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MICROBIAL TRANSFORMATION OF OLIVETOL BY *FUSARIUM ROSEUM*

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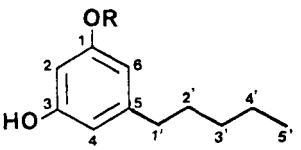
Microbial transformation studies with cannabinoids have resulted in the identification of metabolites modified on the *n*-pentyl side-chain and/or monoterpene units (1). Metabolism of the resorcinol moiety has not been seen. However, such metabolism occurs in mammals, where glucuronide conjugates of the phenolic hydroxyl group of cannabidiol have been found (2). In a continuing investigation of the pathways of metabolism of cannabinoids by microorganisms, a study of the biotransformation of olivetol (**1**) by *Fusarium roseum* was undertaken. Olivetol serves as an experimental model of the *n*-pentyl resorcinol moiety of cannabinoids (3). *F. roseum* appears to metabolize only the aromatic portion of this molecule.

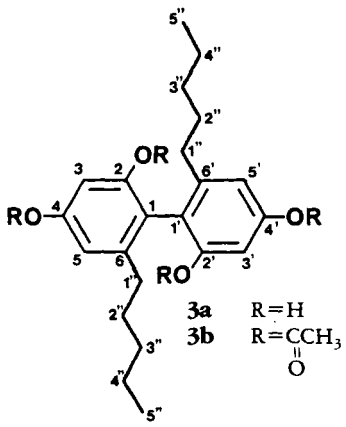
Initial screening studies with *F. roseum* indicated that this organism was capable of biotransforming olivetol to form both more and less polar metabolites than the starting material. After a time-course study indicated the optimal length of incubation, a preparative-scale fermentation was performed to isolate sufficient quantities of metabolites for structure elucidation. Two metabolites of olivetol were isolated and identified as monomethyl olivetol (**2**) and 2,2′,4,4′-tetrahydroxy-6,6′-di-*n*-pentyl-biphenyl (**3a**).



1 R=H
2 R=CH_3

The less polar metabolite was readily identified as monomethyl olivetol based on a comparison of its spectral properties with those of olivetol (3). The ms of **2** indicated a molecular ion of *m/z* 194 and molecular formula $C_{12}H_{18}O_2$. Modification of the aromatic moiety is suggested by the base peak of *m/z* 138, which arises via benzylic cleavage with proton transfer back to the aromatic ring. This is compared to the base peak of *m/z* 124 for olivetol (3). The ^1H nmr of **2** showed a singlet at 3.75 ppm (3 H) indicating the presence of a methoxyl group. No change in the side-chain resonances was seen relative to olivetol. A quartet at 55.46 ppm in the ^{13}C-nmr spectrum further confirmed the presence of a methoxyl group. In addition, six aromatic resonances are evident in this ^{13}C-nmr spectrum, since the symmetry of the aromatic unit has been destroyed due to methylation. The metabolite was also synthesized from olivetol by treatment with CH_2N_2 and the spectral data (^1H nmr, ms, uv, ir) are identical with those of the isolated metabolite.

The more polar metabolite was identified as the olivetol dimer **3a**. The ms



3a R=H
3b R=CCH_3 (=O)

showed a molecular ion of *m/z* 358 and indicated a molecular formula $C_{22}H_{30}O_4$. A sample of the metabolite was acetylated and gc/ms analysis of this peracetylated derivative **3b** showed a molecular ion of *m/z* 526 indicating the presence of four hydroxyl groups. Based on this evidence, the dimer must have been formed via coupling between two carbon atoms. The ^1H nmr showed an AB quartet at 6.32-6.22 ppm ($J=2.5$ Hz) for the aromatic protons, suggesting that the dimer was formed by oxidative coupling at carbons *ortho* to both a phenol and the *n*-pentyl side-chain. The other possible symmetrical dimer, formed by coupling at the carbons *ortho* to both phenols, would be expected to give a singlet for the aromatic protons, inasmuch as they would be equivalent. The dimeric metabolic is weakly optically active ($[\alpha]^{24}D$ +4.60°) due to atropisomerism or restricted rotation about the bond joining the aromatic rings (4).

