Studies on the Biosynthesis of Phenols in Fungi

PRODUCTION OF 4-METHOXYTOLUQUINOL, EPOXYSUCCINIC ACID AND A
 DIACETYLENIC ALCOHOL BY SURFACE CULTURES OF *LENTINUS DEGENER*
 I.M.I. 110 525

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1. 4-Methoxytoluquinol was secreted into the medium by surface cultures of the basidiomycete *Lentinus degener* Kalchbr. (approx. 100mg./l. of medium). In addition, epoxysuccinic acid (150–200mg.) and a long-chain diacetylenic alcohol (3mg.) were also secreted. Epoxysuccinic acid has previously been found in the culture medium of some Fungi Imperfecti. These metabolites were all synthesized during the early phase of growth but maximum production occurred some time later. 2. Supplementation of the medium with cycloheximide or 8-azaguanine inhibited the production of epoxysuccinic acid. 3. Sodium [^{14}C]acetate and 6-methyl[^{14}C]salicylic acid were not incorporated into 4-methoxytoluquinol, but [^{14}C]tyrosine and [$\text{Me-}^{14}\text{C}$]methionine were incorporated to the extent of 0.55 and 4.75% respectively (minimum values). Degradation studies established that the aromatic ring and *O*-methyl group were derived from the ring and β -carbon atom of tyrosine; the *O*-methyl group alone was formed from methionine.

As part of a study of the biosynthesis of phenols in fungi (Packter & Glover, 1965; Packter, 1966a; Steward & Packter, 1968), the culture medium of the basidiomycete *Lentinus degener* Kalchbr. has been examined. Anchel, Hervey, Kavanagh, Polatnick & Robbins (1948) had previously isolated 4-methoxytoluquinone from the medium after incubation with this organism. However, it had been shown that the phenol fumigatol is the principal metabolite secreted by *Aspergillus fumigatus* (Packter & Glover, 1962, 1965). The corresponding quinone may also be present to a small extent but this arises from fumigatol by oxidation in the medium (Packter, 1965, 1966a). Results obtained by Pettersson (1964a,b) and Yamamoto, Nitta, Tango, Saito & Tsuchimuro (1965) have confirmed this. The material obtained by Pettersson (1964a), however, was not characterized or purified. Further, gliorosein (1,6-dihydro-3,4-dimethoxy-6-methyltoluquinone) is the actual metabolite secreted by *Gliocladium roseum* and the corresponding quinol, quinhydrone and quinone are derived from this in the medium after isomerization and subsequent oxidation (Packter & Steward, 1967).

Thus it seemed probable that 4-methoxytoluquinol (I) was the actual metabolite secreted by *L. degener*. Further, it was possible on structural grounds that this was an instance of a phenolic metabolite derived via the acetate-plus-malonate

pathway, and 6-methylsalicylic acid (II) could therefore be the primary aromatic intermediate. Indeed, Birch (1961), in a preliminary report, indicated that both acetate and 6-methylsalicylic acid were incorporated into 4-methoxytoluquinone by this organism. [Moreover, after this work had commenced, Pettersson (1966a,b) confirmed these results and also claimed that the quinol form was secreted. The quinol, however, was neither characterized nor purified.]

Preliminary work from this laboratory on the biosynthesis of 4-methoxytoluquinol has been reported (Packter, 1966b). The results indicated that neither acetate nor 6-methylsalicylic acid was involved in its biosynthesis. Since compounds related to shikimic acid metabolites might therefore be utilized, [^{14}C]tyrosine was tested. This substrate was effectively incorporated. The present paper reports in detail the results obtained when

[1-¹⁴C]acetate, 6-methyl[¹⁴C]salicylic acid and [U-¹⁴C]tyrosine were tested for activity as precursors of 4-methoxytoluquinol. The origin of the C-methyl group was also investigated.

METHODS

Organisms and culture conditions. *Penicillium griseofulvum* Dierckx (I.M.I. 75832) and *L. degener* (I.M.I. 110525) were obtained from the Commonwealth Mycological Institute (Kew, Surrey). Stock cultures were maintained on potato-dextrose agar (Difco) slopes and subcultured every 1–2 months. *P. griseofulvum* was grown as described by Packter (1965). *L. degener* was incubated as surface cultures in Roux bottles containing 200 ml. of Raulin–Thom medium (Anslow & Raistrick, 1938), supplemented with 0.2% yeast extract (Difco) and 0.1% agar. Occasionally it was incubated in 500 ml. conical flasks containing 125 ml. of medium. The agar was added to prevent waterlogging of the mycelium. Submerged cultures were grown in conical flasks (1 l. capacity; 400 ml. of medium including agar) in an incubated shaker. The organism displayed a long lag phase and visible mycelium began to form after about 10 days. Maximum yield of mycelium and metabolites occurred after 4–5 weeks at 28–30° in each case. Rates of growth and actual yields of metabolites, however, varied from one experiment to another. Cambie, Gardner, Jones, Lowe & Read (1963) report similar variations for a polyacetylene-producing strain of the basidiomycete *Poria sinuosa*.

