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New Metabolites from *Lentinus degener*

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The present work describes the isolation and identification of 4-methoxy-6-hydroxy-2,5-toluquinone (II) as a metabolic product of three out of four investigated strains of *Lentinus degener;* this pigment has not previously been recognized as a natural product. In two of the strains 4-methoxy-6-hydroxy-2,5-toluquinone was produced along with minor amounts of 4-methoxy-2,5-toluquinone (I). The quinonoid pigments were found to be formed by autoxidation of the corresponding hydroquinones, which could be isolated from young, pigment producing, *L. degener* cultures and were identified by thin-layer chromatography. The non-pigmented strain of the mould was shown to form small amounts of 6-methylsalicylic acid (III).

In relation to investigations on the biosynthesis of fungal benzoquinones it was considered to be of interest to examine the mould species *Lentinus degener*, from which 4-methoxy-2,5-toluquinone (I) has been isolated by Anchel *et al.*,[1] with respect to the presence of minor quinonoid and phenolic secondary metabolites; similar investigations of quinone producing strains of *Aspergillus fumigatus*,[2] *Penicillium spinulosum*,[3] and *Gliocladium roseum* [4] have led to the detection of a number of previously undescribed mould meta-

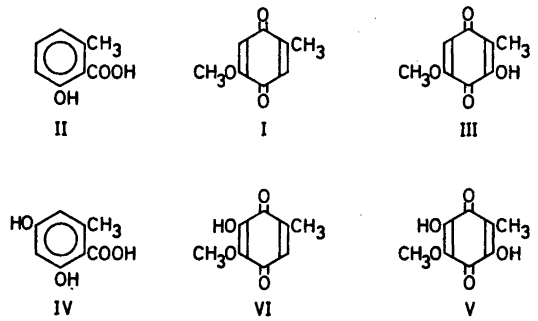

Fig. 1. Metabolic products isolated from *Lentinus degener* (I, II, and III) and *Aspergillus fumigatus* (IV, V, and VI). The quinonoid pigments have also been isolated in the hydroquinone form.

bolic products. Four different strains of *L. degener* (MNHN 279, 1027, 1239, and 1240) were studied in the present work, and the mould was grown as surface cultures on a Raulin-Thom solution. For detection of secondary metabolites ethereal extracts of the culture filtrates were examined by paper and thin-layer chromatography.

4-Methoxy-2,5-toluquinone (see above) was found to be produced by two of the four investigated strains (MNHN 279 and 1027). The major product of these strains was, however, a second pigment, which could be isolated and identified as 4-methoxy-6-hydroxy-2,5-toluquinone (II). The identity was established by studies of the general properties of the pigment, elementary analyses, and direct comparison with authentic material, using mixed m.p., ultraviolet and infrared data, and the chromatographic behaviour as criteria (see experimental section). Small amounts of 4-methoxy-6-hydroxy-2,5-toluquinone were also isolated from strain 1240 (Table 1), whereas no pigments could be detected in the culture filtrates of strain 1239. The three pigment producing strains of *L. degener* did not form any detectable amounts of 4-hydroxy-2,5-toluquinone, 6-methylsalicylic acid (III), *m*-cresol, orsellinic acid (IV), or orcinol. On the other hand, the non-pigmented strain (MNHN 1239) was found to produce a phenolic substance that was isolated and identified as 6-methylsalicylic acid.

Previous investigations on the biosynthesis of fungal benzoquinones (see above) have established that the pigments are present almost exclusively in the hydroquinone form during the larger part of the production phase, a fairly slow, non-enzymatic, conversion into the corresponding quinones taking place at a late stage in the development of the mould. This appeared to be valid also for the pigments produced by *L. degener*. When the dark-brown medium from 3 weeks old cultures of *L. degener*, MNHN 1027, was made alkaline by the addition of dilute sodium hydroxide the colour immediately changed to intense purple, due to dissociation of the hydroxyl group in 4-methoxy-6-hydroxy-2,5-toluquinone. Iodometric titration of the medium from old cultures confirmed that the pigments were present exclusively in the quinone form. No quinones could, however, be detected on iodometric titration of freshly separated filtrates of young (6—10 days) cultures of the mould, and the pale-yellow solutions did not give the colour reaction with alkali characteristic of 4-methoxy-6-hydroxy-2,5-toluquinone. On the other hand, when the alkaline filtrates of young cultures were shaken with air or oxygen the colour rapidly changed to purple. The amounts of oxygen absorbed during this process corresponded well to the amounts of quinones formed (as indicated by iodometric titration). Furthermore, after treatment with a slight excess of sodium dithionite the aerated solution showed an ultraviolet absorption spectrum which was indistinguishable from that of the non-aerated filtrates. These observations indicate that the pigments are present exclusively in the hydroquinone form in young cultures of the mould. Confirmatively, both 4-methoxy-2,5-toluhydroquinone and 4-methoxy-6-hydroxy-2,5-toluhydroquinone could be identified on thin-layer chromatograms of ethereal extracts of young *L. degener* cultures.

