667. *Studies in Mycological Chemistry. Part XIII.* Averufin, a Red Pigment from* Aspergillus versicolor *(Vuillemin) Tiraboschi.*

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The mycelium of *Aspergillus versicolor* (Vuill.) Tiraboschi contains 10—15 pigments. The isolation of one of the red pigments, averufin, in a crystalline state is described. Analytical and spectroscopic evidence indicates that averufin has structure (V).

The mould, *Aspergillus versicolor* (Vuill.) Tiraboschi, has long been known [1] to produce red colouring material, but present chemical knowledge concerning the constituent pigments is fragmentary. From the mycelium of one particular strain, Hatsuda and Kuyama isolated [2] a crystalline orange-yellow pigment, $C_{15}H_{10}O_6$, m. p. 282°, termed versicolorin, which they suggested had structure (Ia) or (Ib). However, synthetical experiments by Venkataraman and his colleagues [3] have shown that neither suggestion is correct. Birkinshaw *et al.* isolated [4] an orange pigment, m. p. 233—234°, from a strain of this mould, but the quantity obtained was insufficient for structural studies. During investigations [5] on sterigmatocystin (II), a yellow xanthonoid metabolite of *A. versicolor*, we accumulated some mixed red pigments from the strain which we were using and we now record the results of our investigations on them.

By a long and tedious procedure, involving successive extraction, adsorption chromatography, multiple fractional extraction, and paper chromatography (see below), we have shown that the mycelium of one strain of *A. versicolor* probably contains, in addition to sterigmatocystin, between ten and fifteen pigments. One of these, for which we propose the name, averufin, has been isolated as bright orange-red laths, m. p. 280—282° (decomp.). We have obtained *ca.* 600 mg. of this substance on which the work to be described has been performed.

Averufin, $C_{20}H_{16}O_7$, possesses no methoxyl group. Kuhn–Roth determinations (for *C*-Me groups) on the compound gave, unaccountably, results which varied from 3% to 9% (one *C*-Me group in $C_{20}H_{16}O_7$ requires 4·1%). Although averufin itself possesses almost no detectable optical activity, its acetate is lævorotatory. Averufin is stable to acid but unstable to prolonged treatment by alkali. It gives a positive test for a hydroxyquinone (with zinc dust and aqueous sodium hydroxide) and its ultraviolet absorption spectrum indicates that it is a hydroxyanthraquinone (for a suitable list of spectra for comparison, see ref. 6).

The hydroxylation pattern was deduced from the following observations: (i) averufin contains three hydroxyl groups since it readily gives a triacetate; (ii) a solution of averufin in glacial acetic acid does not fluoresce, indicating the probable absence [7] of a 1,4-dihydroxy-pattern; (iii) averufin is not a mordant dye and it gives a negative zirconium nitrate test,[8a] proving the absence of a 1,2-dihydroxy-grouping; (iv) it is soluble in aqueous sodium

* Part XII, *J.*, 1963, 829.

[1] Thom and Raper, "A Manual of the Aspergilli," Williams and Wilkins Coy., Baltimore, 1945, p. 190.

[2] Hatsuda and Kuyama, *J. Agric. Chem. Soc. Japan*, 1954, **28**, 989; Hatsuda, Kuyama, and Terashima, *ibid.*, 1955, **29**, 11; *Chem. Abs.*, 1956, **50**, 15,522; Thomson, "Naturally Occurring Quinones," Butterworth's Scientific Publications, London, 1957, p. 205.

[3] Ayyangar, Joshi, and Venkataraman, *Tetrahedron*, 1959, **6**, 331; Venkataraman, *J. Indian Chem. Soc.*, 1960, **37**, 247.

[4] Birkinhsaw and Hammady, *Biochem. J.*, 1957, **65**, 162.

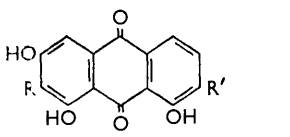
[5] Bullock, Roberts, and Underwood, *J.*, 1962, 4179, and previous papers.

[6] Birkinshaw, *Biochem. J.*, 1955, **59**, 486.

