Bacterial Metabolism of Substituted Phenols. Oxidation of 3-Methyl-4-(methylthio)phenol by *Nocardia* sp. DSM 43251

Hans Georg Rast, Gabriele Engelhardt, Peter R. Wallnöfer,* Linthard Oehlmann, and Klaus Wagner

3-Methyl-4-(methylthio)phenol is oxidized by the soil isolate *Nocardia* sp. DSM 43251 in the presence of a carbon source supporting growth. When the bacteria were grown on acetate the compound was mainly oxidized via the 2,3-("meta")-fission pathway without accumulation of intermediate metabolites. In the presence of sucrose as growth substrate, however, 3-methyl-4-(methylthio)catechol and 4-methyl-5-(methylthio)catechol accumulated as transient intermediates. The products of ring fission of these metabolites were 2,5-dihydro-3-(methylthio)-4-methyl-5-oxofuran-2-acetic acid and 2,5-dihydro-2-methyl-3-(methylthio)-5-oxofuran-2-acetic acid, which resulted from a 1,2-("ortho")-fission of the aromatic nucleus. The compounds were identified by their mass and NMR spectra and were not further degraded. Crude extracts of cells grown on acetate as cosubstrate contained considerable catechol 2,3-dioxygenase activity and low catechol 1,2-dioxygenase activity; those of cells grown with sucrose, however, had a higher activity of 1,2-cleavage than of 2,3-cleavage.

Hydrolysis and sulfoxidation are common pathways for the degradation of organophosphorus insecticides in soil. From thioaryl-substituted insecticides (Figure 1) the corresponding phenols and their sulfinyl and sulfonyl derivatives are released as main products of decomposition (Fest and Schmidt, 1973; Bull et al., 1976). These phenolic compounds are strongly adsorbed to the soil organic matter and are therefore assumed to be responsible for the formation of bound residues. Mineralization of methylthiophenols has not yet been described although much information is available on the microbial metabolism of substituted phenols.

Recently we reported the oxidation of 4-(methylthio)- and 4-(methylsulfinyl)phenol by the soil isolate *Nocardia* sp. DSM 43251 (Engelhardt et al., 1977). The compounds are cometabolized by hydroxylation of the benzene ring with the formation of the substituted catechol followed by a 2,3-ring cleavage ("meta" fission) to give 2-hydroxy-5-(methylthio)- or 2-hydroxy-5-(methylsulfinyl)muconic semialdehyde. Oxidation of 4-(methylthio)phenol to 4-(methylsulfinyl)phenol represented a bypath of 4-(methylthio)phenol metabolism. The present report describes the oxidation of 3-methyl-4-(methylthio)phenol, which is released by hydrolysis from the insecticide fenthion and the nematicide fenamiphos (Figure 1), with particular emphasis on the structure of intermediates and the enzymes and mode of ring fission involved.

MATERIALS AND METHODS

Chemicals. 3-Methyl-4-(methylthio)phenol and 4-(methylthio)phenol were purchased from EGA Chemie, Steinheim, Germany. 3-Methyl-4-(methylthio)catechol and 4-methyl-5-(methylthio)catechol were isolated from growth medium after a 2-day incubation period. Coenzymes were purchased from Boehringer, Mannheim, Germany. All other chemicals are commercially available compounds.

Culture Conditions. Maintenance of *Nocardia* sp. DSM 43251 was performed as described (Engelhardt et al., 1976). Degradation studies were performed using Hegeman's mineral base (Hegeman, 1966) modified by addition of 0.02% yeast extract. Cultures were incubated on a rotary shaker (New Brunswick G 10) at 28 °C and 220 rpm. Liquid cultures were grown in 100-mL Erlenmeyer flasks containing 25 mL of mineral base with the addition of 0.01% of 3-methyl-4-(methylthio)phenol and 0.4% of acetate, sucrose, fumarate, succinate, glycerol, or ethanol, respectively, as carbon source. For large-scale preparations of metabolites, cultivation was performed in 2-L Erlenmeyer flasks with 1 L of medium.

