

Studies on the Metabolic Products of *Aspergillus fumigatus* (J-4). Chemical Structure of Metabolic Products

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Several new pigments produced by a fungus *Aspergillus fumigatus* (J-4) were studied. The structures were determined as 2-chloro-1,3,8-trihydroxy-6-methylanthrone (9) (I), 2-chloro-1,3,8-trihydroxy-6-methylanthraquinone (II), 2-chloro-1,3,8-trihydroxy-6-hydroxymethylanthraquinone (III) and emodin. The chlorinated monoanthrone derivative is the first case in natural products. When Cl⁻ was replaced by Br⁻ in the medium, the fungus produced corresponding brominated anthrone (IV).

The fungus, *Aspergillus fumigatus* (J-4) which was isolated from jam was presumed to produce some strange coloured materials. This fungus was cultivated on a medium of malt extract-glucose-peptone in tap water (standard medium) at 27° for 2-3 weeks. The cultivation period was determined by the colour and amount of the mycelium and by the pH value of the medium.

The culture broth was usually reddish yellow (pH 3.4-3.8) and citric acid was isolated from it. The yellowish green mycelium was dried, powdered and extracted with petroleum ether, ether and methanol, successively. Ergosterol was obtained from petroleum ether extract (yield, 0.03% of mycelium) and considerable amount of mannitol (1.8%) was isolated from the methanol extract.

By short extraction of the mycelium with ether at room temperature, an yellow pigment was detected as the major constituent on thin-layer chromatogram, whereas it could not be isolated by common procedure using Soxhlet's apparatus, and replaced by a dark coloured material. It was found that this yellow pigment was unstable to raised temperature, boiling with polar solvents, and particularly to light or alkaline medium. So the extraction, concentration and chromatography were performed in a dark room avoiding such conditions. The ether extract was treated with benzene by refluxing to remove insoluble dark coloured pigments and the benzene solution was chromatographed on a silicagel column. The desired yellow pigment (I), mp 227-229°, was eluted with benzene (yield, 0.8% of mycelium). Further elution with benzene containing 3% of ether liberated two compounds, *i.e.* orange prisms, mp 254-255° (yield, 0.1%) and orange-red needles (II), mp 271-273° (yield, 0.13%). The former was identified with emodin by comparing with authentic sample, and its acetate, mp 196° was also identified with emodin acetate. Orange-red powder (III), mp 297°, was isolated by successive elution with ethyl acetate. II and III were supposed to be anthraquinone derivatives from the colour test, UV- and IR-spectra. The compound I, II and III obtained by careful procedure were supposed to be intact pigments produced by the fungus, although the dark coloured material was yielded by decomposition of them. The isolation procedure is shown in Fig. 1.

The pure compound I was not so sensitive to light, but it was decomposed partially by boiling in polar solvents and completely in alkaline medium. It was optically inactive, and no methoxyl group was detected. The infrared absorption at 1619 cm⁻¹ suggested the presence of carbonyl group of 1,8-dihydroxyanthraquinone-type (*cf.* emodin, 1617 cm⁻¹)

1) Location: Takaramachi 13, Kanazawa.

