Pharmaceutical Research, Vol. 6, No. 11, 1989

Report

Microbial Models of Mammalian Metabolism: Stereoselective Metabolism of Warfarin in the Fungus *Cunninghamella elegans*

Yiu W. J. Wong[1] and Patrick J. Davis[1,2]

Received April 3, 1989; accepted June 5, 1989

Biotransformation stereoselectivity of warfarin was studied in the fungus *Cunninghamella elegans* (ATCC 36112) as a model of mammalian metabolism. This organism was previously shown to produce all known phenolic mammalian metabolites of warfarin, including 6-, 7-, 8-, and 4'-hydroxywarfarin, and the previously unreported 3'-hydroxywarfarin, as well as the diastereomeric warfarin alcohols, warfarin diketone, and aliphatic hydroxywarfarins. Using *S*-warfarin and *R*-warfarin as substrates, and an HPLC assay with fluorescence detection to analyze metabolite profiles, the biotransformation of warfarin was found to be highly substrate and product stereoselective. Both aromatic hydroxylation and ketone reduction were found to be stereoselective for *R*-warfarin. Ketone reduction with the warfarin enantiomers exhibited a high level of product stereoselectivity in that *R*-warfarin was predominantly reduced to its *S*-alcohol, while *S*-warfarin was reduced primarily to the corresponding *R*-alcohol.

KEY WORDS: microbial models of mammalian metabolism; warfarin; hydroxylation; reduction; stereoselective metabolism; *Cunninghamella elegans*.

INTRODUCTION

The synthetic coumarin *RS*-warfarin (**1**; Fig. 1) is a vitamin K antagonist which has been used extensively both as a clinically effective oral anticoagulant in man and as a rodenticide. Recent interest in warfarin stems from its use as an *in vivo* and *in vitro* "metabolic probe" for determining cytochrome P-450 isozyme profiles in diverse mammalian metabolic systems (1–6).

The mammalian metabolism of warfarin has been the subject of intensive investigations for two decades. Aromatic hydroxylation yields 6-hydroxywarfarin (**2**), 7-hydroxywarfarin (**3**), 8-hydroxywarfarin (**4**), and 4'-hydroxywarfarin (**5**), while aliphatic hydroxylation yields 9-hydroxywarfarin (**7**) or 10-hydroxywarfarin (**8**) (7,8). Warfarin is chiral (based on the presence of a chiral center at the C-9 position), and oxidative metabolism of this drug has been shown to be substrate stereoselective (9,10). The overall regioselectivity (i.e., position of hydroxylation) and substrate stereoselectivity observed (i.e., which enantiomer is preferentially hydroxylated) has been shown to be dependent on the specific cytochrome P-450 isozymes present. The correlation between specific isozymes and metabolites generated can be further investigated using selective P-450 inducers and inhibitors. Thus, by examining metabolic profiles, similarities and differences in cytochrome P-450 isozymes from different sources can be compared (11).

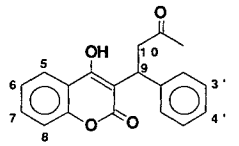
In addition to cytochrome P-450 mediated oxidative metabolism, warfarin also undergoes reduction of the ketonic side-chain to yield alcoholic products (**9–12**) (12). Since a new chiral center is created at the C-11 position, this reaction can be both substrate and product stereoselective, theoretically yielding four stereoisomeric alcohols representing two diastereomeric sets of enantiomers: 9*R*-warfarin-11*R*-alcohol (**9**) /9*S*-warfarin-11*S*-alcohol (**10**), and 9*R*-warfarin-11*S*-alcohol (**11**) /9*S*-warfarin-11*R*-alcohol (**12**).

Recent studies in our laboratory have focused on the use of microorganisms as model systems for studying mammalian drug metabolism. This discipline, "microbial models of mammalian metabolism," was first formalized by Smith and Rosazza (13), and is based upon demonstrated parallels in the metabolism between these diverse biological systems. The area has been the subject of several reviews (14–21). A principle goal of these studies is to define a microbial model, consisting of either one single culture or a composite of cultures exhibiting the broad range of mammalian phase-1 biotransformations, for application in the early stages of drug development and toxicological evaluation. Such a model can be used in predicting metabolite candidates and utilized for the preparation of sufficient quantities of metabolites for structure elucidation, biological evaluation, and use as analytical standards in mammalian metabolism studies.

