671. *Studies in Mycological Chemistry. Part XVII.* Averythrin, an Anthraquinonoid Pigment from* Aspergillus versicolor (*Vuillemin*) *Tiraboschi*

By John C. Roberts and Patrick Roffey

The isolation of a red pigment from the mycelium of a strain (" T.R.L., No. 2543 ") of *Aspergillus versicolor* (Vuill.) Tiraboschi is described. This pigment, now called averythrin, is shown to be *trans*-2-hex-1′-enyl-1,3,6,8-tetrahydroxyanthraquinone (I).

The metabolic products of *A. versicolor* (Vuill.) Tiraboschi (strain " T.R.L., No. 2543 ") were first investigated by Birkinshaw *et al.*[1] They isolated three metabolites, namely, sterigmatocystin (a xanthone whose structure has since been elucidated [2]), an orange pigment called " Product B," and mannitol. The nature of " Product B " was not established but the possibility of its being an anthraquinone derivative was considered. A reinvestigation of this partiular strain has led, in our hands, to the production of sterigmatocystin, a red anthraquinonoid pigment which is different from " Product B," and mannitol. We now describe the isolation and structural elucidation of this red pigment which we name averythrin.

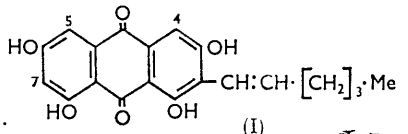
* Part XVI, B. W. Bycroft, J. C. Roberts, and P. M. Baker, *J.*, 1964, 2289.

[1] J. H. Birkinshaw, I. M. M. Hammady, and M. M. M. Abou-Zeid, *Biochem. J.*, 1957, **65**, 162.

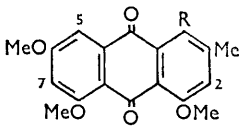
[2] (*a*) J. E. Davies, D. Kirkaldy, and J. C. Roberts, *J.*, 1960, 2169; (*b*) J. C. Roberts and J. G. Underwood, *J.*, 1962, 2060; E. Bullock, J. C. Roberts, and J. G. Underwood, *J.*, 1962, 4179.

This strain of the mould, when grown in surface culture on a standard liquid medium, readily produced a thick, green mycelial felt with a reddish-brown reverse. Successive extraction of the dried, powdered mycelium with the following solvents led to the products shown in parentheses: light petroleum (b. p. 60—80°) (sterigmatocystin); ether (a red oil); acetone (mannitol). Pure averythrin was isolated from the red oil by a long and troublesome procedure (see below).

Averythrin, m. p. 229—231° (decomp.), crystallises from methanol (as the hemihydrate, $C_{20}H_{18}O_6,\frac{1}{2}H_2O$) in small, red prisms. It is optically inactive (cf. " Product B ") and

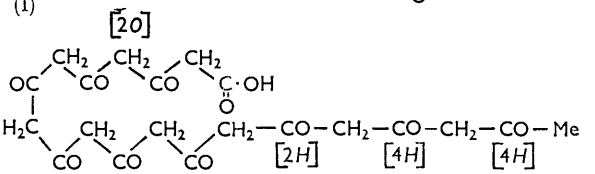


(I)



(IIa: R = H)
(IIb: R = OMe)

(III)



possesses no methoxyl group. 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 polyhydroxyanthraquinone (for a suitable list of spectra for comparison, see ref. 3). Averythrin contains four hydroxyl groups since it gives a tetra-acetate and a tetra-*O*-methyl derivative. Catalytic hydrogenation of averythrin yields (after aerial oxidation of the concomitantly produced anthraquinol system) a dihydro-derivative which crystallises as the hemihydrate, $C_{20}H_{20}O_6,\frac{1}{2}H_2O$. This compound, which also yields a tetra-acetate and a tetra-*O*-methyl derivative, has essentially the same ultraviolet absorption spectrum as that of averythrin. Reductive acetylation of tetra-*O*-acetyldihydroaverythrin (by the Brockmann technique [4]) yields inhomogeneous material with an ultraviolet absorption spectrum approximating to that of a penta-acetoxyanthracene. We assume that the first-formed hexa-acetate suffers partial reductive deacetoxylation (cf. ref. 4).

