

## Yellow Pigments of *Aspergillus niger* and *Asp. awamori*

### Part I. Isolation of Aurasperone A and Related Pigments

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From mycelia of *Asp. niger* and *Asp. awamori* aurasperones A, B and C along with related two yellow pigments have been isolated.

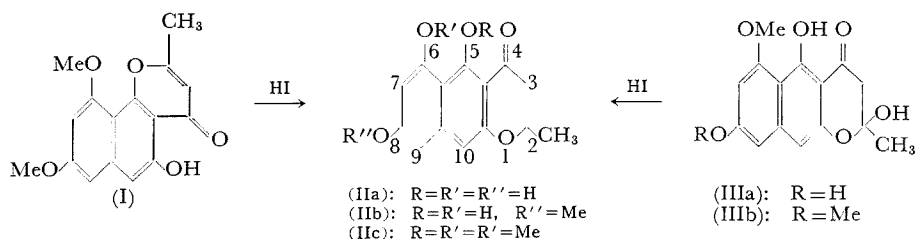
Aurasperone A,  $C_{32}H_{26}O_{10}$ , is obtained in yellow prisms; m.p.  $207^{\circ}C$ ;  $[\alpha]_D -136^{\circ}$ ; gives the diacetate and the dimethyl ether and is assumed to be a dimeric 2-methyl-5-hydroxy-6,8-dimethoxy-4H-naphtho [2,3-b] pyran-4-one (IV). Aurasperone B,  $[\alpha]_D +46.3^{\circ}$ , is the main yellow metabolite, m.p.  $186^{\circ}C$ , and affords aurasperone A on hydrochloric acid-treatment. It has molecular formula  $C_{32}H_{30}O_{12}$  and is supposed to have the structure (V). The other yellow pigments have been found to be also congeners of aurasperone A.

Though the formation of yellow pigments in the mycelium of *Aspergillus niger* has been observed by many investigators<sup>1)</sup> in relation to the humus-formation and the minerals in the culture media, the pigments have not been chemically characterized besides flavasperone (formerly asperxanthone).

Flavasperone (I), a pale yellow angular naphtho- $\gamma$ -pyrone, was first isolated from the

mycelium of *Asp. niger* by Lund et al.<sup>2)</sup> in 1953 and has been found by Bycroft et al.<sup>2)</sup> to undergo Wessely-Moser rearrangement with demethylation reacted with hydriodic acid to give nor-rubrofusarin (IIa), a linear naphthopyrone.

Recently, we have referred the stability of linear naphtho- $\gamma$ -pyrone in connection to the structure of rubrofusarin (IIb).<sup>3)</sup>



Since flavasperone has been isolated, the presence, in the mycelium, of stable linear

1) W. W. Reid, *Nature*, **165**, 190 (1950); A. E. Oxford, *J. S. African Chem. Inst.*, **11**, 105 (1950); E. Küster, *Z. Pflanzen-ernähr. Düngung. Bodenk.*, **57**, 51 (1952); J. E. Zajic and H. H. Kuehn, *Myco l. Appl.*, **17**, 149 (1962).

2) N. A. Lund et al., *J. Chem. Soc.*, **1953**, 2434; B. W. Bycroft et al., *ibid.*, **1962**, 40; B. W. Bycroft and J. C. Roberts, *ibid.*, **1963**, 4868.

3) H. Tanaka and T. Tamura, *Tetrahedron Letters*, No. 4, 151 (1961); *This Journal*, **26**, 767 (1962). H. Tanaka, Y. Ohne, N. Ogawa and T. Tamura, *ibid.*, **27**, 48 (1963); H. Tanaka and T. Tamura, *ibid.*, **27**, 249 (1963).

