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Microbial Transformations of Warfarin: Stereoselective Reduction by *Nocardia corallina* and *Arthrobacter* Species

PATRICK J. DAVIS* AND JINEE D. RIZZO

Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Texas at Austin, Austin, Texas 78712

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The microbiological metabolism of warfarin was examined as a model of metabolism in higher organisms, including humans, and to determine the chirality of microbial reductases for application in organic synthesis. Nineteen cultures were examined based on their reported abilities to reduce ketonic substrates, and several were shown to catalyze the desired reaction. *Nocardia corallina* (ATCC 19070) exhibited complete substrate and product stereoselectivity as it reduced *S*-warfarin to the corresponding *S*-alcohol. *Arthrobacter* species (ATCC 19140) exhibited marked substrate and complete product stereoselectivity since *S*-warfarin, and to a lesser extent *R*-warfarin, were reduced to the corresponding *S*-alcohols. These reductions parallel those reported to occur in mammalian species.

Racemic warfarin [3-α-(acetonylbenzyl)-4-hydroxycoumarin, I; see Fig. 1], a vitamin K antagonist, has been utilized for more than 2 decades as an oral anticoagulant and as a rodenticide. Its two enantiomers (chiral center at C-9) do not exhibit equivalent anticoagulant activity due to complex factors, including different intrinsic activities as well as differences in pharmacokinetics and metabolism (22, 27). Recent interest in warfarin stems from its use as a "metabolic probe" for studying complex patterns of metabolism involving regio- and stereoselectivity (9–11, 14, 15, 20, 21, 27).

Two major phase 1-type reactions are observed in the mammalian metabolism of warfarin, namely, reduction of the side chain ketone and hydroxylation in aromatic and aliphatic positions. Since warfarin represents a racemic mixture (*RS*-I), ketone reduction theoretically yields four stereoisomers: two diastereomeric sets of enantiomers, 9*R*-warfarin–11*R*-alcohol (II)/9*S*-warfarin–11*S*-alcohol (IV), and 9*R*-warfarin–11*S*-alcohol (III)/9*S*-warfarin–11*R*-alcohol (V) (2, 16, 29). The substrate and product stereospecificities depend on the animal species examined (12, 16–18, 20, 27–29); however, the majority of animal systems exhibit *R*-substrate stereospecificity and *S*-product stereospecificity, leading to a predominence or exclusive production of *R*-warfarin–*S*-alcohol (III). A further cyclodehydration product has also been observed (1, 6), as a result of spontaneous dehydration during workup or an in vivo activation process (20).

Hydroxylation yields stereoisomers of 6-hydroxywarfarin (VI), 7-hydroxywarfarin (VII), 8-hydroxywarfarin (VIII), 9-hydroxywarfarin (benzylic-hydroxywarfarin, IX) which can be dehydrated to 9,10-dehydrowarfarin (X), and 4′-hydroxywarfarin (XI) (7, 11, 13, 14, 20, 21).

Previous studies in our laboratory have focused on the use of microbiological systems in organic synthesis, particularly where stereoselectivity of metabolism exhibits advantages over classical synthetic methods (4, 5). An ultimate goal of such studies is to develop microbial systems as predictable reagents for conducting synthetic reactions (24). Other studies involve the use of microorganisms as models of metabolism in higher organisms, including humans (3); (R. V. Smith, S. A. G. Milton, and P. J. Davis, submitted for publication; R. V. Smith, P. J. Davis, and K. M. Kerr, submitted for publication). Such studies, referred to as "microbial models of mammalian metabolism" (26), are based on comparative biochemistry, since the same types of phase 1 reactions occur in both microorganisms and mammals (23, 25). The goal of these studies was to demonstrate similarities and differences between these biological extremes in the types of enzymes and mechanisms involved in xenobiotic metabolism.