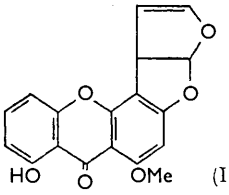
[7] Raistrick, Robinson, and Todd, *Biochem. J.*, 1934, **28**, 567; cf. Birkinshaw and Gourlay, *ibid.*, 1961, **81**, 618.

[8] Feigl, "Spot Tests in Organic Analysis," Elsevier, Amsterdam, 1960 (6th English edn.), pp. (*a*) 210. (*b*) 207.

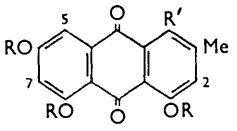
carbonate solution and thus contains at least one β-hydroxyl group; (v) the position of the long-wavelength absorption band (453 mμ) in the ultraviolet spectrum of averufin indicates the presence [6,9] of at least two α-hydroxyl groups; (vi) the infrared absorption spectrum of averufin possesses well-defined bands at 1675 (C=O) and 1622 cm.$^{-1}$ (hydrogen-bonded C=O), proving [10] that there cannot be more than two α-hydroxyl groups and that these are disposed 4,5 (or 1,8); (vii) the nuclear magnetic resonance spectrum of averufin derivatives (see below) indicates that averufin possesses two hydroxyl groups situated *meta* to each other; (viii) the colour reactions of averufin (with aqueous alkali and with ferric chloride) are very similar to those of emodin (IIIa). We conclude that averufin is a derivative of 1,6,8-trihydroxyanthraquinone.



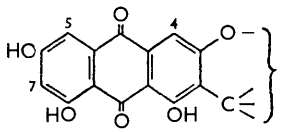
(IIa: R = CH_2·OH, R′ = H)
(IIb: R = H, R′ = CH_2·OH)



(II)

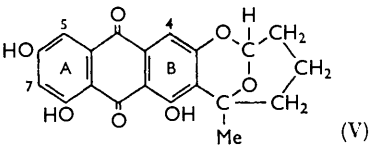


(IIIa: R = R′ = H)
(IIIb: R = Me, R′ = H)
(IIIc: R = Me, R′ = OMe)

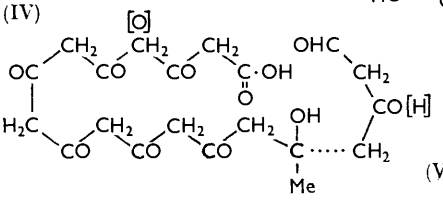


$C_5H_{10}O$

(IV)



(V)



(VI)

The instability of averufin towards alkali apparently accounts for our failure to prepare fully methylated averufin by the normal (Robertson–Robinson) method. Tri-*O*-methylaverufin was prepared, with difficulty, by prolonged treatment of averufin, at room temperature, with an excess of silver oxide and methyl iodide.

A comparison of the aromatic proton resonances in the nuclear magnetic resonance spectrum of tri-*O*-methylaverufin with those in the spectra of related compounds (see Table 1) reveals that in the averufin molecule (see structure V) one nucleus (A) is substituted by two hydroxyl groups situated *meta* to each other. It is also apparent that the other aromatic nucleus (B) is trisubstituted and that its lone aromatic proton occupies the 4-position. The substitution pattern in the second aromatic nucleus (B) may be further

TABLE 1.

Magnetic resonance absorptions of aromatic protons (solvent, deuterochloroform).

	τ Scale.	*J* in c./sec.			
Compound	H-2	H-4	H-5	H-7	*J*(H-5/H-7)
Tri-*O*-methylemodin (IIIb)	2·90	2·36	2·67	3·23	*ca.* 2·5
Tetra-*O*-methylcatenarin (IIIc)	2·83	—	2·75	3·26	*ca.* 2·2
Tri-*O*-methylaverufin (V; OMe for each OH) ...	—	2·43	2·55	3·10	*ca.* 2·5

defined as follows. First, it is now commonly accepted [11] that the ultraviolet absorption spectrum of a fully acetylated hydroxyquinone resembles that of the parent (unsubstituted) quinone; however, the spectrum of tri-*O*-acetylaverufin (see Table 2) resembles that of

[9] Briggs, Nicholls, and Paterson, *J.*, 1952, 1718.
[10] Bloom, Briggs, and Cleverley, *J.*, 1959, 178.
[11] Astill and Roberts, *J.*, 1953, 3302, and references there quoted.

