Studies in the Biochemistry of Micro-organisms

112. ANTHRAQUINONE PIGMENTS OF STRAINS OF *CLADOSPORIUM FULVUM* COOKE

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The phytopathogenic fungus *Cladosporium fulvum* Cooke was shown by Hasper (1925) to produce a pigment giving a purple reaction with alkalis. During mutation experiments, Day (1957) observed that a few colonies obtained from spores irradiated with X-rays or ultraviolet light differed in pigmentation from normal colonies. Two types of mutants giving a red colour with alkali were obtained in addition to green mutants giving no reaction. On further investigation Day & Sherratt (1958) found that yellow pigments could be extracted with organic solvents from the red mutants and from the purple wild-type of organism grown on oatmeal slopes. The absorption spectra of these extracts were recorded; these results, together with solubilities and colour reactions, suggested that the pigments were hydroxylated anthraquinones.

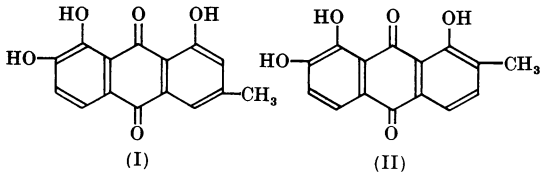
Through the courtesy of Dr P. R. Day, who furnished cultures, we were enabled to examine pigment production by three strains of *Cladosporium fulvum*, the wild strain no. 144 and two mutants nos. 40A–1 and 31–8. Various media were employed; the best growth and pigment production of strains 144 and 40A–1 were obtained on a Czapek–Dox medium supplemented by Marmite. No pigment was obtained from strain 31–8 on any of the media tried (including oatmeal as suggested by Dr Day).

The dried defatted mycelium of mutant 40A–1 yielded a pigment of m.p. 262–263°, which proved to be frangula-emodin, as obtained from the higher fungus *Cortinarius sanguineus* (Kögl & Postowsky, 1925; Birkinshaw & Gourlay, 1961), and shown to be identical with an authentic specimen.

The wild strain (144) produced a pigment crystallizing in orange needles from benzene, m.p. approx. 310° (decomp.), giving the following colour reactions: with concentrated sulphuric acid, strong purple–violet becoming pure violet after 3 min.; with aqueous sodium hydroxide, strong purple–blue becoming violet–blue and stable after 3 min. It had the empirical formula $C_{15}H_{10}O_5$ and contained one *C*-methyl group. It had three hydroxyl groups, since acetylation gave a triacetyl derivative. On methylation di- and tri-methyl derivatives were obtained. These facts, together with the light-absorption spectrum, indicated that the product was a new trihydroxymethylanthraquinone isomeric with emodin. We have allocated to this product the trivial name *cladofulvin*.

The absorption peak in the visible region at λ449 mμ indicated that there are two α-hydroxy groups in the cladofulvin molecule. By oxidizing the *O*-dimethyl derivative of cladofulvin with potassium permanganate in acetone, hemipinic acid was obtained. The arrangement of the hydroxyl groups in one aromatic ring was thus decided: they must be in the 1,2-positions. The third hydroxyl group and the methyl group must be substituents of the other aromatic ring of the anthraquinone structure. The placing of this third hydroxyl group, which must occupy an α-position, can be inferred from the infrared spectrum of cladofulvin. This shows two peaks in the carbonyl-stretching frequency region, at 1655 and at 1615 cm.$^{-1}$. The former peak is evidently due to an unassociated carbonyl group, the latter to a hydrogen-bonded carbonyl group. A similar double peak in this region is shown by aloe-emodin, which has bands at 1674 and 1626 cm.$^{-1}$ (Hay & Haynes, 1956) and by nataloe-emodin with bands at 1662 and 1626 cm.$^{-1}$ (Haynes & Henderson, 1960). The *O*-dimethyl derivative of cladofulvin also shows two bands (1657 and 1624 cm.$^{-1}$) in this region, but in the *O*-trimethyl derivative where hydrogen-bonding is excluded the band corresponding to the higher-frequency peak lies at 1661 cm.$^{-1}$ and there is no peak at about 1620 cm.$^{-1}$.

The hydroxyl groups are thus located in the 1,2,8-positions in the cladofulvin molecule. If it is assumed that the methyl group occupies a β-position as commonly found in the fungal anthraquinones and as indicated by the reduction of cladofulvin to β-methylanthracene with zinc dust, the methyl group could occupy either the 6- or the 7-position. On biogenetic grounds, the position 6 might be preferred, but the structure (I) thus obtained has already been allocated to nataloe-emodin (Haynes & Henderson, 1960). This structure was confirmed by synthesis (Haynes, Henderson & Tyler, 1960). Since cladofulvin clearly differs in properties from nataloe-emodin, the structure of cladofulvin should be 1,2,8-trihydroxy-7-methylanthraquinone (II).



(I) (II)

EXPERIMENTAL

Combustion analyses were by Dr A. Schoeller. The infrared spectra were determined by Miss I. Tanner of Parke, Davis and Co. Ltd. All melting points are uncorrected.

