SYNTHESIS OF HIGH SPECIFIC ACTIVITY R- AND S-WARFARIN-^{3}H

C. E. Cook, C. Ray Tallent, Noel H. Ballentine, George F. Taylor, and John A. Kepler
Chemistry and Life Sciences Group
Research Triangle Institute
Research Triangle Park, North Carolina 27709, U.S.A.

SUMMARY

4-Hydroxy-3-[3-oxo-1-(3-bromophenyl)butyl]-2H-1-benzopyran-2-one (3'-bromowarfarin) was synthesized and resolved into its enantiomers by formation of the d-10-camphorsulfonate diastereoisomers, which were separated by liquid chromatography and hydrolyzed. The pure enantiomers were reduced with tritium gas to yield R- and S-4-hydroxy-3-[3-oxo-1-(phenyl-3-^{3}H)butyl]-2H-1- benzopyran-2-one (R- and S-warfarin-3'-^{3}H) of specific activity 31.2 and 24.9 Ci/mmole, respectively. The compounds have been used to develop a stereoselective radioimmunoassay for warfarin enantiomers.

Key Words: Warfarin, Enantiomers, Optical Isomerism, Tritium, Catalytic Reduction, Bromine

INTRODUCTION

Warfarin [4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one] is probably the most commonly used oral anticoagulant drug (1). A large number of studies have dealt with the metabolism, pharmacokinetics, and pharmacological effects of the enantiomers of warfarin. In 1974 Breckenridge et al. reported that S-warfarin is more potent as an anticoagulant and has a shorter half-life in man than the R-isomer and conclude that "the kinetic, metabolic, and functional differences between the enantiomers of warfarin emphasize that these compounds are, in many respects, quite dissimilar, and suggest that when racemic warfarin is given to man, one is administering not one, but two drugs" (2). In order to investigate the disposition of the enantiomers after administration of the racemic drug, we have been developing immunoassay procedures which are stereoselective for each enantiomer. This required the synthesis of high specific activity tritium labeled R- and S-warfarin, which is described in this report.

0362-4803/79/0416-0623$01.00

Received September 20, 1978

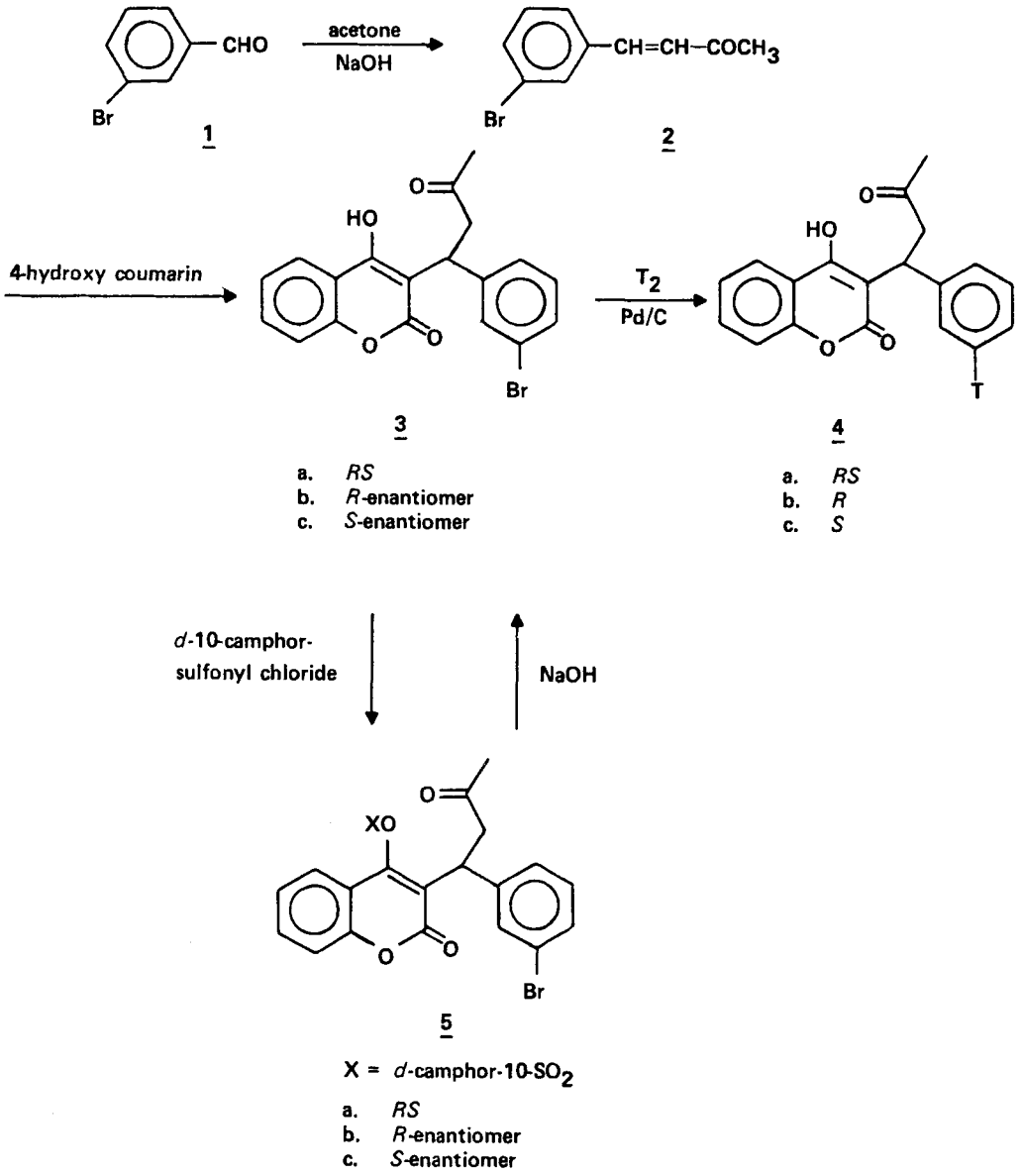
CHO
Br
1
acetone
NaOH
CH=CH–COCH3
Br
2
4-hydroxy coumarin
HO
O
O
O
Br
3
a. RS
b. R-enantiomer
c. S-enantiomer
T2
Pd/C
HO
O
O
O
T
4
a. RS
b. R
c. S
d-10-camphor-
sulfonyl chloride
NaOH
XO
O
O
O
Br
5
X = d-camphor-10-SO2
a. RS
b. R-enantiomer
c. S-enantiomer

