J Comput Aided Mol Des (2010) 24:553–573
DOI 10.1007/s10822-010-9335-7

Warfarin: history, tautomerism and activity

William R. Porter

Received: 3 March 2010 / Accepted: 13 March 2010 / Published online: 30 March 2010

Abstract The anticoagulant drug warfarin, normally administered as the racemate, can exist in solution in potentially as many as 40 topologically distinct tautomeric forms. Only 11 of these forms for each enantiomer can be distinguished by selected computational software commonly used to estimate octanol–water partition coefficients and/or ionization constants. The history of studies on warfarin tautomerism is reviewed, along with the implications of tautomerism to its biological properties (activity, protein binding and metabolism) and chemical properties (log P, log D, pK_a). Experimental approaches to assessing warfarin tautomerism and computational results for different tautomeric forms are presented.

Keywords Tautomers · Warfarin · Keto-enol tautomerism · Ring-chain tautomerism

Introduction

In 2007, warfarin was the most frequently prescribed oral anticoagulant drug and the overall sixteenth most prescribed drug in the United States from a single manufacturer (as the generic form marketed by Teva Pharmaceutical Industries Ltd., Petach Tikva, Israel) [1]. 3-(α-Phenyl-β-acetylethyl)-4-hydroxycoumarin, as it was called by its discoverer, Miyoshi Ikawa [2], working in the laboratory of Professor Karl Paul Link at the University of Wisconsin during the early 1940's, was subsequently shown to be orally active as an anticoagulant by bioassay in rabbits that were administered the compound neat in a gelatin capsule [3]. The bioassay study described the anticoagulant properties (or lack thereof) for 106 different compounds. The substance later known as warfarin was #42 on this list of 106 compounds, and for many years it was simply referred to as Compound 42, shown in Fig. 1. Compound 42 had a "relative anticoagulant index" of 20 when 5 mg doses were administered to rabbits weighing ~2.5 kg. The "relative anticoagulant index" was defined to be the increase in prothrombin time (in seconds, a measure of ability of blood to coagulate) multiplied by a scaling factor and then by the millimolar amount of test compound administered. The scaling factor was set so that the "relative anticoagulant index" would be equal to 100 when an administered dose of 0.75 mg of the reference compound 3,3′-methylenebis(4-hydroxycoumarin), later named dicumarol (USAN) or dicoumarol (INN). The authors [3] noted that compound 42, and some structurally related congeners, had a more prolonged effect than the reference compound. Maximum anticoagulant activity was observed 3 days after administration, whereas the activity of the reference compound peaked after 2 days. Also tested in the same screening experiment was the methyl ether of Compound 42, identified as 3-(α-phenyl-β-acetylethyl)-4-methoxycoumarin, Compound 62. This derivative of Compound 42 did have some anticoagulant activity, but its "relative anticoagulant index" was only 3.8 when dosed at 50 mg per 2.5 kg rabbit. Curiously, an isomeric compound, 2-methyl-2-methoxy-4-phenyl-5-oxodihydropyrano-(3,2-c)(1)benzopyran, Compound 63, was even more effective than Compound 42, with a "relative anticoagulant index" of 60 when dosed at only 5 mg per 2.5 kg rabbit. Compounds 62 and 63 are methyl ethers prepared by chemically modifying different tautomeric forms of

W. R. Porter (✉)
Global Pharmaceutics and Life Cycle Technology, Dept. R43D, Bldg. AP31, Abbott Laboratories, 200 Abbott Park Road, Abbott Park, IL 60064, USA
e-mail: william.r.porter@abbott.com

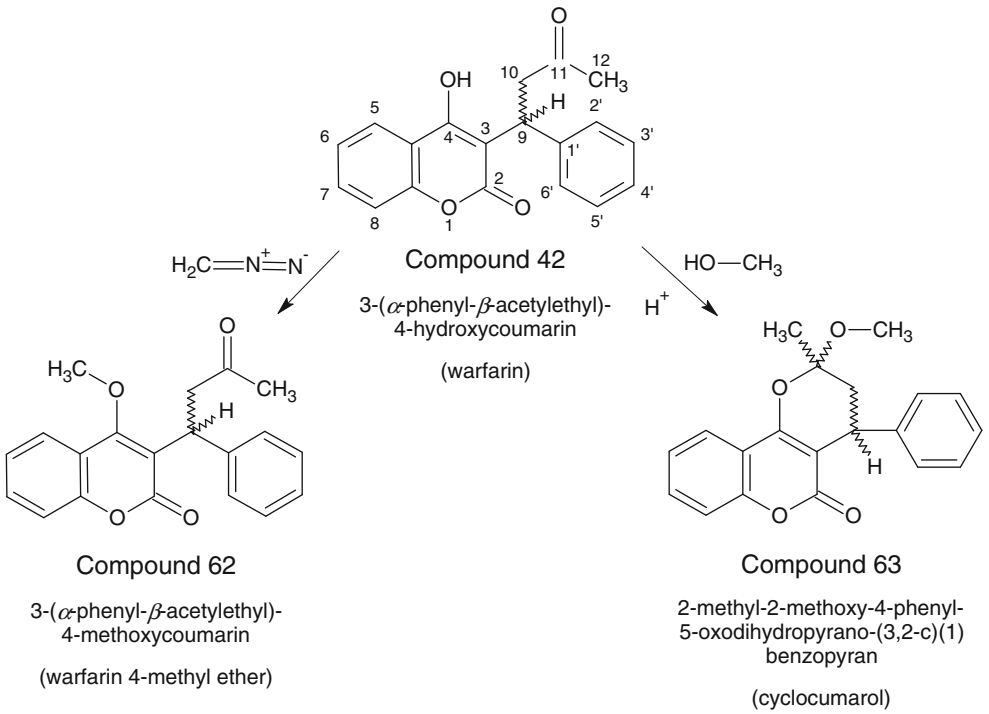


Fig. 1 Compound 42 and its methyl ethers, Compound 62 and Compound 63

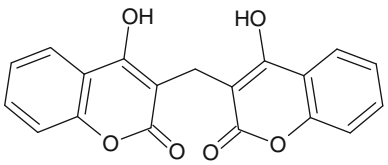


Fig. 2 Dicumarol (USAN) or dicoumarol [INN], the first coumarin oral anticoagulant drug

Compound 42 (Fig. 1). Compound 42 as first prepared was synthesized as a racemate, and was very difficult to purify. The yield was poor, only 40%, but Ikawa was finally able to make as much as 50 grams with m.p. 161 °C. Compound 62 with m.p. 127 °C was obtained by treatment of Compound 42 with diazomethane in ether (Fig. 1). Diazomethane preferentially methylates the enantiomers of the tautomeric form of warfarin identified as Tautomer A later in this report (Fig. 12). Compound 63 was formed in 83% yield by refluxing 10 g of Compound 42 in absolute methanol containing 4% HCl for only 15 min (Fig. 1). The material isolated was eventually demonstrated to be a racemic mixture of methyl cyclic ketals formed by methylation of the tautomeric form of warfarin identified as Tautomer J later in this report (Fig. 21).

What prompted these studies? In the early 1920s, there was an outbreak of a bizarre bleeding disorder in cattle in the northern United States and Canada; cattle were hemorrhaging and bleeding to death after minor surgical procedures, such as dehorning, and sometimes with no apparent cause [4, 5]. As the story goes [6–8], the summer of 1933 was hotter and wetter than usual in Wisconsin, and farmers were unable to properly dry their hay. Farmer Ed Carlson, from Deer Park, Wisconsin, had put up some slightly damp sweet clover hay in his silo, and as winter set in, he began feeding it to his cows. They began hemorrhaging shortly after starting their new feed. So, farmer Carlson packed up a dead heifer, a bucket of blood, and a pile of sweet clover hay in his truck and drove to Madison, Wisconsin where he visited Professor Karl Paul Link in his laboratory at the Department of Biochemistry, Wisconsin Agricultural Experiment Station, University of Wisconsin. (In some retellings, farmer Carlson drove to Madison in a raging blizzard, but this detail is lacking from earlier versions of this tale [6].) Studies on the sweet clover malady were already well underway in Madison at this time, but scientists had thought that the problem lay in the source of the hay, and were trying selective breeding experiments to develop a strain of sweet clover that would not cause hemorrhaging in livestock. Some years later, Link's students finally isolated the active hemorrhagic agent [9], which turned out to be produced by microbial fermentation during spoilage of the hay. This substance was later characterized as 3,3′-methylenebis(4-hydroxycoumarin) (Fig. 2) [10]; it was tested and found useful as a drug and was marketed for many years. Clinical studies began at Wisconsin immediately after its discovery [11].

After the discovery of dicumarol, Link's students began an extensive investigation of analogs, resulting in the testing of the 106 compounds noted previously. Compound 42, due to its longer duration of action, was later developed as a rodenticide. It was named warfarin (*WARF* + -arin, from coumarin) in honor of the Wisconsin Alumni Research Foundation, to which Professor Link signed over his patent rights in gratitude for the financial support he had received to fund the work leading to the discovery of dicumarol and warfarin. The use of warfarin as a rodenticide may stem from the observation that both it and Compound 63 (later named cyclocumarol) were much more toxic to mice and rats than to rabbits or dogs on a weight basis. The median lethal dose of Compound 42 in rats was found to be between 0.125 and 0.25 mg/kg/day, while that for Compound 63 was found to be between 0.35 and 0.5 mg/kg/day. The animals died with extensive lung and urinary damage, with some showing paralysis of the hind quarter before death. Intestinal and subcutaneous hemorrhage, noted in dicumarol poisoning (median lethal dose $\sim$5 mg/kg/day in rats) was not as prominent in rats poisoned with Compounds 42 and 63 [12].

