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On the Biosynthesis of Toluquinones from *Aspergillus fumigatus*

IV. Biogenetic Relationships of the Pigments*

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The interrelationships of the toluquinonoid pigments produced by *A. fumigatus*, L.S.H.T.M. A 46, has been investigated by observing the pattern of incorporation of 3-hydroxy-2,5-toluhydroquinone (I) and 3,4-dihydroxy-2,5-toluhydroquinone (III). The former compound was found to be converted as a unit into 3,6-dihydroxy-2,5-toluhydroquinone (II), a reaction that is analogous to the hydroxylation of fumigatin hydroquinone (V), by which spinulosin hydroquinone (VI) appears to be formed. 3,4-Dihydroxy-2,5-toluhydroquinone was incorporated into all of the pigments that contain a methoxyl group. Further experiments, including short-term studies of the utilization of 1-^{14}C-acetate for pigment formation, established that 3,4-dihydroxy-2,5-toluhydroquinone is a possible natural precursor of, for instance, fumigatin.

Six closely related toluquinonoid pigments (Nos. I—VI in Table 1), orsellinic acid (VII) and its decarboxylated product, orcinol, have been isolated from certain strains of *Aspergillus fumigatus*.[1,2] Previous investigations have shown that the pigments are biosynthesized by the acetate-polymalonate pathway, with intermediate formation of orsellinic acid.[1-4] Further, it appears well established that autoxidation of the corresponding hydroquinones is the final step in the toluquinone biosynthesis;[5] the pigments were, in fact, found to be present in the hydroquinone form during the entire active phase of production.[6] If any interconversions of the pigments take place (which might be anticipated from their structures), these would, therefore, be expected to involve the hydroquinone forms.

The mould has previously been shown to be capable of hydroxylating one of the pigments; fumigatin hydroquinone (V) was found to be directly (without primary degradation to acetate) converted into spinulosin hydroquinone (VI).[4]

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Table 1. Pigments isolated from *Aspergillus fumigatus*, L.S.H.T.M. A 46.

No.	Derivative of 2,5-toluquinone	
I	3-Hydroxy-	
II	3,6-Dihydroxy-	
III	3,4-Dihydroxy-	
IV	3-Methoxy-4-hydroxy-	
V	3-Hydroxy-4-methoxy-	(fumigatin)
VI	3,6-Dihydroxy-4-methoxy-	(spinulosin)

For this reason, 3-hydroxy-2,5-toluhydroquinone (I) would be expected to function as a precursor of 3,6-dihydroxy-2,5-toluhydroquinone (II), as well as of 3,4-dihydroxy-2,5-toluhydroquinone (III). The latter compound might, further, be a common precursor of the pigments that contain a methoxyl group. These possibilities, which are indicated in Figs. 1 and 2, have now been

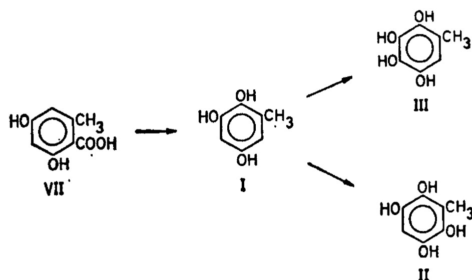

Fig. 1. Possible relationships of the pigments (shown in the hydroquinone form) that lack a methoxyl group.

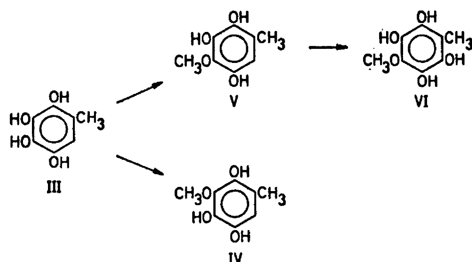

Fig. 2. Possible pathway for the formation of the pigments (shown in the hydroquinone form) that contain a methoxyl group.

tested by observing the incorporation of activity into the different toluquinones produced by *A. fumigatus*, L.S.H.T.M. A 46, when biosynthesized in the presence of ^{14}C-labelled 3-hydroxy-2,5-toluhydroquinone, 3,6-dihydroxy-2,5-toluhydroquinone, and 3,4-dihydroxy-2,5-toluhydroquinone, respectively.

