654. *Studies in Mycological Chemistry. Part I. Flaviolin, 2(or 3) : 5 : 7-Trihydroxy-1 : 4-naphthaquinone, a Metabolic Product of* Aspergillus citricus *(Wehmer) Mosseray.*

By B. D. Astill and John C. Roberts.

The isolation of flaviolin, a metabolic product of *Aspergillus citricus*, is described. From a consideration of its properties and of the general properties of its triacetyl and *O*-trimethyl derivatives, together with the demonstration that its triacetyl derivative can be degraded to 3 : 5-dihydroxyphthalic acid, it is concluded that flaviolin is 2(or 3) : 5 : 7-trihydroxy-1 : 4-naphthaquinone.

Aspergillus citricus (Wehmer) Mosseray is a mould belonging to the *A. niger* group (Thom and Raper, "A Manual of the Aspergilli," Williams and Wilkins Coy., Baltimore, 1945). The particular strain of *A. citricus* used in the present work was originally used in these laboratories because of its ability to produce citric acid from glucose (B. Shaw, Thesis, London, 1950). When this mould was grown on an aqueous medium containing glucose and inorganic salts the substrate became gradually yellow (between approximately the fifth and the eleventh day of incubation at 29°) but, later, the colour changed rapidly

(within one day) to a dark port-wine red. This rapid colour change was found to be coincident with the virtual disappearance of glucose from, and the alkalinisation of, the substrate (see Fig. 1). This paper records our investigations on the pigment responsible for the production of this red colour.

Ether-extraction of the acidified, concentrated substrate yielded a dark red paste. Purification of this material by partition chromatography on columns of powdered cellulose, impregnated with a phosphate buffer, produced an amorphous, red substance which separated slowly from solutions in dioxan, or benzene–dioxan, as small, garnet-red rhombs which, under the normal conditions for determination of m. p., decomposed at about 250°. (Prolonged maintenance at considerably lower temperatures also led to decomposition.) A total of about 2 g. of crystalline pigment was obtained for this work from approximately 800 l. of substrate.

We propose the name, flaviolin, for this pigment because of its remarkable colour properties in aqueous solution (see below). Crystalline flaviolin apparently contained

FIG. 1.

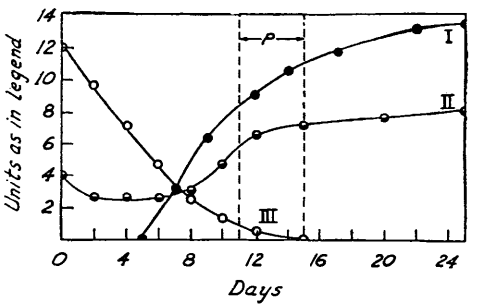


FIG. 2.

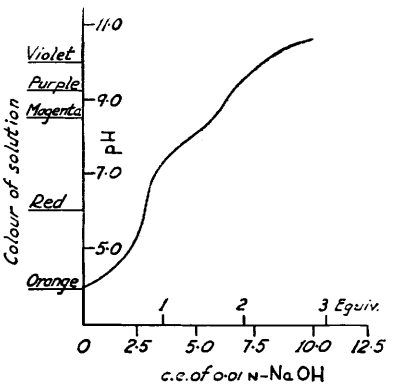


FIG. 1. *Course of flaviolin production. I, Concn. of flaviolin (mg./l.). II,* pH. *III, Concn. of glucose monohydrate (g./200 c.c.). P, Period during which substrates of individual flasks changed from yellow to red.*

FIG. 2. *Titration curve for flaviolin* ($C_{10}H_6O_5,\frac{1}{3}H_2O$, *equiv. to* 3NaOH).

dioxan of crystallisation as well as a small quantity of water of crystallisation. Loss of dioxan, which occurred quite readily, led to an amorphous product. The water of crystallisation was more tenaciously held. These properties invalidated any attempts to determine the molecular weight by crystallographic or other methods. Analysis detected no elements other than carbon and hydrogen and gave zero results for *C*-methyl (Kuhn–Roth) and methoxyl groups (Zeisel). With the aid of information, concerning the structure of flaviolin, which was later acquired from a study of its derivatives, we ascribe to amorphous flaviolin the molecular formula $C_{10}H_6O_5,\frac{1}{3}H_2O$.

