# MICROBIAL TRANSFORMATIONS OF NATURAL ANTITUMOR AGENTS. 17. CONVERSIONS OF LAPACHOL BY *CUNNINGHAMELLA ECHINULATA*

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ABSTRACT.—Microbial transformation of lapachol with *Cunninghamella echinulata* (NRRL 3655) yielded three metabolites which were isolated and identified as lomatiol, lomatiol lactate ester, and lapachol-β-glucoside. The metabolites were characterized by infrared, nuclear magnetic resonance, and mass spectral analyses. Lomatiol lactate ester and lapachol-β-glucoside were chemically and/or enzymatically hydrolyzed, and the products were characterized by comparison to authentic compounds. Lomatiol has been identified as one of the mammalian metabolites of lapachol.

Lapachol (**1**) is a naturally occurring naphthoquinone derivative found in the heartwood of several species of *Bignoniaceae* and *Verbenaceae* (1). It has antitumor (2, 3), antibiotic (3), and antimalarial (4) activities and has been examined for its potential as an antitumor agent in phase I clinical trials (5). The mammalian metabolism of lapachol has been studied in experiments with dogs, and three metabolites were isolated: lomatiol, its glucuronide, and a carboxylic acid derivative (1,4-dihydro-3-hydroxy-α-methyl-1,4-dioxo-2-naphthalene-crotonic acid) (6).

Microorganisms have been used to study the metabolism of a number of naturally occurring antitumor compounds (7). These transformations are con-

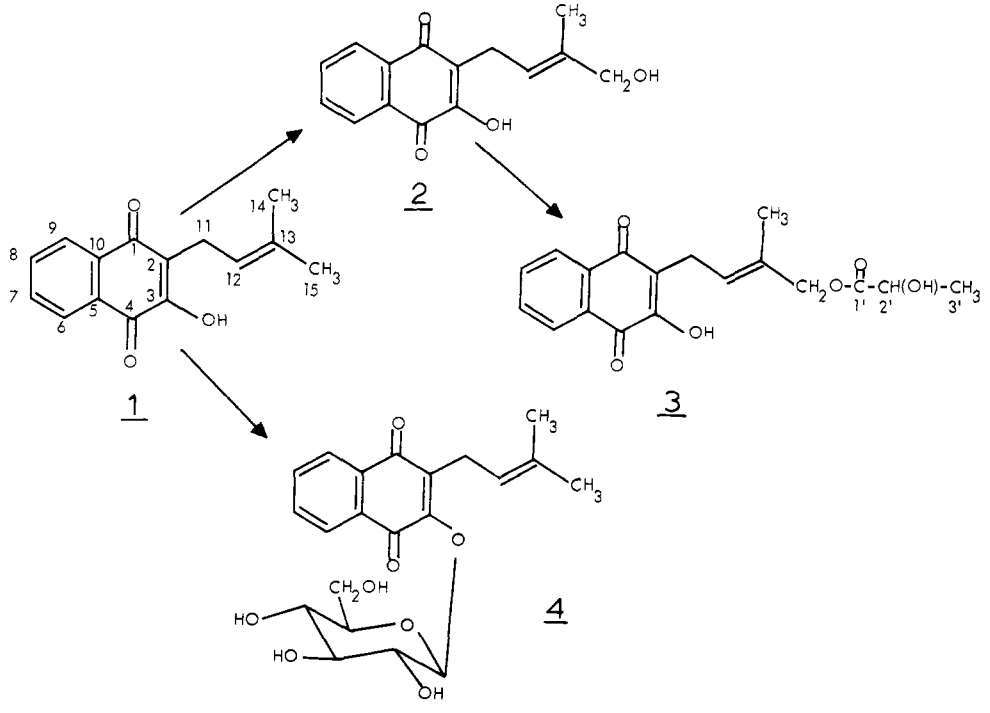


FIG. 1. The structures of lapachol (**1**), lomatiol (**2**), lomatiol lactate (**3**) and lapachol-β glucoside (**4**).

ducted to produce metabolites for biological testing and structure determination and to identify novel metabolic pathways which may also occur in mammalian metabolic systems (8, 9).

Previous reports describe a novel oxidative ring fission of lapachol by *Penicillium notatum* (10) and the oxidative cyclization of lapachol to dehydro-α-lapachone by *Curvularia lunata* (11). This report describes the microbial transformation of lapachol to lomatiol (**2**), lomatiol lactate ester (**3**), and lapachol-β-glucoside (**4**) by *Cunninghamella echinulata* (NRRL 3655).

