Studies on the Biosynthesis of Quinones in Fungi

INCORPORATION OF 6-METHYLSALICYLIC ACID INTO FUMIGATIN AND RELATED COMPOUNDS IN *ASPERGILLUS FUMIGATUS* I.M.I. 89353

By N. M. PACKTER

Department of Biochemistry, University of Leeds

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1. Orsellinic acid has been detected as a metabolite of *Aspergillus fumigatus*. 2. The other principal aromatic components of the medium are fumigatin and the quinol, fumigatol. Fumigatol has been shown to be dihydrofumigatin after oxidation to the quinone followed by acetylation. 3. ^{14}C -labelled 6-methylsalicylic acid can be hydroxylated in *A. fumigatus* to form orsellinic acid and decarboxylated to give *m*-cresol. 4. ^{14}C -labelled 6-methylsalicylic acid is incorporated into fumigatin and fumigatol (1.0–1.5%), but the conversion does not occur until about 2–3 days after supplementation of the medium. At this stage of growth, the organism has already synthesized approx. 20 times as much fumigatol as fumigatin and this ratio is reflected in the much lower specific activity of the quinol. 5. Supplementation of the medium with either orsellinic acid or orcinol, in addition to ^{14}C -labelled 6-methylsalicylic acid, greatly decreases the latter's incorporation into fumigatin. At the same time, the cultures containing these substances are stimulated to produce another quinone with relatively high specific activity. 6. 6-Methylsalicylic acid has not been detected in the medium of normal cultures. The results indicate that 6-methylsalicylic acid itself is not a direct precursor of fumigatin and fumigatol but that it is converted into a true intermediate, probably after hydroxylation to orsellinic acid. 7. Supplementation of the medium with 6-methylsalicylic acid (15–25 mg./200 ml.) greatly affects the metabolism of *A. fumigatus*. Growth is inhibited and the synthesis of fumigatol is markedly depressed in these cultures. The inhibitory effects may possibly be related in some way to the production of *m*-cresol.

It has been established by degradative studies that many phenolic substances, secreted by fungi into the culture medium, are derived through condensation of C_2 units (Birch, Massy-Westropp & Moye, 1955; Birch, 1957; Birkinshaw & Gowlan, 1962). More recent work has shown that many of these substances, e.g. 6-methylsalicylic acid (I) and orsellinic acid (II), are in fact synthesized from 1 mol. of acetyl-CoA plus a number of malonyl-CoA units (Bu'Lock, Smalley & Smith, 1962; Mosbach, 1961). Indeed, it is probable that all phenolic compounds formed directly from acetate arise in this manner. Further, Birkinshaw & Gowlan (1962) have demonstrated that sodium [^{14}C]-butyrate (and hence acetoacetate) is also incorporated into orsellinic acid in *Penicillium madriti*. However, their results indicate that the butyrate is initially metabolized to 2 mol. of acetyl-CoA and that the C_4 acid is not directly utilized.

Methoxyphenol derivatives have also been demonstrated as metabolites of fungi. Birch,

Fryer & Smith (1958) and Birch (1961) have shown that aurantiogliocladin (3,4-dimethoxy-6-methyl-2,5-toluquinone), which is produced by the mould *Gliocladium roseum*, and 4-methoxytoluquinone (secreted into the medium by *Lentinus degener*) are also synthesized from acetate. In addition, they have established that the 1-methyl substituent attached to the ring is actually derived from the methyl group of acetate. The structure of the phenolic acids (I) and (II) is such that they might possibly be intermediates in the biosynthesis of these toluquinones. Hydroxylation followed by decarboxylation and oxidation would give rise to the basic toluquinone structure.

Another toluquinone, fumigatin (3-hydroxy-4-methoxy-2,5-toluquinone; III), has been isolated from the medium of a strain of *Aspergillus fumigatus* (L.S.H.T.M. A 46) by Anslow & Raistrick (1938a). It has already been established that both fumigatin and its dihydro derivative, fumigatol (IV), are synthesized from [^{14}C]acetate in excellent yield

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(I) R = H
(II) R = OH

(III)

(IV)

(Packter & Glover, 1965a). Further, the amount of fumigatol secreted by the organism is much greater than that of the quinone. As experience had already been gained with *A. fumigatus* in the above studies and others (Packter & Glover, 1962, 1965a,b), it was decided to test the precursor activity of 6-methylsalicylic acid for the synthesis of fumigatin and fumigatol. Preliminary results on some of this work have already been reported (Packter & Steward, 1965).

EXPERIMENTAL

Microbiological methods

Organisms. The fungi used during this work were obtained from the Commonwealth Mycological Institute (Kew, Surrey) and were: *A. fumigatus* (L.S.H.T.M. A 46; I.M.I. 89353), *Penicillium griseofulvum* (I.M.I. 75832) and *Penicillium barnense* (I.M.I. 40590). Stock cultures of these organisms were maintained on potato-dextrose agar (Difco) at 4° and subcultured every 4–6 weeks. *A. fumigatus* was incubated in Roux bottles containing 200 ml. of Raulin-Thom medium at 28° for 7–8 days. *P. griseofulvum* and *P. barnense* were grown in flasks containing 500 ml. of Czapek-Dox medium at 28° for 12–14 days. All the media were sterilized in an autoclave at 15 lb./in.² for 15 min. before inoculation with a spore suspension.