Chromatography. Details of adsorbents for column chromatography and t.l.c. have been given previously (Packter, 1965). Dicarboxylic acids were resolved on ascending paper systems as given by Martin & Foster (1955).

Isolation of the C-methyl group (Kuhn–Roth oxidation). This procedure was carried out by the method of Barthel & Laforge (1944). The C-methyl group was converted into sodium acetate from which the *p*-nitrobenzyl ester was prepared and purified (Steward & Packter, 1968).

Degradation of the isolated acetic acid. In the experiment with tyrosine as substrate (Table 5), the sodium acetate obtained from the Kuhn–Roth procedure was treated in the Schmidt reaction, by the method of Phares (1951), to obtain separate values for the methyl and carboxyl groups. The CO₂ liberated during the reaction with NaN₃ was counted as BaCO₃; methylamine sulphate was isolated as 5-methylamino-2,4-dinitrotoluene (Grout, Benn, Imaseki & Geissman 1966).

Isolation of the O-methyl group. The radioactivity in the O-methyl group of 4-methoxytoluquinol was determined by conversion into methyl iodide by the Zeisel reaction (Steward & Packter, 1968). The tetramethylammonium iodide thus formed was dissolved in water and a sample was counted for assay of radioactivity.

Spectroscopy. The u.v.-absorption measurements were carried out in ethanol (unless stated otherwise) in a Unicam SP.800A recording spectrophotometer. All u.v.-absorbing substances were assayed spectrophotometrically. The i.r.-absorption spectra were recorded by a Unicam SP.200 instrument as a Nujol mull or a thin film between NaCl cells. The n.m.r. spectrum of epoxysuccinic acid was determined with a Varian Associates A-60 spectrometer. Tetramethylsilane was used as the internal reference (10 τ).

Assay of radioactivity. Radioactivity was measured with a Nuclear–Chicago Corp. gas-flow counter at 20% efficiency.

Melting points. These were determined in unsealed capillary tubes in a Gallenkamp melting-point apparatus and are uncorrected.

Analyses. C and H analyses were kindly performed by the Micro-analytical Laboratory, Department of Organic Chemistry, University of Leeds.

MATERIALS

Isolation and identification of metabolites of *L. degener*. The medium (2 l.) from surface cultures was acidified with HCl and extracted four times with ether, immediately after removal from the incubator. The extracts were combined, dried over Na₂SO₄ and evaporated to dryness to give a brown gum (1.2 g.), which partly dissolved in warm chloroform. Chromatography of the ether-extractable material of the medium indicated that 4-methoxytoluquinol was present in good yield. However, other metabolites were present; the major component (besides the quinol) could be readily resolved as it was insoluble in chloroform. It was characterized as (–)-*trans*-epoxysuccinic acid (oxiran-1,2-dicarboxylic acid). A diacetylenic primary alcohol was also isolated.

4-Methoxytoluquinol. The chloroform-soluble material of the original ether extract (670 mg.) was chromatographed on silicic acid (26 g.)–Celite (13 g.), and the column was developed with 10% E/P.* A fraction containing a diacetylene was eluted with 25% E/P. A brown crystalline solid with an aromatic smell was eluted with 60% E/P; this was taken up in chloroform and the soluble material (250 mg.) was rechromatographed. Pure 4-methoxytoluquinol (I) was obtained as a white crystalline solid (110 mg.) after crystallizations from chloroform. It had m.p. 125–126° [Luff, Perkin & Robinson (1910) reported m.p. 124° and Anchel *et al.* (1948) gave m.p. 122–124°]. The u.v.- and i.r.-absorption spectra have not been recorded previously. The quinol gave $\lambda_{\max}$ 294 nm. ($E_{1\%}^{1\text{cm}}$ 280) (Found: C, 61.9; H, 6.3. Calc. for C₈H₁₀O₃: C, 62.3; H, 6.5%). The i.r.-absorption spectrum showed $\nu_{\max}$ at 3350 (OH), 1635, 1535 (aromatic C=C) and 865 cm.^{–1} (single aromatic H). The yellow quinone formed on oxidation with FeCl₃ was identical (by m.p., u.v. and i.r. spectra) with synthetic 4-methoxytoluquinone as prepared by the method of Woodward, Sondheimer, Taub, Heusler & McLamore (1952). It gave m.p. 172° and $\lambda_{\max}$ (cyclohexane) 258 nm. ($E_{1\%}^{1\text{cm}}$ 1470). Further, 4-methoxytoluquinol (10 mg./50 ml. of autoclaved medium) was completely converted into the quinone within 24 hr.

In all the incorporation experiments, the quinol was purified by two or three crystallizations from chloroform to give material with m.p. 125–126°, followed by oxidation with FeCl₃ (Vischer, 1953). The mixture was then extracted with chloroform and 4-methoxytoluquinone was purified by t.l.c. It possessed the same specific radioactivity as the crystalline quinol.