An aqueous solution of flaviolin showed a spectacular series of colour changes as the pH of the solution was varied; in acid solution (pH <2·8) the colour was pure yellow, at neutrality red, and in alkaline solutions (pH >10) deep violet (see also Fig. 2). This, together with the general physical and chemical properties (see the Experimental section), suggested that flaviolin was a hydroxylated quinone of strongly acidic character. An electrometric titration revealed the presence of one strongly acidic group (pK'_a 4·7 at 21°) and two much weaker acidic groups (Fig. 2). The ultra-violet absorption spectrum (Fig. 3) revealed strong absorption in the 270—290- and 400—500-mμ regions, a characteristic of, among other compounds, hydroxyanthraquinones and hydroxy-1 : 2- and -1 : 4-naphthaquinones.

Attempts to acetylate flaviolin by most of the normal procedures led to intractable gums. However, acetic anhydride, with perchloric acid as catalyst, gave a good yield of a yellow, beautifully crystalline compound [triacetylflaviolin, $C_{10}H_3O_2(OAc)_3$] from which,

by gentle alkaline hydrolysis, flaviolin could be regenerated—proving that a Thiele reaction had not taken place.

It is well established (Macbeth, Price, and Winzor, *J.*, 1935, 325; Lugg, Macbeth, and Winzor, *J.*, 1937, 1597; Cooke, Macbeth, and Winzor, *J.*, 1939, 878; Arnstein and Cook, *J.*, 1947, 1023) that the ultra-violet absorption spectra of acetoxylated quinones closely approximate to the spectra of the parent unsubstituted quinones.* The absorption spectrum of the triacetate (Fig. 4) indicated it to be, beyond doubt, a derivative of 1 : 4-naphthaquinone (see Friedel and Orchin, " Ultraviolet Spectra of Aromatic Compounds," John Wiley and Sons Inc., New York, 1951, graph No. 254) and it showed a close similarity

FIG. 3. FIG. 4.

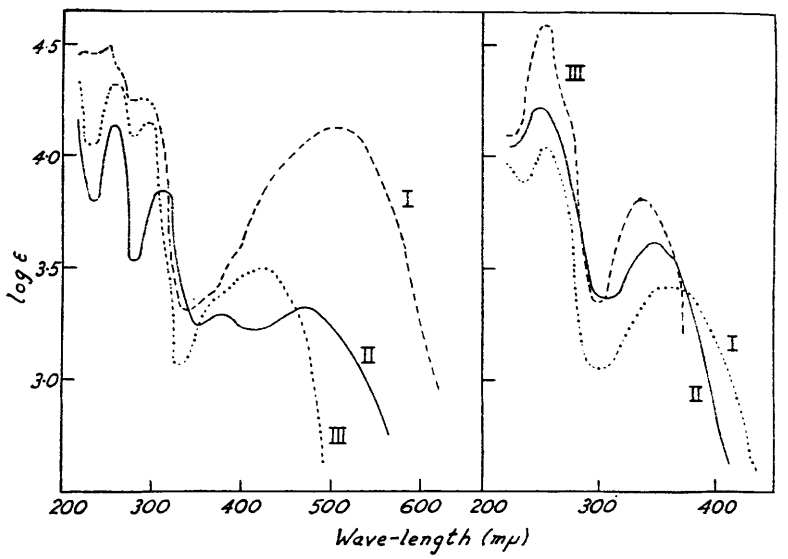
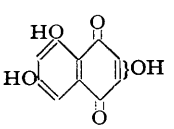
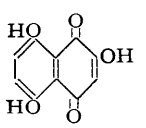


FIG. 3. *Ultra-violet absorption spectra of: I, naphthapurpurin; II, flaviolin; III,* O-*trimethylflaviolin.*

FIG. 4. *Ultra-violet absorption spectra of: I, triacetylnaphthapurpurin; II, triacetylflaviolin; III, diacetylquinizarin.*

to the spectrum of triacetylnaphthapurpurin (Fig. 4 also shows, for comparison, the spectrum of an acetoxylated anthraquinone). Flaviolin is, therefore, a trihydroxy-1 : 4-naphthaquinone.

