Two New Benzoquinones from *Gliocladium roseum*

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Two hitherto undescribed mould metabolic products have been isolated from cultures of *Gliocladium roseum* grown on Raulin-Thom medium. Their structures are determined as 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone (II) and 2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone (III), respectively. These pigments have been converted, *in vitro*, into aurantiogliocladin (I), the major metabolic product of the same mould.

A group of three closely related antibiotics, named aurantiogliocladin, rubrogliocladin and gliorosein, has been isolated from the culture medium of *Gliocladium roseum* Bainer.[1] Aurantiogliocladin (I) was shown to be 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone, and rubrogliocladin is the corresponding quinhydrone.[1,3] Gliorosein, which is one of the two possible dihydro-aurantiogliocladins, originally formed about one-fifth of the total crystalline product obtained after extraction, but in later experiments the organism had ceased to produce it and only aurantiogliocladin was present in the culture filtrates.[2]

During the investigation of a number of quinone producing fungi the author observed that the medium of three weeks old submerged cultures of *G. roseum* showed a purple colour after removal of aurantiogliocladin by extraction with petroleum ether. This colour is not given by any of the compounds previously isolated from the mould. A chromatographic investigation of the culture medium was, therefore, undertaken, which gave evidence for the presence of two purple pigments, called compound A and compound B. These pigments were isolated by extraction with ether and separated by paper chromatography.

Compound A was produced in a rather low yield (5—10 mg/l) by all of the cultures that formed aurantiogliocladin. It could readily be obtained in the crystalline form (m.p. 70°) by sublimation or by recrystallization from petroleum ether. The yield of compound B was still lower (3—5 mg/l). It was produced, along with aurantiogliocladin and compound A, very regularly by the original strain of *G. roseum* (A.C.C. 650). Some quinone producing subcultures of the latter strain, however, failed to form compound B in detectable amounts. Compound B seemed less stable than compound A, and was

destroyed completely by aeration of the culture filtrates at pH 10 for 15 min. It partly decomposed on sublimation, but could be isolated as orange needles, m.p. 182°, by recrystallization from petroleum ether.

Both the compounds showed typical quinone behaviour. They reacted with carbonyl reagents, liberated iodine from an acid solution of potassium iodide, and were readily reduced (with decolourisation) by, for instance, sodium dithionite. Aqueous solutions of the compounds in the reduced state rapidly took up oxygen from the air; aeration at pH 7 immediately restored the purple colour of the oxidized form of the pigments.

Further evidence for a quinonoid structure of the new pigments was obtained from the infra-red absorption spectra, which are very similar in the double-bond stretching region to those of benzoquinones.[4],[7] Thus aurantioglioclandin, for instance, shows two strong bands at 6.05 μ (C=O) and 6.19 μ (C=C), and compound A (B) the corresponding bands at 6.10 μ (6.09 μ) and 6.18 μ (6.19 μ). The bands show characteristic splitting due to Fermi-resonance interaction.[8] The intensity of the C=O band is approximately half of that of the C=C band (they are almost equally intense in the infra-red spectrum of aurantioglioclandin) which could indicate the presence of hydroxyl groups. Confirmatively, compound A (B) shows absorption attributable to hydroxyl groups at 2.95 μ (2.96 μ). The presence of at least one methoxyl group in compound A is strongly indicated by the characteristic weak absorption bands in the 3.5 μ region (similar to those of aurantioglioclandin and other methoxybenzoquinones); these bands are absent in the infra-red absorption spectrum of compound B.

In alcoholic solution compound A shows two absorption bands in the ultra-violet: I. λ_{max} 278 mμ; ε = 16 200, II. λ_{max} 442 mμ; ε = 650. The ultra-violet absorption spectrum of compound B (I. λ_{max} 277 mμ; ε = 11 800, II. λ_{max} 445 mμ; ε = 410) is similar. These spectra are characteristic of benzoquinones,[9] and definitely exclude naphtha- or anthraquinonoid structures, which would give more than two absorption bands. The position of the absorption bands clearly indicates that the two benzoquinonoid pigments are heavily substituted.[9]