Although the above microbially mediated reactions are unprecedented in cannabinoid transformation studies, they have been observed with other phenolic substrates. For example, pentachlorophenol is methylated by the fungus *Trichoderma viride* and 2,4-dichlorophenol is oxidatively coupled by a laccase from the fungus *Rhizoctonia praticola* (5). Aryl-aryl coupling has also been observed with non-phenolic substrates. *Streptomyces rimosus* oxidatively couples primaquine-*N*-acetate to form a diastereomeric mixture of symmetrical dimers linked at the 5 position of a quinoline nucleus (6).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir, uv, and ^{13}C-nmr spectra and optical rotations were determined as previously detailed (3). ^1H-nmr spectra were determined in the stated solvents with TMS as the internal standard on a Bruker HX90 (90 MHz) instrument equipped for pulse mode with Fourier transform analysis or on a Bruker WP80 (80 MHz) instrument. Chemical shifts are reported in ppm (δ) and coupling constants in Hz. High-resolution mass spectra were obtained with a Kratos MS-30 mass spectrometer. Gc/ms spectra were obtained with a Finnigan 4021 gc/ms instrument.

CHROMATOGRAPHY.—Tlc was performed on 0.25 mm silica gel 60F-254 plates (E. Merck). Chromatograms were visualized by fluorescence quenching under 254 nm uv light and by spraying with a 0.5% aqueous solution of Fast Blue BB salt (Aldrich Chemical Co.). Column chromatography was performed using the specified amount of Si gel 60 (230-240 mesh, E. Merck). The time-course of biotransformation was monitored by tlc analysis of culture extracts using a $CHCl_3$-EtOH (92:8) solvent system and revealed the presence of the two metabolites in addition to olivetol (monomethyl olivetol (**2**) Rf=0.55, olivetol (**1**) Rf=0.25, olivetol dimer (**3a**) Rf=0.18). Maximum conversion, as judged by tlc monitoring, was seen approximately 8 days after substrate addition.

CULTURE METHODS.—*F. roseum* culture was obtained from the Department of Plant Pathology and Weed Science, Mississippi State University, Mississippi State, Mississippi. It is currently maintained in the OSU College of Pharmacy culture collection by serial transfer on Czapek Solution Agar (Difco) slants.

Shaken cultures for biotransformation experiments were generated by a two-stage fermentation procedure in a medium consisting of (per liter of H_2O): Pharmamedia, 10 g; yeast extract, 5 g; D-glucose, 20 g; NaCl, 5 g; K_2HPO_4, 5 g. Stage I cultures were initiated by pipetting an aqueous spore suspension prepared from 5-day-old slant cultures of *F. roseum* into 100 ml of medium in a 500-ml Erlenmeyer flask. The Stage I cultures were incubated for 2 days (250 rpm, 25°) and then 10 ml of culture was withdrawn to inoculate each Stage II culture. The Stage II cultures were used for olivetol biotransformation experiments. Controls consisted of cultures grown without olivetol, incubations containing medium and substrate without microorganism, and incubations containing substrate and autoclaved organism. Neither of the transformation products were found by tlc analysis of controls.

OLIVETOL.—Spectral data for olivetol have been described elsewhere (3).

TRANSFORMATION OF OLIVETOL BY *F. ROSEUM*.—Olivetol was dissolved in absolute EtOH (1.66g/33.2 ml) and distributed evenly among 83 48-h-old Stage II cultures. The cultures were incubated (250 rpm, 25°) for 8 days and were then harvested by filtration. The filtrate was acidified to pH 3 with concentrated HCl and extracted consecutively with hexane (twice), $CHCl_3$ (three times), and EtOAc (three times), in a ratio of 4 parts filtrate to 3 parts extraction solvent. The hexane extraction removed ergosterol

and other lipids which interfered with the chromatography of the metabolites. The $CHCl_3$ and EtOAc extracts were both subjected to a precolumn purification step, which consisted of flushing the extract through 10 g of Si gel 60 (230-400 mesh, E. Merck) with EtOAc as eluent. This removed very polar non-phenolic components from the extracts.

The EtOAc extract (1.25 g, after precolumn) was applied to a Si gel 60 column (120 g, 69×2.3 cm), and eluted with hexane-EtOAc (18:7) (4.9 ml fractions). Fractions 33-44 contained pure metabolite **2** (58.5 mg, 3.3% yield).

The $CHCl_3$ extract (1.480 g, after precolumn) was applied to a Si gel 60 column (120 g, 69×2.3 cm), and eluted with $CHCl_3$-EtOH (92:8) (5 ml fractions). Fractions 91-116 (81.5 mg) contained impure **3a** and were chromatographed on a Si gel 60 column (8 g, 30×0.8 cm), using $CHCl_3$-MeOH (94:6) as eluent (0.8 ml fractions). Fractions 34-42 from this column were pooled to give 9.8 mg of still impure **3a**.