2-methoxyanthraquinone rather than that of anthraquinone itself. Secondly, the ultraviolet absorption spectrum of tri-*O*-methylaverufin is very similar to that of 1,3,6,8-tetramethoxy-2-methylanthraquinone. We may thus write for averufin the partial structure (IV).

An examination of all the three different kinds of spectra of averufin and its derivatives reveals no indication of the presence of olefinic bonds in the averufin molecule. A comparison of the infrared spectrum of averufin with that of emodin reveals the presence in the former, but not in the latter, of two well-defined bands (at 1099 and 1129 cm.$^{-1}$) in the cyclic ether (C–O–C stretching) region. Valency analysis of averufin, on the assumption that the $C_5H_{10}O$ part of the molecule (see IV) contains no unsaturation and that the oxygen atom is ethereal, leads to the conclusion that averufin contains five rings in all.

TABLE 2.

Ultraviolet absorption spectra (in ethanol).

Compound	$\lambda_{max.}$ in mμ; $10^{-3}\varepsilon$ (in parentheses)					
Tri-*O*-acetylaverufin (V; OAc for each OH)	214(26·5),	246(15·0),	278*(36·4),	284(37·4),	339(4·27),	379(4·38)
2-Methoxyanthraquinone †	208(20·1),	248(17·6),	269(33·0),	281(24·6),	332(3·50),	369(2·89)
Anthraquinone	—	252(43·5),	263(17·2),	272(14·6),	327(4·60)	—
Tri-*O*-methylaverufin ‡ (V; OMe for each OH)		223(33·0),	286(37·1),	345(4·1),	409(4·67)	
1,3,6,8-Tetramethoxy-2-methylanthraquinone [12]		222(32·5),	281(37·3),	350(5·24),	405(3·88)	
O-Methylaversin [12]		224(33·1),	285(36·8),	350(4·58),	409(4·20)	
1,3,6,8-Tetramethoxyanthraquinone [12]		223(44·2),	281(31·1),	—	412(5·82)	
Tri-*O*-methylemodin (IIIb)		221(37·6),	276(26·2),	340(3·1),	404)7·3)	
Tetra-*O*-methylcatenarin (IIIc)		224(36·8),	274(23·5),	347(2·86),	406(7·75)	

* Inflexion. † Another peak at 241(16·1). ‡ Another peak at 247(10·0).

The nuclear magnetic resonance spectrum of tri-*O*-methylaverufin shows (in addition to signals due to three aromatic protons, see Table 1): (i) two singlets at 5·93 τ (intensity 3) and 5·89 τ (intensity 6) (corresponding to 3 × O·CH_3); (ii) a signal at 4·5 τ (intensity 1) which is of a blunted cone shape and is an ill-defined triplet or quadruplet with a band width of *ca.* 7 c./sec. at " half-height "; (iii) a " humped " region extending from 7·6 to 8·47 τ with one sharp (unsplit) peak superimposed at 8·36 τ. The area enclosed, by extrapolation downwards of the sides of the sharp peak, represents *ca.* 4½ protons. The peak therefore corresponds to one methyl group. This " hump " and peak (together of intensity *ca.* 9) is reminiscent of absorptions found in the spectra of steroids [13] where sharp peaks due to the protons of methyl groups are superimposed on " humps " due to the protons of methylene and methine groups.

We consider that the above observations uniquely define the structure of averufin as (V).

The τ-value (4·5) for the methine proton in the bicyclic side-chain is that to be expected [14] for a proton attached to a carbon atom directly flanked by two oxygen atoms. The τ-value for a rather similarly situated proton in dihydroclerodin-I [15] is 4·51. The signals due to the six protons of the three methylene groups lie in the body of the " hump " between 7·6 and 8·47 τ. The low τ-value (8·36) for the unsplit C–CH_3 absorption is ascribed to the situation of the methyl group β to both oxygen and to phenyl.

