

Degradation of Methoxylated Benzoic Acids by a *Nocardia* from a Lignin-Rich Environment: Significance to Lignin Degradation and Effect of Chloro Substituents

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Strain A81 of *Nocardia corallina* hydroxylates or demethylates *p*-anisic acid to *p*-hydroxybenzoic acid and isovanillic acid. It demethylates veratric acid to a mixture of vanillic and isovanillic acids. These are both demethylated to protocatechuic acid, which undergoes ring cleavage to afford β -carboxy-*cis-cis*-muconic acid. The intermediacy of protocatechuic acid in the catabolic pathway of veratric acid was confirmed by blocking ring cleavage with an additional substituent in the ring: 5-chlorovanillic acid was demethylated to 5-chloro-protocatechuic acid, which accumulated. Chloro substituents in the ring of other methoxylated benzoic acids also arrested their normal metabolism by the *Nocardia*: an *ortho*-chloro substituent thwarted both demethylation and ring-opening. *ortho*-Hydroxylation of *p*-methoxybenzoic acid to isovanillic acid was unaffected by a chlorine *ortho* to the methoxyl group. Washed whole cells of veratric acid-grown *N. corallina* A81 produced no detected structural changes in an isolated lignin. The implications of this observation are discussed.

There is a growing interest in the potential use of microorganisms to convert or destroy plant materials or residues or industrial by-products derived from lignocellulosics. Substrates for such bioconversions include cattle manure from feedlots (2, 4), wastepaper (44), and pulpmill wastes (1).

Lack of success of most bioconversion attempts of this kind can be ascribed to the lignin content of the substrate. The intimate association of plant polysaccharides with lignin (13) presents accessibility barriers and metabolic blocks to digestion of the carbohydrates by microorganisms (16). Efficient bioconversion of lignified plant materials is therefore contingent upon ability of an organism to overcome the lignin barrier. Some microorganisms, mainly holobasidiomycetes associated with white rot of wood, are capable of decomposing lignin together with wood polysaccharides. Other microorganisms associated with brown rot decay can degrade wood polysaccharides, but they leave a residue of modified lignin (23).

Lignin is a peculiar natural polymer in that it does not have a readily hydrolyzable bond

recurring at periodic intervals along a linear backbone. Instead, it is a three-dimensional amorphous copolymer derived from plain and mono- and di-methoxylated *p*-coumaryl alcohols by a process of phenol oxidation that leads to a random distribution of carbon-carbon and strong ether linkages between units, there frequently being more than one linkage between two neighboring phenylpropanoid monomers (15, 39). This structure is not amenable to chemical hydrolysis (15, 39). In its biodegradation, therefore, hydrolytic depolymerization cannot be the initial step.

How biodegradation of lignin occurs is still largely a matter of speculation (23). It has been established from work with lignin models that phenol oxidases can cleave some carbon-carbon bonds in certain lignin structures (25, 26). However, recent work with lignin itself has shown that these enzymes cause only removal of peripheral groups while polymerizing the core of lignin (21). An alternative pathway for possibly altering lignin with microorganisms without initial depolymerization is via demethylation of its methoxyl substituents and subsequent

cleavage of its aromatic rings (15). These are activities commonly associated with bacteria capable of dissimilating substituted aromatic compounds (9). Possibly bacteria can attack similar structures within the lignin polymer. We therefore screened environments rich in lignin for bacteria capable of growth on wood flour and on simple methoxylated aromatic compounds. Vanillic and veratric acids were chosen for screening because they have the ring substitution patterns found, respectively, in a majority of nonetherified and etherified structural elements of conifer lignin (Fig. 1) (15, 39). One of the most promising strains in terms of growth rate and range of aromatic compounds used for growth has been studied in detail as to its effects on veratric acid and related compounds and an isolated lignin. Because it is noncellulolytic, this organism was possibly growing on lignin or lignin-derived structures during enrichment on wood flour.

The organism, designated as *Nocardia corallina* A81, demethylates veratric acid to a mixture of vanillic and isovanillic acids; these are metabolized via protocatechuic acid. *p*-Anisic acid is both hydroxylated to isovanillic acid and demethylated to *p*-hydroxybenzoic acid. Data establishing these transformations are presented and preliminary experiments with a spruce cellulase lignin are discussed.

The intermediacy of protocatechuic acid in the catabolism of veratric acid was established by blocking ring cleavage with an additional substituent in the ring: 5-chlorovanillic acid was demethylated to 5-chloroprotocatechuic acid, which was not metabolized. The success of this approach led us to examine effects of chloro substituents on demethylation and ring cleavage in related compounds. Results on cometabolism of these compounds are presented.