The mycelial tissue was exhaustively extracted with EtOH to give 10.84 g of a brown oil. The oil was partitioned between $CHCl_3$ and H_2O (300 ml each, three times). The $CHCl_3$ extract (4.32 g) was further partitioned between hexane and 90% MeOH (200 ml each, three times). The 90% MeOH solubles (0.88 g) were partitioned between $CHCl_3$ and 70% MeOH (150 ml each, three times). The $CHCl_3$ extract (397 mg, after precolumn) contained some **3a** and was therefore chromatographed on a Si gel 60 column (35 g, 40×1.5 cm), using $CHCl_3$-EtOH (92:8) as eluent (3.5 ml fractions). Fractions 19-25 from this column contained impure **3a** (70 mg).

The impure fractions of **3a**, from beer and mycelia, were combined and subjected to preparative tlc in CH_2Cl_2-EtOH (92:8) to give 12.9 mg of **3a** (0.4% yield).

CHARACTERIZATION OF MONOMETHYL OLIVETOL (**2**).—Compound **2** exhibited the following spectral properties: uv λ max (EtOH) (log ε) 279 (3.15), 273 (3.16), 200 (4.35); ir ν max ($CHCl_3$) 3595, 3300, 2925, 2850, 1600, 1190, 1160, and 1050 cm^{-1}; ^1H nmr ($CDCl_3$, 80 MHz) δ 0.88 (3H, t, J=6, 5′CH_3), 1.25-1.65 (6H, m, 2′-, 3′-, 4′-CH_2), 2.51 (2H, t, J=7, 1′-CH_2), 3.75 (3H, s, -OCH_3), 5.07 (1H, br s, exchanges with D_2O, -OH), 6.25-6.32 (3H, m, 2-, 4-, 6-ArH); cmr ($CDCl_3$), 14.07 (q, C-5′), 22.66 (t, C-4′), 30.87 (t, C-2′), 31.70 (t, C-3′), 36.22 (t, C-1′), 55.46 (q, -OCH_3), 99.24 (d, C-2), 107.20 (d, C-6), 108.36 (d, C-4), 145.98 (s, C-5), 156.92 (s, C-3), 161.18 (s, C-1); ms *m/z* 194.1308 (25.39%, calcd. for $C_{12}H_{18}O_2$ 194.1308), 138.0665 (100%, calcd. for $C_8H_{10}O_2$ 138.0681), 137.0609 (16.13%, calcd. for $C_8H_9O_2$ 137.0602).

SYNTHESIS OF MONOMETHYL OLIVETOL (**2**).—Olivetol (106 mg, 0.59 mmol) was dissolved in 2 ml MeOH. To this was added an excess of freshly distilled ethereal CH_2N_2. The flask was stoppered and allowed to stand at ambient temperature for 10 h. Excess solvent was evaporated and the resulting yellow oil (113 mg) was purified by preparative tlc in $CHCl_3$-EtOH (92:8) to give 31 mg monomethyl olivetol, identical in all respects (^1H nmr, ms, uv, ir, co-tlc) to the metabolite **2**.

CHARACTERIZATION OF OLIVETOL DIMER (**3a**).—Metabolite **3a** exhibited the following spectral properties: uv λ max (EtOH) (log ε) 282 (3.47), 208 (4.25); ir ν max (neat) 3400, 2920, 2860, 1590, 1460, and 1150 cm^{-1}; ^1H nmr (CD_3OD, 90 MHz) δ 0.81 (6H, t, J=6, 5″-CH_3), 1.10-1.50 (12H, m, 2″-, 3″-, 4″-CH_2), 2.18 (4H, t, J=7, 1″-CH_2), 6.24 (2H, d, J=2.5, 5-, 5′-ArH), 6.30 (2H, d, J=2.5, 3-, 3′-ArH); ms *m/z* 358.2135 (40.4%, calcd. for $C_{22}H_{30}O_4$ 358.2144), 302.1485 (17.3%, calcd. for $C_{18}H_{22}O_4$ 302.1518), 287.1272 (33.9%, calcd. for $C_{17}H_{19}O_4$ 287.1283), 41.0391 (100%, calcd. for C_3H_5 41.0391); $[\alpha]^{24}D$ +4.60° (c 0.4 MeOH).

SYNTHESIS OF **3b**.—Metabolite **3a** (1 mg) was dissolved in 0.5 ml pyridine and 0.5 ml Ac_2O was added. The mixture was stirred at ambient temperature for 17 h and then 5 ml MeOH was added. The mixture was evaporated to dryness to give 1.5 mg, which was submitted for gc/ms analysis. The gc was equipped with a 3% SP2100 column (glass, 6 ft×2 mm) and a temperature program (200° for 0.1min, increasing to 250° at 5°/min, injector 270°) was used. Ms (70 eV) *m/z* 526 (0.5%), 484 (20.7%), 442 (46.2%), 400 (50.2%), 358 (49.0%), 287 (24.2%), 42 (100%).

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