Hydroxybenzoquinones show a considerable bathochromic shift with alkali of the wave-length of maximum absorption in the visible region, due to dissociation of the hydroxyl group. The corresponding change in colour from faint yellow to intense red, purple, or blue (according to the nature, number, and position of the substituents in the hydroxyquinone nucleus) is most characteristic and has been made use of for structural determinations.[10] Alkyl and/or alkoxyl substituted hydroxybenzoquinones usually give pK_1 values ranging from 4 to 6 (the "Brunnenwasser" reaction [11]). 2,5-Dihydroxy-benzoquinones form a separate group and titrate as dibasic acids ($pK_1 \sim 3.5$; $pK_2 \sim 5.5$) with reversible fading of the purple colour in the second dissociation step; other dihydroxybenzoquinones are not decolourized until in strong alkaline solutions (more or less irreversibly).[12],[13] The colour reactions with alkali of compound A, as well as of compound B, gave further evidence for a heavily substituted hydroxybenzoquinonoid structure. In aqueous solution compound A titrated as a monobasic acid with pK_1 = 5.0 (the extinction/pH curve is shown in Fig. 1), and the bathochromic shift with alkali (from 430 mμ
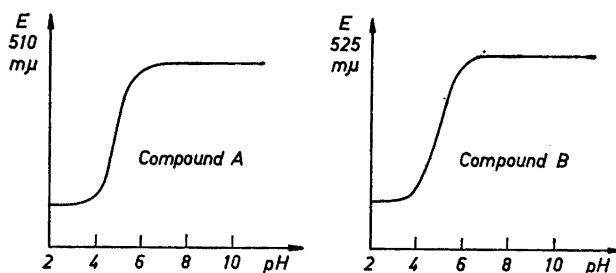
Fig. 1. Extinction/pH curves for the new benzoquinonoid pigments from *G. roseum*.

to 512 mμ) lead to a change in colour from yellow to bluish purple. The colour did not fade in strong alkali (pH 12), for which reason a 2,5-dihydroxyquinonoid structure must be excluded. Similarly compound B showed a bluish purple colour in alkaline solutions (bathochromic shift from 435 mμ to 522 mμ), almost indistinguishable from that of compound A. The shape of the extinction/pH curve obtained (pK_1 = 4.9; see Fig. 1) excludes that compound B is a derivative of 2,5-dihydroxybenzoquinone. On the other hand, the susceptibility of compound B to oxidative decomposition might indicate a 2,3- or 2,6-dihydroxyquinonoid configuration.

Conclusive evidence for the presence of one hydroxyl group in compound A and two hydroxyl groups in compound B was obtained by the preparation of the corresponding mono- and dimethyl ether, respectively (see below). Compound A (m.p. 70°) further contains one methoxyl group (Zeisel) and two methyl groups (Kuhn-Roth), calculated on a molecular weight of 185 (Rast); analyses were in good agreement with the molecular formula $C_9H_{10}O_4$. (Found: C 59.60; H 5.58. Calc.: C 59.34; H 5.53.) It is without question a monohydroxy-monomethoxyxyloquinone, of which there are three possible isomers. 3-Hydroxy-5-methoxy-2,6-dimethyl-1,4-benzoquinone has previously been described in the literature,[14] and is not identical with compound A since it has a different melting-point (116°) and since it separated from compound A in several chromatographic systems tested. On demethylation compound A yielded a dihydroxyquinone which was identical with compound B in mixed melting-point determinations and chromatographic tests. It further showed an extinction/pH curve indistinguishable from that of compound B, and is thus no 2,5-dihydroxybenzoquinone. Consequently, compound A cannot be 2-hydroxy-5-methoxy-3,6-dimethyl-1,4-benzoquinone; it is only possible that compound A is 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone (II). Confirmatively, treatment of compound A with ethereal diazomethane gave a monomethyl ether which proved to be identical with aurantiogliocladin (I).

Fig. 2. Metabolic products of *G. roseum:* Aurantiogliocladin (I), compound A (II), and compound B (III).

I $R_1 = R_2 = CH_3$

II $R_1 = H$ $R_2 = CH_3$

III $R_1 = R_2 = H$
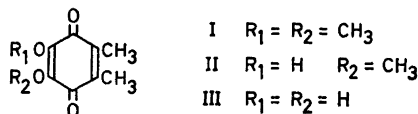
Compound B contains, besides two hydroxyl groups, two methyl groups (Kuhn-Roth) calculated on a molecular weight of 175 (Rast), and is thus one of the three possible dihydroxyxyloquinones. (Found: C 57.44; H 4.89; OCH_3 nil. Calc. for $C_8H_8O_4$: C 57.14; H 4.80.) Compound B (m.p. 182°) seems not to be identical with dihydroxy-p-xyloquinone (m.p. 245°) or with dihydroxy-m-xyloquinone (m.p. 167°).[15],[16] The former quinone must, further, be excluded as it is a 2,5-dihydroxybenzoquinone (see above) and the latter quinone since it separated from compound B in chromatographic tests. Compound B should, consequently, be 2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone (III), which is also consistent with its formation by demethylation of compound A. Confirmatively, compound B yielded a dimethyl ether that proved to be identical with aurantiogliocladin (I).

