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ARTICLES

Microbial Models of Mammalian Metabolism: Production of 3′-Hydroxywarfarin, a New Metabolite of Warfarin Using *Cunninghamella elegans*

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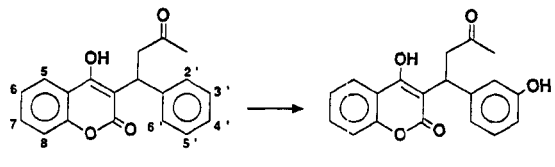
Abstract □ Warfarin, an anticoagulant and "metabolic probe" for cytochrome P-450 isozyme multiplicity, was metabolized by the fungus *Cunninghamella elegans* (ATCC 36112) to yield the previously unreported metabolite 3′-hydroxywarfarin. This metabolite was isolated from cell suspension cultures and characterized by analytical (HPLC) and spectral (EI-MS, PMR) comparisons with synthetic 3′-hydroxywarfarin.

The coumarin anticoagulant warfarin (1) has found extensive use both as a rodenticide and as a clinically effective agent for the treatment of a number of hematological disorders including thrombophlebitis, pulmonary embolism, and myocardial infarction.[1] In recent years, warfarin has also been employed as a "metabolic probe" for determining cytochrome P-450 isozyme profiles in diverse in vitro and in vivo mammalian metabolic systems.[2–18]

The metabolism of warfarin has been extensively studied for over two decades. Metabolites resulting from aromatic hydroxylation in the 4′-, 6-, 7-, and 8-positions, as well as aliphatic hydroxylated products, have been isolated from mammals, including humans.[9–11] The overall regioselectivity of warfarin hydroxylation (i.e., position of hydroxylation) and substrate stereoselectivity observed (i.e., which enantiomer is preferentially hydroxylated) have been shown to be dependent on the specific P-450 isozymes present; this serves as the basis for its use as a metabolic probe for examining cytochrome P-450 isozyme profiles in a wide range of biological systems. Other metabolic pathways of warfarin include reduction of the ketonic side chain to yield diastereomeric alcoholic products[19] and oxidative cleavage of the coumarin ring.[20,21]

Recent investigations in our laboratory have focused on the applications of microorganisms as models to study metabolism of drugs and other xenobiotics in mammals, including humans. Such studies are based on comparative metabolism, as the same types of phase-1 biotransformations observed in mammals are paralleled in selected microbial species. This discipline, referred to as "microbial models of mammalian metabolism",[22] has been the subject of several recent reviews.[23–27] While the aim of these studies is to demonstrate similarities and differences between these biological extremes in the types of enzymes and mechanism involved in xenobiotic metabolism, an ultimate practical goal is the development of a model consisting of one or more organisms exhibiting the broad range of mammalian phase-1 biotransformations for applications in the early stages of drug development and toxicological evaluation. Such a model could be used to predict metabolite candidates of new drugs, to explore unknown metabolic pathways, and to prepare sufficient quantities of specific metabolites for structure elucidation, biological evaluation, and for use as analytical standards in mammalian metabolism and pharmacokinetic studies.

Because of its diverse metabolic profile and the well-characterized enzymology associated with its metabolism, warfarin (1; Scheme I) was selected as a model xenobiotic to explore parallels in microbial versus mammalian metabolism. Previous studies have demonstrated distinct parallels in the metabolism of this agent.[28–30] In particular, the conversion of warfarin to 4′-hydroxywarfarin by the fungus *Cunninghamella bainieri* has been shown to involve the NIH shift (arene oxide) pathway, consistent with a cytochrome P-450-mediated hydroxylation mechanism.[30] We have recently observed that the fungus *Cunninghamella elegans* (ATCC 36112) is capable of producing the full profile of known



Warfarin (1) 3′-Hydroxywarfarin (2)

Scheme I—Structure and numbering system for warfarin (**1**), and conversion to 3′-hydroxywarfarin (**2**) by *Cunninghamella elegans*.

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mammalian metabolites of warfarin,[31] and that these biotransformations are also stereoselective.[32] These studies have also led to the observation that this fungus is capable of producing a previously unreported phenolic metabolite of warfarin, 3'-hydroxywarfarin (2). The isolation and characterization of this new metabolite is the subject of this report.