Chromatographic methods

Column chromatography. All the metabolites isolated from the various culture media were initially purified by chromatography on columns of silicic acid (Mallinckrodt A.R.; 100 mesh)–Celite 535 (Johns-Manville Ltd.) (2:1, w/w). The ether-extractable material (approx. 200 mg.) was chromatographed on columns 10 cm. × 3 cm. The unsaponifiable lipids obtained from the mycelium of *A. fumigatus* was chromatographed on acid-washed alumina (Woelm; from L. Light and Co., Colnbrook, Bucks.) (Mervyn & Morton, 1959).

Thin-layer chromatography. Fractions containing phenolic substances were resolved by chromatography on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) held on glass plates (20 cm. × 20 cm.). The solvent system benzene-methanol-acetic acid (10:2:1, by vol.) was used. Plates were activated before use for 1 hr. at 110° and equilibrated with the solvent for 1 hr. before development. Phenolic compounds were detected as described by Curtis, Harries, Hassall & Levi (1964).

Analytical methods

Spectrophotometry. Ultraviolet-absorption measurements were carried out in cyclohexane ('special for spectroscopy'; British Drug Houses Ltd., Poole, Dorset) or ethanol in a Unicam SP.500 spectrophotometer. All the substances isolated were characterized and their $E_{1\text{cm}}^{1\%}$ values determined. Assays were based on amounts measured spectrophotometrically and by weight (in most instances). Infra-red-absorption spectra were determined in a Unicam SP.200 spectrophotometer as a Nujol mull or as a thin film between NaCl cells.

Assay of radioactivity. Radioactivity was measured by counting portions of the various fractions on nickel planchets in a Nuclear-Chicago Corp. gas-flow counter (25% efficiency). Equal amounts (usually 0.2 ml.) were estimated spectrophotometrically. All specific activities were corrected for background and to infinite thinness and are quoted as counts/min./mg.

Melting points. These were measured in a Gallenkamp melting-point apparatus and are uncorrected.

Isolation and identification of metabolites in the medium

Fumigatin (III). This substance was isolated from the culture medium of *A. fumigatus* and purified as described by Packter & Glover (1962, 1965a) (see also Scheme 1). The medium was always extracted with ether immediately after removal from the incubator to minimize oxidation of fumigatol. Most of the quinone found in the ether extract must therefore have been present in the medium originally (Table 2). Great care must be taken when handling fumigatin as this quinone readily decomposes and volatilizes at above 30°. Fumigatin obtained after chromatography and three crystallizations from cyclohexane gave m.p. 113–114°, which agrees with that (114°) determined by Anslow & Raistrick (1938a) and that (112°) obtained after a chemical synthesis by Seshadri & Venkatasubramaniang (1959) (Found: C, 56.6; H, 4.9. Calc. for C₈H₆O₄: C, 57.1; H, 4.8%). The acetyl derivative of fumigatin was prepared as described by Baker & Raistrick (1941) except that it was purified by chromatography on Kieselgel G (R_f 0.63) before crystallization from light petroleum. The pure substance had m.p. 97° and showed λ_{max} in cyclohexane 262 and 380 m μ ($E_{1\text{cm}}^{1\%}$ 662 and 50 respectively).

It has been demonstrated (Craven, 1931) that benzoquinones with at least one unsubstituted position in the ring yield, with ethyl cyanoacetate, a blue colour that gradually changes through green to red. Ethyl cyanoacetate (0.1 ml.) followed by excess (3 ml.) of ethanolic NH₃ [ethanol-aq. NH₃ (sp.gr. 0.88) (1:1, v/v)] was added to 0.2 mg. of fumigatin and a number of other quinones that were

Scheme 1. Procedure for the isolation of various metabolites from the ether-extractable material of the medium of *A. fumigatus*. Experimental details are described in the text.

Table 1. *Colour of products after reaction of various quinones with ethyl cyanoacetate*

Experimental details are given in the text.

Substance	Colour produced immediately	Colour produced after 1 min.
Benzoquinone	Blue	Red
Toluquinone	Blue	Blue
4-Methoxytoluquinone*	Blue	Blue
Ubiquinone-0 (3,4-dimethoxy-toluquinone)*	Blue	Blue
Fumigatin	Purple	Yellow-green
Compound 'Q'	Blue	Yellow-green
Acetylfumigatin	Red	Red
4-Hydroxytoluquinone*	Red	Red
2-Methylnaphthaquinone	Blue	Blue
Aurantiochlorin (3,4-dimethoxy-6-methyltoluquinone)	Pale yellow	Pale blue

* Packter & Glover (1965b).

available and the colours produced are reported in Table 1. Most of the quinones tested gave an immediate blue colour, but aurantiochlorin (which is fully substituted) initially gave a negative result. Within 1 min., however, a pale-blue colour appeared that was presumably formed after partial hydrolysis of this quinone under the alkaline conditions. Characteristic colour reactions were also obtained when one of the substituents in the ring was a hydroxyl group.

Attempts to purify the ^{14}C -labelled fumigatin on thin-layer plates of Kieselgel G were unsuccessful, as this quinone decomposed during the chromatography. In most cases, however, the purified fumigatin (m.p. 110°) from the incorporation experiments was acetylated. The acetylfumigatin thus produced had the same specific activity as the original fumigatin (when corrected for the difference in mol. wt.), confirming that this substance was indeed radioactive and that the crystalline fumigatin was radiochemically pure.