The final proof was given by synthesis of 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone and 2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone (neither of which has been described previously), which were found to be identical with compound A and compound B, respectively (see following paper).

EXPERIMENTAL

Culture conditions. Gliocladium roseum, A.C.C. 650, the original culture isolated by Brian *et al.*,[1] was obtained through the kindness of Dr. Brian, Imperial Chemical Industries Ltd., Akers Research Laboratories, Welwyn, Herts., England. Three subcultures of the above strain (*G. roseum*, C.M.I. 93065) were obtained from the Commonwealth Mycological Institute, Kew, Surrey, England. All of the strains were maintained on malt agar slopes at 20°; temperatures above 25° prevented growth of the organism. Experimental cultures were grown on a Raulin-Thom solution (glucose, 50 g; tartaric acid, 3 g; ammonium tartrate, 3 g; $(NH_4)_2SO_4$, 0.2 g; $(NH_4)_2HPO_4$, 0.4 g; K_2CO_3, 0.4 g; $MgCO_3$, 0.3 g; $FeSO_4$, $7H_2O$, 0.01 g; $ZnSO_4$, $7H_2O$, 0.05 g; distilled water to 1000 ml) either as surface cultures (in Fernbach flasks) at 20°, or as submerged cultures (in shaken Erlenmeyer flasks) at 25°.

Surface cultures of the mould were found to grow slowly (maximum yield of quinones after about 4 weeks) and the mycelium was to a large extent submerged. The mould failed to grow at temperatures above 20°. In submerged cultures growth was rapid, also at 25°. A maximum yield of quinones was generally obtained after 10 − 14 days of cultivation, but submerged cultures often failed to form any quinones at all when inoculated from agar slope cultures. This difficulty could be overcome by using the mycelium from quinone producing shake cultures for inoculation.

The pattern of quinone production was not considerably affected by alterations of the culture conditions; aurantiogliocladin, compound A, and compound B always occurred together, in submerged as well as in surface cultures. Two of the three cultures obtained from the C.M.I. collection, however, failed to form compound B, while aurantiogliocladin and compound A were produced in the normal amounts.

Isolation of the quinones. Two weeks old submerged cultures of *G. roseum*, C.M.I. 93065, were used for the isolation of the different quinones. The filtered metabolism solution (pH 5 − 6; intensely yellow in colour) was extracted three times with one-tenth of the volume of petroleum ether (40 − 60°), which removed all of the aurantiogliocladin. Evaporation of most of the petroleum ether yielded aurantiogliocladin as orange needles, m.p. 60 − 62°, which were removed by filtration and recrystallized from petroleum ether, giving a constant m.p. of 64°.

After extraction with petroleum ether the culture filtrates became purple in colour, changing to yellowish brown on acidification with conc. HCl. The filtrates (pH 2.0) were extracted with half the volume of ether, the ether was removed in a vacuum, and the residue chromatographed on Whatman No. 3 MM paper, using butanol-propanol-2 M ammonium hydroxide (1:6:3 by vol.) as the solvent.[17] Traces of aurantiogliocladin (R_F 0.95)

Table 1. 100 × R_F values for aurantiogliocladin (I), compound A (II), and compound B (III) on thin-layer chromatograms.

Solvent system:	I	II	III
Chloroform	57	26	0
Chloroform-benzene (3:1)	46	21	0
Chloroform-xylene (3:1)	49	11	0
Ethanol-conc. ammonia (5:1)	92	59	45
Ethanol-butanol-2 M ammonium hydroxide (3:5:3)	95	65	41
Propanol-butanol-2 M ammonium hydroxide (6:1:3)	98	65	40

gave a yellow zone near the solvent front, while compound A (R_F 0.72) and compound B (R_F 0.53) appeared as well separated bluish-purple zones, which were cut out of the paper, moistened with 1 M HCl, and extracted with ether. When rechromatographed all of the quinones gave single spots; it can definitely be excluded that compound A or compound B are arteficial products of the isolation procedures.

The combined ethereal eluates (of compound A and B, respectively) from a large number of chromatograms were concentrated *in vacuo* and extracted with 0.1 M aqueous NaHCO₃, which became intensely purple. The purple solution was acidified with conc. HCl, when it became brownish yellow, and was reextracted with ether. Removal of the ether yielded a crystalline material which was purified by recrystallization from petroleum ether (40 — 60°). Compound A separated as reddish-brown prisms, m.p. 70°, and compound B as micro-bunches of orange needles, m.p. 182° (decomp.) with softening and darkening from 178°. For analysis a sample of compound A was sublimed at 100° in a vacuum (1 mm Hg). No decomposition occurred and the melting-point was unchanged. Compound B, also, sublimed readily but with slight decomposition.