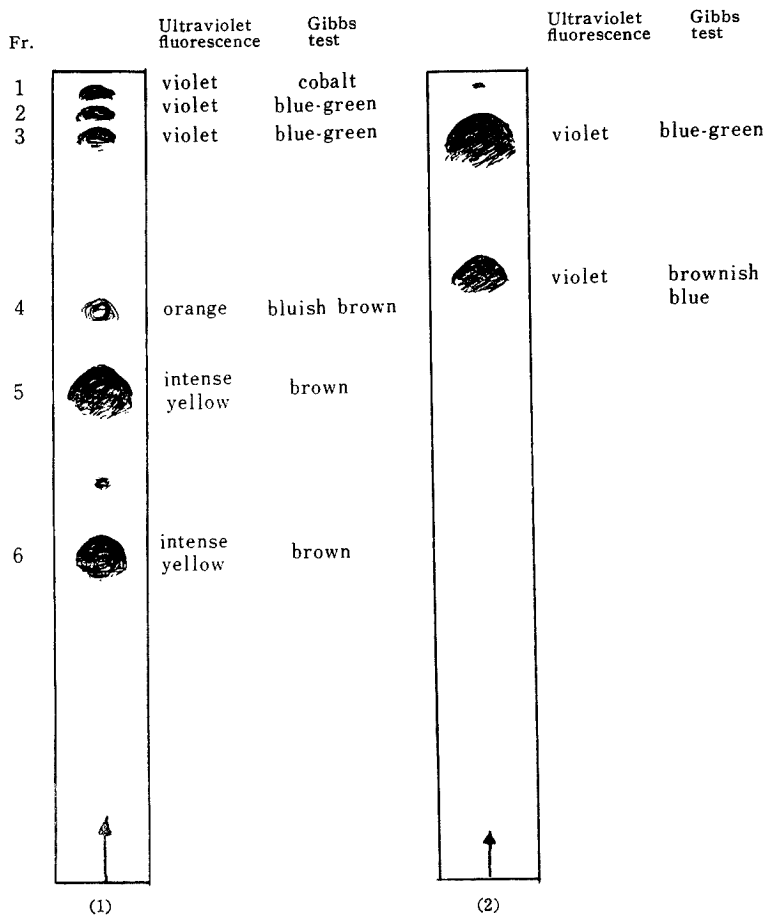


FIG. 1. Paper Chromatograms of the Pigments-Extracts of Mycelium of *Asp. niger* 2126 before (1) and after (2) HCl-Treatment.

naphthopyrone might be expected.<sup>4)</sup>

Thus, we decided to investigate pigments of *Asp. niger* and closely related fungus, *Asp. awamori*.

The defatted ether-extracts of the mycelia were shown by paper chromatography to consist of more than six yellow pigments (Fractions 1~6) with brown-green ferric re-

actions (Fig. 1). The extracts were repeatedly chromatographed on calcium hydrogen phosphate to separate the pigments. The weakly blue-fluorescent band (Fr. 1) was first eluted with benzene to give light yellow prisms, m.p. 205~206°C. This compound,  $C_{16}H_{14}O_5$ , had an ultraviolet spectrum of angular naphthopyrone and was identified to be flavasperone (I)<sup>2)</sup> As the second fraction yellow prisms (Fr. 2) melting at 222°C were obtained. Though the ultraviolet spectrum indicated Fr. 2 to be a linear naphthopyrone it was not examined further owing to its poor yield. The deep yellow band (Fr. 3) was then eluted

4) It is interesting that flavasperone is isolated<sup>2)</sup> also from *Asp. fonsecaeus*, while its ultraviolet mutant produces mainly fonsecin (IIIa), 2-methyl-2,5,8-trihydroxy-6-methoxy-2,3-dihydro-4H-naphtho [2,3-b] pyran-4-one; fonsecin is readily converted to  $\gamma$ -pyrone (IIa) by simultaneous demethylation and dehydration with hydriodic acid. O. L. Galmarini, F. H. Stodola, K. B. Raper and D. L. Fennell, *Nature* **195**, 502 (1962); O. L. Galmarini and F. H. Stodola, *J. Org. Chem.*, **30**, 112 (1965).

