

## Microbial Production of a Crisnatol Metabolite

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Microbial metabolism studies of crisnatol (1), a new DNA intercalator, has resulted in the isolation and characterization of a major metabolite identified as the C-1 hydroxylated crisnatol (2). The structure of the metabolite was established by comparison of its spectral data to that of crisnatol. Complete <sup>13</sup>C-NMR assignments for crisnatol and its C-1 hydroxylated metabolite were also made.

**KEY WORDS:** crisnatol metabolite; crisnatol C-13 nuclear magnetic resonance (NMR) assignments; microbial metabolism; two-dimensional (2D) NMR techniques.

### INTRODUCTION

Crisnatol (1) is a novel DNA intercalator that has been shown to be very active against an array of murine tumors (1-3) (Scheme I). Its disposition, excretion, and metabolism have also been reported (4,5). Microorganisms, especially fungi, have been shown to be excellent predictors of mammalian drug metabolites and have the capability of producing metabolites in quantities sufficient for complete structure elucidation studies and further biological evaluation (6,7). In view of the low yield of metabolites obtained (4,5), crisnatol was subjected to a microbial metabolism study which has resulted in the efficient production of a major metabolite of crisnatol. The procedure for preparation of this metabolite and the rigorous establishment of its structure by two-dimensional nuclear magnetic resonance (2D-NMR) techniques are reported herein.

### MATERIALS AND METHODS

**Chemicals.** All solvents were analytical grade. Crisnatol mesylate was provided by Burroughs Wellcome Company.

**Instrumentation.** The melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. <sup>1</sup>H- and <sup>13</sup>C-NMR spectral data were obtained on a Varian VXR-300 FT spectrometer operating at 300 and 75 MHz, respectively. The chemical shift values are reported as parts per million (ppm) and the coupling constants as hertz (Hz). Abbreviations for NMR signals are as

follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; m, multiplet; and br, broad. Standard pulse sequences were used for COSY, HETCOR, DEPTGL, NOESY, and APT experiments. The LR-HETCOR was optimized for  $J = 5$  and 10 Hz.

**Microorganisms.** *Cunninghamella elegans* ATCC 9245 was obtained from the culture collection at The University of Mississippi, Department of Pharmacognosy, and originally purchased from the American Type Culture Collection (ATCC). It was maintained on Mycophil agar slants stored at 4°C.

**Chromatographic Procedure.** Thin-layer chromatography (TLC) was performed on precoated Silica gel G-25 UV<sub>254</sub> plates (0.25 mm, Brinkmann Instruments Inc., West Germany) using methylene chloride-methanol-ammonia (85:10:1; v/v) as eluting solvent. The  $R_f$  values in this solvent system were as follows: crisnatol, 0.74; metabolite a (2), 0.60; and metabolite b, 0.32. The plates were visualized by viewing under UV light and spraying with iodoplatinate reagent. Low-pressure column chromatography was performed using a 2.5 × 50-cm Aldrich class column packed with 130 g of kieselgel 60 (230-400 mesh, E. Merck, Darmstadt, Germany). The column was eluted with 2% methanol/methylene chloride with increasing concentrations of methanol (up to 10%). The liquid chromatography/mass spectrometry (LC/MS) system used was the same as that described previously (8).

**General Fermentation Procedure.** A two-stage fermentation procedure was used. In the first stage a medium consisting of 20 g dextrose, 5 g yeast extract (BBL), peptone (Difco), 5 g K<sub>2</sub>HPO<sub>4</sub>, 5 g NaCl, 1000 ml distilled water, pH adjusted to 6.8 with 2 N HCl was used. The medium was autoclaved in individual flasks for 15 min. Nutrient broth was used as the medium for the second-stage fermentation. Fermentations were performed using stainless-steel capped 125-ml Bellco-Delong culture flasks containing 25 ml of medium using a G-10 Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) operating at 150 rpm (room temperature.)