Flaviolin   Naphthapurpurin

Methylation of flaviolin by means of methyl sulphate gave a poor yield of the *O*-trimethyl ether, $C_{10}H_3O_2(OMe)_3$, which crystallised in deep-yellow prisms (use of diazomethane led to no pure product), the ultra-violet absorption spectrum of which (Fig. 3) was similar to, but simpler than, that of flaviolin. This ether, when gently warmed with sodium hydroxide solution, dissolved to give a red solution—a reaction undoubtedly due to the production of the sodium salt of a 2(or 3)-hydroxy-dimethoxy-1 : 4-naphthaquinone. The assumption of the presence of a hydroxyl group in the 2(or 3)-position of flaviolin was also necessary to account for the strongly acidic nature of the pigment. It thus appeared that flaviolin contained at least one hydroxyl group in the quinonoid nucleus.

* In this connection it is instructive to note that the effect of an acetoxyl group (in contrast to that of a hydroxyl or methoxyl group) on the oxidation–reduction potential of quinones is very small (see Fieser and Fieser, " Organic Chemistry," 2nd Edn., D. C. Heath & Co., Boston, 1950, p. 755).

Oxidation of triacetylflaviolin with chromic acid in acetic anhydride–acetic acid solution yielded an acidic product which, after gentle hydrolysis to remove the acetyl groups, gave positive colour reactions for 3 : 5-dihydroxyphthalic acid. This hydrolysed oxidation product was, however, impure and could not be crystallised. Its identity was confirmed by conversion into 3 : 5-dimethoxyphthalic anhydride.

Flaviolin is, therefore, 2(or 3) : 5 : 7-trihydroxy-1 : 4-naphthaquinone. Attempts to synthesise the *O*-trimethyl ethers corresponding to the two possible structures are in progress.

So far as we are aware, this is the first recorded instance of the isolation of a 1 : 4-naphthaquinone derivative from a member of the *Aspergillus* genus. Three 1 : 4-naphthaquinone derivatives have been reported as metabolic products of certain *Fusarium* species : javanicin (Arnstein and Cook, *J.*, 1947, 1021), solanione (Weiss and Nord, *Arch. Biochem.*, 1949, **22**, 288), and fusarubin (Ruelius and Gauhe, *Annalen*, 1950, **569**, 38). Solanione and javanicin may be identical.

A 0·1% solution of flaviolin was inactive against a number of *Mycobacteria* (including *M. tuberculosis*). We are indebted to Messrs. Boots Pure Drug Co. Ltd. and Dr. D. A. Peak for this information.

Experimental

Isolation of Flaviolin.—*Aspergillus citricus* (National Collection of Type Cultures, No. 1692, Ac. 72) was kept in sub-culture on Czapek–Dox agar slopes. For production of the pigment the mould was grown in surface culture on either of the two following liquid media : (A) glucose monohydrate (50 g.), ammonium nitrate (2·25 g.), potassium dihydrogen phosphate (0·30 g.), hydrated magnesium sulphate (0·25 g.), and tap-water to 1 l.; (B) glucose monohydrate (5%) and inorganic salts (Czapek–Dox formula) in tap-water. There was little to choose between the two media from the point of view of pigment-production. Flat, round culture-flasks (see *Biochem. J.*, 1944, **38**, 456), each filled with 500 c.c. of medium, were sterilised (10 lb./sq. in. excess steam pressure for 20 min.) and, after having been inoculated with a heavy aqueous spore suspension, were kept at 29° ± 1° for 21—25 days. The substrate (60—70 l. from 140 flasks) was filtered through cotton-wool and concentrated to about 8 l. by passage through a recyclising, climbing-film evaporator. (The mycelium contained no appreciable amount of pigment and was discarded.) The syrupy concentrate was acidified (250 c.c. of concentrated hydrochloric acid) and separated from a dark brown sludge, either by centrifugation in a Sharples "Super Centrifuge," or by decantation after 24 hr. The clear, yellow liquid was extracted with ether. The pigment was removed by shaking the ethereal solution with successive quantities of 20-c.c. portions of a saturated solution of sodium hydrogen carbonate until the aqueous layer no longer became purple. The combined aqueous layers were strongly acidified and extracted with ether. Removal of the ether *in vacuo* yielded 1—2 g. of a dark red paste having a strong odour of valerian. Attempts to purify this crude material by crystallisation were normally unsuccessful. Attempted purification by adsorption chromatography, using a variety of adsorbents, or through the preparation of crystalline derivatives also failed.

