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A New Warfarin Metabolite: Structure and Function

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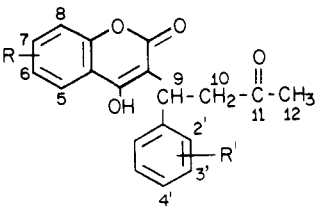
Received February 27, 1978

The metabolism of the clinically utilized, anticoagulant warfarin [4-hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-1-benzopyran-2-one] by rat liver microsomes has been investigated. The structure of a new warfarin metabolite [4-hydroxy-3-(3-oxo-1-phenyl-1-butenyl)-2*H*-1-benzopyran-2-one] (dehydrowarfarin) has been determined by mass spectral comparison with the chemically synthesized compound. The formation of dehydrowarfarin is catalyzed by cytochrome P-450 and is unusual in that the final product is effectively dehydrogenated warfarin.

Warfarin [4-hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-1-benzopyran-2-one] (**1**) is a vitamin K_1 antagonist widely employed as a rodenticide and as a therapeutic drug for the treatment of such coagulation disorders as thrombophlebitis, pulmonary embolism, and myocardial infarction. Human[1] and rodent[2,3] resistance to warfarin has been encountered, however, and clinical complications have arisen as a consequence of its administration with other medications.[4,5]

The clinical and environmental importance of warfarin has prompted a number of investigations to determine its pharmacological and biological fate. The *R* and *S* optical enantiomers of warfarin have been resolved.[6] (*S*)-Warfarin is five to eight times more potent an anticoagulant than (*R*)-warfarin in both man[7-10] and the rat.[11-14] In man (*S*)-warfarin is metabolized faster than the *R* isomer, whereas in the rat (*R*)-warfarin is metabolized more rapidly.[12-14] These observations suggest that the rate of metabolism of the warfarin enantiomers is not solely responsible for their different hemorrhagic potencies. Barker et al.[15] found little unmetabolized warfarin in the urine of rats administered the drug but were able to identify four monohydroxylated metabolites (**1a–d**) as well as a cyclized



1. R = R' = H
1a, R = 6-OH; R' = H
1b, R = 7-OH; R' = H
1c, R = 8-OH; R' = H
1d, R = H; R' = 4'-OH

derivative, 2,3-dihydro-2-methyl-4-phenyl-5-oxo-4*H*-pyrano[3,2-*c*]-2*H*-benzopyran. Lewis and Trager[16] isolated a mixture of diastereoisomeric, aliphatic warfarin alcohols from the urine of human volunteers. Subsequent in vitro

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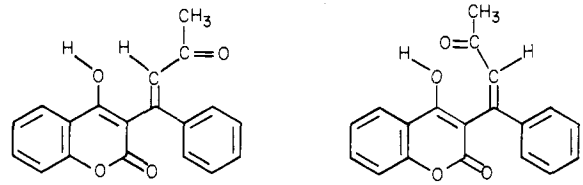
and in vivo investigations also demonstrated the formation of these metabolites by the rat.[17,18] Recently, Pohl et al.[19] identified "benzylic" hydroxywarfarin and Thonnart et al.[20] small amounts of ring-opened compound 6-(1-hydroxy-2-oxo-3-phenylhexylidene)-2,4-cyclohexadien-1-one as products of the NADPH-dependent, rat hepatic microsomal metabolism of warfarin. Salicylic acid has also been identified as a biological degradation product of warfarin[21] and conjugates of many warfarin-hydroxylated metabolites have been reported.[22-24] However, with the exception of 4'-hydroxywarfarin and the warfarin alcohols, which possess greatly diminished anticoagulant activity relative to warfarin,[15,25] none of the currently identified metabolites of warfarin is pharmacologically active.[15] The question as to whether warfarin or one of its presently unidentified metabolites is responsible for antivitamin K_1 activity has not been resolved.

We have recently reported the in vitro formation of a previously undetected metabolite of warfarin catalyzed by rat liver microsomes.[26] The rate of formation of this metabolite was stereoselective for the pharmacologically more potent *S* enantiomer, and its concentration was comparable to many of the known warfarin metabolites produced by the rat hepatic microsomal system. We report here on the structure, synthesis, and biochemical and pharmacological properties of this new warfarin metabolite.