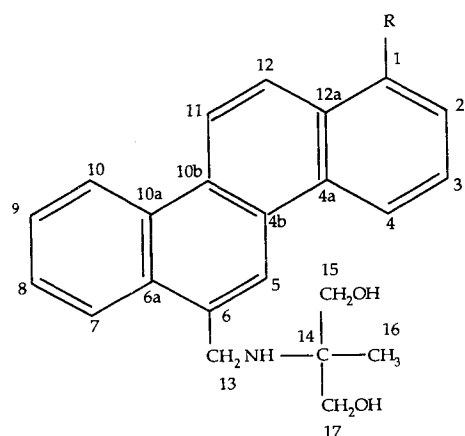
First-stage flasks were inoculated from slants of *C. elegans* (ATCC 9245) under aseptic conditions. After a 48-hr incubation period, a 3-ml portion of the stage I medium was used to inoculate the stage II medium. For the screening-scale studies, the incubation was continued for 10 hr prior to phenobarbital (inducer) addition, and 24 hr later the substrate (crisnatol mesylate) was added as a solution of DMF (0.2 mg/ml of medium). Samples were extracted three times with ethyl acetate and the organic layer was dried *in vacuo* (40°C). The samples were analyzed using thin-layer chromatography.

**Preparative-Scale Fermentation.** Preparative-scale fermentation of crisnatol (1) by *C. elegans* (ATCC9245) was performed using a two-stage fermentation procedure. Stage I was conducted in the medium described for screening. Eight liters of nutrient broth was used for stage II (400 ml/2-liter flask). These cultures were grown for an additional 10 hr before the addition of the inducer, phenobarbital sodium (60 mg in 1.0 ml 70% EtOH). Twenty-four hours later the substrate, crisnatol mesylate (600 mg/2.0 ml DMF), was evenly distributed into the culture flasks. Metabolism of the sub-

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1 R = H

2 R = OH

Scheme I

strate was monitored by TLC as described above, and the fermentation was terminated after 15 days by the addition of ethyl acetate to the flasks. The cultures were harvested by filtration, pooled, and exhaustively extracted with ethyl acetate. The organic phase was dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated *in vacuo* ( $40^\circ\text{C}$ ) to obtain a dark brown viscous residue. A total of 1.61 g of the residue was adsorbed on 1.5 g of silica and loaded on a kieselgel column (230–400 mesh,  $2.5 \times 50$  cm), eluted with 2%  $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$  (8-ml fractions were collected, and TLC was used for pooling fractions). A second chromatography on the still impure metabolite was performed on a small silica gel column ( $1 \times 20$  cm) using the same solvent as described previously. Metabolite a (2) crystallized as pale yellow needles (methanol/acetone) (45 mg) having mp  $222\text{--}224^\circ\text{C}$ ;  $\text{C}_{23}\text{H}_{23}\text{O}_3\text{N}$ ; LC/MS  $\text{M}^+ + \text{H} = 362$  (100%);  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR (see Table I).

The small amount of metabolite b (~10 mg) was obtained from more polar fractions but was determined to be impure and somewhat unstable. The LC/MS and  $^1\text{H}$ -NMR data suggested the 1,2-dihydrodiol metabolite (4).

## RESULTS AND DISCUSSION

Crisnatol (1) was subjected to a microbial metabolism study as described previously (6). Screening-scale studies using 40 organisms revealed that only *Cunninghamella elegans* (ATCC9245) showed the ability to biotransform crisnatol. A preparative-scale fermentation using this same organism provided 45 mg of a crystalline metabolite identified as the C-1 hydroxylated metabolite (2) and a smaller amount of a metabolite that was still impure and tentatively identified as the 1,2-dihydrodiol (4). The LC/MS technique previously used with arteether proved to be very useful in aiding metabolite identification in this study as well (8).

The molecular formula of the crystalline metabolite was suggested as  $\text{C}_{23}\text{H}_{23}\text{O}_3\text{N}$  from the mass spectral data. The additional oxygen present in the molecular formula, compared to that of crisnatol, was clearly present as a phenolic hydroxyl group as determined by  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data. The location of the hydroxyl group at C-1 (2) was determined

Table I.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR Assignments for Crisnatol (1) and Metabolite (2)<sup>a</sup>