In normally developed cultures the conversion of the hydroquinone form of the pigments into the quinone form started on the 10th to the 16th day

of cultivation, and was completed within 2—3 days. The latter process showed all the characteristics of a non-enzymatic reaction (autoxidation of the hydroquinones).[5] The oxidation rate was thus considerably increased on removal of the mycelium, and aeration of the culture medium (pH 4—5) led to a quantitative formation of quinones within some minutes. At lower pH-values the oxidation rate was gradually decreased, being negligible below pH 1. Preincubation of the culture filtrates at pH 0 and 100° for 10 min had no effect on the rate of quinone formation at pH 4. It may thus be concluded that the toluquinonoid pigments produced by *L. degener* are formed by autoxidation of the corresponding hydroquinones, analogous to what has been described for other quinone producing moulds.

While 4-methoxy-2,5-toluquinone and 6-methylsalicylic acid are well-known fungal metabolites,[6] 4-methoxy-6-hydroxy-2,5-toluquinone has not previously been recognized as a natural product. The latter compound is, obviously, closely related to the toluquinonoid pigment spinulosin (V), which is known to be produced along with fumigatin (VI) by *A. fumigatus* and *P. spinulosum* (see Fig. 1).[2,3] Since fumigatin hydroquinone has been found to be incorporated as a unit into spinulosin in *A. fumigatus*,[7] one might expect that a similar relationship exists between the pigments formed by *L. degener*. Attempts to show this by incorporation of radioactive 4-methoxy-2,5-tolu-hydroquinone into 4-methoxy-6-hydroxy-2,5-toluquinone were, however, unsuccessful.

EXPERIMENTAL

Culture conditions. Four strains of *Lentinus degener* (MNHN 279, 1027, 1239, and 1240) were obtained through the courtesy of Prof. R. Heim, Museum National d'Histoire Naturelle, Paris; the strain (MNHN 523) from which Anchel *et al.* isolated 4-methoxy-2,5-toluquinone [1] was, unfortunately, no longer available. The different strains were maintained on malt-agar slopes at 20°, and experimental cultures were grown at 25° in 2000 ml Fernbach flasks, holding 500 ml portions of Raulin-Thom solution (glucose, 50 g; tartaric acid, 3 g; ammonium tartrate, 3 g; $(NH_4)_2HPO_4$, 0.4 g; $(NH_4)_2SO_4$, 0.2 g; K_2CO_3, 0.4 g; $MgCO_3$, 0.3 g; $FeSO_4.7H_2O$, 0.05 g; $ZnSO_4.7H_2O$, 0.05 g; distilled water, 1000 ml).

The production pattern was not changed when the mould was grown as submerged, shaken-flask, cultures (500 ml Erlenmeyer flasks holding 150 ml portions of the above medium), whereas the yield of quinones was considerably decreased when the Raulin-Thom solution was exchanged for a Czapek-Dox medium.

Chromatographic methods. For routine chromatographic examinations of ethereal extracts of the culture filtrates the solvent system propanol-butanol-2 M ammonium hydroxide (6:1:3 by vol.) was used. This system is particularly suitable for the analysis of benzoquinones, which appear as intensely coloured, self-indicating, spots and can be detected in amounts of some μg.[8] For detection of phenolic metabolites the chromatograms were sprayed with the reagents described by Reio.[9] The above solvent system was also used for the larger-scale paper chromatographic preparation of 4-methoxy-6-hydroxy-2,5-toluquinone (see below). The techniques (paper and thin-layer chromatography) and solvent systems employed for chromatographic identification of the quinonoid metabolites (and their quinonoid derivatives),[8] as well as of the corresponding hydroquinones,[10] have been described previously. The paper chromatographic behaviour of 6-methylsalicylic acid was studied in the solvent systems described by Reio.[9]

Isolation and characterization of 4-methoxy-2,5-toluquinone. The combined, yellowish-brown, filtrates (7000 ml) of several 10 days old surface cultures of *L. degener*, MNHN 1027, were adjusted to pH 9 by the addition of dilute sodium hydroxide, and were vigor-

ously aerated for 5 min to oxidize the hydroquinone form of the pigments. The alkaline solution, which rapidly became intense purple in colour during the aeration, was extracted twice with 1000 ml of petroleum ether (b.p. 40—60°), which removed most of the 4-methoxy-2,5-toluquinone; the purple aqueous solution was then used for the isolation of 4-methoxy-6-hydroxy-2,5-toluquinone (see below).