(–)-*trans*-Epoxysuccinic acid. The chloroform-insoluble residue (430 mg.) was dissolved in a minimum volume of ether and chromatographed on silicic acid (26 g.)–Celite (13 g.); brown material that was insoluble in ether (50 mg.) was discarded. An aliphatic acid and some 4-methoxytoluquinol were eluted together with 60% E/P. The chloroform-

* Abbreviation: E/P, solution of diethyl ether in light petroleum (b.p. 40–60°).

insoluble fraction (290 mg.) was crystallized from ether-chloroform (2:1, v/v) to give white needle crystals (100 mg.), m.p. 186° (Found: C, 36.7; H, 2.8. Calc. for $C_4H_4O_5$: C, 36.4; H, 3.0%). They did not absorb in the u.v. region. The i.r.-absorption spectrum showed prominent $\nu_{\max}$ at 1700 (aliphatic CO), 1420, 1305 (C-O) and 1245 and 850 cm^{-1} (epoxide). On titration with 0.05 M-NaOH it gave equiv.wt. 64, pK_{a1} 2.60 and pK_{a2} 3.45. It had $[\alpha]_D^{25} -110 \pm 5^\circ$ (c 0.22 in water). Thus this metabolite is (-)-*trans*-epoxysuccinic acid (III) [Birkinshaw, Bracken & Raistrick (1945) quote $[\alpha]_D^{25} -106^\circ$ (with m.p. 186°) and Martin & Foster (1955) -100°].

The n.m.r. spectrum was taken in D_2O (31 mg. in 0.5 ml.) and showed two singlets at 5.2 and 6.15 τ [due to HDO and oxiran (ethylene oxide) protons respectively]. Since the oxiran peak was a singlet, the coupling constant (J) between the vicinal H atoms is zero. The dihedral angle between the *trans*-CH groups is therefore approx. 90°.

Diacetylene. The u.v. spectrum of the 25% E/P fraction showed a typical ene-diyne chromophore, unchanged on addition of alkali (Anchel, 1952; Cambie *et al.* 1963). The component with this prominent spectrum was purified by rechromatography followed by chromatography on magnesia-Celite. The column was developed with 5% E/P and a colourless oil was eluted with 15% E/P. It gave $\lambda_{\max}$ 227, 238, 251, 265.5 and 281 nm. ($E_{1\%}^{1\text{cm}}$ 92, 196, 416, 605 and 480 respectively). The i.r.-absorption spectrum (thin film) had $\nu_{\max}$ 3430 (OH), 2250, 2160 (C:C), 1380 (CH_3), 1065 (primary C-OH) and 960 cm^{-1} (*trans*-CH:CH). Absorption around 3300 cm^{-1} was absent, suggesting lack of a free ethynyl hydrogen. This was confirmed by the relative stability of the compound. Bands due to vinyl, allene or carbonyl groups were also absent.

Pooled samples were retained from various experiments. The purified diacetylene (15 mg.) was esterified by treatment with *p*-phenylazobenzoyl chloride and pyridine (Burgos, Hemming, Pennock & Morton, 1963). The ester was purified by t.l.c. on Kieselgel G with benzene-cyclohexane (1:1, v/v) as eluent (R_F 0.23) and crystallized from ethanol. It had m.p. 106–107° and $\lambda_{\max}$ 324 nm. ($E_{1\%}^{1\text{cm}}$ 615). The mol.wt. was approx. 226 as calculated from the mean molecular extinction value 26770 of esters with this chromophore (Burgos *et al.* 1963). The above findings suggest, therefore, that this is a C_{16} ene-diyne primary alcohol (assuming that one hydroxyl group is present).

The diacetylene was also present in total-lipid and unsaponifiable-lipid fractions. These were obtained from the mycelium of surface cultures as described previously (Packter, 1965, 1966a) during examination of fractions. The lipid was chromatographed on acid-washed alumina (Brockmann grade III) and the diacetylene was eluted with 25% E/P. Approx. 5 mg./21. of medium (17 g. of mycelium) was obtained in each case. Presumably the presence of a large excess of antioxidant (pyrogallol) helped to prevent decomposition.

