823. *The Pigmentation and Cell-wall Material of* Daldinia *Sp.*

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The chromogen of *D. concentrica* sporophores is 4 : 5 : 4′ : 5′-tetrahydroxydinaphthyl, which is oxidised enzymically to 4 : 9-dihydroxyperylene-3 : 10-quinone and to polymer. In the sporophores these oxidation products cross-link the cell-wall material, apparently by combining with non-acetylated amino-groups.

The fungus *Daldinia concentrica* (Bolt.) Ces. and de Not. is one of the larger Ascomycetes, occurring commonly in Britain as a parasite upon ash (*Fraxinus excelsior* L.) in which it causes a " white rot " with black staining and the condition known as " calico wood." At maturity it produces numerous fruit-bodies in the form of black hemisperical lumps up to 10 cm. across and with a characteristic zoned appearance in section. These eventually become light in weight and brittle, but younger specimens are denser and tougher and exude a little milky fluid on fracture; though ethanol extracts of such fruits are not deeply coloured they have a distinctive ultraviolet absorption spectrum and deposit a copious black precipitate on storage. This behaviour was under investigation when Anderson and Murray [1] reported the isolation of 4 : 9-dihydroxyperylene-3 : 10-quinone (I) from the sporophores; since this quinone [2] is almost black and virtually insoluble in most solvents it seemed possible that our own extracts might contain a more soluble precursor responsible for the characteristic absorption spectrum.

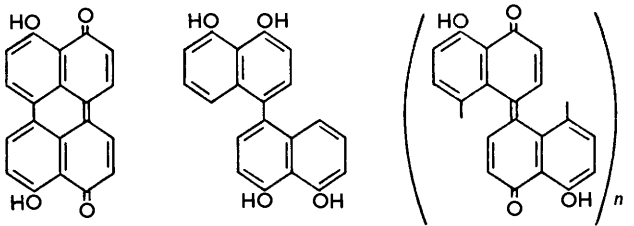
When a batch of sporophores had been broken up and defatted by extraction with light petroleum, ether-extraction afforded a solution ($\lambda_{max.}$ 313, 325, 340 mμ) from which the suspected precursor could be extracted by aqueous sodium carbonate (causing a bathochromic shift of 10—15 mμ) though not by sodium hydrogen carbonate solution. The solution gave an intense grey-green colour with ferric chloride, followed by a black precipitate. By chromatography on " Florisil " in chloroform, with careful exclusion of light and air from the column, a phenol was isolated as light brown crystals, m. p. 225—230° (decomp.). As attempts at further purification led only to deterioration, the compound was acetylated; this gave a stable derivative, m. p. 245°, for which elementary analysis and molecular-weight determination indicated a formula $C_{20}H_{10}(OAc)_4$.

The ultraviolet absorption spectrum of the phenol (cf. Table 1) is characteristic of naphthalene derivatives containing auxochromic substituents only in the α-positions.[3] Now, of the hydroxynaphthalenes of this type, those with two hydroxyl groups absorb at shorter wavelengths whilst 1 : 4 : 5-trihydroxynaphthalene absorbs at longer wavelengths. Since the close relation of the phenol to the perylene derivative (I) (see below) rendered unlikely the presence of substituents other than hydroxyl groups, a symmetrically hydroxylated 1 : 1′-dinaphthyl structure could be deduced from the spectroscopic data. The infrared absorption spectrum of the phenol was very similar to that of 1 : 8-dihydroxynaphthalene, and the presence of hydroxyl groups in *peri*-positions was confirmed by the effect of the phenol in depressing the pH of boric acid solution (see Table 2), the magnitude of the effect being characteristic.[4] This experiment failed to reveal the presence of other ionisable groups.