Figure 1

EXPERIMENTAL

General.--Ultraviolet (uv) spectra were obtained on a Cary 14 recording spectrophotometer, infrared (ir) spectra on a Perkin-Elmer 237B or 467 grating spectrophotometer, optical rotations on a Perkin-Elmer Model 141 polarimeter, mass spectra (ms) on a MS-902 high resolution, LKB Model 9000 or Finnigan 3300 spectrometer, and proton magnetic resonance (pmr) spectra on a Varian HA-100 (100 MHz) or EM-360 (60 MHz) spectrometer. Thin layer chromatography (tlc) was carried out on silica gel F-254 precoated plates (Brinkmann) 250 μm thick. Spots on tlc were visualized by uv analysis at 254 nm and by spraying the plates first with 5% phosphomolybdic acid in 95% ethanol and heating at 110° followed by spraying with 5% $CeSO_4$ in 10% aqueous H_2SO_4 and reheating. R_f values are given in Table 1. Organic extracts were dried by washing with saturated NaCl solution and filtering through Whatmann 1-Phase Separating Paper. Melting points were taken on a Köfler hot stage microscope apparatus and are uncorrected. For purification by chromatography, mixtures were passed through columns of silica gel 60 (35-70 mesh, EM Reagents) prior to liquid chromatography at ca. 80 psi on silica gel 60 prepacked columns (EM Reagents). Fraction cuts of ~20 ml were taken during the chromatographies and similar fractions were combined after tlc analysis. Typical fraction volumes are given in the experimental section. Commercial analytical grade solvents were used. High pressure liquid chromatography was carried out on a Waters Model M-600 equipped with a 254 nm uv detector. Radioactivity was measured in a Packard Model 3375 liquid scintillation spectrometer using a medium containing 0.4 g Omnifluor (New England Nuclear, Boston, MA) per liter of toluene. Correction for quenching was carried out using an external standard procedure.

4-(3-Bromophenyl)-3-butene-2-one (2).--3-Bromobenzaldehyde (14.8 g, 80 mmol, Aldrich Chemical Co.) in 41.2 mL of acetone was cooled to 0°C. Aqueous NaOH (16.4 mL, 5%) was added dropwise over 5-10 min. After 10 min more at 0°, the reaction mixture was stirred at room temperature for 30 min. HCl (16 mL of 6 N) was added and the reaction mixture poured into 100 mL of distilled H_2O. Products were extracted into chloroform which was evaporated to afford 21.46 g of a viscous yellow oil (cf. ref. 3). A modified dry-column chromatography was used to purify the crude product. Silica gel (900 g, activity III according to Brockmann scale,

