The Degradation of Lignin-related Compounds by *Aspergillus flavus*

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Lignin-related compounds containing the β -aryl ether linkage were metabolized by *Aspergillus flavus* as sole carbon source. Growth measurements were made and degradation intermediates were identified using thin-layer chromatography, gas-liquid chromatography, and infra-red, proton nuclear magnetic resonance and mass spectroscopy. Degradation pathways were established by the detection of methanol as the product of demethoxylation reactions, and cleavage of the β -aryl ether bond was demonstrated by the use of a ^{14}C -labelled model compound.

INTRODUCTION

Lignin is an amorphous high-molecular-mass polymer composed of phenylpropane subunits interconnected by a variety of non-hydrolysable bonds (Sarkanen & Ludwig, 1971; Adler, 1977; Higuchi, 1982). Of the relatively few groups of micro-organisms that can degrade the macromolecule the most efficient degraders are the white-rot fungi, which can cleave $\text{C}\alpha\text{--C}\beta$, β -aryl ether, $\text{C1--C}\alpha$ bonds and aromatic rings. They are also able to oxidize α -carbons as well as hydroxylating aromatic rings and demethylating methoxy groups (Ander *et al.*, 1985; Buswell *et al.*, 1982; Eriksson *et al.*, 1984; Kuwahara *et al.*, 1984; Tien & Kirk, 1984). Much of the research on white-rot fungi has centred upon the organism *Phanerochaete chrysosporium*. The brown-rot fungi can only cause limited degradation (Kirk, 1971; Ander & Eriksson, 1978) and the soft-rot fungi usually cause only very slow and minor modifications to lignin (Ander & Eriksson, 1978; Crawford, 1981).

Several other groups of fungi regarded as wood-rotting (Drew & Kadam, 1979; Crawford & Crawford, 1980; Kadam & Drew, 1986), and bacteria (Crawford *et al.*, 1983; McCarthy & Broda, 1984), have been shown to degrade lignin.

To simplify the study of lignin degradation, model compounds are frequently used. These can be monomeric but are often dimeric aromatic compounds containing one or more of the most common linkages found in the macromolecule. The mechanisms underlying the metabolism of these models is then assumed to be applicable to the degradation of lignin. There is some dispute as to the validity of the use of model compounds but much of the present understanding of lignin biodegradation has arisen from their study.

In this paper we provide evidence for the degradation of lignin-related and other aromatic compounds containing the β -aryl ether linkage by *Aspergillus flavus*. This fungus is often found in wood-rotting environments and has been listed as a soft-rot organism (Seeman *et al.*, 1975). It has previously been found to attack whole wood and lignin model compounds (Iyayi & Dart, 1982; Betts *et al.*, 1987*a, b*; Dart *et al.*, 1987).

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METHODS

Maintenance and growth of organism. *Aspergillus flavus* IMI 15959 was obtained from CAB International, Ferry Lane, Kew, Surrey, UK. The organism was maintained on slopes of malt agar (pH 5.2) and was subcultured at intervals of 2 weeks.

The organism was induced to sporulate by growth at 30 °C on a medium containing (1^{-1}): $K_3PO_4 \cdot H_2O$, 2 g; KNO_3 , 2 g; $CaCl_2$, 0.25 g; yeast extract, 5 g; glucose, 10 g; Bacto Casamino acids, 5 g; metals solution, 0.1 ml. The metals solution consisted of (ml^{-1}): $MgSO_4 \cdot 7H_2O$, 0.05 mg; $ZnSO_4$, 0.002 mg; $FeSO_4$, 0.002 mg; $MnSO_4$, 0.001 mg; $CuSO_4$, 0.005 mg. The pH was adjusted to 5.2 before autoclaving.

The fungal growth medium comprised (1^{-1}): $NaNO_3$, 5 g; KH_2PO_4 , 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; yeast extract, 0.1 g; aromatic carbon source, 1 g. The pH of the medium was adjusted to 5.2 before autoclaving. Flasks (1 litre) containing 500 ml medium were inoculated with 1 ml of a spore suspension containing 10^5 spores ml^{-1} and incubated in a rotary shaker at 30 °C and 120 r.p.m. Samples were taken for chemical analysis after 3 d.