Cultures. Three strains of *Cladosporium fulvum*, received from Dr P. R. Day, and having the catalogue nos. indicated in parentheses, were used. These were the wild-type strain (144) and two mutants (40A-1 and 31–8). These mutants are the 'red mutants' examined by Day & Sherratt (1958), so named since they give a red colour with alkali.

Medium. Of various media tried for growth and pigment production, including the oatmeal medium of Dr Day, the most suitable was found to be the standard Czapek–Dox medium with a supplement of 0·15% of Marmite. The medium was distributed in 1 l. conical flasks (350 ml. per flask), sterilized by steaming on 3 successive days, inoculated and incubated at 24°. Only two of the strains, nos. 144 and 40A-1, produced appreciable amounts of pigment; strain 31–8 was ineffective on all media tried.

Emodin from strain 40A-1 (*a mutant*). The dried mycelium was extracted first with light petroleum (b.p. 40–60°) to remove fatty materials, then with ether. The product obtained from the ether was purified by crystallization from acetic acid. It had m.p. 262–263°, unchanged on admixture with authentic frangula-emodin, m.p. 263° (Found: C, 66·6; H, 3·8. Calc. for $C_{15}H_{10}O_5$: C, 66·7; H, 3·7%). Acetylation of the product with acetic anhydride–H_2SO_4 gave the triacetate, m.p. 194–195°, unchanged on admixture with emodin triacetate of the same m.p. This product is the one mentioned by Day & Sherratt (1958) with light-absorption maxima at λ220, 252, 267, 288 and 435 mμ, which are in fair agreement with those recorded for emodin (Birkinshaw, 1955).

Product from strain 144 (*wild type*). In a typical experiment the dried mycelium (300 g.) obtained from 100 flasks was defatted with light petroleum and extracted with ether. The crude orange–yellow pigment, cladofulvin (2·9 g.), thus obtained was purified by recrystallization from benzene, giving orange needles of m.p. over 310° (decomp.). It crystallizes from dioxan in bright-red needles containing solvent of crystallization, which can be removed by drying at 100° in high vacuum. Light-absorption values in ethanol: $\lambda_{max.}$ 235, 270 and 449 mμ; log ε 4·43, 4·43, 4·05 respectively. Infrared peaks in Nujol mull: 3379, 3200, 1655, 1615, 1574, 1273, 1190, 1168, 1151, 1112, 1066, 1015, 889, 872, 849, 824, 772, 749, 722 cm.$^{-1}$ (Found: C, 66·3; H, 3·6; OMe, 0·0; *C*-Me, 4·4. $C_{15}H_{10}O_5$ requires C, 66·7; H, 3·7; 1 *C*-Me, 5·7%). The following colour reactions were given by the product: with conc. H_2SO_4, strong purple–violet becoming stable pure violet after 3 min.; with 2N-NaOH, strong purple–blue, stable violet–blue after 3 min.; with 2N-Na_2CO_3, as for NaOH; in $NaHCO_3$, insoluble; with boroacetic anhydride plus acetic anhydride or benzene, reddish.

Derivatives

Cladofulvin triacetate (1,2,8-*triacetoxy*-7-*methylanthraquinone*). Cladofulvin (0·18 g.) in acetic anhydride (5 ml.) plus conc. H_2SO_4 (0·1 ml.) was kept at 90° for 1 hr. The solid obtained by pouring the cooled mixture into ice–water was recrystallized thrice from methanol and thus yielded the *triacetate* as golden-yellow plates (0·12 g.), m.p. 185–186° (Found: C, 63·7; H, 4·2; Ac, 33·5. $C_{21}H_{16}O_8$ requires C, 63·6; H, 4·1; 3 Ac, 32·5%).

O-*Dimethylcladofulvin* (8-*hydroxy*-1,2-*dimethoxy*-7-*methylanthraquinone*). Cladofulvin (0·97 g.) was suspended in acetone (50 ml.) and treated with excess of diazomethane in ether. After 1 hr. the clear brownish red solution was evaporated to dryness and the product (1 g.) was crystallized several times from acetic acid, then from ethyl acetate. Yellow needles of O-*dimethylcladofulvin* were thus obtained, m.p. 169–171°. Infrared-absorption peaks in Nujol mull: 1657, 1624 cm.$^{-1}$ (Found: C, 68·7; H, 4·7; OMe, 19·9. $C_{17}H_{14}O_5$ requires C, 68·45; H, 4·7; 2 OMe, 20·7%).

O-*Trimethylcladofulvin* (1,2,8-*trimethoxy*-7-*methylanthraquinone*). A solution of cladofulvin (0·48 g.) in anhydrous acetone (150 ml.) was treated with dimethyl sulphate (5 ml.) and refluxed for 14 hr. over K_2CO_3 (10 g.). The solid was removed by filtration and the filtrate evaporated, giving a yellow oily residue. This was shaken with 2N-NaOH (30 ml.) and left for 2·5 days. A yellowish brown product was thus obtained, which, on recrystallization from methanol, gave fine yellow needles of O-*trimethylcladofulvin*, m.p. 212–214°. Infrared peak in Nujol mull: 1661 cm.$^{-1}$ (Found: C, 69·5; H, 4·9; OMe, 29·3; mol.wt. by isothermal distillation, 325. $C_{18}H_{16}O_5$ requires C, 69·2; H, 5·2; 3 OMe, 29·8%; mol.wt. 312).