Isolation and Identification of Metabolite. The majority of hepatic microsome-catalyzed biotransformations of drugs and other xenobiotic compounds initially involves oxidation by cytochrome P-450, the terminal oxidase of the mixed function oxidase system. Investigations with the unknown warfarin metabolite were performed to determine its polarity relative to known monohydroxywarfarin metabolites and thereby gain insight into its structure. Relative polarities were determined from retention times of the metabolites on a reverse-phase μ Bondapak C_{18} high-performance liquid chromatography (LC) column. Under isocratic conditions, the extent of substrate oxidation can be estimated, since, for example, dihydroxy compounds exhibit a more reduced affinity for the hydrophobic column matrix than do analogue monohydroxy compounds. Since the extent of ionization of acidic compounds, such as warfarin, also determines their retention times on a reverse-phase column, initial chromatographic investigations were performed in an acetonitrile–aqueous acetic acid mixture, pH 2.5, to completely protonate all the warfarin derivatives. Under these conditions, the unknown warfarin metabolite migrated as a less polar compound than all of the monohydroxylated metabolites but was more polar than warfarin. This pattern of retention was in sharp contrast to that initially observed in the acetonitrile–NH_4OAc, pH 4.85, LC chromatographic system[26] where the unknown metabolite migrated as the most polar warfarin derivative. The profound pH dependence of its migration indicated that it had a lower pK_a than warfarin and its known metabolites.

LC was utilized to isolate sufficient quantities of the unknown warfarin metabolite for UV spectral and mass spectral analyses. The metabolite was isolated following the metabolism of (*R*)-warfarin in a pregnenolone-16α-carbonitrile (PCN) induced rat hepatic microsomal system employing cumene hydroperoxide as a source of activated oxygen. This system provided the highest levels of the unknown metabolite (see later). The identity of the metabolite isolated from this system, and that produced from uninduced or phenobarbital-induced microsomes with NADPH, was demonstrated by their identical behavior under a variety of liquid chromatographic conditions and by comparisons of UV spectra, as described below.

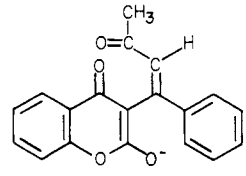
The UV spectrum of the isolated metabolite in acidic solution contained considerably more fine structure than the spectra of warfarin or its monohydroxylated products, with a series of absorbance maxima between 250 and 300 nm, which indicated the presence of a highly conjugated system. The EI mass spectrum yielded a parent ion at *m/e* 306, a loss of 2 H from the structure of warfarin. Collectively, the results of these studies indicated that the most probable structure of the unknown metabolite was 4-hydroxy-3-(3-oxo-1-phenyl-1-butenyl)-2*H*-1-benzopyran-2-one (dehydrowarfarin, **2**), which can occur as the cis and/or trans isomer.



trans cis

2

Dehydrowarfarin was synthesized by the reaction of warfarin with a pyridine solution of cuprous chloride, a reaction recently discovered in our laboratories during investigations of warfarin oxidation in the presence of metal–oxygen complexes, similar to those of Ikeda et al.[27] NMR analysis of the reaction product demonstrated the loss of warfarin benzyl proton at 4.1 ppm and disappearance of the broad methyl–methylene multiplet at 1.3–2.9 ppm, which was replaced by a methyl singlet at 1.95 ppm. A broad singlet at 5.3 ppm superimposed on a singlet at 5.8 ppm represents the 4-OH and the vinyl protons. One of these protons (4-OH) rapidly exchanged, and the other slowly exchanged over a number of hours when D_2O was added to a solution of the compound in chloroform. The mass spectrum contained a parent ion at *m/e* 306, and the IR spectrum and elemental analysis were also consistent with structure **2**. The mass spectrum of dehydrowarfarin after long exposure to D_2O had a parent ion at *m/e* 308, indicating that two protons had exchanged for deuterium. From the mass fragmentation pattern and the NMR spectrum, the exchangeable protons are the 4-OH proton and the vinyl proton. The fine structure in the UV spectrum of protonated dehydrowarfarin in acidic solution is consistent with the presence of a highly conjugated system (**2**). In basic solution, a shift from the anion of the 4-hydroxycoumarin nucleus (**2**) to the anion of the 2-hydroxychromone (**3**) would result in decreased conjugation and fine structure. This was observed when the spectrum was determined in basic solution.