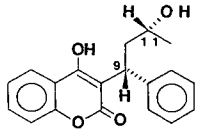
We previously observed the powerful metabolic capability of the fungus *Cunninghamella elegans* (ATCC 36112) in

[1] Division of Medicinal Chemistry, College of Pharmacy, University of Texas, Austin, Texas 78712-1074.
[2] To whom correspondence should be addressed.

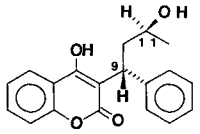
0724-8741/89/1100-0982$06.00/0



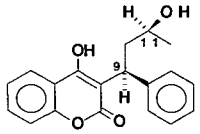
1. Warfarin
2. 6-Hydroxywarfarin
3. 7-Hydroxywarfarin
4. 8-Hydroxywarfarin
5. 4'-Hydroxywarfarin
6. 3'-Hydroxywarfarin
7. 9-Hydroxywarfarin
8. 10-Hydroxywarfarin



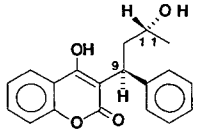
9. 9R,11R-Alcohol



11. 9R,11S-Alcohol



10. 9S,11S-Alcohol



12. 9S,11R-Alcohol

Fig. 1. Structures of warfarin (1) and its metabolites (2–12).

metabolizing warfarin to all known mammalian metabolites plus the previously unreported 3'-hydroxywarfarin (6) (22). We now present data to demonstrate that the metabolism of warfarin by this fungus is also highly stereoselective.

MATERIALS AND METHODS

Materials

All reagents were analytical reagent grade or higher in quality. Solvents for high-performance liquid chromatography (HPLC) were HPLC grade (OmniSolv, MCB Manufacturing Chemists, Cincinnati, OH). *RS*-Warfarin (1) (Sigma Chemicals, St. Louis, MO) was resolved according to the method of West *et al.* (23) by differential crystallization of *S*-warfarin-quinidine and *R*-warfarin-quinine. After liberation from the salt form, *R*-warfarin and *S*-warfarin were recrystallized from acetone-water until constant optical rotations and melting points were obtained as reported earlier (24). Phenolic warfarin metabolites [6-, 7-, 8-, 4'-, and 3'-hydroxywarfarin (2–6)] were synthesized by the methods of Hermodson *et al.* (25) or Bush *et al.* (26) and were fully characterized as described elsewhere (22). Warfarin alcohols (9–12) were synthesized by sodium borohydride reduction of warfarin (1) (27), as were the HPLC internal standards, the stereoisomeric 4'-hydroxywarfarin alcohols (28).

Preparation of Cell Suspensions of *Cunninghamella elegans* (ATCC 36112) and Metabolism of *S*- and *R*-Warfarin

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; sodium chloride, 5 g; potassium phosphate (dibasic), 5 g; yeast extract (Difco), 5 g; distilled water, 1000 ml; pH adjusted to 7.0 with 6 *N* HCl. The medium was sterilized in individual flasks at 121°C for 15 min. After inoculation, the stage-1 flask was incubated for 72 hr 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 hr, the fungal cells were harvested by filtration and rinsed by resuspension in distilled water followed by filtration. This process was repeated three times. 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. In an initial experiment to study the production profile of each metabolite isomer, a total of 10 cell suspension cultures was prepared and divided into two groups of five flasks. After preincubation for 1 hr under the conditions specified above, 6 mg of *R*-warfarin as the potassium salt in 200 μl of sterile water was added to each of five flasks in one group to give a final substrate concentration of 0.3 mg/ml of buffer. The procedure was repeated for the other group of five flasks using *S*-warfarin as the substrate, and the incubation was then resumed. One flask of cell suspension from each group was harvested at 2, 6, 12, 24, and 48 hr after substrate addition. Samples were filtered to remove fungal cells, and filtrates were stored at −20°C until analysis. In all subsequent experiments, the transformation was terminated at 48 hr after substrate addition.