Synthesis of lignin model compounds. 1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol (compound 1), 3,4-dimethoxy- ω -(2-methoxyphenoxy)acetophenone (compound 2) and 4-hydroxy-3-methoxy-phenyl- ω -(2-methoxyphenoxy)acetophenone (compound 3) were synthesized using modifications to the methods of Alder *et al.* (1952) and Landucci *et al.* (1981).

1-(3,4-Dimethoxyphenyl)-2-(2-phenylphenoxy)ethanol (compound 4), 3,4-dimethoxy- ω -(2-phenylphenoxy)acetophenone (compound 5) and 4-hydroxy-3-methoxyphenyl- ω -(2-phenylphenoxy)acetophenone (compound 6) were synthesized by the same methods but 2-phenylphenol replaced guaiacol.

3,4-Dimethoxy- ω -(2-methoxyphenyl)acetophenone was synthesized with the C-2 carbon atom of the side-chain labelled with ^{14}C using modifications to the method of Landucci *et al.* (1981). Sodium [$1-^{14}C$]acetate solution (250 μCi , 9.25 MBq) was freeze-dried to remove the water prior to the addition of glacial acetic acid (1.5 ml). This was subsequently transferred to a three-necked flask fitted with a condenser, an inlet port for BF_3 gas and a drying tube with an outlet connected to a series of traps containing water to absorb excess BF_3 . Guaiacol (5.80×10^{-3} M) was added and the solution was stirred magnetically in an ice bath and a very slow stream of BF_3 gas was then introduced into the reaction vessel. The solution turned red after approximately 15 min; after 2 h the BF_3 supply was disconnected and a 10% (w/v) solution of sodium acetate was poured into the flask. A yellow precipitate formed and the mixture was transferred into a separating funnel and extracted three times with 10 ml portions of chloroform. The chloroform extract was washed with a saturated solution of Na_2CO_3 and water and was then evaporated under vacuum. The crude product was examined by TLC against an authentic sample of acetovanillone using a solvent system of benzene/ethyl acetate (9:1, v/v) and was recrystallized from carbon tetrachloride to give pale yellow crystals of [^{14}C]acetovanillone (yield 43%).

[^{14}C]Acetovanillone (100 μCi , 3.7 MBq; 5.44×10^{-3} M), dimethyl sulphate (2.44×10^{-2} M) and potassium carbonate (1.11×10^{-2} M) in dry acetone (30 ml) were refluxed for 5 h. The reaction mixture was then cooled and filtered. Excess dimethyl sulphate and acetone were evaporated under a stream of nitrogen. The red/brown solid remaining was extracted with hot cyclohexane until TLC (solvent system was described above) showed that no product remained in the residue. The light brown product was then recrystallized from cyclohexane to leave [^{14}C]acetoveratrone as a light brown powder (yield 55%). This was used to synthesize ^{14}C -labelled 3,4-dimethoxy- ω -(methoxyphenoxy)acetophenone in an identical way to the synthesis of the unlabelled compound (Adler *et al.*, 1952).

All compounds were subjected to spectral analysis (IR, ^{13}C -NMR, 1H -NMR, MS) to ensure their purity before use in degradation studies.

Isolation of intermediates. Mycelia were harvested from the medium by filtration and washed well with distilled water. The spent medium and washings were combined, adjusted to pH 2 with 2 M- H_2SO_4 and then exhaustively extracted with ethyl acetate. The extracts were combined and dried over anhydrous Na_2SO_4 . The drying agent was removed by filtration and the extract was evaporated to dryness on a rotary evaporator. The dark brown residue was dissolved in a small volume of acetone to give a 2% solution for application to TLC plates.