In preliminary, small-scale experiments it was shown that the pigment could be separated from impurities by paper chromatography using *n*-butanol and aqueous phosphate buffer solutions. The following R_F values were observed for the pigment :

pH	7·0	7·5	8·0	8·5	9·0
R_F	0·90	0·80	0·75	0·65	0·05

For purification of the material on a larger scale the following method was adopted.

Powdered cellulose ("Solka-Floc") was mixed with sufficient aqueous phosphate buffer (0·5M; pH 8·0) to form a fairly liquid paste, separated by filtration, and "dried-off" in warm air. About 0·7 g. of the crude pigment, dissolved in 20 c.c. of butanol (previously equilibrated with the buffer solution), was chromatographed on a column (20 × 3 cm.) of the "buffered cellulose," and the chromatogram was developed with the same solvent. By elution there were obtained, in order : (i) a reddish-brown band (10 c.c.) and (ii) a cherry-red band (30 c.c.). Band (iii) (brown) remained at the top of the column. Fraction (iii) was washed from the cellulose with N-sodium hydroxide solution and the butanol fractions (i) and (ii) were washed with the same reagent. Each alkaline solution was washed exhaustively with ether to remove butanol. After acidification, ether-extraction, and removal of the solvent *in vacuo* fraction (i)

yielded an intractable gum; fraction (ii) gave a bright-red, odourless powder which crystallised from warm dioxan (or dioxan–benzene) in rhombs; fraction (iii) yielded an amorphous, brown substance which showed similar colour reactions (with alkaline reagents) to the product from fraction (ii). Fractions (i) and (iii) were not further investigated.

The yields of crystalline fraction (ii), *flaviolin*, varied from 50 to 250 mg. per batch of 140 culture-flasks. (On one occasion only was it found unnecessary to purify the crude extract by chromatography and direct crystallisation of this crude material yielded 940 mg. of flaviolin.)

General Properties of Flaviolin.—The pigment could be crystallised only from hot dioxan or dioxan–benzene; use of other solvents always led to gums. *Flaviolin* formed garnet-red rhombs decomposing at *ca.* 250° [Found (in a sample dried at room temperature *in vacuo* over phosphoric oxide): C, 56·1; H, 4·5; loss at 60° *in vacuo* over P_2O_5, 18·7. $C_{10}H_6O_5,\frac{1}{3}H_2O,\frac{1}{2}C_4H_8O_2$ requires C, 56·3; H, 4·2; loss in wt. (for conversion into $C_{10}H_6O_5,\frac{1}{3}H_2O$) 17·2%. Found (in material dried at 60° *in vacuo* over P_2O_5): C, 56·5; H, 3·75. Found (in a sample dried at room temperature *in vacuo* over H_2SO_4): C, 56·9; H, 3·3; OMe, 0; *C*-Me (Kuhn–Roth), 0. $C_{10}H_6O_5,\frac{1}{3}H_2O$ requires C, 56·6; H, 3·2%]. It was insoluble in light petroleum, sparingly soluble in benzene and water, and readily soluble in ether, ethanol, glacial acetic acid, chloroform, and warm dioxan. In concentrated sulphuric acid it gave an intense bright-red solution. An alkaline aqueous solution (originally deep-violet in colour) became yellow or brown after exposure to the air for some days. The colour of a solution of flaviolin in aqueous sodium hydroxide was destroyed by gently warming the solution with zinc dust and was subsequently restored by shaking the solution with air. Sodium hydrogen carbonate solution completely extracted flaviolin from its ethereal solution. Flaviolin yielded a red-violet ferric reaction (in water) and gave an amorphous precipitate with 2 : 4-dinitrophenylhydrazine solution in aqueous-methanolic sulphuric acid.