The current study focuses on the first major reactions outlined above, namely, on the reduction of warfarin to the corresponding alcohols by microorganisms. It was observed that *Nocardia corallina* (ATCC 19070) and *Arthrobacter* species (ATCC 19140) exhibited *S*-reduction of racemic warfarin. *N. corallina* demonstrated complete substrate stereoselectivity in reducing *S*-I to the corresponding *S*-alcohol (IV), whereas the *Arthrobacter* species demonstrated marked stereoselectivity for *S*-I, yielding its *S*-alcohol (IV) as a major product, with minor amounts of reduction of *R*-warfarin to its *S*-alcohol (III)

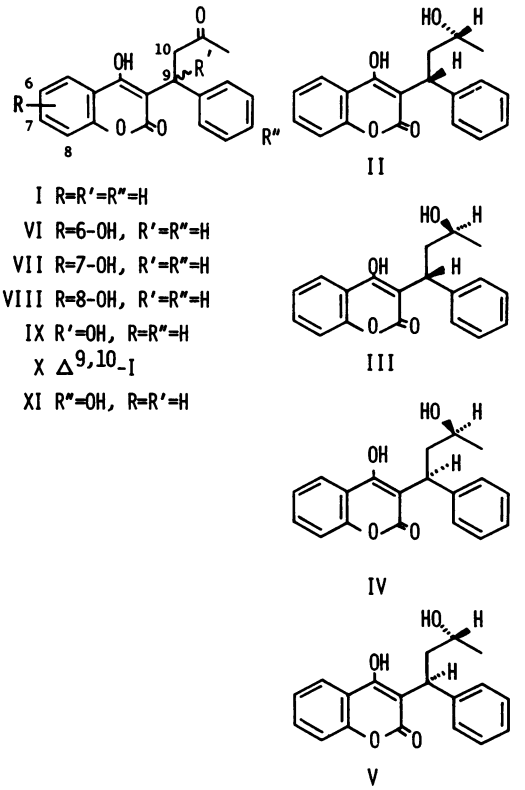

FIG. 1. Structures of warfarin and warfarin derivatives: I, warfarin; II, 9*R*-warfarin–11*R*-alcohol; III, 9*R*-warfarin–11*S*-alcohol; IV, 9*S*-warfarin–11*S*-alcohol; V, 9*S*-warfarin–11*R*-alcohol; VI, 6-hydroxywarfarin; VII, 7-hydroxywarfarin; VIII, 8-hydroxywarfarin; IX, 9-hydroxywarfarin; X, 9,10-dehydrowarfarin; XI, 4'-hydroxywarfarin.

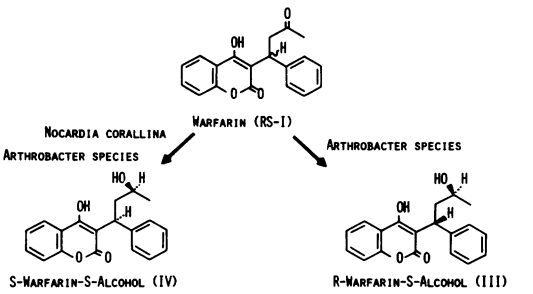

FIG. 2. Microbiological reduction of *RS*-warfarin to *S*-warfarin–*S*-alcohol (IV) by *N. corallina* and *Arthrobacter* species and to *R*-warfarin–*S*-alcohol by *Arthrobacter* species.

(Fig. 2). These results parallel those observed in certain mammalian species (27, 28) and may be useful in applying the *S*-reductase activity of these organisms to organic synthesis in a predictable fashion.

MATERIALS AND METHODS

Instrumentation. Proton nuclear magnetic resonance spectra were generated in $CDCl_3$, using tetramethylsilane as the internal standard, on a Varian HA-100 (100 MHz) spectrometer. Mass spectra were taken on a Dupont model 21491 mass spectrometer by direct probe insertion. Optical rotations were determined at the sodium D-line on a Perkin-Elmer model 141MC polarimeter, using a 1-ml microcell (path length, 1 dm). Melting points were obtained with a Fisher model 355 digital melting point apparatus.

