On the Biosynthesis of Spinulosin in *Penicillum spinulosum*

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The toluquinonoid pigments produced by *Aspergillus fumigatus* (see Table 1) have been shown to be formed by autoxidation of the corresponding hydroquinones, which in their turn are derived from acetate-polymalonate *via* orsellinic acid.[1-5] Some of these pigments appear to be interconvertible in the hydroquinone form; spinulosin hydroquinone may, for instance, arise by O-methylation of 3,4-dihydroxy-2,5-toluhydroquinone in position 4, followed by hydroxylation of the resulting fumigatin hydroquinone in position 6.[6,7] Spinulosin (Fig. 1) was first isolated from
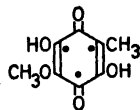

Fig. 1.

Penicillium spinulosum,[8] and the latter mould has now been subjected to investigations in order to compare the biogenetic pathways leading to quinone formation in the two mould species.

Paper and thin-layer chromatographic examinations of culture filtrates of *P. spinulosum* gave evidence for the presence of at least three toluquinonoid pigments; besides spinulosin, 3,4-dihydroxy-2,5-toluquinone and fumigatin were identified by their R_F-values, colours, colour reactions with alkali, and UV-

absorption spectra. All of these pigments have previously been isolated from *A. fumigatus*.[2,6] Furthermore, *P. spinulosum* was found to produce small amounts of orsellinic acid (about 1 mg/l medium) and its decarboxylated product, orcinol (5—8 mg/l). The latter two compounds could always be detected 2—4 days before the pigment production started (in *A. fumigatus* there is no significant production sequence of orsellinic acid and the quinonoid pigments), and were mainly released into the culture medium during this time and in the initial phase of pigment production.

P. spinulosum; was grown as surface cultures, and determinations of the r_H-value of the medium (*cf.* Ref. 4) showed that the pigments formed were present exclusively in the hydroquinone form during the first 3—4 weeks of cultivation. On removal of the mycelium from young cultures of the mould, or on aeration of the culture medium, the pigments were rapidly converted into the quinone form, a reaction which showed all the characteristics of a non-enzymatic air oxidation process;[5] autoxidation of the corresponding hydroquinones thus also appears to be the final step in the toluquinone biosynthesis in *P. spinulosum*.

1-^{14}C-acetate was readily incorporated into all of the pigments produced by *P. spinulosum;* the results of chemical degradations of radioactive spinulosin derived from this precursor supported that the quinone was formed by the acetate-polymalonate pathway (see Table 2 and Fig. 1). Similarly, radioactive orsellinic acid significantly labelled all of the pigments (even though it was mainly decarboxylated by the mould to yield orcinol). With the latter precursor the lipid fraction of the mould cultures remained non-radioactive, strongly indicating that orsellinic acid was incorporated as a unit (without primary degradation to acetate) into the pigments. On the other hand, 3,4-dihydroxy-2,5-toluquinone, fumigatin, and the corresponding hydroquinones (biologically ^{14}C-labelled from acetate) failed to be incorporated into spinulosin, which possibly might be due to technical difficulties with permeability. With exception for this unsuccessful attempt to show that the pigments are interconvertible, all of the observations and experiments described in the present report seem to provide clear evidence that spinulosin is biosynthesized by the

Table 1. Derivatives of 2,5-toluquinone (TQ) isolated from *Aspergillus fumigatus*, L.S.H.T.M. A 46 and A 49.

3-Hydroxy-TQ	(only strain A 46)
3,6-Dihydroxy-TQ	(only strain A 46)
3,4-Dihydroxy-TQ	
3-Methoxy-4-hydroxy-TQ	
3-Hydroxy-4-methoxy-TQ	(fumigatin)
3,6-Dihydroxy-4-methoxy-TQ	(spinulosin)

Table 2. ^{14}C-Distribution in spinulosin derived from 1-^{14}C-acetate. The theoretical distribution of activity, assumed that spinulosin is formed by the acetate-polymalonate pathway as shown in Fig. 1, is indicated by the figures within brackets.

Material	Spec. activity *	Relative total activity	
Spinulosin	6 920	3.00	(3)
Kuhn-Roth ox. carbon dioxide	6 050	1.97	(2)
Kuhn-Roth ox. methyl group of acetic acid	380	0.02	(0)
Kuhn-Roth ox. carboxyl group of acetic acid	17 200	0.93	(1)
Tetramethylammonium iodide	10	0.00	(0)

* counts per min and mg $BaCO_3$.

same pathway in *A. fumigatus* and *P. spinulosum*.

Experimental. P. spinulosum, C.M.I. 91950, which was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England was grown as surface cultures at 25° in 500 ml, Fernbach flasks on 150 ml portions of the modified Czapek-Dox medium described by Birkinshaw and Raistrick.[8]

Identification of metabolic products. The toluquinonoid pigments were identified by methods (see above) that previously have been described in detail;[2] for paper and thin-layer chromatographic comparisons with authentic pigment samples, solvent systems suitable for benzoquinone analysis were used.[9] Orsellinic acid and orcinol were identified by their UV-absorption spectra (bathochromic shift with alkali), and their R_F-values in the solvent systems described by Reio.[10] Orsellinic acid was further identified by submitting it to decarboxylation, which yielded a product that was chromatographically and spectrophotometrically identical with orcinol (*cf.* Ref. 3).

Autoxidation of hydroquinone forms of the pigments. Determinations of the r_H-value of the medium of *P. spinulosum* cultures established that the pigments were present exclusively in the hydroquinone form during the larger part of the production phase.[4] The rate of hydroquinone oxidation in freshly separated filtrates of young cultures of the mould was studied spectrophotometrically at different pH-values (with and without aeration of the solution), and the results obtained strongly indicated that the quinones were formed by an autoxidation process; no evidence for the participation of enzymes was obtained. The techniques used in the above investigations were identical with those employed in similar studies of hydroquinone oxidation in *A. fumigatus.*[5]

Radioactive tracer experiments. The preparation of the ^{14}C-labelled precursors used, and the general techniques employed in these experiments, have been described previously.[1,7] The labelled precursors were added to the culture medium on the tenth day of growth, and after a further 3 days of cultivation ethereal extracts of the culture filtrates were chromatographed (butanol-propanol-ammonium hydroxide[9]) and analyzed in a paper chromatogram scanner. The radioactive spinulosin obtained from 1-^{14}C-acetate (0.5 mC) was eluted from the preparative paper chromatograms with ethanol, diluted with carrier spinulosin (90 mg), and recrystallized to constant specific activity. The distribution of the radioactivity incorporated into the pigment was then determined using the degradation reactions (total combustion, Zeisel demethylation, Kuhn-Roth oxidation, and Schmidt decarboxylation of acetic acid obtained on Kuhn-Roth oxidation) and techniques described for the chemical degradation of fumigatin.[1]

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