66% of radioactivity was recovered in VI, and incorporation into II and Fraction 3 were 1% and 7.3%, respectively. VII administered to Fresenius 4399 was incorporated into Fraction 3 (1.7%) including ethylene oxide dicarboxylic acid (0.5%), and 74% of the added radioactivity was recorvered in VII. These results showed II, VI, and VII were metabolized independently.

When labeled fumigatin quinol was administered to Fresenius 4399, the radioactivity was incorporated into II (10%), VII (11%), VI (0.9%), Fraction 3 (7.7%), and spinulosin (2.5%). Fumigatin oxide (I) isolated was not radioactive, and 38% of radioactivity was recovered in fumigatin quinol. When fumigatin quinol was added on the 4th day and harvested 4 days later, incorporation of radioactivity into spinulosin was slightly increased (4.5% incorporation).[10]

10) G. Pettersson, *Acta Chem. Scand.*, **18**, 335 (1964). In *Aspergillus fumigatus* A 46, fumigatin quinol was well incorporated into spinulosin, when administered on the 5th day of cultivation and harvested 7 days later, but no incorporation was observed, when administered on the 10th day or later.

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When labeled spinulosin was administered to Fresenius 4399, it was converted to VII in high extent (70%), but no radioactivity was detected in fumigatin oxide (I), fumigatin quinol, II nor VI. This was the same in the case of DH 413, thus, radioactive spinulosin added (although DH 413 did not produce spinulosin) was converted to VII at 45% extent, but not to II nor VI, and 4% of radioactivity was recorvered in spinulosin.

In the strain Fresenius 4399, the production of spinulosin seemd to be more prosperous in the eary stage of cultivation. Although II was not incorporated into spinulosin under the usual experimental conditions, it was also presumed that II might be transformed to spinulosin in the eary stage. However, on the contrary to this speculation, no radioactivity was incorporated into spinulosin even when administered on the 3rd day of cultivation and harvested 4 days later.

The metabolic map presumed from the results above is presented in Chart 4.