Experimental Section

Solvent, Reagents, and Standard Compounds—All reagents were analytical reagent grade or higher in quality. Solvents for HPLC were HPLC grade (OmniSolv, MCB Manufacturing Chemists, Cincinnati, OH). Water for HPLC and media preparation was deionized and double distilled in glass. Racemic warfarin (1) was obtained from Sigma Chemical Company (St. Louis, MO). The synthetic precursors 4-hydroxycoumarin and 3-hydroxybenzaldehyde were purchased from Aldrich Chemical Company (Milwaukee, WI). The requisite *meta*-hydroxybenzalacetone was prepared by crossed aldol condensation using acetone and 3'-hydroxybenzaldehyde as described below.[33] The synthetic metabolite 3'-hydroxywarfarin (2) was synthesized by condensation of 4-hydroxycoumarin and *meta*-hydroxybenzalacetone according to the method of Hermodson et al., with appropriate modification (see below).[34] Ethereal diazomethane used in derivatization reactions was generated using Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulphonamide) and a diazomethane-generating apparatus (Aldrich).

Instrumentation—Melting points were taken on a Fisher-Johns melting point apparatus and are not corrected. Proton nuclear magnetic resonance spectra were generated in $CDCl_3$ or $CDCl_3$:$(CD_3)_2CO$, using tetramethylsilane as the internal standard, on a General Electric model GN-500 (500 MHz) or a Nicolet model 200 (200 MHz) spectrometer. Low resolution electron impact-mass spectra (EI-MS) were taken on a Dupont model 21419 mass spectrometer by direct probe insertion.

Chromatographic Procedures—Thin-layer chromatography was performed on plastic-backed 0.25-mm silica gel GF_{254} TLC plates (Polygram, Brinkman, Houston, TX) eluted with ethyl acetate:hexane (40:60). Plates were visualized using UV (254 nm) light.

High-performance liquid chromatography was conducted using a Beckman model 110A pump and a Hitachi model 100-10 variable wavelength detector set at 310 nm. The system was fitted with a Beckman 210 sample injector and either a 250- or 500-μL fixed volume loop. Chromatograms were recorded with a Hewlett-Packard model 3390A reporting integrator. Each solvent system was prepared by filtering individual components through glass filter pads (GF/F grade; Whatman, Clifton, NJ), mixing, and deaerating ultrasonically before use. The following chromatographic systems were used: (A) Spherisorb Si-5 (silica) column (5 μm, 0.45 × 25 cm; Analytical Sciences, Inc., Santa Clara, CA) eluted with hexane:ethyl acetate:acetic acid:methanol (75:25:0.4:0.25) at 1.0 mL/min; (B) Ultrasphere ODS (C-18) column (5 μm, 0.45 × 25 cm; Beckman Associates, Berkeley, CA) eluted with methanol:5 mM phosphoric acid (70:30) at 1.0 mL/min; (C) Ultrasphere ODS (C-18) column (5 μm, 0.45 × 25 cm; Beckman) eluted with phosphate buffer (5 mM, pH 7.0):methanol:tetrahydrofuran (61:32:7), containing 0.01 M tetrabutylammonium phosphate, at 1.0 mL/min;[35] (D) Ultrasphere ODS (C-18) column (5 μm, 0.45 × 25 cm; Beckman) eluted with aqueous ammonium acetate (pH 4.8):acetonitrile (70:30) at 1.4 mL/min[36] [the ammonium acetate buffer (pH 4.8) was prepared by pH adjustment of 1.5% acetic acid with ammonium hydroxide]; (E) Ultrasphere ODS (C-18) column (5 μm, 0.45 × 25 cm; Beckman) eluted with aqueous ammonium acetate (pH 4.8):acetonitrile (50:50) at 1.0 mL/min. Under these conditions, the following retention times (min) were observed for synthetic 3'-hydroxywarfarin (2) and the metabolite, respectively: System A = 15.03 and 15.00; System B = 9.30 and 9.29; System C = 12.84 and 12.80; System D = 10.10 and 10.02. System E was used only for purification.

