LV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LIII. THE CRYSTALLINE COLOURING MATTERS OF *FUSARIUM CULMORUM* (W. G. SMITH) SACC. AND RELATED FORMS

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The genus *Fusarium* (Link) is one of the most important genera of fungi from the economic standpoint. Species in the genus are distributed on plants, animals, raw materials, excreta and in soil and water, and many of them play an important part from the phytopathological point of view as causative agents of plant diseases. During recent years the classification of species in the genus has engaged the attention of many mycologists, notably Appel & Wollenweber [1910], Sherbakoff [1915], Brown & Horne [1924], Wollenweber [1931] and Wollenweber & Reinking [1935]. As one basis of classification the very varied colours of many species of *Fusarium* have been used, the colours ranging from yellow, orange, red, purple to blue according to species. The opinion has been expressed by many investigators that the colour of the culture is a function of the *p*H of the medium, so that one and the same culture may be orange or yellow coloured at an acid reaction, the colour changing to red or blue when the medium becomes alkaline. Several attempts have been made to isolate the different pigments in a state of chemical purity, but, with one possible exception which will be referred to later, without success. Bessey [1904] investigated the conditions of pigment formation by *Fusarium culmorum* (W. G. Smith) Sacc. Bessey isolated a pigment in a crude form and showed that it is yellow in colour when the medium is acid, changing to red and violet when the reaction is neutral or alkaline and *vice versa*. These conclusions were confirmed by Seliber [1910] and Butler [1926] working with other species of *Fusarium*. Bezssonoff [1914], working with an organism to which he gave the name *F. orobanchus* (this organism is not listed by Wollenweber & Reinking [1935]), claimed to have isolated two crystalline pigments, which he described as a yellow anthocyanin-like substance and a red carotene-like substance. No melting points or other physical characteristics other than solubilities were given, nor were any chemical analyses recorded. Further observations on the conditions governing pigment formation by species of *Fusarium* are recorded by Smith & Swingle [1904], Appel & Wollenweber [1910], Wollenweber [1914], Reinking & Wollenweber [1927], Brown & Horne [1924], and Brown [1926; 1935].

In the work about to be described a successful attempt has been made to isolate in a crystalline form and in a state of chemical purity the pigments present in *F. culmorum* (W. G. Smith) Sacc. and related forms. *F. culmorum*

is widely distributed and is one of the causative agents of foot rot in wheat. All the strains and forms related to *F. culmorum* which were examined produced a brilliant carmine-coloured pigment on a variety of different media, the colour of the pigment changing on acid media to a golden yellow colour, and on alkaline media to a purple colour.

Two different pigments were isolated, one a beautifully crystalline red pigment for which the name *rubrofusarin* is proposed, and the other a golden yellow micro-crystalline pigment which has been termed *aurofusarin*. Rubrofusarin and aurofusarin were isolated, in amounts varying with the species used and with the cultural conditions employed, from the dried mycelium of two different strains of *F. culmorum* (W. G. Smith) Sacc., from *F. culmorum* (W. G. Smith) Sacc. var. *cereale* (Cke.) Wr., from *F. culmorum* (W. G. Smith) Sacc. var. *lethaeum* Sherb., and from *F. graminearum* Schwabe. In addition to rubrofusarin and aurofusarin a colourless crystalline substance for which the name *culmorin* is proposed was isolated from the dried mycelium of one strain of *F. culmorum*.

Rubrofusarin, $C_{15}H_{12}O_5$, is a monomethyl ether containing one methyl group and two active hydrogen atoms, and from it the following crystalline derivatives were prepared: the mono- and di-acetates, the mono- and di-methyl ethers, dibromorubrofusarin, nor-rubrofusarin (i.e. demethylated rubrofusarin), nor-rubrofusarin diacetate and the ferrichloride of rubrofusarin dimethyl ether. The opinion is tentatively expressed that rubrofusarin, in spite of its red colour (nor-rubrofusarin is golden yellow in colour) may be the monomethyl ether of a methyltrihydroxyxanthone, isomeric but not identical with ravenelin recently described as a metabolic product of *Helminthosporium Ravenelii* Curtis (Raistrick *et al.* 1936). Rubrofusarin does not give any marked colour changes with different alkalis and hence is not the pigment responsible for the colour changes at different *p*H values of cultures of *Fusarium culmorum*.

Aurofusarin, $C_{30}H_{20}O_{12}$, is a dimethyl ether and the following crystalline derivatives were prepared: the dibenzoate, di-*p*-bromobenzoate and dianisate; reduced aurofusarin, $C_{30}H_{24}O_{12}$, and its hexabenzoate, hexa-*p*-bromobenzoate and hexa-anisate. Owing to the slight solubility of aurofusarin and all its derivatives in almost all solvents, great difficulty was experienced in determining the molecular weight of aurofusarin. The empirical formula $C_{30}H_{20}O_{12}$ is therefore tentatively suggested with the reservation that future work may show the need for altering it. On treating aurofusarin with alkalis or alkaline earths, insoluble highly pigmented compounds were obtained, the colours of which vary from wine-red with MgO to dark blue with KOH. It thus appears fairly certain that the different colours recorded by many authors for cultures of the same strain of *Fusarium culmorum* and related forms under different cultural conditions are due to the presence of aurofusarin, the colour of which in the medium is determined partly by the *p*H of the medium and partly by the available bases present in the medium.

Culmorin, $C_{15}H_{26}O_2$, was obtained in beautiful colourless needles. It is a neutral substance, containing two active hydrogen atoms, and the two oxygen atoms must be present as hydroxyl groups since a crystalline diacetate, and a crystalline di-*p*-bromobenzoate were prepared.