Fumigatol (IV). This quinol was obtained as described by Packter & Glover (1962, 1965a) (see also Scheme 1). The pure material had m.p. 107° . The crystals could be stored at 4° for at least 6 months without deterioration or lowering of m.p. [Anslo & Raistrick (1938a) had reported m.p. 101°] (Found: C, 56.3; H, 6.0. Calc. for $\text{C}_8\text{H}_{10}\text{O}_4$: C, 56.5; H, 5.8%). Fumigatol was further characterized by oxidation. Fumigatol (50 mg.) was dissolved in 5 ml. of ethanol and left at room temperature for 18–20 hr. The solution gradually became dark red over this period. Ether was then added and the total was evaporated to dryness under N_2 . The red oil was chromatographed on a column of silicic acid–Celite and fumigatin was isolated and purified as shown in Scheme 1. The yield was low (approx. 15%), as fumigatin is unstable in ethanol. The resulting quinone had m.p. $113\text{--}114^\circ$ and exhibited ultraviolet and infrared spectra identical with those of authentic fumigatin. The acetyl derivative had m.p. 97° . The mixed m.p. with acetylfumigatin (m.p. 97°) prepared from fumigatin itself was 97° .

The infrared spectra of fumigatin (III), fumigatol (IV) and acetylfumigatin have not been recorded previously and are shown in Fig. 1. Fumigatin showed the following characteristic absorption bands: 3400 cm^{-1} (OH), 1670 , 1645 , 1640 , 1620 cm^{-1} (quinone) and 890 cm^{-1} (one free H attached to the ring). Similarly, acetylfumigatin had bands at 1680 , 1665 , 1620 cm^{-1} (quinone), 870 cm^{-1} (one free H) and, in addition, 1775 cm^{-1} (CO). However, absorption in the region of 3400 cm^{-1} was absent. Fumigatol showed strong bands at 3400 cm^{-1} (OH) and 900 cm^{-1} (one free H) (Bellamy, 1954a).

6-Methylsalicylic acid (I). Procedures for the isolation of 6-methylsalicylic acid from *P. griseofulvum* (Birch *et al.* 1955) and *P. urticae* (Tanenbaum & Bassett, 1958) have already been reported. However, pure 6-methylsalicylic acid could be much more readily obtained if the total ether extract of the medium were chromatographed initially. Thus the medium was acidified to pH 2 and extracted three times with ether. The resulting extracts were pooled, dried over anhydrous Na_2SO_4 and evaporated to dryness to give a pale-yellow solid. This was dissolved in a minimum volume of ether–light petroleum (1:1, v/v) and chromatographed on silicic acid–Celite. Virtually all the 6-methylsalicylic acid present (about 60% of the total ether-extractable material) was eluted with ether–light petroleum (1:9, v/v) to give an off-white crystalline solid, m.p. 167° . Pure 6-methylsalicylic acid was obtained from this material after three or four crystallizations from chloroform at room temperature. It gave m.p. 172° and λ_{max} in ethanol 243 and $311\text{ m}\mu$ ($\epsilon_{1\text{cm}}^{1\%}$ 478 and 265 respectively) (Found: C, 63.3; H, 5.3. Calc. for $\text{C}_8\text{H}_8\text{O}_3$: C, 63.2; H, 5.3%).

Other metabolites were eluted with greater concentrations of ether–light petroleum and appeared to be identical with gentisyl alcohol, gentisic acid and patulin. These substances have all been found previously in *P. urticae* (Birkinshaw, Bracken & Raistrick, 1943; Tanenbaum & Bassett, 1959) and are probably derived from 6-methylsalicylic acid in this organism (Tanenbaum & Bassett, 1959; Gatenbeck & Lönnroth, 1962).

^{14}C -labelled 6-methylsalicylic acid was prepared in a similar manner. Sodium [$1\text{-}^{14}\text{C}$]acetate (The Radiochemical Centre, Amersham, Bucks.) was added to the culture medium of *P. griseofulvum* after 7 days' growth and incubation was continued for a further 5 days. Pure 6-methylsalicylic acid was isolated as above and had m.p. 172° . The mother liquor from the final crystallization exhibited the same m.p. and specific activity as the crystals. Only one zone could be detected on thin-layer chromatograms. 6-Methylsalicylic acid (I) prepared in this way had equal amounts of radioactivity distributed among C-2, C-4, C-6 and C-8 respectively. One-quarter of the total radioactivity therefore resides in the carboxyl carbon atom (Birch *et al.* 1955).

When 6-methylsalicylic acid was isolated from the medium of *A. fumigatus*, the original chromatographic fraction (Scheme 1) was run on thin-layer plates of Kieselgel G. The purified acid was eluted from the plate with ether before dilution with carrier material and subsequent crystallization.

Orsellinic acid (II). As with 6-methylsalicylic acid, pure orsellinic acid could be readily obtained after crystallization of a chromatographic fraction. Accordingly, the culture medium of *P. barnense* (Gatenbeck & Mosbach, 1959; Mosbach, 1960) was acidified to pH 2 and extracted three

Fig. 1. Infrared-absorption spectra of (a) fumigatin (III), (b) acetylfumigatin, (c) fumigatol (IV) (3-hydroxy-4-methoxytoluquinol) and (d) purple crystalline substance (compound 'Q') isolated from cultures of *A. fumigatus* when incubated with medium supplemented with 6-methylsalicylic acid and either orsellinic acid or orcinol (see the text). Spectra (a), (b) and (d) were taken as a mull in Nujol; (c) was determined as a thin film.