MATERIALS AND METHODS

Organism. The bacterium was isolated from a lagoon used for disposal of manure and wood-waste animal bedding (G. S. Fischer, M.S. thesis, Univ. of

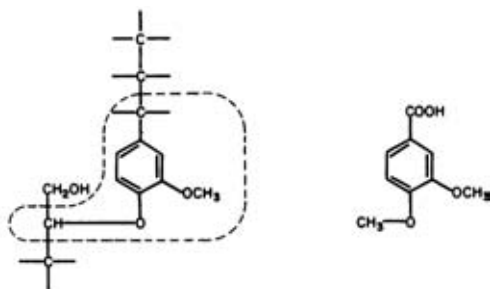


FIG. 1. The relationship between certain common structural elements of lignin and veratric acid.

Wisconsin, Madison, 1968). Lignous materials had accumulated in a thick layer at the bottom of this impoundment, furnishing a natural lignin enrichment.

The organism is coral colored and possesses a characteristic nocardial life cycle. Our isolate is nonmotile, noncellulolytic, and does not liquefy gelatin. It grows on lactose, sucrose, sorbitol, glucose, or mannose as major carbon source with no acid or gas formation. It fails to grow on cellobiose. It produces a slightly alkaline reaction on litmus milk, reduces nitrate to nitrite, and does not produce H_2S . It shows acid-fastness after 8 to 10 h when grown on Difco nutrient agar, but the acid-fast test rapidly becomes negative after 24 h.

This description closely fits that of the species *Nocardia corallina*; however, our identification must still be considered tentative because of the uncertain taxonomy of this group of microorganisms (27, 28). Our isolate is designated *N. corallina* A81.

Semidefined medium, stock cultures, and culture of the organism. In all experiments, cells were grown at 30 C in a basal mineral salts medium containing the following in grams per 100 ml: $(NH_4)_2SO_4$, 0.01; NaCl, 0.01; $MgSO_4 \cdot 7H_2O$, 0.02; $CaCl_2$, 0.001; yeast extract (Difco), 0.001; K_2HPO_4 , 0.1; and KH_2PO_4 , 0.05, at a final pH of 7.2. Aromatic substrates were added to a concentration of 0.25 g/liter. Higher concentrations of some substrates inhibited growth.

Stock cultures were maintained on Difco nutrient agar slants which were stored at 4 C; transfers were made every 2 weeks.

Compounds. 3-Chloro-4-methoxybenzoic acid (XII) (Roman numerals refer to structural formulae shown in Fig. 5 and 6) was purchased from K & K Chemical Co. All nonchlorinated compounds commercially available were purchased from Aldrich Chemical Co. Satisfactory infrared (IR), ultraviolet (UV), and proton magnetic resonance (PMR) spectra were obtained for all compounds.

Isovanillin was oxidized with silver oxide (33, 34), and the product was recrystallized from ethanol-water (85% yield, m.p. 253 C). PMR spectrum (shift, multiplicity, integration ratio, assignment, miscellaneous) in deuteroacetone: $\delta = 3.95$, singlet, three, OCH_3 ; 7.69 and 7.07 double doublets, three, aromatic protons, $J_{5,6} = 9$ Hz and $J_{2,6} = 2.6$ Hz.

5-Chlorovanillin (14) was oxidized with silver oxide (33, 34) to 5-chlorovanillic acid, m.p. 252 C (PMR in $CDCl_3/d_6$ -dimethylsulfoxide [DMSO]: $\delta = 3.92$, singlet, three, OCH_3 ; 6.37, broad singlet, $COOH/OH/H_2O$, removed by D_2O ; 7.45 and 7.70, doublets, one each, aromatic protons, $J_{2,6} = 2$ Hz).

5-Chlorovanillin was methylated (10) to 5-chloroveratraldehyde (m.p. 56 C), which was oxidized (30) to 5-chloroveratric acid (X), m.p. 191 C (PMR in deuteroacetone: $\delta = 3.90$ and 3.97, singlets, three each, two OCH_3 ; 7.58 and 7.66, doublets, one each, aromatic protons, $J_{2,6} = 1.8$ Hz).

Vanillin acetate (m.p. 76 C) was nitrated with fuming, red nitric acid (5) at 5 to 10 C, giving 2-nitrovanillyl acetate (m.p. 85 C). This was reduced with $FeSO_4/NH_3$ (36) to 2-aminovanillin, m.p. 186 C,

which was diazotized (37) and poured into a solution of cuprous chloride to give 2-chlorovanillin, m.p. 127 to 128 C. This was oxidized (33, 34) to 2-chlorovanillic acid (XVI), m.p. 178 to 179 C (PMR in deuteroacetone: $\delta = 4.0$, singlet, three, OCH_3 ; 6.94 and 7.67, doublets, one each, aromatic protons, $J_{5,6} = 9$ Hz). Treatment of the acid with diazoethane and subsequent saponification of the ethyl ester afforded the 4-0-ethyl ether (PMR in deuteroacetone: $\delta = 1.45$, triplet, three, $-(\text{CH}_2)\text{-CH}_3$, $J = 7$ Hz; 3.38, singlet, three, OCH_3 ; 4.21, quartet, two, $-\text{CH}_2\text{-(CH}_3)$, $J = 7$ Hz; 7.06 and 7.70, doublets, one each, aromatic protons, $J_{5,6} = 9$ Hz).