Determination of Warfarin Metabolites in Microbial Cell Suspensions

The microbial cell suspension samples were analyzed using a reverse-phase ion-pair liquid chromatographic method with fluorescence detection as described elsewhere (28). Briefly, all analyses were conducted with a Beckman Model 110A pump and a Schoeffel Model FS 970 fluorometric detector. The excitation wavelength was 290 nm and emission was measured in the presence of an emission filter (No. 389). The reverse-phase column used was a Beckman Ultrasphere C-18 (5-μm particle size; column, 250 × 4.5 mm) eluted at 1.0 ml/min with the following solvent system: methanol (30%, v/v); tetrahydrofuran (7%, v/v); 1.0 *M* aqueous tetrabutylammonium phosphate (1%, v/v); 5 m*M*, pH 7.5, potassium phosphate buffer (62%, v/v). Chromatograms were recorded with a Hewlett-Packard Model 3390A reporting integrator.

Extraction Procedure

The sample filtrate (4 ml) and the internal standard (4'-hydroxywarfarin alcohols, 400 ng as the potassium salt in 100 μl of water) were added to an extraction tube (125 × 16 mm). Samples were then extracted with 10% dichlo-

romethane in cyclohexane (2 × 4 ml). The organic layer was removed by aspiration and the residual aqueous phase extracted with ethyl acetate (5 ml). The organic layer was then back extracted with 0.1 *M* KOH (2 ml), and the organic layer discarded. The aqueous phase was acidified with 5 *N* HCL (1 ml) and extracted with diethyl ether (5 ml). The ethereal layer was taken to dryness under nitrogen and reconstituted with 250 μl of mobile phase. A total of 50 to 200 μl of this solution was injected into the chromatograph. Calibration plots [peak height ratio (analyte/internal standard) versus analyte concentration] were constructed based upon the analysis of samples spiked with 10–100 ng of each phenolic or alcoholic metabolite, and unknowns were calculated by interpolation using these plots.

RESULTS AND DISCUSSION

The stereoselectivity in the metabolism of warfarin (1) by the fungus *Cunninghamella elegans* (ATCC 36112) was investigated by cell suspension transformation of pure enan-

