Anthraquinone Pigments from *Phoma foveata* Foister

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1. Suitable conditions have been determined for growing the fungus *Phoma foveata* in culture for optimum production of pigments. 2. The pigments have been extracted and fractionated according to their solubility in aqueous bicarbonate, carbonate and alkali. 3. The carbonate- and alkali-soluble pigments have been further separated by chromatography and shown to consist of anthraquinone derivatives. 4. The alkali-soluble fraction, which comprised the bulk of the pigment, consists of pachybasin (1-hydroxy-3-methylanthraquinone; II) and chrysophanol (1,8-dihydroxy-3-methylanthraquinone; III) by comparison with authentic samples. 5. The carbonate-soluble fraction contains emodin (1,6,8-trihydroxy-3-methylanthraquinone; V) and a hitherto unrecorded pigment for which the name phomarin and the structure 1,6-dihydroxy-3-methylanthraquinone (IV) are put forward. 6. The identification of emodin and the structural determination of phomarin are based largely on their ultraviolet, visible, infrared, nuclear-magnetic-resonance and mass spectra.

The imperfect fungi *Phoma solanicola* Prill. and Delacr., *Ph. tuberosa* Mel., Ros. and Sch. and *Ph. foveata* cause gangrene in potatoes, and Malcomson (1958) considers them to be indistinguishable in pathological action. The organism associated with potato gangrene in Victoria is described by Chambers (1958) as *Ph. foveata*, and this pathogen also arises periodically in Tasmania during the storage of potatoes after harvesting. During a study of this problem by P. J. Sampson (personal communication), it was observed that aging cultures of the organism formed yellow pigments that frequently crystallized as needles in the medium. We have examined these substances, and found that they belong to the class of anthraquinone pigments.

Some 40 anthraquinone derivatives of fungal or lichen origin have been described (Thomson, 1957; Joshi, 1962; Shibata, 1962). All of them have at least one α -hydroxy group, and with one exception they all contain other oxy substituents, the 1,8-

dihydroxy and the 1,5,8- or 1,6,8-trihydroxy derivatives being especially common. Most of them also have a carbon-containing function, usually methyl, in the 3-position. These substitution patterns are intelligible in terms of Birch & Donovan's (1953) hypothesis of the biosynthesis of anthraquinones from a chain of acetate units, followed by cyclization and secondary reactions; the latter include decarboxylation, introduction of other oxy functions, particularly in α -positions, or loss of the 6- or 8-hydroxy groups initially present. The overall correctness of this hypothesis for helminthosporin (I) (Birch, Ryan & Smith, 1958) and for certain other anthraquinones (Gatenbeck, 1960; Shibata & Ikekawa, 1963) has been demonstrated by tracer experiments with $[^{14}\text{C}]$ acetate. Shibata & Ikekawa (1963) have also shown that, though acetate forms the methyl end of the polyacetate chain, the remainder is formed preferentially from malonate. From analogy with fatty acid biosyn-

thesis, it may be inferred that acetate must first be carboxylated to malonate before incorporation into the remainder of the chain.

MATERIALS AND METHODS

Melting points are corrected. Evaporations were carried out *in vacuo*, and sublimations at 105° and 10⁻⁶ mm. Chromatographic solvents were purified by distillation; column chromatography was carried out on MgCO₃ ('heavy'; May and Baker Ltd., Dagenham, Essex)—Hyflo Super-Cel (Hopkin and Williams Ltd., Chadwell Heath, Essex) (1:1, w/w); for thin-layer chromatography a slurry of water–MgCO₃ (1:1, w/w) (without binder) was spread as thinly as possible for maximum resolution on 8 in.² plates, which were air-dried for 24 hr. and stored over silica gel. They were developed with benzene–chloroform mixtures, and at very low concentrations the positions of the pigments were observed under u.v. light. The u.v. and visible spectra were determined in ethanol on a Perkin–Elmer model 4000A Spectracord Spectrophotometer; i.r. spectra were determined in Nujol mulls, or in hexachlorobutadiene in the regions of Nujol absorption, on a Perkin–Elmer model 221 Spectrophotometer; n.m.r. (nuclear-magnetic-resonance) spectra were determined on a Varian model A60 Spectrometer, with tetramethylsilane as internal reference; mass spectra were determined on a Metropolitan–Vickers model M.S.9 Mass Spectrometer with direct entry system. Microanalyses were carried out by the C.S.I.R.O. Micro-analytical Service, Melbourne, Victoria, Australia.