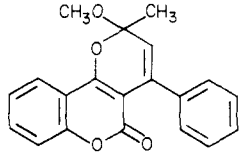
3

Synthetic dehydrowarfarin (**2**) cochromatographed with the unknown warfarin metabolite in two TLC systems [acetonitrile–methylene chloride (1:1); dichloroethane–acetone (7:3)] and in the acetic acid and NH_4OAc–acetonitrile LC system. It also possessed identical UV absorbance spectra to the unknown warfarin metabolite in the range 220–400 nm in both acidic and basic solutions. The EI fragmentation pattern of the unknown warfarin

metabolite corresponded exactly with that of the synthetic compound, except for the relative intensities of the m/e 288 and 263 peaks and for what are probably impurities at m/e 213 and 229. All of these results establish the identity of the unknown metabolite and synthetic dehydrowarfarin.

The assignment of structures to the various major mass ions of dehydrowarfarin is based on the EI fragmentation patterns of warfarin as described by Trager et al.[28] Warfarin exists in solution in an open-chain/hemiketal tautomeric equilibrium and the relative concentrations of the tautomers are largely dependent upon the polarity and pH of the solvent.[29] The base peak ($M^+ - 43$) fragment of warfarin arises from the open-chain form via ionization of the alkyl keto group, followed by loss of the acyl radical to form the dihydrofuranooxonium ion at m/e 265.[28] Fragmentation of warfarin to the vinyl carbonium ion, m/e 145, occurs to a lesser extent, and fragmentation leading to m/e at 251 occurs to a minor extent. In contrast, the fragmentation of dehydrowarfarin (**2**) occurs primarily by dehydration of the hemiketal leading to the major peak at m/e 288 and by loss of the acyl radical (m/e 263) following ionization.

Investigations with synthetic dehydrowarfarin demonstrated that it was very reactive, undergoing chemical reaction with methanol, to produce a nonpolar compound which could not be extracted into basic solution. The product of the reaction was isolated, and mass spectral analysis indicated a molecular weight of 320. This is consistent with the formation of a ketal, compound 4, by



4

analogy with warfarin. Warfarin undergoes addition and cyclization in the presence of methanol to produce cyclocoumarol,[30] but a strong acid catalyst is required to initiate the reaction. The NMR spectrum was also consistent with such a structure with $-OCH_3$ protons at δ 3.33. Dehydrowarfarin was also found to readily undergo reaction with primary amines, presumably to analogue compounds of 4.

In Vitro Metabolic Studies. Rat hepatic microsome-catalyzed formation of dehydrowarfarin and the other oxidized metabolites of warfarin was totally inhibited when molecular oxygen was replaced by nitrogen. An atmospheric mixture of carbon monoxide–oxygen (80:20) partially inhibited the rates of formation of dehydrowarfarin (57%), 4′-hydroxywarfarin (63%), 6-hydroxywarfarin (65%), and 7-hydroxywarfarin (67%). Formation of dehydrowarfarin and the other oxidized warfarin metabolites was markedly better supported by NADPH than NADH. Cumene hydroperoxide supported the microsomal metabolism of warfarin to dehydrowarfarin and its other metabolites to varying extents.[31] This hydroperoxide directly provides activated oxygen to cytochrome P-450 without the intercession of other components of the microsomal system, such as NADPH cytochrome P-450 reductase and phospholipid.[32] Collectively, these data demonstrate that cytochrome P-450 catalyzes the formation of dehydrowarfarin.

Under optimal conditions the microsomal metabolism of (*R*)- or (*S*)-warfarin to multiple products was linear with time for 10 min (Figure 1). Rates of formation of dehydrowarfarin, however, were markedly decreased after 10 min, which was not the case for the other warfarin metabolites (Figure 1). Microsomal metabolism studies using dehydrowarfarin as the substrate demonstrated that it does not undergo rapid biotransformation in vitro, which indicates that the decreased rate of dehydrowarfarin formation is not a consequence of its further metabolism to other products.