The instability of the phenol, with ready conversion into black pigments, has already been remarked upon, and the quinone (I) was identified amongst the oxidation products obtained under a variety of conditions. Thus the product from oxidation with chloranil in phenetole was partly soluble in tetrachloroethane or concentrated sulphuric acid, giving solutions with absorption spectra attributable [2] to the quinone (I) and on vacuum-sublimation afforded crystalline quinone (I), identified by its infrared absorption spectrum.[2]

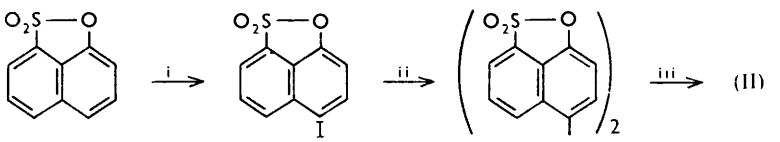
[1] Anderson and Murray, *Chem. and Ind.*, 1956, 376.
[2] Calderbank, Johnson, and Todd, *J.*, 1954, 1285.
[3] Daglish, *J. Amer. Chem. Soc.*, 1950, **72**, 4859.
[4] Hochstein, Stephens, Conover, Regna, Pasternack, and Woodward, *ibid.*, 1953, **75**, 5458.

Similarly the phenol gave a fairly stable pale yellow solution in concentrated sulphuric acid, from which deeply coloured solutions with the absorption spectrum of the quinone (I) were obtained on warming or on addition of nitric acid. The autoxidation of the phenol in dilute aqueous solutions was accelerated not only by alkali but also, more significantly, by the addition of crude mushroom phenolase or of an aqueous extract of *D. concentrica* mycelium; in each case the precipitated product was partly insoluble in concentrated sulphuric acid, and apparently polymeric, and partly soluble, giving a solution with the light absorption of the quinone (I).



(I) (II) (III)

These properties of the phenol suggested its formulation as 4 : 5 : 4′ : 5′-tetrahydroxy-1 : 1′-dinaphthyl (II). Attempts to isolate this compound from the products of the direct oxidative coupling of 1 : 8-dihydroxynaphthalene were unsuccessful, and it was therefore synthesised from 1 : 8-naphthasultone by the route shown. The acetates of the natural and synthetic products were identical in all respects.



Reagents: i, I + HNO_3 in AcOH–H_2SO_4. ii, Activated Cu bronze. iii, KOH fusion.

Oxidation of the tetrahydroxydinaphthyl (II) involves oxidative coupling of a type frequent in Nature,[5] a close analogy being the cyclisation of protohypericin.[6] The primary oxidation product can give rise either to the quinone (I) by internal coupling or to polymers such as (III) by intermolecular reaction. Such polymers, even at a low degree of polymerisation, would be black and highly insoluble. The primary oxidation product might be an extended dinaphthaquinone or a related semiquinonoid free radical; in this connection the very ready oxidative polymerisation of 1 : 8-dihydroxynaphthalene, which cannot form a quinone directly, should be noted.

Since the dinaphthyl (II) is so readily converted into the perylenequinone (I), the question of the occurrence of pre-formed quinone (I) in the sporophores seemed to merit further study. This quinone is virtually insoluble in acetone, yet Anderson and Murray record [1] that on continuous extraction of the sporophores with acetone a black precipitate, affording the quinone (I) on vacuum-sublimation, was deposited from the boiling solvent. They considered that the quinone had been solubilised by other constituents of the extracts. We were able to confirm that limited amounts of the quinone (I) do in fact occur in a free or loosely bound form in the sporophores. Thus cold concentrated sulphuric acid, which as noted above does not oxidise the dinaphthyl (II), extracts material with the absorption spectrum of the quinone (I). Moreover, after powdered sporophores have been exhaustively extracted with light petroleum and ether, further extraction with acetone affords, not only the black precipitate described by Anderson and Murray, but also a reddish-brown solution. This has an absorption spectrum similar to that of the quinone (I) in tetrachloroethane, but shifted some 10 mμ to shorter wavelengths. Warming

[5] Cf. Mason, *Adv. Enzymology*, 1955, **16**, 105, for general discussion.
[6] Brockmann, *Proc. Chem. Soc.*, 1957, 304.

this solution with dilute acid affords a precipitate from which the quinone (I) can be extracted by concentrated sulphuric acid or tetrachloroethane. This apparent solubilisation of the quinone (I) may be effected by some of the more polar constituents of the acetone extract (mannitol, sugars, etc.), either by association or by the formation of easily hydrolysed derivatives.