This investigation is being extended to other species of *Fusarium*, and work is in progress having as its object the elucidation of the molecular structures of rubrofusarin, aurofusarin and culmorin.

Experimental

History of the species used

(1) *Fusarium culmorum* (W. G. Smith) Sacc., L.S.H.T.M. Catalogue No. F. 16, received from Prof. W. Brown, Imperial College of Science and Technology, London, November 1934. Isolated by Dr G. W. Padwick from blighted wheat seedlings in November 1934 (Cat. No. F. 209).

(2) *F. culmorum*, Cat. No. F. 17.

(3) *F. culmorum* (W. G. Smith) Sacc. var. *cereale* (Cke.) Wr., Cat. No. F. 18.

(4) *F. culmorum* (W. G. Smith) Sacc. var. *lethaeum* Sherb., Cat. No. F. 19.

(5) *F. graminearum* Schwabe, Cat. No. F. 29 received from Dr G. W. Padwick, November 1935. Isolated from a wheat ear at New Norway, Alberta in 1929.

Cultures F. 17, F. 18 and F. 19 were purchased from the Centraalbureau voor Schimmelcultures, Baarn, Holland, in March 1935.

Stock cultures of these organisms were maintained throughout the work either on a potato-agar medium of the following composition: broth from 200 g. potatoes; glucose, 15 g.; agar-agar, 20 g.; H_2O, to 1 litre, or on a soil medium containing 200 g. fine sieved soil, 10 g. finely ground maize meal and 80 ml. H_2O in a 500 ml. Erlenmeyer flask. This medium was recommended to us by Dr G. W. Padwick.

Cultural conditions

Preliminary experiments on the optimum conditions for growth of, and pigment production by, *F. culmorum* (Cat. No. F. 16) showed that highly pigmented but differently coloured growths could be obtained on a variety of media over a wide range of cultural conditions. Difficulties were met with owing to the fact, which is so often experienced in working with different species of *Fusarium*, that the cultures very readily gave rise to saltants. The optimum temperature for growth and pigment production was found to be 24°. Good growth and pigment production were observed on both Czapek-Dox and Raulin-Thom media adjusted to an initial *p*H varying from 4 to 9, but rubrofusarin and aurofusarin were found to be produced in larger yield and in a higher state of purity on Raulin-Thom medium than on Czapek-Dox medium. It was also found that while both pigments are produced on Raulin-Thom medium adjusted to an initial *p*H of 4, 5, 6, 7, 8 and 9, rubrofusarin is produced in the largest yields at *p*H 8, while aurofusarin is produced in the largest yields and in a form most easily isolated in a pure condition at *p*H 4.

Preparation, properties and derivatives of rubrofusarin

The medium used for the preparation of rubrofusarin was a Raulin-Thom medium of the following composition: glucose, 75 g.; tartaric acid, 4 g.; diammonium tartrate, 4 g.; $(NH_4)_2HPO_4$, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4,7H_2O$, 0·07 g.; $FeSO_4,7H_2O$, 0·07 g.; distilled water, 1500 ml. 350 ml. of this medium were distributed in each of 100 one-litre conical flasks and after sterilization the requisite amount, previously determined, of sterile 2*N* NaOH was added to each flask to bring the *p*H to 8·0. Each flask was sown with a suspension of *F. culmorum* washed from a potato-dextrose-agar slope about 17 days old, one slope being used for sowing two flasks. The flasks were incubated at 24° in the dark until the glucose content was practically zero, usually 23–25 days. With F. 16 there was a good undulating surface growth of

mycelium varying in colour from yellow, yellow-brown to a dark greenish grey hue with occasional pink or red patches often round the edge in contact with the glass. The mycelium was filtered, washed, pressed, dried *in vacuo* and ground to a fine powder in a coffee mill. It was then extracted with light petroleum, B.P. 40–50°, until the extracts were colourless. Rubrofusarin separated from the petroleum in the form of glistening bronze plates. This was filtered, washed, dried and weighed. The yields of crude rubrofusarin obtained in a number of typical experiments at different initial *p*H values and with different strains of *F. culmorum* and related species are given in Table I. 100 flasks were used in each case. In all about 25 g. of rubrofusarin have been prepared.

Table I

Strain of *F. culmorum* Cat. No.	Initial *p*H of medium	Incubation period in days	Weight of mycelium g.	Weight of crude fat g.	Weight of crude rubrofusarin g.
F. 16	4	39	443	70	0·15
F. 16	5	34	485	85	0·85
F. 16	6	34	480	120	2·30
F. 16	7	23	457	124	3·59
F. 16	8	25	417	101	5·16
F. 16	9	26	480	106	Trace
F. 16	9	28	382	104	0·85
F. 17	8	34	397	89	0·38
F. 18	8	35	321	71	0·14
F. 19	8	38	335	76	0·39
F. 29	4	40	414	75	0·10
F. 29	8	22	520	140	3·09
F. 29	8	22	500	133	3·80
F. 29	8	21	515	150	4·40

The crude rubrofusarin was purified by recrystallization from light petroleum, B.P. 80–100°, benzene or ethyl alcohol, from which solvents it separates in red crystals appearing as glistening plates in the solvent, and as orange-red blunt-ended needles under the microscope. Rubrofusarin contains no N, S or Cl. (Found (Schoeller) on different samples crystallized from different solvents and dried to constant weight *in vacuo* at 105°: (*a*) C, 66·40, 66·26; H, 4·56, 4·38; CH_3O, 11·12, 10·91 %. (*b*) C, 66·17, 66·11; H, 4·62, 4·60; CH_3O, 11·16, 11·18 %. $C_{15}H_{12}O_5$, i.e. $C_{14}H_9O_4(OCH_3)$ requires C, 66·15; H, 4·44; CH_3O, 11·40 %. Mol. wt. cryoscopic in phenol (Dr A. E. Oxford), 274. $C_{15}H_{12}O_5$ requires 272.)