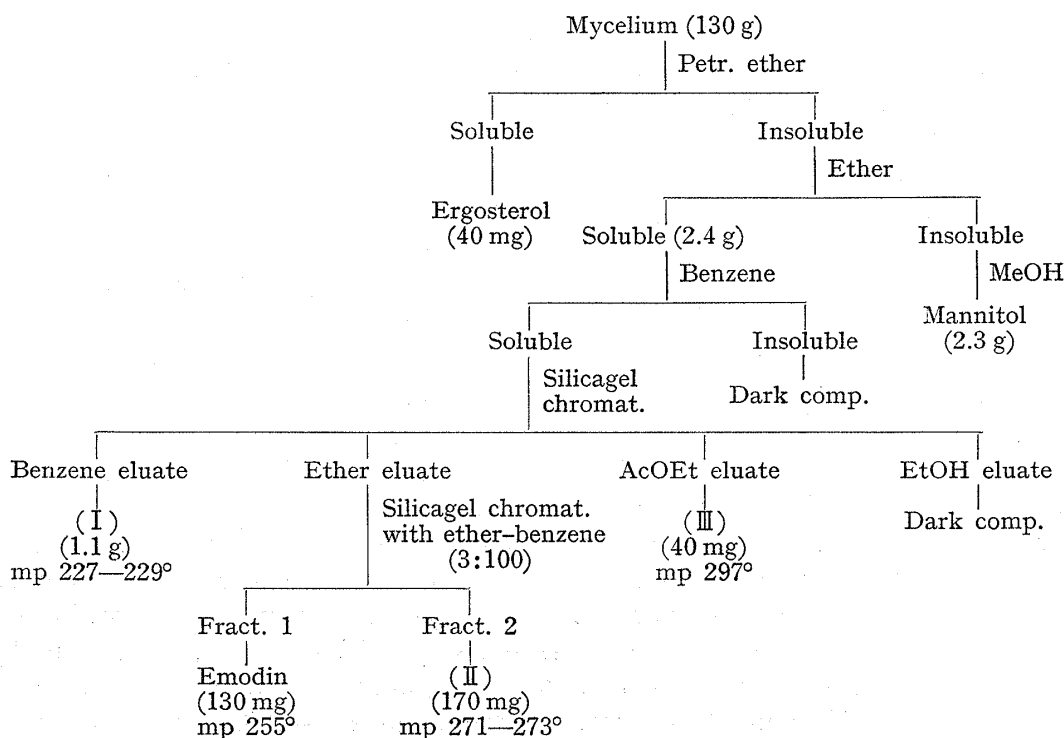
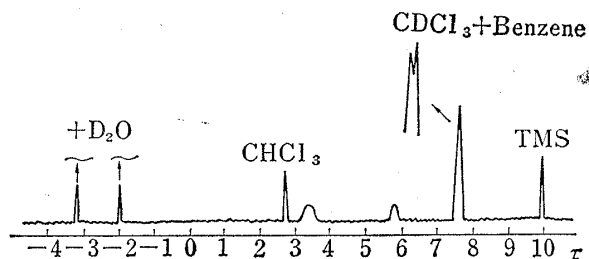


Fig. 1. Isolation Method of Metabolites

and the absorption at 3455 cm^{-1} was assigned to non-chelated hydroxyl group. But UV-spectrum and colour reactions did not correspond with hydroxyanthraquinone derivative. NMR-spectra gave only partial informations for this compound owing to very low solubility, but the presence of CH_2 group (τ value, 5.80) and two chelated hydroxyl group (-1.93 and -3.03) were shown by measurement in acetone at 80° , and methyl group (7.64) was recognized in deuteriochloroform at 80° .

From these results the possibility of anthrone derivative was supposed for I. *p*-Nitrosodimethylaniline test²⁾ gave deep green colour, and oxidation with equivalent amount of chromium trioxide in glacial acetic acid at 60° afforded two materials which were identified with II (main product) and emodin, respectively. Acetylation of I with acetic anhydride in pyridine or by boiling with acetic anhydride for over five minutes were failed, but by boiling with acetic anhydride for 2—3 minutes yellow prisms, mp 231° were obtained. The acetylation of I increased the solubility in deuteriochloroform (ca. 2% at 70°) and NMR-spectrum showed the presence of six protons at 7.63 (CH_3 and OCOCH_3), two protons at 5.76 (CH_2 , s), three ring protons (3.30, bs) and two chelated hydroxyl group (-1.90 and -3.11) as shown in Fig. 2. The overlapping of the signals of methyl and acetyl group at 7.63 was separated by adding of benzene. In IR-spectrum of this acetate, the non-chelated hydroxyl band disappeared and the carbonyl band of 1,8-dihydroxy-chelated-type (1622 cm^{-1}) still remained. So it was a monoacetate and it meant the presence of another group carrying no hydrogen in the nucleus of I.

