Studies on the Compounds Produced by Molds

Part VII. Isolation of Isocoumarin Compounds

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Three isocoumarin compounds (BV 1, 2 and 3) were isolated from the cultural broth of Aspergillus oniki 1784. BV 1 was mellein (3-methyl-8-hydroxy-3,4-dihydroisocoumarin). BV 2 and 3 were assigned to be 3-methyl-4,8-dihydroxy-3,4-dihydroisocoumarin, 3-methyl-3,8-dihydroxy-3,4-dihydroisocoumarin, respectively. These two compounds (BV 2, 3) were newly isolated. Also another component named BV 4 was proved to be 6-methylsalicylic acid.

Aflatoxins,1) metabolites of some strains of Aspergillus flavus, are toxic to most animal species and are known to be carcinogenic for the experimental animals.

As has been previously reported,2) none of the seed molds which are used in the Japanese fermentation food industries produces aflatoxins, although many strains produce blue or green fluorescent compounds which are very similar to aflatoxin B or G in the fluorescence and Rf values on thin-layer chromatograms. These fluorescent compounds which resemble aflatoxin B were proved to be pyrazine compounds, and it was not toxic to mice. Homologous non-fluorescent pyrazine compounds3) were also isolated from cultural broths of the same strain. However, its toxicity was not so great for mice by intra-peritoneal injection. Moreover, a possibility of contamination with these toxic pyrazine compounds produced by some molds in Japanese fermented foods was discussed negatively.4) On the other hand, the chemical component of the spot which resembles that of aflatoxin G was proved to be a compound other than aflatoxin G.5)

On further investigation, however, it became obvious that some of them produced brilliant blue violet fluorescent compounds which are more similar to aflatoxin B than to the above pyrazine compounds in terms of the Rf values and the nature of fluorescence on thin-layer chromatograms.

The mold 1784 (a stock culture strain in our research laboratories) was cultured in a synthetic medium. The filtrate was adjusted to pH 1.0 and then extracted two times with chloroform. The concentrate of this extract was subjected to thin-layer chromatography

Studies on the Compounds Produced by Molds. Part VII

FIG. 1. Thin-layer Chromatograms of BV 1-4.

Adsorbents: (I) Kieselgel G. 0.5 mm prepared from distilled water (II) Kieselgel G. 0.5 mm prepared from 0.1 N oxalic acid sol.

Solvent system: Benzene-Ethylacetate (75:25, v/v).

Fluorescent spot (purple color with ferric chloride).

Non-fluorescent spot (purple color with ferric chloride).

of Kieselgel G with 0.5 mm thickness in benzene-ethylacetate (75:25, v/v). Two bright blue-violet fluorescent bands were observed. These two compounds were numbered in the decreasing Rf order BV 1 and 2 respectively. The both spots gave violet color by spraying with a ferric chloride solution and other two violet color spots newly appeared near the basal line. The latter two compounds were named BV 3 and 4, respectively. In order to obtain good separation and purification of BV 3 and 4, 0.1 N oxalic acid solution was used instead of pure water in the preparation of the layer. However, as shown in Fig. 1, with this layer the Rf values of BV 2 and 3 were reversed and BV 3 appeared as a greenish fluorescent band under an ultraviolet light. The fluorescent bands, BV 1-3, and the non-fluorescent band, BV 4 were eluted with chloroform and then were purified by recrystallization.

BV 1 was recrystallized from acetone/water to give colourless needles mp 56°C, [α]_D^20 = -102.5 (c=1.0, CHCl₃) and its molecular formula C₁₀H₁₆O₂ was assigned by elementary analysis and mass spectrometry. Its absorption spectrum is characteristic; UV λ_max m(μ) (Ε_0) 246 (6480), 315 (3990) and IR ν_max cm⁻¹: 1680, 1620, 1580. These physicochemical and spectral properties are in good agreement with those of mellein.⁶ Therefore, BV 1 was identified as mellein.

BV 2 was recrystallized from hexane to give colorless prisms, mp 121-121.5°C, [α]_D^20 = -40 (c=1.0, CHCl₃) which showed in its IR spectrum a hydroxy absorption at 3400 cm⁻¹ and a carbonyl absorption at 1650 cm⁻¹. It showed UV absorptions at λ_max m(μ) (Ε_0): 244.5 (5430), 315 (4190).