A comparison of the ultraviolet absorption spectrum of tetra-*O*-methyldihydroaverythrin with the spectra of known compounds (Table 1) indicated that dihydroaverythrin was

TABLE 1

Ultraviolet absorption spectra (in ethanol) of tetramethoxyanthraquinones

Compound	$\lambda_{max.}$ (mμ), and $10^{-3}\varepsilon$ in parentheses
Tetra-*O*-methyldihydro-averythrin	222(30·2), 283(36·0), 351(5·79), 403(3·89)
1,3,6,8-Tetramethoxy-2-methylanthraquinone [5]	222(32·5), 281(37·3), 350(5·24), 405(3·88)
1,3,6,8-Tetramethoxyanthraquinone [5]	223(44·2), 281(31·1), — 412(5·82)
Tetra-*O*-methylcatenarin (IIb) [6]	224(36·8), 274(23·5), 347(2·86), 406(7·75)

TABLE 2

Magnetic resonance absorptions of aromatic protons (measured in deuterochloroform)

	τ Scale; *J* in c./sec.				
Compound	H-2	H-4	H-5	H-7	*J* (H-5–H-7)
Tetra-*O*-methyldihydroaverythrin	—	2·47	2·64	3·20	*ca.* 2·5
Tri-*O*-methylemodin (IIa) [6]	2·90	2·36	2·67	3·23	*ca.* 2·5
Tetra-*O*-methylcatenarin (IIb) [6]	2·83	—	2·75	3·26	*ca.* 2·2

[3] J. H. Birkinshaw, *Biochem. J.*, 1955, **59**, 486.
[4] H. Brockmann and G. Budde, *Chem. Ber.*, 1953, **86**, 432.
[5] E. Bullock, D. Kirkaldy, J. C. Roberts, and J. G. Underwood, *J.*, 1963, 829.
[6] D. F. G. Pusey and J. C. Roberts, *J.*, 1963, 3542.

closely related to 1,3,6,8-tetrahydroxy-2-methylanthraquinone. Additional evidence for this substitution pattern in dihydroaverythrin was obtained from a comparison of the proton magnetic resonance spectrum of tetra-*O*-methyldihydroaverythrin with the spectra of related compounds (Table 2). Dihydroaverythrin thus appeared to be a 1,3,6,8-tetrahydroxyanthraquinone carrying a C_6H_{13} substituent at position 2.

The proton magnetic resonance spectrum of tetra-*O*-methyldihydroaverythrin showed resonances, in addition to those of the aromatic protons (see above), which are typical of an Ar–n-hexyl system. The presence of this system was confirmed when it was found that oxidative degradation of dihydroaverythrin, with alkaline hydrogen peroxide, yielded heptanoic acid. Dihydroaverythrin thus appeared to be 2-hexyl-1,3,6,8-tetrahydroxyanthraquinone, and averythrin to be the corresponding 2-hexenyl compound. The proton magnetic resonance spectrum of averythrin, and the spectra of its acetate and *O*-methyl derivatives, all contain an unsymmetrical triplet (at *ca.* 3·3 τ, intensity two) which is difficult to interpret but which, by analogy with a similar feature in the spectrum of isosafrole [7] was assumed to be due to the ethylenic protons of a 1,2-unsaturated side-chain attached to an appropriately oxygenated aromatic nucleus. The tentative conclusion that averythrin was 2-hex-1-enyl-1,3,6,8-tetrahydroxyanthraquinone was confirmed by the following observations: (a) the modified Kuhn–Roth oxidation [8] of averythrin yielded a mixture of acetic, propionic, butyric, and valeric acid, thus indicating the presence of the Me·$[CH_2]_3$·C$\lessdot$ group; (b) ozonolysis of tetra-*O*-acetylaverythrin led to valeraldehyde; (c) ozonolysis of tetra-*O*-methylaverythrin, and isolation of the quinonoid moiety under oxidising conditions, gave 1,3,6,8-tetramethoxyanthraquinone-2-carboxylic acid which was identified by comparison with a synthetic sample. The further observation that the infrared spectrum of averythrin possesses a medium band at 981 $cm.^{-1}$ (ethylenic C–H out-of-plane bending), which is absent from the spectrum of dihydroaverythrin, led finally to the conclusion that averythrin is *trans*-2-hex-1′-enyl-1,3,6,8-tetrahydroxyanthraquinone (I).