Fig. 2. Nuclear Magnetic Resonance Spectrum of I-Acetate (in CDCl_3)

From the results of oxygen- and halogen-analysis the formula of I was determined as $\text{C}_{15}\text{H}_{11}\text{O}_4\text{Cl}$ and it supposed to be a chlorinated trihydroxy-methylanthrone.

2) T. Kariyone, K. Tsukita, and N. Suzuki, *Yakugaku Zasshi*, **74**, 234 (1954).

When I was treated with aqueous 10% sodium hydroxide over-night at room temperature, it was also changed to II and emodin. Reaction of I with diazomethane proceeded with foaming and resulted a substance which was identified with fragilin³⁾ (see later), but did not give a corresponding anthrone derivative.

Compound II showed hydroxyl band at 3350 cm^{-1} (β -OH) and two carbonyl bands at 1616 and 1660 cm^{-1} in IR-spectrum. It afforded triacetate, mp 239 – 240° and its UV-spectrum resembled to that of 2-methylantraquinone. II gave orange-red colour with magnesium acetate whereas no reaction with zirconium nitrate.⁴⁾ These results were completely analogous to emodin (1,3,8-trihydroxy-6-methylantraquinone), and UV-spectra were also very similar. From the elementary analysis the chemical formula $\text{C}_{15}\text{H}_9\text{O}_5\text{Cl}$ was presumed and it was supposed to be chlorinated emodin.

In NMR-spectra of the above metabolites, it was characteristic that one of the chelated hydroxyl group was remarkably shifted to lower magnetic field (-3.03 , -3.11 , -2.60 , I, I-acetate and II, respectively) in comparing with emodin (-1.89 and -2.01 in acetone at 80°), whereas the signals of methyl groups were not different among them (I, 7.64 ; emodin, 7.58). These results supported that chlorine atom was substituted at position 2. For identification with fragilin (2-chloro-1,8-dihydroxy-3-methoxy-6-methylantraquinone) which had been isolated from a lichen (*Spaerophorus fragilis* (L) Ach.), II was methylated with diazomethane and the resulted monomethyl ether, mp 266 – 267° was completely agreed with fragilin in mp, UV- and IR-spectra. Acetate, mp 237 – 238.5° (ref., 234 – 235°) was also identified. Dimethyl derivative of II, mp 243 – 244° was obtained, but trimethyl derivative (ref., mp 208 – 209°) could not be prepared.