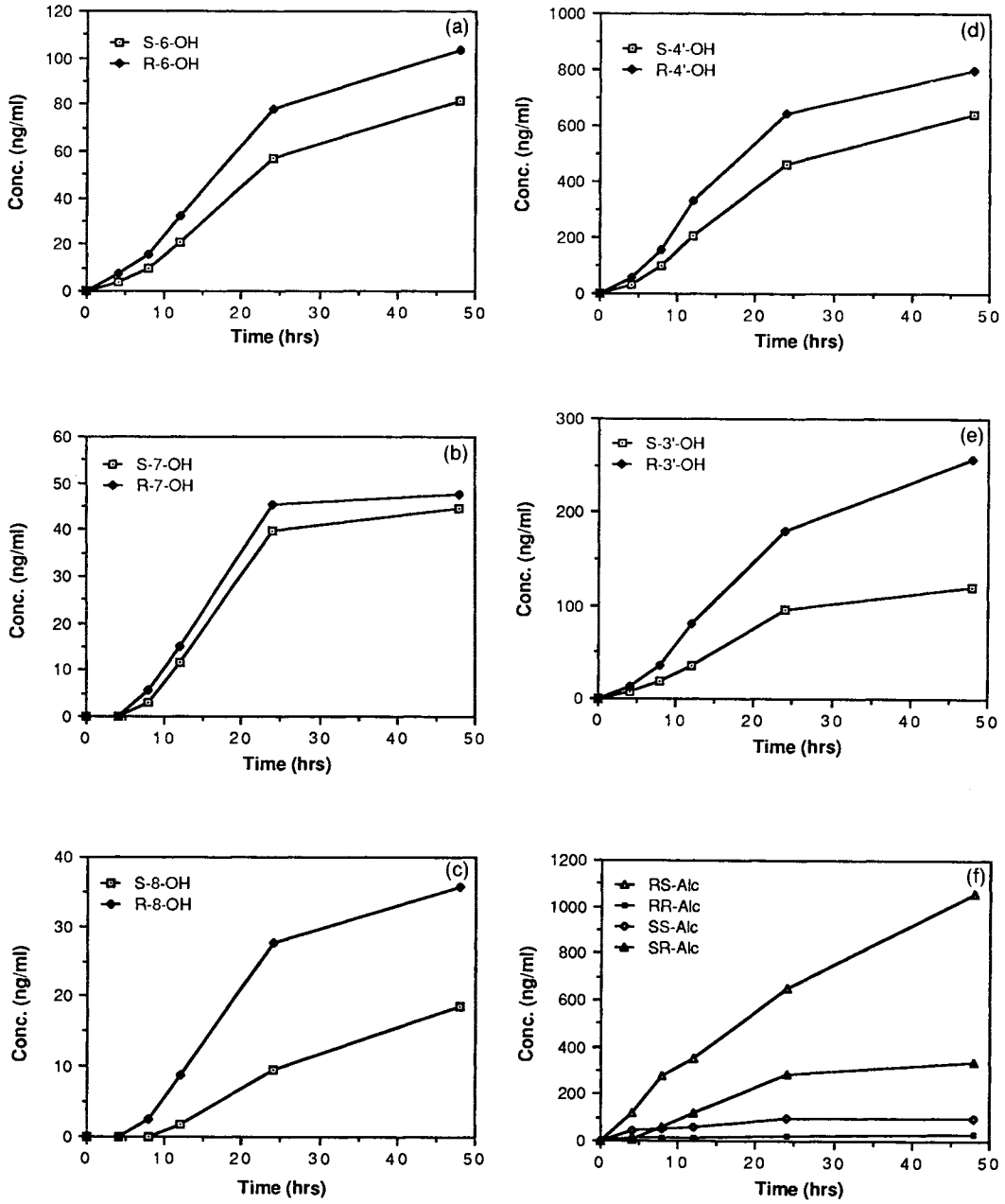


Fig. 2. Production of stereoisomeric warfarin metabolites (phenols and alcohols) using the fungus *Cunninghamella elegans* (ATCC 36112) and using *R*- or *S*-warfarin (1) as the substrate. Figures for the production of phenolic metabolites are as follows: (a) 6-hydroxywarfarin (2); (b) 7-hydroxywarfarin (3); (c) 8-hydroxywarfarin (4); (d) 4′-hydroxywarfarin (5); (e) 3′-hydroxywarfarin (6). (—⊡—) *S*-isomer; (—●—) *R*-isomer. (f) The production of stereoisomeric warfarin alcohols 9–12. (—△—) *RS*-isomer; (—●—) *RR*-isomer; (—◇—) *SS*-isomer; (—▲—) *SR*-isomer.

tiomers of warfarin. Similar methodologies had been used to examine the stereoselective metabolism of warfarin by microsomes isolated from different mammalian sources (4–6).

The production profiles of metabolites from *R*- versus *S*-warfarin are shown in Figs. 2a—f, and the enantiomeric ratios (*R*/*S*) for each metabolite at 48 hr of incubation are summarized in Table I. These results indicate that warfarin is biotransformed by this fungus with a high degree of substrate and, in the case of the alcohol metabolites, product stereoselectivity. Aromatic hydroxylations appear to be stereoselective for *R*-warfarin (*R*-**1**); this was most evident with the production of 3′-hydroxywarfarin (**6**) and 8-hydroxywarfarin (**4**), where the concentrations of the *R*-phenols generated were twofold higher than the corresponding *S*-isomers (*R*/*S* ratio >2). The stereoselectivity in the production of 4′-hydroxywarfarin (**5**) and 6-hydroxywarfarin (**2**) was moderate, with the *R*/*S* ratio observed for both metabolites >1.2. While the observed *R*/*S* ratio of 1.1 for 7-hydroxywarfarin (**3**) suggested a low degree of stereoselectivity, the full production profile (Fig. 2b) revealed that the *R*-isomer was, nevertheless, preferentially produced.

Although the involvement of cytochromes P-450 in these hydroxylations is only suggested at this point, the regioselectivity resulting in multiple phenolic metabolites and the high degree of substrate stereoselectivity observed is strongly suggestive of isozyme multiplicity. Rizzo and Davis (29) have recently demonstrated that the production of 4′-hydroxywarfarin by a related fungus, *Cunninghamella bainieri* (UI 3065), is inhibited by carbon monoxide and does not exhibit a primary isotope effect. Furthermore, the 4′-hydroxylation of 4′-deuterowarfarin by this organism involves an 80% migration and retention of deuterium (29). These results strongly implicate a cytochrome P-450-mediated hydroxylation involving the classic arene oxide (NIH shift) pathway comparable to that observed in mammalian systems. It is anticipated that similar results can be demonstrated with this fungus. Indeed, Cerniglia and coworkers have extensively examined xenobiotic metabolism with the fungal organism used in the current studies (*Cunninghamella elegans*, ATCC 36112) and have implicated a cytochrome P-450 monooxygenase system in the metabolism of polynuclear aromatic hydrocarbons (30).