C No.	$\delta_{\text{C}}$		$\delta_{\text{H}}$	
	1	2	1	2
1	128.4 (1) <sup>1</sup>	153.8 (0)	8.05 (d, 9) <sup>1</sup>	—
2	126.8 (1) <sup>2</sup>	109.7 (1)	~7.0 (m)	7.10 (brd, 8.0)
3	126.5 (1) <sup>2</sup>	127.3 (1)	~7.0 (m)	7.50 (dd, 8.0, 8.0)
4	126.9 (1) <sup>1</sup>	114.0 (1)	8.07 (d, 10) <sup>1</sup>	~8.4 (m)
4a	129.8 (0)	131.4 (0)	—	—
4b	127.2 (0)	127.1 (0)	—	—
5	120.9 (1)	121.7 (1)	8.90 (s)	8.84 (s)
6	136.5 (0)	135.8 (0)	—	—
6a	130.7 (0)	130.7 (0)	—	—
7	124.8 (1)	124.8 (1)	8.41 (brd, 9.3)	~8.4 (m)
8	126.5 (1) <sup>2</sup>	126.5 (1)	~7.7 (m)	~7.7 (m)
9	126.5 (1) <sup>2</sup>	126.5 (1)	~7.7 (m)	~7.7 (m)
10	123.7 (1)	123.8 (1)	8.94 (brd, 9.0)	8.90 (brd, 9.0)
10a	130.3 (0)	130.3 (0)	—	—
10b	127.1 (0)	127.4 (0)	—	—
11	121.3 (1)	119.7 (1)	8.80 (d, 9.0)	8.78 (d, 9.0)
12	123.3 (1)	121.0 (1)	8.94 (brd, 9.0)	~8.4 (m)
12a	131.7 (0)	122.5 (0)	—	—
13	43.9 (2)	43.9 (2)	4.34 (s)	4.35 (s)
14	57.3 (0)	57.7 (0)	—	—
15	64.6 (2)	64.6 (2)	3.50 (s)	3.50 (s)
16	18.5 (3)	18.5 (3)	1.15 (s)	1.19 (s)
17	64.6 (2)	64.6 (2)	3.50 (s)	3.50 (s)

<sup>a</sup> Signal assignments bearing the same numerical superscript may be reversed. The data were obtained on the free bases in DMSO. The numbers in parentheses indicate the number of attached protons (carbon chemical shift) and the multiplicity with the coupling constant ( $J$ ) in Hz (proton chemical shift).

by an intensive study of the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data of crisnatol (1) and the metabolite (2).

The assignment of the  $^1\text{H}$  and  $^{13}\text{C}$ -NMR for crisnatol (1) [free base in dimethyl sulfoxide (DMSO)] was achieved by collecting the  $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR, APT,  $^1\text{H}$ - $^{13}\text{C}$  heterocorrelation (HETCOR),  $^1\text{H}$ - $^1\text{H}$  homocorrelation (COSY), long-range  $^1\text{H}$ - $^{13}\text{C}$  heterocorrelation (LR-HETCOR), and NOESY spectra data. The assignments are listed in Table I. The key experiment for these assignments was the long-range  $^1\text{H}$ - $^{13}\text{C}$  heteronuclear correlation (optimized for  $^3J_{\text{C-H}} = 10$  Hz), which showed correlations from H-13 to carbons C-6a, C-6, and C-5 and H-5 to carbons C-6a and C-4a. Once these assignments were made, the other proton assignments were straightforward, which then allowed all of the carbons to be assigned as well. NOESY correlations were also noted for H-5/H-4 and H-11/H-10.

With assignments for crisnatol (1) in hand, similar analyses were made for the metabolite (2). The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR clearly showed that the extra oxygen was added to the aromatic system most likely as a phenolic hydroxyl group (see Table I) and the only question to be determined was its location. Because there is only one upfield aromatic signal in the  $^1\text{H}$ -NMR which resonates as a broad doublet ( $J = 8.0$  Hz; COSY shows a small cross peak to H-4), the signals for H-11 and H-12 were present, and there was only one protonated signal found significantly upfield (110 ppm or lower) in

the  $^{13}\text{C}$ -NMR, it is suggested that the hydroxyl group is located at C-1, C-4, C-7, or C-10. Carbons 7 and 10 were not shifted when compared with crisnatol. A strong NOESY correlation between H-5 and H-4 (between H-10 and H-11 as well) allowed placement of the hydroxyl at C-1. Placement of the hydroxyl at C-1 is also consistent with the observation that H-12 has been shifted by 0.54 ppm compared to crisnatol. The long-range  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR correlations were in complete agreement with this structure (2) as well as those assignments for crisnatol (1). The  $^1\text{H}$ -NMR assignments are also in agreement with those reported previously (4).

Microorganisms can be used to prepare difficult-to-synthesize drug metabolites as well as serve as *in vitro* models for predictors of mammalian drug metabolism (6,7). This study illustrates another important example of the value of microbial systems as an adjunct to drug metabolism studies.

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