For 2-Chloroisovanillic acid, dry chlorine was passed slowly through a solution of isovanillin in chloroform for 1 h, to produce a copious precipitate of 2-chloroisovanillin. This was filtered off and recrystallized from ethanol-water to give crystals of m.p. 205 to 206 C. This aldehyde was oxidized (33, 34) to 2-chloroisovanillic acid (XIII), m.p. 228 to 229 C (PMR in d_6 -DMSO: $\delta = 3.89$, singlet, three, OCH_3 ; 7.00 and 7.37, doublets, one each, aromatic protons, $J_{5,6} = 9$ Hz).

2-Chloroveratraldehyde (m.p. 72–73 C) was prepared by dropwise addition of 40% aqueous NaOH to an ethanolic equimolar solution of dimethyl sulfate and 2-chloroisovanillin. This was oxidized (30) to 2-chloroveratric acid (XV), m.p. 202 to 203 C (PMR in CDCl_3/d_6 -DMSO: $\delta = 3.85$ and 3.94, singlets, three each, two OCH_3 ; 6.84 and 7.74, doublets, one each, aromatic protons, $J_{5,6} = 9$ Hz; a broad singlet at $\delta = 4.70$ caused by the carboxyl proton disappeared on addition of D_2O).

Attempts to prepare and isolate 5-chloroprotocatechuic acid and its derivatives by direct chlorination of protocatechuic acid and its derivatives in CCl_4 , acetic acid, etc., or by demethylation of 5-chlorovanillic acid and its acetate resulted only in decomposed black materials.

Diazoethane was prepared as a solution in ether-dimethylformamide (24).

Spectroscopy. UV spectra were determined on a Cary model 14 spectrophotometer, IR spectra on a Beckman IR-12 instrument, and PMR spectra on a Varian T-60 instrument.

Gas chromatography. A Hewlett-Packard model 5750 chromatograph with a flame ionization detector was used for gas chromatography. Three columns were used: (i) 2.0-m by 0.206-cm inside diameter stainless steel; silicone gum rubber UCC-W-982, 10% on Chromosorb G, AW-DMCS, 80–100mesh; (ii) 20-m by 0.206-cm inside diameter stainless steel; silicon gum rubber XE-60, 0.5% on Chromosorb G, AW-DMCS, 80 to 100 mesh; (iii) 2-m by 0.206-cm inside diameter stainless steel; silicone gum rubber SE-30, 5% on Chromosorb G, AW-DMCS, 80–100mesh.

Isothermal operating conditions were: injection, 250 C; detector, 250 C; columns, (i) 210 C, (ii) 150 C, and (iii) 180 C. The carrier gas was nitrogen at a flow rate of 25 ml/min.

Compounds were chromatographed as their trimethylsilyl derivatives. Bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane (Regis Chemical Co.) served as derivatizing agent in dimethylformamide.

Thin-layer chromatography. Precoated silica-gel plates (Bakerflex, silica gel IB-F, J. T. Baker Chemical Co.) were used for thin-layer chromatography (TLC). Developing solvents were benzene-methanol-acetic acid (90:16:8 by volume) and chloroform-ethanol (95:5 by volume). Spots were observed under UV light or by spraying with Gibb reagent (1% ethanolic 2,6-dichloroquinone-*N*-chloroimide) followed by exposure to ammonia fumes.

Plates for preparative chromatograms were silica-gel GF Uniplate (Analtech Inc.). The R_f values determined for vanillic acid and isovanillic acid in benzene-methanol-acetic acid were approximately 0.50 and 0.40, respectively, a remarkably efficient separation of these isomers (cf. ref. 22).

Extraction of metabolites from culture filtrates. Ten milliliters of a culture growing on 0.025% aromatic acid substrate served to seed 1 liter of the same medium. This was incubated at 30 C with mechanical agitation for 30 h and then clarified by filtration. The clarified medium was acidified to pH 2.0 with 1.0 N H_2SO_4 and extracted three times with 500-ml portions of chloroform-acetone (1:1 by volume), and finally with 500 ml of chloroform. The pooled extracts were washed with 100 ml of water and dried over anhydrous Na_2SO_4 . Solvents were removed at <50 C in a vacuum evaporator, leaving a residue. A portion of this was analyzed by gas chromatography. The rest was used for purification and further characterization by melting point, spectra, etc.

Chlorinated aromatic substrates were added at a concentration of 0.05 g/liter together with 0.20 g of their nonchlorinated analogues per liter. Incubation was for 72 h at 30 C with mechanical agitation. The nonhalogenated compound served as the carbon and energy source and was totally consumed. Each chlorinated compound was metabolized to another substance that accumulated. Such products were extracted from culture filtrates as above.