Sterols. During the chromatography of the unsaponifiable lipid, some ergosterol was eluted with 20% E/P (characterized by its u.v. and i.r. spectra). The major sterol, however, was eluted from the alumina column with ether. It had $\lambda_{\max}$ 243 nm. with associated subsidiary peaks at 236 and 251 nm. They indicated the presence of a heteroannular diene system similar to that in dehydrotumulosic acid (Cort *et al.* 1954) but lacking an oxo group at C-3 as in polyporenic acid C (Bowers, Halsall, Jones & Lemin, 1953). It was purified by chromatography on magnesia-Celite and was eluted with ethanol-ether (1:9, v/v). The resulting white solid was crystallized from chloroform and then ethanol to give m.p. 270–275° and $\lambda_{\max}$ 243 nm. ($E_{1\%}^{1\text{cm}}$ 185). The i.r.-absorption spectrum showed prominent peaks at 3450, 3350 (OH), 1715 (CO), 1660 (C:C), 1080, 1035 (C-OH), and 895 cm^{-1} ($:\text{CH}_2$). It was analysed as $C_{31}H_{48}O_4$ (N absent) and gave equiv.wt. 478. Its identity as dehydrotumulosic acid (mol.wt. 484) was established by mass spectrometry, after esterification with diazomethane. A parent ion was obtained at m/e 498 with good peaks at $M-18$ ($-\text{H}_2\text{O}$) and $M-31$ ($-\text{OCH}_3$). Similar peaks were also noted at $M-32$ and $M-45$ due to additional loss of CH_2 . The spectrum showed that some tumulosic acid (one double bond) was also present (Cort *et al.* 1954). Dehydrotumulosic acid and the related eburicic acid and dehydro-eburicic acid (no additional hydroxyl) and polyporenic acid C are the common sterols found in many basidiomycetes (Birkinshaw, Morgan & Findlay, 1952).

Succinic acid. The medium (21.) from submerged cultures of *L. degener* was extracted as above to give a dark-yellow oil (200 mg.). Fractions containing the diacetylenic alcohol and succinic acid (20 mg.) were obtained after chromatography (60% E/P). No epoxysuccinic acid or 4-methoxytoluquinol was detected (u.v. and i.r. spectra). The succinic acid was rechromatographed and crystallized from ether, m.p. 183–184°, undepressed by authentic material. The two samples had identical i.r. spectra and R_F values in various solvent systems (Martin & Foster, 1955).

The diacetylene was produced in approximately the same amount as that in surface cultures (3.5 mg./l. of medium).

Production of metabolites by surface cultures of *L. degener*. Preliminary work established the pattern of secretion of the various metabolites. The diacetylene and 4-methoxytoluquinol were measured spectrophotometrically by using their E values at $\lambda_{\max}$ 266 and 294 nm. respectively. The fraction eluted from the silicic acid column with 60% E/P was washed twice with CHCl_3 at room temperature and the residual pale-brown solid was assayed gravimetrically. The i.r.-absorption spectrum showed that this material was almost pure epoxysuccinic acid. Yields of metabolites tended to vary from batch to batch (see also Cambie *et al.* 1963) but it was found that duplicate groups within one experiment usually gave good agreement.

The yields of the major metabolites from one experiment are given in Table 1. The diacetylene was secreted into the medium at an early stage of growth; epoxysuccinic acid and 4-methoxytoluquinol were produced somewhat later. Maximum production of metabolites occurred after 4–5 weeks' growth (groups 4, 5 and 6). The yield of products per g. of mycelium only varied two- to four-fold throughout growth. The relative production in groups 1–6, expressed as wt. of mycelium (g.)/wt. of metabolite (mg.), only ranged from 1:8 to 1:15 (4-methoxytoluquinol), 1:11 to 1:32 (epoxysuccinic acid) and 1:0.3 to 1:1 (diacetylene).

Succinic acid (2mg.) was found in groups 1 and 2; no attempt was made to isolate it from the other groups. Some 4-methoxytoluquinone (2mg.) was detected in the medium of groups 7 and 8. The yield of epoxysuccinic acid was higher than that normally obtained (Table 6).

Solvents. Light petroleum (b.p. 40–60°), ether and ethanol for spectroscopy were purified as described by Packter (1965).

Radioactive compounds. Sodium [1^{14}C]acetate, L-[Me^{14}C]methionine and L-[U^{14}C]tyrosine hydrochloride were obtained from The Radiochemical Centre (Amersham, Bucks.). The [U^{14}C]tyrosine was used without further purification for the preliminary experiments. For the degradation experiment, however, the tyrosine was initially purified by ascending paper chromatography on Whatman 3MM paper with butan-1-ol-acetic acid-water (4:1:5, by vol.) (Parson & Rudney, 1965). The purity of the sample was checked by radioautography with Kodak X-ray film (Kodirex) and by scanning with a Nuclear-Chicago Corp. Actigraph II scanner. Approx. 1% of the total radioactivity was present as impurities. The tyrosine zone (R_F 0.32)

was eluted with warm ethanol-0.1 M-HCl (1:1, v/v) and neutralized with solid NaHCO_3 before use.

6-Methyl[^{14}C]salicylic acid was prepared biosynthetically from sodium [1^{14}C]acetate by cultures of *P. griseofulvum* (Birch, Massy-Westropp & Moye, 1955). It had equal amounts of radioactivity distributed among C-2, C-4, C-6 and C-8 respectively (Birch *et al.* 1955). The ^{14}C -labelled material was isolated as described by Packter (1965).