The mass spectrum of tetra-*O*-methyldihydroaverythrin showed a parent peak corresponding to a molecular ion of *m/e* 412. Additional peaks at *m/e* 397, 383, 369, 355, 341, and 327 attested the stepwise degradation of the alkyl side-chain.

It is noteworthy that averythrin conforms to the acetate theory of biogenesis [9] [see (III)]. Appropriate condensations, reductions, oxidation, and the elimination of the elements of a molecule of water (from the atoms or groups attached to C-1 and C-2 in the side-chain) lead from the " polyacetic acid " system (III) to the averythrin structure (I).

The structural elucidation of two other red pigments called aversin and averufin, obtainable from different strains of *A. versicolor*, has been described previously.[5,6] It is interesting to note that these two pigments, together with averythrin, all have anthraquinone chromophores with identical oxygenation patterns.

Experimental

M. p.s were determined on a Kofler hot-stage apparatus. Optical rotations were measured on an Ericcson E.T.L.-N.P.L. automatic polarimeter, type 143A. Ultraviolet spectra were recorded, for ethanolic solutions, with a Perkin-Elmer spectrophotometer (model 137 UV). Infrared spectra were determined, for compounds in potassium bromide discs, with a Unicam spectrophotometer (S.P. 200). Proton magnetic resonance (p.m.r.) spectra were recorded on an A.E.I. R.S.2 spectrometer; these spectra were calibrated by the side-band technique, tetramethylsilane (for $CHCl_3$ and $CDCl_3$ solutions), or sodium 2,2-dimethyl-2-silapentane-5-sulphonate

[7] " High Resolution NMR Spectra Catalog," N. S. Bhacca, L. F. Johnson, and J. N. Shoolery, Varian Associates, Palo Alto, California, 1962; spectrum No. 252.

[8] (*a*) H. Bickel, H. Schmid, and P. Karrer, *Helv. Chim. Acta*, 1959, **38**, 664; (*b*) C. F. Garbers, H. Schmid, and P. Karrer, *ibid.*, 1954, **37**, 1336.

[9] A. J. Birch, *Proc. Chem. Soc.*, 1962, 3.

(for solutions in D_2O–NaOD), being used as internal references; in the sequel, figures in parentheses, following the statement of the nature of the signal, indicate intensities.

The silica used for thin-layer chromatography (t.l.c.) was " Kieselgel G nach Stahl " (Merck); that used for column chromtography was grade " M.F.C." (Hopkin and Williams).

Isolation of Averythrin.—*A. versicolor* (" T.R.L., No. 2543," from the culture collection of the Biological and Chemical Research Laboratory, Transvaal and Orange Free State Chamber of Mines, Johannesburg, South Africa) was kept in sub-culture on malt–agar slopes. Surface cultures, on a liquid Czapek–Dox medium [1] in " penicillin-type " flasks (see *Biochem. J.*, 1944, **38**, 456), were harvested after incubation at 25° for 3 weeks. The mycelium was collected and dried *in vacuo* at 50°. The finely powdered mycelium was exhaustively extracted (Soxhlet) with (a) light petroleum (b. p. 60—80°), (b) ether, and (c) acetone.

The light petroleum extract, when cooled, deposited long needles which, after purification,[2a] were identified as sterigmatocystin. Evaporation of the acetone extract gave a brown residue which, after having been washed with light petroleum (b. p. 60—80°) and repeatedly crystallised from methanol, yielded mannitol (identified by a comparison of itself and of its acetate with authentic specimens).

Evaporation of the ether extract (from 300 g. batches of mycelium) *in vacuo* gave variable amounts of a dark red oil which would not crystallise. A solution of this oil in chloroform (100 ml.) was poured on to a column (50 × 5 cm.) of heavy magnesium carbonate (Hopkin and Williams), and the chromatogram was developed with chloroform. The following bands (in order from the top) were observed: (i) violet, (ii) bright red, (iii) dark red, (iv) light red, and (v) other, but indistinct, bands due to sterigmatocystin and fatty impurities. Percolation with chloroform was continued until the eluate was colourless and only bands (i)—(iv) remained. The column was extruded and bands (ii)—(iv) were combined. The adsorbent was dissolved in cold 2N-hydrochloric acid, and the liberated pigments were collected in ether. Evaporation of the ether gave an amorphous, dark red solid (*ca.* 250 mg./300 g. of mycelium); t.l.c. [silica plate and benzene–ethyl acetate (2 : 1)] revealed at least six pigments, the one in major quantity having R_F 0·58.