for dry-column chromatography from ICN Pharmaceuticals) was packed dry in a glass column (bed dimensions of 5.5 x 60 cm). The oil was dissolved in 100 ml of chloroform containing 100 g of silica gel. The chloroform was evaporated and the silica gel/sample layered on top of the column. A layer of sand and a plug of glass wool were then added. Chloroform was added rapidly to the top of the column. Fairly pure 2 (4.5 g) was eluted in the 320-1200 mL fraction. Polar reaction products (13 g) were then eluted with 2 L of ethyl acetate/benzene (1/4). An ir of the polar products indicated β-hydroxy ketone: ir (film) 3440 cm^{-1} (broad, -OH). Refluxing the hydroxy ketone for 4 hr with p-toluene sulfonic acid (1.6 g alcohol/25 mg acid) in benzene using a Dean-Stark trap followed by extraction with $NaHCO_3$ solution and evaporation of the benzene afforded more of the desired product. Further chromatography of 4.5 g of 2 on a size C (3.7 x 44 cm) silica gel 60 prepacked EM Reagents column using a gradient of 2 L of benzene to 2 L of ethyl acetate/benzene (1/9) afforded 2.06 g of pure product: ir (CH_2Cl_2) 1670 cm^{-1} (C=O), 1613 (C=C); pmr ($CDCl_3$) δ 2.33 (s, 3, -CO-CH_3), 6.57 (d, 1, J = 17 Hz, -CH-CO-) 7.38 (m, 5, Ar-H and ArCH=) ppm; bp 104-6°C (0.25 mm); mass spectrum (70 eV) m/e (rel intensity) 226 (22, M^+), 224 (24), 211 (58), 209 (58), 183 (21), 181 (22), 145 (79), 102 (100), $C_{10}H_9BrO$ requires m/e 224.

R,S-3'-Bromowarfarin (3a).--Refluxing ketone 2 (3.06 g, 13.7 mmol) and 4-hydroxycoumarin (2.22 g, 13.7 mmol) in dry pyridine (55 mL) for 4 hr followed by partition between 2N HCl and chloroform afforded a thick orange oil after drying and evaporation of the chloroform extract. A modified dry-column was set up as previously described and the oil was eluted with acetone/benzene (1/9) to yield crude coumarin 3a (1.41 g, 27%) in the 720-1600 mL fraction: mp 183.5-5.5°C (after crystallization from benzene); pmr (pyridine-d_5) δ 1.78 (s, 3, -CH_3), 2.41 (m, 2, CH_2), ~4.5 (m, 1, Ar-CH), 7.0-8.0 (m, Ar-H) ppm.

Anal. Calcd for $C_{19}H_{15}BrO_4$: C, 58.93; H, 3.90; Br, 20.64. Found: C, 59.14; H, 4.05; Br, 20.64.

Preparation and Resolution of R,S-3'-Bromowarfarin-d-10-camphorsulfonate (5).--d-10-Camphorsulfonyl chloride (ref. 4, 2.07 g, 8.29 mmol, mp 57-68.5°C) and R,S-3'-bromowarfarin (1.41 g, 3.63 mmol) were dissolved in 7 mL of dry pyridine and set at 10°C for 48 hr (cf. refs. 5 and 6). Pyridine was evaporated under N_2; the resulting

partially crystalline residue was dissolved in 120 mL methanol/ethyl acetate/benzene (1.4/1/6) and quickly chromatographed on a column (2.8 x 20 cm) of 60 g of silica gel 60 [35-70 mesh, ~40 mL/min, ethyl acetate/benzene (1/7)]. The first 800 mL contained the desired diastereoisomers 5. Solvent evaporation afforded a light yellow oil that was dissolved in 20 mL of benzene, divided and chromatographed on two size C silica gel 60 prepacked columns. (Rapid handling of the oily residue was necessary as the diastereoisomeric mixture tends to crystallize). A gradient of 2 L of benzene to 2 L of ethyl acetate/benzene (1/9) eluted the less polar diastereoisomer (S-enantiomer), typically in the 2650-3090 mL fraction, and the more polar diastereoisomer (R-enantiomer) in the 3230-3750 mL fraction. Each diastereoisomer was crystallized three times from hot acetone/hexanes: Less polar (5c, S-enantiomer): 235 mg; $[\alpha]_D^{23}$ -129.7° (c 1.8, $CHCl_3$); mp 122.5-30.5°. More polar (5b, R-enantiomer): 456 mg; $[\alpha]_D^{23}$ +167.4° (c 1.8, $CHCl_3$); mp 140-45.5°.

