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The Biosynthesis of Spinulosin in *Aspergillus fumigatus*

GÖSTA PETTERSSON

Institute of Biochemistry, University of Lund, Sweden

3,4-Dihydroxy-2,5-toluquinone, 3-methoxy-4-hydroxy-2,5-toluquinone, and fumigatin (II) are identified as metabolic products of *A. fumigatus*, L.S.H.T.M. No. A 49, which is known also to produce spinulosin (I). The acetate-malonate origin of fumigatin from *A. fumigatus*, L.S.H.T.M. No. A 46, is shown by degradation of labelled fumigatin obtained after feeding the mould with 2-^{14}C-malonate. Fumigatin hydroquinone (VII) is proved to be an intermediate in the biosynthesis of spinulosin in strain A 49 by the use of fumigatin, specifically ^{14}C-labelled by biosynthesis from 2^{14}-C-malonate or $^{14}CH_3$-L-methionine. A simple technique to show the incorporation, as a unit, of a non-specifically labelled precursor is described and used to prove that fumigatin hydroquinone is a precursor of spinulosin also in strain A 46.

Spinulosin (I) and fumigatin (II) are produced by two different strains of *Aspergillus fumigatus* Fresenius, L.S.H.T.M. Cat. Nos. A 49 and A 46, respectively.[1,2] Spinulosin, obtained from strains of *Penicillium spinulosum* Thom,[3] was the first mould product to be identified as a quinone; it has also been isolated from an unidentified *Penicillium*[4] (possibly *P. spinulosum*), and *P. cinerascens* Biourge.[5] In a recent investigation[6] spinulosin and four other toluquinonoid pigments were shown to be produced along with fumigatin by the strain A 46 mentioned above. The suspicion arose that the same metabolites could be formed in strain A 49, even if in other proportions. A careful chromatographic investigation of the latter strain was therefore undertaken, using the identification methods previously described.[6] Besides spinulosin, fumigatin, 3,4-dihydroxy-2,5-toluquinone (III), and 3-methoxy-4-hydroxy-2,5-toluquinone (IV) were identified in the ethereal extract of the culture medium. Thus there is a strong resemblance in the production pattern of the two strains of *A. fumigatus*, but the following differences may be noted:

(1) 3-Hydroxy-2,5-toluquinone and 3.6-dihydroxy-2,5-toluquinone (V), which are both produced by strain A 46, could not be detected in the culture medium or mycelium of strain A 49.

(2) The relative proportions of fumigatin, 3-methoxy-4-hydroxy-2,5-toluquinone, and 3,4-dihydroxy-2,5-toluquinone are the same in both the

strains, but the proportion of spinulosin is much larger in strain A 49, even in young cultures.

(3) Strain A 49 produces in addition to the toluquinones at least two phenolic pigments, easily detected on paper chromatograms by their yellow colour, changing to red on exposure to ammonia vapour. It is probable that one of these pigments is identical with the yellow solid isolated from strain A 49 by Anslow and Raistrick.[1] No pigments of this type are produced by strain A 46.

Despite these minor metabolic differences between the two strains, it is likely that the toluquinones are formed in the same way in both cases. In strain A 46 orsellinic acid (VI) can be converted to fumigatin,[7] a sequence of reactions involving two hydroxylation steps. As the introduction of a third hydroxyl group would give spinulosin it seemed reasonable to test whether fumigatin is an intermediate in the biosynthesis of spinulosin.

In a preliminary experiment fumigatin, biologically ^{14}C-labelled from acetate, was added to a 5 days old culture of strain A 49. This strain was selected in preference to strain A 46 because it gives a better yield of spinulosin. One week later spinulosin was isolated and found to be radioactive. Little or no incorporation of activity into spinulosin was obtained when more than 10 days old cultures were used, unless ^{14}C-fumigatin was added in the reduced form. These experiments indicate that fumigatin can function as a precursor to spinulosin in young cultures, but that fumigatin hydroquinone (VII) is the natural intermediate. The proposed sequence of reactions leading to fumigatin and spinulosin, shown in Fig. 1, is supported by the fact that fumigatin hydroquinone has been isolated from strain A 46, and that in this strain the ratio fumigatin/fumigatin hydroquinone increases with the age of the culture.[2] Furthermore, a recent investigation of strains of *A. fumigatus*[8] has shown