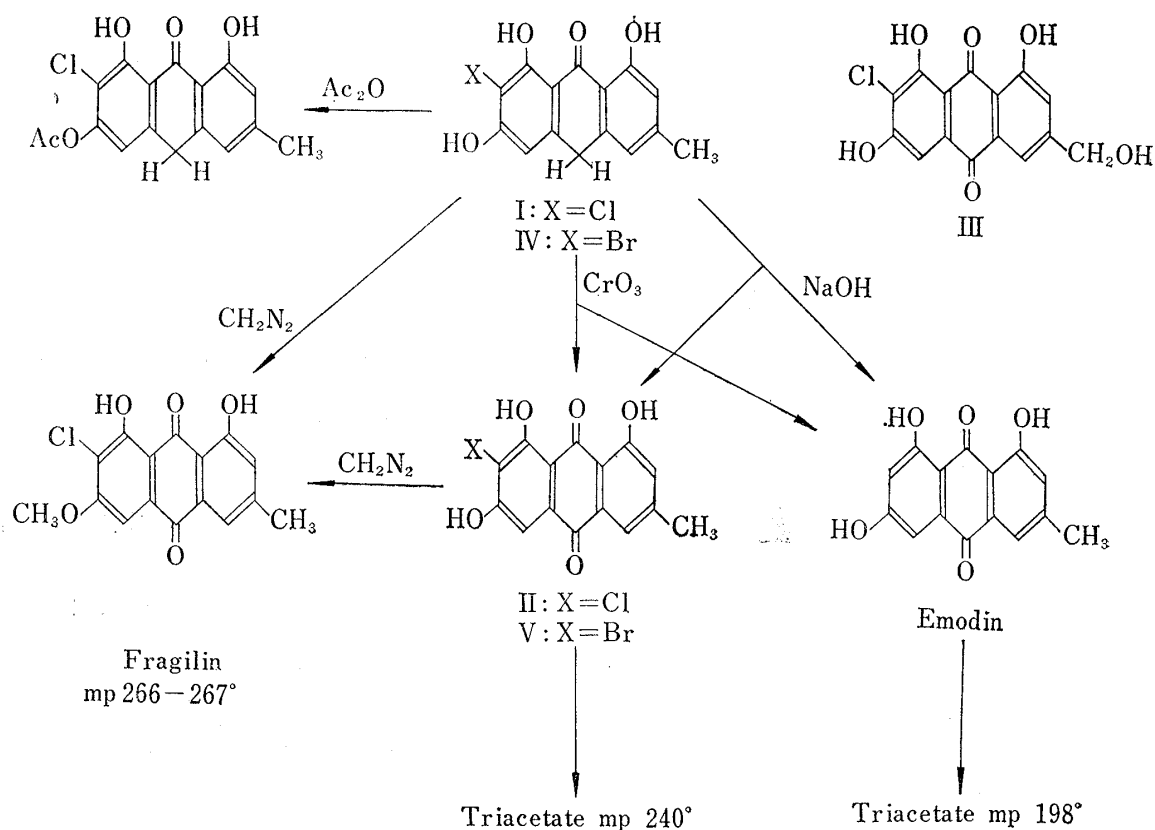


Chart 1

3) T. Bruun, D.P. Hollis, and R. Ryhage, *Acta Chem. Scand.*, **19**, 839 (1965).

4) F. Feigl, "Spot Tests in Organic Analysis," 6th English Edition.

From the above experiments the chemical structures of I and II were determined as 2-chloro-1,3,8-trihydroxy-6-methylanthrone(9) and 2-chloro-1,3,8-trihydroxy-6-methylanthraquinone, respectively.

Compound III also had chlorine atom, and the chemical formula was determined as $C_{15}H_9O_6Cl$ from elementary analysis. It was also supposed to be an anthraquinone derivative by its properties and colour reactions. It showed almost the same UV-spectrum and carbonyl bands (1660 and 1620 cm^{-1}) in IR-spectrum as II. But no C-methyl group was detected by Kuhn-Roth method, it had a sharp absorption of C-OH stretching at 1052 cm^{-1} which was not recognized in II. Its acetate, mp 212°, showed the bands of phenolic acetate (1775 cm^{-1}) and aliphatic acetate (1735 cm^{-1}) (*cf.* aloë-emodin acetate, 1765 and 1730 cm^{-1}). Determination of acetyl group was showed the existence of four acetyl groups. In NMR-spectrum it had signals of four acetyl groups, CH_2-O- group (4.78) and aromatic protons (1.97, 1.84 (d), 2.60 (d) ($J=3$ cps, *meta*-coupling)). The latter two *meta*-coupled signals of aromatic protons were shifted to lower magnetic field than those of II-acetate (2.02 (d), 2.77 (d)) by the same differences of ppm-values, whereas another signal of aromatic proton was little changed.

From the above results it was presumed that III-acetate had CH_2OAc group with two aromatic protons in the same ring, and the chemical structure of III was supposed to be 2-chloro-1,3,8-trihydroxy-6-hydroxymethylanthraquinone.

In the cultivation of the fungus, the source of chlorine atom was not added particularly, so the appearance of the metabolites containing chlorine atom was not expected. Chloride ion was not detected in malt extract, glucose and peptone used (by sodium fusion), but it was contained in tap water (10–12 ppm).