The combined yellow petroleum ether extracts were concentrated in vacuum, and the semi-solid residue was crystallized from 95 % ethanol to give 4-methoxy-2,5-toluquinone as yellow plates (72 mg) of m.p. 172°. (Found: C 63.0; H 5.3; OCH_3 20.1. Calc.: C 63.1; H 5.3; OCH_3 20.4). The mixed m.p. with an authentic sample of 4-methoxy-2,5-toluquinone (m.p. 172—173°), prepared according to the method of Woodward *et al.*,[11] was 172—173°. The ultraviolet and infrared absorption spectra, and the chromatographic behaviour of the natural product were identical with those of authentic sample. Thiele acetylation [12] of the natural product, further, yielded a colourless solid which crystallized in needles of m.p. 92°, not depressed on admixture with an authentic sample of 4-methoxy-2,3,5-triacetoxytoluene (m.p. 92°), prepared according to the method of Aulin and Erdtman.[13]

Isolation and characterization of 4-methoxy-6-hydroxy-2,5-toluquinone. The aerated alkaline culture filtrates, which had been extracted with petroleum ether (see above), were strongly acidified by the addition of conc. hydrochloric acid (150 ml), when the colour changed from intense purple to faint brown. The acid solution was extracted with half the volume of ether, the ether removed in vacuum, and the residue chromatographed on Whatman No. 3 MM papers, using the solvent system described above. The purple pigment appearing on the chromatograms (R_F 0.62—0.67) was eluted with acetone, and removal of the acetone yielded a dark-brown oily residue, which was extracted with a saturated solution of sodium bicarbonate. The purple bicarbonate solution was filtered, acidified, and thoroughly extracted with ether, and the ether solution was dried with anhydrous sodium sulphate. On removal of most of the solvent in vacuum the pigment crystallized in light-brown plates (340 mg), m.p. 190—195°. It was further purified by sublimation (130°; 1 mm Hg), being obtained as brownish-orange prisms of m.p. 202—203° (decomp.).

The identification of the crystalline material as 4-methoxy-6-hydroxy-2,5-toluquinone was based on the following evidence (*cf.* Ref. 14).

1. The substance showed a typical hydroxyquinone behaviour. It titrated as a monobasic acid with pK = 4.8 (the equivalent weight obtained was 173), and showed an intense purple colour in alkaline or neutral aqueous solution, changing to faint yellow on acidification. It reacted with carbonyl reagents, took up bromine in carbon tetrachloride, liberated iodine from an acid solution of potassium iodide, and was readily reduced (with decolourisation) by, for instance, sodium dithionite. An aqueous solution of the reduced form of the pigment rapidly took up oxygen from the air; aeration of the solution at pH 9 immediately restored the purple colour of the oxidized form.

2. Analyses established an empirical formula of $C_8H_8O_4$, and gave evidence for the presence of one O-methyl group (Zeisel) and one C-methyl group (Kuhn-Roth), calculated on a molecular weight of 164 (Rast). (Found: C 57.2; H 5.0; OCH_3 18.5; (C-)CH_3 8.8. Calc.: C 57.1; H 4.8; OCH_3 18.5; (C-)CH_3 8.9).

3. The mixed m.p. with an authentic sample of 4-methoxy-6-hydroxy-2,5-toluquinone (m.p. 203°), prepared according to the method of Aulin and Erdtman,[13] was 202—203°.

4. The ultraviolet and infrared absorption spectra and the chromatographic behaviour of the natural product were indistinguishable from those of authentic material.

5. The pigment (120 mg) formed a monomethyl ether on treatment with dimethylsulphate (1.0 ml) in acetone (5.0 ml). The mixture was boiled for 3 h, during which time potassium carbonate (1 g) was added in five lots. The mixture was cooled, diluted with ether, and the potassium sulphate and excess potassium carbonate were removed by filtration. The solvents were evaporated, leaving a brown oil which was extracted with boiling petroleum ether (b.p. 40—60°). The yellow solid separating on removal of most of the petroleum ether was purified by sublimation (150°; 1 mm Hg), and was obtained as bright yellow needles (25 mg) of m.p. 125°. (Found: C 59.4; H 5.6; OCH_3 33.7. Calc.: C 59.4; H 5.5; OCH_3 34.4). The dimethoxyquinone prepared in this way was found to be identical with an authentic sample of 4,6-dimethoxy-2,5-toluquinone,[15] using mixed m.p., ultraviolet and infrared data as criteria.