The crude material (250 mg.) was dissolved in the minimum of hot ethyl acetate (*ca.* 3 ml.), and benzene (20 ml.) was added. The cooled solution was poured on to a silica column (25 × 2 cm.). Development with benzene gave a dark red band at the top of the column and an indistinct yellow-orange band which was eluted. Benzene–ethyl acetate (4 : 1) then caused the elution of a red-orange band which was collected in 30 ml. fractions. These were screened on thin-layer plates, as above, and those fractions giving a single spot, R_F 0·58, were combined. The solvents were evaporated *in vacuo*, and the red solid remaining was crystallised from methanol, ethyl acetate, or acetic acid to give *averythrin* (*ca.* 125 mg.).

General Properties of Averythrin.—Averythrin crystallised from methanol in small, red prisms, m. p. 229—231° (decomp.); it contained no halogen, nitrogen, sulphur, or methoxyl group (Found: C, 66·3, 66·4, 66·1; H, 5·5, 5·0, 4·9. $C_{20}H_{18}O_6,\frac{1}{2}H_2O$ requires C, 66·1; H, 5·3%); $\lambda_{max.}$ 223, 255sh, 266, 294, 324, 453 mμ (10^{-3} ε 29·2, 13·3, 15·1, 28·2, 10·5, 9·0), $\nu_{max.}$ 3350, 2855, 2800, 1650, 1617, 1580, 1479, 1440, 1412, 1330, 1285, 1261, 1205, 1160, 1140, 1090, 1040, 981, 950, 887, 867, 830 $cm.^{-1}$. The p.m.r. spectrum (D_2O–NaOD) showed: (i) a singlet (1) at 3·16 τ, (ii) two doublets (each 1) at 3·22 and 3·87 τ (J = 2·6 c./sec.), (iii) an unsymmetrical triplet (2) at 3·36 τ, (iv) a multiplet (2) at 7·76 τ, (v) a humped region (4) at *ca.* 8·6 τ, and (vi) a deformed triplet (3) at 9·08 τ.

Averythrin is insoluble in water but easily soluble in most polar organic solvents. It readily dissolves in 2N-sodium hydroxide and in 2N-sodium carbonate to give red-purple solutions, and is sparingly soluble in sodium hydrogen carbonate solution to give a pink colour. It yields a deep purple colour with concentrated sulphuric acid and a red-brown colour, in ethanolic solution, wih ferric chloride.

Tetra-O-*acetylaverythrin.*—Averythrin (0·1 g.), pyridine (1 ml.), and acetic anhydride (5 ml.) were kept at 100° for 3 hr. Evaporation of the solvents *in vacuo* and recrystallisation of the residue from ethanol gave the *acetate* (105 mg.) as yellow needles, m. p. 198—200° (Found: C, 64·1; H, 5·0. $C_{28}H_{26}O_{10}$ requires C, 64·4; H, 5·0%), $\lambda_{max.}$ 213, 272, 348 mμ (10^{-3} ε 24·4, 31·7, 4·77), $\nu_{max.}$ included bands at 1765 (aryl acetate) and 1666 $cm.^{-1}$ (anthraquinone C=O group). The p.m.r. spectrum ($CDCl_3$) showed: (i) a singlet (1) at 2·03 τ, (ii) two doublets (each 1) at 2·01 and 2·64 τ (J = 2·5 c./sec.), (iii) an unsymmetrical triplet (2) at 3·64 τ, (iv) two singlets (each 6) at 7·54 and 7·63 τ, partly obscuring a multiplet (2), (v) a humped region (4) at *ca.* 8·6

τ, (vi) a deformed triplet (3) at 9·06 τ. This acetate could not be sublimed. It was hydrolysed, by cold aqueous ethanolic potassium hydroxide, to averythrin.