This fungus failed to grow on a modified Czapek-Dox medium (KCl was eliminated). When malt extract was added to the modified medium, the fungus showed normal growth (yield of mycelium, 14 g/liter), but the yield of pigments (I, II and emodin) was decreased to almost nil. In this cultivation it was characteristic point that *meso*-erythritol, mp 120°, was isolated in considerable amount (2.2% of mycelium) which was not recognized in the standard cultivation, and mannitol was decreased to about half amount (0.9%).

The relation between the yield of the pigments and the concentration of chloride ion in the culture medium was investigated by adding varying amount of sodium chloride to the standard medium. The curve (Fig. 3) shows that 130 ppm of chloride ion is optimal for the production of the pigments.

When this fungus was cultivated for 3 weeks on Czapek-Dox medium in which potassium chloride was replaced by potassium bromide or sodium bromide and supplemented with malt extract, yellow prisms (IV), mp 242–243°(decomp.), were obtained. It contained bromine atom and the properties in UV- and IR-spectra, anthrone test (deep green) and other colour tests were almost the same at chloroanthrone (I). It was changed to emodin (main product) and bromoanthraquinone (V), mp 272–273°, by treating with aqueous sodium hydroxide, and also afforded bromoanthraquinone (V) by chromium trioxide oxidation.

The properties of the bromoanthraquinone (V) were also very similar to chloroanthraquinone (II). From these results the bromine atom in these metabolites was also supposed to be substituted at position 2.

Compound I was the first example of chlorine containing anthrone derivative and also the first case of mono-anthrone derivative isolated from fungus. Anthrone is supposed to

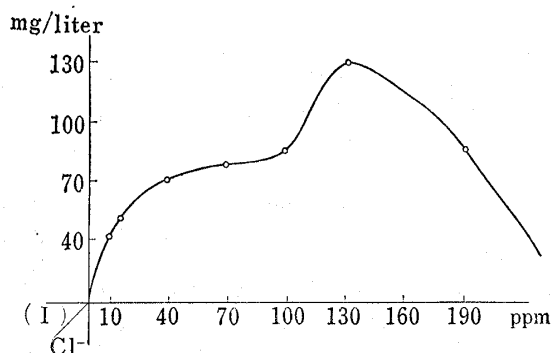


Fig. 3. Relationship between the Yield of (I) and the Concentration of Cl^{-}

be a precursor of anthraquinone biosynthesis, so this fungus which produce the corresponding anthrone and anthraquinone in the same time is also interesting from the biogenetic point of view.

Experimental⁵⁾

Cultivation of *Aspergillus fumigatus* (J-4)—The standard culture medium used was the solution of the following composition: malt extract (Difco Lab.), 20 g; anhydrous glucose (for injection, Daiichi Pharm. Co.), 20 g; peptone (Daigo Eiyu Chem. Co.), 1 g in 1000 ml of tap water. The medium was distributed in 200 ml portions into 500 ml Roux flasks, sterilized, inoculated with spores and cultivated for 2–3 weeks at 27°. The yellowish green coloured mycelium was filtered, dried and ground; yield of mycelium, 7.6 g from 1 liter broth. The culture fluid was usually showed pH 3.0 after 10 days' cultivation and 3.8 at harvest time. Citric acid was isolated from the culture medium.

Extraction of Pigments from Mycelium—The powdered mycelium was extracted with petr. ether (5 hr), ether (15 hr) and methanol (5 hr), successively using Soxhlet's apparatus. The deep yellow ethereal solution was evaporated and the residue (2.4 g from 130 g of mycelium) was treated with benzene. The benzene extract (5 liter) was chromatographed on a column of silicagel (Kanto Chem. Co.; 2.5 × 25 cm) and yellowish eluate was evaporated in vacuum and residual pigment was crystallized from benzene as pale yellow plates (I), mp 227–229° (decomp.), yield, 0.8% of mycelium. The above procedure should be performed in the dark place (Fluorescence lamp also decreased the yield of the pigment.). The pigments adsorbed on the column were eluted with ether, concentrated and the residual mixture was rechromatographed on silica gel (2.5 × 25 cm) with benzene containing 3% of ether. From the orange band eluted first, emodin, mp 254–255° was obtained, yield, 0.1%, and the second red band gave orange-red needles (II), mp 271–273°, yield, 0.13%. The pigments which were not eluted with ether were developed with AcOEt and orange-red powder (III), mp 297°, was isolated, yield, 0.03%. Further elution with EtOH gave black compound.