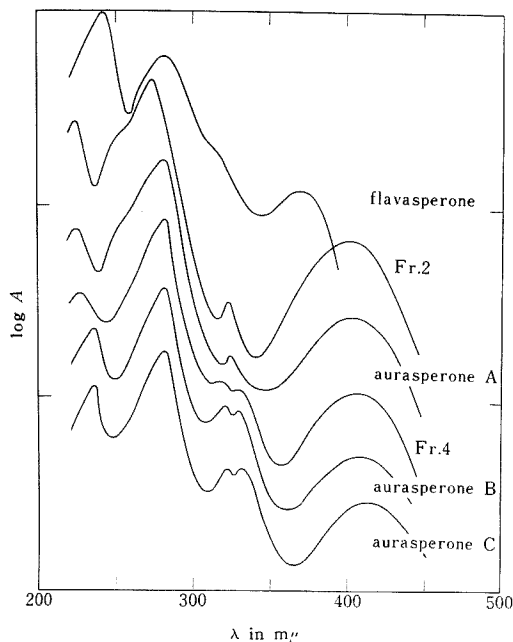


FIG. 2. The Ultraviolet Spectra of the Yellow Pigments.

to afford bright yellow prisms, m.p. 207~208°C,  $C_{32}H_{26}O_{10}$ , for which we propose the name aurasperone A. Molecular weight determinations and the the ultraviolet spectrum (Fig. 2) suggest that it is a dimer of linear naphthopyrone. Recrystallization from alcohol gave needles, m.p. 270°C. Fr. 4 and yellow-fluorescent fractions 5 and 6 were gradually eluted with benzene-ethyl acetate. They are rather unstable and attempts to crystallize them met with difficulty; chromatography on silicic acid in the above solvent mixture gave almost pure semicrystals melting with decomposition at 197°, 186° and 185°C resp. Because of close resemblance to aurasperone A in chemical properties and ultraviolet spectra (Fig. 2) they are presumed to be its congeners. Though *Asp. niger* gave the pigments in good yield compared with *Asp. awamori*, essential difference in the pigment-formation was not observed; two species produced, as the main coloring principles,

yellow-fluorescent Frs. 5 and 6, which were named aurasperones B and C resp. Fig. 3 shows the infrared spectra of aurasperones.

Aurasperone A is laevo-rotatory,  $[\alpha]_D -136^\circ$ , and contains two unreactive carbonyl groups ( $\nu_{\max} 1650\text{ cm}^{-1}$ ) and two C-methyl groups along with four methoxyl groups. It has a brown-green ferric reaction and gives the blue-green coloration with Gibbs reagent. Its two hydroxyl groups are strongly hydrogen-bonded ( $\nu_{\max} \sim 2580\text{ cm}^{-1}$ ); it did not readily react with ethereal diazomethane but reacted with dimethylsulfate to form a dimethyl ether, m.p. 169~173°C, the ultraviolet spectrum of which showed close similarity with that of rubrofusarin dimethyl ether (IIc) (Fig. 4). Acetylation with acetic anhydride-pyridine afforded a diacetate, m.p. 320°  $[\alpha]_D -242^\circ$ . When the pigment was distilled from zinc-dust, anthracene and naphthalene were formed as in the cases of rubrofusarin<sup>3)</sup> and ustilaginoidin A<sup>5)</sup>, the dimer of nor-rubrofusarin with 9, 9' C-C linkage. Aurasperone A dimethyl ether,  $C_{34}H_{30}O_{10}$ , of m.p. 169~173°C possesses  $[\alpha]_D -166^\circ$  and is not identical with ustilaginoidin A hexamethyl ether<sup>5)</sup> of m.p. 252~253°C,  $[\alpha]_D +108^\circ$ . The infrared spectrum of the dimethyl ether is shown in Fig 5.

By above evidences and consideration of the acetate theory aurasperone A is assumed to be a dimeric 2-methyl-5-hydroxy-6,8-dimethoxy-4H-naphtho [2,3-b] pyran-4-one (IV), the linking position of which is different from that of ustilaginoidin A. The optical activity is ascribed to the intramolecular restricted rotation of the C-C linkage. The further evidences for the structure (IV) will be presented in part II.