The fungus was grown initially on potato–dextrose agar in 9 cm. diam. glass Petri dishes, but a modified Sabouraud medium (Robinow, 1963) was later adopted. After 9 days' growth at room temperature without temperature control, the contents of the dishes were dried *in vacuo* at 60°, ground in a Wiley no. 1 mill and extracted in a Soxhlet apparatus with acetone until no further coloured materials were removed. An extract from 400 plates produced 10 g. of sticky red crystalline material on evaporation. This was dissolved in benzene and the solution extracted in a separating funnel with aq. 5% (w/v) NaOH until no further red salts were removed. The alkaline solution was acidified with HCl and the regenerated yellow pigments were extracted with chloroform. The solvent was removed and replaced with benzene, and the solution extracted successively with aq. 5% (w/v) NaHCO₃ and aq. 5% (w/v) Na₂CO₃. After these extractions, the bulk of the alkali-soluble pigment remained in the benzene solution, which on evaporation yielded fraction *R*₁. The aqueous solutions were separately acidified and extracted with chloroform as before; the chloroform solution thus obtained from the carbonate extract yielded fraction *R*₂ on evaporation, and that from the bicarbonate extract fraction *R*₃.

Fraction *R*₁ was chromatographed on a column in light petroleum (b.p. 40–60°). Two coloured bands appeared, and on elution with acetone the lower straw-yellow band (*R*₁*A*) was completely removed. The pigment in the remaining pink band (*R*₁*B*) was recovered by dissolving the MgCO₃ from the column in dilute HCl, then extracting the mixture with chloroform. The acetone and chloroform extracts were evaporated separately, and the homogeneity

of the fractions *R*₁*A* and *R*₁*B* thus obtained was checked by thin-layer chromatography; the fractions were finally purified by sublimation.

Fraction *R*₂, dissolved in light petroleum–acetone (3:1, v/v), was chromatographed similarly to *R*₁. Eight bands appeared, of which *R*₂*A*, the most easily eluted, proved to be identical with *R*₁*A*. This was followed by *R*₂*B* (pale pink), *R*₂*C* (yellow), *R*₂*D* (pale pink) and *R*₂*E* (pink). The column was cut into suitable sections and each pigment recovered as before. Fractions *R*₂*B*, *R*₂*C* and *R*₂*E* only were present in sufficient quantity (1–5 mg.) for further investigation; they were purified as described above.

Fraction *R*₃ has not been examined in detail, but column chromatography revealed at least nine coloured bands, one of which was eluted by benzene, and proved to be soluble in aq. 5% (w/v) NaOH but insoluble in aq. 5% (w/v) NaHCO₃ or aq. 5% (w/v) Na₂CO₃. The amount was insufficient to verify its identity with pigment *R*₁*A*, with whose behaviour it corresponds.

RESULTS

Characterization of pigments. Fractions *R*₁*A*, *R*₁*B*, *R*₂*B*, *R*₂*C* and *R*₂*E* all gave negative zirconium nitrate reactions (Feigl, 1960), indicating the absence of hydroxyl groups *ortho* to one another. Fraction *R*₁*A* was crystallized from methanol and finally obtained as a yellow sublimate, m.p. 176° unchanged by admixture with an authentic sample of pachybasin (II), which was synthesized by the method of Waldmann & Sellner (1938). Light-absorption maxima were (log $\epsilon_{\max}$ in parentheses): 224 (3.74), 252 (4.01), 281 (3.65) and 403 m μ (3.02); the i.r.-absorption maxima were: 3083, 2967, 2933, 2862, 1677, 1642, 1593, 1578, 1403, 1380, 1338, 1311, 1290, 1235, 1148, 1068, 1039, 1002, 929, 872, 805, 793, 769 and 712 cm.⁻¹. The n.m.r. spectrum in CDCl₃ showed δ : 2.45 (Me), 7.17 (H-2; broad, 4 cyc./sec.), 7.55 (H-4; broad, 4 cyc./sec.), 8.20 (H-5 and H-8), 7.74 (H-6 and H-7) and 12.67 p.p.m. (OH) (Found: C, 75.9; H, 4.3; O, 20.8. Calc. for C₁₅H₁₀O₃: C, 75.6; H, 4.2; O, 20.2%).