Contrary to expectations, only one of the pigments was found to be labelled from radioactive 3-hydroxy-2,5-toluhydroquinone; about 2 % of the activity added was incorporated into 3,6-dihydroxy-2,5-toluhydroquinone. The latter compound, which is a minor metabolite of the mould, seemed not to have been labelled *via* the acetate pool, as the major pigment (fumigatin) was non-radioactive and ^{14}C-labelled acetate under similar conditions is known to be readily incorporated into all of the secondary metabolites produced by the

mould. Preliminary experiments, further, established that none of the precursors tested was metabolized when added to culture filtrates of the mould. Consequently, it seems likely that 3-hydroxy-2,5-toluhydroquinone was converted as a unit into 3,6-dihydroxy-2,5-toluhydroquinone by the action of endo-cellular enzyme systems. This process is obviously analogous to (and probably catalyzed by the same enzymes as) the hydroxylation of fumigatin hydroquinone (see above).

The above experiment, further, indicates that 3,6-dihydroxy-2,5-toluhydroquinone (II) is a metabolic end-product; no activity was incorporated into spinulosin (VI), which might be derived from 3-hydroxy-2,5-toluhydroquinone *via* 3,6-dihydroxy-2,5-toluhydroquinone. Similarly, when the latter compound, biologically ^{14}C-labelled from acetate, was tested as a precursor, it failed to be incorporated into any of the toluquinones produced (besides 3,6-dihydroxy-2,5-toluquinone). It, therefore, appears that 3-hydroxy-2,5-toluhydroquinone and 3,6-dihydroxy-2,5-toluhydroquinone should be placed on a side pathway in the sequence of reactions, by which orsellinic acid is converted into 3,4-dihydroxy-2,5-toluquinone and the methoxytoluquinones. This idea receives further support from the fact that the latter pigments are regularly present in quinone producing strains of *A. fumigatus*, while 3-hydroxy-2,5-toluquinone and 3,6-dihydroxy-2,5-toluquinone often fail to be formed; they have, for instance, not been detected in *A. fumigatus*, L.S.H.T.M. A 49.[4]

$^{14}CH_3$-L-methionine has been shown to label the three pigments that contain a methoxyl group.[1] Chemical degradation of fumigatin, derived from this precursor, established that isotope was located at the methoxyl carbon, exclusively.[3] The presence of transmethylating enzymes, acting on polyphenolic substrates and using methionine as a methyl donor, has been established in several biological systems. It, therefore, seemed likely that 3,4-dihydroxy-2,5-toluhydroquinone (III) would be a common precursor of 3-methoxy-4-hydroxy-2,5-toluhydroquinone (IV) and fumigatin hydroquinone (V), and hence also of spinulosin hydroquinone (VI). This pathway, which is shown in Fig. 2, received support from the present experiments; radioactive 3,4-dihydroxy-2,5-toluhydroquinone significantly labelled all of the methoxytoluquinones, while orsellinic acid, orcinol, and 3,6-dihydroxy-2,5-toluquinone were non-radioactive. The incorporation yields were, however, unexpectedly low. In the best experiment 1.6 % of the activity added was present in fumigatin, 0.3 % in 3-methoxy-4-hydroxy-2,5-toluquinone, and 0.1 % in spinulosin. This could, of course, be explained as a result of technical difficulties with permeability, but might also indicate that the observed O-methylation of 3,4-dihydroxy-2,5-toluhydroquinone is of little significance in the biosynthesis of, for instance, fumigatin. Further experiments were, therefore, undertaken in order to study the biogenetic relationship of the latter two pigments, which are the major secondary metabolites in *A. fumigatus*, L.S.H.T.M. A 46.

Even though no significant production sequence of the different pigments could be observed, an early indication that 3,4-dihydroxy-2,5-toluhydroquinone functions as a biosynthetic intermediate was based on the fact that this compound is the major pigment in very young cultures of the mould, whereas fumigatin hydroquinone predominates after one week of growth. As shown in Fig. 3, the concentration of the latter compound (in the culture

medium) was found to increase almost linearly with the age of the culture. The concentration of 3,4-dihydroxy-2,5-toluhydroquinone, on the other hand, seemed to attain a maximum value within a few days, which was essentially maintained (a slight increase could generally be observed) until pigment production ceased. This might indicate that 3,4-dihydroxy-2,5-toluhydroquinone, as soon as steady state conditions are attained, is formed and released into the medium at the same rate as it disappears from the medium by conversion into the methoxytoluhydroquinones (preliminary experiments excluded that it disappeared by non-enzymatic reactions, *e. g.* oxidative destruction). However, since fumigatin hydroquinone is formed at a rate of approximately 10 μM/h (see Fig. 3), one would expect that at least half of the assumed pool

