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On the Biosynthesis of Toluquinones from *Aspergillus fumigatus*

I. The Biogenetic Role of Orsellinic Acid and Orcinol

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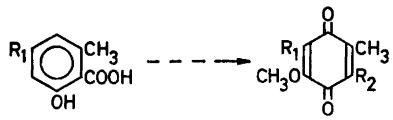
Orsellinic acid (IV) and its decarboxylation product, orcinol, have been isolated from a strain of *A. fumigatus*, known to produce fumigatin (I) and related toluquinones. Evidence was obtained for the intermediate formation of orsellinic acid in the biosynthesis of both orcinol and the toluquinonoid pigments. The pigments seemed not to be formed *via* orcinol.

The metabolism solution of *Aspergillus fumigatus* Fresenius, L.S.H.T.M. A 46, grown as a surface culture on a Raulin-Thom medium is yellowish-brown, but on making alkaline it becomes a strong purple colour. This striking change led Anslow and Raistrick [1] to investigate the solution, from which they isolated a pigment, called fumigatin, which was shown to be 3-hydroxy-4-methoxy-2,5-toluquinone (I). Radioactive tracer studies on the biosynthesis of this pigment were taken up by the author, who found that the methoxyl carbon (C-8) of fumigatin is derived from the C_1-pool, C-7 and C-1 from acetate, and the remaining five carbon atoms from malonate.[2,3] The toluquinonoid nucleus is apparently formed by condensation of one molecule of acetyl-coenzyme A and three of malonyl-coenzyme A, in analogy to the biosynthesis of, for instance, 6-methylsalicylic acid (II).[4]

4-Methoxy-2,5-toluquinone (III) from *Lentinus degener* has been reported to be derived from 6-methylsalicylic acid,[5] as indicated in Fig. 1. Theoretically,



II: $R_1 = H$ III: $R_1 = H$ $R_2 = H$

IV: $R_1 = OH$ I: $R_1 = OH$ $R_2 = H$

V: $R_1 = OCH_3$ $R_2 = OH$

Fig. 1. Reactions that have been reported to take place in moulds: 6-methylsalicylic acid (II) → 4-methoxy-2,5-toluquinone (III), 6-methylsalicylic acid (II) → aurantiogliocladin (V), orsellinic acid (IV) → fumigatin (I).

at least, fumigatin could be formed by similar reactions from orsellinic acid (IV), the acetate-malonate origin of which has been established in studies on the moulds *Penicillium baarnense*,[6,7] *P. cyclopium*,[8] and *P. madriti*,[9] where it functions as a precursor for penicillic acid. Obviously, orsellinic acid would be formed directly by cyclisation of the hypothetical β-polyketo chain obtained from one acetate and three malonate units, while a reduction step is required to yield 6-methylsalicylic acid. For these reasons, orsellinic acid, specifically ^{14}C-labelled in position 2, was tested as a precursor in *A. fumigatus* and proved to be incorporated, as a unit, into fumigatin.[2]

Even though the conversion of orsellinic acid takes place in the mould, some other compound might be the natural intermediate. Birch has claimed that 6-methylsalicylic acid was incorporated into aurantiogliocladin[5] (V), a quinonoid product of *Gliocladium roseum*.[10] The close structural relationship between this fungal metabolite and fumigatin (see Fig. 1) indicates that 6-methylsalicylic acid might also be a possible precursor for the latter quinone. To test this possibility 6-methylsalicylic acid, biologically ^{14}C-labelled from acetate, was added to a 5 days old culture of *A. fumigatus*, L.S.H.T.M. A 46. Even though the labelled 6-methylsalicylic acid seemed to be metabolized (yielding two unidentified products), the fumigatin isolated after a further 7 days of growth was non-radioactive. Furthermore, the labelled 6-methylsalicylic acid was not incorporated into any of the other toluquinones produced by the mould, whereas these are all readily labelled from ^{14}C-orsellinic acid.[11] Consequently, the latter compound seems to be a more probable natural precursor for the pigments than 6-methylsalicylic acid.