Fraction *R*₁*B* formed orange–yellow crystals on sublimation, m.p. 186° alone and 189° on admixture with an authentic sample (m.p. 193°) of chrysophanol (III) (supplied by Fluka A.-G., Buchs, Switzerland, and purified by chromatography as above). The i.r. spectra of *R*₁*B* and chrysophanol were identical (Bloom, Briggs & Cleverley, 1959), as also were the u.v. and visible spectra (Birkinshaw, 1955). The n.m.r. spectrum in CDCl₃ showed δ : 2.45 (Me), 7.12 (H-2; broad, 4 cyc./sec.), 7.40 (H-4; broad, 3 cyc./sec.), 7.80 (H-5, H-6 and H-7), 12.00 (OH) and 12.14 p.p.m. (OH) (Found: C, 71.0; H, 4.1; O, 25.3. Calc. for C₁₅H₁₀O₄: C, 70.9; H, 3.9; O, 25.2%).

Fractions *R*₂*B*, *R*₂*C* and *R*₂*E* were not available in sufficient quantity for rigorous purification. The

first two had similar u.v. and visible spectra, the same n.m.r. spectra, and the same parent peaks and similar fragmentation patterns in their mass spectra. It is considered that they consist of the same substance, *phomarin*, contaminated with different impurities. From the mass spectra, R_2B appeared to contain a higher-molecular-weight waxy substance, and R_2C apparently contains small amounts of R_2E . After further purification by chromatography and sublimation, fraction R_2B was used to determine the light-absorption spectra for *phomarin*, and R_2C for the remaining data.

Phomarin forms a yellow crystalline sublimate that decomposes gradually above 200° without melting. It dissolves in aq. 5% (w/v) sodium carbonate almost without colour change. The light-absorption maxima were: 219, 268, 293 (shoulder) and 410 m μ ; after being refluxed with 1 ml. of acetic acid and 1 ml. of 48% (w/v) hydrobromic acid for 7½ hr., the spectrum of *phomarin* (0.5 mg.) was unchanged. The i.r.-absorption maxima were: 3414, 3310, 2925, 2856, 1677, 1640, 1603, 1579, 1488, 1389, 1305, 1268, 1191, 1153, 1020, 945, 894, 854, 817, 795 and 795 cm.⁻¹. The n.m.r. spectrum in acetone showed δ : 7.10 (broad, 3 cyc./sec.), 7.56 (broad, 3 cyc./sec.), 7.28 (quartet, J_1 8.5, J_2 2.5 cyc./sec.), 7.65 (doublet, J 2.5 cyc./sec.) and 8.14 p.p.m. (doublet J 8.5 cyc./sec.) (Found: mol. wt. from mass spectrum: 254.0561. C₁₅H₁₀O₄ requires 254.0579).

Fraction R_2E was obtained as an orange sublimate that was rechromatographed on a column as before. Two small leading bands (red and violet) were discarded, and the main pink band was cut into three equal sections that were extracted as described above. The three fractions had nearly identical i.r. spectra, which were substantially the same as the recorded spectrum of emodin (Bloom, *et al.* 1959). The u.v. and visible spectra of R_2E were identical with those of emodin and citreorosein (Birkinshaw, 1955). The m.p. of R_2E was 240° and a mixed m.p. with an authentic sample (m.p. 255°) of emodin (supplied by Fluka) was 251°. The n.m.r. spectrum in acetone showed δ : 7.02 (broad,

3 cyc./sec.), 7.49 (broad, 3 cyc./sec.), 7.19 (doublet, J 2.5 cyc./sec.), 6.57 (doublet, J 3 cyc./sec.) and 7.77 p.p.m.; relative intensities were 20, 27, 31, 25 and 15 (Found: mol. wt. from mass spectrum: 270.0519. Calc. for C₁₅H₁₀O₅: 270.0528). In the mass spectrum, in addition to the main parent peak, there was a small peak at mass 254 and an even smaller one at mass 286.