Solvents, reagents, and standard compounds. All solvents were of analytical reagent grade or higher quality. Solvents for high-performance liquid chromatography (HPLC) were of HPLC grade (Lichrosolv; MCB Manufacturing Chemists, Cincinnati, Ohio). Water was deionized and double distilled in glass. All glassware used in extractions was silylated with 2% trimethylsilyl chloride (Pierce Chemical Co., Rockford, Ill.) in toluene, rinsed, and dried before use. Phenprocoumon [3-α-(*N*-propylbenzyl)-4-hydroxycoumarin] was a gift from Organon Corp., West Orange, N.J.

RS-Warfarin (Sigma Chemical Co., St. Louis Mo.) was resolved according to the method of West et al. (30) by differential crystallization of *S*-warfarin–quinidine and *R*-warfarin–quinine. *S*-Warfarin–quinidine was recrystallized from 95% ethanol to constant optical rotation: $[\alpha]_D^{31} = +92.7°$ (c = 1.73, ethanol) [reported (30): $[\alpha]_D^{25} = +87$ (c = 1.7, ethanol)]. *R*-Warfarin–quinine was recrystallized from ethanol-diethyl ether to constant optical rotation: $[\alpha]_D^{31} = -68°$ (c = 1.78, ethanol) [reported (30): $[\alpha]_D^{25} = -71°$ (c = 1.7, ethanol)]. After liberation of the free acids, *S*-I and *R*-I were recrystallized from acetone-water (8:2) until constant optical rotations and melting points were obtained: *S*-warfarin—$[\alpha]_D^{34} = -129.5°$ (c = 0.90, 0.5 N NaOH) [reported (30): $[\alpha]_D^{25} = -148°$ (c = 1.2, 0.5 N NaOH)] and mp 173 to 176°C (reported [30], mp 172 to 173°C); *R*-warfarin—$[\alpha]_D^{34} = +129.1°$ (c = 1.02, 0.5 N NaOH) [reported (30): $[\alpha]_D^{25} = +149°$ (c = 2.0, 0.5 N NaOH)] and mp 172 to 176°C [reported (30), mp 170 to 171°C). Mass spectral data are as follows: [*m/e* (percent relative abundance for *RS*-I, *R*-I, and *S*-I)]: 308 (42), 265 (100), 250 (32), 187 (31), 121 (39), and 93 (15); consistent with the reported spectrum for *RS*-I (29).

The warfarin alcohols (II to V) were synthesized according to the method of Chan et al (2).

Chromatographic procedures. Analytical thin-layer chromatography was performed on plastic-backed 0.25-mm silica gel GF_{254} plates (Polygram; Brinkmann Instruments, Inc., Houston, Tex.) eluted with one of the following solvent systems: A, dichloroethane-acetone (85:15); B, heptane-ethyl acetate-acetic acid (100:50:1); C, chloroform-acetic acid (95:5). R_f values were as follows: warfarin (I), A = 0.71, B = 0.70; warfarin alcohols 1 (III/V), A = 0.59, B = 0.56, C = 0.67; warfarin alcohols 2 (II/IV), A = 0.39, B = 0.44, C = 0.44. Plates were visualized by spraying with 1% hydro-alcoholic sodium hydroxide solution and viewing at 280 nm. Warfarin yielded a blue fluorescence, and the warfarin alcohols yielded an intense white fluorescence.