Electrometric Titration of Flaviolin.—An aqueous solution of the pigment was titrated with 0·01N-sodium hydroxide solution, a glass electrode and a Cambridge pH Meter (Fig. 2) being used.

Course of Flaviolin Production.—Some exploratory experiments were made with substrates containing inorganic salts together with 4 or 10% of glucose monohydrate. A substrate containing 6% of glucose monohydrate and inorganic salts as in medium (A) was chosen for detailed examination.

A batch of 500-c.c. conical flasks, each containing 200 c.c. of medium, was inseminated and incubated at 29° ± 1°. At intervals, the substrates from three flasks (chosen at random) were combined and used for the following estimations. (i) The pH was determined electrometrically. (ii) Glucose was determined volumetrically with Pavy's solution. (iii) The concentration of flaviolin was measured thus: 50 c.c. of substrate were acidified to pH 2·0, filtered, and extracted with ether; the ethereal solution was washed once with water, then dried, and the solvent evaporated; the residue was dissolved in ethanol and the intensity of absorption at 265 mμ measured spectroscopically; from a knowledge of the known absorption of pure flaviolin at 265 mμ the concentration of flaviolin in the alcoholic solution was calculated. The results are shown in Fig. 1.

O-*Triacetylflaviolin.*—One drop of aqueous 60% perchloric acid was added to a solution of flaviolin (30 mg.) in acetic anhydride (2 c.c.). The solution became warm and, after having been kept for 1 hr. (without application of heat), it was poured into water. A crystalline product (40 mg.) separated and was repeatedly crystallised from ethyl acetate, to give O-*triacetylflaviolin* (20 mg.) as yellow, slender prisms, m. p. 160—161° [Found: C, 57·4; H, 3·95; Ac, 39·8%; *M* (Rast), 378; *M*, calculated from density of substance (1·390 g./c.c.) and dimensions of unit cell (8·046 × 12·09 × 16·34 Å) and on the assumption of four molecules per unit cell, 333·1. $C_{10}H_3O_5Ac_3$ requires C, 57·8; H, 3·6; Ac, 38·9%; *M*, 332·3]. This material dissolved immediately in cold 2N-sodium hydroxide to give a deep pink colour, which, when the solution was warmed, became intense blue. Acidification and ether-extraction of this solution yielded a substance which was indistinguishable by colour tests (with solutions of sodium acetate, sodium hydrogen carbonate, and sodium hydroxide) and by paper chromatography from flaviolin. That at least one acetoxy-group (probably one in the 2- or 3-position) was readily hydrolysable was shown by the observation that when an ethereal solution of triacetylflaviolin was shaken with a saturated solution of sodium hydrogen carbonate the aqueous layier slowly developed a pink colour which could be discharged by acidification.

Three attempts at reductive acetylation of flaviolin failed. Neither the use of zinc and acetic acid in presence of acetic anhydride nor the use of magnesium, pyridine, and acetic anhydride led to the production of any pure product. In the third attempt triacetylflaviolin (100 mg.) was heated with acetic anhydride (3 c.c.), acetic acid (3 c.c.), and zinc dust (0·5 g.) on a

water-bath for 3 hr. No colour change was observed and triacetylflaviolin (90 mg.) was recovered.

O-*Trimethylflaviolin*.—A solution of flaviolin (75 mg.) and methyl sulphate (1·5 c.c.) in acetone (10 c.c.), with anhydrous potassium carbonate (1·5 g.), was heated under reflux on a water-bath for 8 hr. The mixture was cooled and the acetone largely removed in an air-stream. Addition of water (30 c.c.) led to the separation of an oil which subsequently crystallised. Recrystallisation (to constant m. p.) from ethanol yielded O-*trimethylflaviolin* (6 mg.) as golden-yellow prisms, m. p. 186—187° (decomp.) [Found : C, 62·3; H, 5·2; OMe, 36·4. $C_{10}H_3O_2(OMe)_3$ requires C, 62·9; H, 4·9; OMe, 37·5%]. In a second preparation (similar quantities) the crude material was dissolved in benzene, filtered, and chromatographed on alumina (10 × 1·2 cm.) (Spence, Type "H" which had been "acid washed"). Development with (i) benzene, (ii) benzene containing 10% v/v of ether, and (iii) benzene containing 2% v/v of dry ethanol produced a fast-moving, yellow band which was eluted (a reddish-brown band remained at the top of the column). Removal of the solvents *in vacuo* from the eluate and crystallisation of the residue from aqueous methanol yielded *O*-trimethylflaviolin (10 mg.) as slender, deep-yellow prisms, m. p. 191° (decomp.) (Found : C, 62·9; H, 4·9; OMe, 34·6%). This ether, when warmed with aqueous alcoholic sodium hydroxide, gave a red solution. Acidification and ether-extraction yielded a yellow ethereal solution which, when shaken with the alkaline reagent, immediately produced a red colour in the aqueous phase.