Warfarin was later developed as a drug. Interestingly, Compound 63 (cyclocumarol) was used clinically first, beginning in May, 1949 [11]. Clinical testing of warfarin for anticoagulant therapy did not begin in the United States until 1953 [11], although by that time it had become popular as a rodenticide, after it was registered for use as such in 1948 [8]. The use of warfarin skyrocketed, eventually replacing dicumarol, after it was used to successfully treat President Eisenhower when he suffered his heart attack in 1955 [8]. Eisenhower may not have been the first head of state to receive warfarin; recent evidence suggests that warfarin, or a related compound, may have been used to poison Soviet leader Joseph Stalin in 1953 [13].

Problems with the isolation and crystallization of warfarin persisted, however. Lester Scheel [12] was able to increase the yield on a molar scale synthesis to 55% by first adding alkali to pH 12, then slowly adding dilute HCl to pH 6.0, followed by recrystallization from dioxane/water. Scheel made no speculations as to why this procedure worked. Work by Scheel and Dorothy Wu demonstrated that Compound 42 had more rapid onset and was about 50 times more potent as a rodenticide than dicumarol [14]. A latter Link student, Martin Seidman, seemed to be on the right track, however. Seidman experimented with catalysts and found that he could increase the yield to 67% by using dioxane as the reaction medium for the Michael condensation used to produce warfarin from benzalacetone and 4-hydroxycoumarin (Fig. 3) with piperidine as the catalyst [15]. In every case, Seidman isolated the product by dumping it into ice water, then collecting the gummy solid that precipitated, which he then recrystallized from acetone/water. Seidman was apparently the first to note (or at least describe on paper) that Compound 42 is a delta hydroxyl ketone, and can therefore exhibit ring-chain tautomerism (Fig. 4). Seidman devoted the second half of his thesis work to studies on the oxidation of sugar mercaptals; since sugars were well-known at that time to exhibit ring-chain tautomerism we can speculate that Seidman's experience with carbohydrate chemistry may have inspired him to recognize the potential for ring-chain tautomerism in the chemistry of warfarin. Seidman went onto synthesize numerous cyclic ketals of Compound 42, using alcohols of varying size and complexity. He as actually able to separate, by fractional crystallization, both the (R,R)/(S,S) and (R/S)/(S,R) diastereomeric racemic ketals of Compound 42 with ethylene chlorohydrin (major product m.p. 189–191 °C, minor product m.p. 132.5–134 °C) and with 2-ethoxy ethanol (major product m.p. 102–103 °C, minor product m.p. 137–138 °C). Despite his efforts to use the then newly emerging technique of infrared spectroscopy to compare 4-hydroxycoumarin with Compound 42 and with their respective methyl ethers and acetate esters, Seidman was unable to clearly demonstrate which form, the open chain tautomer or the ring tautomer, was present in solid Compound 42. It would take more than 20 years for this question to be answered; it awaited the solution of the single crystal structures of racemic warfarin methanol solvate in 1973 [16] and *S*-(−)-warfarin in 1975 [17]. The form of warfarin that Ikawa, Scheel and Seidman prepared is the ring hemiketal tautomeric form of warfarin identified as Tautomer J later in this report (Fig. 21) in which the phenyl and hydroxyl groups are trans to each other and axial while the benzylic hydrogen and the methyl substituent occupy equatorial positions on the dihydropyran ring [18], a fact which was not confirmed by crystallography until 2003! In searching the literature, there is only one publication that describes a form of solid warfarin that does NOT match the form described originally by Link's students, and this was prepared essentially by the same method as that used by Scheel, but without recrystallization using an organic solvent [19]. These workers describe a lower melting form (m.p. 152–154 °C) with two strong carbonyl stretching vibrations in its infrared spectrum (at 1,707 and 1,682 cm^{-1}); the actual structure of this new solid form has not been confirmed, but the infrared spectrum is consistent with it being the tautomeric form of warfarin identified as Tautomer A later in this report (Fig. 12). The form of warfarin isolated by Ikawa [2], Scheel [12] and Seidman [15], with m.p. 160–161 °C, is the cyclic hemiketal tautomer (phenyl and hydroxyl *trans* form of Tautomer J, Fig. 21) described above with a single strong carbonyl stretching vibration at 1,681 cm^{-1} [20].

Seidman prepared solid sodium warfarin, and it is this water-soluble form that was later developed commercially.

Fig. 3 Synthesis of warfarin

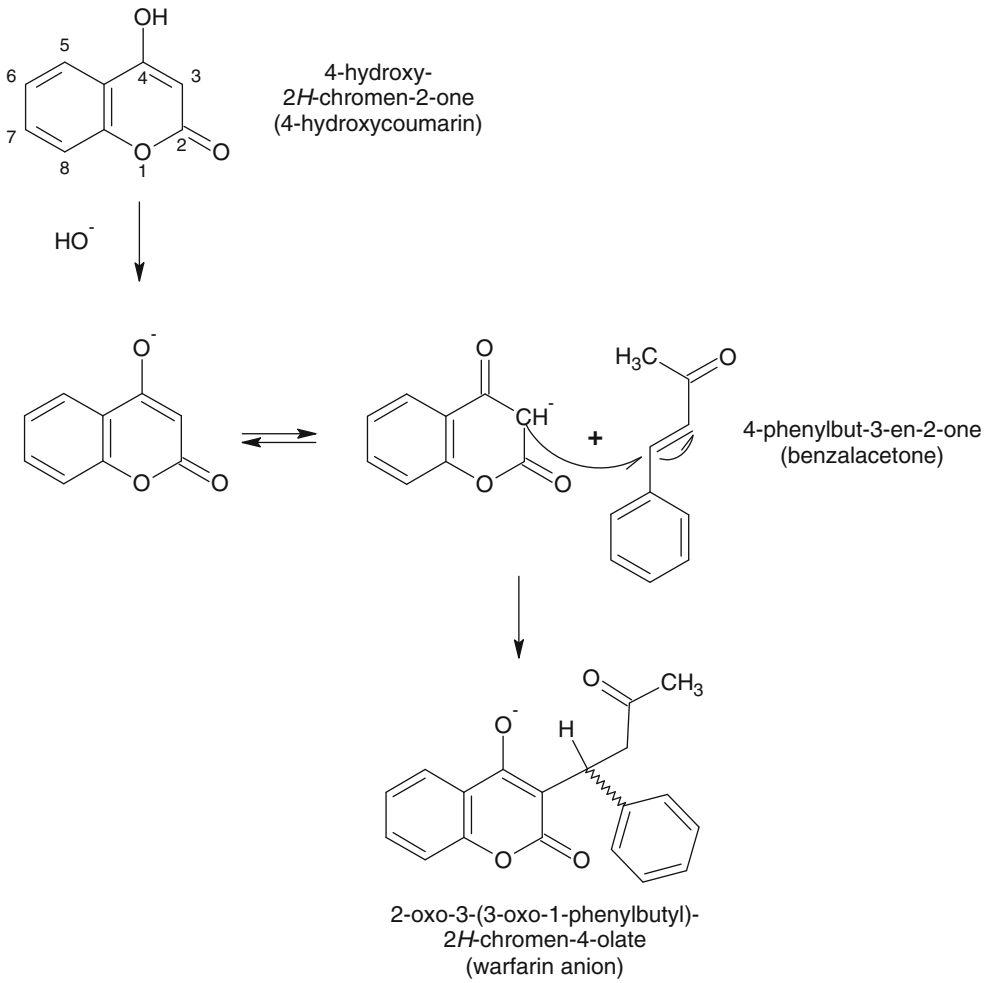


Fig. 4 Open chain and ring tautomeric forms of warfarin as envisioned by Seidman [15]

However, Seidman's preparation (using sodium ethoxide in absolute ethanol) did not produce material of very good quality. Work continued for some years in Professor Link's laboratory to devise a suitable process. A process for making *solutions* of sodium warfarin in water was eventually developed [21]; these could be concentrated and eventually dried to produce an amorphous material. The procedure required very careful pH control, as warfarin tended to degrade in highly alkaline media. It was not until the early 1960s that Link's lab developed the process for making a stable, crystalline form of sodium warfarin, which was described as a clathrate with isopropanol [22]. This material was described as having variable composition, in which the ratio of sodium warfarin to isopropanol and water may vary from 8:4:0 to 8:2:2 [23]. Only recently has it been shown that this material is really just a very hygroscopic solvate of sodium warfarin, with one *R*-(+)-warfarin open chain 4-hydroxycoumarin anion, one *S*-(−)-warfarin open chain 4-hydroxycoumarin anion, two sodium ions and one molecule of isopropanol in each asymmetric unit; the unit cell contains four such asymmetric units [24, 25]. The sodium ions are in close proximity to the oxygen atoms in the 4-position of the coumarin rings. So, it has been firmly established by single crystal X-ray crystallography that

Seidman's structures for the two predominant tautomeric forms of warfarin were indeed correct.

It took 52 years for confirmation by experimental measurements that Seidman's structural assignments were correct. Over this period of time, chemical methods demonstrated that multiple tautomeric forms of warfarin could exist in solution or as different solid forms, in some cases only in small amounts, as evidenced by reaction products that could only arise from particular tautomeric forms as intermediates. Likewise, the physical properties of warfarin could only be understood by accounting for the interconversion of different tautomeric forms. Computational chemists seeking to simulate or predict the chemical and physical properties of a substance exhibiting the level of tautomeric complexity such as warfarin must take consider all of the possible tautomers that could exist and then undertake to evaluate to what extent each tautomeric form may contribute to chemical reactivity and physical properties.

Determining tautomeric equilibria

To calculate properties of a molecule, it is evident that its structure must be known. With molecules that can exist in multiple tautomeric forms, the question rapidly becomes, which structure?