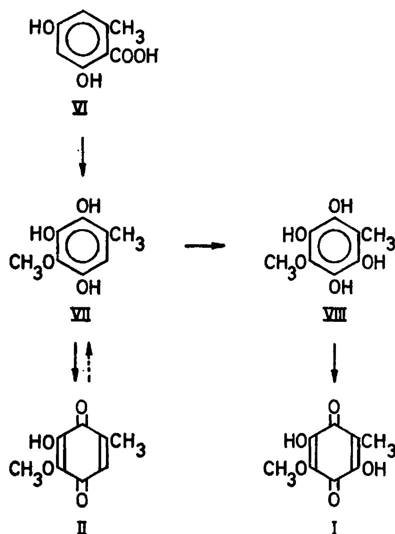

Fig. 1. Biogenetic scheme for spinulosin and fumigatin. The dashed arrow indicates a conversion, possible only in young cultures.

the presence of a phenolase system, which could be responsible for the formation of spinulosin and fumigatin from the corresponding hydroquinones. The observation that young cultures can use fumigatin for spinulosin synthesis is also consistent with the proposed biogenetic scheme. One possible explanation is based on the fact that substitution with a methyl, amine, or hydroxyl group in the quinonoid nucleus leads to a diminished oxidation-reduction potential for the system quinone/hydroquinone.[9] Thus spinulosin hydroquinone (VIII) is able to reduce fumigatin. In young cultures where pigment production is still proceeding, sufficient spinulosin hydroquinone would be formed to reduce the added fumigatin. Theoretically, at least, this reduction is autocatalytic since a new molecule of spinulosin hydroquinone could be obtained from every molecule of fumigatin hydroquinone thus formed.

A possible objection to the previous tracer experiment is that a catabolic degradation of the added ^{14}C-fumigatin probably would label the acetate-pool, and hence spinulosin.[6] The experiment had, therefore, to be repeated with specifically labelled fumigatin followed by degradation of the labelled spinulosin to prove the direct conversion. For practical reasons it is impossible to obtain fumigatin specifically ^{14}C-labelled by chemical synthesis, but there are at least three ways to obtain it by biosynthesis using strain A 46[7]:

(a) 2-^{14}C-Orsellinic acid will give fumigatin labelled almost exclusively in position 4.

(b) $^{14}CH_3$-L-Methionine is incorporated only into the methoxyl carbon (C—8) of fumigatin.

(c) If fumigatin is biosynthesized in accordance with the acetate-malonate theory, which is assumed, but unproved, ^{14}C-malonate would label C—1 and C—2 of fumigatin less then the rest of the quinonoid nucleus.

Of these alternatives only (b) and (c) will give fumigatin with a sufficient high specific activity. 8-^{14}C-Fumigatin was used (expt. A) as it was available from an earlier experiment.[7] However it is possible, even if unlikely, that the methoxyl carbon of fumigatin labels the methoxyl carbon of spinulosin specifically *via* the C_1-pool. Therefore the experiment was repeated also with fumigatin isolated from strain A 46, cultivated on a solution containing diethyl 2-^{14}C-malonate (expt. B). In both cases fumigatin was reduced with glutathione before addition to the cultures of strain A 49.

Further preliminary experiments indicated that conversion of ^{14}C-fumigatin hydroquinone to spinulosin also occurred in strain A 46. These experiments were complicated by the poor yields of spinulosin in this strain and by a much lower incorporation of radioactivity than in strain A 49. It was, therefore, necessary to add relatively large amounts of non-radioactive carrier to the isolated spinulosin, leading to a lower specific activity than is desirable in the chemical degradations. For these reasons the use of specifically labelled fumigatin with the subsequent degradation reactions was not feasible; a new technique had, therefore, to be developed.