## RESULTS AND DISCUSSION

Small-scale screening experiments with 48 microorganisms identified 20 cultures capable of metabolizing lapachol. The most common metabolite was the ketol derivative of lapachol (10), and it was produced by 17 of the 20 active cultures. Several of the microorganisms which produced the ketol derivative as the major product also converted lapachol to other metabolites in lower yield. Lomatiol (**2**) was produced by *Cunninghamella echinulata* (NRRL 3655) and *Sebekia benihana* (NRRL 11111), whereas lomatiol lactate ester (**3**) was produced only by *C. echinulata* (NRRL 3655). Lapachol-β-glucoside (**4**) was produced by six of the cultures examined: *Cunninghamella bainieri* (UI 3065), *Cunninghamella blakesleeana* (X 198, ATCC 8688a, and ATCC 9245), *Cunninghamella echinulata* (NRRL 3655), and *Rhizopus arrhizus* (QM 1032).

In screening experiments, the minor metabolites (**2**, **3**, and **4**) were produced in moderate yields in the early stages of the fermentation (24 to 48 h). The amounts of these metabolites gradually decreased with time, and the quantity of the ketol derivative gradually increased. After 72 to 144 h, the only metabolite remaining was the ketol.

*C. echinulata* (NRRL 3655) was selected for a preparative-scale fermentation to obtain sufficient quantities of metabolites for structure elucidation. Lomatiol (**2**) was identified on the basis of ir, nmr, and mass spectral data. The infrared spectrum of the metabolite contained quinone carbonyl peaks at 1667 and 1632 $cm^{-1}$, and an OH stretching absorption at 3451 $cm^{-1}$. The high-resolution mass spectrum indicated that the molecular weight was 258.08770 for $C_{15}H_{14}O_4$. The base peak at *m/e* 277 could be explained by loss of $CH_2OH$ from the molecular ion. Peaks in the mass spectrum at *m/e* 240, 225, 212, and 197 could result from an initial loss of $H_2O$ from the molecular ion followed by elimination of $CH_3$ and/or CO. Comparison of the nmr spectra of the metabolite (**2**) and lapachol (**1**) suggested that the naphthoquinone ring was unaltered, but that the side-chain had been modified. The signals for the gem-dimethyl group of lapachol were absent and were replaced by singlets at δ1.80 and 3.90 which corresponded to a $CH_3$ group and a $CH_2OH$ group, respectively. These data were consistent with the structure of lomatiol (**2**) and were compatible with published values (1).

Metabolite (**3**) was identified as lomatiol lactate ester. The high-resolution mass spectrum of the metabolite indicated that the molecular weight was 330.10830 and that the molecular formula was $C_{18}H_{18}O_6$. The mass spectrum was qualitatively similar to that of lomatiol (**2**), except that the base peak occurred at *m/e* 45 for $CH_3CHOH^+$. A peak at *m/e* 258 can be rationalized by loss of $C_3H_4O_2$ from the molecular ion to give lomatiol (**2**). The nmr spectrum contained a singlet at δ1.82 ($CH_3$) and a singlet at δ4.55 ($CH_2O$) which indicated that one of the side-chain methyl groups had been oxidized. The presence of a doublet at δ1.40 which was coupled with a quartet at δ4.25 suggested that the metabolite

contained a $CH_3CHOH$ group. The infrared spectrum contained an ester carbonyl absorption band at 1718 $cm^{-1}$ which was in agreement with published values for lactate esters (12, 13). All of these data were consistent with the structure of lomatiol lactate ester (**3**).

The proposed structure was verified by hydrolysis of the metabolite with alcoholic potassium hydroxide. The alcohol was identified as lomatiol on the basis of infrared and mass spectral data which were identical to those of authentic lomatiol. The carboxylic acid was identified as L(+)lactic acid by enzymatic assay with lactate dehydrogenase (14).