When *A. fumigatus*, L.S.H.T.M. A 46, is grown in submerged instead of in surface cultures there is little or no production of pigments. However, an investigation of the metabolism solution of submerged cultures gave evidence for the presence of two phenolic compounds, called A and B, which could be extracted with ether after acidification of the solution, and separated by chromatography. Both compounds yielded intense colours with phenolic reagents such as diazotized *o*-dianisidine (A purple, B brown), diazotized 4-benzoylamino-2,5-dimethoxyaniline (A and B purple), and 2,6-dibromoquinone-4-chloroimide (A and B violet). In addition, compound A gave a positive ferric chloride test (blue-violet) and colour reactions characteristic of carboxylic acids. Titration of compound A indicated the presence of one carboxyl group; the equivalent weight obtained was 175. Carbon dioxide was evolved when an ethanolic solution of compound A was refluxed for 24 h, and this treatment gave in quantitative yield a decarboxylation product, chemically and physically identical with compound B (see below). Compound A could be obtained in the crystalline form by recrystallisation from dilute acetic acid (m.p. 176°, decomp.), and compound B by sublimation in vacuum (m.p. 109°).

Orsellinic acid, which has a molecular weight of 168 and gives the same colour reactions as compound A, also has the same melting-point (176°), which was not depressed by compound A in mixed melting-point determinations. Further evidence for the identity of compound A and orsellinic acid were obtained from their UV- and IR-spectra, and from their chromatographic behaviour; the same R_F-values were obtained in all the different solvent systems

tested,[12] both on paper and thin-layer chromatograms. It immediately follows that the decarboxylation product of compound A, and thus compound B, should be identical with orcinol. Confirmatively, the former two substances showed the same R_F-values and colour reactions as orcinol, and their UV- and IR-spectra were identical with those obtained from the autentic sample. The melting-point of compound B (after sublimation) was unchanged by admixture with anhydrous orcinol (m.p. 109°), and treatment of compound B with an excess of *p*-nitrobenzoylchloride in pyridine yielded a derivative melting at 214°, the melting-point of the di-*p*-nitrobenzoate of orcinol.

Thus it may be concluded that orsellinic acid and orcinol are produced in submerged cultures of *A. fumigatus*, L.S.H.T.M. A 46. A chromatographic investigation showed that they are present also in ordinary, pigment producing, surface cultures of the mould, where they were obtained from the mycelium as well as from the culture medium. Furthermore, both the compounds were detected in a different strain of *A. fumigatus* (L.S.H.T.M. A 49), which is known to produce toluquinonoid pigments[3] (*e.g.* fumigatin and spinulosin).

Orcinol and orsellinic acid were not recognized as mould products until 1958, when they were claimed to have been detected, among other aromatic products, in the culture medium of *Penicillium griseo-fulvum*.[12] This is the only report of orcinol as a fungal metabolite, but orsellinic acid has subsequently been identified in *P. cyclopium*,[7] and isolated from *Chaetomium cochliodes*,[13] *P. baarnense*,[5] and *P. madriti*.[9]

As mentioned above, orsellinic acid is directly converted into fumigatin in *A. fumigatus*, L.S.H.T.M. A 46. Since it now has been shown that orsellinic acid is present as a metabolic product of the mould, it seems likely that it also functions as the natural precursor for fumigatin and the other toluquinones produced. Further evidence in this direction was obtained in radioactive tracer experiments. On addition of 1-^{14}C-acetate to surface cultures of the mould orsellinic acid becomes labelled more rapidly than the pigments. In further studies the variations in time of the specific activities of orsellinic acid and fumigatin, respectively, were determined in experiments where the radioactive substrate (acetate) was replaced by non-labelled Raulin-Thom medium after a very short incubation time. The theory of tracer experiments of this kind has been elaborated by Zilversmit,[14] who found characteristic shapes of the specific activity/time curves of precursor-product systems. A typical example is given in Fig. 2, and the results of the present studies, shown in Fig. 3, support the idea that orsellinic acid functions as the natural precursor for, at least, fumigatin.