If it can be shown that ^{14}C-fumigatin hydroquinone is incorporated into spinulosin without labelling the acetate-pool, the direct conversion of fumigatin hydroquinone is evidenced. Birch *et al.* have in such an indirect way provided some evidence that 6-methylsalicylic acid can function as a precursor of 4-methoxy-2,5-toluquinone[10] produced by *Lentinus degener*.[11] They observed

that acetate labelled the C_1-pool, so that considerable activity appeared in the methoxyl carbon of 4-methoxy-2,5-toluquinone after feeding the mould with ^{14}C-acetate. Radioactive 6-methylsalicylic acid, however, was incorporated as a unit, since this precursor did not label the methoxyl group.

Birch *et al.* also claim to have used the same technique to show that 6-methylsalicylic acid, but not the theoretically more likely orsellinic acid, functions as a precursor of aurantiogliocladin,[10] produced by *Gliocladium roseum.*[12]

Unfortunately this technique can not be applied in general, as the C_1-pool usually is not labelled from acetate to any great extent. For this reason, and since chemical degradation of the isolated spinulosin would still be required, the technique can not be used in the investigation of strain A 46.

Another method to decide whether the acetate-pool is labelled or not, frequently used in our laboratories, is to isolate and examine the lipid fraction of the mould mycelium. In strain A 46, however, there are other acetate-derived compounds more readily available for examination than those of the lipid fraction; these are the toluquinones produced besides fumigatin and spinulosin. If only the latter two quinones are significantly labelled from ^{14}C-fumigatin hydroquinone, and if acetate under the same conditions (the same rate of production of the different pigments, and the same magnitude of unlabelled pools when the precursors are added) labels one or more of the other quinones, it is proved that ^{14}C-fumigatin hydroquinone is incorporated as a unit into spinulosin. Even if some primary degradation to acetate occurs, a comparison of the specific activities of the pigments obtained from ^{14}C-acetate and ^{14}C-fumigatin hydroquinone, respectively, would reveal if there is, in addition, any direct conversion of the latter precursor to spinulosin. A determination of the specific activities will not necessarily require any chemical degradation of the toluquinones, as they can be separated on a paper chromatogram,[13] and the radioactivity determined by scanning the chromatogram with a strip-counter. Alternatively they can be eluted from the chromatogram and measured in a liquid scintillation counter, using a suitable solvent. Quantitative estimation of the intensely coloured compounds can be made spectrophotometrically directly on the chromatograms or after elution.

Three 5 days' cultures of strain A 46, which appeared to be in the same stage of development, were selected and supplied with, respectively, 50 μC of 1-^{14}C-acetate, 50 μC of 2-^{14}C-acetate, and about 20 μC of fumigatin hydroquinone, biologically ^{14}C-labelled from acetate (expt. C). After another 5 days of growth, paper chromatograms of the ether extracts of the culture filtrates were developed and scanned in a strip-counter. Then, after elution, quantitative analysis of the quinones was made spectrophotometrically.

EXPERIMENTAL

Culture conditions. Aspergillus fumigatus Fresenius, L.S.H.T.M. Cat. Nos. A 46 and A 49 were obtained through the courtesy of Dr. G. Smith. They were cultivated as surface cultures under the conditions described by Anslow and Raistrick.[1,2]

^{14}C-Fumigatin. The precursor was added to a 5 days' culture of strain A 46 and growth continued for another 5 days. The culture filtrate, after adjustment to pH 8.0 by the addition of 1 M NaOH, was vigorously aerated for 1 h. By this treatment, 3-hydroxy-2,5-toluquinone and most of the 3,4-dihydroxy-2,5-toluquinone were destroyed. The

solution was then extracted twice with an equal volume of ether after acidification with concentrated HCl. The ether was evaporated *in vacuo* and the crude extract chromatographed on Whatman No. 3MM paper, using butanol-propanol-2 M ammonium hydroxide (1:6:3 by volume) as solvent. From the air-dried chromatogram the part containing fumigatin was cut out, moistened with 1 M HCl and extracted with ether. The syrupy residue remaining after removal of the ether was dried *in vacuo* over P_2O_5 for 24 h, then dissolved in 5 ml of anhydrous ether. Fumigatin was precipitated in an amorphous, but chromatographically pure, form by pouring the solution into 150 ml of boiling petroleum ether (b.p. 40°—60°).