Carbon-13 nmr spectral data for lapachol and lomatiol lactate ester are shown in table 1. All but four of the carbon signals of the metabolite could be readily assigned by comparison with values for lapachol (15). The remaining four signals were assigned by comparison with values published for ethyl lactate (16). Study of the carbon-13 nmr spectra of 2-olefins has shown that $\alpha$-methyl carbons in *cis* isomers are shielded by approximately 6 ppm relative to those in *trans* isomers (17). This effect has also been observed in compounds which contain *gem*-dimethyl groups. For example, in 2-methyl-2-butene the methyl carbon *cis* to C–4 is shielded by 8 ppm relative to the methyl carbon *trans* to C–4 (17). These results allowed assignment of the chemical shifts of the C–14 and C–15 methyl groups of lapachol as 17.8 and 25.6 ppm, respectively. The side-chain double bond of the metabolite was therefore assigned the *E* configuration since the carbon-15 signal was shifted some 44 ppm downfield relative to lapachol; the signal for carbon-14 was relatively unchanged.

The spectral properties of metabolite (**4**) indicated that it was lapachol-$\beta$-glucoside. The high-resolution mass spectrum of the metabolite indicated a molecular weight of 404.14844 for $C_{21}H_{24}O_8$. The molecular ion fragmented by loss of $C_6H_{10}O_5$ to *m/e* 242 for $C_{15}H_{14}O_3$ consistent with the structure of lapachol, which suggested that the metabolite contained a sugar group. The nmr spectrum of the metabolite was also consistent with a glycoside structure. In addition to the usual lapachol resonances, the spectrum contained a broad singlet at $\delta$5.60 which corresponded to an acetal CH-group and an 8-proton multiplet at $\delta$3.18 to 3.87 which was assigned to the side-chain $CH_2$ group and the 2'-, 3'-, 4'-, 5'-, and 6'-protons of the sugar. The metabolite was hydrolyzed with sulfuric acid and $\beta$-glucosidase, and the aglycone gave uv, ir, and mass spectral values identical to those of lapachol. The sugar was examined by paper chromatography and tlc and was chromatographically identical to glucose. The identity of the sugar was confirmed by treatment with a solution of resorcinol in $H_2SO_4$ (18). The visible spectrum of this product was superimposable with the spectrum obtained with authentic glucose.

*C. echinulata* possesses interesting and broad metabolic capabilities. This microorganism catalyzes hydroxylation of acronycine (19), *N*-demethylation of d-tetrandrine (20), and *O*-demethylation of several compounds including papaverine (21) and 9-methoxyellipticine (22). In addition, *C. echinulata* catalyzes oxidative ring cleavage (10), hydroxylation, glucosylation, and esterification of lapachol. Microbial hydroxylation of lapachol to form lomatiol represents a parallel with mammalian metabolism since lomatiol was one of the metabolites isolated in studies with dogs (6). Lomatiol has been tested for antitumor activity and was inactive in the L-1210 and Walker-256 test system (2).

The glucosylation and esterification reactions catalyzed by *C. echinulata* are unusual microbial transformations. Esterifications of xenobiotics have been

TABLE 1. Carbon-13 nmr spectral data for lapachol (**1**) and lomatiol lactate ester (**3**).

| Carbon | Chemical Shift (acetone-$d_6$) | |
|---|---|---|
| | Lapachol (**15**) | Lomatiol lactate ester (**3**) |
| C-1 | δ184.3[a] | δ184.3[a] |
| C-2 | 154.8 | 154.8 |
| C-3 | 123.4 | 122.3 |
| C-4 | 181.2[a] | 181.3[a] |
| C-5 | 130.0[b] | 130.2[b] |
| C-6 | 125.9[c] | 125.9[c] |
| C-7 | 134.2[d] | 134.8[d] |
| C-8 | 132.7[d] | 133.2[d] |
| C-9 | 125.6[c] | 125.1[c] |
| C-10 | 132.2[b] | 132.7[b] |
| C-11 | 22.3 | 22.0 |
| C-12 | 120.8 | 126.2[c] |
| C-13 | 132.2[b] | 131.7[b] |
| C-14 | 17.8 | 13.4 |
| C-15 | 25.6 | 69.7 |
| C-1′ | — | 174.7 |
| C-2′ | — | 66.7 |
| C-3′ | — | 20.1 |

[a,b,c,d]Chemical shift assignments may be interchangeable.

occasionally observed in microbial systems. Acetylations are most common (23), but other conjugative-type reactions have also been reported. For example, chloramphenicol is converted to the 3-propanoate, 3-isobutyrate, and 3-isovalerate by *Streptomyces coelicolor* (24). Reports of microbial glucosylations have been limited primarily to pyrimidine and ribonucleoside substrates (23). Other compounds which undergo glucosylation include chloroquinaldol and several hydroxy- and dihydroxy-anthraquinones which are converted to glucosylated derivatives by *Sporotrichum sulfurescens* (23), and *Streptomyces aureofaciens* (25, 26), respectively. Since many of the naturally occurring antitumor compounds are highly water insoluble, this type of transformation may prove useful for production of water soluble dervatives.