HPLC analyses were conducted with a Beckman

model 110A single-piston pump with a Hitachi model 100-10 variable-wavelength detector and a Beckman model 500 automated sample injector set for 20-μl injections at 15-min intervals with a 6-s flush time. Detection was at 310 nm, and chromatograms were recorded with a Houston Instruments Omni-Scribe single-pen recorder. The mobile phase consisted of aqueous ammonium phosphate (1.5% aqueous acetic acid adjusted to pH 4.7 with ammonium hydroxide) and acetonitrile in a ratio of 690:310 (8). The mobile phase was prepared by filtering individual solvents through glass-fiber pads, GF/F grade (Whatman, Clifton, N.J.), mixing, and degassing ultrasonically before use. The column was a μ-Bondapak C-18, 3.9 by 300 mm (Waters Associates, Milford, Mass.), eluted at 2.0 ml/min. Under these conditions, the following retention times (T_r) were observed for the two sets of enantiomeric alcohols: warfarin alcohols II/IV, 2.96 min; warfarin alcohols III/V, 3.47 min; warfarin (I), 13.11 min; phenprocoumon (internal standard), 8.52 min.

For quantitative experiments, standard curves were generated by dissolving 6.2 mg of warfarin, 2.6 mg of warfarin alcohols II/IV, and 2.6 mg of warfarin alcohols III/V in 10 ml of methanol. Portions of 1,000, 600, 400, 200, 100, and 50 μl were placed in duplicate silylated glass extraction tubes (125 by 16 mm) with PTFE-lined caps, and the solvent was removed under a gentle nitrogen stream. A total of 1 ml of an *N. corallina* culture, 100 μl of 6 N HCl, 50 μl of internal standard solution (phenprocoumon, 10 mg/2 ml in methanol), and 4 ml of diethyl ether were added; the tubes were capped, mixed for 15 min at 18 inversions per min (Lab-Tek aliquot mixer), and centrifuged at 500 rpm (IEC model 7R centrifuge). A total of 3 ml of the organic layer was taken to dryness under nitrogen. For HPLC analyses, the samples were reconstituted with 0.5 ml of methanol-acetonitrile (1:1) and 1.5 ml of the mobile phase and pressure-filtered (Whatman GF/F filters) before injection. The resultant peak heights were used to plot standard curves for the warfarin alcohols and warfarin as peak height ratios (standard/phenprocoumon) versus micrograms of standard per milliliter of culture. The following curves were used in this study: *RR*/*SS*-warfarin alcohols (II/IV), slope = 112, y-intercept = 0.54, $r = 0.996$; *RS*/*SR*-warfarin alcohols (III/V), slope = 145, y-intercept = 1.92, $r = 0.995$; warfarin (I), slope = 546, y-intercept = −2.54, $r = 0.996$. Values for unknowns were determined by extraction and analysis as described above for the standards, followed by extrapolation of the peak height ratios obtained to yield micrograms of each compound per milliliter in the sample. Linear regression and other statistical analyses were conducted with a Texas Instruments model TI 58C computer.

General fermentation procedure. All cultures were maintained on refrigerated (2°C) slants of Sabouraud-maltose agar (Difco Laboratories, Detroit, Mich.), Mycophil agar (BBL Microbiology Systems, Cockeysville, Md.), or American Type Culture Collection (ATCC) medium 5 sporulation agar and transferred every 6 months. The medium used in these studies consisted of the following: dextrose, 20 g; soybean meal (20 mesh; Capital Feeds, Austin, Tex.), 5 g; sodium chloride, 5 g; potassium phosphate (dibasic), 5 g; yeast extract (Difco), 5 g; distilled water, 1,000 ml; pH adjusted to 7.0 with 6 N HCl. The medium was sterilized in individual flasks at 121°C for 15 min. Incubations were conducted in 12-ml Bellco DeLong culture flasks containing 25 ml of the medium, using an NBS model G-25-R environmental shaker (New Brunswick Scientific Co., Edison, N.J.) at 250 rpm and 27°C. For each culture examined, first-stage flasks were initiated by suspending spores from fungal cultures or surface growth for bacteria and yeasts in sterile medium and transferring the suspension to a 125-ml flask under aseptic conditions. After incubation for 72 h, a 2-ml portion was used to inoculate a second-stage flask of the same composition for each organism, and the incubation was allowed to continue for 24 h.