500 μC of 1-^{14}C-acetate was used as the precursor for the biosynthesis of ^{14}C-fumigatin to be used experiment C. 50 μC of diethyl 2^{14}-C-malonate as precursor gave 6 % of incorporation into fumigatin, half of which was submitted to chemical degradation. The other half was used in experiment B.

^{14}C-Spinulosin. ^{14}C-Fumigatin was dissolved in a minimal volume of 0.1 M phosphate buffer, pH 8.0, and treated with solid glutathione until the intense purple colour of fumigatin faded. This solution of ^{14}C-fumigatin hydroquinone was added to a 5 days' culture of strain A 49 and growth continued for a further 5 days, when the culture filtrate was treated as described for ^{14}C-fumigatin. Radioactive spinulosin was eluted from the paper chromatogram, diluted with carrier spinulosin and purified by sublimation *in vacuo*.

In experiment A and B, 0.4 μC and 1.5 μC of ^{14}C-fumigatin hydroquinone was added as precursor, and the isolated spinulosin diluted with 40 mg and 80 mg of non-radioactive carrier, respectively. In both experiments the incorporation of radioactivity into spinulosin was 10—15 %.

Degradation of fumigatin and spinulosin. The chemical degradation of fumigatin has been described previously in detail.[7] The same degradation reactions were used to determine the distribution of radioactivity in spinulosin. All radioactivity measurements in the degradation series were performed in a Baird-Atomic Liquid Scintillation Counter, with the samples as barium carbonate suspended in a gel of Aerosil in a toluene solution of 2,5-diphenyloxazol.

Experiment C. On the 10th day of growth the filtered media from the three cultures were adjusted to pH 8.0 and vigorously aerated for 2 min. By this treatment most of the 3-hydroxy-2,5-toluquinone was destroyed, but 3,4-dihydroxy-2,5-toluquinone only to a small extent. This was done because 3-hydroxy-2,5-toluquinone decomposes in the solvent system used (butanol-propanol-ammonium hydroxide), leading to an undesirably high background activity in part of the chromatograms (R_F 0.0—0.2). After acidification, the culture filtrates were extracted with ether, the ether removed and the crude extracts chromatographed on Whatman No. 1 paper strips. The radioactivity along the air-dried chromatograms was measured in a Baird-Atomic Paper Chromatogram Scanner. For quantitative analysis the strips were moistened with 1 M HCl, the different quinones eluted using ether, and the extinction of the ethereal solutions determined in a Beckman DU Spectrophotometer at the wavelength of maximum absorption (see Table 4).

RESULTS AND DISCUSSION

The ^{14}C-distribution of spinulosin isolated in experiment A is shown in Table 1. 90 % of the total radioactivity of spinulosin is present in the methoxyl carbon, strongly indicating the incorporation of 8-^{14}C-fumigatin hydroquinone as a unit.

Results of the chemical degradation of fumigatin labelled by biosynthesis from 2-^{14}C-malonate are given in Table 2. For acetate-malonate derived compounds Bu'Lock *et al.* have used the ratio

$$k_m = \frac{\text{incorporation from malonate into acetate-derived } C_2\text{-unit}}{\text{incorporation from malonate into malonate-derived } C_2\text{-unit}}$$

Table 1. ^{14}C-Distribution in spinulosin obtained by biosynthesis from 8-^{14}C-fumigatin hydroquinone (expt. A).

Material	Number of carbon atoms	Specific activity*	Total activity	Relative total activity
Spinulosin, total combustion	8	62.4	499.2	1
Tetramethylammonium iodide	4	112.1	448.4	0.90

* counts per minute and mg of $BaCO_3$.

Table 2. ^{14}C-Distribution in fumigatin obtained by biosynthesis from 2-^{14}C-malonate.