Determination of Degradation Rates. Degradation of 3-methyl-4-(methylthio)phenol was assayed by extraction of residual substrate and metabolites, followed by quantitative UV analysis as described (Engelhardt et al., 1977).

Estimations. Growth of the bacteria was assayed by measuring the turbidity of the cultures at 578 nm with a Beckman Model DB spectrophotometer. Protein was calculated by the method of Lowry et al. (1951) using bovine serum albumin as standard. Melting points were determined using a Kofler hot stage (Reichert, Austria). Mass spectra (MS) were recorded on a Varian MAT CH 7A or Varian MAT CH 5 mass spectrometer and nuclear magnetic resonance (NMR) spectra on a Bruker WP 60 Fourier transform instrument. Infrared absorption spectra were obtained using a Perkin-Elmer 521 instrument.

Detection Reagents. Phenolic substances were detected by spraying with Folin-Ciocalteau reagent, diluted 1:3 with deionized H_2O; *o*-dihydroxyphenolic compounds and enols were detected by spraying with 3% $FeCl_3$ in H_2O.

Isolation and Identification of Metabolites. Metabolites were each isolated from a 5-L culture grown with 0.01% 3-methyl-4-(methylthio)phenol and 0.4% sucrose as carbon source. The concentration of the catechol derivatives peaked after 2 days of incubation and that of the ring fission products after 6 days. The cells were separated by centrifugation at 5000*g* and the products isolated.

Preparation of Crude Extracts and Enzyme Assays. Crude extracts were obtained by the alumina grinding technique as described by Englehardt et al. (1977).

Specific activities of crude extracts were determined spectrophotometrically and by measuring O_2 consumption with an oxygen electrode. Spectrophotometrical measurement of catechol 1,2-dioxygenase was performed according to Gibson (1970), of catechol 2,3-dioxygenase according to Kojima et al. (1961) with 4-(methylthio)-catechol as substrate (E = 34 300 at pH 7.5) (Engelhardt et al., 1977). Polarographic oxygen determinations were performed with a WTW-electrode (Wissenschaftlich Technische Werkstätten, Weilheim, Germany). The assay

Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Abt. Pflanzenschutz, Menzingerstr. 54, 8000 München 19, Germany (H.G.R., G.E., P.R.W.), and Forschung und Entwicklung, Bayer AG, Leverkuseng and Abteilung Pflanzenschutz, Anwendungstechnik, Biologische Forschung, Bayer AG, Leverkusen (L.O., K.W.).

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Thioaryl substituted insecticides and nematicides

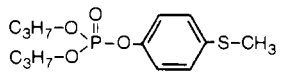
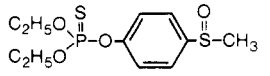
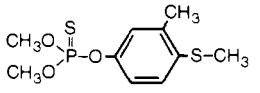
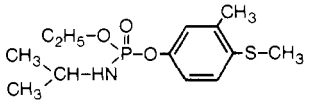
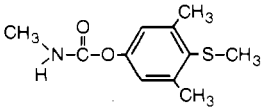
chemical structure	common name
	Propaphos
	Fensulfothion
	Fenthion
	Fenamiphos
	Mercaptodimethur

Figure 1. Thioaryl-substituted insecticides and nematicides.

system contained, in a total volume of 5.2 mL, 500 μmol of phosphate buffer, 1 μmol of substrate, and 0.1 mL of crude extract (14–18 mg of protein/mL). One unit is defined as the amount of enzyme oxidizing 1 μmol of substrate/min at 30 °C. Specific activity is defined as the number of enzyme units per milligram of protein.

RESULTS AND DISCUSSION

Cooxidation of 3-Methyl-4-(methylthio)phenol in the Presence of Different Substrates. Since concentrations of 3-methyl-4-(methylthio)phenol above 0.02% prevented growth of *Nocardia* sp. DSM 43251 entirely and no significant growth of the organism could be observed with 0.01 and 0.02% of the compound as sole source of carbon, oxidation of the compound under the conditions of cometabolism was studied. The rate of metabolism as well as that of accumulation of intermediate products depended greatly on the kind of carbon source used.

