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The Biosynthesis of Fumigatin

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Fumigatin produced by a strain of *Aspergillus fumigatus* in presence of (A) ^{14}C-labelled sodium acetate, (B) $^{14}CH_3$-L-methionine, and (C) 2-^{14}C-orsellinic acid was isolated and the distribution of radioactivity determined. It was shown that (A) fumigatin is biosynthesized in accordance with the acetate theory, (B) the methoxyl carbon of fumigatin is derived from the C_1-pool, and (C) orsellinic acid is an intermediate in the biosynthesis of fumigatin.

Fumigatin, 3-hydroxy-4-methoxy-2,5-toluquinone, was isolated from the metabolism solution of a certain strain of *Aspergillus fumigatus* Fresenius and structurally determined by Raistrick and Anslow [1]. The corresponding quinol was also present in the culture medium, and both were found to be metabolic products, the proportions varying according to the stage of development of the mould. Later, fumigatin has been synthesized in different ways [2-4].

Both the shikimic acid route and the acetate route can formally explain the biosynthesis of fumigatin. However, as the acetate route has been shown to be valid for the toluquinones aurantiogliocladin produced by *Roseum gliocladium* Bainer [5] and 4-methoxy-2,5-toluquinone [6] produced by *Lentinus degener* [7], the same route is the most probable one for the formation of fumigatin.

In order to prove this, *Aspergillus fumigatus* was supplied with $CH_3{}^{14}COONa$ and $^{14}CH_3COONa$, respectively, in two successive experiments (expt. A), and the radioactive fumigatin isolated from the medium was diluted with nonradioactive fumigatin and submitted to the following degradation reactions:

(*a*) Total oxidation to carbon dioxide trapped as barium carbonate, the radioactivity of which was measured.

(*b*) Kuhn-Roth oxidation to carbon dioxide and acetic acid, and a Smith degradation of the acetic acid obtained, giving the radioactivity of C-1 and C-7 of fumigatin.

(*c*) Zeisel demethylation giving methyl iodide collected as tetramethylamine iodide, which was oxidized to carbon dioxide, giving the radioactivity of C-8.

(*d*) Formation of the monoxime of fumigatin by treatment with hydroxylamine and subsequent oxidation yielding 1-methyl-2,3-dihydroxy-4-methoxy-

5-nitrobenzene. Total oxidation of the bromopicrin obtained from a hypobromite degradation of this nitro compound gave the radioactivity of C-5.

The carbon of the methoxyl groups in fungal metabolites has been shown to be generally derived from the C_1-pool. By supplying the mould with $^{14}CH_3$-L-methionine (expt. B), measuring the total radioactivity of the isolated fumigatin and the activity of C-8 as above, this was shown to be the case also for the methoxyl carbon of fumigatin.

6-Methylsalicylic acid has been claimed to be an intermediate in the biosynthesis of both 4-methoxy-2,5-toluquinone and aurantiogliocladin [6]. A more probable primary condensation product of the acetate units in the biosynthesis of fumigatin is orsellinic acid, which has been shown to be a precursor to mycophenolic acid [6].

In a preliminary experiment orsellinic acid, biologically ^{14}C-labelled from acetate, was added to a replacement culture of the mould. By developing an ether extract of the culture medium on paper chromatogram and scanning the chromatogram with a G.M.-counter, a significant incorporation of radioactivity into fumigatin was detected. The lipid fraction from the same culture, obtained by extracting the airdried mycelium with ether, evaporating the ether and washing the residue with water to remove traces of fumigatin, showed to be only negligibly labelled, strongly indicating that practically no degradation of the orsellinic acid to acetate had occurred. To prove that the fumigatin had become labelled by a direct conversion of the orsellinic acid, the experiment was repeated with orsellinic acid, labelled specifically in C-2 position.

EXPERIMENTAL

Culture conditions. Aspergillus fumigatus Fresenius, L.S.H.T.M. Cat. No. A 46, was incubated in flasks containing 500 ml of Raulin-Thom solution as described by Anslow and Raistrick [1]. After 20 – 25 days of incubation the culture medium turned dark purple-red in colour due to decreasing acidity, and was removed by suction. When used for replacement cultures, the mycelium was carefully washed with water and put on a medium containing 5 % glucose.