To select cultures capable of metabolizing warfarin, the following cultures were examined: *Arthrobacter* species (ATCC 19140 and 21237), *Aspergillus niger* (UI-X-172), *Cryptococcus macerans* (Ziffer), *Curvularia falcata* (QM-72a), *Curvularia lunata* (ATCC 13633), *Nocardia corallina* (ATCC 19070, ATCC 19071, ATCC 19148), *Nocardia minima* (ATCC 19150), *Rhodotorula rubra* (ATCC 20129 and sensu), *Saccharomyces cerevisiae* (NRRL Y-2034), *Schizosaccharomyces pombe* (ATCC 2476 and ATCC 20130), *Sporobolomyces pararoseus* (ATCC 11386), *Streptomyces griseus* (UI-1158W and ATCC 10137), and *Streptomyces lavendulae* (NRRL B-2036). The sources of cultures were: ATCC, Rockville, Md.; J. P. Rosazza, College of Pharmacy, University of Iowa (UI), Iowa City, Iowa; Northern Regional Research Laboratories (NRRL), U.S. Department of Agriculture, Peoria, Ill.; Quartermaster Culture Collection (QM), E. G. Simmons, University of Massachusetts, Amhearst, Mass.; H. Ziffer, National Institutes of Health, Bethesda, Md.; A. Nespiak (sensu culture), Department of Botany and Biology, Medical Academy, Wroclaw, Poland. *RS*-I (12.5 mg in 50 μl of dimethylformamide) was added to second-stage cultures of each organism. Three-milliliter samples were harvested after 24, 48, 72, and 144 h of incubation. Each sample was acidified with 1 ml of 2.5 N HCl and extracted with 2.5 ml of chloroform, and the organic layer was examined against the standard compounds by thin-layer chromatography, using the solvent systems described above. Repeat studies with those cultures exhibiting metabolism included control cultures with no substrate added and autoclaved cultures with substrate added.

For analytical studies, 10 second-stage cultures of *N. corallina* (ATCC 19070) and *Arthrobacter* species (ATCC 19140) were used. Substrate addition consisted of the addition of 12.5 mg of *RS*-I, *R*-I, or *S*-I as their sodium salts dissolved in 0.25 ml of sterile water to triplicate flasks for each substrate. The 10th flask for each organism served as a nonsubstrate control. Samples of 1.0 ml were withdrawn after 1, 48, 96, 148, and 192 h of incubation from each flask and quantitatively analyzed as described above.

Mass spectral analysis of the alcohol from *N. corallina* yielded *m/e* = 310 (M^+), 292, 265, 263, and 189, identical with the standard alcohol and consistent with that reported by Trager et al. (29).

RESULTS AND DISCUSSION

Nineteen cultures were screened for their ability to metabolize warfarin based on literature