Presumably the perylenequinone (I) is formed from the dinaphthyl (II) in the fungus by an enzymic oxidation similar to that demonstrable *in vitro*; however, the amount of free or loosely bound quinone in the sporophores is insufficient to account for their intense blackness. After exhaustive extraction with organic solvents and water, the ground sporophores leave a black residue which retains the microscopic structure of the original material and is made up of the heavily pigmented cell-walls. This powder behaves as a quinonoid polymer; it is reversibly bleached by reducing agents and even in the reduced form contains no alkali-soluble phenols. A typical fungal cell-wall material is chitinous,[7] with up to 6·9% of nitrogen, and an equivalent acetyl content, in a poly-(*N*-acetylglucosamine) structure. By contrast, the material from *D. concentrica* contains only 2% of nitrogen and no acetyl groups. Moreover, after prolonged acid hydrolysis, over 30% of the material remains as an insoluble black powder.

These properties are explained if we suppose that the cell-wall polysaccharides contain non-acetylated amino-sugar residues and that in the sporophores these have been cross-linked by combination with monomeric or polymeric quinonoid oxidation products formed by enzymic oxidation of the dinaphthyl (II). The quinone (I) is known to react readily with amines,[2] and the formation of " melanochitin " by such a reaction presents some analogies to the process of " quinone tanning " of cuticular proteins in insects, etc.[8]

The isolation of a 1 : 1′-dinaphthyl derivative from natural sources has not previously been recorded, though some 2 : 2′-dinaphthyls are known as plant products, *e.g.*, diospyrol [9] and gossypol.[10] These, like the phenol (II) and the naturally occurring dianthryl derivatives, have structures based on identical moieties. In this they resemble the erythroaphins, in which the chromophore of the perylenequinone (I) was first detected,[2] though the relation of these compounds to the parent aphis pigments remains uncertain. Recently [11] the same chromophore has been detected in the pigment of various phytopathogenic fungi of a genus, *Elsinöe*, not unrelated to *Daldinia*. The genus *Daldinia* itself comprises a number of species closely similar to *D. concentrica*, and it is probable that the pigmentation of these is essentially similar to that described here. A preliminary communication of this work has already been published; [12] further work includes studies of *D. concentrica* in laboratory cultures.

Experimental

Extraction of D. concentrica *Sporophores.*—The sporophores, collected as available and stored in the dark at −5°, were minced and the coarse powder (875 g.) was steeped overnight in light petroleum, which was then filtered and the solid was continuously extracted with ether (2 l.) for 24 hr.; the ether was then renewed, and after a further 24 hr. a third portion of ether was employed to remove final traces of soluble material. The extracts were stored in the dark at −5° until required. In a typical procedure 350 ml. of the first ether extract (a red solution) were shaken with two portions of aqueous sodium carbonate (100, 50 ml.), and the aqueous extracts were combined and acidified with dilute hydrochloric acid. The solution and the tarry precipitated solids were extracted twice with fresh chloroform (containing 1% of ethanol) (100, 50 ml.), and the combined extracts were concentrated under reduced pressure to *ca.* 50 ml. This solution was put on a column (25 × 3 cm.) made up from a slurry of " Florisil "

[7] Tracey, in Paech and Tracey, " Modern Methods of Plant Analysis," Springer-Verlag, Berlin, 1955, Vol. II, p. 264.
[8] Reviewed by Dennel, *Biol. Rev.*, 1958, **33**, 178.
[9] Loder and Robertson, *J.*, 1957, 2233.
[10] Adams, Morris, Geissman, Butterbaugh, and Kirkpatrick, *J. Amer. Chem. Soc.*, 1938, **60**, 2193.
[11] Weiss, Flon, and Burger, *Arch. Biochem. Biophys.*, 1957, **69**, 311.
[12] Allport and Bu'Lock, *Proc. Chem. Soc.*, 1957, 264.