On oxidation with chromic acid (Kuhn-Roth method) evidence of the presence of one side-chain methyl group was obtained (found: 101·4 and 107·0 % of one $CH_3.COOH$).

Active hydrogen (Roth). In pyridine 5·700 mg. gave 1·07 ml. methane at 21° and 1·20 ml. at 95°, corresponding to 2·28 and 2·56 active hydrogen atoms respectively.

Rubrofusarin melts at 210–211° to a deep red liquid and readily sublimes unchanged in a high vacuum. It is slightly soluble in all the usual organic solvents, forming orange-coloured solutions. 0·5 g. dissolved in 225 ml. boiling alcohol, in 37 ml. boiling benzene and in 680 ml. boiling light petroleum (80–100°) and from these solvents there separated on cooling 0·42, 0·42 and 0·43 g. respectively. It is insoluble in water and HCl. It dissolves in conc. NH_4OH to give a bright yellow solution. It is apparently only slightly soluble in NaOH and KOH, but this is probably due to the slight solubility of the sodium and potassium salts in NaOH and KOH, since the red colour of rubrofusarin changes to yellow on the addition of these alkalis. It gives a colourless or very pale straw

coloured solution in concentrated H_2SO_4 which becomes a deep reddish brown on the addition of $K_2Cr_2O_7$. An alcoholic or aqueous alcoholic solution gives a greenish brown colour with $FeCl_3$.

Rubrofusarin monoacetate $C_{14}H_8O_3(OCH_3)(O.COCH_3)$. Rubrofusarin (0·3 g.), fused sodium acetate (0·4 g.), acetic anhydride (2·5 ml.) and glacial acetic acid (1·2 ml.) were heated at 140–150° for 20 min. The deep red solution became greenish brown in colour. After cooling, water (15 ml.) was added and the greenish yellow solid was filtered, washed and dried, wt. 0·3 g. It was crystallized from glacial acetic acid, benzene and finally from glacial acetic acid, as large golden yellow hexagonal prisms, M.P. 211°, to a red liquid. (Found (Schoeller): C, 64·89, 64·84; H, 4·59, 4·73; OCH_3, 9·24, 9·50 %. $C_{17}H_{14}O_6$ requires C, 64·94; H, 4·49; OCH_3, 9·88 %.) Acetyl estimations (Roth) indicated the presence of one acetyl group.

Rubrofusarin diacetate, $C_{14}H_7O_2(OCH_3)(O.COCH_3)_2$. A mixture of rubrofusarin (0·5 g.), acetic anhydride (7 ml.) and pyridine (4 ml.) was heated for 4 hours on the steam-bath. The yellow crystalline product which separated was filtered after cooling overnight, wt. 0·4 g. It was recrystallized from glacial acetic acid and finally from dioxan; almost colourless rods, M.P. 260°. (Found (Weiler): C, 64·36, 64·12; H, 4·58, 4·37; OCH_3 (macro-estimation), 8·58 %. $C_{19}H_{16}O_7$ requires C, 64·03; H, 4·53; OCH_3, 8·71 %.)

Rubrofusarin monomethyl ether, $C_{14}H_8O_3(OCH_3)_2$. Rubrofusarin (0·5 g.) was dissolved in boiling benzene (250 ml.), the solution cooled and an excess of an ethereal solution of diazomethane added. The orange-coloured solution immediately became a deep brownish purple, and there was a feeble evolution of nitrogen. When it was allowed to stand overnight, the colour became golden yellow. The solution was filtered, the solvents removed *in vacuo* and the residual yellow solid washed with a little cold methyl alcohol which removed some sticky material, wt. 0·33 g. It was recrystallized first from 60 % aqueous dioxan, then from 50 % benzene-light petroleum and finally from 60 % aqueous dioxan with charcoal; clusters of very pale yellow needles, M.P. 203–204° to a dark brown melt. In contradistinction to rubrofusarin itself this monomethyl ether is readily soluble in dilute NaOH giving a reddish orange-coloured solution. It dissolves in conc. HCl to give a crimson solution from which there separates a red crystalline ferrichloride on the addition of a solution of $FeCl_3$ in HCl. (Found (Schoeller): C, 67·16, 67·20; H, 4·90, 5·06; CH_3O, 21·57, 21·56 %. $C_{16}H_{14}O_5$ requires C, 67·11; H, 4·93; CH_3O, 21·69 %.)

Rubrofusarin dimethyl ether, $C_{14}H_7O_2(OCH_3)_3$. Rubrofusarin monomethyl ether (0·3 g.), suspended in acetone (10 ml.) was mixed with dimethyl sulphate (5 ml.); a deep red colour developed. The mixture was shaken with 2*N* NaOH, and during the reaction a crystalline product separated. After dilution with an equal volume of water, rubrofusarin dimethyl ether was filtered, washed with water and dried; wt. 0·28 g. It crystallized from 60 % aqueous methyl alcohol (charcoal) in glistening colourless needles, M.P. 187–188°. It forms a yellow solution with a green fluorescence in methyl alcohol. It is quite insoluble in NaOH even on boiling, but readily dissolves in conc. HCl yielding a crimson solution and gives a crystalline ferrichloride (see below). (Found (Weiler): C, 68·03, 68·28; H, 5·38, 5·57; OCH_3 (macro), 30·54 %. $C_{17}H_{16}O_5$ requires C, 67·98; H, 5·37; OCH_3, 31·01 %.)