## EXPERIMENTAL[1]

CHROMATOGRAPHY.—Thin-layer chromatography was performed on 0.25- or 0.50-mm thick layers of silica gel $GF_{254}$ (Merck) prepared on glass plates with a Quickfit Industries Spreader (Quickfit Industries, London, England). Modified layers were prepared by slurrying silica gel with 4% $KH_2PO_4$ or 0.1 N boric acid. Solvent systems used for tlc are shown in table 2. Chromatograms were visualized by fluorescence quenching under 254-nm ultraviolet light and by spraying with ceric ammonium sulfate [1% $Ce(NH_4)_4{\cdot}(SO_4)_4$ in 50% $H_3PO_4$] (27) or aniline hydrogen phthalate (28) and warming the sprayed plates with a heat gun.

[1]Melting points were determined in open-ended capillary tubes with a Thomas-Hoover capillary melting-point apparatus and were corrected. $^1H$ nmr spectra were obtained with a Varian model T-60 or Jeol 90 MHz spectrometer with tetramethylsilane as an internal standard. $^{13}C$ nmr spectra were obtained with a Bruker HX-90E spectrometer at 22.63 MHz incorporating a time-shared internal deuterium lock, a Bruker SXP high power radiofrequency amplifier, a Nicolet BNC-12 computer, a model 293 I/0 controller for signal averaging and Fourier transformation of the free induction decay. Low-resolution mass spectra were obtained with a Finnigan model 3200 mass spectrometer, and high-resolution mass spectra were obtained through the courtesy of the chemistry department of the Massachusetts Institute of Technology. Infrared spectra were obtained with a Beckman IR-4240 spectrophotometer, and ultraviolet spectra were obtained with a Pye-Unicam SP 1800 spectrophotometer.

TABLE 2. Tlc solvent systems.

| Tlc System | Solvent | Adsorbent |
|---|---|---|
| A | Benzene-Acetic Acid (95:5) | Silica Gel |
| B | Chloroform-Methanol (9:1) | Silica Gel |
| C | Chloroform-Methanol-Diethylamlne (45:3:2) | Silica Gel |
| D | Butanol-Acetic Acid-Ether-Water (9:6:3:1) | Silica Gel |
| E | Hexane-Acetone-Acetic Acid (55:40:5) | Silica Gel-$KH_2PO_4$ |
| F | Methanol-Acetic Acid-Benzene (3:1:1) | Silica Gel-Boric Acid |
| G | Methyl Ethyl Ketone-Acetic Acid-Methanol (3:1:1) | Silica Gel-Boric Acid |

Column chromatography was performed with silica gel (Baker 3405, 60–200 mesh) which was activated for 60 min. at 120° prior to use. To prepare modified silica gel for column chromatography a thick slurry of silica gel with 4% $KH_2PO_4$ was made and dried overnight at 120°. Columns were wet packed in the developing solvent and fractions were collected in a Fractomette 200 instrument.

LAPACHOL.—Lapachol was purchased from Aldrich Chemical Co., and its characteristics have been published (10).

FERMENTATION PROCEDURES.—Cultures used in this work were stored at 4° in sealed screw-capped tubes on Sabouraud-maltose agar slants. Microorganisms were grown according to the previously described two-stage fermentation procedure (19) in soybean meal-glucose medium. Screening experiments were conducted in 125-ml steel-capped Delong culture flasks containing 25 ml of medium. Lapachol (10 mg in 0.1 ml DMF) was added to 24-h stage II cultures, and samples were withdrawn at various time intervals. Samples (4 ml) were acidified to pH 2.0 with 6 N HCl and extracted with 1 ml of ethyl acetate. Approximately 30 μl of the ethyl acetate extracts were examined by tlc (systems A and E).

Controls consisted of fermentations without lapachol, and solutions of lapachol in buffers, including 0.1 M citric acid (pH 3.1), 0.1 M sodium phosphate (pH 6.5), and 0.1 M tris(hydroxymethyl)aminomethane (pH 8.7), all of which were incubated with shaking for the duration of normal fermentations (72 h). Although lapachol decomposed slightly (less than 1% as estimated by tlc) to colorless products under all pH conditions, none of the decomposition products were chromatographically comparable to the observed microbial metabolites.