times with ether. The pooled ether extracts were evaporated to dryness and chromatographed on silicic acid-Celite. A fraction containing penicillic acid was eluted with ether-light petroleum (1:9, v/v), and practically all the orsellinic acid was eluted with ether-light petroleum (3:7, v/v). Cyclohexane-soluble material was removed from this, and the resulting pale-yellow solid was nearly pure orsellinic acid, m.p. 168° and $\lambda_{\max}$, 262 m μ ($E_{1\%}^{1\text{cm}}$, 680). This material was crystallized four times from chloroform to give white crystals, m.p. 182°. Birkinshaw & Gowland (1962) found m.p. 174–176° and Gatenbeck & Mosbach (1959) quoted 176°. It showed $\lambda_{\max}$ in ethanol 262 and 300 m μ ($E_{1\%}^{1\text{cm}}$, 775 and 275 respectively). The solid, however, rapidly gained 1 mol. of water of crystallization and analysed as the monohydrate (Found: C, 51.7; H, 5.4; loss at 100°, 9.4. Calc. for $\text{C}_8\text{H}_8\text{O}_4 \cdot \text{H}_2\text{O}$: C, 51.6; H, 5.4; H_2O , 9.7%).

None of the metabolites eluted with greater concentrations of ether-light petroleum showed any selective absorption in the ultraviolet region and they were not examined further.

When orsellinic acid was isolated from *A. fumigatus*, it was resolved from other components by thin-layer chromatography before dilution with carrier material and subsequent crystallization (Scheme 1).

When *A. fumigatus* was incubated with Raulin-Thom medium the ether-extractable material contained small amounts of orsellinic acid (Table 6). This was characterized by R_F values, colour reactions with FeCl_3 (purple) and the diazonium reagent and by its ultraviolet and infrared spectra, all of which were identical with those of authentic orsellinic acid produced by *P. barnense*. In addition, recent experiments have shown that ^{14}C -labelled orsellinic acid is synthesized from ^{14}C acetate in *A. fumigatus* (N. M. Packter, unpublished work).

m-Cresol. The ether-light petroleum (1:9, v/v) fraction often smelled strongly of cresol. This material was dissolved in cold light petroleum and the soluble fraction exhibited ultraviolet and infrared spectra identical with those of *m*-cresol. Moreover, the R_F value on thin-layer chromatography and colour reaction with the diazonium reagent confirmed the identity of this isomer. The product, after acetylation, was purified by thin-layer chromatography and its infrared spectrum corresponded exactly to that of authentic *m*-cresyl acetate.

m-Cresol was estimated spectrophotometrically. It had $\lambda_{\max}$ in ethanol 275 m μ and an inflexion at 280 m μ ($E_{1\%}^{1\text{cm}}$, 154 and 138 respectively).

The infrared spectra of 6-methylsalicylic acid and orsellinic acid were recorded. They both had bands at about 3400 cm^{-1} (OH-bonded CO_2H), 2560 cm^{-1} (OH from CO_2H stretching), 1640 cm^{-1} (aromatic CO) and 900 cm^{-1} (OH from CO_2H deformation). The position of the 1640 cm^{-1} band is due to internal hydrogen-bonding between the *o*-hydroxyl and carboxyl groups (Bellamy, 1954b).

Isolation and identification of metabolites in the mycelium

Ubiquinone. 6-Methylsalicylic acid might also be effective as a precursor of the toluquinone portion of ubiquinone. Total lipid, unsaponifiable matter and ubiquinone were isolated from *A. fumigatus* as previously described, except that the lipid extract was not washed with water (Packter & Glover, 1962, 1965a). The ubiquinone was further purified

by thin-layer chromatography, with the solvent system methanol-benzene (1:99, v/v). It had R_F 0.65. It was diluted with carrier ubiquinone-50 and crystallized three times from ethanol to give yellow crystals, m.p. 48°.

Ergosterol. It was desirable to determine whether the radioactivity in ubiquinone was present in the aromatic or isoprenoid portion of the molecule. As only a small amount of ubiquinone with low radioactivity was available, this was tested by checking whether any radioactivity had also been incorporated into ergosterol.

The unsaponifiable fraction was dissolved in a minimum volume of light petroleum and crystallized overnight at -20°. The insoluble portion was removed by centrifugation and consisted mainly of ergosterol. This was chromatographed on magnesia ('for adsorption chromatographic analysis'; British Drug Houses Ltd.)-Celite (2:1, w/w). The column was first washed with ether-light petroleum (1:4, v/v), and ergosterol was eluted with ether-light petroleum (1:1, v/v). Pure material (m.p. 160-162°) was obtained from this fraction after repeated crystallizations from cyclohexane at room temperature.

Oxidation of fumigatol in aqueous solution

A solution of fumigatol in ethanol is fairly readily oxidized and this reaction has been the basis of one of the stages in its purification (Scheme 1). A similar conversion might also occur in aqueous solution. Consequently, some or possibly all the fumigatin might have been derived from fumigatol after it had been secreted into the medium. To test this possibility, fumigatol was dissolved in autoclaved medium (70 mg./200 ml.) and the solution was extracted with ether after given time-intervals. The subsequent extract was chromatographed, and fumigatin and fumigatol contents were estimated. The results expressed in Table 2 demonstrate that fumigatol in aqueous solution was oxidized to a significant extent, even over 30 min. However, the amount of fumigatin produced in this time would account for less than one-third of the content of this metabolite as usually estimated (Tables 3, 4 and 6). When fumigatol was left in solution for a long period (72 hr.), only trace amounts of fumigatin could be detected. Most of the quinone that had been formed must have decomposed. About one-third of the fumigatol had been converted into more polar substances that were insoluble in ether.

Table 2. *Oxidation of fumigatol in aqueous solution*

Fumigatol (70 mg.) was dissolved in 200 ml. of autoclaved medium in 500 ml. conical flasks and incubated at 28° for various time-intervals. The actual time required to extract the medium was 10-15 min. in each case.