RESULTS

Testing of ^{14}C -labelled substrates for activity as precursors of 4-methoxytoluquinol. Details of the incorporation of sodium [1^{14}C]acetate, 6-methyl-[^{14}C]salicylic acid, [U^{14}C]tyrosine and [Me^{14}C]methionine into 4-methoxytoluquinol are given in Table 2. Neither of the first two substrates was incorporated to any significant extent. In the group with acetate as substrate, approx. 2% of the initial

Table 1. *Production of metabolites by Lentinus degener*

Roux bottles containing 200 ml. of Raulin-Thom medium were incubated at 28–30°; groups of three bottles were removed at intervals. The results are expressed as total wt./600 ml. of medium. N.D., Not detected (less than 2 mg.).

Group	Time of incubation (days)	pH of medium	Wt. of mycelium (g.)	Ether extract (mg.)	Epoxysuccinic acid (mg.)	Diacetylene (mg.)	4-Methoxytoluquinol (mg.)
1	12	3.7	0.3	12	N.D.	0.3	N.D.
2	20	2.6	0.8	80	9	0.6	6
3	26	2.9	3.0	238	73	1.4	46
4	30	2.5	4.3	363	108	1.3	45
5	34	2.6	5.4	450	171	1.6	60
6	37	2.5	5.9	451	184	1.8	64
7	44	2.5	6.1	362	104	1.7	30*
8	51	2.5	6.4	260	60	1.5	7*

* 2 mg. of 4-methoxytoluquinone was present.

Table 2. *Testing of ^{14}C -labelled substrates for activity as precursors of 4-methoxytoluquinol*

Each group consisted of five Roux bottles and the test substrates were added (in solution in sterile water) at the times indicated. Incubation proceeded for a further 7–11 days. Results are expressed as totals/l. of medium. 6-Methylsalicylic acid had specific radioactivity 8800 c.p.m./ μmole .

Test substrate	Radio-activity added (μC)	Time of addition (days)	Total incubation (days)	Ether extract		4-Methoxytoluquinol			Incorporation into 4-methoxytoluquinol (%)
				Wt. (mg.)	$10^{-3} \times$ Total radio-activity (c.p.m.)	Wt. (mg.)	$10^{-3} \times$ Total radio-activity (c.p.m.)	Sp. radio-activity (c.p.m./ μmole)	
Sodium [1^{14}C]acetate	50	22	30	215	390	68	8.2	18	0.03
6-Methyl[^{14}C]salicylic acid	2.5	23	34	620	271	122	0.6	0.7	0.03
[U^{14}C]Tyrosine	10	25	35	540	115	65	32.5	78	0.73
[Me^{14}C]Methionine	50	29	38	608	2005	155	1057	1050	4.75

Table 3. Incorporation of ^{14}C -labelled substrates into unsaponifiable lipid components

Experimental details are given in Table 2								
Test substrate	Unsaponifiable lipid		Dehydrotumulosic acid		Ergosterol		Fatty acid	
	Wt. (mg.)	Total radio-activity (c.p.m.)	Wt. (mg.)	Sp. radio-activity (c.p.m./ μ mole)	Wt. (mg.)	Sp. radio-activity (c.p.m./ μ mole)	Wt. (mg.)	Sp. radio-activity (c.p.m./ μ mole)
Sodium [1- ¹⁴ C]acetate	115	528 000	9	4060	2	2040	18	1210
6-Methyl[¹⁴ C]salicylic acid	98	500	10	2	0.5	—	—	—
[U- ¹⁴ C]Tyrosine	90	1575	21	4	—	—	6	5
[Me- ¹⁴ C]Methionine	69	156 400	14	2590	—	—	12	140

Table 4. Incorporation of [^{14}C]tyrosine and [$\text{Me-}^{14}\text{C}$]methionine into 4-methoxytoluquinol

[^{14}C]Tyrosine was purified as indicated in the Methods section and added as an aqueous ethanolic solution. The labelled substrates were given after 30 days' growth and incubation proceeded for a further 12 days. Both groups consisted of four Roux bottles and the results are expressed as totals/800 ml. of medium.

Test substrate	Ether extract			4-Methoxytoluquinol			
	Initial radioactivity (μC)	Wt. (mg.)	$10^{-3} \times$ Total radioactivity (c.p.m.)	Wt. (mg.)	$10^{-3} \times$ Total radioactivity (c.p.m.)	Sp. radio-activity (c.p.m./ μmole)	Incorporation (%)
[^{14}C]Tyrosine	20	1605	620	530	541	153	6.2
[$\text{Me-}^{14}\text{C}$]Methionine	40	504	1900	145	1198	1272	6.8

radioactivity was present in the ether-extractable material; of this only 2% was located in 4-methoxytoluquinol. However, [^{14}C]acetate was effectively incorporated into the unsaponifiable lipids (1.9% conversion) (Table 3). Dehydrotumulosic acid was isolated after chromatography on magnesia and was radioactive (12mg.; specific radioactivity 4060 c.p.m./ μmole). In this particular group, ergosterol (2mg.) was isolated and purified (specific radioactivity 2040 c.p.m./ μmole). Similarly, a fatty acid fraction (18mg.) was obtained from the column that had specific radioactivity 1210 c.p.m./ μmole on the basis of the molecular weight for oleic acid. Thus the acetate had been converted into acetyl-CoA and its lack of incorporation into the quinol cannot be due to the organism's inability to perform this reaction.