Synthesis of *meta*-Hydroxybenzalacetone—*meta*-Hydroxybenzaldehyde (2.0 g; 16 mmol) was dissolved in a solution of acetone (10 mL) and 5% NaOH (40 mL). The solution was stirred for 2 h and then acidified with concentrated HCl. The mixture was then extracted with ether (2 × 50 mL). The ethereal layer was pooled and the solvent was removed under reduced pressure to give 2 g of oil (yield 75%) which solidified on standing. The following physical data were obtained: mp 96 °C; low resolution mass spectrum [*m/z* (% relative abundance)] M^+ = 162 (39), 147 (100), 119 (62), 91 (95), 65 (50); 200 MHz ^1H NMR ($CDCl_3$): [δ (multiplicity, *J*, assignment)] 7.49 (d, *J* = 17 Hz, 1H, Ar-CH=), 7.38 (dd, *J* = 8 Hz, 1H, C5-H), 7.1 (d, 1H, *J* = 8 Hz, C6-H), 7.07 (s, 1H, C2-H), 6.92 (m, 1H, C4-H), 6.7 (d, *J* = 17 Hz, =CH-CO-), 2.4 (s, 3H, CH_3).

Synthesis of 3'-Hydroxywarfarin (2)—A solution of 4-hydroxycoumarin (0.5 g, 3.1 mmol) and *meta*-hydroxybenzalacetone (1.23 g, 7.6 mmol) in methanol (50 mL) was refluxed for 3 days, allowed to cool to room temperature, and the methanol was removed under reduced pressure. The residue was dissolved in acetone (10 mL). An equal volume of 6M HCl was added to this solution and the mixture was stirred overnight. A saturated solution of NaCl (20 mL) was added, and the final mixture was extracted with ethyl acetate (2 × 20 mL). The volume of the organic phase was reduced and the product was allowed to crystallize to give 700 mg of the product (yield 76%). The following analytical data were obtained: mp 186 °C [lit. 188–189 °C[34]]; low resolution mass spectrum [*m/z* (% relative abundance)] M^+ = 324 (10), 306 (20), 281 (57), 265 (34), 249 (41), 235 (23), 221 (12), 187 (18), 163 (19), 162 (61), 161 (40), 147 (50), 121 (88), 120 (93), 119 (60), 93 (27); 500 MHz ^1H NMR following methylation of the 4-enol with diazomethane ($CDCl_3$) [δ (multiplicity, *J*, assignment)] 7.65 (dd, *J* = 1.5 & 7.9 Hz, 1H, C5-H), 7.47 (ddd, *J* = 1.6, 7.3, & 8.6 Hz, 1H, C7-H); 7.26 (m, 2H, C6-H & C8-H), 7.12 (dd, *J* = 7.9 Hz, 1H, C5'-H), 7.0 (br d, *J* = 7.9 Hz, 1H, C6'-H), 6.96 (br dd, *J* = ~2.0 Hz, 1H, C2'-H), 6.65 (ddd, *J* = 0.8, 2.5 & 7.9 Hz, 1H, C4'-H), 4.93 (dd, *J* = 9.4 & 5.7 Hz, 1H, C9-H), 4.82 (s, 1H, phenolic-OH), 4.03 (s, 3H, -OCH_3), 3.88 (dd, *J* = 18.1 & 9.4 Hz, 1H, C10-methylene H_a), 3.19 (dd, *J* = 18.1 & 5.7 Hz, 1H, C10-methylene H_b), 2.15 (s, 3H, CH_3).