DISCUSSION

Considerable advantages were found in the use of modified Sabouraud medium (Robinow, 1963) over potato-dextrose agar medium in growing the fungus: the time required to achieve maximum pigment production was thereby reduced from 21 to 9 days, and at the same time considerably more pigment was produced. However, the capacity to produce pigments was rapidly lost on continued subculturing on artificial media, and stock cultures were maintained on potatoes (var. Pink Eye) at 4°. Pigment production in liquid potato-dextrose medium was poor, and the extraction procedure tedious owing to the formation of emulsions.

The two major pigments, pachybasin (R_1A ; II) and chrysophanol (R_1B ; III) were obtained substantially pure and readily identified, but the separation procedures were inadequate for rigorous purification of the minor components. It was clear that the presence of mixtures affected the solubility in alkali of some of the components: thus pachybasin, which when pure is insoluble in carbonate and bicarbonate solutions, was present in the former extract and probably in the latter as well. Moreover, it was evident that discrete coloured bands appearing in a chromatograph column did not necessarily correspond to separate compounds; the bands could be due to mixtures of the same components with different amounts of impurities, as observed in other anthraquinone studies (R. G. Cooke, personal communication).

From its mass spectrum, fraction R_2E consists very largely of a compound C₁₅H₁₀O₅, with smaller amounts of C₁₅H₁₀O₄ and even less of C₁₅H₁₀O₆. The spectral data indicate that R_2E consists mainly of emodin; its melting point is low for emodin, but its identity is confirmed by the following considerations. The visible spectrum of R_2E has an absorption maximum at 435 m μ , which indicates the presence of two α -hydroxyl groups (Briggs, Nicholls & Paterson, 1952; Birkinshaw, 1955). These must be in the 1,8-positions, since a 1,4- or 1,5-arrangement would produce 'conjugate chelation' (Bellamy, 1958; Bloom *et al.* 1959) in both carbonyl groups; the carbonyl absorptions (1677 and 1625 cm.⁻¹) in the infrared spectrum of R_2E show in fact that only one is thus chelated. The aromatic

(II) R = R' = H

(III) R = H, R' = OH

(IV) R = OH, R' = H

(V) R = R' = OH

(VI) R = OMe, R' = OH

(VII) R = O·CH₂·CH:·CMe₂,
R' = OH

portion of the n.m.r. spectrum of fraction R_2E indicates that four aromatic protons are present in the nucleus, two in each ring; further, they are in *meta* positions in each case, since they exhibit long-range coupling with a small coupling constant (Jackman, 1959). The substitution pattern is thus at positions 1, 3, 6 and 8, as for physcion (VI) and madagascin (VII), whose published n.m.r. spectra (Ritchie & Taylor, 1964*a,b*) correspond to that of R_2E in the aromatic region. From the molecular formula $C_{15}H_{10}O_5$ of the major component of R_2E , the two other substituents must be a methyl group and a hydroxyl group, and thus R_2E consists principally of emodin (V). The main impurity in R_2E is evidently chrysophanol (III) from its molecular formula $C_{15}H_{10}O_4$, and from the occurrence of a small peak at δ 7.77 p.p.m. in the n.m.r. spectrum of fraction R_2E , which corresponds to the absorption of the three protons attached to positions 5, 6 and 7 of chrysophanol; the remaining aromatic protons at positions 2 and 4 in the latter give absorptions that overlap with some of the peaks for emodin.