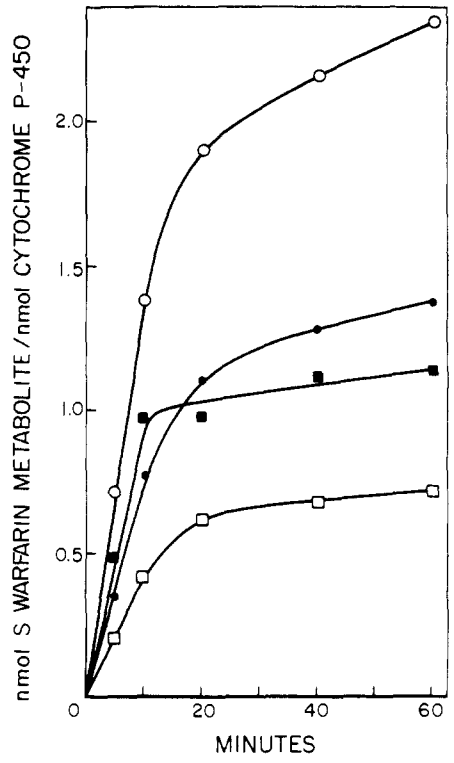


Figure 1. Rates of formation of dehydrowarfarin (■), 6-hydroxywarfarin (●), 7-hydroxywarfarin (□), and 4′-hydroxywarfarin (○) from (*S*)-warfarin, catalyzed by uninduced rat liver microsomal cytochrome P-450. Concentration of cytochrome P-450, 1.0 nmol/mg of protein; concentration of microsomal protein, 2 mg/mL. The reaction was run at 37 °C and pH 7.4. The results are from a single determination using a pool of five rats.

Investigations of the effects of induction of cytochrome P-450 were performed to determine the in vitro conditions which would provide maximal quantities of dehydrowarfarin for structural identification. The results of these metabolic studies with uninduced and phenobarbital-, 3-methylcholanthrene-, and PCN-induced liver microsomes are presented in Figure 2. For the NADPH-supported metabolism by control and induced microsomes, dehydrowarfarin formation was stereoselective for (*S*)-warfarin. With cumene hydroperoxide, however, the stereoselectivity of the reaction was reversed in favor of (*R*)-warfarin. The greatest quantities of dehydrowarfarin were produced by cumene hydroperoxide supported metabolism of (*R*)-warfarin by PCN-induced microsomes. This system was thus utilized as the source of dehydrowarfarin for studies of its structure.

Biological Studies. Dehydrowarfarin was found to be inactive as a vitamin K_1 antagonist ($p < 0.005$), as determined by one-stage clotting assays of plasma from rats administered the synthetic compound. It also was inactive as an antagonist of the synthesis of the individual extrinsic coagulation factors, II, VII, and X, and the intrinsic coagulation factors, VIII and IX. Fibrinogen levels remained normal at least 24 h after dehydrowarfarin administration. Further, rats administered large doses of dehydrowarfarin displayed no physical reaction to the compound.

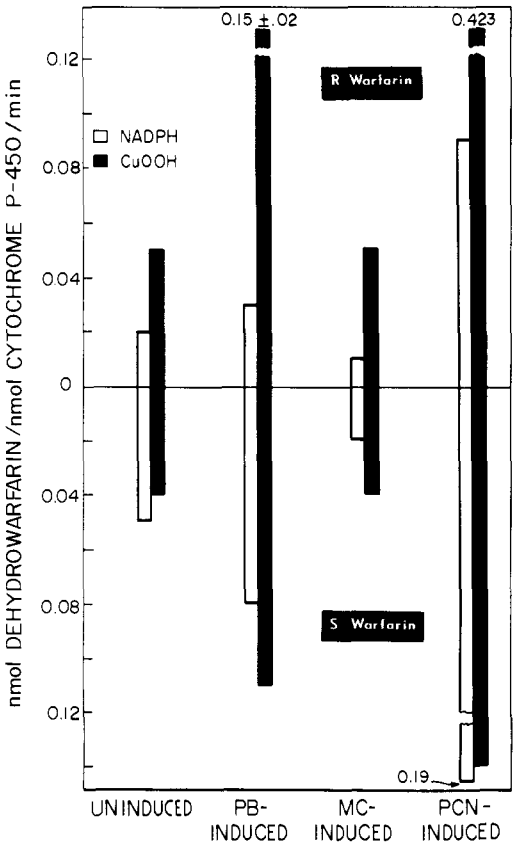


Figure 2. Rates of formation of dehydrowarfarin from (*R*)- or (*S*)-warfarin catalyzed by uninduced and phenobarbital-, 3-methylcholanthrene-, or PCN-induced rat liver microsomes. Reactions were supported by either NADPH or cumene hydroperoxide. Reactions were performed at 37 °C and pH 7.4.