Time of incubation (hr.)	Wt. of ether extract (mg.)	Wt. of fumigatol (mg.)	Wt. of fumigatol (mg.)	Ratio of fumigatin to fumigatol
0.2	68	0.6	61.6	1:103
0.5	67	1.4	50.7	1:36
1	57	2.1	40.1	1:19
20	50	5.0	25.5	1:5
24	49	4.7	20.1	1:4
72	45	0.5	12.9	1:26

No oxidation or decomposition of fumigatol occurred during chromatography on silicic acid-Celite.

Packter & Glover (1962) have reported that some fumigatin present in the medium is attached to a polypeptide. It has been established that quinones that contain unsubstituted positions in the ring readily react with thiol groups of various substances to form addition compounds (Redfearn & Whittaker, 1962). We have confirmed this by showing that an aqueous solution of fumigatin reacts with L-cysteine to give an addition product whose ultraviolet spectrum closely resembles that of fumigatol with $\lambda_{\max}$ in 0.01N-HCl 287 m μ ($E_{1\text{cm}}^{1\%}$ 195) (Fig. 2). Possibly, then, fumigatin is normally stabilized in the medium by combining with this polypeptide through a thiol group.

Purification of solvents

Ether. Ether was distilled over reduced iron immediately before use.

Light petroleum (b.p. 40-60°). This solvent was dried over Na wire and distilled before use. The fraction boiling in the range 40-45° was collected.

Ethanol. Spectroscopically pure ethanol was prepared by refluxing 2 l. of ethanol with 40 g. of KOH and 80 g. of Zn powder for 4 hr. The fraction boiling at 78° was then collected.

RESULTS

Incorporation of ^{14}C -labelled 6-methylsalicylic acid into fumigatin and fumigatol in A. fumigatus. In the first experiment, 17 mg. of ^{14}C -labelled

Fig. 2. Effect of L-cysteine on the ultraviolet spectrum of fumigatin. $\circ$, Spectrum of fumigatin in 0.01N-HCl (0.001%); 5 mg. of L-cysteine was added, and the spectrum again measured after 15 min. ($\blacktriangle$), after 30 min. ($\square$) and after 90 min. ($\bullet$).

6-methylsalicylic acid was added to each of three Roux bottles after 3 days' growth and incubation proceeded for a further 5 days. The ether-extractable material possessed some 10% of the initial radioactivity. The aqueous medium after extraction contained less than 5% of the original radioactivity. Fumigatin was isolated and the purified material (Scheme 1) found to be considerably active. Fumigatol, however, was far less radioactive. Moreover, the amount of fumigatol produced was much less than that in normal cultures (Packter & Glover, 1962, 1965a). Some orsellinic acid was present and this was purified to constant specific activity. *m*-Cresol was also produced and was labelled. Finally, a small amount of residual 6-methylsalicylic acid was recovered from the medium and this accounted for one-quarter of the radioactivity in the ether extract. Its specific activity was identical with that of the original acid.

It was possible that the low radioactivity of the fumigatol might have been caused by an inhibition of synthesis of this substance when 6-methylsalicylic acid (or some metabolite derived from it) was present. Supplementation of the medium with increasing amounts of 6-methylsalicylic acid (Table 6) showed that only a small decrease in growth and production of fumigatol occurred at a concentration of 5 mg./200 ml. Accordingly, in later experiments, 5 mg. of ^{14}C -labelled substrate was used to supplement the medium.

The speed with which 6-methylsalicylic acid was incorporated into fumigatin, fumigatol and orsellinic acid was determined (Table 3). At 5 days' growth (i.e. 2 days after supplementation of the medium), neither fumigatin nor fumigatol was appreciably labelled. Orsellinic acid, however, was radioactive. Fumigatin from group 2 was fairly radioactive but still considerably less so than orsellinic acid. Fumigatol possessed some small but definite radioactivity. Finally, after a total of 9 days' incubation, the radioactivity of fumigatin had again increased. In addition, fumigatol was definitely labelled. Incorporation into fumigatin and fumigatol was 0.6 and 0.8% respectively. The concentration of orsellinic acid in the ether extract of group 3 was considerably lower than in the other two groups and insufficient was present to permit purification.

Some residual 6-methylsalicylic acid was recovered from each of the three groups but the specific activity in each case was practically equal to the initial specific activity.

The maximum content of fumigatin and fumigatol occurred after about 6-7 days' incubation. The weight of the total ether-extractable material, however, increased steadily over the 9-day period. Thus some products were still being synthesized and secreted into the medium.

Table 3. Incorporation of ^{14}C -labelled 6-methylsalicylic acid into orsellinic acid, fumigatin and fumigatol by *A. fumigatus* at different time-intervals

6-Methylsalicylic acid (5 mg./200 ml.; specific activity 27 000 counts/min./mg.) was added to each of nine Roux bottles after 3 days' incubation. Each group consisted of three Roux bottles and values are expressed as totals. The specific activity of 6-methylsalicylic acid and orsellinic acid has been corrected for the addition of carrier material.