With 6-methylsalicylic acid as substrate, approx. 25% of the original radioactivity was present in the various fractions of the ether-extractable material from the medium. No residual acid (or *m*-cresol) was detected. The 6-methylsalicylic acid had therefore been extensively metabolized. [A further experiment established that 6-methylsalicylic acid (10mg./200ml. of medium) was taken up and metabolized within 2 days of supplementation of the medium.] Neither 4-methoxytoluquinol nor the unsaponifiable lipids became labelled.

Both [^{14}C]tyrosine and [$\text{Me-}^{14}\text{C}$]methionine

were well incorporated into the ether extract. The major portion of the radioactivity was present in 4-methoxytoluquinol (30% and 70% respectively). In both cases the quinol was rigorously purified. The crystalline product was then converted into the quinone, which was chromatographed on thin-layer plates of Kieselgel G and crystallized from chloroform. Tyrosine was poorly incorporated into unsaponifiable lipids, and the sterols and fatty acids were not labelled. The situation was different with methionine, however, which was incorporated to the extent of 0.7%. As expected, dehydrotumulosic acid was highly radioactive since methionine is the source of the additional methylene group. Insufficient ergosterol was obtained in these groups to permit purification.

Degradation of 4-methoxytoluquinol derived from [^{14}C]tyrosine and [$\text{Me-}^{14}\text{C}$]methionine. It was next decided to determine the origin of the *O*- and *C*-methyl groups of 4-methoxytoluquinol. Accordingly [^{14}C]tyrosine hydrochloride (purified by paper chromatography as described in the Materials section) and [$\text{Me-}^{14}\text{C}$]methionine were added to the medium after 30 days' growth. Both substrates were effectively incorporated (6.2 and 6.8% respectively) (Table 4). The yield of quinol and the value for the incorporation of tyrosine were both very much increased over that normally obtained (Tables 2 and 6) and may be due to the presence of

ethanol or sodium chloride or both in the tyrosine solution.

The 4-methoxytoluquinol was then degraded by the Kuhn-Roth procedure to yield the *C*-methyl group as sodium acetate, and by the Zeisel reaction to give the *O*-methyl group as tetramethylammonium iodide. In the group with tyrosine as substrate, the sodium acetate was further degraded to give the methyl group as the dinitrotoluene derivative and the carboxyl carbon atom as barium

carbonate. The results clearly showed (Table 5) that the *O*-methyl group was derived from methionine but that the *C*-methyl group was not formed from this substrate. However, results from the tyrosine experiment indicated that the sodium acetate (i.e. the *C*-methyl group) possessed 27% of the total radioactivity in 4-methoxytoluquinol. This corresponds well with the theoretical value of two-sevenths (29%), assuming that both carbon atoms are derived from tyrosine. Moreover, the values for the specific radioactivities of each carbon atom (C-1 and C-7) are approximately equal and correspond to one-seventh each. Thus the *C*-methyl group is formed specifically from the β -carbon atom of tyrosine.

Effect of cycloheximide and 8-azaguanine on the production of metabolites secreted by L. degener. Supplementation of the medium with either inhibitor of enzyme synthesis exerted the same effect. Production of the diacetylene and 4-methoxytoluquinol was not appreciably affected in groups 4 and 6 (inhibitors added after 24 days' growth) (Table 6). Production of diacetylene in groups 3 and 5 was decreased to some extent and that of the quinol most markedly. The amounts quoted for quinol do, however, refer to material that was isolated crystalline and had the same u.v. and i.r. spectra as authentic material. No unusual acetylenes or phenols were detected (by u.v. and i.r. spectra or t.l.c.) during examination of the ether-extractable material of the medium (see the Materials section). The effect on formation of epoxysuccinic acid was very pronounced in all the test groups (3-6). The presence of this metabolite was not detected in these groups; some fumaric acid and succinic acid, however, was present (approx. 5mg.). Production of lipids and sterols was also depressed to some extent in the test groups compared with that in the controls.

Table 5. *Degradation of 4-methoxytoluquinol derived from [U-¹⁴C]tyrosine and [Me-¹⁴C]methionine*

4-Methoxytoluquinol was degraded to obtain values for the specific radioactivities of the *O*-methyl and *C*-methyl groups as described in the Methods section. Sodium acetate was purified as the *p*-nitrobenzyl ester, which was measured spectrophotometrically. In the group with tyrosine as substrate, the sodium acetate was further degraded to give separate values for the methyl and carboxyl carbon atoms. Values in parentheses show the percentages of total radioactivity in 4-methoxytoluquinol. Experimental details are given in Table 4.