As Seidman noted, warfarin is a delta hydroxyl ketone. That is, it is a delta hydroxyl ketone if the core aromatic portion of the molecule shared in common with dicumarol and similar anticoagulants contains a hydroxyl group (Fig. 5). 4-Hydroxycoumarin is just one of two enol tautomeric forms of 2,4-chromandione, a β-keto lactone. The tautomerism of 4-hydroxycoumarins has been studied extensively. Karl Paul Link's students took special interest in this problem [26]. Other investigators used chemical reactivity [27–30], ultraviolet spectroscopy [29, 31–36], infrared spectroscopy [29, 30, 34, 37–40] and proton nuclear magnetic resonance spectroscopy [40] to probe the 4-hydroxycoumarin-2,4-chromadione-2-hydroxychrome equilibrium. X-ray crystallographic studies confirmed in every case studied up through the early 1970s that, at least in the solid state, the 4-hydroxycoumarin form predominates, based on the bond lengths in the pyranone ring [16, 17, 41–47]. However, some authors have claimed that the 2-hydroxychromone tautomer may exist, at least to some extent, in solution [27, 29, 30,–31, 34, 35, 37, 38].

Chemical methods have proven to be entirely unsatisfactory for estimation of the tautomeric composition of 4-hydroxycoumarin derivatives in solution. The kinetics of tautomeric interconversion and chemical derivatization is complex. While the relative rates of derivatization and proton transfer between tautomeric forms and the solvent affect the outcome, inherent in these methods is that the reagent used to derivatize the individual tautomeric forms must react with each tautomeric form faster than a proton can be transferred between tautomeric forms in solution [48, 49], and solvent mediated proton transfer is very fast, indeed. In the case of 4-hydroxycoumarin itself, acid-catalyzed alkylation can yield either *C*-alkylated products (corresponding to alkylation of the chromandione tautomeric form) [50, 51] or either of two *O*-alkylated (coumarin or chromone) products [27, 29, 30, 52].

Ultraviolet spectroscopic studies failed to distinguish between the possible tautomeric forms [29, 31–36, 53–57]. Two model methyl ethers, 4-methoxycoumarin and 2-methoxychromone, both have UV spectra that are blue-shifted relative to the spectra of typical 3-subsituted coumarin anticoagulants. Although the UV spectra of the anticoagulant drugs tested were, on the whole, more similar in appearance to the UV spectrum of 4-methoxycoumarin than that of 2-methoxychromone, it is difficult to draw any quantitative conclusions based on the spectra of these two model compounds.

Proton magnetic resonance spectroscopy is only somewhat helpful. The hydrogen in the 3-position of pmr spectrum of 4-hydroxycoumarin is readily exchangeable with deuterium in deuterium oxide as solvent [51, 58], demonstrating that the chromandione tautomeric form can at least exist to a small extent in solution, but only a single vinyl proton resonance is observed, with no hint of a methylene tautomeric form. Tautomeric interconversion must therefore be rapid on the pmr timescale. In the case of warfarin, all of the side-chain methylene and methyl protons are completely exchanged with deuterium when a solution of warfarin is prepared in deuterium oxide at pD = 7.45, indicating that under these conditions warfarin

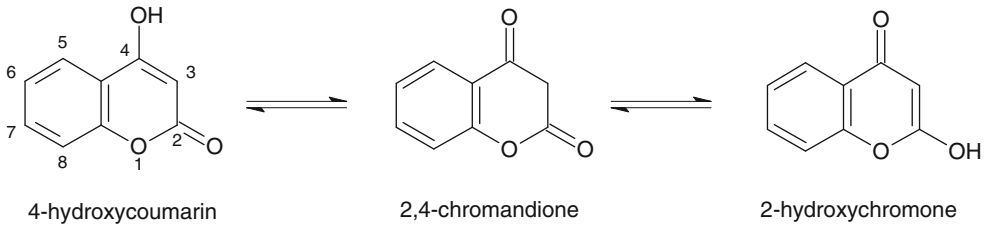


Fig. 5 Tautomerism of 4-hydroxycoumarin

exists at least partially as an open chain tautomeric form with a side chain enolizable ketone moiety with enolic hydrogens on either side [59].

Molecular orbital calculations have been used to predict both UV and pmr spectra [60–65]. Calculations assuming the 4-hydroxycoumarin tautomeric structure have generally been more successful at predicting experimentally observed spectra than calculations assuming the 2-hydroxychromone tautomeric form, but the agreement between theoretical and experimental spectra was only suggestive, not conclusive.

The only truly successful instrumental technique that has been applied to this problem has been infrared spectroscopy, as foreseen by Seidman back in 1950 [15]. This technique has the advantage that it can be used both to study crystalline and amorphous solids as well as the same materials dissolved in a wide variety of solvents. Furthermore, distinctions between coumarin and chromone forms can be easily made [34, 37, 39, 40, 61, 66]. Yet, by the mid 1970s, there was still controversy in the literature over the correct assignment of the carbonyl stretching vibrations of 4-hydroxycoumarin and 2-hydroxychromone. Working in the laboratory of William Trager, and later as an independent collaborator, this author set out to end the confusion by synthesizing 4-hydroxycoumarin derivatives specifically labeled with ^{13}C in the 2-position [67–69]. A band ranging from 1,664 to 1,718 cm^{-1} was identified as the C = O stretching vibration for the carbonyl group in position 2 of the coumarin ring for crystalline solid 4-hydroxycoumarin derivatives that had been confirmed to have this structure by X-ray crystallography [69]. The highest frequency band in the 1,550–1,750 cm^{-1} region of the spectra of these same analogs in solution also is shifted when labeled with ^{13}C in the 2-position of the coumarin ring. The position of this band is consistent within the group of 4-hydroxy and 4-alkoxy coumarin derivatives studied; it is also consistent with the observed carbonyl stretching vibration frequency for 4-methoxy coumarin, but not 2-methoxy chromone [68]. However, the 3-deutero and 2-^{13}C-labeled anions obtained from 4-hydroxycoumarin by the addition of alkali have infrared spectra suggesting that the charge on the anion is delocalized [69].

Having in hand specifically labeled 2-^{13}C-4-hydroxycoumarin analogs, it was then possible to unequivocally assign the ^{13}C resonances for the C2, C3, and C4 carbons in the ^{13}C magnetic resonance spectra of these compounds [70–74]. Warfarin and its 4′-hydroxy, 6-hydroxy, 7-hydroxy and 8-hydroxy metabolites all exist in DMSO-d_6 solution as a mixture of the open chain 4-hydroxy coumarin tautomer (9–13%, Fig. 4 left and Fig. 12), the (*R,R*)-/(*S,S*)—[that is, methyl and phenyl *trans* and axial] coumarin hemiketal ring tautomer (61–68%, Fig. 4 right and Fig. 21 left) and the (*R,S*)-/(*S,R*)—[that is, methyl and phenyl *cis* and equatorial] coumarin hemiketal ring tautomer (23–28%, Fig. 4 right and Fig. 21 right). Only 5-hydroxywarfarin (not known to be a warfarin metabolite) exists in solution solely in the open chain 4-hydroxycoumarin tautomeric form in this solvent. In $CDCl_3$ solution, warfarin exists in the same three tautomeric forms, but the open chain form comprises ~15% of the total, while the *trans* hemiketal accounts for only 45% and the *cis* hemiketal makes up 40% of the total [71]. In $CDCl_3$ solution, unlike in DMSO-d_6 solution, 5-hydroxywarfarin is only 50% in the open chain tautomeric form, while 22% is in the cyclic *trans* hemiketal and 28% is in the cyclic *cis* hemiketal tautomeric form. The tautomeric equilibrium is solvent-dependent. Not only is the ratio of open-chain to ring forms dependent on solvent polarity, but the less polar solvent $CDCl_3$ tends to favor equilibration to form proportionally higher amounts of the *cis* hemiketal tautomer. Circular dichroism studies of the enantiomers of warfarin and phenprocoumon (3-(1-phenylpropyl)-4-hydroxycoumarin, Fig. 9 top, a warfarin analog lacking the side-chain carbonyl moiety, and thus incapable of forming a ring tautomer) demonstrated that warfarin exists predominantly in an open chain tautomeric form at physiological pH in water (where it is mostly present as its anion) [59], but predominately in the ring hemiketal forms in *n*-octanol [75]. Warfarin also exists predominantly in ring hemiketal forms in water at low pH, where it is not ionized [59]. The ionization state of warfarin is a critical factor in establishing the predominant tautomeric forms present in aqueous solution as a function of pH. In studies of 4-hydroxycoumarin itself, Andrew Obaseki observed [73] that the ^{13}Cmr spectrum of 4-hydroxycoumarin and its methyl and ethyl ethers in DMSO-d_6 showed that the resonance of C4 was ~166–167 ppm downfield from Me_4Si, whereas the resonance of C2 was only 162–163 ppm downfield. The ^{13}Cmr spectra of 2-methoxychromone and 2-ethoxychromone differed inasmuch as the chemical shifts for C2 and C4 were shifted to ~168 and 179 ppm downfield. Interestingly, the ^{13}Cmr spectrum of 4-hydroxycoumarin anion had chemical shifts for C2 and C4 intermediate between those for 4-hydroxycoumarin and its alkyl ethers and for the 2-alkoxychromone, with chemical shifts of 166 and 176 ppm. Titration of 4-hydroxycoumarin in a mixture of 30% DMSO-d_6 and 70% D_2O by addition of NaOD showed that the ^{13}Cmr chemical shift of C4 changes smoothly during titration from 173 to 193 ppm, while the chemical shift of C2 changes smoothly during titration from 166 to 184 ppm in this cosolvent mixture [73]. These results are consistent with delocalization of charge in the anion. It would be interesting to perform a similar study using warfarin itself, but this has not yet been accomplished to this author's knowledge.