Dissolved in conc. hydrochloric acid, aurasperone A gives a crimson solution which regenerates, on addition of water, the pigment unchanged, whilst aurasperones B and C as

5) S. Shibata, A. Ohta and Y. Ogihara, *Preceeding of Vth Japan Symposium on the Chemistry of Natural Products* (July, 1962) P. 42; S. Shibata, A. Ohta and Y. Ogihara, *Chem. Pharm. Bull.* (Tokyo), **11**, 1174 (1963); S. Shibata, Y. Ogihara and A. Ohta, *ibid.*, **11**, 1179 (1963); S. Shibata and Y. Ogihara, *Tetrahedron Letters*, No. 26, 1777 (1963).

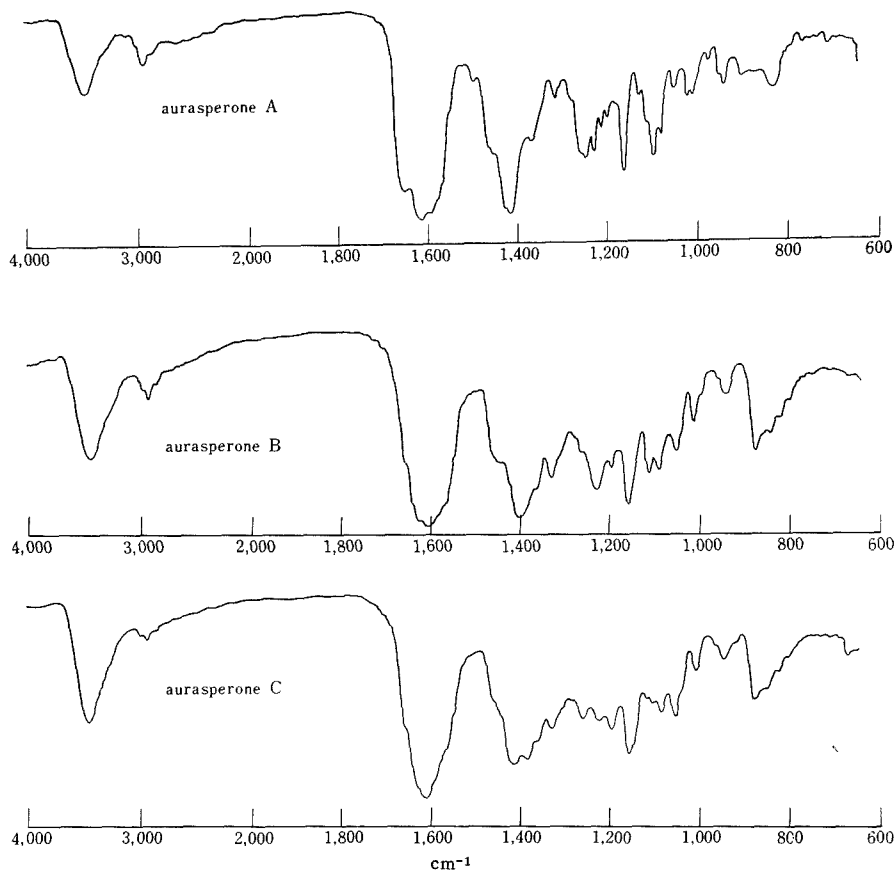


FIG. 3. Infrared Spectra of Aurasperones A, B and C.

well as Fr. 4 suffer with structural changes on that treatment; on addition of water to scarlet solutions aurasperone B and Fr. 4 afforded aurasperone A, while aurasperone C gave a yellow compound, m.p.  $>300^{\circ}\text{C}$ , with low solubility and  $R_F$  value (0.75) compared with aurasperone A (Fig. 1).