Substrate ...	Sp. radioactivity (c.p.m./ μ mole)	
	Tyrosine	Methionine
4-Methoxytoluquinol (C-1-C-8)	153 (100)	1272 (100)
Kuhn-Roth acetate (<i>C</i> -CH ₃)		
(a) Total as ester (C-1, C-7)	41 (27)	1 (0)
(b) C-7 as dinitrotoluene derivative	24 (15)	—
(c) C-1 as BaCO ₃	20 (13)	—
<i>O</i> -CH ₃ group (C-8) as (CH ₃) ₄ NI	0.4 (0)	1306 (103)

Table 6. *Effect of cycloheximide and 8-azaguanine on the production of metabolites by Lentinus degener*

Cycloheximide (100 μ g./ml.) and 8-azaguanine (1 mg./ml.) were added to surface cultures of *L. degener* in conical flasks (500ml. capacity; 125ml. of medium) after 18 or 24 days' growth. Incubation proceeded for a total of 32 days in all groups. Each experimental group consisted of three flasks and the results are expressed as totals/375 ml. of medium. N.D., Not detected (less than 2mg.).

Group	Inhibitor	Time of addition (days)	Mycelium			Medium		
			Dry wt. (g.)	Lipid (mg.)	Sterol (mg.)	Diacetylene (mg.)	4-Methoxytoluquinol (mg.)	Epoxysuccinic acid (mg.)
1	None	—	2.9	48	12	1.1	68	48
2	—	—	2.5	60	10	1.6	54	52
3	Cycloheximide	18	1.8	21	4	0.5	6	N.D.
4	—	24	3.2	24	4	1.4	35	N.D.
5	8-Azaguanine	18	2.0	29	4	0.8	2	N.D.
6	—	24	3.1	22	6	1.2	33	N.D.

DISCUSSION

There is a small but continuous production of 'secondary' metabolites by *L. degener* during the period of rapid growth, but their synthesis is maximal during later stages of growth. However, the yield of metabolites per g. of mycelium varies only over a two- to four-fold range over this period (Table 1).

Although epoxysuccinic acid is structurally related to the tricarboxylic acid-cycle metabolites, it has been isolated previously from only three fungi, *Penicillium vermiculatum*, *Paecilomyces varioti* (Sakaguchi & Inoue, 1938, 1940) and a strain of *A. fumigatus* (L.S.H.T.M. M400) (Birkinshaw *et al.* 1945; Martin & Foster, 1955). [The former two organisms were renamed by K. B. Raper (see Martin & Foster, 1955) from those originally described by Sakaguchi & Inoue (1938, 1940)]. Its immediate precursor in *A. fumigatus* is fumarate and the epoxide oxygen is derived from molecular oxygen (Wilkoff & Martin, 1963). In all cases this acid has been produced by Fungi Imperfecti. This is the first instance in which it has been found in a genus within the basidiomycetes. Many polyacetylenes, however, have been isolated from the growth medium of basidiomycetes, and also from the Compositae and Umbelliferae families among higher plants (Jones, 1960). This is the first reported occurrence of a polyacetylene in the genus *Lentinus*. They can all be formally derived from linoleic acid via its acetylenic 12,13-dehydro derivative, crepenynic acid (Bu'Lock & Smith, 1967).

4-Methoxytoluquinol and epoxysuccinic acid were not detected in the medium of submerged cultures. However, the diacetylene was produced in approximately the same yield as that in surface cultures.

Possible ^{14}C -labelled substrates were next examined for activity as precursors of 4-methoxytoluquinol in surface cultures. The results given in Tables 2 and 3 indicate that acetyl-CoA and 6-methylsalicylic acid are not involved in its biosynthesis although they are both extensively metabolized. [^{14}C]Tyrosine, however, is effectively incorporated. Degradation studies have established that the aromatic ring and β -carbon atom of tyrosine are converted directly as a $\text{C}_6\text{-C}_1$ unit into the ring and *C*-methyl group of 4-methoxytoluquinol (Tables 4 and 5). The *O*-methyl group alone is derived from methionine.

These results differ considerably from those of two previous studies on the biosynthesis of 4-methoxytoluquinone (Birch, 1961; Pettersson, 1966*a,b*). Birch (1961), in a preliminary report that did not give any experimental details, and Pettersson (1966*a,b*) indicated that both [^{14}C]-

acetate and 6-methyl[^{14}C]salicylic acid could act as precursors for this quinone. However, the extent of incorporation was very low compared with that obtained with related metabolites (Birch, Fryer & Smith, 1958; Packter, 1966*a*; Packter & Steward, 1967). Moreover, Pettersson (1966*a,b*) quoted a lower value for the incorporation of 6-methylsalicylic acid than his previously reported one for acetate. The reasons for the apparent incorporation of acetate and 6-methylsalicylic acid are not known, but in the present work rigorous purification of 4-methoxytoluquinol for radiochemical assay and subsequent degradation was essential for meaningful results. The specific radioactivities (c.p.m./mg.) of 4-methoxytoluquinol derived from 6-methyl[^{14}C]salicylic acid, for example, at various stages of purification were 642 (chromatographic fraction), 290 (rechromatography), 50 (first cryst.), 6 (second cryst.), 3 (third cryst.) and 3 (oxidation to quinone; t.l.c.).