Preparation of Cell Suspensions of *Cunninghamella elegans*—The fungus *Cunninghamella elegans* (ATCC 36112) was maintained on refrigerated (4 °C) slants of Sabouraud-maltose agar (Difco Laboratory, Detroit, MI) and transferred to fresh slants every 6 months to maintain viability. This fungus was grown according to a two-stage fermentation procedure. The surface growth of a slant was used to inoculate one Stage-1 Bellco-Delong flask (1 L) containing 200 mL of growth medium. The medium used in these experiments consisted of the following: dextrose, 20 g; soybean meal (20 mesh; Capitol Feeds, Austin, TX), 5 g; NaCl, 5 g; potassium phosphate (dibasic), 5 g; yeast extract (Difco), 5 g; distilled water, 1000 mL (pH was adjusted to 7.0 with 6M HCl). The medium was sterilized in individual flasks at 121 °C for 15 min. After inoculation, the stage-1 flask was incubated for 72 h at 27 °C and 250 rpm in a G-25 Environmental Shaker (New Brunswick Scientific Company, Edison, NJ) at which time 10 mL of culture was used to inoculate each Stage-2 flask (1 L) containing 200 mL of fresh medium. After incubation for 24–48 h, the fungal cells were harvested by filtration. The residual culture medium was removed by repeated rinsing with distilled water, and the cell pellets were collected by filtration under reduced pressure. Each cell suspension culture was prepared by suspending 4 g of cell pellets in 20 mL of pH 6.7 phosphate buffer (0.5 M) in a 125-mL Bellco-Delong flask.

Production of 3'-Hydroxywarfarin (2) with *Cunninghamella elegans*—A total of 100 flasks of cell suspension were prepared as described above. After incubation for 1 h, 6 mg of warfarin as the potassium salt in 100 μL of sterile distilled water was added to each flask. The incubation was allowed to continue for 48 h (27 °C, 250 rpm). The cell suspensions were filtered to give a total of 2 L of filtrate. A total of 100 mL of filtrate was injected with the aid of a 10-mL glass syringe into a Sep-Pak C-18 Cartridge (Waters Associates, Inc., Milford, MA) which had been preconditioned with methanol (5 mL) and pH 4.7 ammonium acetate buffer (5 mL). The metabolites were eluted from the cartridge with 5 mL of acetone. The remaining culture filtrate was similarly processed. The acetone eluants were pooled, and the solvent was taken to dryness under reduced pressure. The residue was dissolved in ethyl acetate (200 mL), partitioned with water (2 × 200 mL), and the aqueous phase discarded. The organic phase was back extracted with 0.5 M KOH (2 × 100 mL). The resulting aqueous phase was acidified with concentrated HCl and extracted with ethyl acetate (2 × 200 mL). The organic phase was taken to dryness under reduced pressure to give the crude extract which was dissolved in acetonitrile (5 mL). To this solution was added pH 4.7 ammonium acetate buffer (5 mL). The mixture was sonicated and centrifuged at 3000 rpm for 5 min. The metabolites were readily separated from warfarin by repeated 500-μL injections of the clear supernatant into the HPLC (System E). The fractions containing all metabolites were collected and pooled. The eluant was then diluted with an equal volume of water. The resulting solution was extracted

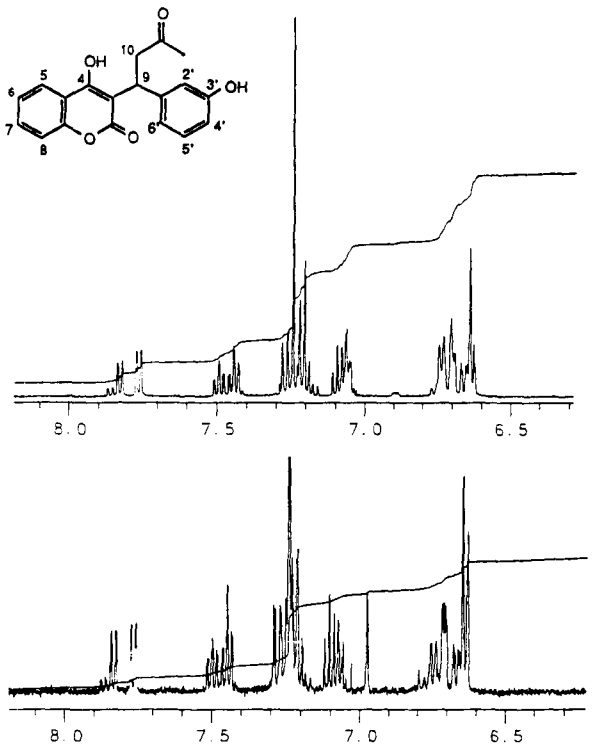
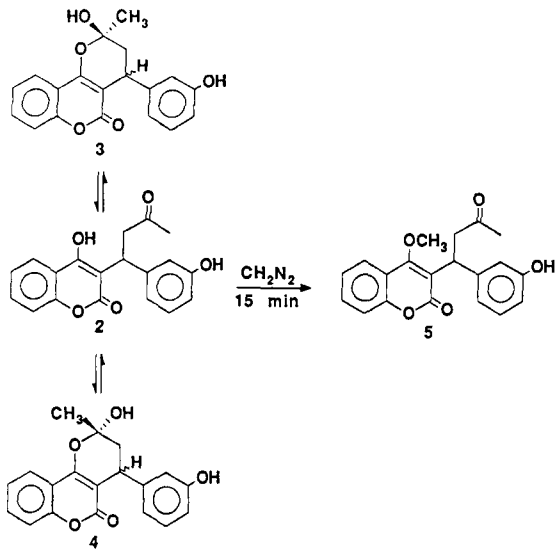