Reduction of warfarin to the corresponding alcohols was also highly substrate stereoselective with *Cunninghamella elegans*, with *R*-warfarin (*R*-**1**) being reduced more extensively than *S*-warfarin (*S*-**1**) (*R*/*S* ratio = 2.6; Table I). The product stereoselectivity of this reaction was also interesting; while *R*-warfarin was almost exclusively reduced to the corresponding *S*-alcohol (**11**) (9*R*,11S/9*R*,11R ratio = 35.8), *S*-warfarin was preferentially reduced to the corresponding *R*-alcohol (**12**)) (9*S*,11R/9*S*,11S ratio = 3.3). The reverse product stereoselectivity in the reduction of the two warfarin enantiomers suggests that two different reductases might be involved in the metabolism of warfarin. It is interesting to note that previous studies on the bacterial (rather than fungal) reduction of warfarin indicated a somewhat different stereochemical course (24). Specifically, *Nocardia corallina* exhibited complete substrate and product stereoselectivities in that only *S*-warfarin was reduced, and only to the corresponding *S*-alcohol (**10**). *Arthrobacter* species exhibited marked substrate and complete product stereoselectivity in reducing *S*-warfarin (and, to a minor extent, *R*-warfarin) to the corresponding *S*-alcohols (i.e., principally **10**, with minor amounts of **11**).

The stereoisomer profiles for all phenolic and alcoholic metabolites generated in cell suspension cultures 48 hr after substrate addition are summarized in Figs. 3a and 3b.

Since the regio- and stereoselectivities of mammalian warfarin metabolism are dependent upon the profile of cytochrome P-450 isozymes present in a given biotransformation system, it is not surprising that varying patterns of metabolites have been observed in the wide range of *in vivo* and *in vitro* systems explored to date. It is the correlation of stereoisomeric phenolic metabolites with specific isozymes (by differential induction, inhibition, or the use of purified enzymes) that serves as the basis for using warfarin as a cytochrome P-450 probe. Nevertheless, it is interesting that *Cunninghamella elegans* produces the full array of mamma-

Table I. Stereoisomeric Ratios (*R*/*S*) Observed for Warfarin Metabolites[a]

Metabolite	*R*/*S* ratio[b]	*RS*/*RR* ratio	*SR*/*SS* ratio
6-Hydroxywarfarin (**2**)	1.27 (±0.11)[c]		
7-Hydroxywarfarin (**3**)	1.11 (±0.13)[c]		
8-Hydroxywarfarin (**4**)	2.05 (±0.17)[c]		
4′-Hydroxywarfarin (**5**)	1.27 (±0.11)[c]		
3′-Hydroxywarfarin (**6**)	2.20 (±0.25)[c]		
Warfarin alcohols (**9–12**)	2.62 (±0.39)[d]	35.9 (±1.98)[e]	3.33 (±0.52)[f]

[a] Metabolites generated in cell suspension and measured 48 hr after substrate addition.

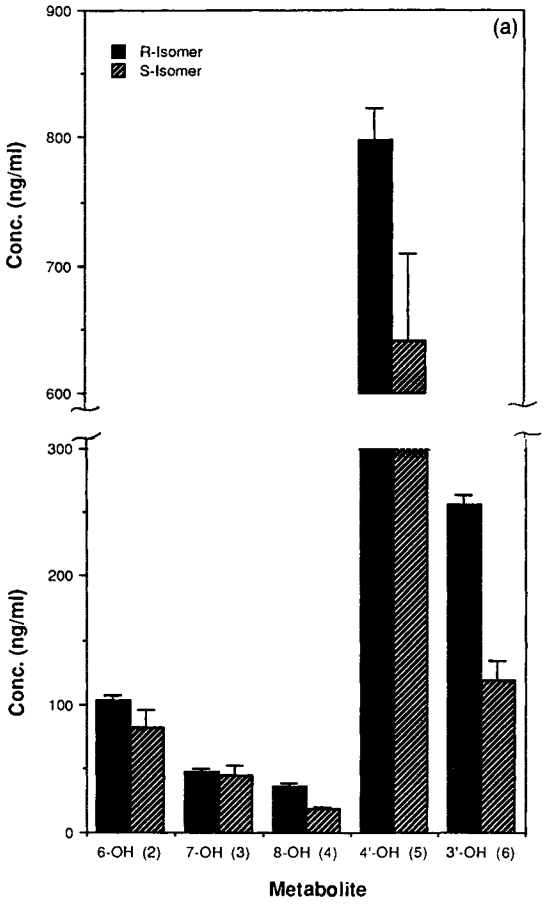
[b] Data shown = *R*/*S* ratio (±SE) for $N = 3$.