Tri-O-*methylaverythrin*.—Averythrin (57 mg.), dry acetone (10 ml.), anhydrous potassium carbonate (0·5 g.), and dimethyl sulphate (0·15 ml.) were heated under reflux for 6 hr. The mixture was filtered, and the solvent was removed *in vacuo*, to give a crystalline orange-yellow residue; t.l.c. [silica plate and benzene–ethyl acetate (19 : 1)] indicated the presence of two compounds (R_F 0·55 and 0·16) corresponding respectively to the tri- and tetra-*O*-methyl derivatives. A solution of the crude residue in benzene (10 ml.) was chromatographed on a silica column (20 × 2 cm.). Development with benzene gave a broad orange band which was eluted. Removal of the solvent *in vacuo*, and recrystallisation of the residue from ethanol, gave the *ether* (26 mg.) as orange needles, m. p. 184—186° (Found: C, 69·6; H, 6·4. $C_{23}H_{24}O_6$ requires C, 69·7; H, 6·1%), $\nu_{max.}$ included bands at 1661 (quinone C=O) and 1620 cm.$^{-1}$ (hydrogen-bonded quinone C=O). The p.m.r. spectrum ($CHCl_3$) included (*inter alia*) three singlets (each 3) at 5·97, 6·00, and 6·10 τ ($3OCH_3$).

Tetra-O-*methylaverythrin*.—Averythrin (0·1 g.), dry acetone (60 ml.), anhydrous potassium carbonate (3 g.), and dimethyl sulphate (1·5 ml.) were heated under reflux until t.l.c. (as above) showed the presence of only one compound, R_F 0·16 (*ca.* 8 hr.). The mixture was filtered and the solvent was removed *in vacuo* from the filtrate. A solution of the resulting dark oil in benzene (25 ml.) was chromatographed on a silica column (20 × 1·5 cm.). Benzene–ethyl acetate (9 : 1) eluted a wide green-yellow band. Evaporation of the solvents from the eluate and crystallisation of the residue from methanol gave the *ether* as yellow needles (73 mg.), m. p. 176—177° [Found: C, 70·5; H, 6·3%; *M* (mass spectrometry), 410. $C_{24}H_{26}O_6$ requires C, 70·2; H, 6·4%; *M*, 410], $[\alpha]_D^{20} \not> -1·8°$ (*c* 0·40 in $CHCl_3$), $\lambda_{max.}$ 228, 293, 374 mμ (10^{-3} ε 30·5, 40·3, 10·0), $\nu_{max.}$ included a band at 1662 cm.$^{-1}$ (quinone C=O) but no band near 1620 cm.$^{-1}$ (absence of hydrogen-bonded C=O). The p.m.r. spectrum ($CDCl_3$) showed: (i) a singlet (1) at 2·47 τ, (ii) a doublet (1) at 2·67 τ, (iii) a multiplet (3) at 3·18—3·27 τ, (iv) three singlets (3, 3, and 6) at 5·93, 5·97, and 6·01, respectively, (v) a multiplet (2) at 7·7 τ, (vi) a humped region (4) at *ca.* 8·5 τ, and (vii) a deformed triplet (3) at 9·0 τ.

Dihydroaverythrin.—A solution of averythrin (22 mg.) in ethyl acetate (8 ml.) was shaken, in an atmosphere of hydrogen, with palladised charcoal (10%; 25 mg.); *ca.* 2·1 mol. of hydrogen was absorbed. Filtration, evaporation of the solvent from the filtrate, and chromatography of a benzene solution of the residue on a column (10 × 1 cm.) of silica, gave an orange band at the top of the column. Ethyl acetate–benzene (4 : 1) eluted an orange band. Evaporation *in vacuo* of the solvents from the eluate, and crystallisation of the residue from methanol, gave *dihydroaverythrin* (16 mg.) as small, red prisms, m. p. 253° (Found: C, 65·3, 66·0; H, 5·7, 5·6. $C_{20}H_{20}O_6,\frac{1}{2}H_2O$ requires C, 65·7; H, 5·7%), $\lambda_{max.}$ 223, 255, 265sh, 293, 325, 453 mμ (10^{-3} ε 28·0, 11·93, 12·9, 30·6, 9·35, 8·89), $\nu_{max.}$ included bands at 3335, 2850, 2800, 1650, and 1614 cm.$^{-1}$. The p.m.r. spectrum (D_2O–NaOD) showed: (i) a singlet (1) at 3·15 τ, (ii) two doublets (each 1) at 3·15 and 3·86 τ (J = 2·5 c./sec.), (iii) an ill-defined triplet (2) at 7·42 τ, (iv) a humped region (8) at *ca.* 8·5 τ, (v) a deformed triplet (3) at 9·05 τ. A mass-spectrometric determination of the molecular weight on a sample of this phenol which had been sublimed (at *ca.* 240°/10^{-2} mm.) gave a value of 356 ($C_{20}H_{20}O_6$ requires *M*, 356).