A more definite proof for the important biogenetic role of orsellinic acid was obtained in investigations on a mutant of *A. fumigatus*, L.S.H.T.M. A 49. This mutant failed to yield aromatic secondary metabolites when grown on an ordinary Raulin-Thom solution. A normal production of toluquinonoid pigments (and of orcinol) was, however, obtained on addition of orsellinic acid to the medium. Several other compounds (*e.g.* 6-methylsalicylic acid and orcinol) were tested, but none of them could substitute for orsellinic acid in this respect. ^{14}C-labelled acetate and malonate, added to the culture medium containing orsellinic acid, were not incorporated into the secondary products formed. Orsellinic acid, therefore, appears to be the source of carbon utilized

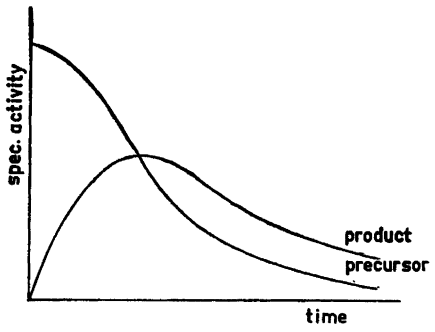


Fig. 2. Specific activity/time curves for a precursor-product system, according to Zilversmit.

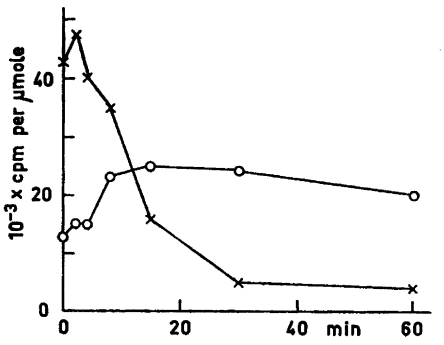


Fig. 3. Specific activity/time curves, orsellinic acid ×, fumigatin O.

for pigment formation in the mutant, which seems to be unable to carry out the acetate-malonate condensation yielding orsellinic acid, while the enzyme systems performing the subsequent modifications of this precursor, obviously, are present in an active form.

The occurrence of orsellinic acid and orcinol in the same organism points to a similar origin for the two compounds; this is also indicated by the high incorporation rate of acetate into orcinol. The latter compound may, however, be formed either *via* orsellinic acid or independently of the production of orsellinic acid. Theoretically, the former route is the most likely one, and the significance of this route has been confirmed by several experiments. About 8 % of the radioactivity added was, for instance, recovered in compound B (orcinol) in the tracer experiment with 2-^{14}C-orsellinic acid, mentioned above. Since this precursor labelled fumigatin in a specific way, the acetate-pool must have remained non-radioactive, and orcinol can not have been labelled *via* acetate. It may, further, be recalled that the mutant strain of *A. fumigatus*, L.S.H.T.M. A 49, failed to produce orcinol unless orsellinic acid was added to the culture medium. These observations show that orsellinic acid functions as a precursor (above the acetate-malonate level) for orcinol. The non-oxidative removal of nuclear carboxyl groups has been established in several microbial systems.[15–18] It, therefore, appears that orcinol is formed by a direct decarboxylation of orsellinic acid.

Orsellinic acid is readily submitted to non-enzymatic decarboxylation, even under fairly mild conditions. It could, therefore, be suspected that orcinol was formed as an artefact during the isolation procedures rather than as a metabolic product of the mould. Experiments showed, however, that no significant amounts of orcinol were formed when a solution of orsellinic acid in the Raulin-Thom medium was treated in a similar manner as the metabolism solutions.

A decarboxylation step must, obviously, be involved in the conversion of orsellinic acid into the toluquinonoid pigments produced by *A. fumigatus*. It could, therefore, be suspected that orcinol, which is present in the cultures

of the mould, were formed as an intermediate in this conversion. It may be mentioned that both orsellinic acid and orcinol recently have been found to function as precursors for phoenicin,[19] a quinonoid pigment from *Penicillium phoenicium*. However, the mutant strain of *A. fumigatus*, L.S.H.T.M. A 49, was unable to utilize orcinol (in contrast to orsellinic acid) for pigment formation, even though a great deal of the orcinol added was recovered from the mycelial part of the cultures. Furthermore, ^{14}C-labelled orcinol, supplied to young cultures of *A. fumigatus*, L.S.H.T.M. A 46, was not incorporated into any of the pigments produced, whereas these are all readily labelled from radioactive acetate, malonate, or orsellinic acid. Nothing points to that the mycelial membranes are impermeable to orcinol; about 4 % of the radioactivity added was recovered from the mycelium.