BV 3 was recrystallized from chloroform/hexane in colorless plates mp 109-109.5°C, [α]_D^20 = 0 (c=1.0, CHCl₃) and its absorption spectrum was very similar to those of BV 1 and 2: UV λ_max m(μ) (Ε₄): 246 (5970), 315 (3880), IR ν_max cm⁻¹: 3360, 1650, 1620, 1580.

Elementary analysis and mass spectrometry revealed that the molecular formula of BV 2 was C₁₀H₁₆O₄.

This close similarities in the physicochemical and spectral properties of BV 2 and 3 to those of BV 1 indicated that these components were also probably isocoumarin derivatives. Moreover, the NMR spectrum of BV 2 indicated the presence of a methin proton at the benzylic position (δ 4.56, 1H, m, J=20 cps) and a secondary methyl proton (δ 1.58, 3H, d, J=5 cps).

While the spectrum of BV 3 indicated the presence of two benzylic protons (δ 3.24, 2H, s) and a tertiary methyl proton (δ 1.80, 3H, s).

The structures of BV 2 and 3 were confirmed by converting them into mellein by means

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⁶ E. Nisikawa, Nippon Nogeikagaku Kaishi, 9, 772 (1933); T. Yabuta and Y. Sumiki, ibid., 9, 1264 (1933).
Refluxing of BV 2 with iodine and red phosphorus gave 3-methyl-8-hydroxyisocoumarin in low yield, whereas a reaction with 90% formic acid and sodium carbonate gave 3-methyl-8-hydroxyisocoumarin in good yield (93%).

Catalytic reduction of 3-methyl-8-hydroxyisocoumarin gave mellein as shown in chart 1.

From these results the structures of BV 2 and 3 can be assigned to be 3-methyl-4,8-dihydroxy-3,4-dihydroisocoumarin and 3-methyl-3,8-dihydroxy-3,4-dihydroisocoumarin respectively. Hence, BV 2 and 3 are new compounds.

BV 4 was identified as 6-methylsalicylic acid by comparing its mp and IR spectrum with those of an authentic sample.

The LD₅₀ values of BV 1, 2 and 3 for mice (dd strain) as measured by intraperitoneal injection were 250~500 mg for BV 1, 1000~1500 mg for BV 2, 262 mg for BV 3 per kg of body weight.

These compounds were produced by two

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7) J. D. Dutcher, J. Biol. Chem., 232, 785 (1957).
strains out of seven strains of *Aspergillus ochraceus* tested. Therefore, it appears that these isocoumarin compounds are produced by many strains of *Aspergillus ochraceus* species. So far, similar isocoumarin compounds have been isolated from cultural broths of certain fungal strains. It has been reported that mellein (ochracin) was isolated from a cultural broth of *Aspergillus melleus* and 3,4-dihydro-6,8-dihydroxy-3,4,5-trimethylisocoumarin-7-carboxylic acid from *Aspergillus terreus*. Recently, ochratoxins, toxic metabolites of *Aspergillus ochraceus* Wilhelm, have been paid attention by many researchers because of their high hepatotoxocities.

Kubota et al. have obtained an isocoumarin compound named sclerolide that can regulate plant growth from a cultural broth of *Sclerotinia sclerotiorum*. Sassa et al. have obtained Sclerotinin A and B from the same fungal strain that have the same activity as sclerolide. Moreover, oospolactone and oosponol and oospoglycol have been reported to be metabolites of some species of *Oospora* that was isolated from the air of an asthmatic patient's room. It is interesting to note that similar compounds have been isolated from various kinds of microorganisms. 6-Methylsalicylic acid has been isolated from various kinds of the mold.

**EXPERIMENTAL**

**Organism and culture.** The strain of *Aspergillus oniki*, 1784, which has been stocked in our research laboratories was used as a biological source. The composition of the medium was as follows: sucrose 50 g, NH₄NO₃ 10 g, KH₂PO₄ 5 g, MgSO₄·7H₂O 2.5 g distilled water 1 liter, pH 6.0. One and half liter of the medium autoclaved in a five liter Fernbach flask was inoculated with the organisms from a seed culture and cultured for 3 weeks at 30°C.

