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Microbial Transformations of Natural Antitumor Agents: Oxidation of Lapachol by *Penicillium notatum*

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The naphthoquinone lapachol (1) is readily metabolized by several fungi and streptomycetes. Preparative-scale fermentations with *Penicillium notatum* (UI 1602) provided a major polar metabolite (4), which was isolated and identified as an intermediate of the Hooker oxidation. The metabolite was synthesized by reacting lapachol with hydrogen peroxide under alkaline conditions.

Lapachol (1) is a naturally occurring naphthoquinone derivative found in the heartwood of several plants. Lapachol was first isolated (15) and characterized (10) during the late 19th century, and the compound has been synthesized (4, 11). Considerable recent attention has been focused on lapachol and its analogs, since these compounds possess antimalarial (5), antitumor (Walker 256 rat carcinosarcoma [16] and Yoshida sarcoma [9]), antibiotic, and antischistosomal (7, 9) properties. Lapachol itself has been examined for its potential as an antitumor agent in phase I clinical trials (3).

Little is known about the microbial metabolism of naphthoquinones including lapachol. Microbial transformation studies were initiated with lapachol in order to determine pathways of metabolism and to prepare metabolites in sufficient quantity for biological testing. This report is concerned with the production of a polar lapachol metabolite by *Penicillium notatum* (UI 1602).

MATERIALS AND METHODS

Melting points were determined in open-ended capillary tubes in a Thomas Hoover capillary melting-point apparatus and were corrected. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian T-60 spectrometer, using TMS as an internal standard. Low-resolution mass spectra were obtained with a Finnigan model 3200 mass spectrometer. High-resolution mass spectral data was obtained through the courtesy of the chemistry department of the Massachusetts Institute of Technology, Cambridge. Thin-layer chromatography (TLC) was performed on 0.25- or 1.0-mm-thick layers of Silica Gel GF_{254} (Merck) prepared on glass plates with a Quickfit Industries spreader.

Lapachol. The lapachol used as substrate in this study was obtained from Aldrich Chemical Co. (lot no. 120427); mp 139 to 140.5°C (140°C in reference 14); NMR (14) ($CDCl_3$), ppm, 1.67 (3H, s, CH_3), 1.78 (3H, s, CH_3), 3.30 (2H, d, CH_2), 5.23 (1H, t, —CH═), 7.68 (2H, m, 6— and 7—H), 8.08 (2H, m, 5— and 8—H); mass spectrum (14) *m/e* (percent relative abundancy) 242 (15), 228 (16), 227 (100).

Fermentation procedure. Cultures used in this work are maintained in The University of Iowa, College of Pharmacy culture collection. All cultures were stored on Sabouraud-maltose agar slants in sealed screw-capped tubes at 4°C except for *Streptomyces platensis* and *S. punipalus*, which were stored on a sporulation agar (1). Cultures were grown by the previously described two-stage fermentation procedure (2) in a soybean meal-glucose medium of the following composition: soybean meal, 5 g; glucose, 20 g; yeast extract, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; distilled water, 1,000 ml, pH adjusted to 7.0 with 6 N HCl. The medium was sterilized in an autoclave at 121°C for 15 min before use.

Fermentations were conducted on rotary shakers (model G-25, New Brunswick Scientific Co.) operating at 250 rpm describing a 1-inch (about 2.5-cm) stroke, at 27°C in cotton-plugged Erlenmeyer flasks containing one-fifth of their volumes of medium. Fermentations were initiated by suspending the surface growth from slants in 5 ml of sterile medium and using the suspension to inoculate stage I cultures. Thick 72-h stage I cultures were used to inoculate stage II fermentations. The inoculum was 10% of the volume of medium held in stage II culture flasks. Lapachol was added as substrate to 24-h stage II cultures as a solution in dimethyl formamide (DMF) (100 mg of lapachol/ml of DMF). Substrate concentrations in stage II cultures were 0.4 mg/ml for small-scale fermentations and 0.5 mg/ml for preparative-scale fermentations.