R- and S-3'-Bromowarfarin (3b and 3c).--The bromowarfarin camphorsulfonate of negative $[\alpha]_D$ (5c, 200 mg, 0.33 mmol) was dissolved in 1.25 mL of dioxane. Sodium hydroxide (0.652 mL, 5% solution) was added and the reaction stirred at room temperature for 24 hours (cf. refs. 5 and 6). The reaction mixture was then adjusted to a pH of 3 with 0.1 N HCl and the resulting white precipitate extracted into ethyl acetate. The residue resulting from drying and evaporation of the ethyl acetate was chromatographed on a 2.2 x 10.5 cm (20 g) column of silica gel 60 (35-70 mesh). Ethyl acetate eluted S-bromowarfarin in the first 100 mL fraction.

Three crystallizations from acetone-hexanes yielded 27 mg (21%) of pure S-enantiomer 3c: mp 170-73.5°C; $[\alpha]_D^{23}$ -148.3° (c 1.2, 0.5 N NaOH); mass spectrum (70 eV) m/e (rel intensity) 388 (34, M^+), 386 (34), 345 (96), 343 (100).

Anal. Calcd. for $C_{19}H_{15}BrO_4$: C, 58.93; H, 3.90. Found: C, 58.88; H, 3.99.

The more polar 3'-bromowarfarin diastereoisomer 5b (400 mg, 0.67 mmol) in a similar manner yielded 36 mg (14%) of pure R-enantiomer 3b: mp 168-73.5°C; $[\alpha]_D^{23}$ +145.8 (c 1.2, 0.5 N NaOH).

Anal. Found: C, 59.03; H, 3.93.

Catalytic reduction of a sample of crude S-bromowarfarin 5c [5% Pd/C, triethylamine/dioxane (1/9)] followed by purification of the product by high pressure

liquid chromatograpy on Partisil-10 (see below) yielded S-warfarin: $[\alpha]_D^{25}$ -143.3° (c 1.2, 0.5 N NaOH) [lit (6) $[\alpha]_D^{25}$ -147°].

R- and S-Warfarin-3'-^{3}H (4b and 4c).--S-3'-Bromowarfarin (3c, 10 mg, 0.026 mmol) was reduced using a modified Glascock microhydrogenation apparatus at room temperature and pressure with 5 Ci of $^3H_2$ in 1.75 mL of triethylamine/dioxane (1/9) over 6 mg of 5% Pd/C catalyst ($^3H_2$ uptake was 0.028 mmol, theoretical = 0.026 mmol). After filtration of the solution through celite to remove the catalyst, the radioactive product was purified by continuous horizontal chromatography for 16 hr on a 20 x 20 x 0.025 cm silica gel 60 F-254 plate [ethyl acetate/benzene (2/98)]. Radioscanning indicated one peak of R_f equivalent to warfarin. The specific activity (uv analysis) was 29.3 Ci/mmole. High performance liquid chromatography (hplc) of this product (inseparable in various tlc systems) on silica [Partisil-10; 250 x 9.4 mm column; uv detector, 254 nm; ethyl acetate/heptane/methanol (1/39.2/9.8), 9.9 mL/min; 25-50 µg injection in chloroform; retention time of warfarin = 7.82 min; retention time of bromo analog = 9.55 min] indicated 14% of unreacted 3'-bromowarfarin. Reduction of this mixture with H_2 gas yielded pure S-(-)-warfarin-3'-^{3}H (134 mCi, 21% chemical yield, 18% radiochemical yield) of specific activity 24.9 Ci/mmol. R-(+)-warfarin-3'-^{3}H, similarly prepared (332 mCi, 41% chemical yield, 44% radiochemical yield), had a specific activity of 31.2 Ci/mmol. The labeled compounds were stored in benzene at 5°C at concentrations of 1-3 mCi/mL. Little decomposition occurred over a period of a year under these conditions.

Determination of Optical Purity of S-Warfarin-3'-^{3}H (4c).--Compound 4c was added to 203 mg of R,S-warfarin dissolved in chloroform/methanol. Solvents were evaporated under N_2 to yield a white crystalline residue of specific activity 11.0 µCi/mmol. The d-10-camphorsulfonates were synthesized and separated as described above. The less polar diastereoisomer [S-(-)-enantiomer] exhibited a specific activity of 20.9 µCi/mmol; the more polar diastereoisomer 0.2 µCi/mmol.