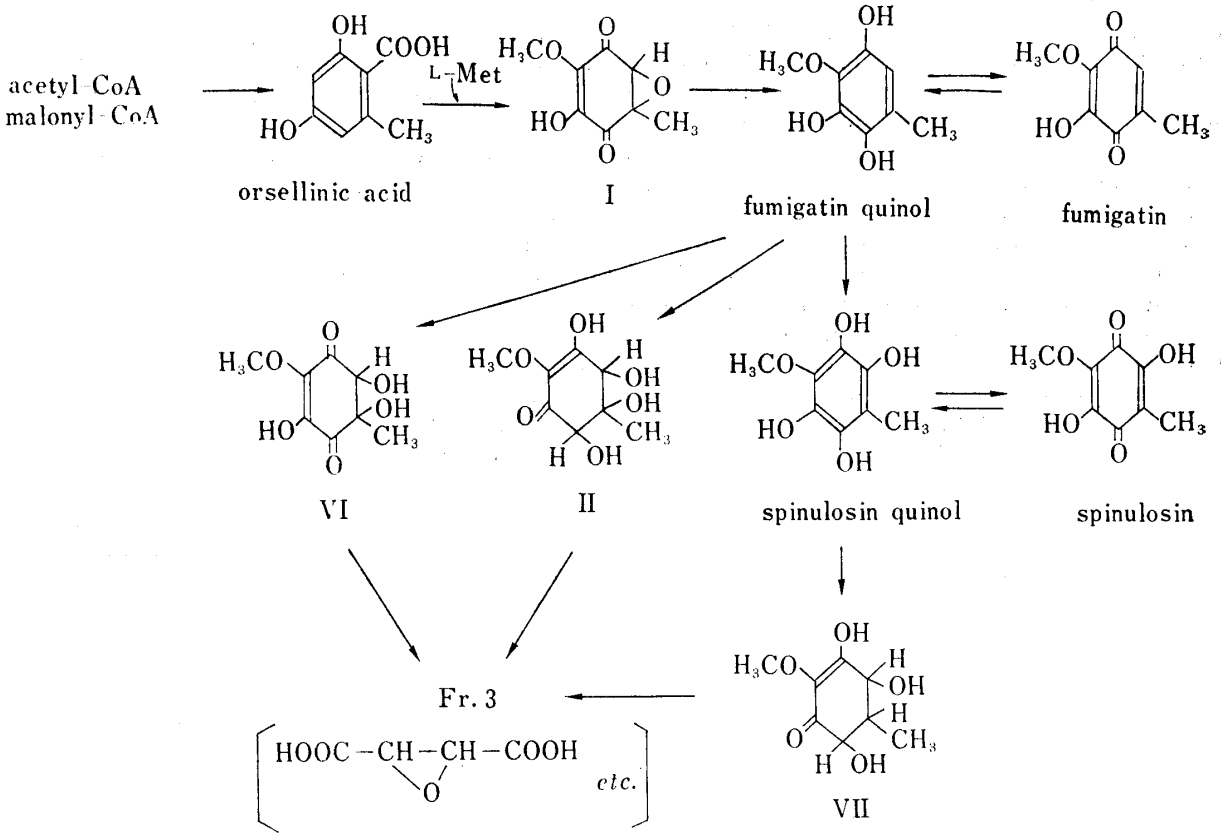

Chart 4. Metabolic Map of Metabolites in *Aspergillus fumigatus*

As described above, the strain DH 413 produced little spinulosin and compound VII, mp 166°, but still kept the ability to metabolize spinulosin to VII. So it became clear that the enzymatic ability to convert fumigatin quinol to spinulosin (presumably quinol) was deficient in DH 413.

It is said there are different strains in *Aspergillus fumigatus* from the point of spinulosin production. DH 413 and L.S.H.T.M. No. A 46 are "spinulosin unproducing strain" and Fresenius 4399 and A 49 are "producing strain." The difference between them might depend on the ability of hydroxylation of fumigatin quinol to spinulosin quinol as shown in this study.

Experimental

Cultural Conditions——*Aspergillus fumigatus* Fresenius 4399 was obtained by courtesy of the Institute for Fermentation (Osaka). *Aspergillus fumigatus* DH 413 and Fresenius 4399 were both cultivated under the conditions described in Part I.

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Isolation of Metabolites——After the culture broth was extracted with AcOEt, the aqueous layer was adsorbed on a column (3×50 cm) of Dowex-1, formate, and washed with H_2O. The adsorbed metabolites were eluted with 0.005N HCl and effluent was collected in 20 ml portions. After Fraction **1a** (tube No. 50—200) and **1b** (200—326) were collected, the developing solvent was changed to 0.01N HCl, and Fraction **2** (tube No. 400—450) and **3** (500—600) were collected. Each fraction was evaporated below 40° under reduced pressure, and resulted syrupy residue was crystallized from organic solvent.

Spinulosin Quinol-hydrate (Compound II, mp 191°)——It was crystallized from EtOH as colorless plates or needles (yield, *ca.* 500 mg from 1 liter of 14 days' culture). It was soluble in H_2O and polar solvents, insoluble in nonpolar solvents such as ether, $CHCl_3$ and benzene. *Anal.*. Calcd. for $C_8H_{12}O_6$: C, 47.06; H, 5.92; OCH_3, 15.2; mol. wt., 204. Found: C, 46.91; 47.08; H, 5.79, 5.75; OCH_3 (Zeisel), 15.3; mol. wt. (titration), 205.2.

Acetylation of II——The mixture of II (140 mg) and fused AcONa (70 mg) in Ac_2O (5 ml) was refluxed for 1 hr and poured into H_2O. The resulting precipitates were collected and recrystallized from EtOH as colorless rhombic prisms melting at 192° (yield, 96 mg), which was identified with tetraacetate of spinulosin quinol. *Anal.* Calcd. for $C_{16}H_{18}O_9$: C, 54.24; H, 5.12. Found: C, 54.35; H, 5.11.