In addition, the strain (M.N.H.N. 1027) of *L. degener* used by Pettersson (1966*a,b*) appeared to be different from that used in this work (I.M.I. 110525) as it produced 4-methoxytoluquinone in much lower yield than the 6-hydroxy derivative. Moreover, it was also unusual since it reached maximum production of metabolites after 7–10 days' growth, in contrast with the 4–5 weeks noted here (Table 1) and the lag phase of approx. 10 days. This is in good agreement with the original work by Anchel *et al.* (1948) (5–6 weeks) and that of Gardner, Jones, Leeming & Stephenson (1960), for example, on polyacetylene-producing strains of different genera of basidiomycetes (4–8 weeks).

The results presented in this paper do not indicate which pathway of tyrosine catabolism is utilized for the synthesis of 4-methoxytoluquinol. Possible routes in *Lentinus* sp. are via cinnamic acid derivatives or *p*-hydroxyphenylacetic acid. Power, Towers & Neish (1965) have shown that many basidiomycetes, including *L. lepideus*, possess phenylalanine and tyrosine ammonia-lyase activity; the products formed are cinnamic acid and *p*-coumaric acid respectively. Shimazono (1959) has shown that methyl *p*-coumarate is converted (57%) into methyl *p*-methoxycinnamate (Shimazono, Schubert & Nord, 1958) by *L. lepideus*. Methyl isoferulate (IV) was also identified in the culture medium. Power *et al.* (1965) have demonstrated that [^{14}C]phenylalanine is converted into an ester of isoferulic acid

to the extent of 0.5–1.2%, but [^{14}C]tyrosine was not incorporated (0.002%). It was suggested that the preferential incorporation of phenylalanine in this organism may be the result of the higher phenylalanine ammonia-lyase activity compared with that of the tyrosine enzyme. In replacement cultures, however, both phenylalanine and tyrosine were converted into *p*-hydroxyphenylacetic acid. This product can be readily visualized as a precursor of 4-methoxytoluquinol; decarboxylation followed by two hydroxylation steps and an *O*-methylation would give this quinol. Alternatively, tyrosine may be converted into *p*-coumaric acid, *p*-methoxycinnamic acid and then isoferulic acid (or their esters). Cleavage at the double bond and reduction of the benzoic acid derivative thus formed (Farmer, Henderson & Russell, 1959) through to the methyl stage would give 2-hydroxy-4-methoxytoluene. A final hydroxylation reaction at C-6 would produce 4-methoxytoluquinol (2,6-dihydroxy-4-methoxytoluene).

Another factor to be considered is whether the intermediates involved in the production of 4-methoxytoluquinol are derived for this secondary metabolic process via shikimic acid-pathway metabolites directly (e.g. *p*-hydroxyphenylpyruvic acid or *p*-hydroxybenzoic acid), rather than as degradative products of tyrosine. In view of the comparatively low incorporation values usually obtained with tyrosine, it would seem that it is not itself the primary distal precursor.

4-Methoxytoluquinol is not synthesized via the acetate-plus-malonate pathway, despite its structural similarity to other toluquinols such as fumigatol and gliorosein (keto form) (Bentley & Lavate, 1965; Packter, 1966a; Packter & Steward, 1967). Derivatives of orsellinic acid are the aromatic precursors. Terreic acid (3,4-epoxy-6-hydroxytoluquinone) (Sheehan, Lawson & Gaul, 1958) is also formed from acetate with 6-methylsalicylic acid as the first aromatic intermediates (Read & Vining, 1968). Ubiquinone is formed in yet another manner; the aromatic portion is derived from *p*-hydroxybenzoic acid (Parson & Rudney, 1965). The *C*-methyl group attached to the ring arises by transfer of an intact methyl group from methionine (Parson & Rudney, 1965; Threlfall, Whistance & Goodwin, 1967). Thus the mere structures of apparently related compounds may give a most unreliable guide as to the nature of their biogenesis.

The effects of cycloheximide and 8-azaguanine on the production of epoxysuccinic acid were quite marked (Table 6). Presumably, the oxygenase enzyme required to convert fumaric acid into this metabolite (Wilkoff & Martin, 1963) is absent under these conditions. The formation of 4-methoxytoluquinol and the diacetylene, however, were not appreciably affected when the inhibitors were

added at the later stage of growth. It seems therefore that the enzymes required for their synthesis are constitutive. Any change in the pattern of dehydrogenase or dehydratase activity during the biosynthesis of the diacetylene would have resulted in an acetylenic or olefinic product with a different chromophore.

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