During growth on 0.4% acetate, 0.01% of 3-methyl-4-(methylthio)phenol was completely removed from the medium within 10 days. The only metabolite detected in the medium was 3-methyl-4-(methylsulfinyl)phenol (Figure 2). Although crude extracts of cells contained considerable amounts of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities, neither a substituted catechol nor ring cleavage products of 1,2- and 2,3-fission accumulated in the medium. When incubated with 0.4% sucrose, 0.01% of 3-methyl-4-(methylthio)phenol was oxidized completely within 4 days of growth (Figure 3). The compound was transformed to 3-methyl-4-(methylthio)catechol (I) and 4-methyl-5-(methylthio)catechol (II) with a maximum of accumulation after 2 days. The products of ring cleavage of these intermediates were 2,5-dihydro-3-(methylthio)-4-methyl-5-oxofuran-2-acetic acid (III) and 2,5-dihydro-2-methyl-3-(methylthio)-5-oxofuran-2-acetic acid (IV), which result from 1,2-fission of the aromatic nucleus. Small amounts of 3-methyl-4-(methylsulfinyl)phenol and the sulfinyl analogues of the above metabolites were detected also.

The same metabolites in minor concentrations were identified when the organism was grown in the presence of fumarate, succinate, and glycerol as carbon sources,

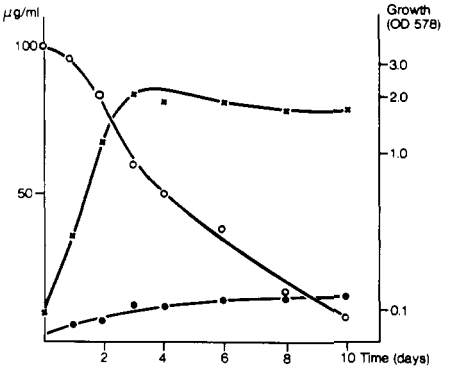


Figure 2. Degradation of 3-methyl-4-(methylthio)phenol in the presence of acetate as carbon source. Symbols: (x–x) growth (OD_{578}), (○–○) oxidation of 3-methyl-4-(methylthio)phenol, (●–●) accumulation of 3-methyl-4-(methylsulfinyl)phenol.

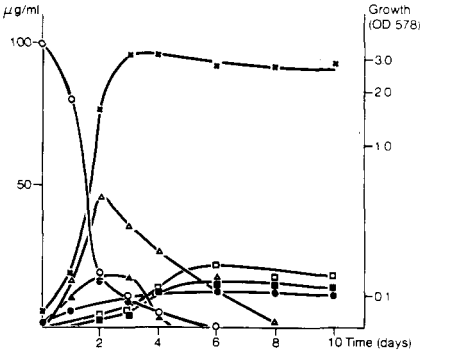


Figure 3. Degradation of 3-methyl-4-(methylthio)phenol in the presence of sucrose as carbon source. Symbols: (x–x) growth (OD_{578}), (○–○) oxidation of 3-methyl-4-(methylthio)phenol, (●–●) accumulation of 3-methyl-4-(methylsulfinyl)phenol, (△–△) transient accumulation of 3-methyl-4-(methylthio)catechol, (▲–▲) transient accumulation of 4-methyl-5-(methylthio)catechol, (□–□) formation of 2,5-dihydro-3-(methylthio)-4-methyl-5-oxofuran-2-acetic acid, (■–■) formation of 2,5-dihydro-2-methyl-3-(methylthio)-5-oxofuran-2-acetic acid.

respectively. Ethanol, however, totally depressed the rate of oxidation of 3-methyl-4-(methylthio)phenol.

Identification of Metabolites. All metabolites found during cooxidation with sucrose as cosubstrate were identified by means of their physical properties and mass and NMR spectra. TLC data, UV absorption maxima, melting points and mass spectral data for all metabolites are summarized in Table I.

Figure 4 shows the proton NMR spectra of the two isomeric catechols 3-methyl-4-(methylthio)catechol (I) and 4-methyl-5-(methylthio)catechol (II) in hexadeuteriobenzene as solvent. In the top diagram it will be seen that in the aromatic range there is an AB system for the two ortho protons centered at 6.55 ppm, which corresponds to structure I. In the bottom diagram two singlets appear in the aromatic range at 6.33 and 6.60 ppm for the two para protons, which correspond to structure II.