Material	Carbon atoms isolated	Number of carbon atoms	Activity* of the material	Total activity of the material
Tetramethylammonium iodide	8	4	20	80
Total combustion	all	8	1985	15 880
Kuhn-Roth oxidation, carbon dioxide	2, 3, 4, 5, 6, 8	6	2340	14 040
Kuhn-Roth oxidation, total combustion of acetic acid	1,7	2	670	1 340

* counts per minute and mg of $BaCO_3$.

as a measure of the conversion of malonate to acetate.[14] Analogously the ratios k_1 and k_2 defined as

$$k_1 = \frac{\text{incorporation from 1-}^{14}\text{C-acetate into methyl-derived carbon}}{\text{incorporation from 1-}^{14}\text{C-acetate into carboxyl-derived carbon}}$$

$$k_2 = \frac{\text{incorporation from 2-}^{14}\text{C-acetate into carboxyl-derived carbon}}{\text{incorporation from 2-}^{14}\text{C-acetate into methyl-derived carbon}}$$

may be used as a measure of the conversion of 1^{14}-C-acetate to 2-^{14}C-acetate (or more strictly the extent to which the carboxyl group of acetate is converted to the methyl group) and *vice versa*. Values of k_m ranging from 0.00 to 0.65 have been reported, and calculations on results from degradations of acetate-derived compounds generally give $k_1 = 0.00$ or occasionally up to 0.05. k_2 is of the same order as k_1, but most often slightly higher (0.00—0.10). The experimental figures obtained for fumigatin from strain A 46 [7] give $k_1 = 0.01$ and $k_2 = 0.09$.

As a measure of the incorporation of activity into C—1 and C—7 of fumigatin it is convenient to use the ratio R defined as $R = (a_1 + a_7)/(a_1 + a_2 + ... + a_7)$, where a_i stands for the activity of carbon i of fumigatin. When 2-^{14}C-acetate is used as a precursor one has $R = (1 + k_2)/(4 + 3k_2)$. Substituting the value of $k_2 = 0.09$ gives $R = 0.26$, which is also the experimentally obtained figure.[7] Similarly 1-^{14}C-acetate as precursor will give $R = (1 + k_1)/(3 + 4k_1) = 0.33$ (if $k_1 = 0.01$). The experimental determination [7] gave $R = 0.32$.

From the figures of Table 2 one obtains $R = 0.085$, significantly lower than the value $R = 0.26$ calculated for 2-^{14}C-acetate. This means that malonate labels the C_2-unit consisting of C—1 and C—7 of fumigatin to a considerably lower extent than the other C_2-units of the quinone. It can be concluded that, consistent with the acetate-malonate theory, fumigatin is formed from one molecule of acetyl-CoA (giving C—1 and C—7) and three molecules of malonyl-CoA. The value of k_m can be obtained as $k_m = 3R/(1-R) = 0.28$, assuming that $a_3 = a_5 = 0$, $a_2 = a_4 = a_6$, and $a_1 = k_2a_7 = k_2k_ma_2$.

The results of the chemical degradation of spinulosin, isolated in experiment B, are listed in Table 3. If spinulosin were labelled *via* the acetate pool, the value of R would be between 0.26 and 0.33, calculated for 2-^{14}C-acetate and 1-^{14}C-acetate, respectively. The figures of Table 3 give $R = 0.104$, significantly

Table 3. ^{14}C-Distribution in spinulosin obtained by biosynthesis from malonate-labelled fumigatin hydroquinone (expt. B).

Material	Carbon atoms isolated	Number of carbon atoms	Activity* of the material	Total activity of the material
Tetramethylammonium iodide	8	4	7	28
Total combustion	all	8	232	1856
Kuhn-Roth oxidation, carbon dioxide	2, 3, 4 5, 6, 8	6	269	1614
Kuhn-Roth oxidation, total combustion of acetic acid	1,7	2	95	190

* counts per minute and mg of $BaCO_3$.

lower than 0.26, and close to $R = 0.085$ calculated for the added ^{14}C-fumigatin hydroquinone. Thus it is proved that the latter compound can function as a precursor of spinulosin in strain A 49. The high degree of incorporation (at least 10 %) makes it unreasonable to doubt that it also is the natural intermediate in the biosynthesis of spinulosin.