The n.m.r. spectrum of phomarin indicates the presence of five aromatic protons; there are thus three substituents, and, since the molecular formula is $C_{15}H_{10}O_4$, these must be two hydroxyl groups and a methyl group. This conclusion is supported by a comparison of the fragmentation patterns obtained from chrysophanol and phomarin by mass spectroscopy. The main peaks were in corresponding positions although they differed in intensity. A peak at mass 239 ($p - 15$)⁺ is attributed to loss of a methyl group from the parent ion, one of mass 237 ($p - 17$)⁺ to loss of a hydroxyl group and one of mass 169 to the fragment ($p - C_3O_3H$)⁺, which was recorded in the mass spectra of all dihydroxyanthraquinones examined by Beynon & Williams (1960). From the carbonyl absorptions (1677 and 1640 cm^{-1}) in the i.r. spectrum of phomarin, one of the hydroxyl groups is in an α -position (Bloom *et al.* 1959), and from the very strong aromatic bands in the 1560–1620 cm^{-1} region, more intense than the carbonyl bands, the other hydroxyl group is in a β -position (Tanaka, 1958). These assignments for the two hydroxyl groups are confirmed by the light-absorption peak at 410 $m\mu$, which indicates that one hydroxyl group only is in an α -position (Briggs *et al.* 1952; Birkinshaw, 1955). The hydroxyl groups cannot be in the same ring, otherwise the protons attached to it would produce doublets in the n.m.r. spectrum, and the aromatic region would show not more than one singlet absorption. Two broad singlets in fact appear at δ 7.10 and 7.56 p.p.m. (each 3 $cyc./sec.$); they are present in approximately the same positions in all the spectra of the anthraquinones from *Ph. foveata*, and also in the recorded spectra of physcion

(VI) and madagascin (VII) (Ritchie & Taylor, 1964*a,b*). These absorptions seem to be typical of *meta*-grouped protons H-2 and H-4 exhibiting long-range coupling through a methyl group. The spectral evidence thus places a methyl group *meta* to an α -hydroxyl group, and points to phomarin having a 1,6- or 1,7-dihydroxy-3-methylanthraquinone structure. This is further confirmed by the remaining aromatic portion of the n.m.r. spectrum. The quartet at δ 7.28 p.p.m. (J_1 8.5, J_2 2.5 $cyc./sec.$) is assigned to a β -proton split by both α -protons attached to the same ring, one *ortho* and one *meta* to it; the β -proton in its turn splits each of the α -ones, one *ortho* at δ 8.14 p.p.m. (J 8.5 $cyc./sec.$) and one *meta* at δ 7.65 p.p.m. (J 2.5 $cyc./sec.$). The n.m.r. evidence does not differentiate between the above-mentioned alternative structures for phomarin, but a distinction can be drawn from their recorded i.r. spectral data, which are limited to the 1560–1620 cm^{-1} region: a Nujol mull of the 1,7-dihydroxy compound showed one very strong absorption band at approx. 1580 cm^{-1} , whereas for the 1,6-dihydroxy compound the aromatic absorption was split into two bands at approx. 1600 and 1580 cm^{-1} (Tanaka, 1958). Phomarin shows two intense bands at 1603 and 1579 cm^{-1} in this region, and it is therefore assigned the structure 1,6-dihydroxy-3-methylanthraquinone (IV). This structure is also preferred on biogenetic grounds, and on this basis both phomarin and chrysophanol have deoxymodoin structures.

Pachybasin (II) and chrysophanol (III) have been isolated previously from the fungus *Pachybasium candidum* (Sacc.) Peyronel by Shibata & Takido (1955), and chrysophanol in addition from *Penicillium islandicum* Sopp. by Howard & Rastick (1950). Emodin (V) has been isolated from the latter mould (Shibata, 1962), and also from *Cortinarius sanguineus* (Wulf) Fries (Kögl & Postowsky, 1925) and from a mutant strain of *Cladosporium fulvum* Cooke (Agosti, Birkinshaw & Chaplen, 1962). In addition, emodin and chrysophanol have been found in many higher plants (Karrer, 1958).

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