Tetra-O-*acetyldihydroaverythrin*.—This was prepared in the same way as tetra-*O*-acetylaverythrin (above). The *acetate* of the dihydro-derivative crystallised from methanol in yellow needles, m. p. 176—177° (Found: C, 64·5, 64·2; H, 5·5, 5·0. $C_{28}H_{28}O_{10}$ requires C, 64·1; H, 5·4%), $\lambda_{max.}$ 212, 264, 342 mμ (10^{-3} ε 29·7, 42·5, 6·35), $\nu_{max.}$ included bands at 1765 (aryl acetate) and 1670 cm.$^{-1}$ (unchelated quinone C=O). The p.m.r. spectrum ($CDCl_3$) showed: (i) a singlet (1) at 2·01 τ, (ii) two doublets (each 1) at 2·01 and 2·65 τ (J = 2·5 c./sec.), (iii) four singlets (each 3) at 7·47, 7·50, 7·56, and 7·61 τ, partly obliterating a multiplet (2), (iv) a humped region (8) at *ca.* 8·6 τ, (v) a deformed triplet (3) at 9·1 τ.

Reductive Acetylations.—The above acetate (100 mg.) was reductively acetylated by the Brockmann technique,[4] and the material produced was purified by chromatography in chloroform solution on a silica column (45 × 0·6 cm.). The product separated from methanol as a semicrystalline, pale yellow material (*ca.* 50 mg.) ($\lambda_{max.}$ 345, 363, 384, 404 mμ) which was shown by t.l.c. to contain two compounds having almost the same R_F values. Further purification was impossible. (1,4,5,9,10-Penta-acetoxyanthracene [4] has $\lambda_{max.}$ 349, 367, 385, 407 mμ.) Similar results were obtained in the reductive acetylation of tetra-*O*-acetylaverythrin.

Tri-O-*methyldihydroaverythrin*.—Dihydroaverythrin (0·1 g.), dry acetone (35 ml.), anhydrous

potassium carbonate (1·7 g.), and dimethyl sulphate (0·9 ml.) were heated under reflux for ½ hr.; t.l.c., as used in the preparation of tri-*O*-methylaverythrin, above, gave a single spot, R_F 0·66. Isolation and purification of the product (by chromatography in benzene solution on silica) in the usual way gave the *ether* (40 mg.) which crystallised from methanol in orange needles, m. p. 176—178° (Found: C, 69·0; H, 6·5. $C_{23}H_{26}O_6$ requires C, 69·3; H, 6·6%), $\nu_{max.}$ included bands at 1660 (C=O) and 1620 cm.$^{-1}$ (hydrogen-bonded C=O).

Tetra-O-methyldihydroaverythrin.—Dihydroaverythrin (35 mg.) was methylated in a similar way to that used for tetra-*O*-methylaverythrin (above). After *ca.* 9 hr., t.l.c. indicated the presence of only one compound, R_F 0·22. The *ether* (27 mg.) crystallised from methanol in yellow needles, m. p. 151—152° [Found: C, 70·2; H, 7·1%; *M* (mass spectrometry), 412. $C_{24}H_{28}O_6$ requires C, 69·9; H, 6·9%; *M*, 412]; $\nu_{max.}$ included a band at 1657 but no band in the region of 1620 cm.$^{-1}$. The p.m.r. spectrum ($CDCl_3$) showed (in addition to the resonances of the aromatic protons; see above): (i) two singlets (each 6) at 5·9 and 6·2 τ, (ii) an ill-defined triplet (2) at 7·2 τ; (iii) a humped region (8) at *ca.* 8·6 τ, (iv) a deformed triplet (3) at 9·09 τ.