TRANSFORMATION OF LAPACHOL BY *Cunninghamella echinulata* (NRRL 3655).—Stage II cultures of *C. echinulata* were grown according to the usual fermentation protocol in 30 1-liter Delong flasks. Lapachol (**1**) was dissolved in dimethylformamide (3 g/30 ml) and distributed evenly among the 24-h-old stage II flasks. The cultures were incubated with shaking at 28°. Tlc analysis (system E) 40 h after substrate addition indicated that about 25% of the added lapachol had been converted to metabolites (**2**), (**3**), and (**4**); the cultures were harvested by filtration. The filtrate was acidified to pH 2 with 6 N HCl and exhaustively extracted with ethyl acetate. The combined extract was dried over anhydrous $Na_4SO_4$ and concentrated to a brown non-drying oil (10.0 g).

The mixture was adsorbed onto 17.5 g of silica gel and applied as a dry powder to the top of a silica gel column (300 g, 48 × 4 cm). The column was eluted with hexane-acetone mixtures of increasing polarity (98:2 to 4:6) at a flow rate of 3 ml/min while 850 18-ml fractions were collected. Lomatiol (**2**) and its lactic acid ester (**3**) were eluted with hexane-acetone (7:3) in fractions 420–510 (2.1 g). Lapachol-β-glucose (**4**) was eluted with hexane-acetone (1:1) in fractions 600–666 (450 mg) and with hexane-acetone (4:6) in fractions 667–729 (549 mg), 730–780 (817 mg), and 781-820 (155 mg).

The mixture of lomatiol and lomatiol lactate ester (2.1 g) was adsorbed onto 13 g of silica gel and applied to a silica gel column (250 g buffered with 25 g of $KH_2PO_4$, 58 × 4 cm) which was eluted with chloroform-methanol (99:1) at a flow rate of 1 ml/min while 275 12-ml fractions were collected. The ester was eluted in fractions 42–57, and lomatiol was eluted in fractions 58–105.

CHARACTERIZATION OF LOMATIOL (**2**).—The metabolite (**2**) was further purified by preparative tlc (silica gel-$KH_2PO_4$ in chloroform-methanol [98:2] ) and, when crystallized from acetone-hexane, yielded yellow needles of pure lomatiol (15 mg); mp 119.5–123.5°C [lit. (1)

127°]; λ max (EtOH) (1) 252, 277, 332, and 383 nm (ε15,856, 16,394, 2,634, and 1,424); νmax (KBr) (1) 3451, 1667, 1632, and 1590 $cm^{-1}$; δ (acetone-$d_6$) 1.80 (s, 3H, $CH_3$), 3.32 (d, 2H, $CH_2$), 3.90 (s, 2H, $CH_2O$), 5.49 (m, 1H, -CH=), 7.80 (m, 2H, aromatic), 8.01 (m, 2H, aromatic); *m/e* 258.08770 (calc. for $C_{15}H_{14}O_4$: 258.08921); *m/e* 240 (17%), 227 (100), 225 (10), 212 (5), 197 (15), 188 (20), 115 (32), 105 (38), 77 (44).

CHARACTERIZATION OF LOMATIOL LACTATE ESTER (**3**).—The metabolite (**3**) crystallized from acetone-hexane as yellow needles of pure lomatiol lactate ester (48 mg); mp 113–114°C; λmax (EtOH) 251, 276, 332, and 382 nm (ε14,852, 18,152, 2,379, and 1,293); νmax (KBr) 3510, 3350, 1718, 1660, 1640, and 1590 $cm^{-1}$; δ ($CDCl_3$) 1.40 (d, 3H, *J* 7.5 Hz, $CH_3$), 1.82 (s, 3H, $CH_3$), 3.35 (d, 2H, *J* 8 Hz, $CH_2$), 4.25 (q, 1H, *J* 7.5 Hz, CH-O), 4.55 (s, 2H $CH_2O$), 5.57 (t, 1H, *J* 8 Hz, -CH=), 7.72 (m, 2H, aromatic), 8.08 (m, 2H, aromatic); *m/e* 330.10830 (calc. for $C_{18}H_{18}O_6$: 330.11034); *m/e* 258 (5%), 240 (65), 227 (48), 225 (40), 223 (25), 212 (25), 197 (47), 115 (37), 105 (51), 77 (48), 45 (100).