Dehydration of II with Ethanolic HCl——The mixture of II (78 mg) in EtOH (7 ml) added with 5 drops of conc. HCl was refluxed for 3 hr. The brownish solution was evaporated under reduced pressure, added with H_2O and extracted with ether. The ether extract was purified by sublimation. The sublimate at 120—130° at 8 mmHg was identified with spinulosin (32 mg) and the sublimate at 140—170° at 8 mmHg was identified with spinulosin quinol (17 mg).

Methylation of II——To the etherial solution of CH_2N_2 (20 ml, CH_2N_2 was prepared from 1.5 g nitrosomethylurea) 675 mg of finely ground II was added. It was reacted under foaming without dissolving. After standing for 5 hr, the resulting powder was collected and recrystallized from $CHCl_3$ as colorless prisms (III) melting at 131.5—132.5° (yield, 455 mg, 67%). It was optically inactive and easily soluble in H_2O, EtOH, benzene, acetone and AcOEt. *Anal.* Calcd. for $C_9H_{14}O_6$: C, 49.54; H, 6.47. Found: C, 49.71; H, 6.58.

Dehydration of Methylether (III)——III (200 mg) was boiled with ethanolic HCl (5 drops of conc. HCl in 10 ml EtOH) for 5 hr. The orange-red solution was concentrated under reduced pressure, added with H_2O and extracted with ether. The ether extract was treated with petr. benzin. From the soluble part, spinulosin was obtained (56 mg). *Anal.* Calcd. for $C_8H_8O_5$: C, 52.18; H, 4.38. Found: C, 52.10; H, 4.40. Insoluble part in petr. benzin was purified by sublimation and crystallized from benzene as slightly brownish prisms melting at 166° (40 mg), which was identified with spinulosin quinol by IR spectrum. *Anal.* Calcd. for $C_8H_{10}O_5$: C, 51.61; H, 5.41. Found: C, 51.35; H, 5.53.

2,3-Dimethoxy-5-hydroxy-6-methylbenzoquinone (IV)——One hundred and fifty milligrams of methyl ether (III) was acetylated with AcONa and Zn-powder in Ac_2O (5 ml). The obtained yellowish oil was boiled for 1.5 hr with conc. H_2SO_4 (0.2 ml) in MeOH (3 ml) under N_2-atmosphere according to the method of Anslow and Raistrick.[7] H_2O was added to the reaction mixture and the solvent was removed under reduced pressure. The residue was extracted with small volume of AcOEt and the extract was purified by silicagel chromatography (solvent: benzene) followed by recrystallization from petr. benzin as orange needles (25 mg). It melted at 108—109° (ref. 108°[6]). UV λ_{max}^{EtOH} mμ: 298. *Anal.* Calcd. for $C_9H_{10}O_5$: C, 54.54; H, 5.05. Found: C, 54.79; 54.67; H, 5.17, 5.20. IV was acetylated with Ac_2O (3 ml) and conc. H_2SO_4 (3 drops) by boiling for 10 sec. The reaction mixture was poured into H_2O, neutralized with Na_2CO_3, and extracted with petr. benzin to give colorless syrup which could not be crystallized. UV λ_{max}^{EtOH} mμ: 270. NMR (in $CDCl_3$) τ: 6.20 (6H, $2OCH_3$), 7.70 (6H, CH_3 and CH_3CO).

Spinulosin Dimethylether (V)——IV (80 mg) was treated with etherial solution of CH_2N_2, the solvent was evaporated, and the residue was recrystallized from petr. benzin as orange-red needles melting at 80° (yield, 70 mg), which was identified with the authentic sample obtained from spinulosin.[7] *Anal.* Calcd. for $C_{10}H_{12}O_5$: C, 56.60; H, 5.70. Found: C, 56.43; H, 5.78.

Spinulosin-hydrate (Compound VI, mp 182°)——It was obtained from Fraction 2 by crystallization from EtOH–benzene mixture as slightly yellowish needles, mp 181—182° (decomp.) (yield, *ca.* 50 mg from 1 liter broth). It afforded easily spinulosin by treatment with ethanolic HCl under the same condition in II (yield, 50%).