The products left by concentration of the dried solvent extracts were purified as required by recrystallization or preparative TLC, or both. All products except IX and XIV were identified by comparison with authentic compounds using UV, IR, and PMR spectroscopy. Because IX and XIV (and their acetates) rapidly autoxidized to black products when their extracts were concentrated during efforts to isolate them, they were treated with diazoethane and isolated as their diethyl ethers following saponification. The ethers were characterized by PMR spectroscopy as follows. The PMR (CDCl_3) of the diethyl ether of IX showed $\delta = 1.42$ and 1.48, triplets, three each, two $\text{CH}_3\text{-(CH}_2)$, $J = 6.5$ and 7.0 Hz; $\delta = 4.15$ and 4.21, quartets, two each, two $-\text{CH}_2\text{Me}$, $J = 6.5$ and 7.0 Hz; $\delta = 7.50$ and 7.75, doublets, one each, aromatic protons, $J_{5,6} = 2$ Hz; $\delta = 9.3$, broad singlet, one, COOH , removed with D_2O . The PMR of the diethyl ether of XIV in deuteroacetone showed $\delta = 1.36$ and 1.44, two triplets, three each, $\text{CH}_3\text{-(CH}_2)$, $J = 7$ Hz; $\delta = 4.10$ and 4.19, quartets, two each, two $\text{CH}_2\text{-Me}$, $J = 7$ Hz; $\delta = 7.03$, doublet, one, aromatic proton, $J = 9$ Hz; $\delta = 7.9$, broad singlet, one, COOH , removed by D_2O .

Preparation of cell-free extracts. Ten milliliters of a logarithmic culture growing on 0.025% veratric

acid served to seed 1 liter of the same medium, which was vigorously aerated on a rotary shaker for 24 h at 30 C. The veratric acid was then replenished to approximately its original concentration and incubation continued 8 to 10 h. Cells were harvested by centrifugation at $16,000 \times g$ for 10 min, washed with 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-0.3 M NaCl at pH 6.8, and resedimented. Portions of 1 to 2 g (wet weight) of cells were suspended in 5 vol of 0.1 M Tris-hydrochloride buffer at pH 6.8 and sonically disrupted, with cooling, for a total of 3 min. After centrifugation for 20 min at $17,500 \times g$ to remove cell debris and unbroken cells, the supernatant fluid was used as a crude cell-free extract. All procedures were performed at 0 C or in an ice bath.

Larger batches of cells were prepared in 20-liter carboys aerated by gas dispersion tubes carrying sterile air. Cells were harvested in a Sharples air-driven centrifuge, then washed and frozen in 0.1 M Tris-hydrochloride (pH 6.8) at -20 C until required for the preparation of cell-free extracts or whole-cell suspensions.

Oxygen uptake. Oxygen consumption by cell-free extracts and whole-cell suspensions was observed at 30 C in a Gilson respirometer. Endogenous respiration was subtracted from the total uptake.

Lignin. Lignin was isolated as a water-insoluble residue from spruce wood following vibratory ball-milling and solubilization of the bulk of the carbohydrates with a polysaccharidase enzyme mixture (35). This lignin had a methoxyl content of 14.26% and a carbohydrate content of 6.6% (measured as reducing sugars in an acid hydrolysate against a glucose standard curve; see ref. 31). The lignin was added as a dry powder to sterilized mineral salts medium as described in Results and Discussion.

Recovery of lignin from incubation media. Lignin was recovered by centrifuging the culture medium and dissolution from the sediment in dioxane-water (1:1 volume). Removal of solvent by vacuum evaporation left a residue which was purified according to Björkman (3). The purified lignin was dissolved in 1 to 5 ml of dioxane-water (1:1 by volume), insolubles were removed, and the solution was passed through a column (5 by 67 cm) of Sephadex G25 packed in dioxane-water (1:1). The excluded, polymeric peak was collected (see ref. 29 for details of gel chromatography).

RESULTS AND DISCUSSION

Survey of compounds utilized. *N. corallina* A81 grows on a variety of aromatic compounds. Some of the compounds utilized by this bacterium as a sole source of carbon and energy are as follows.

Acids are 3-hydroxy-4-methoxybenzoic; 4-hydroxy-3-methoxybenzoic; 3,4-dimethoxybenzoic; benzoic; *o*-, *m*-, and *p*-hydroxybenzoic; *m*- and *p*-methoxybenzoic; 3,4-trimethoxybenzoic; 3,4-dihydroxybenzoic; 3-ethoxy-4-hydroxybenzoic; 3-hydroxy-4-ethoxybenzoic; 5-ethoxyveratric; 3-ethoxy-4-methoxybenzoic;

3,4-dimethoxyphenylacetic. Aldehydes are 3-hydroxy-4-methoxybenzaldehyde; 4-hydroxy-3-methoxybenzaldehyde; 3,4-dimethoxybenzaldehyde. Alcohols are 3-hydroxy-4-methoxybenzyl; 3,4-dimethoxybenzyl. Miscellaneous compounds are creosol (4-hydroxy-3-methoxytoluene), catechol, guaiacol (2-methoxyphenol), phenol. Isolated cellulase lignin and all of the chlorinated aromatic acids used in this study failed to support growth.