Table 1

R_f Values of Compounds[a]

Compound	Solvent[b]	R_f
2	A	0.44
3	A	0.15
	B	0.59
4	A	0.15
5b	A	0.24
5c	A	0.29

[a]Silica gel 60 F-254

[b]Solvent A = benzene/ethyl acetate (7/1, v/v)
Solvent B = benzene/acetone (4/1, v/v)

RESULTS AND DISCUSSION

For synthesis of the required warfarin enantiomers it appeared to us most efficient to prepare enantiomeric precursors which could be converted, preferably by reduction with tritium gas, to the desired compounds. 3'-Bromowarfarin appeared to be a suitable precursor, as replacement of the bromine by tritium would yield a compound with a stable tritium atom under the conditions of immunoassay. Furthermore, in view of the lack of reported metabolism at this position, the 3'-tritiowarfarin compounds might be useful for other pharmacological studies. The required 3'-bromowarfarin was readily synthesized by conversion of 3-bromobenzaldehyde to the bromophenylbutenone 2 by reaction with acetone and sodium hydroxide. A large proportion of the reaction product was obtained in the form of the aldol condensation product which could be converted to the desired enone by acid catalyzed dehydration. Reaction of the enone 2 with 4-hydroxycoumarin by a standard procedure yielded 3'-bromowarfarin (3a) which was characterized by its pmr spectrum and by analysis.

Although warfarin may be resolved by the use of alkaloid salts (5, 6), this is inconvenient for the preparation of analog enantiomers on a small scale. We therefore chose to convert the bromowarfarin to its 4-(d-10-camphorsulfonyloxy) derivative 5. Kloss (5) and Preis (6) had earlier reported that warfarin could be converted to the d-camphorsulfonate by use of the sulfonyl chloride in pyridine. Although separation of the resulting diastereoisomers by crystallization is

difficult, we have found that they may be readily separated by liquid chromatography and have used this for the preparation of warfarin of high optical purity (submitted for publication). This technique also worked well in the case of the bromo analog, and after chromatography, the resulting individual diastereoisomers had 99% or greater optical purity (readily determined by thin layer or high pressure liquid chromatography). Basic hydrolysis of the camphorsulfonates yielded the pure bromowarfarin enantiomers. The optical rotations observed for these compounds were very similar to those of warfarin itself, and this permitted a tentative assignment of stereochemistry. Confirmation was obtained by catalytic reduction of the isomer of negative rotation to yield S-warfarin.

Reduction of the bromo compounds with tritium gas in the presence of palladium on charcoal catalyst led to the formation of the desired tritium labeled enantiomers. As the asymmetric center is benzylic, there was some concern as to whether it might undergo racemization under the conditions of the catalytic reduction. Therefore, the optical purity of the S-enantiomer was checked by addition to excess R,S-warfarin, formation of the d-10-camphorsulfonates, their separation by chromatography, and determination of specific activity. When this was done, the sulfonate of the R-enantiomer contained only 1% of the radioactivity found in the sulfonate of the S-enantiomer. Thus, little or no racemization occurred under the conditions of catalytic reduction.

The above procedure thus constitutes a useful method for the synthesis of the tritium labeled enantiomers of warfarin. Emphasis throughout was placed upon purity of the products rather than yields, and the yields could be considerably increased by reworking mother liquors and chromatography fractions. The resulting compounds have been used to develop stereoselective radioimmunoassays for the enantiomers of warfarin after administration of the racemic drug (7).

ACKNOWLEGEMENTS

This work was supported by Grant No. 5-R01-GM23197 with the Pharmacology-Toxicology Program, National Institute of General Medical Sciences, NIH. We thank Fred Williams and Dr. D. Rosenthal (Research Triangle Center for Mass Spectrometry supported by the Biotechnology Resources Branch, Division of Research Resources, National Institutes of Health, Grant No. PR-330) for mass spectrometry.

REFERENCES

1. Yacobi, A. and Levy, G. - J. Pharmacokin. and Biopharm. 2: 239 (1974).
2. Breckenridge, A., Orme, M., Wesseling, H., Lewis, R. J. and Gibbons, R. -Clin. Pharmacol. Therap. 15:424 (1974).
3. Drake, N. L. and Allan, P. - "Organic Synthesis," Coll. Vol. I (2nd Edition), Blatt, A. H., Editor, John Wiley and Sons, Inc., 1958, pp. 77-78.
4. Smiles, S. and Hilditch, T. P. - J. Chem. Soc. 91: 519 (1907).
5. Kloss, R. - Ph.D. Thesis, University of Wisconsin, 1956.
6. Preis, S. - Ph.D. Thesis, University of Wisconsin, 1957.
7. Cook, C. E., Ballentine, N. H., Seltzman, T. B. and Tallent, C. R. - Pharmacologist 19 (2): Abstract 6 (1977).