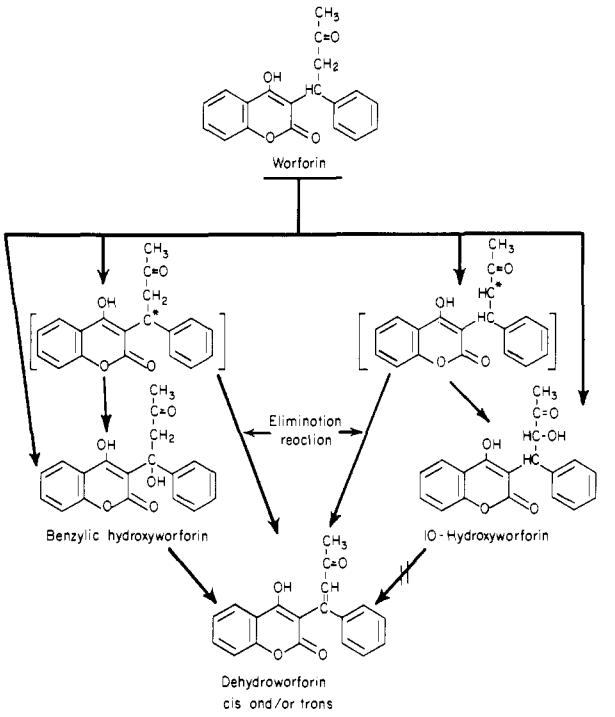
Both the sodium salt and the free acid of dehydrowarfarin were administered at ten times the dose of warfarin required to produce a high degree of hypoprothrombinemia.

The fact that dehydrowarfarin reacts with primary alcohols and amines to form stable, cyclized compounds (see structure **4**) suggested that it might undergo reaction with cellular macromolecules producing possible necrotic and mutagenic effects. Dehydrowarfarin was, therefore, tested in the DNA reversion assay of Ames,[33] but no mutagenic activity was observed. This lack of reactivity in vivo suggests that either dehydrowarfarin does not reach the ultimate site on the DNA required for mutagenic activity or, because of its acidity, it does not exist in the protonated form under physiological conditions and, therefore, cannot react with cellular components. Indeed, the sodium salt of dehydrowarfarin does not react with methanol.

Discussion

The cytochrome P-450 catalyzed oxidation of aromatic compounds has been thoroughly investigated and the vast majority of these biotransformations shown to involve arene oxide intermediates.[34] The mechanism of cytochrome P-450 catalyzed oxidation at saturated carbon atoms, however, is much less clearly understood. Presumably, the saturated carbon atom of the substrate must be activated by loss of hydride, hydrogen atom, or a proton to form a carbonium ion, radical, or carbanion (abstraction) or the transfer of the equivalent of singlet oxygen atom to the substrate must occur (insertion).[35] Enzymatic hydroxylations at saturated carbon atoms often display a primary isotope effect and precede with a high degree of

Scheme I



* Enzymatically activated carbon atom (see Discussion).

retention of configuration.[35] The insertion mechanism has been commonly accepted for aliphatic hydroxylation by cytochrome P-450,[36] but recently Hjelmeland et al.[37] have suggested an alternative radical abstraction–recombination mechanism.

The cytochrome P-450 catalyzed formation of dehydrowarfarin is thus unusual, in that the final result is effectively a dehydrogenation of warfarin. The most plausible mechanisms for dehydrowarfarin formation are illustrated in Scheme I.