Analytical TLC plates (Whatman LK6 DF silica gel) were used to determine R_F values. For preparative TLC, 1 mm thick Kieselgel 60 GF₂₅₄ on 20 $\times$ 20 cm glass plates was used. The solvent systems were benzene/dioxane/acetic acid (90:25:4, by vol.) and benzene/ethyl acetate (9:1, v/v). Visualization was by UV light. Bands were scraped from preparative plates and the silica gel was extracted five times with acetone. The extract was then filtered using a sintered disc funnel and reduced to a small volume on a rotary evaporator. A control without substrate and an uninoculated control was treated in an identical manner.

Methanol produced from compounds (2) and (5) was determined by GLC. The system used was a Phase-Sep chromatograph with a Spectra-Physica SP4290 integrator. An FID detector was fitted to the instrument and the glass column was packed with 10% PPG on Chromosorb W, 80–100 mesh. The oven temperature was 70 °C and the carrier gas was N_2 (8 p.s.i.). Butanol was used as the internal standard.

Samples of the growth medium (1 ml) were taken at 24 h intervals during growth of *A. flavus* on compounds (2) and (5). These aqueous samples were used to inject directly into the column. Methanol was identified by

comparison of the retention times of peaks from samples with the retention time of an aqueous methanol standard. A control lacking substrate and an uninoculated control were also examined.

The R_f values of intermediates were determined and compared with those of authentic samples. Isolates and authentic samples were also co-chromatographed. IR spectra were obtained on a Perkin-Elmer SP200 spectrophotometer, ^1H -NMR spectra were recorded using a Perkin-Elmer R32 spectrometer and mass spectra were obtained on a Kratos MS 50/DS-55 mass spectrometer. Spectra were always compared with those of authentic samples.

Identification of ^{14}C -labelled intermediates. Triplicate 250 ml flasks, each containing 1 μCi (37 kBq) ^{14}C -labelled compound (3) in 100 ml of growth medium, were incubated with *A. flavus* for 3 d under similar conditions to those used with unlabelled compounds. A sealed system was prepared allowing flasks to be gassed with sterile air. The outlet was attached to a trap containing 20% (w/v) KOH to capture any $^{14}\text{CO}_2$ emitted. The contents of flasks were filtered and extracted using the procedure described above. The concentrated extract was diluted with exactly 1 ml of redistilled acetone to allow application to analytical TLC plates for quantitative radio-analysis. Measured volumes of the acetone solution were applied to TLC plates together with a range of unlabelled standards. The plates were developed and visualized as described above. Spots corresponding to standards were outlined, scraped from the plates and extracted with acetone. Scintillation fluid (5 ml of Cocktail T) was added to the residue after evaporation of the acetone. The radioactivity was measured using a scintillation counter. Uninoculated and substrate controls were used as indicated above.

RESULTS

Identification of intermediates

Extracts from media in which *A. flavus* was grown on compound (1) showed six major bands on TLC. Preparative TLC and spectral analysis of the isolates showed that these corresponded to: 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol (compound 1, unused substrate); 3,4-dimethoxy- ω -(2-methoxyphenoxy)acetophenone (compound 2); 4-hydroxy-3-methoxy- ω -(2-methoxyphenoxy)acetophenone (compound 3); guaiacol; vanillic acid; and protocatechuic acid. The sequence in which the compounds were formed was determined by a replacement experiment using metabolites of compound (1) as the sole carbon source. The results are shown in Table 1 and the proposed degradation sequence is shown in Fig. 1.

Extracts from media in which *A. flavus* was grown on compound (4) also showed six major bands on TLC. Preparative TLC and spectral analysis of the isolates showed that these corresponded to: 1-(3,4-dimethoxyphenyl)-2-(2-phenylphenoxy)ethanol (compound 4, unused substrate); 3,4-dimethoxy- ω -(2-phenylphenoxy)acetophenone (compound 5); 4-hydroxy-3-methoxy- ω -(2-phenylphenoxy)acetophenone (compound 6); 2-phenylphenol; vanillic acid; and protocatechuic acid. The sequence in which the compounds were formed was determined by a replacement experiment using metabolites of compound (4) as the sole carbon source. The results are shown in Table 1. The proposed degradation sequence is shown in Figure 1.