So, a clear pathway can be established, linking solid state and solution studies of tautomeric equilibrium: Use single crystal X-ray diffraction to establish the crystal and molecular structure of the material suspected to tautomerize in solution. Identify carbon atoms in the molecule that are bonded to the heteroatom responsible for tautomerization and replace them one at a time as needed with ^{13}C. Use the isotopic shift in infrared absorbance bands introduced by inserting the specific label to identify characteristic absorbance bands of potential tautomeric species in solution in appropriate polar and nonpolar infrared-transparent solvents. Confirm, using ^{13}C magnetic resonance spectroscopy, the proportion of different tautomeric forms in solution, bearing in mind that the tautomeric equilibrium is likely to be affected by the polarity and hydrogen bond donating or accepting properties of the solvent.

Biological implications

Warfarin is commonly cited as an example for drug interactions, both with other drugs and with environmental and dietary factors [76]. Not only are both enantiomers of warfarin strongly bound to serum albumin, each enantiomer is extensively metabolized in the liver, both by various cytochrome P-450 isoenzymes (with different susceptibility for different isoforms of cytochrome P-450 for each enantiomer) as well as by other hepatic enzyme systems. Finally, both enantiomers act with similar effectiveness at the biological target for inhibition of coagulation, an enzyme required for recycling an essential cofactor in the post-translational modification of proenzymes participating in the complex enzymatic cascade responsible for blood clotting. Because of these various factors, adequately titrating patients with warfarin and maintaining an appropriate level of anticoagulation is complex, and requires constant monitoring and adjustment.

Does tautomerism play a role in the biological fate of warfarin?

Warfarin and vitamin K epoxide reductase

The clotting of blood is a complex process operating under tight enzymatic control to produce hemostasis [77]. Many of the enzymes involved in the clotting cascade are synthesized as proenzymes, lacking critical calcium ion binding sites. Conversion of these proenzymes to their active forms requires post-translational modification by a membrane-bound enzyme located in the cellular rough endoplasmic reticulum that functions in an oxidative process requiring molecular oxygen to add carbon dioxide to glutamic acid side chains at critical locations in the proenzymes into γ-carboxyglutamic acid moieties. The reaction can proceed only in the presence of adequate levels of vitamin K hydroquinone, an essential cofactor. During the process of converting each glutamic acid moiety to a γ-carboxyglutamic acid moiety, one molecule of vitamin K hydroquinone is consumed and converted to vitamin K 2,3-epoxide (Fig. 6) [78]. This epoxide form of vitamin K must be reduced, in a two-step reaction, to vitamin K quinone and thence to vitamin K hydroquinone before the cycle can continue. The enzyme responsible for these two reductions is vitamin K epoxide reductase (VKOR) [79]. This enzyme is inhibited by 4-hydroxycoumarin anticoagulants, like dicumarol and warfarin, and by structurally similar compounds [80].

VKOR seems equally susceptible to inhibition by both enantiomers of warfarin [81], and each enantiomers competes effectively with the other enantiomer for binding to the oxidized form of VKOR [81, 82], although when sufficiently high levels of reducing agents such as dithiothreitol are provided in vitro the *S*-enantiomer of warfarin is released more quickly than the *R*-enantiomer. Studies with other inhibitors confirm, for example, that salicylic acid, which structurally mimics to some extent the 4-hydroxycoumarin portion of the warfarin molecule, can inhibit VKOR [83]. Thus, questions about the tautomeric form of warfarin that binds to its target, VKOR, seem moot. However, warfarin analogs that either cannot ionize at all (e.g., by replacing the 4-hydroxy group with a chloro moiety) or that are weaker acids (e.g., by replacing the 4-hydroxy group with a 4-thio group) are ineffective as inhibitors of VKOR [84], so this evidence suggest that 4-hydroxy coumarins bind to VKOR as their anions. As has been established, the anionic form of warfarin is predominantly the open chain 4-hydroxycoumarin tautomeric form.

But, what about Compound 62 and Compound 63? How do these inhibit VKOR? There are no reported studies in the literature on the metabolic fate of these compounds in vivo; the only in vitro study used a method of analysis that cannot distinguish between Compounds 62 or 63 and warfarin itself [85], so it remains a matter for speculation whether both Compound 62 (warfarin 4-methyl ether) and Compound 63 (cyclocumarol; warfarin methyl ketal) serve as prodrugs for warfarin, or have any activity of their own.

Warfarin binding to human serum albumin

Warfarin is strongly bound ($\sim$99%) to human serum albumin [86]. Human serum albumin has at least four binding sites that bind warfarin with varying affinity [87]. There is at least one site to which warfarin is strongly bound, although a model with two strong binding sites and two weaker binding

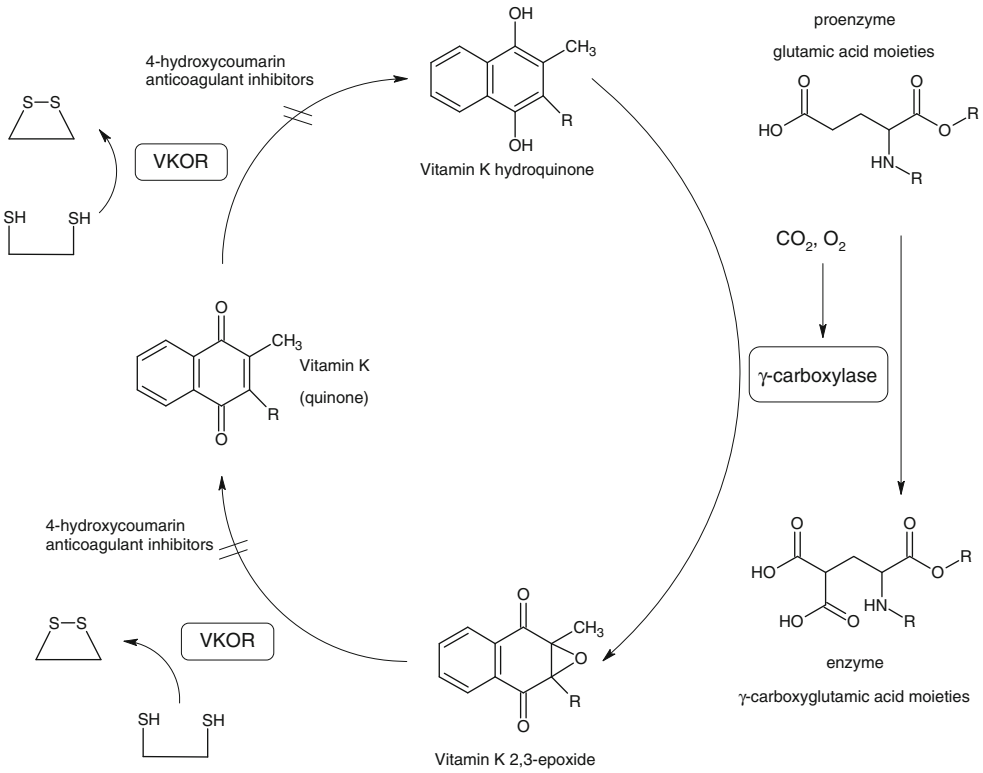


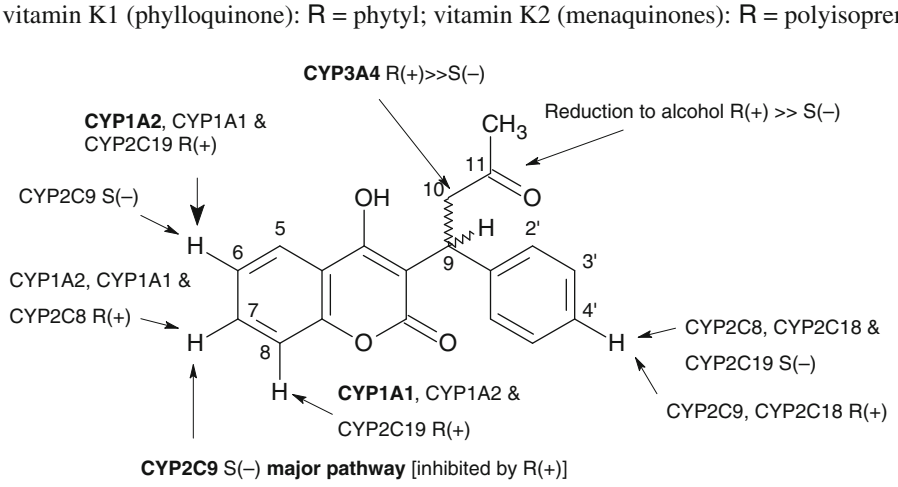
Fig. 6 The vitamin K cycle vitamin K1 (phylloquinone): *R* phytyl; vitamin K2 (menaquinones): *R* polyisoprene, $n \sim 8$

sites also fits the data equally well. Spectroscopic evidence suggests that all of the warfarin binds to human serum albumin as the ionized form of the open chain tautomer. As noted previously, in aqueous media the open chain form is predominantly present as the 4-hydroxycoumarin tautomeric form. Both *R*-(+) and *S*-(−) isomers of warfarin bind to the same site when co-crystallized with human serum albumin and myristic acid [88]; both enantiomers bind in the open-chain tautomeric form in the crystal. The electrostatic interactions between the oxygen moieties in the coumarin/chromandione/chromone ring system with amino acids in albumin are consistent with localization of the negative charge on the warfarin anion on the oxygen attached to C4 in the coumarin ring.