The mass spectra of both compounds are identical. Therefore, it is not possible to distinguish between the two structures.

The simple proton NMR spectrum of 2,5-dihydro-2-methyl-3-(methylthio)-5-oxofuran-2-acetic acid (Figure 5) (III) shows a signal at 1.60 ppm as a singlet, which corresponds to a methyl group bound to a quaternary carbon atom. The next singlet at 2.50 ppm belongs to the methylthio group. An AB system with the center at 2.83 ppm (J_{AB} geminal = 16 Hz) indicates a methylene group, and the olefinic proton appears at 5.80 ppm. The infrared spectrum in benzene as solvent shows the typical carbonyl

Table I. Physical Data of the Discussed Compounds

compound	R_f values[a] A	B	C	mp, °C	UV max (methanol)	molar extinction coeff. (methanol)	mass spectrum, M^+
3-methyl-4-(methylthio)phenol	0.50	0.82		52-54	232	8700	154 m/e (100%)
3-methyl-4-(methylsulfinyl)phenol		0.22	0.39	134-135	246	10600	170 m/e (79%)
I	0.35	0.70		42	290	2300	170 m/e (100%)
II	0.26	0.66		20	298	3100	170 m/e (100%)
III		0.34	0.54	114-116	274	10500	202 m/e (68%)
IV		0.32	0.49	139-141	264	12800	202 m/e (63%)

[a] A, benzene/acetic acid (9:1); B, benzene/dioxane/acetic acid (90:25:4); C, ethyl acetate/chloroform/acetic acid (60:40:5).

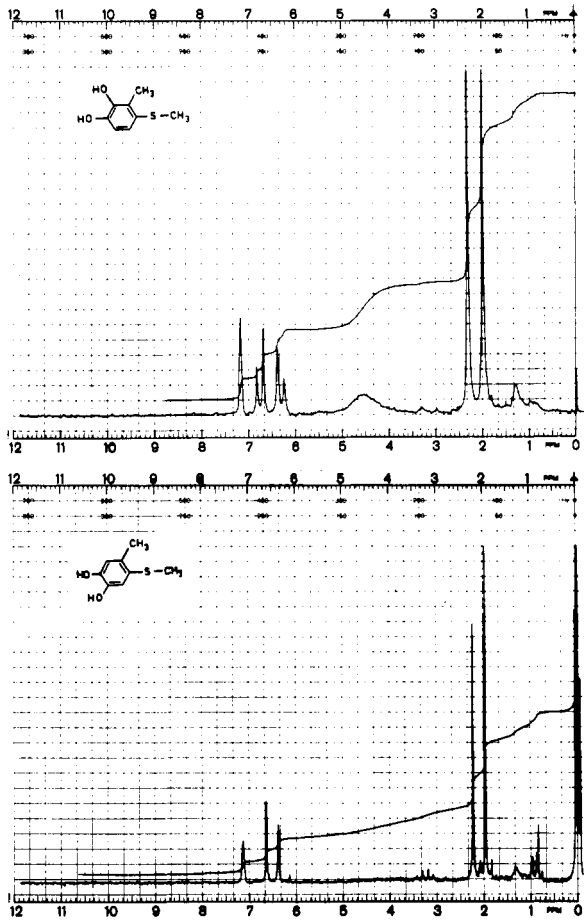


Figure 4. 60-MHz ^1H NMR spectra of 3-methyl-4-(methylthio)catechol (I) and 4-methyl-5-(methylthio)catechol (II) in C_6D_6.