Investigations in our laboratory have indicated that 10-hydroxywarfarin (Scheme I) is a metabolite of warfarin, the formation of which is catalyzed by cytochrome P-450. This metabolite does not undergo dehydration to dehydrowarfarin in dilute acid or under the conditions of the microsomal metabolism of warfarin or our LC assay. Benzylic hydroxywarfarin has been identified by Pohl et al.[19] as a metabolite of warfarin. It is, however, likely that benzylic hydroxywarfarin would form a six-membered ring by hydrogen bounding between the benzylhydroxy hydrogen and the 11-keto group of the warfarin side chain. This ring system would undergo either spontaneous or mild acid-catalyzed dehydration to dehydrowarfarin. The most probable explanation for the slow loss of the vinyl proton of dehydrowarfarin is that D_2O adds across the 9,10 double bond to yield benzylic hydroxywarfarin with deuterium on C_{10}. This molecule then spontaneously dehydrates back to dehydrowarfarin with retention of the deuterium and loss of the original proton. In view of this apparent spontaneous dehydration of benzylic hydroxywarfarin to dehydrowarfarin, it is probable that the metabolic pathway for formation of dehydrowarfarin is also via benzylic hydroxywarfarin.

Experimental Section

NMR spectra were recorded with a Varian Model EM-360 NMR spectrometer using TPS [3-(trimethylsilyl)propanesulfonic acid sodium salt] as internal standard. Mass spectra were recorded on a AEI MS-30 employing the direct insertion system; t = 100–250 °C. UV spectra were recorded on an Aminco DW-2 spectrophotometer. Reagents used in microsomal metabolism

studies were purchased from Sigma. Cumene hydroperoxide was obtained from Pfaltz and Bauer, Inc. Experimental animals were male Wistar rats (250 ± 10 g) from a colony maintained in this Division. Microanalyses were carried out by Instramal Laboratory, Inc., Rensselaer, N.Y., and were ±0.04% of the theoretical values.

Synthesis. Dehydrowarfarin (2). Warfarin (1) (10 g, 0.033 mol) was dissolved in pyridine (40 mL) at 37 °C. To this was added freshly prepared, powdered CuCl (5.0 g, 0.05 mol), and the mixture was incubated at 37 °C overnight with gentle shaking. The reaction was diluted with water (500 mL), acidified with concentrated HCl (50.0 mL), and extracted with dichloromethane (2 × 300 mL). The extracts were combined and extracted with 1.0 M NaOH (3 × 100 mL), and, after acidification (concentrated HCl), the aqueous phase was extracted with diethyl ether (2 × 300 mL). The combined extracts were dried (Na_2SO_4), filtered, and concentrated to approximately 100 mL. A pale yellow solid precipitated, which was recovered by filtration and recrystallized (acetone–H_2O) to yield compound **2**: 3.5 g (33%); mp 219–220 °C dec; NMR ($CDCl_3$) δ 1.95 (3 H, s), 5.3 (1 H, s), 5.8 (1 H, s), 7.35 (9 H, br m); mass spectrum parent ion *m/e* 306; IR (KBr) 5.90, 6.16, 6.25, 6.50, 6.70 μ. Anal. ($C_{19}H_{15}O_4$) C, H.

The sodium salt of dehydrowarfarin was prepared by adding a molar excess of compound **2** to dilute sodium hydroxide with stirring. The suspension was clarified by filtration and the filtrate lyophilized to yield a yellow powder.

Compound 4. The synthesis of compound 4 was essentially that of Link et al. for the preparation of cyclocoumarol.[30] Dehydrowarfarin (0.5 g, 0.002 mol) was dissolved in methanol (20 mL). After 24 h the solution was concentrated to 10.0 mL and cooled at 5 °C overnight. The crystalline, white solid was recovered by filtration to yield 0.25 g (50%) of compound **4**: mp 92–94 °C; NMR (Me_2SO-d_6) δ 1.87 (3 H, s), 3.33 (3 H, s), 5.77 (1 H, s), 7.43 (5 H, s), 7.8 (4 H, br m); mass spectrum parent ion *m/e* 320.

Microsomal Studies. Rat hepatic cytochrome P-450 levels were induced by ip administration of phenobarbital, PCN, or 3-methylcholanthrene once daily for 3 days (100, 100, and 25 mg/kg/day, respectively). Phenobarbital was administered in physiological saline, and both PCN and 3-methylcholanthrene solutions were administered in corn oil. Twenty-four hours after the last injection, the rats were sacrificed by cervical dislocation, and their hepatic microsomes were isolated by a modification of the method of Tangen et al.[38] The protein concentration of the microsomal suspensions was determined by the method of Schacterle and Pollack,[39] and the cytochrome P-450 concentrations were determined by the method of Omura and Sato.[40]