Dibromorubrofusarin, $C_{15}H_{10}O_5Br_2$. To a boiling solution of rubrofusarin (0·1 g.) in glacial acetic acid (8 ml.) were added 4 ml. of approximately *N*/2 Br in glacial acetic acid. The bromo-derivative started to separate immediately and HBr was evolved. After cooling it was filtered and crystallized from glacial

acetic acid and then from dioxan; long red needles, M.P. 244° (decomp.). The crystals from dioxan separate with ½ molecule of dioxan of crystallization which is lost on drying at 120–125° in high vacuum. (Found (Schoeller), on material dried to constant weight at 120–125° *in vacuo*: C, 41·67; H, 2·34; Br, 37·52; CH_3O, 7·09%. $C_{15}H_{10}O_5Br_2$ requires C, 41·87; H, 2·34; Br, 37·17; CH_3O, 7·22%.)

Nor-rubrofusarin, $C_{14}H_{10}O_5$. Rubrofusarin (0·4 g.) and concentrated HI (20 ml.) were heated in the Zeisel apparatus at a bath temperature maintained at 95–100° for 5 hours. The cold mixture was poured into water, filtered, triturated with sodium thiosulphate solution, washed with water and dried, wt. 0·37 g. The product crystallized from aqueous dioxan (75% dioxan) in long golden yellow rods which blackened but did not melt at 280°. It is readily soluble in cold dilute NaOH, forming a deep yellow solution, and in Na_2CO_3 to give a deep yellow solution which soon darkens in air to a very dark brown. It is insoluble in sodium acetate and in sodium bicarbonate. Its golden-coloured alcoholic solution becomes a dark brownish green on addition of dilute $FeCl_3$. (Found (Schoeller): C, 64·95, 64·95; H, 3·92, 4·06%. OCH_3, nil. $C_{14}H_{10}O_5$ requires C, 65·10; H, 3·91%.)

Active hydrogen (Roth). In pyridine 6·525 mg. gave 1·70 ml. methane at 21° and 1·74 ml. at 95°, corresponding to 3·00 and 3·07 active hydrogen atoms respectively.

Nor-rubrofusarin diacetate, $C_{14}H_8O_5(COCH_3)_2$. Nor-rubrofusarin (0·2 g.), fused sodium acetate (0·4 g.), acetic anhydride (1·4 ml.) and glacial acetic acid (0·8 ml.) were heated at 140–150° for 20 min. The cooled melt was treated with water (30 ml.) and the product filtered, washed and dried; wt. 0·25 g. It was crystallized from glacial acetic acid, then benzene and finally glacial acetic acid; clusters of deep golden yellow flat needles, soluble in NaOH, M.P. 204° to a red liquid. (Found (Schoeller): C, 63·01, 63·04; H, 4·30, 4·34%. $C_{18}H_{14}O_7$ requires C, 63·14; H, 4·12%.) Acetyl estimations (Roth) indicated the presence of two acetyl groups.

Nor-rubrofusarin dimethyl ether (identical with rubrofusarin monomethyl ether). Nor-rubrofusarin (0·35 g.), finely powdered and suspended in benzene (250 ml.) was treated with an excess of ethereal diazomethane and allowed to stand for 2 days. About half of the nor-rubrofusarin dissolved. After filtration the deep yellow solution was evaporated *in vacuo*, the residue rubbed up with a little cold methyl alcohol and the product purified as described for rubrofusarin monomethyl ether; clusters of very pale yellow needles, M.P. 203°, alone or mixed with rubrofusarin monomethyl ether.

Rubrofusarin dimethyl ether ferrichloride. A solution of ferric chloride (0·3 g.) in conc. HCl (3 ml.) was poured into a stirred solution of the dimethyl ether (0·1 g.) in cold glacial acetic acid (5 ml.). The ferrichloride crystallized at once in dark red prisms. It crystallized from glacial acetic acid in maroon-coloured prisms, M.P. 183–184° decomp. (Found (Schoeller): C, 41·14; H, 3·57; Fe, 10·91, 10·89%. $C_{17}H_{17}O_5FeCl_4$ requires C, 40·90; H, 3·44; Fe, 11·19%.)

Preparation, properties and derivatives of aurofusarin

The medium used for the preparation of aurofusarin was the same as was used for the preparation of rubrofusarin except that the *p*H (about 4·0) was not adjusted. The cultural conditions were also the same and the flasks were taken off after about 40 days. There was a good thick corrugated surface felt with colours ranging through red, pink and salmon to yellow. The dried powdered mycelium was first exhaustively extracted with light petroleum, B.P. 40–50°, to extract fat and small amounts of rubrofusarin. It was then air-dried and

reground. 175 g. portions of this material were shaken for 2 min. with 600 ml. of cold $CHCl_3$ and then filtered. The process was repeated once. On standing, there separated from the cold $CHCl_3$ extracts considerable amounts of almost pure aurofusarin. The mycelium was now exhaustively extracted with boiling $CHCl_3$ under reflux until the whole of the pigment had been extracted. The chloroform solutions were evaporated *in vacuo* and set aside to crystallize. The yields of crude aurofusarin obtained in a number of typical experiments at different initial *p*H values are given in Table II. 100 flasks were used in each case. It was found, on treating the light petroleum-extracted mycelium from the preparation of rubrofusarin, i.e. mycelium grown on a medium with an initial *p*H of 7–8, that the chloroform extracts were very dark in colour and contained aurofusarin in solution as a salt or other metallic complex. On shaking the chloroform solution with excess of mineral acid the colour of the solution lightened considerably and it was found possible to isolate fair amounts of aurofusarin, but in a very much less pure condition than the material isolated from mycelium grown at an initial *p*H of 4·0. Of all the strains used *F. graminearum*, Cat. No. 29, is the only one which gives good yields of both rubrofusarin and aurofusarin when grown at an initial *p*H of 8.