Sampling procedure. Samples (4 ml) of substrate-containing fermentations were withdrawn at various time intervals, acidified to approximately pH 2 with a few drops of 6 N HCl, and extracted with 1 ml of ethyl acetate. Approximately 30 μl of the extracts was spotted on TLC plates, which were developed in one of two solvent systems: (A) benzene-glacial acetic acid (95:5) or (B) chloroform-ethanol-glacial acetic acid (88:7:5). Chromatograms were visualized by fluorescence detection under 254-nm ultraviolet light or by spraying developed plates with cerric ammonium sulfate reagent [1% $Ce(NH_4)_4(SO_4)_4$ in 50% H_3PO_4] and

warming the sprayed plates with a heat gun.

Screening. Small-scale fermentations were used to screen 48 microorganisms for their abilities to metabolize lapachol. Cultures were grown by the general fermentation procedure in 125-ml Erlenmeyer flasks. Lapachol (10 mg in 0.1 ml of DMF) was added to each 24-h stage II culture, and samples were withdrawn at 24, 48, and 72 h after substrate addition. Several cultures converted lapachol to one or more metabolites (Table 1). The initial results were confirmed in a second experiment, which was conducted using suitable controls. Controls consisted of cultures grown without lapachol and those grown with lapachol suspended in buffers including 0.1 M sodium phosphate (pH 6.5), 0.1 M citric acid (pH 3.1), and 0.1 M tris(hydroxymethyl)aminomethane (pH 8.7). Although lapachol decomposed slightly (less than 1% by TLC estimation) to colorless products under all pH conditions, none of the decomposition products were chromatographically comparable to the observed microbial metabolites. Ten of the cultures listed in Table 1 produced a common, polar metabolite [(4), R_f 0.25 in solvent system B), and *P. notatum* was selected for the preparative-scale conversion of lapachol to this metabolite for purposes of isolation and identification.

Preparation of (4) from lapachol (1) by P. notatum (UI 1602). *P. notatum* was grown according to the usual fermentation procedure, and stage II fermentations were conducted in 30 1-liter Erlenmeyer flasks. A total of 3 g of lapachol was distributed evenly among the 24-h stage II cultures. After 48 h of incubation, nearly all of the lapachol had been consumed, and the fermentation was harvested by filtration. The filtrate was acidified to pH 2.0 with 6 N HCl and was exhaustively extracted with ethyl acetate. The extracts were combined, dried over anhydrous Na_2SO_4, and concentrated to a nondrying oil (6.0 g). The mixture was adsorbed onto silica gel (15 g) and then applied to a silica gel column (300 g, Baker 3405, 60 to 200 mesh, column dimensions 66 by 4.4 cm). The column was eluted with chloroform-ethanol-acetic acid (84:16:0.1) at a flow rate of 1 ml/min while 15-ml fractions were collected.

TABLE 1. *Lapachol-metabolizing microorganisms*

Culture[a]	Metabolites produced		
	(4)	A	Others
Aspergillus alliaceus (NRRL 315)	−	−	+
Cunninghamella echinulata (ATCC 9244)	+	−	+
Cunninghamella blakesleeana (ATCC 8688a)	+	−	−
Cunninghamella echinulata (NRRL 3655)	+	−	+
Curvularia lunata (NRRL 2178)	−	+	−
Helicostylum piriforme (QM 6945)	+	−	−
Mucor mucedo (Wisc 4605)	+	−	+
Penicillium notatum (UI 1602)	+	−	−
Stemphylium consortiale (Wisc 4136)	+	−	+
Streptomyces platensis (ATCC 13865)	+	−	+
Streptomyces punipalus (NRRL 3529)	+	−	+
Streptomyces rimosus (ATCC 23955)	+	−	+
Streptomyces griseus (UI 1158)	−	−	−

[a] ATCC, American Type Culture Collection, Rockville, Md.; NRRL, Northern Regional Research Laboratories, Peoria, Ill.; UI, University of Iowa, College of Pharmacy Culture Collection; Wisc, University of Wisconsin, School of Pharmacy culture collection; QM, Quartermaster Culture Collection, U.S. Army Laboratories, Natick, Mass.