Metabolic studies were performed either with NADPH as a source of reducing equivalents or cumene hydroperoxide as a source of activated oxygen for cytochrome P-450. For studies employing NADPH, incubation flasks contained (*R*)- or (*S*)-warfarin sodium salt (1.0 mg, 3.0×10^{-6} mol) in 0.1 mL of water, a NADPH generating system [NADP (1.0 mg of sodium salt, 1.25 μmol), glucose 6-phosphate (2.0 mg of disodium salt monohydrate, 62.1 μmol)] in 1.0 mL of 0.01 M TES [*N*-tris(hydroxymethyl)-methyl-2-aminoethanosulfonic acid]·0.002 M $MgCl_2$ buffer, pH 7.4, and microsomal protein (8.0 mg). The volume was finally adjusted to 4.0 mL with the TES·$MgCl_2$ buffer, pH 7.4. The mixture was incubated at 37 °C for 1.0 min and the enzymatic reaction initiated by the addition of glucose-6-phosphate dehydrogenase (yeast, 5.0 units). After 10 min the reaction was terminated by filtration through a Millipore filter pad, as previously described.[26] For incubations where cumene hydroperoxide was employed, the NADPH generating system was omitted, and the enzymatic reaction was initiated by the addition of cumene hydroperoxide (3.3×10^{-5} mol). Reactions were terminated after 5 min. Experiments conducted under controlled atmospheres were performed as described, except that flasks were sealed with a rubber septum and equilibrated under the desired atmosphere for 15 min in ice before incubation at 37 °C.

Separation and quantitation of the various warfarin metabolites were performed by a modification of the previously reported LC method[26] employing a Hewlett-Packard 3380A recording integrator calibrated in the external standard mode.

Preparative-scale microsomal incubations were conducted in an Erlenmeyer flask containing PCN-induced microsomes (200 mL, 2.0 mg of protein/mL) in TES·$MgCl_2$ buffer, pH 7.4, and (*R*)-warfarin sodium salt (0.05 g, 1.5×10^{-4} mol). The flasks were incubated with gentle shaking at 37 °C for 5 min, and the reaction was initiated by the addition of cumene hydroperoxide (1.6×10^{-4} mol). After 10 min cumene hydroperoxide (1.6×10^{-4} mol) was again added, and the reaction was stopped after 30 min by cooling in ice. The microsomes were removed by centrifugation at 100 000g for 40 min, and the soluble protein was removed by filtration through an Amicon PM 10 membrane. A portion of the filtrate (60 mL) was loaded onto a μ Bondapak C_{18} preparative column (7.8 mm i.d. × 30 cm, Waters Associates) equilibrated in water. A flow rate of 5.0 mL/min was used throughout. The column was washed with water for an additional 10 min and finally with 1.5% NH_4OAc (pH 4.85)–acetonitrile (9:1) for 10 min. A nonlinear gradient (no. 7, Waters Model 660 solvent programmer) was then run over 5 min to 1.5% NH_4OAc (pH 4.85)–acetonitrile (68:32), and dehydrowarfarin was collected. The collected fractions from three runs were combined, diluted with 2 vol of 1% HOAc, and again loaded on the preparative μ C_{18} column, this time equilibrated in water–acetonitrile (9:1). The column was washed with the same solvent for 5 min, and gradient no. 7 was run over 5 min to water–acetonitrile (55:45). The dehydrowarfarin peak was collected and diluted with 3 vol of water, and concentrated HCl was added to a final concentration of 0.1 M. This solution was extracted with dichloromethane (2 × 100 mL); the solvent fractions were combined, dried (Na_2SO_4), and evaporated to near dryness. A portion of this material was placed in a capillary tube and evaporated to dryness for mass spectral analysis. UV scans were obtained from the remainder of the dichloromethane fraction, which was evaporated to dryness and then redissolved in acetonitrile.

Biological Studies. Mutagenicity tests of dehydrowarfarin were performed with the sodium salt, both with and without liver microsomes added to the agar medium. The bacterial test strains were *Salmonella* type TA 98, TA 100, TA 1535, and TA 1537.