Warfarin binding to and metabolism by hepatic cytochrome P450 mixed-function oxidases

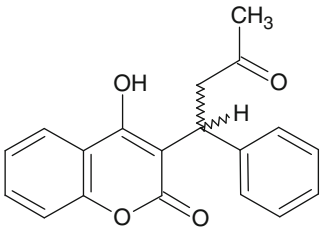
Both enantiomers of warfarin are extensively metabolized in the liver, primarily by oxidation accomplished by various isoforms of cytochrome P450; the side-chain ketone is also reduced by other hepatic enzymes to enantiomeric alcohols (Fig. 7) [89]. The *S*-(−)-enantiomer of warfarin is

Fig. 7 Metabolism of warfarin



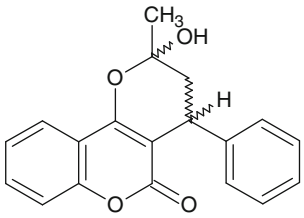
primarily metabolized (more than 80% of metabolic clearance) to 7-hydroxy-(*S*)-warfarin by cytochrome P450 isoform CYP2C9 [90]. Indeed, genetic polymorphisms in this particular isoform account for significant differences in the metabolism of warfarin for patients treated with this drug [91, 92]. It is some interest, therefore, that a crystal structure for this enzyme was published with *S*-(−)-warfarin bound to it [93]. The crystal structure shows *S*-(−)-warfarin bound as the open chain tautomer, albeit at a site removed somewhat from the active heme iron, and in a position ill-suited for generation of the major metabolite, 7-hydroxy—*S*-(−)-warfarin. Indeed, these authors show that a second molecule of warfarin could in principle bind to the active site, oriented in precisely the right way to facilitate generation of the known metabolite. Numerous studies with warfarin itself and with model compounds strongly suggest that the tautomeric form of *S*-(−)-warfarin that is actually metabolized by CYP2C9 is a ring tautomer [85]; the *R*-(+) enantiomer of warfarin is a potent inhibitor ($K_i = 7$ μM) of 7-hydroxylation of *S*-(−)-warfarin by CYP2C9 (Fig. 8, $K_m = 4.3$ μM), as are both enantiomers of phenprocoumon (Fig. 9, an analog of warfarin that can

Fig. 8 Inhibitors of CYP2C9: warfarin and warfarin analogs



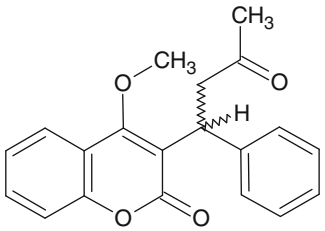
Warfarin (4-hydroxycoumarin open chain form)

9*R*: K_i = 7 μM



Warfarin (4-hydroxycoumarin hemiketal form)

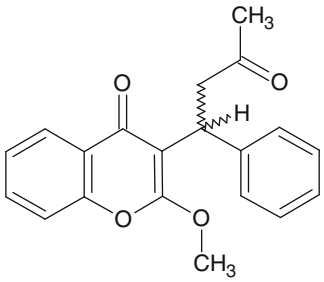
9*S*,11*S*: K_m = 4.3 μM



Warfarin 4-methyl ether (coumarin form)

9*R*: K_i = 10 μM

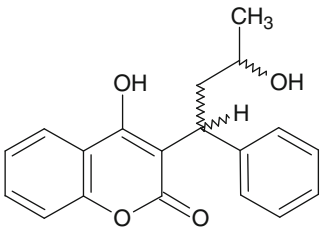
9*S*: K_i = 11 μM



Warfarin 2-methyl ether (chromone form)

9*R*: K_i = 29 μM

9*S*: K_i = 21 μM



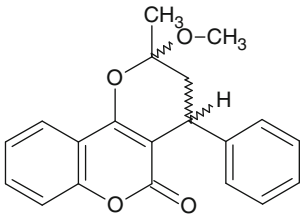
Warfarin alcohol (4-hydroxycoumarin form)

9*S*, 11*R*: K_i = 0.1 μM

9*S*, 11S: K_i = 1 μM

9R, 11S: K_i = 14 μM

9R, 11*R*: K_i = 37 μM



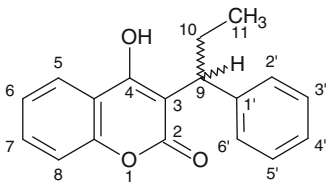
Cyclocumarol (4-hydroxycoumarin form)

9*S*, 11*R*: K_i = 12 μM

9*S*, 11S: K_i = 20 μM

9R, 11S: K_i = 7 μM

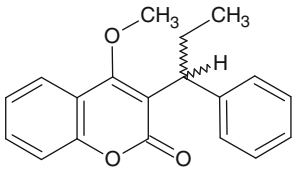
9R, 11*R*: K_i = 10 μM



Phenprocoumon

9*R*: K_i = 0.5 μM

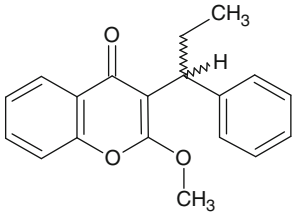
9*S*: K_i = 0.6 μM



Phenprocoumon 4-methyl ether (coumarin form)

9*R*: K_i = 13 μM

9*S*: K_i = 17 μM



Phenprocoumon 2-methyl ether (chromone form)

9*R*: K_i = 14 μM

9*S*: K_i = 8 μM

Fig. 9 Inhibitors of CYP2C9: phenprocoumon and phenprocoumon analogs

exist only in open chain tautomeric forms, $K_{iS} = 0.60$ μM; $K_{iR} = 0.52$ μM). The open chain coumarin and chromone methyl ethers of the enantiomers of warfarin (Fig. 8), the warfarin alcohols formed by reduction of the side chain carbonyl moiety (Fig. 8) and phenprocoumon (Fig. 9) are all inhibitors of the 7-hydroxylation of *S*-(−)-warfarin by CYP2C9 with $K_i \sim$ 10–30 μM. The metabolic profiles for the metabolism of *S*-(−)-warfarin and *S,S*-cyclocumarol (Fig. 8) are identical with respect to regioselectivity. Although both *S*- and *R*-phenprocoumon are metabolized to some small extent by CYP2C9 (at a rate considerably slower than *S*-(−)-warfarin), the major metabolic product in each case is the 4′-hydroxy metabolite, with lesser amounts of the 7- and 6-hydroxy metabolites, suggesting that when phenprocoumon binds to CYP2C9, the phenyl ring binds where the coumarin ring of *S*-(−)-warfarin binds, and the coumarin ring of phenprocoumon occupies the site where the phenyl ring of *S*-(−)-warfarin binds. The 2-methoxychromone methyl ether analogs of the warfarin enantiomers are metabolized following the same regioselective pattern as phenprocoumon enantiomers. The metabolism of the 4-methoxycoumarin ether analogs of the warfarin enantiomers is more confusing; 4′-hydroxylation is comparable to that of the 2-methoxychromone ether analogs of the warfarin enantiomers, but the 4-methoxycoumarin ether analogs of the warfarin enantiomers also show substantial 7′-hydroxylation (but no 6′-hydroxylation, unlike *S*-(−)-warfarin itself). The general pattern of metabolic inhibition and metabolism of congeners supports the hypothesis that the binding of *S*-(−)-warfarin and *S,S*-cyclocumarol to the active site of CYP2C9 are identical and different from the binding of *S*-(−)-phenprocoumon and the 2-methyoxychromone methyl ether of *S*-(−)-warfarin.

These inhibitory patterns have been used to model the active site of CYP2C9 [94]. A structural model was initially generated using the 3D-QSAR method CoMFA using SYBYL and MOPAC 93 software [94]. Critical amino acids were then identified that were suspected to participate in binding and mutant CYP2C9 proteins were constructed to test whether or not changes at these sites would alter the kinetics of metabolism of *S*-(−)-warfarin [95]. Based upon the site-directed mutagenesis studies, the structural model was refined and expanded [96]. This model was recently expanded using crystallographic data for CYP2C9 to identify two different sites (A and B) for inhibitors of the 7-hydroxylation of *S*-(−)-warfarin and to classify inhibitors as to which site on CYP2C9 they bound [97]. Ring-chain and coumarin-chromone tautomerism played critical roles in establishing the parameters of these models.

Chemical implications

Tautomerism plays a role in understanding the chemical reactivity of organic compounds. For example, a minor tautomeric form could serve as a key intermediate in a chemical reaction. This is illustrated in Fig. 10 for the degradation of *R*-(+)-warfarin under mildly alkaline conditions; the *S*(−)-isomer degrades similarly. Warfarin is