Fractions containing (4) (69 through 209) were combined, evaporated to dryness, dissolved in chloroform, and further purified by partitioning against saturated $NaHCO_3$ solution. The metabolite, which was soluble in aqueous $NaHCO_3$, was reclaimed by acidification, extraction with chloroform, and concentration to dryness. The crude metabolite (0.375 g) was crystallized as white needles from benzene: mp 155 to 156°C, decomposition; mass spectrum, *m/e* (percent relative abundancy) 276.10080, calculated for $C_{15}H_{16}O_5$ 276.09977, 258 (6), 231 (24), 213 (7), 208 (54), 189 (9), 171 (68), 162 (70), 148 (31), 144 (26); NMR (MeOH-D_4 ppm), 1.33 (3H, s, CH_3), 1.47 (3H, s, CH_3), 2.13 to 2.87 (2H, m, $-CH_2-$), 4.82 (3H, s), 4.97 (1H, t, J=8, $-CH=$), and 7.52 (4H, m, aromatic-H); semicarbazone, mp 195 to 196°C, decomposition.

Synthesis of the metabolite (4) (reference 6). A solution of 1.2 g of Na_2CO_3 in 25 ml of water was added with stirring to 2.42 g of lapachol (0.01 M) in 25 ml of dioxane. The resulting red solution was stirred under nitrogen while the reaction temperature was maintained at 70°C in a water bath. Hydrogen peroxide (30%, 2 ml) was added dropwise to the solution over a period of several minutes. The reaction mixture gradually turned to a light orange color during a 3-h period, and TLC (solvent system B) indicated that the reaction was complete. After cooling to room temperature, the reaction mixture was diluted with 75 ml of distilled water and acidified to pH 2 with concentrated HCl. The product (4) separated from solution as colorless crystals, which were dried and recrystallized from benzene: mp 154°C decomposition (156 to 157°C in reference 6); mixture mp with the metabolite was undepressed at 156°C; semicarbazone, mp 194 to 195°C decomposition (195 to 196°C, decomposition, in reference 6); mixture mp with the metabolite semicarbazone was undepressed at 194 to 195°C, decomposition; methyl ester mp 154 to 156°C (156°C in reference 6). Analysis of methyl ester: calculated for $C_{16}H_{18}O_5$, C 66.20, H 6.25; found C 65.88, H 5.87. NMR and mass spectra were identical to those obtained with the microbial metabolite.

RESULTS AND DISCUSSION

Small-scale screening experiments were initially conducted with 48 cultures to identify those capable of metabolizing lapachol. Cultures were selected from the following genera (num-

bers of species examined): *Alternaria* (1), *Aspergillus* (7), *Calonectria* (1), *Cunninghamella* (6), *Curvularia* (1), *Cylindrocarpon* (1), *Gliocladium* (1), *Helicostylum* (1), *Microsporum* (2), *Mucor* (3), *Nocardia* (1), *Ophiobolus* (1), *Penicillium* (2), *Pseudomonas* (2), *Rhizopus* (1), *Saccharomyces* (1), *Schizosaccharomyces* (1), *Sporobolomyces* (1), *Stemphylium* (2), *Streptomyces* (8), *Stysanus* (1), *Trichophyton* (1), *Trichothecium* (1), and *Whetzelinia* (1). Of these, 13 cultures produced significant amounts of one or more metabolites (Table 1). TLC indicated that the most common metabolite (4) was produced in highest yield in an uncomplicated reaction by *P. notatum*, and this organism was selected for the preparative-scale fermentation to obtain sufficient quantities of the metabolite for structure elucidation.

The metabolite (4) was isolated and purified by column chromatography. The acidic nature of the metabolite was demonstrated by its solubility in saturated $NaHCO_3$. This property was used to advantage in its purification. Initial comparisons of the NMR spectral data of lapachol and the metabolite revealed that the 3-methyl-2-butenyl side chain was still intact, and that the metabolite possessed at least one aromatic ring with 4-protons. The high-resolution mass spectrum indicated that the metabolite had a molecular weight of 276 for $C_{15}H_{16}O_5$. Peaks in the mass spectrum at *m/e* 231, 208, and 163 could be explained by the loss of COOH and $—CH_2—CH=C(CH_3)_2$ fragments from the molecular ion, confirming the presence of a carboxyl group.