Group	Total incubation time (days)	Final pH of medium	Ether extract (mg.)	Total radio-activity in ether extract (counts/min.)	Sp. activity of ether extract (counts/min./mg.)	6-Methylsalicylic acid (mg.)	Sp. activity of 6-methylsalicylic acid (counts/min./mg.)	Orsellinic acid (mg.)	Sp. activity of orsellinic acid (counts/min./mg.)	Fumigatin (mg.)	Sp. activity of fumigatin (counts/min./mg.)	Fumigatol (mg.)	Sp. activity of fumigatol (counts/min./mg.)
1	5	2.3	171	94 050	550	0.9	27 150	0.9	135	6.5	5	79	1
2	7	2.5	225	91 125	405	0.6	28 900	0.8	410	6.8	180	118	8
3	9	4.1	260	85 280	328	0.5	25 450	0.2	—	6.0	415	104	31

Table 4. *Effect of orsellinic acid and orcinol on the incorporation of ^{14}C -labelled 6-methylsalicylic acid into various metabolites secreted into the culture medium by A. fumigatus*

Group	Final pH of medium	Ether extract (mg.)	Total radio-activity in ether extract (counts/min.)	Sp. activity of ether extract (counts/min./mg.)	Residual 6-methyl-salicylic acid (mg.)	Fumi-gatin (mg.)	Sp. activity of fumigatin (counts/min./mg.)	Fumi-gatol (mg.)	Sp. activity of fumigatol (counts/min./mg.)	Com-pound 'Q' (mg.)	Sp. activity of compound 'Q' (counts/min./mg.)	Orsellinic acid (mg.)	Sp. activity of orsellinic acid (counts/min./mg.)
1	2.4	367	163 600	446	0.8	10.8	340	169	1	—	—	1.3	435
2	2.4	325	141 200	465	0.6	8.6	95	147	3	2	3400*	1.3	210
3	2.5	392	155 200	396	0.8	9.5	12	203	1	3	4400*	1.5	405

* The specific activity of dihydro-compound 'Q' isolated from groups 2 and 3 was 135 and 145 respectively.

Orsellinic acid and orcinol (both 20 mg./200 ml.) were added to each of four Roux bottles in groups 2 and 3 respectively after 3 days' growth. 6-Methylsalicylic acid (5 mg./200 ml.; specific activity 27 000 counts/min./mg.) was added to each bottle 4 hr. later. Incubation proceeded for a further 5 days. All values are expressed as totals/four Roux bottles. The specific activity of orsellinic acid has been corrected for the addition of carrier material.

Effect of orsellinic acid and orcinol on the incorporation of ^{14}C -labelled 6-methylsalicylic acid into fumigatin. The structure of orsellinic acid and the comparative specific activities determined (Table 3) were compatible with the former (II) being an intermediate in the synthesis of fumigatin from 6-methylsalicylic acid. Again, if orsellinic acid is indeed a precursor, the next stage in a possible conversion into fumigatin might be decarboxylation to orcinol. Both these substances were tested. Growth of the mycelia was similar in all the three groups and this was confirmed by the fact that the final pH of the media was the same and that the ether-soluble material, fumigatin, fumigatol and orsellinic acid were all synthesized to much the same extent (Table 4).

The results indicated that orsellinic acid and orcinol had diluted out the radioactivity incorporated into fumigatin. However, another quinone (compound 'Q', see below) was isolated from the ether extracts of groups 2 and 3 and it had a very high specific activity. This compound was eluted from the column immediately after fumigatin and was initially purified by rechromatography (Scheme 1).

The fumigatol fraction was rechromatographed, crystallized from benzene and oxidized (see the Experimental section). No radioactivity was present in the fumigatol of any of the groups in this experiment. Some compound 'Q' was obtained from the oxidation products of the fumigatol fraction in groups 2 and 3 respectively. It possessed appreciable radioactivity but its specific activity was considerably lower than that of the quinone isolated as such from the ether extract (Table 4).

Although the specific activities of the fumigatin in the control and orcinol-supplemented groups (1 and 3 respectively) were very different, the specific activities of the orsellinic acid in these two groups were nearly equal. The specific activity of the orsellinic acid in group 2, however, was only half this value. The same amount of this acid (1 mg.) was present in the ether extract of the medium of the three groups. The content of endogenous orsellinic acid, from the relative specific activities, was about 70 mg.

Small amounts of *m*-cresol (1.0–1.5 mg./four Roux bottles) were found in the ether–light petroleum (1:9, v/v) fractions of the three groups, but insufficient was present to allow purification and estimation of specific activity.

Preliminary identification of compound 'Q'. A purple solid was obtained from the chromatography of the ether extracts of the orsellinic acid and orcinol-supplemented groups. This was rechromatographed and crystallized twice from benzene–cyclohexane (1:1, v/v) (Scheme 1) to yield deep-purple crystals with m.p. 153–155° and λ_{max} in

ethanol 262 and 475 m μ ($E_{1\text{cm}}^{1\%}$ 710 and 42 respectively) [cf. fumigatin with λ_{max} in ethanol 263 and 440 m μ ($E_{1\text{cm}}^{1\%}$ 730 and 55 respectively)]. The second absorption maximum with a low extinction coefficient is a typical feature of the ultraviolet spectra of quinones. The infrared spectrum was also very similar to that given by fumigatin (Fig. 1) and showed the following strong absorption bands: 3425 cm.⁻¹ (OH), 1635 cm.⁻¹ (quinone) and 880 cm.⁻¹ (one unsubstituted position in the ring). The presence of these groups was confirmed by the response the substance gave in the ethyl cyanoacetate reaction (Table 1). Compound 'Q' gave a red solution in ethanol that became yellow on making it alkaline, suggesting that the hydroxyl group was directly attached to the ring. Both fumigatin and the related quinone, spinulosin (6-hydroxyfumigatin), form purple products under these conditions (Anslow & Raistrick, 1938a,b).