Treatment of flaviolin with an excess of ethereal diazomethane for 2 hr., and removal of the excess of reagent, gave a fluorescent solution (possibly due to the formation of a naphthindazole) which yielded, after evaporation of the ether, a residue from which no pure product was isolatable.

Degradation of O-*Triacetylflaviolin*.—To a solution of *O*-triacetylflaviolin (300 mg.) in acetic anhydride (9 c.c.) and acetic acid (5 c.c.) were added 5 c.c. of a solution made by dissolving chromic anhydride (2·5 g.) in water (2 c.c.) and acetic acid (25 c.c.). The mixture was kept at 60—70° for 1 hr., cooled, and diluted with water. The solution was extracted with chloroform (3 × 25 c.c.), and the chloroform extracts (which yielded a small amount of unchanged acetate) were discarded. The aqueous solution was extracted with ether (3 × 50 c.c.). The combined ethereal solutions were washed with water, then dried ($MgSO_4$), and the solvent was removed. The residue (120 mg.) of impure, partly crystalline, diacetoxyphthalic acid was dissolved in 0·2N-sodium hydroxide (17·5 c.c.) and heated on a water-bath for ¾ hr. This solution, after filtration, acidification, exhaustive extraction with ether, and removal of the solvent, yielded a discoloured, partly crystalline product (100 mg.). This material was readily soluble in sodium hydrogen carbonate solution; it gave a reddish-purple ferric reaction and an immediate magenta colour (fading slowly) with an aqueous solution of bleaching powder. This product was undoubtedly impure 3 : 5-dihydroxyphthalic acid but attempts at purification were unsuccessful. It was therefore converted into dimethoxyphthalic anhydride as follows. To a neutralised solution of the acid in water (20 c.c.) was added 35% lead acetate solution (25 c.c.). The washed lead salt was triturated with successive portions of dilute sulphuric acid (10%), and the organic acid recovered (75 mg.) by ether-extraction. To an ethereal solution of the acid was added an excess of ethereal diazomethane. After 16 hr. a negative ferric reaction was obtained and the excess of diazomethane and the ether were removed. The residue was gently heated under reflux with 0·5N-aqueous sodium hydroxide (10 c.c.) for 1 hr. Acidification and ether-extraction yielded crude 3 : 5-dimethoxyphthalic acid (60 mg.). This acid was heated to 170—180° for 15 min. and the product sublimed at 140—150°/0·25 mm. A considerable amount of non-sublimable material remained. The sublimate was purified by two further sublimations, by two crystallisations from benzene–light petroleum (b. p. 60—80°), and finally by sublimation. The product (10 mg.) [Found : C, 57·5; H, 4·3; OMe, 28·9. Calc. for $C_8H_2O_3(OMe)_2$: C, 57·7; H, 3·9; OMe, 29·8%] formed white needles, m. p. 146—147·5° which changed to 146·5—148·5° after admixture with synthetic 3 : 5-dimethoxyphthalic anhydride (Fritsch, *Annalen*, 1897, **296**, 344) of m. p. 147—148·5°.

We thank Professor F. E. King for his interest and encouragement and Dr. S. C. Wallwork for determining the unit-cell dimensions of triacetylflaviolin. One of us (J. C. R.) thanks the Leverhulme Trustees for the award of a Research Fellowship during the tenure of which some of the preliminary work leading to this investigation was performed.

THE UNIVERSITY, NOTTINGHAM. [*Received, June 4th*, 1953.]