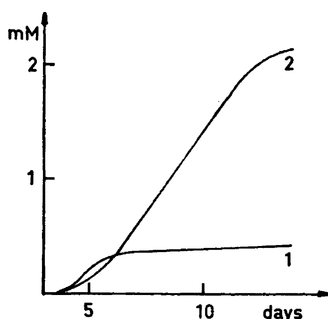

Fig. 3. Concentration of 3,4-dihydroxy-2,5-toluhydroquinone (curve 1) and fumigatin hydroquinone (curve 2) in the medium of surface cultures of *Aspergillus fumigatus*, as a function of time.

of 3,4-dihydroxy-2,5-toluhydroquinone (about 0.4 mM) would be turned over within 3 days, for which time the mould was cultivated in the presence of radioactive 3,4-dihydroxy-2,5-toluhydroquinone. This is not consistent with the low incorporation yields obtained with the latter precursor; it can be concluded that only a minor fraction of the fumigatin formed is derived from 3,4-dihydroxy-2,5-toluhydroquinone present in the culture medium.

Consequently, it appears that 3,4-dihydroxy-2,5-toluhydroquinone is released into the medium at a considerably decreased rate after about one week of cultivation (probably as a consequence of a gradual induction of the O-methyltranspherase system). This was confirmed by studies of the utilization of 1-^{14}C-acetate for pigment formation in 10 days old cultures of the mould; the fumigatin isolated from the medium (mycelium) 4 h after addition of the labelled precursor contained 1.3 (0.1) % of the activity added, whereas only 0.06 (0.01) % were present in 3,4-dihydroxy-2,5-toluquinone. However, when the quinones were isolated from the mycelium a very short time after the addition of 1-^{14}C-acetate an entirely different pattern of labelling was observed. Although the pigments were not found to be sequentially labelled, the total activity incorporated into 3,4-dihydroxy-2,5-toluhydroquinone after 3 min (it was not feasible to obtain reliable radioactivity determinations until then) was in the same order as, or slightly exceeded, that incorporated into fumigatin hydroquinone. At this time no labelled pigments could be detected in the culture medium.

These observations are in consistence with the pathway shown in Fig. 2. Assumed that the rate of *de novo* formation of the pigments can be estimated

from the observed extent of acetate incorporation during the first few minutes, it may, in fact, be concluded that 3,4-dihydroxy-2,5-toluhydroquinone functions as a biosynthetic intermediate, yielding fumigatin hydroquinone as the probable major product. The failure to detect any significant production order in time of the two pigments, as indicated by the appearance of activity, would then indicate that the actual pool of 3,4-dihydroxy-2,5-toluhydroquinone is very small; the latter pigment must be assumed to be O-methylated rapidly after its formation, possibly while still being enzyme-bound. On the other hand, the results obtained do not establish that 3,4-dihydroxy-2,5-toluhydroquinone is the (only) immediate precursor of fumigatin. For instance, it cannot be excluded that 2,3,4,5-tetrahydroxy-6-methylbenzoic acid, which is a possible immediate precursor of 3,4-dihydroxy-2,5-toluhydroquinone, functions as the natural substrate for the O-methyltranspherase system; the radioactive 3,4-dihydroxy-2,5-toluquinone isolated in the short-term studies of acetate incorporation might partly be artificially derived from the above phenolic acid, which would give a wrong estimate of the rate of *de novo* formation of the pigment. Even though it, for the present, is not possible to distinguish between these alternatives, the experiments described seem to provide strong evidence that two hydroxylation steps precede the O-methylation process in the conversion of orsellinic acid into fumigatin.

EXPERIMENTAL

Culture conditions. All experiments described below involved surface cultures of *Aspergillus fumigatus*, L.S.H.T.M. A 46, grown in 500 ml Fernbach flasks on 150 ml portions of a modified Raulin-Thom solution, the composition of which has been given elsewhere.[7]

Preparation of labelled precursors. Radioactive 3-hydroxy-2,5-toluquinone was obtained by oxidation of biologically ^{14}C-labelled orcinol (the preparation of which has been described previously [2]) according to the method of Musso.[8] Radioactive 3,4-dihydroxy-2,5-toluquinone and 3,6-dihydroxy-2,5-toluquinone were obtained by biosynthesis from 1-^{14}C-acetate (1.0 mc) using *A. fumigatus*, strain A 46; the pigments were isolated from the culture medium by ether extraction, and separated by paper chromatography as previously described for the preparation of biosynthetically ^{14}C-labelled fumigatin.[4] The labelled toluquinones were converted into the corresponding hydroquinones by treatment with a slight excess of aqueous sodium dithionite, immediately before administration to the mould cultures.