Table II

Strain of *F. culmorum* Cat. No.	Initial *p*H of medium	Incubation period in days	Weight of mycelium g.	Weight of crude fat g.	Weight of crude aurofusarin g.
F. 16	4	39	443	70	19·7
F. 16	5	34	485	85	13·2
F. 16	6	34	480	120	5·85
F. 16	7	34	435	80	1·25
F. 16*	6	34	181	38	0·80
F. 17	4	47	289	50	2·55
F. 18	4	61	324	45	9·1
F. 19	4	51	396	70	7·6
F. 29	4	40	414	75	18·1
F. 29	8	22	520	140	19·4
F. 29	8	22	500	133	19·0

* Potato 1·5% dextrose medium.

The crude aurofusarin was purified by repeated recrystallization from glacial acetic acid using charcoal in one of the recrystallizations. It separates from this solvent in characteristic orange-yellow blunt-ended prisms. It contains no N, S or Cl.

Analysis of aurofusarin. Great difficulties were experienced in arriving at an empirical formula for aurofusarin because of the impossibility of removing quantitatively the solvent of crystallization, and also because of the conflicting results obtained in molecular weight estimations (see later). Five different samples, dried to constant weight *in vacuo* at 140°, gave the following results (Schoeller), each sample being analysed in duplicate: C, 61·31% (average of 10 analyses—limits 61·08–61·44%); H, 3·41% (average of 10 analyses—limits 3·28–3·53%); OCH_3, 10·45% (average of 5 analyses—limits 10·36–10·52%). $C_{30}H_{20}O_{12}, H_2O$ requires C, 60·99; H, 3·76; two OCH_3, 10·52%.

The alkoxy-group was identified as methoxyl by the formation of the typical crystalline trimethylanilinium iodide, having the theoretical iodine content, with dimethylaniline.

Active hydrogen (Roth): (*a*) in pyridine 7·907 mg. gave 0·51 ml. methane at 22° and 0·60 ml. at 95°; (*b*) in anisole 7·366 mg. gave 0·47 ml. methane at 22°

and 0·56 ml. at 95°. These values correspond to 1·99 and 2·00 active hydrogen atoms in pyridine and anisole respectively for the formula $C_{30}H_{22}O_{13}$.

On heating, aurofusarin darkens in colour at high temperatures but does not melt below 360°. It is only very slightly soluble in most of the usual solvents and is insoluble in water. It dissolves to the extent of 0·1 % in boiling glacial acetic acid, 0·05 % in boiling chloroform or dioxan and about 5 % in boiling nitrobenzene. It dissolves readily in molten phenol, *m*-cresol and guaiacol, giving deep blood-red solutions. It gives a deep brownish red colour when dissolved in cold concentrated H_2SO_4.

On treatment with various alkalis it does not appear to dissolve, but the colour of the crystals changes from orange-yellow to colours varying from red to blue according to the alkali used. Aurofusarin gives a characteristic reaction under the following conditions. A saturated solution in chloroform, golden orange in colour, was shaken with equal volumes of solutions or suspensions of various alkalis. The highly coloured emulsions so formed were centrifuged. With NaOH, $Ca(OH)_2$, $Ba(OH)_2$ and $Mg(OH)_2$ the aqueous phase was colourless while the chloroform layer was intense violet, reddish pink, violet and intense scarlet in colour respectively. With NH_4OH both the aqueous and $CHCl_3$ layers were deep dull red in colour. With KOH, which gave a deep blue emulsion, the chloroform layer was colourless while the aqueous layer was pale reddish brown in colour. It will be noticed that in almost all cases a characteristic colour is present in the chloroform solution, indicating that the metallic complex, whether a salt or otherwise, is more readily soluble in chloroform than in water. This offers an explanation for the fact recorded on p. 391 that when mycelia grown at *p*H 8 are extracted with chloroform, the aurofusarin, as a salt or other metallic complex, is readily extractable.

Aurofusarin benzoate. A suspension of aurofusarin (1 g.) in pyridine (10 ml.) was treated with benzoyl chloride (4 ml.) for ½ hour at room temperature. The aurofusarin soon dissolved and the solution became very dark. After filtration, the filtrate was poured on to ice, and the dark brown oil separating soon hardened. It was filtered, rubbed with ether, and the yellow solid so obtained was ground with bicarbonate solution, filtered, washed with water and dried *in vacuo*. The crude product, which was readily soluble in $CHCl_3$, was crystallized from benzene (charcoal), in which it is also very soluble. The substance which separated from the very dark solution was twice crystallized from benzene (charcoal) in which its solubility had now decreased considerably. It was obtained as yellow plates, which gave a negative chloroform-NaOH reaction. On drying to constant weight at 155–160° it shrank and melted to a gum at 212–215°. It apparently contained 1 molecule of benzene of crystallization which was lost on drying. (Found (Schoeller) on material dried to constant weight *in vacuo* at 155–160°: C, 67·31, 67·34; H, 3·38, 3·36; OCH_3 (macro), 7·81, 7·78 %. $C_{44}H_{28}O_{14}$, i.e. dibenzoate of $C_{30}H_{20}O_{12}$, requires C, 67·67; H, 3·62; OCH_3, 7·95 %.)