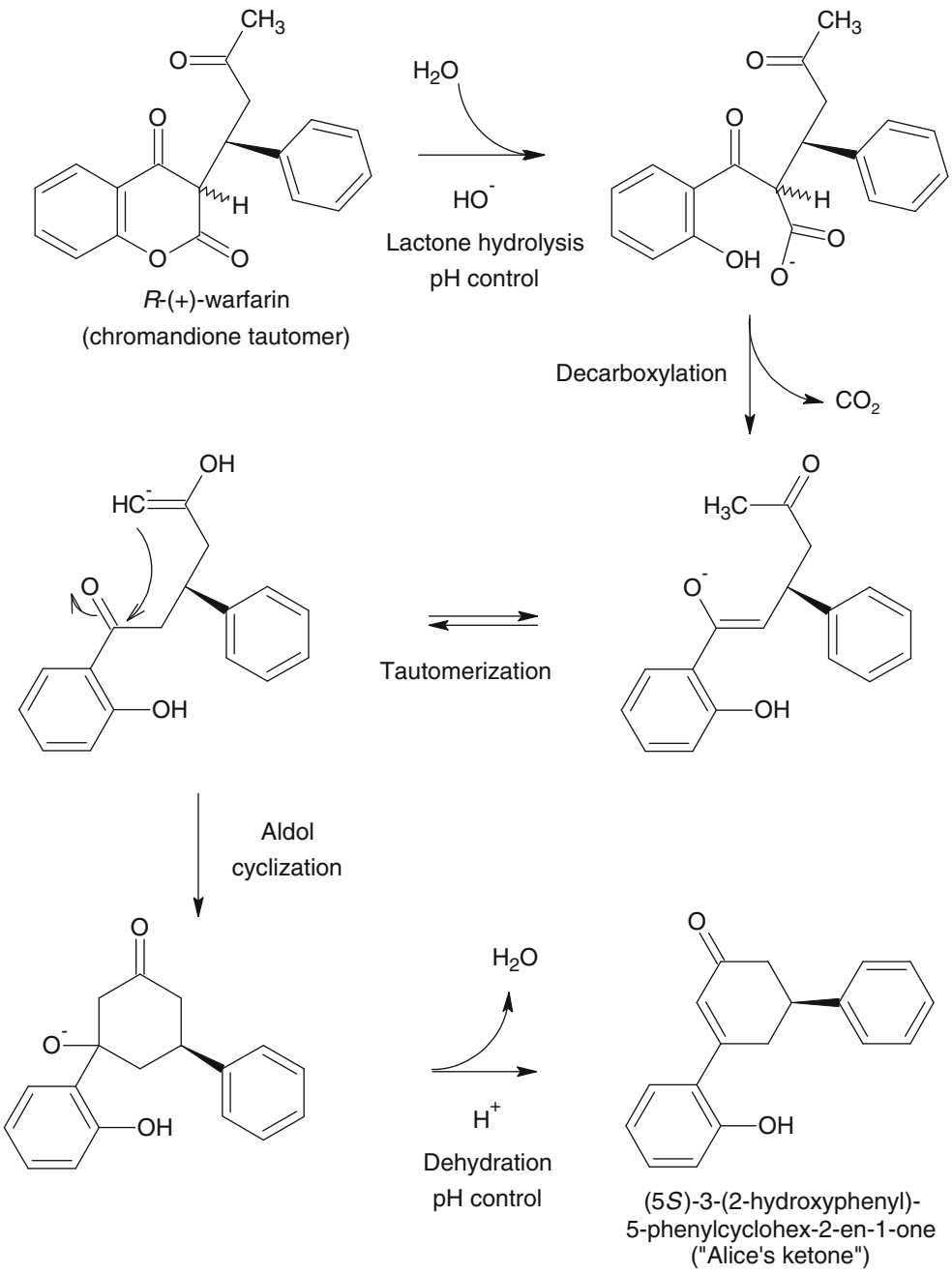


Fig. 10 Degradation of warfarin in mildly alkaline media

hydrolyzed, decarboxylated, and then undergoes cyclyzation by an internal aldol condensation followed by dehydration to produce 3-(2-hydroxyphenyl)-5-phenylcyclohex-2-en-1-one, a compound known originally as "Alice's ketone," after its discoverer, Alice Robertson, one of Karl Paul Link's students [98, 99]. This compound was alleged to be the primary odiferous substance responsible for bait-shyness by rats toward baits containing warfarin. Recently, however, investigators discovered that a manufacturing impurity, benzalacetone (4-phenylbut-3-en-2-one, one of the starting materials for the manufacture of warfarin, Fig. 3), caused bait avoidance by rats at 100 ppm while "Alice's ketone" at 1,000 ppm did not; neither affected the lethality of warfarin [100]. Nevertheless, much time, money and scientific effort was spent over many years in attempts to improve the manufacturing process for warfarin baits to avoid formation of "Alice's ketone." An organic chemist, trying to understand the conditions under which formation of "Alice's ketone" is likely to be favored, needs to understand the role of tautomerism in this process in order to

design a manufacturing process for rodenticide baits that would avoid pH conditions that lead to formation of undesired impurities. Ideally, the chemist would like to know the ionizability of each of the proposed intermediates in the reaction scheme outline in Fig. 10; this cannot be achieved using laboratory measurements, since the properties of individual tautomeric forms are in question. Likewise, a chemist bent upon improving conditions for the synthesis of warfarin itself (Fig. 3) needs to know and understand the ionizability of the reactants, one of which is an unfavored tautomeric form of 4-hydroxycoumarin. Computational chemistry methods can, however, provide some insight into the reactivity of these various intermediate species by providing estimates for ionization constants for each suspected intermediate.

Computational chemistry methods can also be used to estimate physicochemical properties of substances in silico before sufficient quantities of material are prepared to confirm these prediction in vitro. The problem with this approach is that, for tautomeric compounds, different assumed tautomeric forms may lead to different predictions. Solution properties, such as octanol/water partition coefficients (P) or distribution coefficients (D) at specified aqueous pH values, prediction of ionization constants (pK_a), and prediction of other properties that can be correlated with log P, log D, and/or pK_a are all amenable to prediction in silico. Warfarin property predictions have been reported using several different computational software packages. For example:

- Oral drug absorption, blood–brain barrier permeability, Caco-2 cell permeability and immobilized liposome chromatography capacity factor were predicted using several different computational approaches in which warfarin was included as part of the training set [101].
- Warfarin was also included in the training set used to develop a software tool for rapid prediction of polar surface area [102].
- Caco-2 cell permeability data for warfarin was used as part of training set of 17 compounds used to develop MS-WHIM descriptors [103].
- Protein binding of 33 drugs, including warfarin, was predicted using a combination of predicted physicochemical properties estimated using three different software packages [104].
- A comparison of nine software programs for predicting pK_a values included warfarin as one of 197 compounds used in the evaluation [105].

None of the authors of these papers indicated that any structure other than the classic structure of the 4-hydoxycoumarin tautomer described by Ikawa [2] was considered.

One problem confronting computational chemists who wish to evaluate the impact of tautomerism on the prediction of physicochemical properties is that the tautomeric equilibria involved in phenomena such as partitioning between organic solvents and water or dissociation of acids or bases in water are generally not known. Most reports of experimental measurements of log P, log D or pK_a for warfarin ignore tautomerism completely. This may account for much of the discrepancy in reported experimental values measured in different laboratories. Box et al. introduced a technique for measuring ionization constants using what they called "chasing equilibrium" titration methodology [106]. Warfarin was used as an example of a compound that "chases equilibrium" when an acid titrant is used to precipitate warfarin from a solution of its sodium salt in water adjusted to a constant ionic strength with added KCl. The kinetic solubility obtained using this approach was higher than the equilibrium solubility of the unionized form. In their experiment, they report that warfarin pK_a equals 4.94 (25 °C, 0.15 M ionic strength.) As acid titrant is added, unionized warfarin begins to precipitate with a kinetic solubility equal to119 μg/mL at pH 5.8. The pH is rapidly adjusted to keep the pH constant as more precipitate forms at a pH of ~7. Eventually, precipitation continues until the equilibrium solubility, 5.25 μg/mL is reached. We know, from X-ray crystallographic data, that the equilibrium solubility corresponds to the solubility of racemic (*R*,*R*)/(*S*,*S*)-warfarin hemiketal with axial phenyl and methyl groups reported by Wheeler [59]. The solubility at the point of initial precipitation is therefore most likely the solubility of the presumed open chain tautomer precipitated at pH 6 by Shazadi et al. [19], who were careful to keep the pH steady at this value.

Other values for the pK_a of warfarin have been reported; these, of course, also impact $\log_{10} D^{n\text{-octanol/buffer}}$ measurements as they relate to $\log_{10} P^{n\text{-octanol/water}}$ values. Otagiri et al. reported $pK_a = 5.10 \pm 0.04$ (ionic strength not reported) and $\log_{10} D^{n\text{-octanol pH 7.4}} = 1.037 \pm 0.004$ [107]; these same workers reported for phenprocoumon $pK_a = 4.30 \pm 0.05$ and $\log_{10} D^{n\text{-octanol pH 7.4}} = 1.233 \pm 0.003$. Illum et al. used a reported value for warfarin $pK_a = 5.1$, but measured $\log_{10} P^{n\text{-octanol/buffer}} = 2.70$ by spectrophotometric shift in apparent pK_a [108]. Stella et al. reported the pK_a determination for warfarin using solubility vs. pH measurements and found $pK_a = 5.06$. They also reported $pK_a = 5.03 \pm 0.01$ for warfarin and $pK_a = 3.77 \pm 0.01$ for phenprocoumon, both of which were determined spectrophotometrically in aqueous buffers maintained at 0.1 M ionic strength [109]; these workers then argued that, because of the similarity in structure between the 4-hydroxy enol tautomeric form of warfarin and phenprocoumon, the difference in ionization constants must reflect the presence of the cyclic hemiketal tautomers of warfarin in solution. They estimated the ratio of cyclic tautomers to the open chain form equal to ~ 20:1. My laboratory

measured the ionization of warfarin spectrophotometrically in 30 mM zwitterionic buffers saturated with *n*-octanol at 25 °C and found $pK_a = 5.15 \pm 0.04$ and $\log_{10} P^{n\text{-octanol/water}} = 2.82 \pm 0.06$ [110]. Walter and Kurz cited a literature value for the ionization of warfarin ($pK_a = 5.00$) but measured $\log_{10} D^{n\text{-octanol/pH 7.0}} = 1.46$ [111]. Baars et al. measured the ionization of warfarin enantiomers by spectrophotometry in buffers adjusted to 0.5 M ionic strength and found $pK_a = 5.0$ and $\log_{10} D^{n\text{-octanol/pH 7.4}} = 1.20$ [112]. Ishihama et al. used a capillary electrophoresis technique to measure the ionization of warfarin in 0.05 M ionic strength buffers and found $pK_a = 5.03$ [113]. Workers supplying data to the European Commission Health & Consumer Protection Directorate-General reported for warfarin $pK_a = 5.19 \pm 0.03$ @ 20 °C using an unspecified method and $\log_{10} D^{n\text{-octanol/pH 7.0}} = 0.7$ @ 30–36 °C [114]. Völgyi et al. reported the potentiometric titration of warfarin in water adjusted to 0.15 M ionic strength with KCl ($pK_a = 5.01 \pm 0.01$ at 25 °C) and by Yasuda-Shedlovsky extrapolation of pK_a determinations in mixed methanol-dioxane-acetonitrile–water systems of varying water content (pK_a @ 100% water $= 4.98 \pm 0.02$) [115]. Box et al. described a new "fast UV" technique recently, and reported for warfarin $pK_a = 4.86$ [116]. These values are plotted in Fig. 11, where they are compared with the predictions made using the nine software programs reviewed by Liao and Nicklaus [105]. Experimental values for both pK_a and $\log_{10} D^{\text{n-octanol/buffer}}$ depend somewhat on the experimental technique used. In particular, the presence of organic solvents, such as *n*-octanol, can shift the tautomeric equilibria [75]. However, it is remarkable how closely the experimental values around the median $pK_a = 5.03$. The values obtained using commercially available physicochemical property estimation software, on the other hand, are more widely dispersed about their median value, $pK_a = 5$, obtained using both ADME Boxes 4.9 and Pipeline Pilot 5.0 (Fig. 11).