Compound I—Pale yellow plates, mp 227–229° (decomp.), were moderately soluble in acetone and CHCl₃, slightly soluble in EtOH and benzene, and insoluble in H₂O. With NH₄OH or Na₂CO₃ it showed violet colour after standing for a while. UV $\lambda_{\text{max}}^{\text{EtOH}}$ m μ (log ϵ): 226 (4.44), 273 (4.01), 360 (4.16). Anal. Calcd. for C₁₅H₁₁O₄Cl: C, 61.97; H, 3.81; O, 22.02; Cl, 12.20. Found: C, 62.01, 62.04; H, 3.95, 3.81; O, 22.68; Cl, 11.65.

Acetate of Compound I—I (64 mg) was boiled for 3 min with 2 ml of Ac₂O. Ac₂O was evaporated in vacuum at room temperature and the residue was crystallized from benzene as yellow leaflets, mp 228–231° (decomp.), yield, 38 mg. UV $\lambda_{\text{max}}^{\text{EtOH}}$ m μ : 263, 269, 300, 358. Anal. Calcd. for C₁₇H₁₃O₅Cl: C, 61.36; H, 3.94; O, 24.04. Found: C, 61.40; H, 4.00; O, 25.17.

Oxidation of Compound I with CrO₃—To the solution of I (100 mg) in 115 ml of AcOH, the solution of CrO₃ (105 mg) in 10 ml of 50% aqueous AcOH was added dropwise under stirring at 60° during 30 min and the reaction was continued for further 30 min. After cooling, 1000 ml of H₂O was added and extracted with ether. The ethereal solution was concentrated in vacuum to about 20 ml and red pigment mixture was collected by filtration. The mixture was purified by silicagel chromatography with benzene containing 3% of ether. Emodin (10 mg) was eluted first and II (70 mg) was obtained from the next eluate. These were identified by IR, TLC, elementary analysis and mixed mp of their acetate.

Alkaline Degradation of Compound I—I (90 mg) was dissolved in 10% NaOH (30 ml) and kept overnight. The reaction mixture was acidified with 10% H₂SO₄ and extracted with ether. When the ethereal extract was separated on preparative TLC, three pigments were obtained. From the upper spot orange fine crystals were obtained (10mg in pure state) and identified with emodin by IR and TLC. Anal. Calcd. for C₁₅H₁₀O₅: C, 66.67; H, 3.75. Found: C, 66.42; H, 3.72. The middle spot gave the pigment (2 mg) which was identified with II on TLC. The third pigment(s) (70 mg) could not be purified.

Methylation of Compound I with Diazomethane—I (50 mg) was added to the excess of ethereal CH₂N₂. It reacted immediately with foaming and the colour became more intense. A small amount of fragilin was obtained (5 mg) and unknown yellow pigment which was positive for Cl atom and negative for anthrone test was yielded as the main product.

Compound II—It was soluble in acetone and EtOH, slightly soluble in CHCl₃ and benzene. It was purified from benzene. It had no optical activity nor methoxy group. UV $\lambda_{\text{max}}^{\text{EtOH}}$ m μ (log ϵ): 217.5 (4.49), 258 (4.24), 312 (4.22), 324 (sh), 437 (3.87), 510 (3.72). Anal. Calcd. for C₁₅H₉O₅Cl: C, 59.13; H, 2.98; O, 26.26; Cl, 11.64. Found: C, 59.11, 58.99; H, 2.97, 2.96; O, 26.86; Cl, 11.36.