Aurofusarin p-*bromobenzoate.* A suspension of aurofusarin (1 g.) in pyridine (10 ml.) was treated with 4 g. of *p*-bromobenzoyl chloride and was stirred occasionally during 5 hours. Pyridine (5 ml.) was then added and the mixture left for a further 2½ hours. It was filtered and the filtrate poured into ice. The solid so obtained was washed with water, then with ether, ground with bicarbonate solution, filtered, washed with water and ether and dried *in vacuo* over sulphuric acid. The crude product which was obtained in very good yield was crystallized thrice from benzene, exactly as described for the benzoate, and was obtained in yellow prisms which decomposed at 304°. It was apparently free from solvent of crystallization and gave a negative chloroform-NaOH

reaction. (Found, on substance dried to constant weight *in vacuo* at 160° (Weiler): C, 56·12, 56·32; H, 2·77, 2·94 %. (Schoeller): C, 56·33, 56·44; H, 2·64, 2·62; Br, 16·82, 16·80; OCH_3 (macro), 6·65 %. $C_{44}H_{26}O_{14}Br_2$, i.e. the di-*p*-bromobenzoate of $C_{30}H_{20}O_{12}$, requires C, 56·28; H, 2·79; Br, 17·04; OCH_3, 6·62 %.)

Aurofusarin anisate. A suspension of aurofusarin (1 g.) in pyridine (10 ml.) was treated with anisoyl chloride (5 ml.) and stirred for 1½ hours. The mixture was filtered and worked up as described for the benzoate and gave a very good yield of crude product. It was dissolved in hot benzene in which it was exceedingly soluble, and hot petroleum (B.P. 80–100°) was carefully added until there was only a faint turbidity; this was cleared by a drop of benzene, and the whole was allowed to cool slowly. The product separated as a heavy dark brown oil, which crystallized after standing for 2 days. The solid was removed, washed with benzene and crystallized thrice from benzene as previously described. The anisate formed small yellow prisms, which apparently contained benzene of crystallization. After drying to constant weight *in vacuo* at 155–160°, the product shrinks to a gum at 205°. The chloroform-NaOH reaction was negative. (Found (Weiler) on substance dried to constant weight *in vacuo* at 160°: C, 65·66, 65·67; H, 3·82, 3·87; OCH_3 (macro), 14·89 %. $C_{46}H_{32}O_{16}$, i.e. the dianisate of $C_{30}H_{20}O_{12}$, requires C, 65·70; H, 3·84; OCH_3, 14·77 %.)

Reduced aurofusarin. Aurofusarin (5·05 g.) dissolved in *m*-cresol (110 ml.) was hydrogenated in the presence of palladized charcoal. The reaction required 20–30 min., and 385 ml. of H_2 (at N.T.P.) were absorbed, after allowing for the blank of the *m*-cresol. Calculated for an uptake of 4H by $C_{30}H_{20}O_{12}, H_2O \rightarrow C_{30}H_{24}O_{12}$, 383 ml. Several repeat experiments gave similar results. The very dark coloured solution was filtered, the charcoal washed with *m*-cresol, and the reduction product carefully precipitated by the addition of ether; yield, almost theoretical. The crude reduction product crystallized from dioxan in dark chocolate-brown elongated rhomboids which contained dioxan of crystallization and did not melt below 360°. (Found (Schoeller) on 3 different samples dried to constant weight *in vacuo* at 110°: C, 62·27 % (average of 6 analyses—limits 62·13–62·49 %); H, 3·86 % (average of 6 analyses—limits 3·80–3·94 %); OCH_3 (macro), 10·82 % (average of 4 analyses—limits 10·71–10·91 %). $C_{30}H_{24}O_{12}$ requires C, 62·47; H, 4·20; $2OCH_3$, 10·76 %.)

Reduced aurofusarin is slightly soluble in alcohol, giving a brown-coloured solution which becomes definitely darker on the addition of $FeCl_3$. A $CHCl_3$ solution of reduced aurofusarin, when shaken with aqueous solutions of alkalis or alkaline earths, gives a range of colours, both in the $CHCl_3$ and aqueous layers, which is very similar to that given by aurofusarin under the same conditions.

Action of phenylhydrazine on aurofusarin. Aurofusarin (0·5 g.) dissolved in cold *m*-cresol (10 ml.) was treated with phenylhydrazine (1 g.) and the mixture stirred. Almost at once there was an evolution of gas, which was not CO_2. The mixture was left for 1 hour and then heated to 100° for 15 min. After cooling, ether was added and the product was worked up exactly as described under reduced aurofusarin. The substance so obtained appeared to be identical in every respect with reduced aurofusarin, both in its analysis and in its colour reaction with chloroform and alkali. It crystallized from dioxan in the same shaped crystals which contained dioxan of crystallization, and the latter was lost on exposure to air. It contained no nitrogen. (Found (Schoeller) on material dried to constant weight *in vacuo* at 100°: C, 62·28, 62·43; H, 3·79, 3·87; OCH_3 (macro), 11·00 %. $C_{30}H_{24}O_{12}$ requires C, 62·47; H, 4·20; $2OCH_3$, 10·76 %.)