Liao and Nicklaus [105] reported values using software configured to predict experimental pK_a values; however, many of these programs can be configured to report values for individual tautomeric forms. Warfarin in its open chain forms (Fig. 4, left) has both a β-keto lactone moiety and a

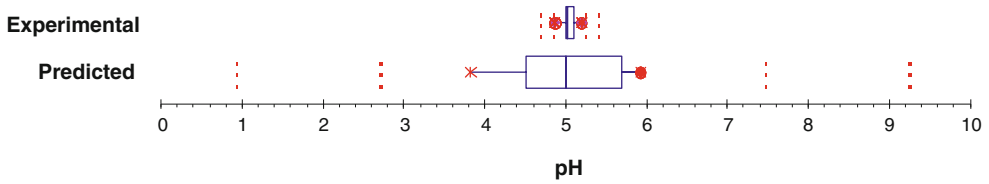
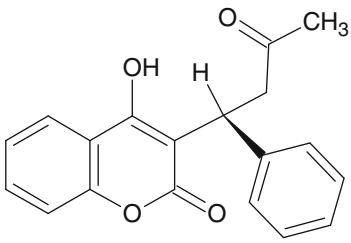


Fig. 11 Box plot of experimentally measured [106–116] and computationally predicted [105] values for the pK_a of warfarin. *Box plots* are a simplified way of depicting statistical distributions that aid in the identification of suspect values. Conventionally, a *box plot* representation of a statistical distribution consists of a rectangle bounded at each end by the 25th and 75th percentiles (encompassing the interquartile range) of the distribution of the data. The *central bar* in each box indicates the 50th percentile (median). The *lines* extending horizontally (whiskers) span the range of the data (lowest to highest values). The *vertical dots* (inner and outer fences) represent deviations of 1.5 × and 3.0 × multiples of the interquartile range. Data points located between the inner and outer fences are suspect outliers; data points outside the outer fences are highly suspect outliers. The smallest experimental pK_a value is just inside the lower inner fence, while all of the other extreme values of each range are well inside the inner fences, so none of the data are suspect outliers

Fig. 12 *R*-(+)-Warfarin coumarin open chain tautomer with side-chain ketone. **a** Not calculated

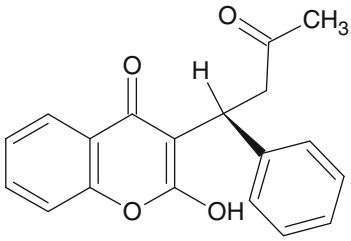
Tautomer A



4-hydroxy-3-[(1R)-3-oxo-1-phenylbutyl]-2*H*-chromen-2-one
CC(=O)C[C@@H](C1=C(O)c2ccccc2OC1=O)c3ccccc3

Software	pK_a	log*P*	log*D*	Other tautomers?
ClogP	[a]	2.90	[a]	[a]
SlogP	[a]	2.23	[a]	[a]
ACD/Labs	4.50	3.13	0.33	AC
ADMET Predictor	5.75	3.05	1.48	[a]
Pipeline Pilot	5.00	[a]	2.13	[a]
Chemaxon Marvin	6.33	2.74	1.66	ABCDEFGHI
Epik	5.92	[a]	[a]	[a]

Tautomer B



2-hydroxy-3-[(1*R*)-3-oxo-1-phenylbutyl]-4H-chromen-4-one
CC(=O)C[C@@H](C=1C(=O)c2ccccc2OC=1O)c3ccccc3

Software	pK_a	log*P*	log*D*	Other tautomers?
ClogP	[a]	2.23	[a]	[a]
SlogP	[a]	2.46	[a]	[a]
ACD/Labs	4.50	2.76	-0.04	ABC
ADMET Predictor	6.37	2.91	1.86	[a]
Pipeline Pilot	7.28	[a]	3.029	[a]
Chemaxon Marvin	6.85	3.52	2.86	ABCDEFGHI
Epik	6.51	[a]	[a]	[a]

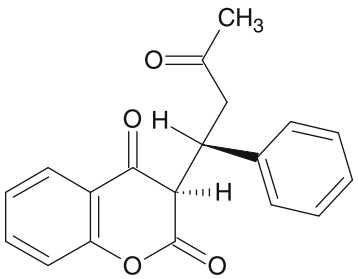
Fig. 13 *R*-(+)-Warfarin chromone open chain tautomer with side-chain ketone. **a** Not calculated

side-chain ketone moiety, each of which can independently exhibit keto-enol tautomerism. The β-keto lactone moiety can exist in any of the three tautomeric forms for 4-hydroxycoumarin illustrated in Fig. 5. However, because warfarin contains a center of asymmetry in the side-chain bonded at position #3 in the 4-hydroxycoumarin ring system, there are actually two possible diastereomeric 2,4-chromandione tautomers for each warfarin enantiomer. Likewise, the side-chain keto group can tautomerize to two different enol forms, one involving the terminal methyl group, and the other involving the interior methylene group. The latter, if formed, has two possible geometric isomers, an *E*-configuration and a *Z*-configuration. Thus, for each warfarin enantiomer there are four topologically distinct forms for tautomerization of the side-chain ketone moiety and four topologically distinct forms for tautomerization of the β-keto lactone moiety, giving rise to the possibility that as many as 16 topologically distinct open chain tautomeric forms can exist for each warfarin enantiomer, for a total of 32 open chain tautomeric forms. Likewise, warfarin in its ring forms (Fig. 4, right) has a newly created center of asymmetry that is formed as a result of ring closure. Furthermore, ring closure can at least in principle occur either to form the 4-hydroxycoumarin cyclic hemiketal (with two possible diastereoisomers for each enantiomer of warfarin) or to form the 2-hydroxychromone cyclic hemiketal (again, with two possible diastereoisomers for each enantiomer of warfarin). Thus a total of eight additional ring tautomeric forms are theoretically possibly, leading to a total of 40 potential tautomeric forms for warfarin.

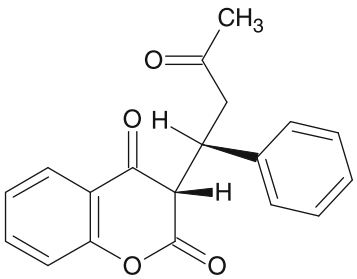
Not all of these forms can be distinguished from one another by current predictive software available to this author and colleagues for testing. While 40 tautomeric forms of warfarin are theoretically possible, the software evaluated in this study do not distinguish enantiomers, diastereomers, or E/Z configurational isomers, leaving only 11 computationally distinct forms of warfarin, shown in Figs. 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 and 22. For simplicity, the *R*-(+)-isomer of warfarin was used to generate all tautomeric structures, so that only 20 of the 40 warfarin tautomers are illustrated in Figs. 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 and 22. Ionization constants and/or partition coefficients were calculated for each of these 11 forms using ClogP (version 4.0, Biobyte Corp., Claremont, CA), SlogP (running under MOE2009.10, Chemical Computing Group, Montreal, QC), ACD/Labs Physicochemical Properties Suite (version 12, Advanced Chemistry Development, Toronto, ON), ADMET Predictor (version 4.0, Simulations Plus, Inc., Lancaster, CA), Pipeline Pilot (version 7.5.2, Accelrys, Inc., San Diego, CA), MarvinSketch with cxcalc calculations (version 5.3.0.1, ChemAxon Kft., Budapest, Hungary) and Epik (version 1.5, running under MAESTO 9.0.211, Schrödinger, New York, NY). The chemical structures, IUPAC names, SMILES notation and calculated results for each software for each of the computationally distinct tautomeric forms of warfarin are reported in Figs. 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 and 22. Some software tested could only compute ionization constants (Fig. 23), some could compute only partition coefficients (Fig. 24), while some could do both, and were therefore capable of calculating octanol-buffer distribution at a particular pH (Fig. 25). Two software (ACD/Labs and ChemAxon Marvin) also have an option to report whether a particular structure as drawn has any additional tautomeric forms. The ACD/Labs software offers advice as to which tautomeric form may predominate in some cases; in other cases, it simply reports a set of tautomers as "condition dependent forms." ChemAxon Marvin software reported all possible open-chain tautomeric forms as options whenever

Fig. 14 *R*-(+)-Warfarin chromandione open chain tautomer with side-chain ketone. **a** Not calculated

Tautomer C



(3*R*)-3-[(1*S*)-3-oxo-1-phenylbutyl]-2*H*-chromene-2,4(3*H*)-dione
CC(=O)C[C@H](c1ccccc1)[C@@H]2C(=O)c3ccccc3OC2=O

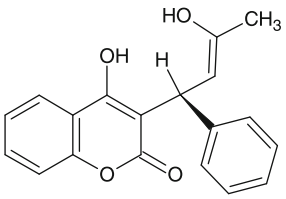


(3*S*)-3-[(1*S*)-3-oxo-1-phenylbutyl]-2*H*-chromene-2,4(3*H*)-dione
CC(=O)C[C@H](c1ccccc1)[C@H]2C(=O)c3ccccc3OC2=O

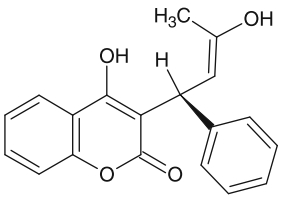
Software	pK_a	log*P*	log*D*	Other tautomers?
ClogP	[a]	1.97	[a]	[a]
SlogP	[a]	2.89	[a]	[a]
ACD/Labs	8.88	1.99	1.98	AC
ADMET Predictor	3.78	2.37	0.65	[a]
Pipeline Pilot	6.11	[a]	2.83	[a]
Chemaxon Marvin	4.74	3.35	1.36	ABCDEFGHI
Epik	8.51	[a]	[a]	[a]