Aurasperone B,  $\text{C}_{32}\text{H}_{30}\text{O}_{12}$ , is dextro-rotatory,  $[\alpha]_D 46.3^{\circ}$ , and its ultraviolet spectrum resembles that of fonsecin monomethyl ether (IIIb)<sup>41</sup> (Fig. 2, Table I). Thus, it is assumed to be a dihydrate of aurasperone A and have the probable structure (V).

Above facts provided a convenient method for the preparation of aurasperone A; the

hydrochloric acid treated pigments-extracts were mostly composed of aurasperone A and the compound with  $R_F$  0.75 and devoid of Fr. 4 and aurasperone B and C. Accordingly, pure aurasperone A was obtained through a single chromatography in better yield. The mechanism of this transformation might be  $\gamma$ -pyrone formation due to acid catalysed dehydration of 2-hydroxy-2,3-dihydropyrone system (cf. fonsecin<sup>41</sup>).

The fact that aurasperone B is the principal yellow pigment of *Asp. niger* and *Asp. awamori* and is easily converted to aurasperone A suggests that the latter may be an artefact. Nevertheless, we are convinced that aurosp-

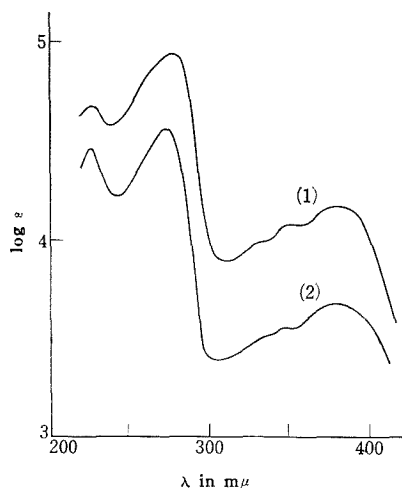


FIG. 4. Ultraviolet Spectra of Aurasperone A Dimethyl Ether (1) and Rubrofusarin Dimethyl Ether (2).

one A is also a natural product by the following reasons; 1) the pigments-extracts at mild conditions also gave the spot on paper chromatogram, 2) difference in the pigments composition was observed among varieties of the fungi. Aurasperone B is regarded as a precursor of aurasperone A.

The finding of these naphthol derivatives in dimeric forms in the mycelia of the fungi is interesting in relation to the humus formation.

### EXPERIMENTAL

Melting points were uncorrected; fluorescence was observed under ultraviolet-illumination; ultraviolet spectra were determined on a Hitachi EPS-2 in 95% alcohol; infrared spectra, on a Hitachi EPI-2 in KBr disks; and paper chromatography, on Toyo-Roshi No. 50 paper with benzene-acetic acid-water (2:1:1), unless otherwise stated.

#### Pigments-Production of *Asp. niger* and *Asp.*

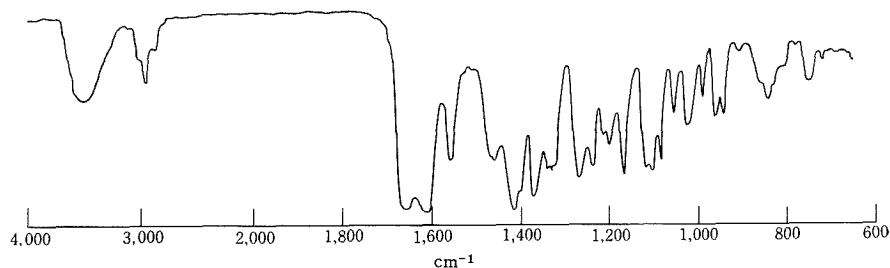
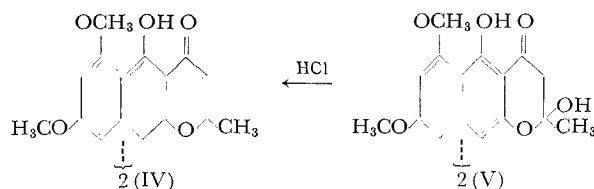


FIG. 5. Infrared Spectrum of Aurasperone A Dimethyl Ether.