Figure 1—*The 500 MHz ^1H NMR spectra of 3′-hydroxywarfarin* (**2**) standard (top) and isolated metabolite (bottom).

with ethyl acetate (2 × 50 mL). The organic phase was taken to dryness under reduced pressure and the residue was dissolved in the mobile phase (10 mL, HPLC system D). After dilution with 10 mL of pH 4.7 buffer, the unknown metabolite was separated from the other phenolic and alcoholic metabolites of warfarin on HPLC system D by repeated 500-μL injections of this solution into the HPLC and collection of the fraction containing this metabolite. The collected eluant was diluted with an equal volume of pH 4.7 buffer and the solution was extracted with an equal volume of ethyl acetate. The organic phase was taken to dryness under nitrogen to give ≤1 mg of metabolite.

High-Resolution (500 MHz) Proton Nuclear Magnetic Resonance Analysis of 3′-Hydroxywarfarin (2)—The ^1H NMR spectra of 3′-hydroxywarfarin (2) and the isolated metabolite were generated in



Scheme II—Proposed open-chain/hemiketal tautomeric equilibrium of 3′-hydroxywarfarin (**2**) and methylation to yield the enol-ether **5**.

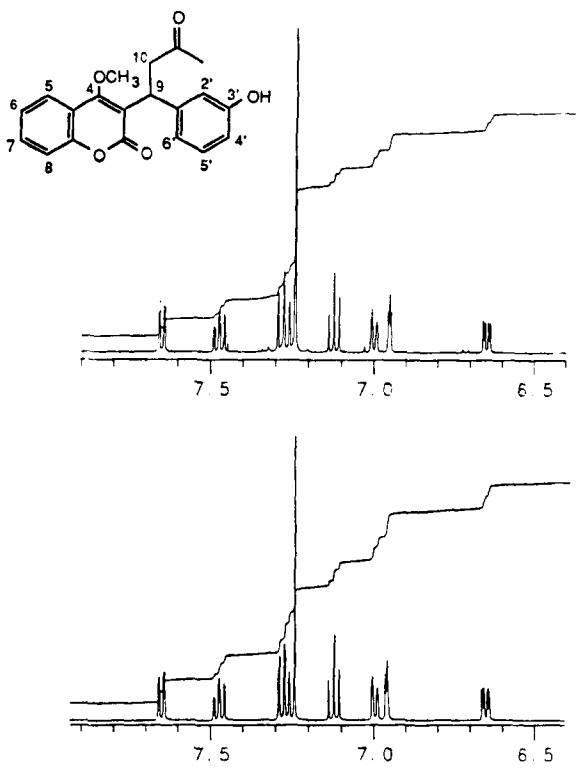


Figure 2—The 500 MHz ^1H NMR spectra of methylated derivatives of 3′-hydroxywarfarin (**5**) standard (bottom) and isolated metabolite (top).

$CDCl_3$:$(CD_3)_2CO$. Proton NMR spectra were also generated for the corresponding methylated derivatives (5), which were generated by adding 1 mL of ethereal diazomethane to the metabolite (or standard 3′-hydroxywarfarin). The derivatization was allowed to proceed for 15 min, and excess diazomethane was removed under nitrogen. The residue was dissolved in 100 μL of methylene chloride. The solution was layered with a capillary tube as a thin zone on the baseline of a TLC plate which was developed twice in ethyl acetate:hexanes (40:60). The plate was visualized under UV light (254 nm) and the band containing the monomethylated product was scraped off the plate and the silica dispersed in acetone (2 mL). The mixture was filtered through a small plug of cotton wool in a 9″ pasteur pipette. The filtrate was taken to dryness under nitrogen. The product was then dissolved in a minimum amount of $CDCl_3$ (100 atom %D) for 500 MHz ^1H NMR analysis.