Metabolism of veratric and *p*-anisic acids.

When *N. corallina* A81 is grown on 3,4-dimethoxybenzoic acid (veratric acid) or 4-methoxybenzoic acid (*p*-anisic acid), catabolic products are soon extractable from the growth medium. Compounds present after 30 h of incubation were identified by gas and TLC.

Both isomeric demethylation products occur in growth liquors of cells grown on veratric acid. These are 4-hydroxy-3-methoxybenzoic acid (vanillic acid, III) and 3-hydroxy-4-methoxybenzoic acid (isovanillic acid, IV). Gas chromatographic comparisons of trimethylsilyl derivatives of compounds extracted from culture filtrates with those of known compounds were made with columns (i) and (ii) (see Materials and Methods); however, only column (ii) separated III and IV. The presence of both isomers was confirmed by TLC.

Both *p*-hydroxybenzoic acid (V) and isovanillic acid (N) were found in culture filtrates of cells grown on *p*-anisic acid (11). The presence of both products was confirmed by gas chromatography using columns (i) and (iii). Thus *N. corallina* A81 hydroxylates the aromatic nucleus *ortho* to the *p*-methoxyl group and demethylates *p*-anisic acid before further metabolism.

These demethylating and hydroxylating reactions give protocatechuic acid (VI); however, under normal incubation conditions this compound did not accumulate in sufficient quantity for chromatographic detection. When 1 g (wet weight) of cells harvested from an early stationary-phase culture growing on veratric acid was suspended in 200 ml of fresh veratric acid medium containing 1 mM 2,2'-dipyridine, trace amounts of protocatechuic acid could be detected by gas chromatography in the growth fluid after 24 h of incubation.

To confirm that protocatechuic acid is an intermediate, a halogen substituent was introduced into the ring, because it was suspected from other work (20) that this would suppress ring cleavage.

Ten liters of medium containing 0.02% vanillic acid (III) and 0.005% 5-chlorovanillic acid (VIII) was inoculated with 10 ml of a culture

growing on vanillic acid. This was incubated at 30 C for 48 h with vigorous aeration. The culture filtrate was acidified with 5 ml of concentrated H_2SO_4 , and metabolites were extracted into chloroform-acetone (1:1) and chloroform. The solvent was reduced to approximately 10 ml in a vacuum evaporator, at which point a darkening of the solution due to decomposition of an oxygen-labile compound became apparent.

Many futile attempts were made to recover pure crystalline 5-chloroprotocatechuic acid (IX), the putative main product in such concentrates. This compound proved to be too readily autoxidizable to purify. Unless kept in acid solution, it darkened rapidly on exposure to air to a black pigment. Analogous experiences were encountered in attempts at chemical preparation (see Materials and Methods).

Diazoethane was added to such a slightly darkened 10-ml concentrate until a strong yellow color of diazoethane persisted, and the solution was held for 24 h in the dark. After removal of solvents, a solution of 200 mg of NaOH in aqueous ethanol was added and the mixture was refluxed for 45 min. The resultant solution was filtered, cooled, and acidified. The acid that precipitated was recrystallized from ethanol-water, giving crystals of m.p. 126 to 127 C. Characterization by PMR spectroscopy proved that these crystals were 5-chloro-3,4-diethoxybenzoic acid, i.e., the diethyl ether of 5-chloroprotocatechuic acid (see Materials and Methods).

Thus, the 5-chloro substituent does not hinder demethylation of 5-chlorovanillic acid, but does prevent ring opening. This causes accumulation of the diphenolic intermediate which is normally removed rapidly by ring-opening oxygenases. Ichihara et al. (20) observed a similar phenomenon with oxidation of *meta*-substituted benzoate derivatives by a benzoate oxidase system when corresponding catechol derivatives accumulated.

Simultaneous adaptation experiments verified the pathway of veratric acid (I) catabolism through vanillic acid (III), isovanillic acid (IV), and protocatechuic acid (VI). When cells grown on veratric acid were placed in media containing vanillic, isovanillic, or protocatechuic acids, oxygen uptake began without a lag. Cells grown on glucose did not catalyze immediate oxidation of vanillic, isovanillic, or protocatechuic acids. This implies that veratric acid or its catabolites bring about the induction of enzymes for the oxidation of these compounds (Fig. 2).