TABLE I. ULTRAVIOLET ABSORPTION SPECTRA

Compound	(λ <sub>max</sub> in mμ; 10 <sup>3</sup> × ε in parentheses)				
Fonsecin (IIIa) <sup>4)</sup>	233 (26.4)	275 (31.4)	321 (7.30)	332 (8.03)	400 (8.78)
Fonsecin monomethyl ether (IIIb) <sup>4)</sup>	232 (28.5)	277 (40.5)	317 (9.10)	330 (10.0)	395 (8.40)
Aurasperone B (V)	235 (51.0)	282 (87.7)	321 (20.1)	334 (20.0)	410 (13.6)

**awamori.** Preliminary examination on pigments-formation was carried out using the following strains; *Asp. niger* BL-5-1 and 2126, and *Asp. awamori* var. *fumeus* NI 5592. These mycelia obtained by culture described below were extracted with ether and the amounts of pigments reduced to aurasperone A were colorimetrically estimated using absorption at 405 m $\mu$ . The yields were 0.5, 0.14 and 0.22% resp. to the dried mycelia. These fungi produced almost identical pigments (Frs. 1~6) which were detected by paper chromatography; Fr. 1=flavasperone,  $R_F$  0.97; Fr. 2,  $R_F$  0.93, Fr. 3=aurasperone A,  $R_F$  0.91; Fr. 4,  $R_F$  0.70; Fr. 5=aurasperone B,  $R_F$  0.62; and Fr. 6=aurasperone C,  $R_F$  0.37 (Fig. 1). Aurasperone B was the main coloring metabolite in each strain examined. Pigments contents-ratio, however, varied in the strains; on paper chromatography the extracts of *Asp. niger* 2126 and *Asp. awamori* showed clearly six spots corresponding to Frs. 1~6, while that of the BL-5-1 strain gave aurasperones B and C as the main spots. *Asp. awamori* var. *fuscus* NI 5592 was also examined to give the almost identical result with *fumeus* strain.

**Culture Medium and Isolation of Pigments.** The medium was composed of glucose 8,217 g,  $\text{NH}_4\text{NO}_3$  40.5 g,  $\text{KH}_2\text{PO}_4$  6.48 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  5.4 g and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.9 g in 90 liters of tap water. Each 1 liter flask, containing 500 ml of this medium, was inoculated with mycelium of *Asp. niger* BL-5-1 and incubated for three weeks at 25°C. The air-dried and pulverized mycelium (540 g) was extracted with ether in a Soxhlet apparatus. Ether was removed and the dark brown residue (33 g) was added with hexane. The precipitate (6.53 g) was collected, dissolved in benzene-ethyl acetate and subjected to chromatography on  $\text{CaHPO}_4$ . The light yellow band was first eluted with benzene and concentrated to give crystals. Recrystallization from benzene-hexane furnished pale yellow needles of flavasperone, m.p. 204~205°C, yield 15 mg.

The following bands which consisted of Fr. 2, Fr. 3=aurasperone A and Fr. 4 were eluted with benzene and further separated by chromatography on  $\text{CaHPO}_4$  and silicic acid to give these fractions in poor yields. Frs. 2 and 4 were obtained from mycelium of the 2126 strain, which that of the *fumeus* strain (321 mg) furnished aurasperone A in the first eluted bright yellow band; the eluate was concentrated and recrystallized from benzene-hexane to give yellow prisms (15 mg), m.p. 207°C, to which the name aurasperone A was assigned.

The next band with intense yellow fluorescence was gradually eluted with benzene-ethyl acetate (10:1). The eluate was concentrated at reduced pressure to give crude Fr. 5 (2.34 g) and was further purified on a column of silicic acid in the above solvents mixture; by concentration followed by slow addition of hexane the eluate afforded yellow semicrystals, m.p. 186°C (dec.) (710 mg), to which we named aurasperone B.

The last yellow fluorescent band (Fr. 6) was slowly eluted and purified like aurasperone B to give yellow semicrystals; aurasperone C; m.p. 185°C (dec.) (380 mg).