Degradations

Oxidation of dimethylcladofulvin. The dimethyl ether (1·25 g.) dissolved in acetone was treated gradually, with shaking, with powdered $KMnO_4$ (8 g.). The mixture was filtered. From the MnO_2, by extraction with water, acidification and transference of the product to ether, a brown gum was obtained which on sublimation *in vacuo* yielded colourless needles, m.p. 150–155°. From the acetone, unoxidized material was recovered (0·36 g.) which was retreated with $KMnO_4$. The material was worked up as before. The combined sublimates were recrystallized from ethyl acetate as needles (20 mg.), m.p. 165–165·5°, unchanged on admixture with authentic hemipinic anhydride (m.p. 164–165°) (Found: C, 57·7; H, 4·1; OMe, 29·5. Calc. for $C_{10}H_8O_5$: C, 57·7; H, 3·9; 2 OMe, 29·8%).

Heating cladofulvin with zinc dust. Cladofulvin (0·20 g.) was intimately mixed with zinc dust (10 g.) and heated in a tube closed at one end and drawn out to a capillary. The condensate on the cool part of the tube was collected. This material, giving a yellow-green fluorescence in ultraviolet light, was dissolved in light petroleum (b.p. 40–60°) and chromatographed on alumina. On elution with benzene (2%, v/v) in light petroleum and evaporation of the solvent a very small amount of product was obtained, m.p. 175°, which did not depress the m.p. of authentic 2-methylanthracene. Light-absorption values in ethanol: $\lambda_{max.}$ 253·4, 325, 340, 357, 377; for 2-methylanthracene 253·2, 325, 340, 357, 377 mμ.

SUMMARY

1. Three strains of *Cladosporium fulvum* Cooke, a wild strain and two mutants, were grown on Czapek–Dox medium supplemented by Marmite, and the mycelial pigments were examined.

2. The pigment produced by one mutant was frangula-emodin; no pigment was obtained from the other mutant examined.

3. The wild strain produced a new trihydroxymethylanthraquinone named cladofulvin, which yielded a triacetate and di- and tri-*O*-methyl derivatives. Distillation with zinc dust yielded 2-methylanthracene; oxidation of the dimethyl derivative of cladofulvin gave hemipinic acid.

4. These facts, together with ultraviolet- and infrared-absorption spectra, indicated that cladofulvin has the structure 1,2,8-trihydroxy-7-methylanthraquinone.

We thank Miss I. Tanner of Parke, Davis and Co. Ltd. for the infrared determinations. For the Uvispek instrument used we are indebted to a grant from the Central Research Fund of London University.

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Biochemical Studies on the Developing Avian Embryo

5. UBIQUINONE AND SOME OTHER UNSAPONIFIABLE LIPIDS*

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The presence of ubiquinone-50 in embryonic chick heart and liver was reported by Brand, Dahl & Mahler (1960). The ubiquinone content of mitochondria was of the same order as that of similar preparations from adult organisms. It was of interest to know whether the egg provided the developing embryo with all the ubiquinone it required, or if the embryo was capable of synthesizing the quinone. This paper describes work on the presence of ubiquinone in egg yolk and on the increasing amount found in the embryo during development.

A preliminary experiment showed that in hen's eggs the unsaponifiable lipids are concentrated in the yolk, whereas negligible amounts are present in the albumin. Lipids are a very important constituent of the yolk and in fact make up some 33% of its total weight. Of the lipids present, triglycerides amount to 62·3% and phospholipids to 32·8%, sterol, vitamins and provitamins adding up to the remaining 4·9% (Romanoff & Romanoff, 1949). In this study of avian-embryo lipids, the components examined include sterol, ergosterol, vitamins A and E, as well as ubiquinone, all or some of which may play fundamental roles in the development of the embryo and in hatching.

* Part 4: Brand, Dahl & Mahler (1960).

MATERIALS

Cornish White Rock hatching eggs were obtained from the Farm Bureau Hatchery, Harrisburgh, Pa., U.S.A. Column chromatography was carried out on neutral alumina (type AG7, Bio-Rad Laboratories, Richmond, California, U.S.A.), and silica gel G (E. Merck, Darmstadt, Germany) was used as adsorbent for thin-layer chromatography. Light petroleum (b.p. 30–60°) was dried over sodium wire and distilled. Analytical grade anhydrous diethyl ether was obtained from Mallinckrodt (New York). A supply of tocopherols (DL-tocol, DL-α-tocopherol, DL-β-tocopherol, D-γ-tocopherol and DL- 2-tocopherol) were kindly given by Dr J. Green of Vitamins Ltd., Tadworth, Surrey.

METHODS

Spectrophotometry. Ultraviolet-absorption spectra were measured in either the Cary 11 or the Cary 14 recording spectrophotometer with cyclohexane (Fisher Spectroanalysed Certified Reagent) as solvent. The Bausch and Lomb Spectronic 20 colorimeter was used for measurements in the visible range.