[c] Ratio reflects *substrate* stereoselectivity in the production of phenolic metabolites and is calculated based upon the concentration of the *R*-phenol/*S*-phenol produced as measured at 48 hr of incubation.

[d] Ratio reflects *substrate* stereoselectivity in the production of alcohol metabolites and is calculated based upon the total concentration of 9R,11*R*-alcohol (**9**) + 9R,11*S*-alcohol (**11**)/9S,11*S*-alcohol (**10**) + 9S,11*R*-alcohol (**12**).

[e] Ratio reflects *product* stereoselectivity in the reduction of *R*-warfarin and is calculated based upon the concentration of the 9*R*,11S-alcohol (**11**)/9*R*,11R-alcohol (**9**) produced.

[f] Ratio reflects *product* stereoselectivity in the reduction of *S*-warfarin and is calculated based upon the concentration of the 9*S*,11R-alcohol (**12**)/9*S*,11S-alcohol (**10**) produced.



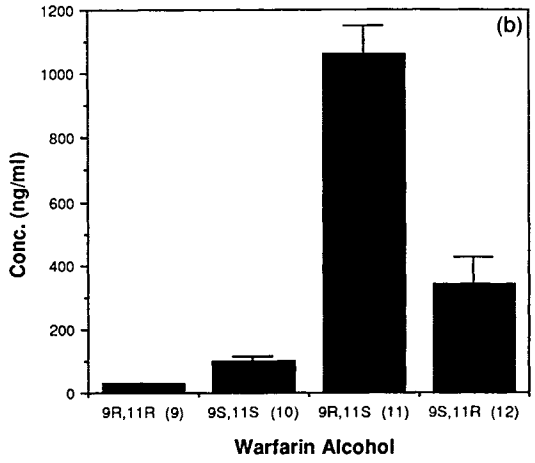


Fig. 3. Concentration of stereoisomeric warfarin metabolites at 48 hr of incubation in cell suspensions of *Cunninghamella elegans* (ATCC 36112) [mean ±SE), $N = 3$]. (a) Phenolic metabolites **2–6**; (b) alcohol metabolites **9–12**.

lian metabolites (including the previously unreported 3′-phenol, **6**) (22) and that the substrate stereoselectivity observed in aromatic hydroxylation (i.e., for *R*-warfarin) is remarkably similar to that reported by Kaminsky *et al.* (11) for rat liver microsomes, a widely used model system for studying drug metabolism. In that study, microsomes derived from uninduced male Sprague–Dawley rats catalyzed the 6-, 7-, and 8-hydroxylation of warfarin with a stereoselectivity for the *R*-enantiomer; the formation of 4′-hydroxywarfarin was nonstereoselective. Following *in vivo* induction with phenobarbital, the generation of all four phenols was stereoselective for *R*-warfarin. Current studies focus on determining whether warfarin metabolism in *Cunninghamella elegans* responds to classic cytochrome P-450 inducers and inhibitors in a manner similar to that described for warfarin metabolism in mammals, in order to clarify further the similarities (and differences) between this broad-based microbial model and classical mammalian biotransformation systems.

ACKNOWLEDGMENTS

The research was executed by Y.W.J. Wong in partial fulfillment of the requirements for the Ph.D. degree and was supported in part by PHS/NIH Grant RO1-ES04200.