Confirmation of this pathway was obtained by measuring oxygen consumption by cell-free

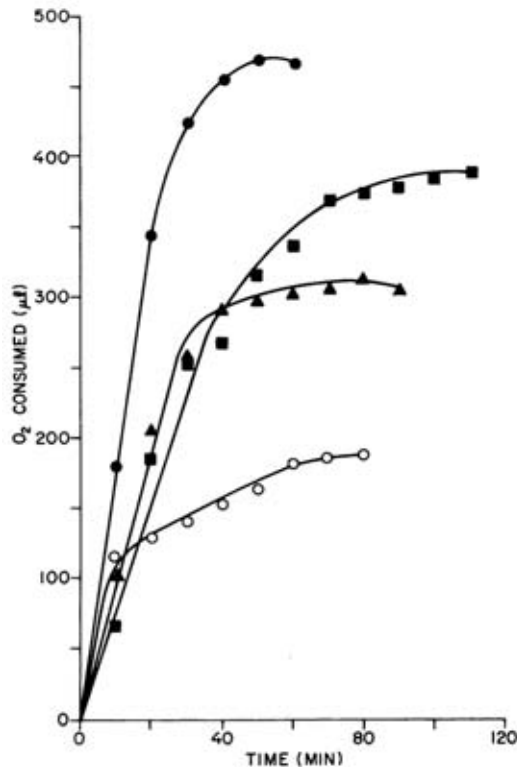


FIG. 2. Oxidation of aromatic substrates by veratric acid-grown cells. Each flask contained a total volume of 3.0 ml. The main well contained approximately 0.02 mg of cells (dry weight) and 2.3 ml of 0.1 M Tris-hydrochloride, pH 6.8. The center well contained 0.2-ml of 20% (wt/vol) KOH and the side arm contained 2 μ mol of substrate in 0.5 ml of buffer. The control flask contained buffer without substrate. The reaction was initiated by tipping substrate into the main well. Symbols: ●, veratric acid; ■, vanillic acid; ▲, isovanillic acid; ○, protocatechuic acid.

extracts with suspected intermediates. Extracts of veratric acid-grown *N. corallina* A81 oxidize veratric, vanillic, isovanillic, and protocatechuic acids with an immediate and rapid uptake of oxygen (Fig. 3). Such cell-free enzyme preparations were occasionally found to be inactive toward methoxylated substrates; this may have resulted from demethylase inactivation by air (7, 38). Oxidation of methoxylated intermediates (observed spectrophotometrically) was enhanced by adding reduced nicotinamide adenine dinucleotide, indicating a mixed-function oxidase activity (38, 43). However, the demethylase system was not rigorously characterized. Brief heating of cell-free extracts in attempts to denature all but the oxygenase (41) resulted in total inactivation.

In order to determine which of the known

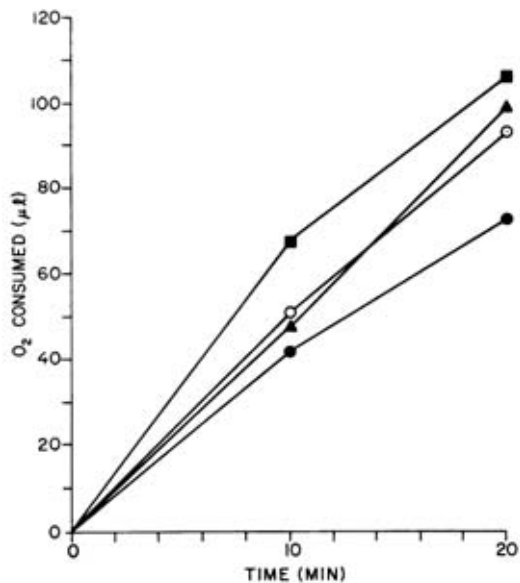


FIG. 3. Oxidation by cell-free extracts (from veratric acid-grown cells) of proposed products of veratric acid degradation. Each flask contained a total volume of 2.0 ml. The main well contained 0.5 ml of cell-free extract and 0.8 ml of 0.1 M Itis-hydrochloride, pH 6.8. The center well contained 0.2 ml of 20% (wt/vol) KOH. The side arm contained 2 μ mol of substrate dissolved in 0.5 ml of 0.1 M Itis-hydrochloride, pH 6.8. The control flask lacked cell-free extract, which was replaced by buffer. The reaction was initiated by tipping substrate into the main well. Symbols: ●, isovanillate; ■, protococatechuate; ▲, vanillate; ○, veratric acid.

patterns of ring cleavage (9) follows production of protocatechuic acid from veratric acid by *N. corallina* A81, the following experiment was conducted. Protocatechuic acid (13 μ mol) was dissolved in 2.0 ml of Tris-hydrochloride buffer (0.1 M, pH 6.8), and its UV spectrum was recorded by using a 1.0-mm cuvette. Then one drop of a concentrated cell-free extract was added to the reaction vessel containing the protocatechuic acid solution as well as to a reference solution (no protocatechuic acid). Spectra were recorded 1 and 30 min later. Figure 4 shows the observed rapid decrease in absorbance at 290 nm and corresponding increase at 270 nm. This is the basis for an assay of protococatechuate-3,4-oxygenase (32). After 30 min the conversion of protocatechuic acid to its oxidation product was complete. The final curve remained unchanged for 12 h. The spectrum of the accumulated product is that shown by Ornston and Stanier (32) to be characteristic of β -carboxy-*cis-cis*-muconic acid (VII). Thus, ring cleavage of protocatechuic acid is by an

ortho-cleavage mechanism, involving a protococatechuate-3,4-oxygenase (9). The metabolic pathways suggested by these experiments are illustrated in Fig. 5.