Results and Discussion

During investigations on the microbial metabolism of warfarin using the fungus *Cunninghamella elegans* (ATCC 36112), a metabolite was detected which did not correspond to any of the known mammalian metabolites of this drug. Cell suspension incubations followed by chromatographic purification allowed for the isolation of sufficient quantities of this material for identification. The structure of this metabolite was established as 3′-hydroxywarfarin by comparisons with synthetic material using HPLC, electron-impact mass spectroscopy (EI-MS), and ^1H NMR spectroscopy, as described below. To our knowledge, the only published reports on this compound include the synthesis described by Hermodson et al.,[34] and the NMR study of Obaseki and Porter,[37] addressing multiple tautomeric forms of hydroxylated warfarins in solution.

The EI-MS spectrum of the isolated metabolite yielded a parent ion at *m/z* 324 indicative of a monohydroxylated derivative of warfarin. The fragmentation pattern was similar to that observed and rationalized for 4′-hydroxywarfarin,[8] including fragments establishing hydroxylation in the phenyl ring rather than the coumarin ring (e.g., the fragment

at *m/z* 187 rather than 203 indicating the coumarin ring is not hydroxylated, and the fragment at *m/z* 147 rather than 131 indicating hydroxylation of the phenyl ring). Since the metabolite was chromatographically distinct from 4′-hydroxywarfarin, the possibilities were narrowed to 2′-hydroxywarfarin or 3′-hydroxywarfarin (2). Following the successful synthesis of 3′-hydroxywarfarin, it was observed that the retention times of the isolated metabolite were consistent with those of synthetic 2 in four HPLC systems (see *Experimental Section*). In addition, the EI-MS spectra of the two compounds were identical.

Final structure proof was carried out using 500 MHz ^{1}H NMR spectroscopy. Figure 1 shows the ^{1}H NMR spectra of the isolated metabolite and of synthetic 2. While the two spectra were essentially identical, definitive assignment of signals was not possible due to the presence of multiple tautomeric forms in solution with considerable overlapping of signals. The three sets of doublets between δ 7.7 and 7.9, corresponding to C5-H and indicating that 3′-hydroxywarfarin (2) exists in solution in an open-chain/hemiketal tautomeric equilibrium (2–4; see Scheme II), are similar to that observed with warfarin.[38] This was described previously for this compound in a study by Obaseki and Porter,[37] addressing the relative populations of the three tautomers in various hydroxylated warfarins. In order to simplify the ^{1}H NMR spectrum of 3′-hydroxywarfarin (2), the open form of this compound was trapped by methylation of the 4-enol (5; Scheme II).[38] Again, identical spectra were obtained for the methylated derivatives of 3′-hydroxywarfarin standard and metabolite (Figure 2).

Members of the fungal genus *Cunninghamella* have demonstrated close parallels with mammalian liver in phase-1 biotransformations of a wide range of drugs and other xenobiotics, including model aromatic substrates,[39,40] *N*-*n*-propylamphetamine,[41] pargyline and deprenyl,[42] pyrilamine,[43] triprolidine,[44] thenyldiamine,[45] methapyrilene,[45] tripelennamine,[45] bornaprine,[46] propranolol,[47] phenelzine,[48] pheniprazine,[48] various aryl alkylamines,[49] and polycyclic aromatic hydrocarbons.[50] Consistent with this precedent, *Cunninghamella elegans* (ATCC 36112) produces the full array of phenolic, alcoholic, and ring cleavage metabolites observed with warfarin in mammalian systems,[31,32] and, as reported herein, produces the previously unreported phenol 3′-hydroxywarfarin (2). Members of this and related fungal genera thus show excellent potential for development as broad-based models to facilitate mammalian (including human) xenobiotic metabolism studies.

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