Administration of labelled precursors. Aqueous solutions of 3-hydroxy-2,5-toluhydroquinone (3 mg; 1.8 μc), 3,4-dihydroxy-2,5-toluhydroquinone (2 mg; 8.6 μc), and 3,6-dihydroxy-2,5-toluhydroquinone (0.1 mg; 1.8 μc), respectively, were added to the medium of 5 days old surface cultures of the mould. Growth was continued for a further 3 days, when the culture filtrates were vigorously aerated at pH 8.0 for 5 min (which destroyed 3-hydroxy-2,5-toluquinone) and thoroughly extracted with ether at pH 1.0. The concentrated ethereal pigment extracts were then separated by paper chromatography, using solvent systems (3 in all) suitable for analysis of hydroxyquinones;[9] the pigments were identified by their R_F-values, colours, and colour reactions on exposure to ammonia vapour. The incorporation of activity into the different toluquinones was measured in a paper chromatogram scanner and quantitatively estimated by comparison with paper chromatogram scans of pigment samples of known activity. All of the labelled products obtained could be identified as pigments; when these were eluted and rechromatographed (in the above systems and the solvent systems B—E of Reio [10]) a single peak of activity, associated with the pigment eluted, was always obtained.

Concentration curves. The formation of fumigatin hydroquinone and 3,4-dihydroxy-2,5-toluhydroquinone in surface cultures of the mould was followed by withdrawal of

5 – 10 ml portions of the medium at fixed intervals. The samples withdrawn were thoroughly extracted with ether at pH 1, and the pigment extracts were separated by paper chromatography, using propanol-butanol-2 M ammonium hydroxide (6:1:3 by vol.) as the solvent. The above two pigments were then eluted with ethanol, and the pigment concentration was determined spectrophotometrically at the wave-length of maximum absorption in the ultra-violet.[1,9]

Even though different cultures varied slightly with respect to the time-scale and the total amount of pigments formed, the general production pattern (indicated in Fig. 3) was the same; in the initial production phase the two major pigments were released into the medium at comparable rates, but after a few days the concentration of 3,4-dihydroxy-2,5-toluhydroquinone remained essentially constant, while the concentration of fumigatin hydroquinone steadily increased for a further 7 – 10 days.

Short-term studies of acetate incorporation. Two 10 days old cultures of the mould, that seemed to be at the same stage of development (as indicated by the general appearance, and the pH and r_H-value of the medium), were each supplied with an aqueous solution of sodium 1-^{14}C-acetate (50 μc). In order to obtain a rapid and even distribution of the precursor, which was added to the culture medium, the culture flasks were gently shaken for a few moments.

3 min later one of the cultures was filtered. The mycelium was briefly rinsed with water, dipped into 25 ml of 1 M hydrochloric acid, and cut into pieces by the aid of a Waring blendor. The "homogenate" was then thoroughly extracted with ether, which removed the pigments. All these operations were carried out as quickly as possible. The incorporation of activity into the two major pigments was determined as described above, and showed that both of them were significantly labelled; 3,4-dihydroxy-2,5-toluquinone contained 0.001 – 0.003 % of the activity added (three separate experiments were undertaken), and generally slightly less activity (0.0005 – 0.003 %) was incorporated into fumigatin. No labelled pigments could be detected on examination of the corresponding culture filtrates (although radioactive orsellinic acid was released into the culture medium already 1 min after the addition of 1-^{14}C-acetate).

The second culture was treated in a similar way 4 h after addition of the labelled precursor. The incorporation of activity into 3,4-dihydroxy-2,5-toluquinone was measured as described above, while the activity of fumigatin was determined in a liquid scintillation counter after conversion of the pigment into barium carbonate by the wet combustion technique of van Slyke and Folch.[11]

Preliminary experiments showed that incorporation of activity from 1-^{14}C-acetate into the two major pigments proceeded continuously during the first 2 – 4 h. However, already after some 10 min the rate of labelling of fumigatin hydroquinone by far exceeded that of 3,4-dihydroxy-2,5-toluhydroquinone. Orsellinic acid, which is a more immediate precursor of the pigments than acetate, could not be used for short-term studies of the pigment formation; it was found to be incorporated at a much slower rate than acetate, possibly as an effect of permeability difficulties.

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