Table 1. Replacement experiments to establish the sequence of degradation of compounds (1) and (4) by *A. flavus*

Substrate	Compounds isolated					
	Compound 1	Compound 2	Compound 3	Guaiacol	Vanillate	Proto-catechuate
Compound 1	+	+	+	+	+	+
Compound 2	—	+	+	+	+	+
Compound 3	—	—	+	+	+	+
Substrate	Compound 4	Compound 5	Compound 6	2-Phenyl-phenol	Vanillate	Proto-catechuate
	Compound 4	Compound 5	Compound 6	2-Phenyl-phenol	Vanillate	Proto-catechuate
Compound 4	+	+	+	+	+	+
Compound 5	—	+	+	+	+	+
Compound 6	—	—	+	+	+	+

Fig. 1. Proposed sequence of degradation of compounds (1) and (4) by *A. flavus*.

Fig. 2. Methanol production by *A. flavus* during growth on compounds (2) and (5). Samples of the growth medium containing either compound (2) (initial amount 332 μmol ; ●) or compound (5) (initial amount 387 μmol ; ○) as carbon source were taken at 24 h intervals and assayed for methanol by GLC as described in Methods.

When the aqueous medium which contained compound (2) as sole carbon source was assayed for methanol by GLC, a positive result was obtained. Its identity was confirmed by co-chromatography with an authentic sample. Methanol production over a 7 d growth period is shown in Fig. 2. No methanol was detected in the controls. The maximum amount of methanol produced during metabolism of compounds (2) and (5) was almost equivalent to the theoretically calculated amounts from three and four methoxyl groups respectively (Fig. 2).

Identification of ^{14}C -labelled intermediates

The first monomeric units produced during metabolism of compound (2) by *A. flavus* were shown to be vanillic acid and guaiacol (Table 1). When $[2-^{14}\text{C}]$ compound (2) was used as substrate, $[2-^{14}\text{C}]$ compound (2) (unused substrate), $[2-^{14}\text{C}]$ compound (3) and $[^{14}\text{C}]$ guaiacol were detected after degradation by *A. flavus* for 3 d (Table 2). No other radiolabelled intermediates were detected.

Table 2. Radioactive compounds isolated after incubation of *A. flavus* with [2-¹⁴C]compound (2)

R_F values were obtained on Whatman LK6 DF silica gel plates. Solvent system 1 was benzene/ethyl acetate (9:1, v/v); solvent system 2 was benzene/dioxane/acetic acid (90:25:4, by vol.). R_F values of authentic compounds are shown in parentheses.

Compound isolated	R_F values		Percentage of original activity
	Solvent system 1	Solvent system 2	
Compound (2)	0.18 (0.18)	0.75 (0.74)	63.76
Compound (3)	0.12 (0.11)	0.66 (0.65)	5.08
Guaiacol	0.50 (0.48)	0.75 (0.76)	3.86

DISCUSSION

The first step in the degradation of compounds (1) and (4) by *A. flavus* is the oxidation of the α -hydroxyl group to a carbonyl group. This may be analogous to the process by which white-rot fungi attack lignin and model compounds as they tend to produce a modified lignin with increased amounts of C α -carbonyl groups compared to undecayed lignin (Kirk & Chang, 1974). Examples of this type of reaction are also found in non-white-rot fungi and bacteria (Crawford *et al.*, 1973; Higuchi, 1980; Pelmont *et al.*, 1985). However, the enzyme systems involved in this oxidation may differ considerably with different organisms and include an NAD⁺-dependent dehydrogenase (Pelmont *et al.*, 1985), a H₂O₂-dependent extracellular enzyme (Renganathan *et al.*, 1986), and a phenol oxidase (Shimada, 1980). Several lignin-degrading *Fusarium* species have been reported to secrete an enzyme which catalyses the oxidation of the primary alcohol group in the α,β -unsaturated side-chain of guaiacylglycerol- β -coniferyl ether to the corresponding aldehyde. The enzyme appeared to be an aromatic alcohol oxidase (Janshekar & Fiechter, 1983). The oxidation state at the α -carbon is thought to be important to the mechanisms by which the C α -C β bond between monomers is broken (Fenn & Kirk, 1984).

