Methoxyhydroquinone, an Intermediate of Vanilllate Catabolism by *Polyporus dichrous*

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Vanilllate was metabolized by *Polyporus dichrous* in liquid culture via methoxyhydroquinone. Protocatechuate, gentisate, and hydroquinone were not affected by vanilllate-metabolizing mycelial pellets that readily degraded methoxyhydroquinone.

Data in the literature indicate that vanilllate (4-hydroxy-3-methoxybenzoate) is metabolized via protocatechuate (3, 4-dihydroxybenzoate) by the lignin-destroying fungus *Polyporus (= Polystictus, Coriolus) versicolor* L. ex Fr. Flaig and Haider (3) reported that an extracellular protocatechuate-3, 4- oxygenase was elaborated by this organism when grown with vanilllic acid, and Cain et al. (1) demonstrated intracellular, but no extracellular, protocatechuate-3, 4-oxygenase in *P. versicolor* grown with vanilllic acid. In connection with studies of the catabolism of ethers of vanilllate and similar 4-hydroxybenzoates by the related lignin-destroying fungus *P. dichrous*, we were surprised to find no intracellular, extracellular, or even significant activity by whole cells against protocatechuate in cultures metabolizing vanilllate. Vanilllate was metabolized via methoxyhydroquinone instead.

**MATERIALS AND METHODS**

*P. dichrous* Fr. (isolate FP 106899-Sp), obtained from the Center for Forest Mycology Research, Forest Service, U.S. Department of Agriculture, Madison, Wis., was maintained on moist, ground aspen wood supplemented with 2.5% peptone. Thirty milliliters of a thick blend of this decaying wood in sterile nutrient medium was used to seed 600 ml of sterile medium in 2-liter Erlenmeyer flasks. The medium contained (grams/liter): glucose, 4; yeast extract (Difco), 1; peptone (Difco), 1; MgSO₄ - 7H₂O, 1; and vanilllic acid, 0.125. Seeded flasks were incubated on a rotary shaker at 75 rpm and 28 C, the optimal temperature for the fungus. Growth, in the form of numerous small pellets, was apparent after 1.5 to 2 days, and the vanilllate, estimated from the absorption at 257 nm of clear culture filtrates, began disappearing after about 55 h. Its maximum rate of disappearance in this system was 10 to 15 mg/h.

Conversion products of the aromatic acids in cultures were detected as follows. Cultures were adjusted to pH 2 and extracted three times with chloroform-acetone (1:1) and then once with chloroform; each extraction was with a volume of solvent equal to the volume of the culture. The combined extracts were washed with a small amount of water, and the solvents were removed under vacuum. Residues were examined by gas chromatography. For this they were dissolved in 100 µl of dimethylformamide and silylated by the addition of 100 µl of bis(trimethylsilyl)trifluoracetamide plus 1% trimethylchlorosilane (Regisil, Regis Chemical Co., Chicago). Gas chromatography was with two different columns, both 2.06-mm inside diameter by 200-cm stainless-steel columns, one packed with SE-30 and the other packed with OV-17, on Chromasorb G, AW-DMCS, 80/100 mesh.

Methoxyhydroquinone was first identified in an extract of a culture of *Lenzites trabea* Pers. ex Fries to which vanilllic acid had been added. The culture extract, obtained as above, was acetylated rather than silylated. For this, 3 to 4 ml of acetic anhydride and 0.2 to 0.3 ml of pyridine were added to the residue and the solution was held overnight. About 25 ml of water was added and the mixture was adjusted to pH 9 and extracted with ether. The ether solution was washed with water, and the ether was removed by vacuum distillation. Methoxyhydroquinone diacetate was separated from the residue by preparative thin-layer chromatography (TLC) (Merck silica gel HF₃₅, solvent, chloroform; *Rₖ* = 0.74; located in ultraviolet [UV] light). The product, eluted from the silica gel with acetone, gave a single peak in gas chromatograms. It was identical to the authentic diacetate by gas chromatography (both columns), by TLC (system above), by UV spectroscopy (ethanol, maximum 272.5 nm, shoulder 277 nm), and by nuclear magnetic resonance spectroscopy ([CDCl₃], 62.23 and 2.27 (two s, 6 protons total, COCH₃); 83.80 (s, 3, OCH₃); 86.8 (center of m, 3, aromatic)).