Table 1. Average amounts (mg/l medium) of quinonoid metabolites in 10 days old surface cultures of *Lentinus degener*.

Strain	Product (derivative of 2,5-toluquinone)	
	4-methoxy-	4-methoxy-6-hydroxy-
MNHN 279	13	35
MNHN 1027	10	50
MNHN 1240	—	8

The above procedures could also be used for the isolation of pigments from *L. degener*, MNHN 279 and 1240; the average yields of toluquinones in the different strains are given in Table 1.

Isolation and characterization of 6-methylsalicylic acid. The combined filtrates (4500 ml) of a large number of 15 days old surface cultures of *L. degener*, MNHN 1239, were adjusted to pH 2 with 2 M hydrochloric acid and extracted with half the volume of ether. The ethereal extract was concentrated to 100 ml, washed with water, and extracted with a saturated solution of sodium bicarbonate. The bicarbonate solution was strongly acidified by the addition of conc. hydrochloric acid and reextracted with ether. The ether was then dried with anhydrous sodium sulphate and evaporated, leaving a semi-crystalline residue which was crystallized from 1 M hydrochloric acid to give 6-methylsalicylic acid as colourless needles (20 mg), m.p. 168°, unchanged on admixture with an authentic sample (m.p. 168°). (Found: C 63.2; H 5.3. Calc.: C 63.2; H 5.3). The infrared and ultraviolet absorption spectra and the chromatographic behaviour of the natural product were identical with those of authentic material.

Hydroquinone forms of the pigments. The general techniques used in order to establish in which form the pigments are present during the growth of *L. degener*, and in order to study the oxidation process by which the quinonoid pigments are formed from the corresponding hydroquinones, have been described previously in relation to similar investigations of the pigments produced by *A. fumigatus*.[5,10] These studies showed that the pigments from *L. degener* are present almost exclusively in the hydroquinone form during the first 10 days of cultivation; there was no difference between the three pigment producing strains in this respect. *L. degener*, MNHN 1027, was used for the isolation of 4-methoxy-2,5-toluhydroquinone and 4-methoxy-6-hydroxy-2,5-toluhydroquinone. The filtered medium of a 7 days old surface culture of the latter strain was acidified by the addition of conc. hydrochloric acid, and was extracted with an equal volume of ether. After removal of the ether in vacuum the syrupy residue was chromatographed on thin-layer plates in an atmosphere of nitrogen, using chloroform-methanol-acetic acid as the solvent.[10] All of these operations were performed quickly, and as far as possible in the absence of air. Both 4-methoxy-2,5-toluhydroquinone and 4-methoxy-6-hydroxy-2,5-toluhydroquinone could be detected and identified on the chromatograms; the identity of the compounds was established by simultaneously running authentic samples (obtained by treatment of the corresponding quinones with sodium dithionite), and by two-dimensional chromatography. In the latter case alkaline solvent systems suitable for separation of the quinone forms of the pigments [8] were used for the second dimension, the hydroquinones being oxidized to the corresponding quinones by exposure to ammonia vapour in the air before the chromatograms were run for the second time.

Preparation and administration of ^{14}C-labelled 4-methoxy-2,5-toluhydroquinone. Sodium 1-^{14}C-acetate (1.0 mC), which is known to be incorporated into 4-methoxy-2,5-toluquinone,[6] was supplied to the medium of two 6 days old surface cultures of *L. degener*, MNHN 279. Growth was continued for a further 4 days, when radioactive 4-methoxy-2,5-toluquinone (14 mg; 11 μC) was isolated as described above. The quinone was dissolved in 20 ml of water and reduced by treatment with a slight excess of ascorbic acid. Immediately after being prepared, the latter solution of radioactive 4-methoxy-2,5-toluhydroquinone was administered to a 6 days old surface culture of *L. degener*, MNHN 1027. Four days later 4-methoxy-6-hydroxy-2,5-toluquinone was isolated from the culture medium as described above. Radioactivity determinations showed that less than 0.01 %

of the activity added had been incorporated into the latter pigment. After further purification of the quinone by paper chromatography, followed by recrystallization from acetic acid, there were no detectable counts in the sample of 4-methoxy-6-hydroxy-2,5-toluquinone.

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