The second degradation step for compounds (1) and (4) is the demethylation of the 4-position methoxyl group. Lignin does not contain 4-methoxyl groups but the use of model compounds which include these is common. The demethylation of other methoxyl groups in lignin is typical in many groups of micro-organisms which attack the lignin polymer.

Demethylation mechanisms have not received as much attention as those for cleavage of the β -aryl ether in lignin research but there are several mechanisms proposed in the literature. Dagley (1978) proposed a hydroxylation of the methyl ether to form a hemiacetal. This is thought to undergo spontaneous aldol cleavage to give formaldehyde and the corresponding phenol. Donnelly & Dagley (1981) and Kersten *et al.* (1982) devised a mechanism in which the ether linkage was converted via a series of reactions to an ester which was hydrolysed to release methanol and give various intermediates of aromatic ring degradation. More recently, Kersten *et al.* (1985) have proposed a demethylation by the ligninase system involving a one-electron oxidation of the methoxybenzene by the oxidized enzyme and subsequent addition of water to the cation radical. This is followed by or simultaneous with methoxy elimination as methanol. Previous work on the demethylation of coniferyl alcohol by *A. flavus* detected methanol but not formaldehyde (Iyayi & Dart, 1982).

Metabolism of compounds (3) and (6) involved cleavage of the dimer in each case to yield guaiacol and vanillic acid from compound (3) and 2-phenylphenol and vanillic acid from compound (6). These products could have resulted from cleavage of either the aryl ether bond or the C α -C β bond. Direct cleavage of the C α -C β bond could have produced veratrole and vanillic acid with subsequent demethylation of veratrole to guaiacol (Fig. 3). Although veratrole was not found, this could be explained by a very rapid demethylation to guaiacol. Conversely, cleavage of the aryl ether bond of compound (3) may have resulted in guaiacol and a C6-C2 compound which was rapidly converted to vanillic acid. A similar explanation may be appropriate for the products of the degradation of compound (6).

The detection of radiolabelled guaiacol as a degradation product of [2-¹⁴C]compound (2)

Fig. 3. Alternative hypothesis for cleavage of compound (3) by *A. flavus*.

indicated that the $C\alpha-C\beta$ bond had been cleaved and not the aryl ether bond, with a demethylation reaction occurring either before or after the cleavage. Demethylation before $C\alpha-C\beta$ cleavage could have resulted in compound (7) (Fig. 3).

The quantity of methanol detected during growth of *A. flavus* on compound (2) also indicated that cleavage of the $C\alpha-C\beta$ bond occurred. The maximum amount of methanol detected (Fig. 2) was almost equivalent to the theoretically calculated amount derived from four methoxyl groups. Three of these are present in compound (2) itself. A further methoxyl group could have arisen from cleavage of the $C\alpha-C\beta$ bond. This could have appeared in veratrole if the $C\alpha-C\beta$ bond of compound (3) had been cleaved, or in guaiacol if compound (3) had been converted to compound (7) prior to $C\alpha-C\beta$ cleavage. Neither veratrole nor compound (7) was detected as an intermediate, but this may have been due to a rapid metabolism as explained above. The maximum amount of methanol produced during metabolism of compound (5) (Fig. 2) also almost equalled the theoretically calculated amount. This also supports a $C\alpha-C\beta$ cleavage of compound (5).

No further degradation products of guaiacol or 2-phenylphenol were detected, but protocatechuic acid was found, suggesting that vanillic acid had been demethylated. Conversion of vanillic acid to protocatechuic acid and its subsequent cleavage in *A. flavus* has been previously established (Iyayi, 1981).

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