**Properties of Yellow Pigments.** Fr. 1 = flavasperone<sup>2)</sup>; it was obtained as the first eluate. This light yellow pigment gave a dark green color with alcoholic ferric chloride and a cobalt color on the Gibbs test. It dissolved in conc. sulfuric acid with intense blue fluorescence. *Anal.* Found: C, 66.83; H, 4.98. Calcd. for  $\text{C}_{16}\text{H}_{14}\text{O}_5$ : C, 67.12; H, 4.93%. Fr. 2; this was separated from the mycelium of the 2126 strain in small yield; bright yellow needles; m.p. 220~222°C (ethyl acetate-hexane). Paper chromatography gave a spot with a violet color under UV-illumination. It produced a dark green ferric color and gave a green-blue color on the Gibbs test. Colorless solution in conc. sulfuric acid showed strong blue fluorescence. The UV spectrum indicates that Fr. 2 is a linear naphtho- $\gamma$ -pyrone;  $\lambda_{\text{max}}$  225, 254, 324 and 403 m $\mu$  ( $E_{1\text{cm}}^{1\%}$  1060, 918, 1739, 120 and 249 resp.)  $\nu_{\text{max}}$  ~2580, 1645  $\text{cm}^{-1}$ .

Fr. 3 = aurasperone A; this was afforded in better yield from mycelium of *Asp. awamori* var. *fumeus*; bright yellow prisms; m.p. 207°C (benzene-hexane);  $[\alpha]_D^{25} -136^\circ$  (2.6, chloroform). Recrystallization from alcohol afforded needles melting at 270°C.

Aurasperone A gave a yellow spot with  $R_F$  0.91 which showed a violet color under UV-illumination. Ultraviolet spectrum is given in Fig 2;  $\lambda_{\text{max}}$  225, 258, 280, 325 and 405 m $\mu$  ( $\log \epsilon$  4.71, 4.73, 5.00, 3.94 and 4.11 resp.). Zinc-dust distillation gave anthracene and naphthalene which were detected by the UV absorption. *Anal.* Found: C, 67.22; H, 4.73; MeO, 20.7; M.W., 533 (Signer). Calcd. for  $\text{C}_{32}\text{H}_{26}\text{O}_{10}$ : C, 67.36; H, 4.59; 4MeO, 21.8%; M.W., 570.5. Fr. 4; it was afforded from the mycelium of the 2126 strain in semicrystalline form, m.p. 196~197°C (chloroform-hexane). Paper chromatography gave a spot with  $R_F$  0.70 which showed a bright orange color under UV light. By HCl-treatment or sublimation this was converted to a substance with  $R_F$

0.94 which was identical with aurasperone A. Dissolved in conc. sulfuric acid it gave an orange solution. It has a dark green ferric reaction and gives a bluish brown color on the Gibbs test. Fr. 4 is assumed to be an aurasperone A hydrate;  $\lambda_{\max}$  229, 281.5, 319, 329, and 407  $m\mu$  ( $E_{1\text{cm}}^{1\%}$  724, 1495, 225, 207 and 196 resp.).

Fr. 5=aurasperone B; this metabolite was the main yellow pigment and obtained in semicrystalline form, m.p. 186°C (dec.),  $[\alpha]_D^{25} +46.3^\circ$  ( $c=1.4$ , chloroform). Paper chromatography showed a yellow spot with intense yellow fluorescence. It gave an analytical values for  $C_{32}H_{30}O_{12}$  and converted to aurasperone A by HCl-treatment; the benzene solution (85 mg, 30 ml) was extracted with conc. HCl ( $3 \times 20$  ml) and the scarlet extracts were diluted with water (150 ml) to give yellow precipitate, which was dissolved in benzene and purified by chromatography on  $CaHPO_4$ ; yellow prisms melting at 207°C (benzene-hexane) were obtained (42 mg) and identified to be aurasperone A.