A small amount of methoxyhydroquinone diacetate was also prepared, as above, from the extract residue of a culture of *P. dichrous* to which vanilllic acid had been added. The compound was identical to the authentic sample by TLC, by gas chromatography (both columns), and by UV spectroscopy.

Authentic methoxyhydroquinone (mp 89–90 C) was
obtained from this Laboratory. The diacetate was prepared as above. The other compounds were obtained commercially, except for 3-O-methylgallic acid, which was prepared by the method of Scheline (7).

Vanillate-metabolizing mycelial pellets were recovered from 600-ml cultures by centrifugation (15 min at 10,500 \times g average; 5 C). They were immediately suspended in 100 ml of 0.05 M phosphate buffer (pH 5.5, 5 C, containing 0.1 nmol of vanillate) and were recentrifuged. The pellets, 15 to 20 g (wet weight), were held on ice and used within 3 to 4 h.

RESULTS AND DISCUSSION

Attempts to detect protocatechuic acid in extracts of cultures metabolizing vanillate were not successful. Adding 2,2'-bipyridine (2) or limiting the oxygen during incubation did not lead to detectable quantities. In recent experiments with Nocardia corallina strain A81, the intermediacy of protocatechuate in vanillate catabolism was demonstrated by incubating 5-chlorovanillate with vanillate-metabolizing cells; 5-chloroprotocatechuate accumulated quantitatively (R. L. Crawford, M.S. thesis, Univ. of Wisconsin, Madison, 1972). Although P. dichrous metabolized 5-chlorovanillate under the conditions described above, no 5-chloroprotocatechuate (or other products) were detected.

Elucidation of the immediate metabolic fate of vanillate with P. dichrous came from experiments conducted with Lenzites trabea. This fungus, like P. dichrous, is a wood-degrading basidiomycete, but merely modifies rather than completely degrades lignin in wood (6). When L. trabea was grown with vanillate under the same conditions used with P. dichrous, a single major product accumulated in an approximately quantitative amount after approximately 72 h and then slowly disappeared. This product was identified as methoxyhydroquinone.

Cultures of P. dichrous metabolizing vanillate were then examined for methoxyhydroquinone. A 600-ml culture containing vanillate was supplemented 62 h after seeding, when about one-third of the vanillate remained, with 150 mg of vanillic acid (15 ml of solution of sodium vanillate, pH 5.5). After an additional 6 h, when about one-third of this vanillate was gone, the culture was extracted, the solvent-free extract residue was acetylated, and 0.7 mg of methoxyhydroquinone diacetate was separated by TLC and identified.

Washed mycelial pellets that were metabolizing vanillate at the rate of 10 to 15 mg (of vanillic acid) per h per 600-ml culture rapidly metabolized methoxyhydroquinone, but protocatechuate and 3-O-methylgallate (3,4-dihydroxy-5-methoxybenzoate) were only slightly depleted (Fig. 1). Inclusion of 28 \mu g of cycloheximide per ml (8) in the assay system (see caption, Fig. 1) to inhibit protein synthesis had little effect on their ability to metabolize methoxyhydroquinone or vanillate.

Although methoxyhydroquinone was readily degraded by the vanillate-metabolizing pellets, hydroquinone and carboxyhydroquinone (gen-
tisic acid) were not affected (Fig. 1). This illustrates a specificity for the methoxyl-substituted compound.

Washed mycelial pellets from control cultures (grown in the absence of vanillate) became adapted to vanillate and to methoxyhydroquinone but not protocatechuate (Fig. 2). When 28 \( \mu g \) of cycloheximide per ml was included (8) in the assay system, the pellets did not deplete any of the compounds.

The conversion of vanillate to methoxyhydroquinone seems to be similar to the recently reported oxidative decarboxylation by various aspergilli of 4-methoxybenzoate to produce 4-methoxyphenol (4). An oxidative decarboxylation also occurs in the bacterial metabolism of salicylate (2-hydroxybenzoate), which is converted to catechol prior to ring cleavage (9). Benzoic acid is oxidatively decarboxylated by some microorganisms, but two hydroxyls rather than one are introduced; catechol is the first product detected (5).

The metabolism of methoxyhydroquinone by \( P. \) dichrous has not been elucidated. Numerous attempts to obtain cell-free activity have thus far failed. Even grinding the cells under liquid nitrogen (1) destroyed their activity against the compound. Concentrates of the protein from culture filtrates of vanillate-metabolizing cultures also were without effect on methoxyhydroquinone.

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