Even though negative results should be treated with great caution, there are other indications that orcinol is formed from orsellinic acid as a by-product rather than as an intermediate in the pigment biosynthesis. Thus the concentration of orcinol in the medium of cultures of *A. fumigatus*, strain A 46, steadily increases, at a rate proportional to the rate of pigment formation, while the concentration of orsellinic acid remains almost constant from the seventh day of cultivation until autolysis begins. It has also been shown [2] that at least one of the pigments, fumigatin, can not be derived from symmetrical intermediates; the other pigments may be expected to be formed by similar mechanisms. For these reasons it must be considered as unlikely that free orcinol functions as an intermediate in the biosynthesis of the toluquinones from *A. fumigatus*.

EXPERIMENTAL

The culture conditions and the isolation of fumigatin have been described elsewhere.[2]

Preparation of ^{14}C-labelled 6-methylsalicylic acid. 0.5 mC of 1-^{14}C-acetate was added to a 4 days' submerged culture of *Penicillium urticae*. Growth was continued for another 4 days, when radioactive 6-methylsalicylic acid was isolated from the culture medium as described by Gatenbeck and Lönnroth.[20]

Preparation of ^{14}C-labelled orcinol. Orsellinic acid, biologically labelled from 1.0 mC of 1-^{14}C-acetate using *Penicillium baarnense*, was isolated as described by Mosbach.[6] A solution of the radioactive orsellinic acid in ethanol was refluxed for 24 h, by which treatment orcinol was obtained in an almost quantitative yield. After evaporation of the ethanol in vacuum the crude, radioactive, orcinol was purified by paper chromatography.

Isolation of orsellinic acid and orcinol. The medium from 14 days old submerged cultures of *A. fumigatus*, L.S.H.T.M. A 46, was acidified and extracted with ether. The residues of the ether extracts were separated on paper chromatograms (Whatman 3 MM) with butanol-propanol-2 M ammonium hydroxide (1:6:3 by vol.) as the solvent, and orcinol (R_F 0.90) and orsellinic acid (R_F 0.55) eluted from the paper with acetone. Orsellinic acid was further purified by recrystallization from dilute acetic acid (m.p. 176°), and orcinol by sublimation at 100° in vacuum (m.p. 109°).

Tracer experiments according to Zilversmit. *A. fumigatus*, L.S.H.T.M. A 46, was cultivated in a Petri dish (15 cm diam.) containing 100 ml of Raulin-Thom medium. After 5 days of growth 50 μC of 1-^{14}C-acetate were added to the culture. 10 min later the radioactive medium was removed by suction and 30 ml of fresh Raulin-Thom solution added after washing the mycelium briefly with distilled water. Equal pieces of the mycelium were then cut out at different times after the replacement of the medium, dipped into a small volume of conc. HCl, and thoroughly extracted with ether. The residues of the extracts were chromatographed on Whatman No. 1 paper and the total radioactivity of fumigatin and orsellinic acid, respectively, measured in a paper chromatogram scanner.

After elution the concentration of each compound was determined in a Beckman DU Spectrophotometer at the wave-length of maximum absorption, enabling a calculation of the molar specific activities.

Non-producing mutant of A. fumigatus, *L.S.H.T.M. A 49.* The original strain was obtained by the courtesy of Dr. G. Smith, London. During experimental work with the mould a non-producing, spontaneously arisen, mutant was recognized. This mutant was identified as an *A. fumigatus* Fresenius by Centraalbureau voor Schimmelcultures, Baarn. It was morphologically identical with the original strain, and yielded a similar pattern of production when grown on a Raulin-Thom solution containing orsellinic acid (25 mg/l).

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