Isolation of fumigatin. The culture filtrate (1 litre) was vigorously aerated for 1 h to oxidize the quinol form of fumigatin, acidified with 5 ml of concentrated HCl, and extracted 4 times with 300 ml of ether. After concentration to 50 ml, the ethereal extract was slowly poured into 500 ml of boiling petroleum ether (b.p. 40° – 60°C), and the dark-brown precipitate obtained filtered off, dried over P_2O_5 for 24 h and chromatographed on a silica gel (100 – 200 mesh) column with anhydrous ether as eluent. The first dark-brown coloured fraction was collected, concentrated, and poured into 300 ml of boiling petroleum ether. After cooling, about 50 mg of fumigatin could be filtered off as an amorphous brown powder, m.p. 116°C with softening from 112°C. The isolated fumigatin gave all the reactions described by Anslow and Raistrick [1] and proved to be identical with synthetic fumigatin.

Experiments A, B, and C. Two cultures were grown for 10 days. At that time a solution containing (A) 0.5 mC of radioactive sodium acetate, (B) 0.05 mC of $^{14}CH_3$-L-methionine, and (C) about 0.02 mC of 2-^{14}C-orsellinic acid was added to each flask, and growth continued for another 15 days. Fumigatin was isolated as described above and diluted with (A) 0.8 g, (B) 0.2 g, and (C) 0.2 g of carrier fumigatin before purification on a silica gel column.

Radioactive assay. The radioactivity was determined after conversion of the material to barium carbonate. In expt. A the measurements were made on the same amount (20 mg) of barium carbonate with a Tracerlab Autoscaler in conjunction with a Tracerlab TGC-2 Geiger tube, and in expts. B and C with a Baird-Atomic Liquid Scintillation

Counter, the barium carbonate being suspended in a gel of Aerosil in a toluene solution of 2,5-diphenyloxazol.

Combustion. Fumigatin, tetramethylamine iodide, and bromopicrin were oxidized using the van Slyke-Folch method [8] and the carbon dioxide evolved was trapped in a carbonate free barium hydroxide solution.

Kuhn-Roth oxidation. The Kuhn-Roth oxidation of fumigatin (80 mg) gave in quantitative yield carbon dioxide, that was trapped in a barium hydroxide solution, and acetic acid, isolated by steam distillation and degraded in a Smith reaction. The methylamine formed was oxidized to carbon dioxide with alkaline permanganate.

Zeisel demethylation. Fumigatin (80 mg) was demethylated with hydrogen iodide ($d = 1.70$) and the methyl iodide evolved was trapped in a solution of 0.5 ml of trimethylamine in 15 ml of 70 % ethanol. The trapping solution was evaporated to dryness at 100° in vacuum, giving a crystalline residue of tetramethylamine iodide.

3-Hydroxy-4-methoxy-2,5-toluquinone-5-monoxime. A mixture of 70 mg of fumigatin, 50 ml of ethanol and 80 mg of hydroxylamine hydrochloride was heated on a steam bath for 2 h and then concentrated to 10 ml. The fumigatin monoxime was precipitated with 40 ml of ether, filtered off, washed with ether and recrystallized from water, giving 80 mg of light brown crystals, m.p. 131°C. (Found: N 7.85. Calc. N 7.75).

The oxime was insoluble in benzene and petroleum ether, sparingly soluble in water, ether and chloroform, and easily soluble in ethanol, sodium hydroxide and concentrated sulfuric acid, giving dark reddish brown solutions.

1-Methyl-2,3-dihydroxy-4-methoxy-5-nitrobenzene. A suspension of 100 mg of fumigatin monoxime in 25 ml of HNO_3 ($d = 1.2$) was allowed to stand at room temperature for 24 h. After neutralization with solid barium hydroxide it was immediately submitted to a hypobromite degradation without isolation of the nitro compound.

Hypobromite degradation. 60 ml of cold aqueous barium hypobromite (from 2.8 g of barium hydroxide and 0.4 ml of bromine in 60 ml of water) were added to the suspension of the nitro compound in water. After keeping the mixture at room temperature for 1 h, the bromopicrin was isolated by steam distillation, washed with dilute HCl and water, and oxidized to barium carbonate as above (yield 15 % from monoxime).