Veratric acid-grown *N. corallina* A81 also cleaves protocatechuic acid to yield a product that gives a positive Rothera test for β -keto acids using the whole-cell, toluenization technique described by Stanier et al. (40). This provides further evidence that veratric acid is being degraded via the β -keto adipate pathway.

Attempts to demonstrate attack on lignin by *N. corallina* A81. Experiments indicated that veratric acid-grown *N. corallina* A81 does not alter isolated spruce lignin. Fifteen grams of

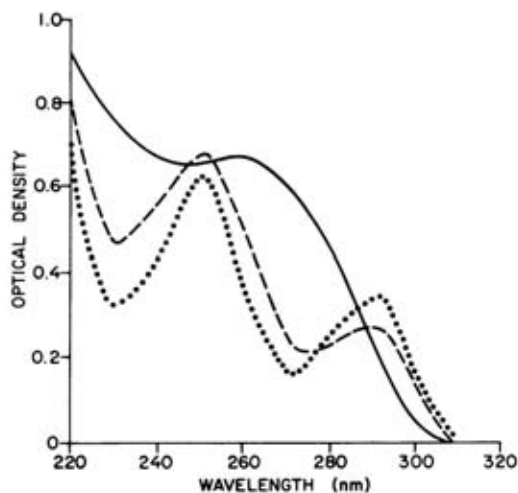


FIG. 4. Spectrophotometric observation of protococatechuate oxidation by a cell-free extract. Dotted line, protococatechuate spectrum; dashed line, 1 min after addition of cell-free extract; solid line, 30 min after addition of cell-free extract.

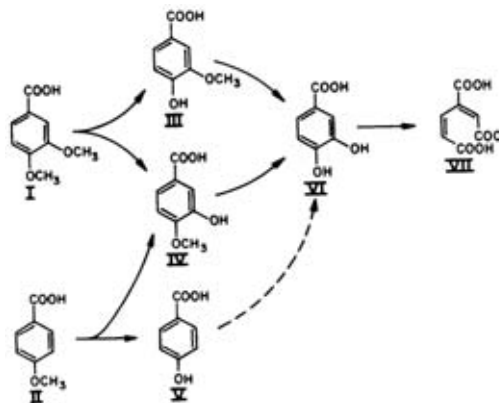


FIG. 5. Metabolism of veratric acid and *p*-methoxybenzoic acid by *Nocardia corallina* A81.

bacterial cells (wet weight) was harvested from veratric acid medium during early stationary growth and suspended in 500 ml of 0.1 M Tris-hydrochloride-0.3 M NaCl (pH 6.8) to which 1.0 g of lignin had been added. This mixture was shaken at 30 C for 48 h and then clarified by centrifugation. Contamination was not encountered. The sedimented solids were suspended in 200 ml of dioxane-water (1 : 1) to extract lignin from the cell mass. The mixture was recentrifuged, and the supernatant fluid was retained. Dioxane-water was removed by vacuum evaporation, and the lignin residue was purified (see Materials and Methods). A control was carried through an identical series of treatments without bacterial cells. The purified cultured and control lignins were compared by methoxyl analysis (control, 13.5%; cultured lignin, 14.0%) and IR spectroscopy. No substantial differences were observed.

Though *N. corallina* A81 was able to use a variety of aromatic compounds that are structurally related to lignin as sole carbon and energy source (see above), it is apparently not able to alter isolated lignin. Our inability to detect chemical changes in the lignin after incubation with veratric acid-grown *N. corallina* A81 could conceivably have resulted from several causes. First, the purification procedure may have selectively eliminated the altered part. However, to avoid this possibility we left out one of the usual purification steps, precipitation in water (3), because the expected actions of the *Nocardia* would be to increase water solubility by demethylation and oxidative degradation of the aromatic rings. It is unlikely that such changes in the polymer would have resulted in elimination of the modified portions during the other purification steps. Second, the lignin may be inaccessible when suspended in water. Even though the *Nocardia* might possess the capacity to alter lignin in plant materials where lignin is distributed in a polysaccharide matrix, this activity might be thwarted because the isolated polymer is "collapsed" and inaccessible to enzymes. However, degradation of a similar isolated lignin, including a reduction in methoxyl content, by lignin-degrading fungi in liquid culture has been reported (23). Third, the bacterium may be unable to attack the polymer despite its demonstrated versatility in metabolizing low-molecular-weight aromatic compounds related to lignin. This last possibility seems most reasonable, since decomposition of lignin requires extracellular enzymes which may not be possessed by our *Nocardia*.

Metabolism of chlorinated methoxybenzoic

acids. The chlorinated compounds used here did not support growth of *N. corallina* A81. In fact, at concentrations greater than 0.06 g/liter no growth occurred even in the presence of nonchlorinated, growth-supporting substrates. A concentration of 0.05 g of chlorinated compounds per liter did not prevent growth on analogous nonchlorinated substrates.

The transformations of chlorinated methoxybenzoic acids effected by *N. corallina* A81 are summarized in Fig. 6. The results of experiments with 5-chlorovanillic acid have been given above.