Reaction with acetic anhydride-pyridine gave rise to afford aurasperone A diacetate. Aurasperone B gave a dark green color with alcoholic ferric chloride and a brown color on the Gibbs test. Above evidences indicate that it is a dihydrate of aurasperone A.  $\lambda_{\max}$  235, 282, 321, 334 and 410  $m\mu$  ( $\log \epsilon$  4.71, 4.94, 4.30, 4.30 and 4.13 resp.); *Anal.* Found: C, 63.74; H, 5.07; MeO, 19.5. Calcd. for  $C_{32}H_{30}O_{12}$ : C, 63.36; H, 4.99, 4MeO, 20.5%.

Fr. 6=aurasperone C; this was given as an almost pure precipitate; m.p. 185°C (dec.). Attempts to crystallize were failed. It gave a dark green ferric color and a brown color on the Gibbs test. HCl-treatment gave a yellow compound with  $R_F$  0.75, m.p.  $>300^\circ\text{C}$ , which gave a dark blue color on the Gibbs test and showed absorption maxima at 225, 282, 326 and 410  $m\mu$  ( $E_{1\text{cm}}^{1\%}$  705, 1720, 196 and 238 resp.). Aurasperone C has an almost identical UV spectrum with aurasperone B and is supposed to be closely related compound;  $\lambda_{\max}$  236, 283.5, 323, 336 and 412  $m\mu$ .

**Preparation of Aurasperone A.** It was obtained in better yield by short treatment of the defatted

pigments-extracts (2.5 g from 294 g of dried mycelium of the fumeus strain) with conc. HCl; a benzene solution of the extracts was shaken with the acid and the deep red HCl-extracts were filtered to remove any suspended flavasperone. The filtrate was promptly diluted with ice-water to afford yellow precipitate which was collected, washed with water and chromatographed on  $CaHPO_4$  in benzene. Yellow band afforded bright yellow needles (91 mg) which was identical with aurasperone A obtained without this treatment.

**Derivatives of Aurasperone A.** Dimethyl ether; to the pigment (101 mg) dissolved in 4 ml of acetone, anhydrous potassium carbonate (2 g) and dimethyl-sulfate (2 g) were added and the mixture was stood in a dark place for a week. The product was chromatographed on  $CaHPO_4$  in benzene. The yellowish blue-fluorescent band was eluted with benzene-ethyl acetate (5:1) to give pale yellow needles with intense blue fluorescence; m.p. 169~173°C (aq. alc.); yield 40 mg. The ether was negative for ferric test. Ultraviolet spectrum is shown in Fig. 3;  $\lambda_{\max}$  227, 274.5, 330, 348 and 380  $m\mu$  ( $\log \epsilon$  4.69, 4.95, 3.97, 4.01 and 4.09 resp.).  $[\alpha]_D^{25} -166^\circ$  ( $c=2.4$ , chloroform). *Anal.* Found: C, 67.19; H, 5.38; MeO, 29.5. Calcd. for  $C_{34}H_{30}O_{10}$ : C, 68.22; H, 5.05; 6MeO, 31.1%. Diacetate; aurasperone A (195 mg) was dissolved in 1 ml of pyridine, added with 2 ml of acetic anhydride and refluxed for 2 hrs. at 120°C (oil bath). The resultant solution was cooled and poured onto ice to stand overnight. Pale yellow crystals were collected, and chromatographed on  $CaHPO_4$  in benzene blue-fluorescent band which was eluted with chloroform gave pale yellow needles from aq. alcohol; m.p. 320°C (dec.) (170 mg).  $[\alpha]_D^{25} -242^\circ$  (1.0, chloroform);  $\lambda_{\max}$  228, 274, 332, 347 and 377  $m\mu$  ( $\log \epsilon$  4.71, 4.96, 3.88, 4.10 and 4.14 resp.),  $\nu_{\max}$  1755 and 1650  $\text{cm}^{-1}$ ; *Anal.* Found: C, 65.95; H, 4.92; MeO, 20.9. Calcd. for  $C_{36}H_{30}O_{12}$ : C, 66.05; H, 4.62; 4MeO, 19.0%.

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