REFERENCES

1. L. S. Kaminsky, M. J. Fasco, and F. P. Guengerich. *J. Biol. Chem.* 255:85–91 (1980).
2. F. P. Guengerich, S. T. Dannan, S. T. Wright, M. V. Martin, and L. S. Kaminsky. *Biochemistry* 21:6019–6030 (1982).
3. L. S. Kaminsky, F. P. Guengerich, G. A. Dannan, and S. P. Aust. *Arch. Biochem. Biophys.* 255:398–404 (1983).
4. P. P. Wang, P. Beaune, L. S. Kaminsky, G. A. Dannan, F. F. Kadlubar, D. Larrey, and F. P. Guengerich. *Biochemistry* 22:5375–5383 (1983).
5. L. S. Kaminsky, D. A. Dunbar, P. P. Wang, P. Beaune, D. Larrey, F. P. Guengerich, R. G. Schnellmann, and E. G. Sipes. *Drug Metab. Dispos.* 12:470–477 (1984).
6. A. E. Rettie, L. Heimark, R. T. Mayer, M. D. Burke, W. F. Trager, and M. R. Juchau. *Biochem. Biophys. Res. Commun.* 126:1013–1021 (1985).
7. W. F. Trager, R. J. Lewis, and W. A. Garland. *J. Med. Chem.* 13:1196–1204 (1970).
8. M. J. Fasco, P. P. Dymerski, J. D. Wos, and L. S. Kaminsky. *J. Med. Chem.* 21:1054–1059 (1978).
9. L. R. Pohl, R. Bales, and W. R. Trager. *Res. Commun. Chem. Path. Pharmacol.* 15:233–256 (1976).
10. L. R. Pohl, S. D. Nelson, W. R. Porter, W. F. Trager, M. J. Fasco, F. D. Baker, and J. W. Fenton. *Biochem. Pharmacol.* 25:2153–2162 (1976).
11. L. S. Kaminsky, M. J. Fasco, and F. P. Guengerich. *J. Biol. Chem.* 254:9657–9662 (1979).
12. T. A. Moreland and D. S. Hewick. *Biochem. Pharmacol.* 24:1953–1957 (1975).
13. R. V. Smith and J. P. Rosazza. *J. Pharm. Sci.* 64:1737–1759 (1975).
14. R. V. Smith, D. Acosta, and J. P. Rosazza. *Adv. Biochem. Eng.* 5:69–100 (1977).
15. J. P. Rosazza. *Recent Results Cancer Res.* 63:58–68 (1978).
16. J. P. Rosazza and R. V. Smith. *Adv. Appl. Microbiol.* 25:169–208 (1979).
17. R. V. Smith and J. P. Rosazza. In J. P. Rosazza (ed.), *Micro-*

bial Transformations of Bioactive Compounds, Vol. 2, CRC Press, Boca Raton, FL, 1982, pp. 1–42.

18. R. V. Smith and P. J. Rosazza. *J. Nat. Prod.* **46**:79–91 (1983).
19. A. M. Clark, J. D. McChesney, and C. D. Hufford. *Med. Res. Rev.* **5**:231–253 (1985).
20. P. J. Davis. In S. B. Lamba and C. A. Walker (eds.), *Antibiotics and Microbial Transformations,* CRC Press, Boca Raton, FL, 1987, pp. 47–70.
21. P. J. Davis. *Dev. Indust. Microbiol.* **29**:197–219 (1988).
22. Y. W. J. Wong. Ph.D. dissertation, University of Texas at Austin, Austin, 1988.
23. B. D. West, S. Preis, C. H. Schroeder, and K. P. Link. *J. Am. Chem. Soc.* **83**:2676–2679 (1961).
24. P. J. Davis and J. D. Rizzo. *Appl. Environ. Microbiol.* **43**:884–890 (1982).
25. M. A. Hermodson, W. M. Barker, and K. P. Link. *J. Med. Chem.* **15**:1265–1270 (1972).
26. E. Bush and W. F. Trager. *J. Pharm. Sci.* **72**:830–831 (1983).
27. K. K. Chan, R. J. Lewis, and W. F. Trager. *J. Med. Chem.* **15**:1265–1270 (1972).
28. Y. W. J. Wong and P. J. Davis. *J. Chromatogr.* **469**:281–291 (1989).
29. J. D. Rizzo and P. J. Davis. *J. Pharm. Sci.* **78**:183–189 (1989).
30. C. E. Cerniglia. *Adv. Appl. Microbiol.* **30**:31–71 (1984).