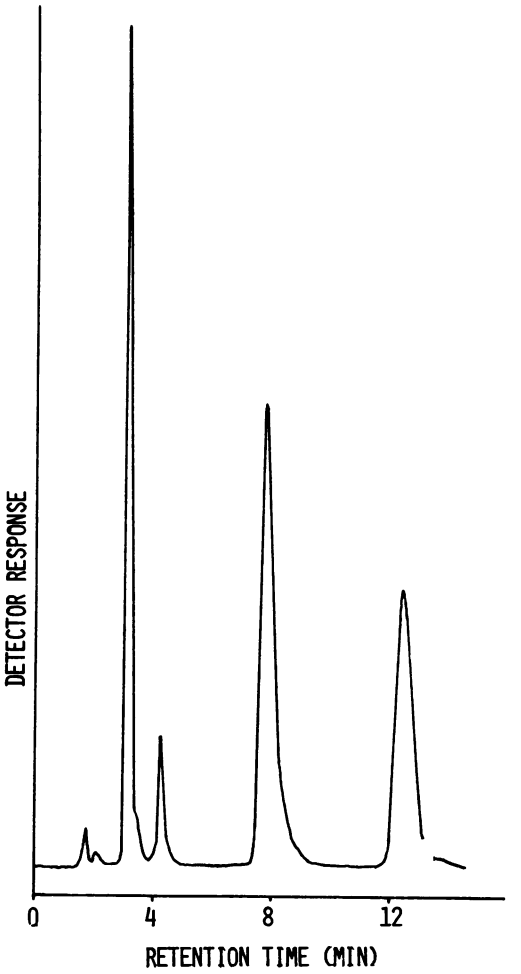

FIG. 3. HPLC chromatogram of *RS*-warfarin metabolism by *N. corallina*. Assigned peaks are: T_r = 3.00, *S*-warfarin–*S*-alcohol (IV); T_r = 8.50, phenprocoumon (internal standard); T_r = 13.00, warfarin (I).

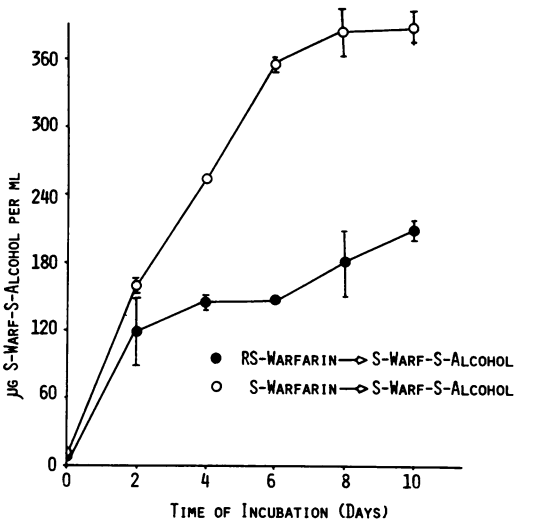

FIG. 4. Time course of the metabolism of warfarin enantiomers by *N. corallina* (ATCC 19070). Symbols: (●) *RS*-warfarin reduction to *S*-warfarin–*S*-alcohol (IV); (○) *S*-warfarin reduction to *S*-warfarin–*S*-alcohol (IV).

reports that they were capable of reducing ketonic substrates. Of these, *Arthrobacter* species (ATCC 19140) and *N. corallina* (ATCC 19070) were found to reduce *RS*-I to warfarin alcohols. *Arthrobacter* species produced the lower R_f alcohol (II or IV or both) as the major product and the upper R_f alcohol (III or V or both) as a minor product by thin-layer chromatographic analysis. *N. corallina* produced only a lower R_f alcohol. Determinations of the substrate and product stereoselectivities of these reactions were approached by the use of the pure warfarin enantiomers as substrates, followed by HPLC analyses of the resultant alcohol(s). Since the two diastereomeric sets of enantiomeric alcohols (*S*-warfarin–*S*-alcohol/*R*-warfarin–*R*-alcohol [IV/II] and *R*-warfarin–*S*-alcohol/*S*-warfarin–*R*-alcohol [III/V]) are resolved by HPLC (T_r = 2.90 and 3.50 min, respectively), as reported by Fasco et al. (8), the complete evaluation of substrate and product stereoselectivity was possible. For example, if *S*-I was used as the substrate, reduction to the *S*-alcohol would yield a peak at 2.90 min corresponding to alcohol IV. Conversely, a peak at 3.50 min would result from reduction to the *R*-alcohol (V). The results of these microbiological studies are summarized in Fig. 2 and are rationalized in the following discussion.

N. corallina metabolized *RS*-I only to a T_r = 2.90 peak by HPLC (Fig. 3); the time course of the metabolism of warfarin stereoisomers is shown in Fig. 4. Racemic warfarin (*RS*-I) was metabolized at an initial rate (linear portion of the curve) of 2.5 μg/ml per h. If only one enantiomer of warfarin was reduced to either II or IV, the extent of theoretical conversion observed was 85%. The stereochemical course of metabolism was determined by the observation that *S*-I was reduced to only the T_r = 2.90 peak, which corresponds to *S*-warfarin–*S*-alcohol (IV). The initial rate of metabolism observed (see Fig. 4) was 2.4 μg/ml per h, with a 78% theoretical conversion. *R*-I exhibited no reduction by this microorganism (i.e., <3%, presumably due to enantiomeric impurity; see below). Hence *N. corallina* exhibited complete substrate (*S*) and product (*S*) stereoselectivity. It is also apparent that the presence of *R*-warfarin (in the *RS* experiment) inhibited neither the rate nor the extent of metabolism of the *S*-enantiomer. In addition, the mass spectral analysis of the alco-