Acetate of Compound II—The mixture of II (25 mg) and AcONa (200 mg) in Ac₂O (3 ml) was boiled for 10 min and poured into water. The precipitates were collected and crystallized from EtOH as yellow

5) All mp were not corrected. All thin-layer chromatography were carried out on silicagel (Wakogel B-5, Wako Chem. Co.) with ether as solvent.

needles, mp 239—240° (decomp.), yield, 23 mg. NMR τ : 7.63, 7.62, 7.53, 7.52, 2.77 (d), 2.02 (d) and 2.00. *Anal.* Calcd. for $C_{21}H_{15}O_8Cl$: C, 58.55; H, 3.60. Found: C, 58.50, 58.69; H, 3.54, 3.44. UV λ_{max}^{EtOH} $m\mu$: 261, 278 (sh), 342.

Methylation of II with Diazomethane (Identification with Fragilin)—The ethereal solution of 102 mg of II was added to the large excess of ethereal CH_2N_2 and kept over-night. The solvent was evaporated and the residue was purified by silicagel chromatography. The benzene eluate was concentrated and purified by sublimation and recrystallization from $CHCl_3$ as orange-yellow needles, mp 266—267°. It was identified with fragilin (2-chloro-1,8-dihydroxy-3-methoxy-6-methylanthraquinone, mp 267—268°) by IR, UV ($CHCl_3$) and TLC. *Anal.* Calcd. for $C_{16}H_{11}O_5Cl$: C, 60.29; H, 3.48. Found: C, 60.07; H, 3.73.

Acetylation of II Methylether (Fragilin)—Methylether of II (fragilin) (53 mg) was dissolved in the mixture of pyridine (3 ml) and Ac_2O (1 ml), and stood over-night. The reaction mixture was poured into water and the precipitates were collected by filtration and crystallized from acetone as pale yellow needles, mp 237—238.5° (ref.,³) 234—235°, yield, 52 mg. *Anal.* Calcd. for $C_{20}H_{15}O_7Cl$: C, 59.64; H, 3.75. Found: C, 59.87; H, 4.01.

Methylation of Compound II Monomethylether (Fragilin) with Dimethyl Sulfate—Fragilin (110 mg) was dissolved in acetone (300 ml), and K_2CO_3 (1 g) and $(CH_3)_2SO_4$ (1 ml) was added in 5 portions. After refluxing for 16 hr, insoluble salt was removed by filtration and concentrated. The residue was treated with 2% NaOH and $CHCl_3$, and $CHCl_3$ layer was concentrated. The resulted precipitates were crystallized from the mixture of MeOH and $CHCl_3$ as pale yellow needles, mp 243—244°, (yield, 50 mg) with recovery of 50 mg of starting material. *Anal.* Calcd. for $C_{17}H_{13}O_5Cl$: C, 61.36; H, 3.94; OCH_3 , 18.65. Found: C, 60.96; H, 3.85; OCH_3 , 19.08. The reaction period was prolonged to 40 hr but dimethylether was the only product.

Compound III—It was eluted with AcOEt from the silicagel column and recrystallized from the same solvent as orange-red powder, mp 297°. It had no C- CH_3 group, but UV-spectrum was very similar to that of II as follows: UV λ_{max}^{EtOH} $m\mu$ (log ϵ): 220 (4.50), 249 (sh), 306 (4.16), 332 (sh), 435 (3.96), 453 (sh), 520 (3.62). *Anal.* Calcd. for $C_{15}H_9O_6Cl$: C, 56.18; H, 2.83. Found: C, 56.19; H, 3.02. It was insoluble in benzene, slightly soluble in ether and $CHCl_3$, and moderately soluble in acetone, EtOH and AcOEt.