Benzoate of reduced aurofusarin. Reduced aurofusarin (1 g.) suspended in pyridine (10 ml.) was treated with benzoyl chloride (4 ml.). After standing

for 2 hours at room temperature the crude benzoate (1·85 g.) was isolated as described for aurofusarin benzoate. It was crystallized from nitrobenzene-ether, pyridine-ether, and finally from pyridine (charcoal); glistening, almost colourless, diamond-shaped plates, M.P. decomp. 368–369°. (Found (Weiler): C, 71·97, 71·97; H, 4·00, 4·18; OCH_3 (macro), 5·28%. $C_{72}H_{48}O_{18}$, i.e. hexabenzoate of $C_{30}H_{24}O_{12}$, requires C, 71·97; H, 4·03; OCH_3, 5·17%.) An identical benzoate, crystallizing in exactly the same form, was obtained by a similar reaction from the product obtained from aurofusarin by the action of phenylhydrazine; M.P. with decomp. 368–369°; mixed M.P. decomp. 368°. (Found (Weiler): C, 71·89, 71·96; H, 4·06, 4·10; OCH_3 (macro), 5·24%.)

p-*Bromobenzoate of reduced aurofusarin.* This was prepared in a similar way from reduced aurofusarin (1 g.), pyridine (10 ml.) and *p*-bromobenzoyl chloride (4 g.). The reaction mixture was allowed to stand overnight, and was worked up as described for the benzoate; yield good. This bromobenzoate is readily soluble in hot nitrobenzene or hot pyridine, and it was crystallized from both solvents containing some ether. It forms clusters of cream-coloured prisms, M.P. decomp. 357°. (Found (Weiler): C, 51·86, 51·59; H, 2·71, 2·58; Br, 28·41, 28·64; OCH_3 (macro), 3·73%. $C_{72}H_{42}O_{18}Br_6$, i.e. hexa-*p*-bromobenzoate of $C_{30}H_{24}O_{12}$, requires C, 51·62; H, 2·53; Br, 28·65; OCH_3, 3·71%.)

Anisate of reduced aurofusarin. A mixture of reduced aurofusarin (1 g.), pyridine (10 ml.) and anisoyl chloride (5 ml.) was allowed to stand at room temperature overnight and worked up as described for the benzoate; yield good. The substance was crystallized first from nitrobenzene-ether and then from pyridine-ether; nearly colourless clusters of needles, M.P. decomp. 338°. (Found (Schoeller): C, 67·78, 67·60; H, 4·34, 4·28; OCH_3 (macro), 18·01%. $C_{78}H_{60}O_{24}$, i.e. hexa-anisate of $C_{30}H_{24}O_{12}$, requires C, 67·81; H, 4·38; OCH_3, 17·99%.)

Molecular weights of aurofusarin and its derivatives

As previously mentioned, very conflicting results were obtained in molecular weight estimations on aurofusarin and its derivatives. The results obtained are summarized in Table III, in which the probable values in column 3 are calculated on the assumption that anhydrous aurofusarin has one of the empirical formulae (*a*) $C_{30}H_{20}O_{12}$ or (*b*) $C_{15}H_{10}O_6$.

These results call for the following comments:

(*a*) Estimation 1. This estimation of the molecular weight of aurofusarin by X-ray analysis was very kindly carried out for us by Miss D. Crowfoot, Somerville College, Oxford, to whom we offer our best thanks. Miss Crowfoot reported "The crystals of aurofusarin are triclinic and the cell molecular weight from the X-ray measurements and density is 1140 ± 26. This may be divided among *n* molecules since there are no symmetry limitations in the triclinic system. The probable molecular weights corresponding to 2, 3 or 4 molecules present are therefore 570 ± 13, 380 ± 9, 285 ± 7. The result is unsatisfactory from the crystallographic point of view owing to the unusually difficult crystal structure adopted."

(*b*) Estimations 4, 6, 7, 8, 9, 12 and 13 were carried out by different analysts by the Rast camphor method. These results are again unsatisfactory since on the same substance, e.g. estimations 6 and 7 and estimations 8 and 9 respectively, the two different analysts found widely different values. They do, however, on the whole, point in favour of the double formula $C_{30}H_{20}O_{12}$ and against the single formula $C_{15}H_{10}O_6$.

(*c*) Estimations 2, 3 and 10 were carried out by Dr A. E. Oxford cryoscopically either in phenol or *o*-cresol. Superficially these results seem to favour

Table III

Estimation	Substance	Probable mol. wt.	Analyst	Method used	Result found
1	Aurofusarin (hydrated)	590 or 295	Miss D. Crowfoot	X-ray analysis	1140 ± 26, 570 ± 13, 380 ± 9, 285 ± 7
2	,,	590 or 295	Dr A. E. Oxford	Cryoscopic in *o*-cresol	396
3	,,	590 or 295	,,	Cryoscopic in phenol	261, 347, 383
4	Aurofusarin benzoate	780 or 390	Schoeller	Rast camphor method	1240, 1420
5	,,	780 or 390	Dr A. E. Oxford	Cryoscopic in nitrobenzene	661, 696
6	Aurofusarin *p*-bromobenzoate	938 or 469	Weiler	Rast camphor method	912
7	,,	938 or 469	Schoeller	,,	1375, 1275
8	Aurofusarin anisate	840 or 420	Weiler	,,	856
9	,,	840 or 420	Schoeller	,,	1205, 930, 1010
10	Reduced aurofusarin	576 or 288	Dr A. E. Oxford	Cryoscopic in phenol	315, 389, 393
11	Reduced aurofusarin bromobenzoate	1674 or 837	,,	Cryoscopic in nitrobenzene	1451
12	,,	1674 or 837	Schoeller	Rast camphor method	654, 677
13	Reduced aurofusarin anisate	1380 or 690	,,	,,	736, 729

the single formula $C_{15}H_{10}O_6$ but they point definitely to the conclusion that the molecular weight of aurofusarin increases with the concentration of the solution. We are agreed with Dr Oxford that because of the well-known properties of these solvents, these results should be disregarded.