So far as we are aware, this is the first claim that a natural product contains a 1,3-benzodioxan system. Some synthetic examples of such systems, although they contain an acetal grouping, are known to be acid-stable and alkali-labile.[16]

Averufin must be closely related to versicolorin since there are many similarities in their infrared absorption spectra.

[12] Bullock, Kirklady, Roberts, and Underwood, *J.*, 1963, 829.
[13] Shoolery and Rogers, *J. Amer. Chem. Soc.*, 1958, **80**, 5121.
[14] Lemieux, Kullnig, Bernstein, and Schneider, *J. Amer. Chem. Soc.*, 1958, **80**, 6098.
[15] Barton, Cheung, Cross, Jackman, and Martin-Smith, *J.*, 1961, 5061.
[16] Chattaway and Calvet Y Prats, *J.*, 1927, 685.

Structure (V) for averufin is acceptable from a biogenetic standpoint. We postulate that a molecule of the metabolite originates from eight " acetate residues " and one molecule of acetoacetaldehyde by suitable aldol-type condensations, followed by cyclic acetal formation and appropriate oxidation and reduction (see VI).

Experimental

M. p.s were determined on the Kofler block. Optical rotations were measured on an Ericsson E.T.L.-N.P.L. automatic polarimeter, type 143A. Ultraviolet spectra were determined on substances in ethanolic solution. Infrared spectra were taken for substances in potassium bromide discs. Proton magnetic resonance spectra (of substances in deuteriochloroform solution were recorded on an A.E.I. (RS 2) spectrometer; these spectra were calibrated by the side-band technique, tetramethylsilane being used as internal reference.

R_F values refer to Whatman No. 1 paper and an ascending solvent composed of chloroform (2 vol.) and light petroleum (b. p. 60—80°; 3 vol.).

Separation of the Pigments and Isolation of Crystalline Averufin.—The dried mycelium of *A. versicolor* (Vuill.) Tiraboschi (Commonwealth Mycological Institute, No. 49,124) was obtained and extracted as previously described.[5] A chloroform solution of the extract was chromatographed on heavy magnesium oxide in order to eliminate sterigmatocystin. The column was extruded and the adsorbent with overlapping brown and purple bands (at the top of the column) was collected and treated with an excess of 2N-hydrochloric acid. The brown amorphous precipitate was collected and dried (*ca.* 12 g./kg. of dry mycelium). The mixed pigments (12 g.) were extracted with chloroform (1 l.), and the insoluble residue (1 g.) removed. The chloroform solution (100 ml.) was poured on a column (30 × 5 cm.) of heavy magnesium carbonate, and the chromatogram was developed with chloroform. The following bands (in order from the top of the column) were observed: (i) brown, (ii) red, (iii) pink, and (iv) additional bands which varied in number (4—6) and colour with the batch of mycelium used. Elution with chloroform was continued until only bands (i), (ii), and (iii) remained. The column was extruded and the material in the red band was separated. (Material containing overlapping brown and red bands was treated with 2N-hydrochloric acid, and the recovered pigments were re-chromatographed.) The adsorbent in the red band was dissolved in 2N-hydrochloric acid, and the liberated pigments were extracted with ether. The combined ethereal extracts were washed with water and dried. Removal of the solvent gave reddish-brown amorphous material (*ca.* 600 mg./kg. of dry mycelium).

A solution of this material (200 mg.) in ether (150 ml.) was equilibrated with 0·5N-sodium carbonate (125 ml.). The two filtered layers were charged into tubes 2—6 of a 100-tube multiple-fractional-extraction apparatus containing 0·5N-sodium carbonate as lower phase and ether as upper phase. A typical result, after 100 transfers, was as follows:

Tube no.	Colour of phases upper/lower	R_F values of contained pigments
2—14	Yellow/purple	0·2, 0·4, and 0·8
15—22	Yellow/dark red	0·4 and 0·8
37—70	Yellow/mauve	0·8
77—99	Yellow/pale mauve	1·0

The combined pigment material in both layers of tubes 40—68 from three runs (total load 600 mg.) was collected in ether, washed with water, and dried ($MgSO_4$). Removal of the solvent gave crude averufin (200 mg.). Crystallisation of this material from acetone gave pure *averufin* (115 mg.). The overall yield from dried mycelium was *ca.* 0·012%.