A substance with properties similar to those of the metabolite had been reported in the literature (6). This compound (4) was reported to be an intermediate in the Hooker oxidation, which occurs with lapachol and related naphthoquinones. Verification of the identity of the metabolite as (4) was obtained by synthesis from (1), using H_2O_2 under alkaline conditions (6). Synthetic (4) and the microbial metabolite gave identical physical properties, derivatives, and spectral data.

The Hooker oxidation involves an initial epoxidation of lapachol followed by base (OH^-)-catalyzed epoxide ring opening as shown in Fig. 1. *P. notatum* strains and other fungi have been shown to undergo epoxidation reactions with other types of substrates (12). It is conceivable that *P. notatum* UI 1602 produces (4) by initial epoxidation followed by ring opening in analogous fashion to the chemical oxidation process. Attempts were made to prepare the proposed epoxide intermediate for purposes of verifying its intermediacy in the microbial reaction. However, reactions conducted with lapachol and

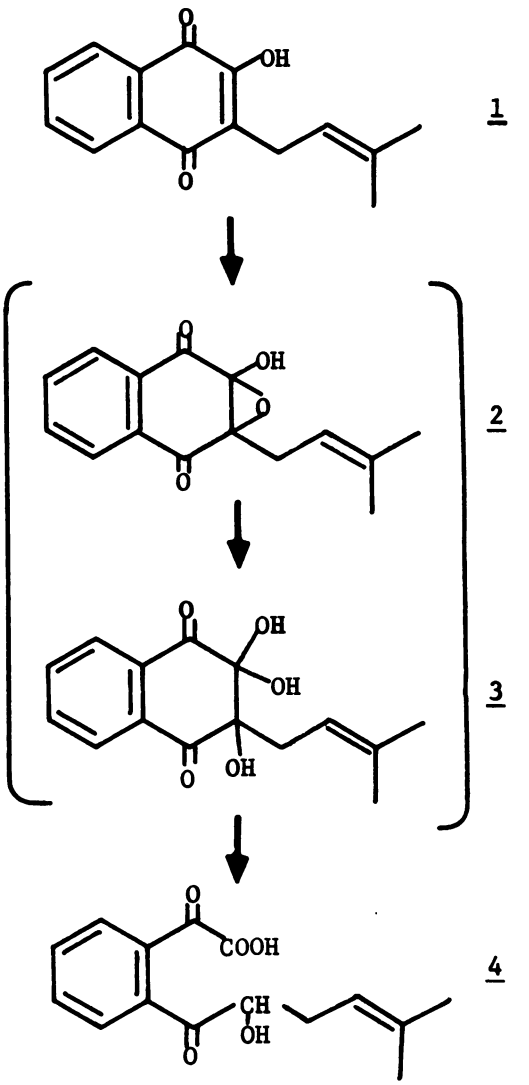

FIG. 1. *Pathway of metabolism of lapachol by Penicillium notatum.*

H_2O_2 under neutral conditions or with *m*-chloroperbenzoic acid resulted in complex mixtures of products. Epoxide derivatives such as (2) are notoriously unstable.

Some microorganisms possess a partial catalase deficiency (8) which might result in the accumulation of H_2O_2 in culture media. If *P. notatum* were such a microorganism, (4) could conceivably be produced from lapachol, especially in alkaline medium. Several experiments were performed to rule out this possibility. Qualitative examination of the mycelium from submerged fermentations and agar slants demonstrated that *P. notatum* possessed vigorous catalase activity (8). Catalase (Sigma C-30, beef

liver) added to cultures at a level of 2,000 U/ml resulted in no apparent inhibition of the conversion of lapachol to (4). The pH of fermentations containing lapachol remained slightly acidic (pH 6.9) during the 24-h period when lapachol was totally converted to (4). Furthermore, H_2O_2 could not be detected in fermentation media by the pyrogallol-purpurogallin method (13), which could detect 60 μg of H_2O_2 (Sigma horseradish peroxidase, P-8125, type I) per ml. All of these results suggest that (4) is produced enzymatically and that it is not an artifact arising from H_2O_2 oxidation in culture media.

We believe this report to be the first example of the oxidative microbial transformation of a naphthoquinone, and we are in the process of studying the metabolism of lapachol with other microorganisms.

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