Fumigatin Quinol-hydrate (Compound VII, mp 166°)——It was obtained from Fraction **1a**, and recrystallized from acetone (yield, *ca.* 100 mg from 1 liter broth). It was easily soluble in EtOH and H_2O, soluble in ether, acetone and $CHCl_3$. UV $\lambda_{max}^{H_2O(pH\ 2)}$ mμ (log ε): 270 (4.09); $\lambda_{max}^{H_2O(pH\ 7)}$: 295 (4.32). IR cm^{-1}: 3400, 2860, 1660 *etc.* *Anal.* Calcd. for $C_8H_{12}O_5$: C, 51.06; H, 6.43; mol. wt. 188.2. Found: C, 51.16; H, 6.50; mol. wt., 196 (titration), 188 (mass spectrum). $[\alpha]_D = -93°$ ($c=1.6$, H_2O).

Ethylene Oxide α,β-Dicarboxylic Acid——Fraction 3 was concentrated under reduced pressure to about 50 ml and extracted continuously with ether for 2—3 days. The solvent was distilled off and the resulting crystalls were collected and recrystallized from ether–petr. ether mixture as colorless prisms melting at 176—177° (ref. 180°[5]) (yield, 50 mg from 4 liter broth). *Anal.* Calcd. for $C_4H_4O_5$: C, 36.37; H, 3.05. Found: C, 36.85; H, 3.19. It was identified with the authentic sample by IR spectrum. Oxalic acid was also obtained from the ether extract (yield, 13 mg from 1 liter broth).

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Fumigatin Chlorohydrin (III)——The ether extract above was re-extracted with petr. benzin under refluxing and the extract was crystallized from petr. benzin as slightly yellowish prisms melting at 170—171°. The formula $C_8H_9O_5Cl$ was determined by mass spectrometry. UV λ_{max}^{EtOH} mμ: 250, 354. IR cm^{-1}: 3360 (OH), 3200 (OH), 2820 (OCH_3), 1660 (C=O). $[\alpha]_D^{20°} = -160°$ (c=1.0, H_2O).

Synthesis of Fumigatin Chlorohydrin (VIII)——Dry HCl gas was bubbled into the solution of 100 mg of fumigatin oxide (I) in ether (4 ml) under ice-cooling with occasional shaking. When the reaction mixture was saturated with HCl gas, the mixture was evaporated in a vacuum desiccator. The residue was crystallized from ether–petr. benzin mixture. It was identified with specimen obtained from Fraction 3 by IR and mass spectra.

Chlorofumigatin (IX) from Fumigatin Chlorohydrin (VIII)——VIII (20 mg) was added to conc. H_2SO_4 (2 ml) and stood for 1 hr at room temperature. The blue solution was poured into H_2O and extracted with $CHCl_3$. Solvent was evaporated and the residue was crystallized several times from petr. benzin as purple-red prisms, mp 92°. This compound was presumed to be chlorofumigatin.

Preparation of Labeled Compounds——$CH_3{}^{14}COONa$ (1 mCi) was administered to DH 413 on the 4th day of cultivation and cultivated further 10 days. All metabolites were isolated according to the method described in Part I of this series and in this paper. Labeled VII was obtained from the culture medium of Fresenius 4399 by administration of $CH_3{}^{14}COONa$ under the same condition as in DH 413. Radioactive spinulosin was prepared from radioactive spinulosin quinol-hydrate (II), thus, II (166 mg) was added to ethanolic HCl (15 drops of conc. HCl added to 15 ml of EtOH) and refluxed for 6 hr and the resulting mixture of spinulosin and its quinol was shaken with 10% NaOH for 1 hr. The reaction mixture was acidified and extracted with ether. The extract was purified by sublimation and then recrystallization from benzene (yield, 112 mg, 75%).

Administration Experiments and Determination of Radioactivity——Several millions cpm of each labeled metabolite was administered to the culture according to the method described in Part III. Harvest, isolation of the metabolites, determination of radioactivity in the tracer experiments were carriedout by the method described in the Part III and this paper.

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