**Isolation of isocoumarins.** After 3 weeks mycelia (1070 g) were removed by filtration. The filtrate (13.6 liters pH 5.68) was adjusted to pH 1.0 and then extracted two times with chloroform. The extracts were concentrated to a small volume. The concentrate was subjected to thin-layer chromatography (0.5 mm thickness of Kieselgel G prepared from 0.1 N oxalic acid solution, benzene (75), ethylacetate (25)). Fluorescent zones were eluted with chloroform and then were further purified by recrystallization. Yield: BV 1, 45 mg, BV 2, 16 mg, BV 3, 27 mg per 13.6 liters filtrate.

**General properties of BV 1, 2, 3 and 4.** BV 1 was crystallized from acetone/water as colorless prisms, mp 56°C, [α]_D^24 = -102.5 (c=1.0 CHCl₃). Anal. Found: C, 66.99; H, 5.44. Calcd. for C₅H₅O₃: C, 67.41; H, 5.66%, UV λ<sub>max</sub> m<sub>s</sub> (Es): 246 (6480), 315 (3990). IR ν<sub>max</sub> cm⁻¹: 1680, 1620, 1580. NMR _{CDCl₃}: 1.52 (3H, d, J=5 cps), 2.93 (2H, d, J=7.5 cps), 4.73 (1H, m, J=20 cps).

BV 2 was crystallized from hexane in colorless prisms, mp 121-121.5°C. [α]_D^26 = -40 (c=1.0 CHCl₃). Anal. Found: C, 61.33; H, 5.17. Calcd. for C₉H₈O₄: C, 61.85; H, 5.19%. UV λ<sub>max</sub> m<sub>s</sub> (Es): 244.5 (5430), 315 (4190), IR ν<sub>max</sub> cm⁻¹: 1680, 1620, 1580. NMR _{Me₄Si}: 1.38 (3H, d, J=5 cps), 4.56 (1H, m, J=20 cps).

BV 3 was crystallized from colorless plates, mp 109-109.5°C. [α]_D^25 = 0 (c=1.0 CHCl₃). Anal. Found: C, 61.95; H, 5.14. Calcd. for C₉H₈O₄: C, 61.85; H, 5.19%. UV λ<sub>max</sub> m<sub>s</sub> (Es): 246 (5970), 315 (3880), IR ν<sub>max</sub> cm⁻¹: 3360, 1620, 1620, 1580. NMR _{Me₄Si}: 1.80 (3H, s), 3.24 (2H, s).
BV 4 was crystallized from chloroform in colorless needles, mp. 171°C. *Anal.* Found: C, 63.04; H, 5.30. Calcd. for C₈H₈O₃: C, 63.15; H, 5.30%.

These four compounds were soluble in acetone, ethyl acetate, ether and ethanol, scarcely soluble in hexane, and insoluble in water. The compounds gave purple color with aqueous ferric chloride on thin-layer chromatograms, or in ethanol solutions.

*Conversion of BV 2 to 3-methyl-8-hydroxyisocoumarin.*
A solution of 17 mg of BV 2 in 0.5 ml of glacial acetic acid was added to a solution containing 3 mg of iodine and 3 mg of red phosphorus in 1 ml of glacial acetic acid. The mixture was refluxed for 2 hr and then filtered through a sintered glass into 30 ml of 1% aqueous solution of sodium bisulfite. A crude crystal formed was filtered off and washed with water. The yield of the dried material was 7.8 mg. Needles from methanol, mp 101–102°C. *Anal.* Found: C, 68.5; H, 4.19. Calcd. for C₁₀H₈O₃: C, 68.18; H, 4.58%.

*Conversion of BV 3 to 3-methyl-8-hydroxyisocoumarin.*
A solution of 50 mg of BV 3 in 2 ml of 90°c formic acid, to which 4.0 mg of sodium carbonate was added, was refluxed for 1 hr and then cooled to room temperature. The crystals were collected on a filter, washed, dried and recrystallized from methanol to give 42 mg (93%) of crystals. The crystalline compound was identical with the compound obtained by dehydration of BV 2 with iodine and red phosphorus.

*Catalytic hydrogenation of 3-methyl-8-hydroxyisocoumarin.*
A solution of 42 mg of 3-methyl-8-hydroxyisocoumarin of methanol was hydrogenated at room temperature under atmospheric pressure in the presence of a small amount of prereduced Adam’s catalyst. A molar equivalent of hydrogen was absorbed in about 180 min. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure to give 18 mg (42% yield) of crystals; colorless prisms from acetone/water mp 56°C. IR spectrum and mp of this compound were identical with those of mellein.

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