Determination of radioactivity in the lipid components of the mycelium. Only 30% of the initial radioactivity was recovered from the ether-extractable material in the medium (Table 4). A further 5% was found in the total lipid extracts of each of the three groups (i.e. control, orsellinic acid- and orcinol-supplemented groups) and was presumably due to the presence of 6-methylsalicylic acid itself or some similar product. Most of the ¹⁴C-labelled 6-methylsalicylic acid must therefore have been metabolized to ¹⁴CO₂ or water-soluble components in the mycelium. The unsaponifiable matter retained only a small proportion of the radioactivity present in the total lipid fraction and no radioactivity could be detected in the ergosterol. Further, some fatty acids were extracted from the unsaponifiable matter with ether and were isolated during chromatography. No radioactivity was present in this fraction.

Parson & Rudney (1964) have demonstrated that hydroxybenzoate is incorporated specifically into the aromatic portion of ubiquinone in a variety of tissues. The methyl group attached to C-1 of the ring is derived from methionine. To test

whether 6-methylsalicylic acid could also act as a precursor of ubiquinone, the mycelia from the groups incubated for different times were saponified and then extracted with ether. Ubiquinone was isolated from the unsaponifiable matter of the first group (tested 2 days after supplementation of the medium) but it was not labelled (Table 5). Ubiquinone obtained at later stages of growth did possess some small but definite radioactivity. The incorporation into ubiquinone, however, was very low (0.025%). Ergosterol had no appreciable radioactivity at any time.

Effect of supplementing the medium with 6-methylsalicylic acid. It has been mentioned above that the presence of 6-methylsalicylic acid inhibits the synthesis of fumigatin. The effect of different concentrations of this substrate was examined (Table 6). Group 2 grew almost as well as the controls and this was confirmed by the similar amounts of cell material and ether extract in these two cultures. The other groups grew rather poorly and secreted correspondingly less material into the medium.

The ether-light petroleum (1:9, v/v) fraction of the control group was chromatographed on Kieselgel G and the zone corresponding to the 6-methylsalicylic acid was eluted. No 6-methylsalicylic acid was detected. A small amount of residual 6-methylsalicylic acid was determined in each of the other groups. Similarly, no *m*-cresol was present in the control cultures. Groups 2-5, however, contained significant quantities of this substance and maximum production occurred when the medium had been supplemented with 15-20 mg. of substrate. Orsellinic acid was found in each group and a possible stimulation in synthesis was found in groups 3 and 4.

The fumigatin content of all the test cultures (estimated in absolute terms) was decreased to some extent, but the amount found in group 4 was still one-half of the control value. By contrast, the presence of 15 mg. of 6-methylsalicylic acid in the medium during incubation caused a pronounced

Table 5. *Incorporation of ¹⁴C-labelled 6-methylsalicylic acid into the unsaponifiable matter and ubiquinone in A. fumigatus*

Experimental details are given in Table 3. The unsaponifiable lipid components were isolated as described in the text. The specific activity of the ubiquinone has been corrected for the addition of carrier material.

Group	Total incubation time (days)	Dry wt. of mycelium (g.)	Unsaponifiable matter (mg.)	Total radioactivity in unsaponifiable matter (counts/min.)	Ubiquinone (mg.)	Sp. activity of ubiquinone (counts/min./mg.)	Ergosterol (mg.)	Sp. activity of ergosterol (counts/min./mg.)
1	5	3.8	71.4	1310	1.1	0	9	0
2	7	4.7	99.8	1820	1.4	55	12	0
3	9	4.8	104.1	1590	1.2	85	16	3

Table 6. *Effect of different concentrations of 6-methylsalicylic acid on the growth of A. fumigatus and on the synthesis of certain metabolites*

6-Methylsalicylic acid was added to each of the Roux bottles in groups 2-5 respectively after 3 days' growth. Incubation was continued for a further 5 days at 28°. All groups consisted of three Roux bottles and results are expressed as totals.

Group	6-Methyl-salicylic acid (mg.)	Final pH of medium	Ether extract (mg.)	Residual 6-methyl-salicylic acid (mg.)	m-Cresol (mg.)	Orsellinic acid (mg.)	Fumigatin (mg.)	Fumigatol (mg.)	Ratio of fumigatin to fumigatol	Dry wt. of mycelium (g.)	Orsellinic acid (mg./g. dry wt.)	Fumigatin (mg./g. dry wt.)	Fumigatol (mg./g. dry wt.)
1	None	2.7	321	None	None	1.1	9.8	180	1:18	5.1	0.22	1.9	35.3
2	15	2.5	231	0.6	0.9	0.9	6.7	117	1:17	4.6	0.20	1.5	25.4
3	45	2.2	144	1.2	5.3	1.3	4.7	41	1:9	4.0	0.32	1.2	10.2
4	60	2.5	141	1.6	7.0	0.9	4.8	19.5	1:4	3.1	0.30	1.5	6.3
5	90	2.5	93	4.1	2.6	0.3	1.3	2.9	1:2	2.4	0.13	0.5	1.2

inhibition of the synthesis of fumigatol. This effect became increasingly apparent as the concentration of test substance was raised.