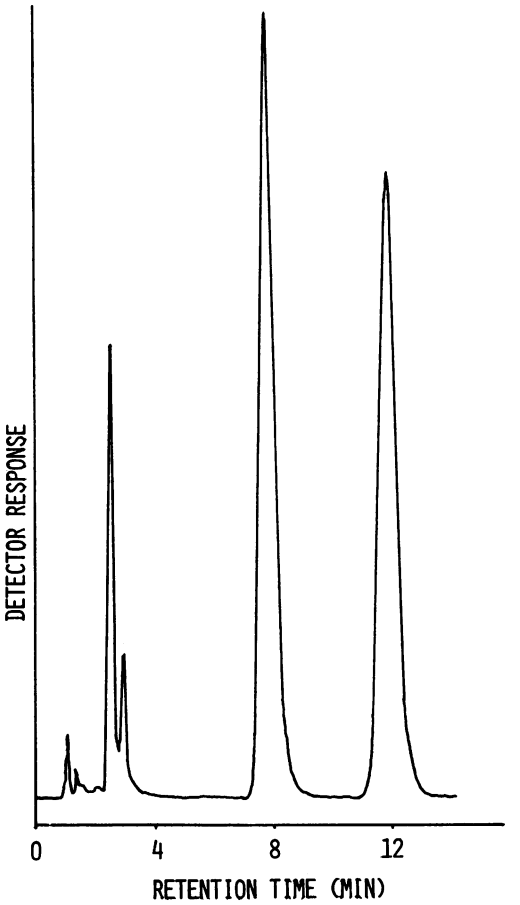

FIG. 5. HPLC chromatogram of *RS*-warfarin metabolism by *Arthrobacter* species. Assigned peaks are: T_r = 3.00, *S*-warfarin–*S*-alcohol (IV); T_r = 3.50, *R*-warfarin–*S*-alcohol (III); T_r = 8.50, phenprocoumon (internal standard); T_r = 13.00, warfarin (I).

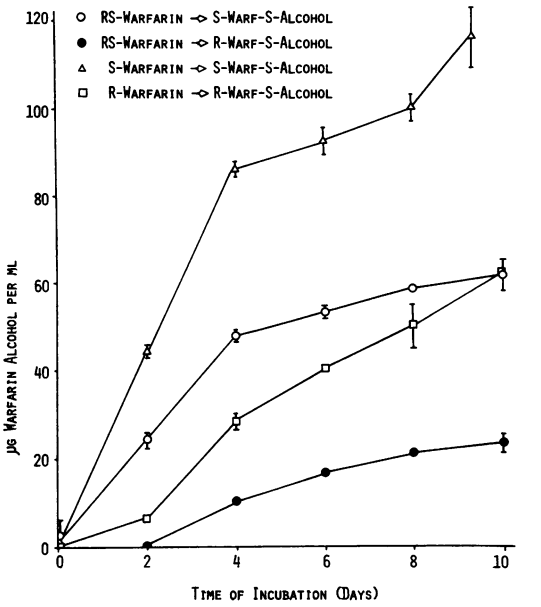

FIG. 6. Time course of the metabolism of warfarin enantiomers by *Arthrobacter* species (ATCC 19140). Symbols: (○) *RS*-warfarin reduction to *S*-warfarin–*S*-alcohol (IV); (●) *RS*-warfarin reduction to *R*-warfarin–*S*-alcohol (III); (△) *S*-warfarin reduction to *S*-warfarin–*S*-alcohol (IV); (□) *R*-warfarin reduction to *R*-warfarin–*S*-alcohol (III).

hol from *RS*-I is consistent with the data of Trager et al. (29) and identical to the standard alcohol.