(Floridin Co., U.S.A.) in chloroform which had been boiled and stirred for 30 min. to expel air; the column was wrapped in black paper. Failure to exclude light and air led to deposition of insoluble purple pigment on the adsorbent. The column was eluted with chloroform, and the fractions giving a positive ferric chloride reaction were combined and evaporated under reduced pressure. The brown, partly solid residue was dissolved in ethanol (100 ml.), water (100 ml.) added, and the solution washed with light petroleum (2 × 50 ml.) to remove lipids. Most of the ethanol was then distilled off and the aqueous residue extracted with ether (3 × 50 ml.). The ether extracts were dried and evaporated, to give 4 : 5 : 4′ : 5′-tetrahydroxy-1 : 1′-dinaphthyl (II) as pale yellow crystals (0·3 g.) [ultraviolet spectrum, Table 1; infrared maxima (Nujol mull) at 760, 820, 896, 1040, 1140, 1320, 1395, 1530, 1590, 1608, 1631, and 3200 cm.$^{-1}$]. By spectroscopic assay, the content of dinaphthyl (II) in the initial ether extracts was found to be *ca.* 1% of the weight of sporophores.

TABLE 1. *Ultraviolet absorption spectra* ($\lambda_{max.}$ *in* mμ).

Compound	In EtOH	In alkali
1 : 8-Dihydroxynaphthalene	305, 320, 333	(317), 329, 342
4 : 5 : 4′ : 5′-Tetrahydroxy-1 : 1′-dinaphthyl (II)	313, 325, 340	(340) 350
1 : 4 : 5-Trihydroxynaphthalene	316, 333, 348	(310) 350

Values in parentheses denote inflexions.

Acetylation of the Dinaphthyl (II).—The phenol (II) (20 mg.) was heated under reflux in a nitrogen atmosphere with purified pyridine (1 ml.) and redistilled acetic anhydride (2 ml.); after 1 hr. water was added and the mixture repeatedly extracted with ether. The combined extracts were washed with dilute hydrochloric acid, aqueous sodium carbonate, and water, dried, and evaporated, giving a yellow solid (25 mg.), which was purified by chromatography on deactivated alumina in acetone, treated with charcoal, and recrystallised from ethyl acetate to give 4 : 5 : 4′ : 5′-*tetra-acetoxy*-1 : 1′-*dinaphthyl*, m. p. 245° [Found: C, 69·15; H, 4·75%; *M* (Barger's method of isothermal distillation in acetone), 495 ± 15. $C_{28}H_{22}O_8$ requires C, 69·13; H, 4·56%; *M*, 486].

Borate Complexes.[4]—A Cambridge pH meter and calomel electrode were used to measure the pH difference between a solution containing 5 ml. of 0·5M-boric acid and 2 ml. of ethanol, and a solution containing 5 ml. of 0·5M-boric acid and the indicated quantity of the compound investigated dissolved in 2 ml. of ethanol. The results are shown in Table 2.

TABLE 2.

Compound	pH difference
(II) (10^{-3} mole)	3·9
1 : 8-Dihydroxynaphthalene (2 × 10^{-3} mole)	4·0
Catechol (2 × 10^{-3} mole)	1·3

4 : 9-*Dihydroxyperylene*-3 : 10-*quinone* (I).—The crude phenol (II) (240 mg. from 100 ml. of ether extract) was heated under reflux with chloranil (400 mg.) in phenetole (100 ml.); after 2½ hr. the solution was cooled and decanted into an excess of light petroleum; the black precipitate was washed with ethanol and benzene, dried, and sublimed at 260—300°/10^{-5} mm. to give dark red crystals of the quinone (I) (ultraviolet and infrared spectra as recorded by Calderbank *et al.*[2]).