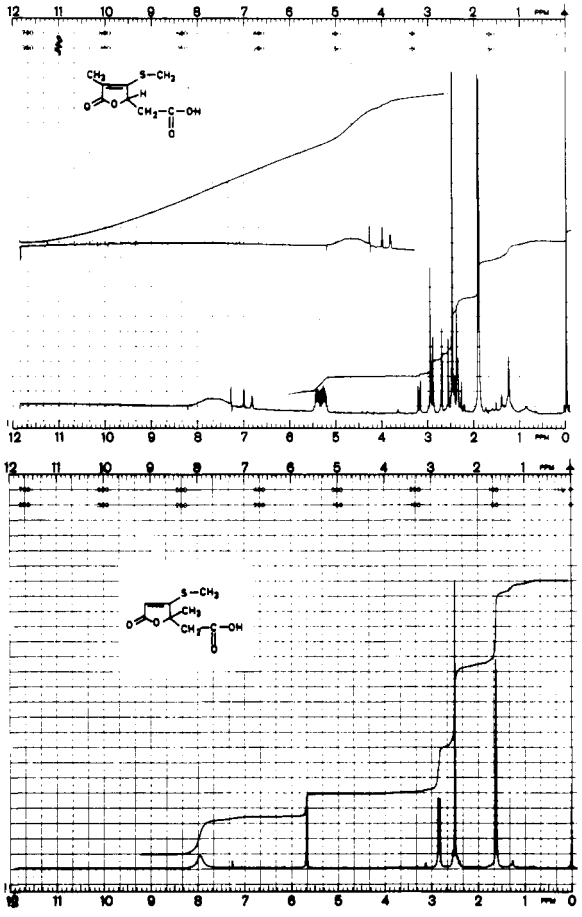


Figure 5. 60-MHz ^1H NMR spectra of 2,5-dihydro-3-(methylthio)-4-methyl-5-oxofuran-2-acetic acid (III) and 2,5-dihydro-2-methyl-3-(methylthio)-5-oxofuran-2-acetic acid (IV) in $CDCl_3$.

bands of five-membered unsaturated ring lactones at 1760 and 1715 cm^{-1}.

In contrast to the NMR spectrum of the lactone just discussed, the proton NMR spectrum of 2,5-dihydro-3-(methylthio)-4-methyl-5-oxofuran-2-acetic acid (Figure 5) (IV) shows a doublet at 1.90 ppm, the SCH_3 group at 2.48 ppm and an AB system centered at 2.80 ppm, which is further split by the 2-H with the coupling constants J_{H,CH_2} = 3.8 Hz and J_{H,CH_2} = 8.0 Hz. The 2-H appears at 5.33 ppm as a doublet of multiplets. The infrared spectrum in chloroform also shows the C=O absorption bands at 1760 and 1715 cm^{-1}.

Oxidations Catalyzed by Extracts. Cells grown with sucrose contained considerable catechol 1,2-dioxygenase activity (0.0068 unit/mg of protein) and low catechol 2,3-dioxygenase activity (0.006 unit/mg of protein), whereas acetate-grown cells contained a sixfold higher "meta"-fission activity (0.0036 unit/mg of protein) and a lower "ortho"-fission activity (0.0022 unit/mg of protein).

Cells and crude extracts containing high catechol 1,2-dioxygenase activity and no catechol 2,3-dioxygenase activity (0.56 unit/mg of protein, cells grown on benzoate as source of carbon) catalyzed the oxidation of I and II to III and IV, respectively. In contrast, cells and crude extracts with high catechol 2,3-dioxygenase activity and no catechol 1,2-dioxygenase activity (0.06 unit/mg of protein, cells grown with 4-(methylsulfinyl)phenol plus fumarate as source of carbon [(Engelhardt et al., 1977)] oxidized I and II with no significant accumulation of ring fission products.

These results might explain the different accumulation of degradation products of 3-methyl-4-(methylthio)phenol during growth with acetate or sucrose as cosubstrate, respectively. In the presence of acetate, larger amounts of the "meta"-pathway enzymes were induced, which led to complete oxidation of the compound as was shown for

4-(methylthio)phenol (Engelhardt et al., 1977), but without accumulation of intermediates. "Ortho" cleavage of 3-methyl-4-(methylthio)phenol during growth on sucrose, however, led to the formation of two metabolites (III and IV) which underwent no further degradation in our experimental conditions.