Table 1. ^{14}C-Distribution of fumigatin from $CH_3{}^{14}COONa$.

Material	Carbon atoms isolated	Number of carbon atoms	Radioactivity* of the material	Total radioactivity of the material	Number of COOH groups found	Number of COOH groups calculated
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Tetramethylamine iodide	8	4	0	0	0.00	0
Total combustion	all	8	454	3632	3	3
K.-R. oxidation, carbon dioxide	2, 3, 4 5, 6, 8	6	405	2430	2.01	2
K.-R. oxidation, total combustion of acetic acid	1, 7	2	583	1166	0.96	1
K.-R. oxidation, methyl group of acetic acid	7	1	10	10	0.01	0
K.-R. oxidation, carboxyl group of acetic acid	1	1	1150	1150	0.95	1
Bromopicrin	5	1	1172	1172	0.97	1

* Counts per minute and 20 mg of $BaCO_3$.

Table 2. ^{14}C-Distribution of fumigatin from $^{14}CH_3COONa$.

Material	Carbon atoms isolated	Number of carbon atoms	Radio-activity* of the material	Total radio-activity of the material	Number of COOH groups found	Number of COOH groups calculated
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Tetramethylamine iodide	8	4	22	88	0.07	0
Total combustion	all	8	674	5392	4	4
K.-R. oxidation, carbon dioxide	2, 3, 4 5, 6, 8	6	685	4110	3.05	3
K.-R. oxidation, total combustion of acetic acid	1, 7,	2	678	1356	1.01	1
K.-R. oxidation, methyl group of acetic acid	7	1	1240	1240	0.92	1
K.-R. oxidation, carboxyl group of acetic acid	1	1	98	98	0.07	0
Bromopicrin	5	1	135	135	0.10	0

* Counts per minute and 20 mg of $BaCO_3$.

Synthesis of biologically ^{14}C-labelled orsellinic acid

Penicillium baarnense was supplied with $CH_3{}^{14}COONa$ and orsellinic acid isolated from the culture medium as described by Mosbach[9].

Synthesis of 2-^{14}C-orsellinic acid. Starting with $CH_3{}^{14}COCH_2COOC_2H_5$ purchased from New England Nuclear Corporation, the synthesis was carried out as described by Mosbach [9].

RESULTS AND DISCUSSION

Experiment A. The results from the experiment with $CH_3{}^{14}COONa$ are listed in Table 1. The figures of column (7) in Table 1 are calculated from the pattern of labelling according to the acetate theory as indicated in Fig. 1. As the value of the total radioactivity of the total combustion of fumigatin is least

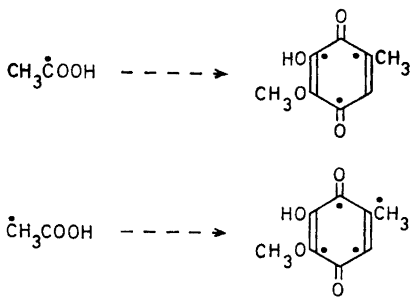

Fig. 1. ^{14}C-Distribution of fumigatin from labelled acetate according to the acetate theory.

Table 3. ^{14}C-Distribution of fumigatin from $^{14}CH_3$-L-methionine.

Material	Number of carbon atoms	Specific activity*	Total radio-activity	Relative total radio-activity
Total combustion	8	745	5960	1
Tetramethylamine iodide	4	1530	6120	1.03

* Counts per minute and mg $BaCO_3$.

subjected to experimental errors, it is put equal to the theoretical value 3, and the other figures of column (6) have been calculated in relation to this value. The results from the experiment with $^{14}CH_3COONa$ are analogously given in Table 2. As seen from the tables, the experimental figures are in good agreement with the theoretical ones, clearly indicating the acetate origin of fumigatin.