Chromatographic methods. Paper and thin-layer chromatography was used for identification studies of the new pigments and of their quinonoid derivatives; the general techniques in chromatographic analysis of benzoquinones, as well as the R_F values of a large number of methyl or methoxyl substituted hydroxybenzoquinones in suitable solvent systems, have been described elsewhere.[17] The R_F values of aurantiogliocladin, compound A, and compound B on thin-layer chromatograms in the corresponding solvent systems are listed in Table 1.

Light absorption measurements. The ultra-violet absorption spectra of the quinones were determined in a Perkin-Elmer Model 137 UV Spectrophotometer, with ethanol as the solvent, and the infra-red absorption spectra in a Perkin-Elmer Infracord Spectrophotometer, the quinones being dissolved in carbon tetrachloride. The extinction/pH curves (Fig. 1) in aqueous solution were determined in a Beckman DU Spectrophotometer. The pH was varied by titration with dilute HCl or NaOH, and was measured electrometrically.

General properties of compound A and B. Compound A (B) gives the following reactions:

(a) A solution in absolute ethanol gives with one drop of 1 M alcoholic FeCl₃ an intense blue (blue-violet) colour, which changes to light brown on the addition of water.

(b) With 1 M NaOH it gives a bluish-purple colour, changing to brown on standing overnight.

(c) With aqueous NaHCO₃, in which it is immediately soluble, it gives a colour indistinguishable from that in 1 M NaOH. This colour is unchanged overnight.

(d) With cold conc. H₂SO₄ it immediately gives a deep blue (cherry red) colour, which is unchanged overnight.

(e) It liberates I from an acidified solution of KI.

(f) It dissolves readily in aqueous Na₂S₂O₄ to give an almost colourless solution. On standing in air the upper layer of the solution quickly becomes purple, due to autoxidation of the reduced form of the pigment.[18]

Compound A is readily soluble in most organic solvents, as well as in water. Compound B is readily soluble in acetone, ether, chloroform, ethyl acetate, and ethanol, fairly soluble in water, and slightly soluble in benzene, petroleum ether, and carbon tetrachloride.

Monomethyl ether of compound A. Compound A (55 mg) was dissolved in anhydrous ether (4 ml) and ethereal diazomethane was added in a slight excess. There was immediate and vigorous evolution of nitrogen and the brownish red solution lightened considerably in colour. The orange crystals (45 mg) separating on removal of most of the solvent were recrystallized from petroleum ether (40 − 60°) and were obtained as long, orange needles, m.p. 64°. (Found: C 61.32; H 6.17; OCH$_3$ 31.0. Calc. for C$_{10}$H$_{12}$O$_4$: C 61.21; H 6.16; OCH$_3$ 31.6.) The melting-point of aurantiogliocladin (64°) was not depressed by admixture with the methyl ether of compound A, and both materials gave identical R_F values when chromatographed in the solvent systems listed in Table 1. The methyl ether of compound A, further, gave ultra-violet and infra-red absorption spectra which were indistinguishable from those of aurantiogliocladin.

Dimethyl ether of compound B. Compound B (40 mg) was methylated by heating for 3 h with acetone (4 ml) and anhydrous K$_2$CO$_3$ (0.2 g). The mixture was diluted with ether (20 ml) and filtered. On evaporation the crude dimethyl ether of compound B crystallized, It was extracted with petroleum ether (40 − 60°) and sublimed in a high vacuum to give an orange material, m.p. 58°. This was recrystallized from petroleum ether to give orange needles (10 mg) with a constant m.p. of 64°, undepressed by admixture with aurantiogliocladin. (Found: OCH$_3$ 30.9.) The dimethyl ether of compound B further proved to be chromatographically identical with aurantiogliocladin, and both materials gave identical ultra-violet and infra-red absorption spectra.

Methylation of compound B with ethereal diazomethane gave a poor yield of the dimethyl ether (identified by chromatography); the main product with this reagent was a yellow material (uncharacterized) containing nitrogen.

Demethylation of compound A. Compound A (25 mg) was dissolved in 1 M NaOH (2 ml) and the solution was boiled for 1 min. After acidification and extraction with ether the crude extract was chromatographed on paper.[17] Besides small amounts of unchanged compound A (2 mg) only one, purple, quinonoid substance could be detected on the chromatograms (R_F values as compound B). This substance was eluted with ether and purified by extraction with aqueous NaHCO$_3$, which was acidified and thoroughly extracted with petroleum ether (40 − 60°). After evaporation of most of the solvent the demethylation product of compound A crystallized as orange micro-crystals (6 mg), m.p. 182°, undepressed by admixture of compound B. Both materials showed identical ultra-violet and infra-red absorption spectra.

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