The structure of (**3**) was verified by hydrolysis of the ester (4 mg/0.5 ml $H_2O$) with 0.5 N KOH/EtOH (0.1 ml). After 2 h at room temperature, the reaction mixture was acidified to pH 2 with 6 N HCl and extracted with chloroform. The organic layer was concentrated to dryness; and the residue, when recrystallized from acetone-hexane, yielded yellow needles (0.8 mg). The ir and mass spectra and chromatographic behavior were identical to those obtained with lomatiol (**2**).

Lactic acid was enzymatically determined by the measurement of the increase in absorbance at 340 nm ($NAD^+ \rightarrow NADH, H^+$) as lactic acid is oxidized by lactate dehydrogenase (Sigma Chemical Co., Kit No. 826-A) (14). The ester (1.58 mg/1 ml $H_2O$) was hydrolyzed with KOH and extracted as described above. An aliquot (0.2 ml) of the aqueous layer was added to a cuvette containing 2.8 ml of the enzyme solution (14). Controls containing boiled enzyme or standard lactic acid solution (0.40 mg/ml) were also prepared, and the cuvettes were incubated for 45 min at room temperature. The absorbance at 340 nm was measured against an enzyme solution blank with a Gilford 240 spectrophotometer: $A_{340}$ 1.657 (aqueous layer), 0.020 (boiled enzyme), and 1.340 (standard lactic acid).

PURIFICATION AND CHARACTERIZATION OF LAPACHOL-β-GLUCOSIDE (**4**).—Fractions containing lapachol-β-glucoside were purified separately by silica gel column chromatography with chloroform-methanol (97:3). The crude metabolite was further purified by crystallization from acetone-hexane or by preparative tlc [chloroform-methanol (9:1)]. A total of 390 mg of lapachol-β-glucoside was obtained, which when recrystallized from acetone-hexane gave yellow needles of pure metabolite (242 mg); mp 178–180° (decomp.); λmax (EtOH) 214, 250, 275, and 334 nm (ε14,621, 22,701, 16,673, and 3,271); νmax (KBr) (29) 3400, 1660, 1615, 1590, and 1065 $cm^{-1}$; δ ($CD_3OD$) 1.67 (s, 3H, $CH_3$), 1.80 (s, 3H, $CH_3$) 3.18 to 3.87 (m, 8H, $CH_2$ and C 2', -3', -4', -5', and -6'H), 5.17 (m, 1H, -CH=), 5.60 (br s, 1H, acetal CH), 7.80 (m, 4H, aromatic); *m/e* 404.14844 (calc. for $C_{21}H_{24}O_9$: 404.14712; *m/e* (29), 242 (13%), 228 (16), 227 (100), 199 (12), 181 (12), 179 (12), 152 (18), 115 (32), 105 (28), 77 (34).

Lapachol-β-glucoside (5 mg/0.25 ml MeOH) was hydrolyzed by refluxing with 2 N $H_2SO_4$ (0.25 ml) for 30 min. The mixture was extracted with ethyl acetate, and the organic layer was examined by tlc (systems A, B, and C) which indicated the metabolite had been converted to a mixture of lapachol and β-lapachone. The aqueous layer was adjusted to pH 7 with saturated $Na_2CO_3$ solution and examined by tlc (systems D, F, and G) and by paper chromatography [Whatman No. 1, butanol-pyridine-water-benzene (5:3:3:1) and phenol-water (4:1)]. The analyses indicated that the aqueous phase contained a single compound that was chromatographically identical to glucose. The identity of the sugar was confirmed by measurement of the spectral absorption of the colored product formed by treatment of the sugar with a solution or resorcinol in $H_2SO_4$ (18). An aliquot (10 μl) of the aqueous layer was added to a resorcinol solution (5 mg/2 ml $H_2O$), and 91% $H_2SO_4$ (5.5 ml) was added. After 45 min at room temperature, the visible spectrum was measured against a blank solution lacking the sugar; λmax 430, 495, and 558 nm.

The metabolite (**4**) was also hydrolyzed enzymatically with β-glucosidase (350 μ/mg, ICN Nutritional Biochemicals Corp.). A solution of lapachol-β-glucoside (5 mg/20 μl DMF) was dissolved in acetone (0.25 ml) and 1 ml of β-glucosidase (1 mg/ml) was added. The mixture was incubated at 37° for 6 h and then extracted with ethyl acetate. When the organic layer was concentrated to dryness, it yielded the aglycone (2 mg) which gave uv, ir, and mass spectra identical to those obtained with lapachol.

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