$^{14}CH_3COONa$ entering the citric acid cycle will give rise to oxaloacetic acid and malic acid, labelled in all four carbon atoms. Decarboxylation of one of these, and oxidative decarboxylation of the pyruvic acid obtained, will give acetic acid labelled in both the carbon atoms. This route could explain why, as seen from Table 2, the radioactivity from $^{14}CH_3COONa$ is incorporated to a slight, but significant, extent into the carboxyl derived carbons 1, 3, and 5 of fumigatin. Taken into account that the labelling of the C_1-pool is higher with $^{14}CH_3COONa$ than with $CH_3{}^{14}COONa$, it is clear that the latter is more fitted for proving the acetate origin, a finding which is valid also for other fungal metabolites, for instance the anthraquinones [10].

Experiment B. Table 3 shows the results of the degradation reactions. All the radioactivity incorporated into fumigatin from $^{14}CH_3$-L-methionine was

Table 4. ^{14}C-Distribution of fumigatin from 2-^{14}C-orsellinic acid.

Material	Carbon atoms isolated	Number of carbon atoms	Specific activity*	Total radio-activity	Relative total radio-activity
Tetramethylamine iodide	8	4	170	680	0.02
Total combustion	all	8	3 885	31 080	1
K.-R. oxidation, carbon dioxide	2, 3, 4 5, 6, 8	6	4 770	28 620	0.92
K.-R. oxidation, total combustion of acetic acid	1,7	2	595	1 190	0.04
Bromopicrin	5	1	26 530	26 530	0.85

* Counts per minute and g $BaCO_3$.

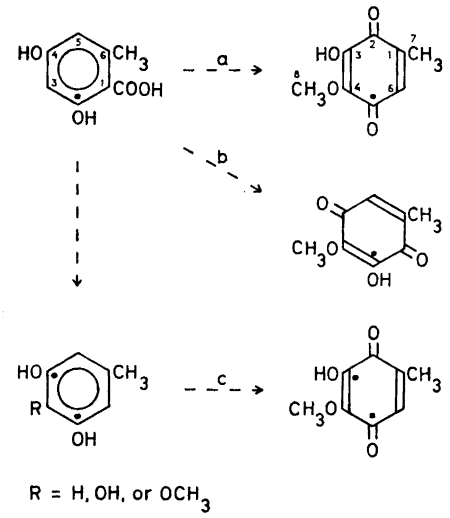

Fig. 2. The three theoretically possible ways of formation of the quinonoid structure of fumigatin.

refound in the methoxyl carbon, which together with the high degree of incorporation (6 %) proves that the methoxyl carbon of fumigatin is derived from the C_1-pool.

Experiment C. The results are given in Table 4. As the acetic acid from the Kuhn-Roth oxidation is almost free from radioactivity, and as 85 % of the total radioactivity is refound in position 5 of fumigatin, it is clear that the radioactivity of fumigatin has been incorporated by a direct conversion of the added orsellinic acid, which means that orsellinic acid can function as a precursor to fumigatin.

It has been shown [11] that orsellinic acid produced by *Penicillium baarnense* is formed in accordance with the malonate theory, an extension of the acetate theory. If orsellinic acid is the natural intermediate for the formation of fumigatin in *Aspergillus fumigatus*, it should presumably be synthesized in a similar manner and the resulting fumigatin therefore derived from three molecules of malonyl-CoA and one molecule of acetyl-CoA, where C-1 and C-7 of fumigatin are derived from acetyl-CoA.

The mode of formation of the quinonoid structure of fumigatin is of interest. Carbon 5 of fumigatin could theoretically be derived in three different ways from orsellinic acid (Fig. 2):

(*a*) From carbon 2 by hydroxylation at position 5 while the molecule is still held asymmetrical by the carboxyl group in position 1.

(*b*) From carbon 4 by an oxidative decarboxylation at position 1, analogous to the oxidation of salicylic acid to catechol.

(*c*) From the carbons 2 and 4 by hydroxylation at positions 1 and 5 when the molecule is symmetrical after decarboxylation.

In experiment C alternative (*a*) would give fumigatin labelled only in C-5, alternative (*b*) only in C-3. Alternative (*c*) would give fumigatin equally labelled in C-5 and C-3. As seen from Table 4, as much as 85 % of the total

radioactivity of fumigatin is refound in C-5, which is only consistent with alternative (*a*).

During the course of this work, the mould was found to produce other quinones than fumigatin. The relation of these to the biosynthesis of fumigatin is under investigation.

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