General Properties of Averufin.—Averufin crystallises from acetone in orange-red laths, m. p. 280—282° (decomp.) which, however, varies with the rate of heating, $[\alpha]_D^{22} > 1°$ (*c* 0·30 in EtOH) [Found: C, 65·5, 64·9, 65·4; H, 4·4, 4·3, 4·3; OMe, 0; N, 0; *C*-Me, 3·5, 6·2, 6·3, 9·4%; *M* (calc. from density of crystal, 1·560 g./ml., and on the assumption of four molecules per cell of volume 939·7 Å^3), 366·5. $C_{20}H_{16}O_7$ requires C, 65·2; H, 4·4; 1*C*-Me, 4·1%; *M*, 368·3], $\lambda_{max.}$ 223, 256(sh), 266, 286(sh), 294, 324, and 453 mμ ($10^{-3}\varepsilon$ 34·7, 16·3, 18·3, 27·2, 33·0, 9·53, and 10·75, respectively), $\nu_{max.}$ 3588, 3381, 3002, 2948, 1675, 1622, 1611, 1598, 1569, 1474, 1461, 1437, 1428, 1402, 1386, 1372, 1326, 1301, 1286, 1270, 1249, 1210, 1189, 1165, 1129, 1113, 1099, 1064, 1028, 998, 977, 955, 941, 893, 883, 877, 871, 860, 839, 787, 772, 752, 746, and 711 cm.$^{-1}$.

Averufin is insoluble in water and sparingly soluble in most organic solvents. It dissolves in 2N-sodium carbonate and in 2N-sodium hydroxide to give a purple colour. Averufin gives a cherry-red colour with concentrated sulphuric acid and an orange-brown ferric reaction in aqueous ethanol. It gives a negative test for a methylenedioxy-group.[8b]

No acid other than acetic acid could be detected by paper chromatography [17] in the distillates obtained in the Kuhn–Roth estimations mentioned above.

Reaction of Averufin towards Alkali and Acids.—(i) A solution of averufin (5 mg.) in 2N-sodium hydroxide (5 ml.) was kept overnight at *ca.* 90°. Acidification of the solution then yielded material which was shown, by paper chromatography, to be a mixture of two substances of which neither was averufin. Under similar reaction, emodin was recovered unchanged.

(ii) To a solution of averufin (10 mg.) in 50% aqueous dioxan (20 ml.) was added concentrated hydrochloric acid (2 ml.), and the mixture was kept at *ca.* 90° for 12 hr. The cooled mixture deposited unchanged averufin (identified by m. p. and infrared spectrum).

(iii) Averufin remained unchanged after it (5 mg.) had been heated under reflux for 4 hr. with a 50% solution of hydrogen bromide in glacial acetic acid (10 ml.). Under similar conditions, tri-*O*-methylemodin gave physcion (O^6-monomethylemodin) which was identified by comparison with an authentic specimen obtained from the lichen, *Xanthoria parietina*.

Tri-O-*acetylaverufin.*—A mixture of averufin (115 mg.), acetic anhydride (4·5 ml.), and pyridine (0·5 ml.) was kept at 90—95° for 4 hr. and then poured on ice (100 g.). The *product* (130 mg.), isolated in the usual way, separated from hot ethanol in yellow needles (112 mg.), m. p. 210—214°, unchanged by further crystallisation from ethanol or ethyl acetate [Found: C, 63·1; H, 4·3; Ac, 25·5%; *M* (Rast), 367, 427. $C_{20}H_{13}O_7(CO{\cdot}CH_3)_3$ requires C, 63·2; H, 4·5; Ac, 26·1%; *M*, 494·4], $[\alpha]_D^{22}$ −14·9° (*c* 0·424 in $CHCl_3$), $\nu_{max.}$ included bands at 1777 and 1768 (aryl acetate groups) and 1678 and 1666 cm.$^{-1}$ (anthraquinone C=O).