In experiment C (strain A 46) no significant difference in the pattern of labelling (Fig. 2) from 1-^{14}C-acetate and 2-^{14}C-acetate could be detected when the chromatograms were scanned. 2-^{14}C-acetate gives a slightly higher specific

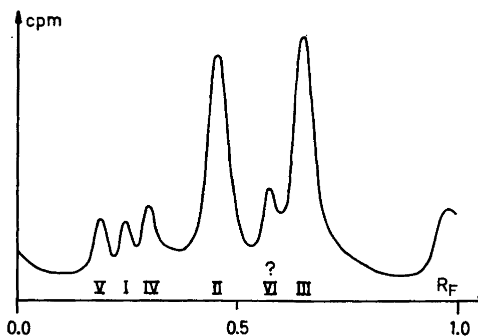

Fig. 2. Incorporation of acetate in strain A 46.
I = spinulosin
II = fumigatin
III = 3,4-dihydroxy-2,5-toluquinone
IV = 3-methoxy-4-hydroxy-2,5-toluquinone
V = 3,6-dihydroxy-2,5-toluquinone
VI = orsellinic acid

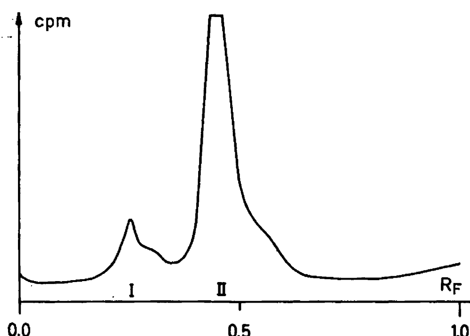

Fig. 3. Incorporation of fumigatin hydroquinone in strain A 46.
I = spinulosin
II = fumigatin

activity in all the pigments. This would be expected on theoretical grounds since the carboxyl group of the intermediate orsellinic acid is lost in the sequence of reactions leading to the different toluquinones. An entirely different pattern of labelling is, however, obtained from ^{14}C-fumigatin hydroquinone, as shown in Fig. 3. The quantitative estimation of the quinones (Table 4) showed that they were produced in approximately the same proportions in the three cultures. Thus, if spinulosin had become labelled from ^{14}C-fumigatin hydroquinone *via* the acetate-pool, 3,4-dihydroxy-2,5-toluquinone should, for instance, give a peak in Fig. 3, at least as high as that of spinulosin. As this

Table 4. Quantitative analysis of the quinones produced in expt. C.

Derivative of 2,5-toluquinone	Number in the text	Wave-length in mμ	Molecular extinction coefficient $cm^{-1}M^{-1}$	Amount in mg present in the culture* (a)	(b)	(c)
3,6-Dihydroxy-	V	282	1.5×10^4	3	2	1
3,6-Dihydroxy-4-methoxy-	I	294	5.2×10^3	4	4	6
3-Methoxy-4-hydroxy-	IV	283	4.8×10^3	8	9	10
3-Hydroxy-4-methoxy-	II	285	4.9×10^3	55	62	—
3,4-Dihydroxy-	III	279	8.0×10^3	60	57	82

* (a) 1-^{14}C-acetate added. (b) 2-^{14}C-acetate added. (c) ^{14}C-fumigatin hydroquinone added.

is not the case, the direct conversion of fumigatin hydroquinone to spinulosin in strain A 46 is proved by the experiment.

The technique used is fairly rapid and simple, and can be varied in details if needed. It requires neither laborious degradation reactions, nor specifically labelled precursors, which are often difficult or impossible to synthesize with a sufficient high specific activity. Furthermore, it seems to be of general applicability in mould studies of this kind, as a great number of moulds (*e.g.* *Penicillium patulum*,[15] *P. brevi-compactum*,[16] *P. griseofulvum*,[17] *P. islandicum*,[18] and *Paecilomyces victoriae*[19]) produce several related acetate-derived compounds at the same time. Any of these compounds could be biologically labelled from acetate, isolated, and retested in the organism to see if it represents an end-product, or if it can function as a precursor for any of the other compounds produced. Such an investigation of the remaining toluquinones from *A. fumigatus*, strain A 46, is in progress. Obviously, the technique is of no use if the compound to be tested is a precursor to all the acetate-derived secondary products of the mould (*e.g.* orsellinic acid, which is incorporated in all the pigments from strain A 46), but this seems to be the only theoretical limitation of the method.

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