DISCUSSION

The experiments involving oxidation of fumigatol in aqueous solution were performed with autoclaved medium, but it is probable that the conditions did not truly represent those that normally occur in the culture medium during growth. If they were the same, very little fumigatol would remain to be extracted even after 2-3 days. Oxidation of fumigatol would usually be greatly decreased because the mycelium would limit the contact of the medium with oxygen. There would be a large concentration of carbon dioxide within the Roux bottles during growth and this would also tend to prevent oxidation. However, more fumigatin was present in the medium than could be accounted for by oxidation during the extraction procedures. Thus some fumigatin must have been secreted into the medium as such or derived from fumigatol during incubation by oxidation, and it is presumably maintained in a stable form that prevents decomposition. It has been reported that some fumigatin in the medium is bound to a polypeptide during growth (Packter & Glover, 1962). Reaction with this substance could most probably occur by condensation with the thiol group of a cysteine residue, for example (see the Experimental section and Fig. 2).

The results from the experiments with 6-methylsalicylic acid show that this substance may be effectively incorporated into fumigatin and to an apparently smaller extent into fumigatol. 6-Methylsalicylic acid also acts as a precursor of orsellinic acid. No 6-methylsalicylic acid, however, was found in the medium of control cultures. Further, ^{14}C -labelled 6-methylsalicylic acid recovered from the medium exhibited the same specific activity as that of the initial substrate. Thus no endogenous material had been newly synthesized and then secreted into the medium. The results indicated that 6-methylsalicylic acid must be converted into another metabolite before it can act as a precursor; this substance is probably orsellinic acid. The content of orsellinic acid in the mycelium of *A. fumigatus* is approx. 20mg./200ml. The molecular weight of orsellinic acid is identical with that of fumigatin (both $\text{C}_8\text{H}_8\text{O}_4$) and, as decarboxylation must be a prerequisite for conversion into fumigatin, one-quarter of the radioactivity will be lost as carbon dioxide. The carboxyl group, of course, is the same as that in 6-methylsalicylic acid and arose from the ^{14}C carboxyl group of sodium $[1\text{-}^{14}\text{C}]$ acetate (see the Experimental section). If orsellinic acid (II) is indeed an inter-

mediate in the synthesis of fumigatin and fumigatol from 6-methylsalicylic acid, its specific activity should be at least four-thirds of the value of any decarboxylation product. This condition was, in fact, satisfied. Further, recent work in this Laboratory has shown that biosynthetically prepared ^{14}C -labelled orsellinic acid was very effectively incorporated (70%) into both fumigatin and fumigatol. The specific activities of these two substances were the same (N. M. Packter, unpublished work).

It has been shown that 6-methylsalicylic acid passes through the mycelium readily as only 5% remained in the medium 2 days after supplementation. Despite this, neither fumigatin nor fumigatol became appreciably labelled during this time. By contrast, both these substances possessed significant radioactivity when they were isolated after longer incubation periods. In all cases, the specific activity of the quinone was considerably higher. These results may be explained by suggesting that ^{14}C -labelled fumigatin and fumigatol are not produced until about 3 days after the ^{14}C -labelled substrate was added. At this stage of growth, the organism has already secreted into the medium approx. 100mg. of fumigatol (/three Roux bottles). Thus the specific activity of the newly synthesized material will be low. On the other hand, the corresponding amount of fumigatin present in the medium at this time is only 6mg. and the resulting specific activity will therefore be much higher. Moreover, it is evident that some ^{14}C -labelled fumigatin must have been secreted as such as this material could not have arisen from oxidation of fumigatol. Similar reasoning would account for the relatively high specific activity of compound 'Q' compared with its dihydro derivative.

Degradative studies could not be performed on these metabolites because of their low specific activities. Consequently, the results might also be consistent with their synthesis from acetyl-CoA after initial breakdown of the benzene nucleus, as distinct from direct incorporation of the aromatic substrate. This possibility, however, was eliminated by showing that no radioactivity was present in ergosterol, a product derived from acetyl-CoA via mevalonic acid. Further, no radioactivity was detected in the fatty acid fraction. Thus, fumigatin and fumigatol are synthesized directly from 6-methylsalicylic acid, probably after the intermediate formation of orsellinic acid followed by decarboxylation, hydroxylation and *O*-methylation (but not necessarily in the order stated). Presumably, the *C*-methyl group remains intact throughout.

Similarly, the radioactivity determined for ubiquinone cannot have arisen from acetyl-CoA. However, 6-methylsalicylic acid is decarboxylated in its conversion into *m*-cresol and fumigatin. The $^{14}\text{CO}_2$ generated might be incorporated by fixation

into β -methylglutaconyl-CoA and then β -hydroxy- β -methylglutaryl-CoA (Coon, Kupiecki, Dekker, Schlesinger & Del Campillo, 1959), and hence into isoprenoid compounds such as ergosterol and ubiquinone. As ergosterol was not radioactive, it is evident that it had not been synthesized from $^{14}\text{CO}_2$ either. Presumably, the same would apply to the isoprenoid portion of ubiquinone, which has a much lower turnover rate. The results therefore indicate that a limited conversion of the 6-methylsalicylic acid into the aromatic portion of ubiquinone does occur (approx. 0.025%) without initial degradation to acetyl-CoA. This order of incorporation is very low compared with that obtained with *p*-hydroxy[^{14}C]benzoate (Parson & Rudney, 1964; Burton & Glover, 1965). It is comparable, however, with that determined by Olson *et al.* (1963) and Olson, Dialameh, Bentley, Springer & Ramsey (1965) with [^{14}C]tyrosine in animal studies. It seems likely that 6-methylsalicylic acid is first oxidized and decarboxylated to a gentisic acid-type compound before conversion into the aromatic precursor of ubiquinone in *A. fumigatus*. This latter acid (2,5-dihydroxybenzoic acid) is probably derived from 6-methylsalicylic acid in *P. urticae* (Tanenbaum & Bassett, 1959; Gatenbeck & Lönnroth, 1962).

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