Synthesis of the Phenol (II).—Glacial acetic acid (5 ml.), concentrated sulphuric acid (1·5 ml.), iodine (1·6 g.), and recrystallised 1 : 8-naphthasultone (2·0 g.; m. p. 156—157·5°) were stirred together whilst concentrated nitric acid (0·4 ml.) was added dropwise; the mixture was then heated at 70° for 45 min. until the iodine colour became permanent. The mixture was poured into water and the solid precipitate washed with aqueous sodium hydrogen sulphite and water, and recrystallised from acetic acid and benzene, affording 4-*iodo*-1 : 8-*naphthasultone* (2·6 g.), m. p. 205·5—206·5° (Found: C, 36·4; H, 1·6; I, 37·8. $C_{10}H_5O_3IS$ requires C, 36·05; H, 1·8; I, 38·15%). This product (0·8 g.) was heated in an oil-bath (220°) whilst activated copper bronze [13] (1·0 g.) was added during 45 min. with occasional stirring; heating was continued for a further 2 hr., and the mixture then cooled and extracted repeatedly with boiling benzene. The combined extracts were treated with charcoal and evaporated; the solid residue (0·24 g.) recrystallised from benzene to give 1 : 1′-*dinaphthyl*-4 : 5 : 4′ : 5′-*disultone*, m. p. 289—290° (Found: C, 58·9; H, 2·6. $C_{20}H_{10}O_6S_2$ requires C, 58·5; H, 2·5%). The disultone (0·2 g.),

[13] Kleiderer and Adams, *J. Amer. Chem. Soc.*, 1933, **55**, 4225.

mixed with potassium hydroxide (2·0 g.) and water (0·5 ml.), was heated under nitrogen to 240—280°, and, after frothing had subsided, to 310°; after 15 min. the black melt was cooled, dilute hydrochloric acid added, and the mixture extracted with ether, evaporation of which gave the crude dinaphthyl (II) with the correct ultraviolet absorption. The crude product was acetylated as described above, to give the tetra-acetyl derivative, m. p. and mixed m. p. 245°, the infrared spectrum of which, in Nujol mull, was identical with that of the naturally derived compound.

Attempted Oxidative Coupling of 1 : 8-*Dihydroxynaphthalene.*—1 : 8-Dihydroxynaphthalene was obtained from 1 : 8-naphthasultone by Erdmann's method;[14] in ethanol solution, it showed absorption max. at 333, 320, and 305 mμ. Addition of neutral aqueous potassium ferricyanide to the ethanol solution led to a green precipitate which was centrifuged and washed repeatedly with water. The solid residue, in ethanol solution, showed absorption max. attributable to the dinaphthyl (II), at 339, 324, and 311 mμ, but no useful yield of this compound could be obtained by this method.

Examination of Cell-wall Material.—The solid remaining after exhaustive ether-extraction of the sporophores was continuously extracted with acetone until no more material dissolved. The red acetone extract showed absorption max. at *ca.* 405 (infl.), 437, 470, (infl.), 520, and 560 mμ [in tetrachloroethane the quinone (II) shows max. at 419, 444, 493, 525, and 567 mμ], and when warmed with dilute hydrochloric acid deposited a black substance, partly soluble in concentrated sulphuric acid or tetrachloroethane to give solutions with the absorption spectra of the dinaphthyl (II). The black powder remaining after acetone-extraction was found, after drying, to contain 2·0% of nitrogen, and its acetyl content, determined by Scarisbrick's method for the acetyl content of chitinous substances,[7] was negligible; after the prolonged acid-hydrolysis in the acetyl determination, *ca.* 35% of the sample remained undissolved. When shaken with aqueous sodium dithionite the black powder assumed a light tan colour, rapidly becoming black on exposure to air.

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[14] Erdmann, *Annalen*, 1888, **247**, 356.