Acetate of Compound III—III (30 mg) was added to the mixture of pyridine (5 ml) and Ac_2O (2 ml) and stood for 2 days. The precipitates were collected by filtration and crystallized from EtOH as pale yellow needles, mp 212°, yield, 28 mg. *Anal.* Calcd. for $C_{23}H_{17}O_{10}Cl$: C, 56.51; H, 3.51; $OCOCH_3$, 4 mol. Found: C, 56.64, 56.41; H, 3.76, 3.53; $OCOCH_3$, 3.37 mol. NMR τ : 7.80, 7.56, 7.54, 7.49 ($OCOCH_3$); 4.78 (CH_2); 2.60 (d), 1.84 (d) ($J=3$ cps, *meta*-coupling), 1.97 (s) (arom. H). UV λ_{max}^{EtOH} $m\mu$: 262.5, 280 (sh), 341. IR (KBr) cm^{-1} : 1775 (phenol acetate), 1735 (alcohol acetate).

Cultivation on Cl- Eliminated Medium—Spores were inoculated on the KCl-eliminated-Czapek-Dox medium added with malt ext. (20 g/liter) and cultivated for 12 days at 27°. Pale gray coloured mycelium was obtained (yield, 66.5 g from 4.8 liter medium). It was extracted in the same manner as in the standard cultivating condition. Ergosterol (49 mg) and a little amounts of II (8 mg), III (3 mg) and emodin (8 mg) were obtained. The methanol extracts were treated with charcoal powder and colourless components were fractionated with acetone. From the insoluble part mannitol, mp 163—164°, was obtained (600 mg) and colourless prisms, mp 120—121°, were obtained from the soluble fraction (1.5 g). The latter had no optical activity ($c=10$, H_2O) and identified with *meso*-erythritol by mixed mp and IR. *Anal.* Calcd. for $C_4H_{10}O_4$: C, 39.34; H, 8.25. Found: C, 39.20; H, 8.11.

Bromoanthrone (IV)—The fungus was cultivated for 20 days on Czapek-Dox medium in which KCl was replaced by KBr (0.8 g/liter). From the mycelium pale yellow plates (IV), mp 242—243° (from benzene), were obtained by the same isolation method as described in chloroanthrone (I), yield, 10 mg from 12 g of mycelium. When NaBr was used in place of KBr, 34 mg of bromoanthrone (IV) was obtained from 39 g of mycelium. *Anal.* Calcd. for $C_{15}H_{11}O_4Br$: C, 53.75; H, 3.31. Found: C, 53.66, 53.52; H, 3.01, 3.04. IR (KBr) cm^{-1} : 3425, 1610. UV λ_{max}^{EtOH} $m\mu$: 226, 273, 360.

Degradation of Bromoanthrone (IV) with 10% NaOH—IV (10 mg) was dissolved in 10% NaOH (10 ml) and stood for 10 min. The reaction mixture was acidified with dil. H_2SO_4 and extracted with ether. The solvent was evaporated and the residue was dissolved in benzene and chromatographed on silicagel column as described in chloroanthrone (I). Emodin was obtained as the main product (8 mg) and the yield of bromoanthraquinone (V) was very low (1 mg).

Oxidation of Bromoanthrone (IV) with CrO_3 —Bromoanthrone (IV) (20 mg) was oxidized with equivalent amount of CrO_3 at 60° as described in chloroanthrone (I). The reaction mixture was purified by chromatography. In this case emodin was not recognized and bromoanthraquinone (V) was obtained (20 mg). It was purified from benzene as orange-red needles, mp 272—273°. The colour reactions with $Mg(OAc)_2$, NaOH, NH_4OH and conc. H_2SO_4 were the same as II. *Anal.* Calcd. for $C_{15}H_9O_5Br$: C, 51.56; H, 2.60. Found: C, 51.61; H, 2.71. IR (KBr) cm^{-1} : 3365, 1662 (free C=O), 1618 (chelated C=O). UV λ_{max}^{EtOH} $m\mu$: 220, 255 (sh), 274, 283, 310 (sh), 330 (sh), 455 (sh), 520 (sh).

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