(*d*) Estimations 5 and 11 were carried out by Dr A. E. Oxford cryoscopically in nitrobenzene. Both these estimations indicate quite definitely the double formula $C_{30}H_{20}O_{12}$ for aurofusarin.

We believe that the above results indicate, although they certainly do not prove conclusively, that aurofusarin has the empirical formula $C_{30}H_{20}O_{12}$, an opinion which is supported by the high melting-point of aurofusarin. This formula is therefore tentatively advanced.

Preparation, properties and derivatives of culmorin

Culmorin has so far been isolated from the dried mycelium of only one strain of *F. culmorum*, i.e. L.S.H.T.M. Cat. No. F. 17. Batches of 100 flasks of Raulin-Thom medium (see p. 387), initial *p*H about 4·0, were sown with F. 17, and incubated for 50–60 days. The dried, ground mycelium was exhaustively extracted for some days with light petroleum (B.P. 40–50°). Crude culmorin contaminated with traces of rubrofusarin separated from the extracts. Experimental details of 7 batches are given in Table IV.

The crude culmorin so obtained melted at 173–175°. It crystallized from benzene in beautiful thick colourless needles, M.P. 174–175°, and sublimed at 95–100° in a high vacuum, giving a product which melted at 175°. (Found (Schoeller): C, 75·65, 75·65; H, 10·74, 10·84%. OCH_3, N, S, Cl, nil. Mol. wt. (cryoscopic in dioxan, Dr A. E. Oxford), 265. $C_{15}H_{26}O_2$ requires C, 75·57, H, 11·00%. Mol. wt. 238.) Optical rotation; in $CHCl_3$ ($c = 1·003$) $[\alpha]_{5461}^{20°}$ −14·45°.

Culmorin contains two active hydrogen atoms since in a Zerewitinoff estimation (Roth) it afforded 2·01 mol. of CH_4 in pyridine and 2·03 mol. in anisole.

Table IV

Incubation period in days	Weight of mycelium g.	Weight of crude fat g.	Weight of aurofusarin g.	Weight of culmorin g.
47	289	50	2·55	14·2
65	277	46	2·1	6·0
62	302	47	2·1	3·45
51	304	52	1·9	8·7
54	322	50	1·2	4·1
51	223	48	—	20·9
52	307	52	—	5·4

Culmorin is insoluble in water, acids and alkalis. It gives only a negligible titration figure in alcoholic solution either with cold or hot NaOH, even on standing. An alcoholic solution gives no coloration with ferric chloride and it does not reduce Fehling's solution. It dissolves in concentrated sulphuric acid forming a golden yellow solution. Attempts to prepare a phenylhydrazone and a semicarbazone failed.

Culmorin diacetate, $C_{15}H_{24}O_2(CO.CH_3)_2$. Pure culmorin (0·6578 g.) was acetylated in a quantitative estimation by the Peterson-West method [1927] by treatment at 37° for 4½ days with 5 g. of a mixture of pyridine (2 pts.) and acetic anhydride (1 pt.). Acetic anhydride equivalent to 5·73 ml. *N* NaOH was fixed. Theoretical for two acetylatable groups = 5·53 ml.

The diacetate was filtered from the titration solution, washed with water, and dried; wt. 0·85 g., M.P. 86–90°. It is extremely soluble in all organic solvents, including light petroleum. It was finally crystallized by dissolving in cold glacial acetic acid and adding cold water until a very faint turbidity appeared. The diacetate crystallized in colourless elongated prisms, M.P. 90–91°. It sublimes very easily in a high vacuum at 55° and melts at 93°. (Found (Schoeller): C, 70·74, 70·63; H, 9·37, 9·25 %. $C_{19}H_{30}O_4$ requires C, 70·75; H, 9·38 %.)

Culmorin di-p-bromobenzoate. A mixture of culmorin (0·2 g.) and *p*-bromobenzoyl chloride (0·5 g.) was heated at 130–140° for ½ hour. After cooling, the sticky product was dissolved in cold pyridine and water was added slowly. The white sticky product slowly hardened on standing and stirring with dilute Na_2CO_3. It was finally powdered, washed with water, dried and crystallized from alcohol; thick colourless prisms, M.P. 102–103°. (Found (Schoeller): C, 57·66, 57·75; H, 5·36, 5·26; Br, 26·33, 26·48 %. $C_{29}H_{32}O_4Br_2$ requires C, 57·59; H, 5·34; Br, 26·48 %.)

SUMMARY

The colouring matters present in the mycelium of cultures of *Fusarium culmorum* (W. G. Smith) Sacc. and related forms have been investigated. Two hitherto undescribed crystalline colouring matters have been isolated from all forms of *F. culmorum* examined. They are *rubrofusarin*, $C_{15}H_{12}O_5$, glistening red plates, M.P. 210–211°, and *aurofusarin*, $C_{30}H_{20}O_{12}, H_2O$, orange-yellow prisms, M.P. above 360°. Various crystalline derivatives of rubrofusarin and aurofusarin have been prepared and are described. A third substance, *culmorin*, $C_{15}H_{26}O_2$, M.P. 175°, thick colourless needles, was isolated from one strain of *F. culmorum*.

We wish to thank our colleague Dr A. E. Oxford, for much work on the determination of the molecular weight of aurofusarin and its derivatives. We also express our indebtedness to the Department of Scientific and Industrial Research for a grant to one of us (J. N. A.).

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