Modified Kuhn–Roth Oxidation of Averythrin.—Averythrin (30 mg.) was added to the standard [8b] chromic–sulphuric acid solution (20 ml.). Alternate distillations (of 2 ml. of liquid) and replacements (of 2 ml. of water to the reaction mixture) were carried out until *ca.* 25 ml. of distillate had been collected. This was neutralised (2N-sodium hydroxide), and the solution was evaporated to dryness. A solution of the residue in a minimum of water was shaken with Zeo-Karb-225 and the mixture was filtered. The acids in the filtrate were identified by circular paper chromatography (Whatman No. 1 paper) using ethanol–aqueous ammonia (*d* 0·880) (24 : 1) as the developing solvent and 0·5% aqueous solutions of the lower aliphatic acids to provide "markers." The zones were detected by spraying the paper with a solution made by diluting Universal Indicator (British Drug Houses) with four times its volume of water and adjusting the pH to 8—9. The solution from the oxidation process gave zones at R_F 0·32, 0·45, 0·57, and 0·69. Acetic, propionic, n-butyric, and n-valeric acids gave zones at R_F 0·32, 0·47, 0·57, and 0·69, respectively.

Ozonolysis of Tetra-O-acetylaverythrin.—The acetate (0·1 g.) was dissolved in methylene dichloride (40 ml.) and the solution was cooled to −40°. Ozonised oxygen was bubbled through the solution for 2 hr. Removal of the solvent *in vacuo* gave a light yellow solid which was mixed with a suspension of zinc dust (0·3 g.) in acetic acid (30 ml. of a 25% aqueous solution). The mixture was then steam-distilled until the distillate no longer gave a precipitate with a saturated solution of 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid. The derivative was collected and, after having been purified by chromatography in benzene solution on a silica column (14 × 0·6 cm.), was crystallised from methanol to give yellow needles (2 mg.), m. p. 107°. This compound was proved to be n-valeraldehyde 2,4-dinitrophenylhydrazone by comparison with an authentic specimen, m. p. 107° (mixed m. p. and chromatographic behaviour).

Oxidation of Dihydroaverythrin.—To a solution of the pigment (120 mg.) in 2N-sodium hydroxide (25 ml.) was carefully added hydrogen peroxide (15 ml. of a 15% solution). After the initial evolution of oxygen had ceased, the solution was heated on a water-bath for ½ hr. Further portions of the hydrogen peroxide solutions (2 × 10 ml.) were added until the colour had faded to a very pale red. The cooled, acidified solution was extracted with ether. Evaporation of the ethereal solution gave an acidic yellow syrup. The acid was converted into its *p*-bromoanilide. A solution of this anilide in benzene was chromatographed on a column of silica (15 × 0·7 cm.). Development with benzene–ethyl acetate (9 : 1) gave a light brown band which was eluted. Evaporation of the solvents from a 5 ml. fraction of the eluate immediately following the light brown band gave colourless rods (2·5 mg.), m. p. 95—97·5°. This compound was identified as *p*-bromohexananilide by comparison with an authentic specimen, m. p. 96—98° (mixed m. p. and chromatographic behaviour).

Ozonolysis of Tetra-O-methylaverythrin.—A stream of ozonised oxygen was passed for 2 hr. through a cold (−30°) solution of the ether (0·1 g.) in methylene dichloride (40 ml.). Removal of the solvent *in vacuo* yielded a reddish oil which was stirred for 12 hr. with water (50 ml.) and hydrogen peroxide (2 ml. of a 30% solution). Further quantities (3 × 2 ml.) of hydrogen peroxide were then added at ½ hr. intervals. A saturated solution of sodium hydrogen carbonate (50 ml.) was added and the mixture was filtered from some oily material. Acidification of the filtrate gave a solid which crystallised from methanol as yellow needles, m. p. 228—231°.

This compound was proved to be 1,3,6,8-tetra-*O*-methylanthraquinone-2-carboxylic acid by comparison with a synthetic sample, m. p. 229—231° (mixed m. p. and infrared spectra).

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