The stereospecificity of *Arthrobacter* species was more complex. The administration of *RS*-I yields the T_r = 2.90 peak as the major metabolite and the T_r = 3.50 peak as a minor metabolite. (Fig. 5). The time course shown in Fig. 6 for *RS*-I as substrate represents an initial rate of 0.50 μg/ml per h, with a 25% theoretical conversion to the first alcohol, and 0.13 μg/ml per h to the second alcohol. The use of *S*-I as the substrate (Fig. 6) yielded production of only the *S*-warfarin–*S*-alcohol (IV; T_r = 2.90; 0.90 μg/ml per h; 23% theoretical conversion), whereas *R*-warfarin was converted to the *R*-warfarin–*S*-alcohol (III; T_r = 3.50; 0.26 μg/ml per h; 12% theoretical conversion). In the latter case, a minor (i.e., less than 3%) apparent reduction of *R*-warfarin to the first alcohol, which might be interpreted as production of *R*-warfarin–*R*-alcohol (II), was assumed to be the result of minor *S*-I contamination in *R*-warfarin, leading to minor production of *S*-warfarin–*S*-alcohol (III). Indeed, both cultures exhibited a diminution of the artifactual alcohol (III) formation as *R*-warfarin was repeatedly recrystallized. Other investigators have also had considerable difficulty in obtaining pure warfarin enantiomers because of the fractional crystallization procedures needed (18, 20). Hence, the *Arthrobacter* species exhibited a marked substrate stereoselectivity for *S*-warfarin, with some *R*-warfarin metabolism, but complete product stereoselectivity to yield only the corresponding *S*-alcohols (III and IV). In addition, it is apparent that the metabolism of each enantiomer in the racemic mixture is effectively halved by the presence of the other enantiomer, but with no ultimate effect on the extent of conversion, suggesting the involvement of a common reductase.

The product stereospecificity exhibited by the two organisms utilized in these studies parallels the majority of mammalian studies reported to date, in that reduction to an *S*-alcohol predominates, regardless of the stereochemistry of the substrate. With the in vivo studies in humans (2) and with human and rat liver in vitro systems

(18, 20), the major metabolic route of *R*-warfarin involves reduction to the *S*-alcohol (III), whereas lesser amounts of the *R*-alcohol (II) are produced. Since aromatic hydroxylation of *S*-warfarin is the predominant pathway, the reduction represents a minor route of metabolism. Nevertheless, the reduction of *S*-warfarin to the corresponding *S*- and *R*-alcohols (IV and V, respectively) has been observed, with *S*-reductase activity predominating (2, 12, 16, 18). In one case (19), the in vivo reduction of warfarin in the rat occurs with a marked substrate selectivity for *S*-warfarin and a marked product stereoselectivity (reductase) for the production of *S*-alcohols. Thus, these cultures exhibit reductase activity (product stereoselectivity) closely paralleling mammalian systems. As mentioned above, the substrate stereoselectivity is more complex, since a major reason that *R*-warfarin reductase activity predominates in mammals is that the major metabolic route of *S*-warfarin in such systems is aromatic hydroxylation (20). The cultures examined in this study do not allow this type of reaction; thus, *S*-warfarin reduction predominates and yields IV as the major product in both cases.

The use of microorganisms for type-specific reactions of synthetic interest is facilitated by the determination of the stereochemical course of metabolism. Since the enzymes involved in the catalysis may be considered as "chiral reagents," one often observes a high degree of substrate and product stereoselectivity. In the present case, no "R-organisms," i.e., cultures exhibiting *R*-reduction of warfarin, were observed, although the demonstrated *S*-reductase activity of *N. corallina* and *Arthrobacter* species suggests their potential utility in stereochemical organic synthesis.

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