For anticoagulant activity tests both the sodium salt in physiological saline and the free acid of dehydrowarfarin in corn oil were administered ip to six rats at a dose of 100 mg/kg. Twelve hours after injection, rats were anesthetized with Diabutal and 4.5 mL of blood was withdrawn from the posterior vena cava with a 5.0-mL syringe containing 3.8% trisodium citrate (0.5 mL). The plasma obtained by centrifugation at 2000g for 10 min was assayed for clottability by the one-stage method using a fibrometer. The plasma concentration of the individual extrinsic and intrinsic coagulation factors and fibrinogen was determined as described by Raymond and Dodds.[41] Control rats received corn oil or physiological saline or the sodium salt of racemic warfarin which was administered ip at a dose of 10 mg/kg.

Acknowledgment. This work was supported by Grant HL 19772 from the National Institutes of Health, PHS/DHEW. The authors wish to thank Dr. W. J. Dodds and Mrs. A. Moynihan for coagulation factor analysis and Dr. T. Miller for performing the Ames test.

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Folate Antagonists. 13. 2,4-Diamino-6-[(α,α,α-trifluoro-*m*-tolyl)thio]quinazoline and Related 2,4-Diamino-6-[(phenyl- and naphthyl)thio]quinazolines, a Unique Class of Antimetabolites with Extraordinary Antimalarial and Antibacterial Effects[1,2]

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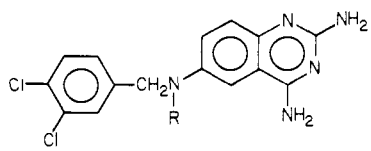
An array of nonclassical thioquinazoline analogues (VIII) of methotrexate was prepared by cyclization of the requisite 2-amino-5-(arylthio)benzonitrile with chloroformamidine hydrochloride (28–79%). The aminonitrile precursors were obtained by $SnCl_2$–HCl reduction (28–99%) of the corresponding 2-nitro-5-(arylthio)benzonitriles, which were synthesized by the condensation of the appropriate 5-chloro-2-nitrobenzonitriles with various arylthiols (36–83%). Many of the thioquinazolines (VIII) showed suppressive antimalarial activity comparable with or superior to chloroquine, cycloguanil, and pyrimethamine against drug-sensitive lines of *Plasmodium berghei* in mice and *Plasmodium gallinaceum* in chicks, and several displayed potent prophylactic activity against *P. gallinaceum*. Moreover, the thioquinazolines retained potent antimalarial effects against chloroquine-, cycloguanil-, pyrimethamine-, and DDS-resistant lines of *P. berghei* in mice and against chloroquine- and pyrimethamine-resistant strains of *Plasmodium falciparum* in owl monkeys. The most active compound, namely, 2,4-diamino-6-[(α,α,α-trifluoro-*m*-tolyl)thio]quinazoline, was designated for preclinical toxicological studies. Numerous substances exhibited in vitro activity against a broad spectrum of pathogenic bacteria at concentrations of <0.25 μg/mL. The thioquinazolines also proved to be potent folate antagonists, causing 50% inhibition of *Streptococcus faecalis* R (ATCC 8043) at drug concentrations ranging from 0.2 to 2.0 ng/mL. Structure–activity relationships are discussed.

A plethora of 2,4-diamino-6-[(benzyl)amino]quinazoline antifolates, exemplified by 2,4-diamino-6-[(3,4-dichlorobenzyl)amino]quinazoline (Ia),[4,5] 2,4-diamino-6-[(3,4-dichlorobenzyl)nitrosamino]quinazoline (Ib),[6,7] and 2,4-diamino-6-[(3,4-dichlorobenzyl)methylamino]quinazoline (Ic),[8] exhibit strong antimalarial effects against sensitive and drug-resistant lines of *Plasmodium berghei* in mice, *Plasmodium gallinaceum* in chicks, and *Plasmodium cynomolgi* and *Plasmodium knowlesi* in rhesus monkeys.[4–8] In contradistinction, oxygen and sulfur bioisosteres such as 2,4-diamino-6-[(*p*-chlorobenzyl)oxy]quinazoline (IIa)[9] and 2,4-diamino-6-[(*p*-chlorobenzyl)thio-, sulfinyl-, and sulfonyl]quinazoline (IIb–d)[10] were either much less potent than the triaminoquinazolines Ia–c or lacked appreciable antimalarial activity altogether.[9,10] However, it is noteworthy that potent antimalarial activity was restored when the methylene bridge of IIa was extruded.[9] Thus, 2,4-



Ia, R = H
b, R = NO
c, R = CH_3

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