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Trifluralin Degradation and Binding in Soil

Willis B. Wheeler,* George D. Stratton, Robert R. Twilley, Li-Tse Ou, David A. Carlson, and James M. Davidson

The herbicide trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) was applied to Webster silty clay loam (fine-loamy, mixed, mesic Typic Haplaquoll) and Cecil sandy loam (clayey, kaolinitic, thermic Typic Hapludult) at 10, 1000, and 20 000 μg per gram of soil (ppm) as technical grade material and as a commercial formulation, both fortified with [^{14}C]trifluralin. All treatments were incubated approximately 12 weeks under aerobic conditions. Soil samples were taken biweekly and analyzed for parent trifluralin, metabolic products, and "bound" ^{14}C. The mono- and didealkyl products were detected as well as two benzimidazole derivatives. The percentage of "bound" ^{14}C increased with time; Webster soil bound a higher percentage of ^{14}C than did Cecil soil. No observable differences were detected in rates or pathways of metabolism.

Trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine), a selective preemergence, soil incorporated herbicide, is used widely to control numerous grasses and broadleaf weeds in soybeans and cotton. Degradation, persistence, photodecomposition, volatility, and other biological and physiochemical properties of trifluralin and related compounds have been reviewed by Helling (1976) and Probst et al. (1969, 1975). In general, trifluralin when incorporated into soil at agricultural application rates has been reported to be moderately persistent (Parka and Tepe, 1969; Probst et al., 1967; Rahman, 1977; Savage, 1973). Degradation rate was directly correlated to temperature and moisture content of soil (Zimdahl and Gwynn, 1977). Kearney et al. (1976) reported that 3, 5, and 7 months postapplication of [^{14}C]trifluralin ($^{14}CF_3$) recoveries of applied ^{14}C were 91, 78, and 69%, respectively. Messersmith et al. (1971) also reported that 3–5% of ^{14}C-labeled trifluralin (propyl-1-^{14}C) was mineralized to $^{14}CO_2$ during 30 days of incubation in soils after application at a rate of 1 μg/g of soil.

Volatility is an important mechanism for losses from soil; high soil temperature and moisture enhance such losses (Helling, 1976; Parochetti and Hein, 1973).

The degradation pathways of trifluralin have been reviewed by Probst et al. (1975). Probst et al. (1967) and Kearney et al. (1976) reported that trifluralin was dealkylated to the *N*-propyl and to the unsubstituted α,α,α-trifluoro-2,6-dinitro-*p*-toluidine and reduced to the 2-amino and 2,6-diamino analogues. Kearney et al. (1976) also detected two benzimidazole derivatives, 2-ethyl-7-nitro-1-propyl-5-(trifluoromethyl)benzimidazole and 2-ethyl-7-nitro-5-(trifluoromethyl)benzimidazole in Metapeake Loam soil. Leites and Crosby (1974) also reported a number of benzimidazole derivatives from the ultraviolet irradiation of trifluralin in water or in aqueous methanol.

Kearney et al. (1976) reported further that a certain percentage of the material applied as [^{14}C]trifluralin was unextractable or "bound". At 3, 5, and 7 months this represented 14, 24, and 25%, respectively, of the applied ^{14}C.

The fate of pesticides when applied at high levels to soils has not been extensively investigated. Stojanovic et al. (1972) used CO_2 evolution as an indication of trifluralin degradation after application to soil at 5 tons/acre (5000 ppm). It was reported that analytical grade trifluralin did not degrade, but the formulated material did.

A recent report by Ou et al. (1978) suggests that CO_2 evolution may not accurately reflect degradation. Addition of ^{14}C pesticide and the monitoring for $^{14}CO_2$ is a more reliable indicator.

Wolfe et al. (1973) and Staiff et al. (1975) reported that organophosphate insecticides parathion and azinphos methyl showed increased persistence in soil when applied at rates of 35 000 to 95 000 ppm. Parathion residues were

University of Florida, IFAS, Pesticide Research Laboratory, Gainesville, Florida 32611 (W.B.W., G.D.S., R.R.T.), University of Florida, IFAS, Soil Science Department, Gainesville, Florida 32611 (L.-T.O., J.M.D.), and Insects Affecting Man Research Laboratory, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Gainesville, Florida 32604 (D.A.C).

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