The proton magnetic resonance spectrum showed: (i) a singlet at 2·32 τ (H-4); (ii) two doublets at 2·69 and 2·00 τ, J = 2·5 c./sec. (H-7 and H-5); (iii) a quadruplet or triplet (intensity 1) at 4·68 τ (proton of methine group); (iv) two singlets at 7·44 τ (intensity 6) and 7·54 (intensity 3) (3 × $CO{\cdot}CH_3$); (v) a humped region (total intensity 9) between 7·8 and 8·5 τ with a sharp peak at 8·35 τ (methylene group protons and protons of $\geqslant C{\cdot}CH_3$).

This acetate could not be sublimed. In benzene solution, it chromatographed as a single yellow band on a column of silica. It was hydrolysed by cold 2N-sodium hydroxide to give unchanged parent compound (identified by paper chromatography).

Tri-O-*methylaverufin.*—Averufin (108 mg.), methyl iodide (30 ml.), and dry, freshly prepared silver oxide (1·5 g.) were stirred at room temperature for 80 hr. Removal of the solids and evaporation of the excess of methyl iodide gave an oil which, when mixed with benzene (*ca.* 7 ml.), partially solidified. The mixture was filtered and the solid residue was shown to be incompletely methylated material since its infrared absorption spectrum contained a strong band at 1630 cm.$^{-1}$ (hydrogen-bonded C=O). The filtrate was chromatographed on an acid-washed alumina column (25 × 2 cm.). Development of the chromatogram with benzene gave a pale yellow band which was eluted. The remaining yellow-orange material was eluted slowly by benzene, and the eluate was collected in 100-ml. fractions. The solvent from each fraction was evaporated, and the residue was taken up in hot methanol. The second fraction yielded a crystalline product (20 mg.) which was recrystallised from methanol to give *tri*-O-*methylaverufin* as yellow prisms (12 mg.), m. p. 190—191° [Found (on a sample dried *in vacuo* at 110° for 4 hr.): C, 67·2; H, 5·0. $C_{23}H_{22}O_7$ requires C, 67·3; H, 5·4%], $\nu_{max.}$ included 1671 (C=O), and 1095 and 1126 cm.$^{-1}$ (cyclic ether C–O–C stretch). The other spectra have been described above.

An attempt to prepare this ether by the acetone–methyl iodide–anhydrous potassium carbonate technique gave a dark-brown oil from which no pure compound could be separated.

Tri-O-*methylemodin.*—This was prepared from emodin by the acetone–methyl iodide–anhydrous potassium carbonate method. Recrystallised from methanol, it formed yellow needles, m. p. 225—226° (lit.,[18] m. p. 225°).

Tetra-O-*methylcatenarin.*—In our hands the earlier described synthesis [19] of catenarin gave poor results.

Crude 1,4-dihydroxy-6,8-dimethoxy-3-methylanthraquinone was synthesised [20] and was

[17] Reid and Lederer, *Biochem. J.*, 1951, **50**, 60.
[18] Fischer, Falco, and Gross, *J. prakt. Chem.*, 1911, **83**, 212.
[19] Anslow and Raistrick, *Biochem. J.*, 1941, **35**, 1006.
[20] Chandrasenan, Neelakantan, and Seshadri, *Proc. Indian Acad. Sci.*, 1960, **51**, *A*, 296.

chromatographed (in chloroform solution) on heavy magnesium carbonate. The orange-coloured band was eluted with chloroform. Evaporation of this eluate and crystallisation of the residue from ethanol gave the pure compound, m. p. 221—222° (lit.,[20] m. p. 212—213°). Methylation of this compound, by the usual method, gave tetra-*O*-methylcatenarin, m. p. 194—196° (lit.,[21] m. p. 190—191°), in good yield.

2-*Methoxyanthraquinone*.—This was produced by methylation of 2-hydroxyanthraquinone which had been obtained [22] from 2-aminoanthraquinone. The crude ether was purified by chromatography, in chloroform solution on acid-washed alumina, and was then recrystallised from methanol to give pale yellow needles, m. p. 196—197° (lit.,[23] m. p. 195—196°).

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[21] Anslow and Raistrick, *Biochem. J.*, 1940, **34**, 1124.
[22] Perkin and Whattam, *J.*, 1922, **121**, 289.
[23] Kaufler, *Ber.*, 1904, **37**, 65.