When *N. corallina* A81 was grown in the presence of veratric acid (I) and 5-chloroveratric acid (X), the latter was demethylated quantitatively to 5-chloroisovanillic acid (XI), which accumulated in the culture medium and was identified by the PMR spectrum of its 4-0-ethyl ether (PMR in 9:1 CsCl₂/d₆-DMSO: $\tau = 1.47$, triplet; three, $-(CH_2)-CH_3$, $J = 7$ Hz; 3.94, singlet, three, OCH₃; 4.17, quartet, two, $-CH_2-$

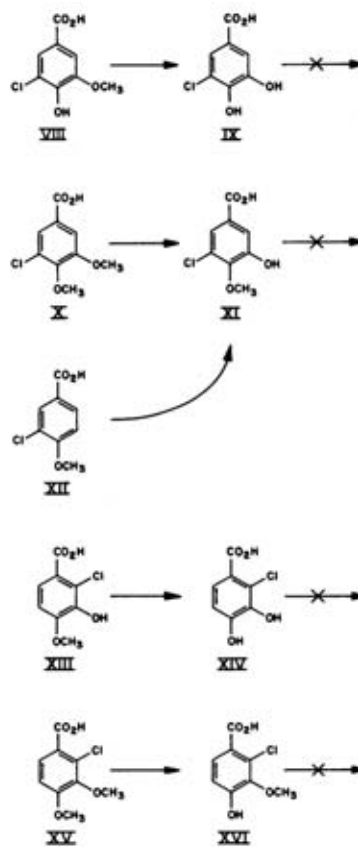


Fig. 6. Metabolism of some chlorinated aromatic acids by *Nocardia corallina* A81.

(CH₃), J = 7 Hz; 7.57 and 7.77, doublets, one each, aromatic protons, J_{2,6} = Hz; a broad singlet of τ = 6.65 due to the carboxyl proton and water disappearing on addition of D₂O). With 2-chloroveratric acid (XV) plus veratric acid (I) as substrates, 2-chlorovanillic acid (XVI) accumulated. 3-Chloro-4-methoxybenzoic acid (XII) was co-oxidized in the presence of *p*-methoxybenzoic acid (II) to 5-chloroisovanillic acid (XI). 5-Chlorovanillic acid (VIII) was converted, in the presence of vanillic acid (III), to 5-chloroprotocatechuic acid (IX). Similarly, 2-chloroisovanillic acid (XIII), in the presence of isovanillic acid (IV), was converted to 2-chloroprotocatechuic acid (XIV). All the metabolic products other than 5-chloroisovanillic acid were isolated as such or as their ethyl ethers, and were identified on the basis of UV, IR, and PMR spectra and retention time as trimethylsilyl derivatives compared with standards on gas chromatography.

These results indicate that introduction of a chlorine atom into the ring of these aromatic ethers arrests their normal metabolism by our *Nocardia*. Both demethylating and ring-opening enzyme systems are evidently affected. A methoxyl group ortho to a chlorine atom is not demethylated. However, if the chlorine is oriented *meta* to the methoxyl, demethylation does occur. Evidently an ortho chlorine atom sterically and/or electronically interferes with the demethylating enzyme system. *ortho*-Hydroxylation is not prevented by a chlorine adjacent to a methoxyl.

Dealkylation of alkoxy groups is not prevented by an *ortho*-chloro substituent with all microorganisms. Evans et al. (11) reported dealkylation of 2,4-dichlorophenoxyacetic acid and 2-chlorophenoxyacetic acid by a soil pseudomonad. Also, we have recently observed conversion of 5-chloroveratric acid to 5-chlorovanillic acid by the fungus *Polyporus dichrous* (unpublished data).

Normally, when structures of the veratryl or guaiacyl type are demethylated, the resultant *ortho*-diphenol is rapidly cleaved by *N. corallina* A81. A chloro substituent ortho to either of the paired hydroxyls suppresses ring cleavage by this strain. Again, the chlorine atom may sterically or electronically hinder the protocatechuate-3,4-oxygenase.

Ring cleavage by both *ortho*- and *meta*-cleaving oxygenases of chlorocatechols (11, 17, 42) occurs with certain other bacteria, although Ichihara et al. (20) reported that chlorocatechol was resistant to ring cleavage by a catechol oxygenase from a strain of *Pseudomonas*

aeruginosa, and other examples of resistance of chlorocatechols are known (18). Cain et al. (6) reported that a *Nocardia* was able to cleave the ring of 2-fluoroprotocatechuic acid.

Our results provide another example of the successful use of cometabolism in elucidating a biochemical pathway (18, 19). By studying the cometabolism of several related, chlorinated, aromatic compounds, we have demonstrated the blocking effect of a chlorine atom ortho but not meta to the ring position involved in the next catabolic reaction. To our knowledge, other investigators have not varied the position of halogen substitution during their studies of cometabolism of halogenated aromatic compounds (12, 18, 20).

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