Fig. 15 *R*-(+)-Warfarin coumarin open chain tautomers with side-chain interior *E* or *Z* enol. **a** Not calculated

Tautomer D



4-hydroxy-3-[(1*R*,2*Z*)-3-hydroxy-1-phenylbut-2-en-1-yl]-2*H*-chromen-2-one
C/C(O)=C/[C@@H](C1=C(O)c2ccccc2OC1=O)c3ccccc3

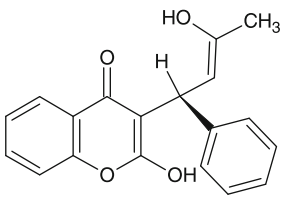


4-hydroxy-3-[(1*R*,2*E*)-3-hydroxy-1-phenylbut-2-en-1-yl]-2*H*-chromen-2-one
C\C(O)=C/[C@@H](C1=C(O)c2ccccc2OC1=O)c3ccccc3

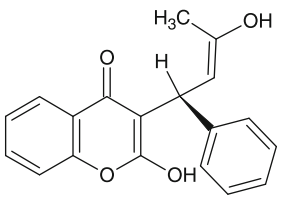
Software	pK_1	pK_2	log*P*	log*D*	Other tautomers?
ClogP	[a]	[a]	3.79	[a]	[a]
SlogP	[a]	[a]	3.59	[a]	[a]
ACD/Labs	4.50	10.75	3.17	0.36	ACD
ADMET Predictor	5.66	10.51	3.44	1.76	[a]
Pipeline Pilot	4.97	[a]	[a]	2.58	[a]
Chemaxon Marvin	6.33	10.12	2.91	1.82	ABCDEFGHI
Epik	6.53	9.90	[a]	[a]	[a]

Fig. 16 *R*-(+)-Warfarin chromone open chain tautomers with side-chain interior *E* or *Z* enol. **a** Not calculated

Tautomer E



2-hydroxy-3-[(1*R*,2*Z*)-3-hydroxy-1-phenylbut-2-en-1-yl]-4*H*-chromen-4-one
C/C(O)=C/[C@@H](C=1C(=O)c2ccccc2OC=1O)c3ccccc3

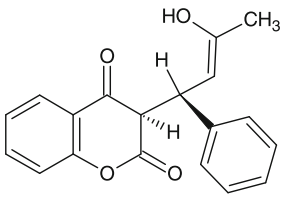


2-hydroxy-3-[(1*R*,2*E*)-3-hydroxy-1-phenylbut-2-en-1-yl]-4*H*-chromen-4-one
C\C(O)=C/[C@@H](C=1C(=O)c2ccccc2OC=1O)c3ccccc3

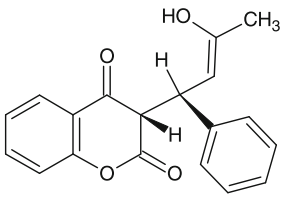
Software	pK_1	pK_2	log*P*	log*D*	Other tautomers?
ClogP	[a]	[a]	3.12	[a]	[a]
SlogP	[a]	[a]	3.82	[a]	[a]
ACD/Labs	4.50	10.74	2.72	-0.09	ACE
ADMET Predictor	6.42	10.55	3.15	2.14	[a]
Pipeline Pilot	7.90	[a]	[a]	3.65	[a]
Chemaxon Marvin	6.85	10.09	3.69	3.03	ABCDEFGHI
Epik	6.90	9.90	[a]	[a]	[a]

Fig. 17 *R*-(+)-Warfarin chromandione open chain tautomers with side-chain interior *E* or *Z* enol. **a** Not calculated

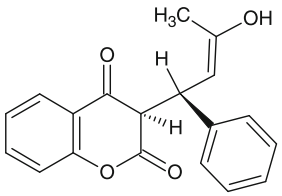
Tautomer F



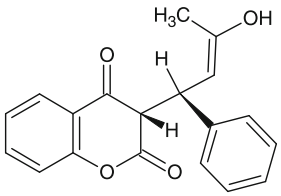
(3*R*)-3-[(1*S*,2*Z*)-3-hydroxy-1-phenylbut-2-en-1-yl]-2*H*-chromene-2,4(3*H*)-dione
C/C(O)=C/[C@H](c1ccccc1)[C@@H]2C(=O)c3ccccc3OC2=O



(3*S*)-3-[(1*S*,2*Z*)-3-hydroxy-1-phenylbut-2-en-1-yl]-2*H*-chromene-2,4(3*H*)-dione
C/C(O)=C/[C@H](c1ccccc1)[C@H]2C(=O)c3ccccc3OC2=O



(3*R*)-3-[(1*S*,2*E*)-3-hydroxy-1-phenylbut-2-en-1-yl]-2*H*-chromene-2,4(3*H*)-dione
C\C(O)=C/[C@H](c1ccccc1)[C@@H]2C(=O)c3ccccc3OC2=O

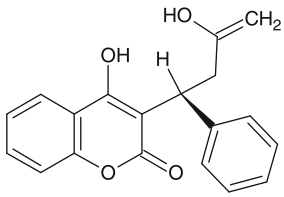


(3*S*)-3-[(1*S*,2*E*)-3-hydroxy-1-phenylbut-2-en-1-yl]-2*H*-chromene-2,4(3*H*)-dione
C\C(O)=C/[C@H](c1ccccc1)[C@H]2C(=O)c3ccccc3OC2=O

Software	pK_1	pK_2	log*P*	log*D*	Other tautomers?
ClogP	[a]	[a]	2.86	[a]	[a]
SlogP	[a]	[a]	3.84	[a]	[a]
ACD/Labs	8.66	12.08	2.61	2.56	ACF
ADMET Predictor	2.89	10.96	2.66	0.26	[a]
Pipeline Pilot	6.20	[a]	[a]	3.42	[a]
Chemaxon Marvin	6.13	10.17	3.52	2.29	ABCDEFGHI
Epik	8.61	9.90	[a]	[a]	[a]

Fig. 18 *R*-(+)-Warfarin coumarin open chain tautomer with side-chain exterior enol. **a** Not calculated

Tautomer G



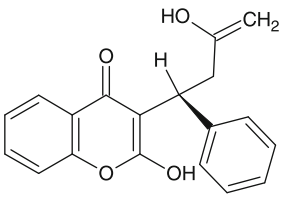
4-hydroxy-3-[(1*R*)-3-hydroxy-1-phenylbut-3-en-1-yl]-2*H*-chromen-2-one
C=C(O)C[C@@H](C1=C(O)c2ccccc2OC1=O)c3ccccc3

Software	pK_1	pK_2	log*P*	log*D*	Other tautomers?
ClogP	[a]	[a]	3.79	[a]	[a]
SlogP	[a]	[a]	3.84	[a]	[a]
ACD/Labs	4.50	10.66	3.05	0.25	ACG
ADMET Predictor	5.54	10.52	3.46	1.70	[a]
Pipeline Pilot	4.30	[a]	[a]	2.70	[a]
Chemaxon Marvin	6.37	10.15	3.05	1.99	ABCDEFGHI
Epik	6.39	10.01	[a]	[a]	[a]

any one particular open chain tautomeric form was drawn; however, it failed to suggest any ring tautomers.

The programs tested showed remarkable variation in results (Figs. 23, 24, 25). Minimum and maximum values often differed by more than two log units. Evidently the computational chemist cannot put too much faith in the accuracy of these reported results. Bear in mind that it is impossible, with current technology, to confirm or deny any of these predictions experimentally, so these computed values are not scientifically testable. Even a careful study of analogs cannot shed much light on the influence of tautomerism on the physical properties of warfarin. As noted previously, Stella et al. [109] argued that the increased acidity of phenprocoumon compared to warfarin could be used to estimate the fraction of warfarin existing in solution as ring tautomeric forms J and K (Fig. 22) but their argument is based on an assumption that all of the difference in acidity could be accounted for entirely by ring-chain tautomerism. It would be useful to experimentally measure the ionization constants and octanol/water partition coefficients of other analogs of warfarin that lack (or have modified) specific oxygen moieties involved in the tautomeric equilibria. Unfortunately, systematic studies of the properties of analogs have yet to be undertaken.

Tautomer H

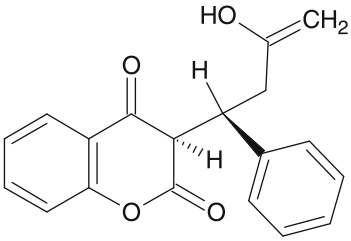


2-hydroxy-3-[(1*R*)-3-hydroxy-1-phenylbut-3-en-1-yl]-4*H*-chromen-4-one
C=C(O)C[C@@H](C=1C(=O)c2ccccc2OC=1O)c3ccccc3

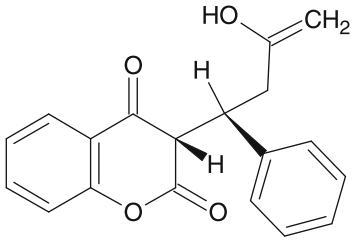
Software	pK_1	pK_2	log*P*	log*D*	Other tautomers?
ClogP	[a]	[a]	3.12	[a]	[a]
SlogP	[a]	[a]	3.90	[a]	[a]
ACD/Labs	4.50	10.65	2.59	-0.22	ACH
ADMET Predictor	6.23	10.54	3.12	1.94	[a]
Pipeline Pilot	7.22	[a]	[a]	3.58	[a]
Chemaxon Marvin	6.89	10.12	3.82	3.20	ABCDEFGHI
Epik	6.76	10.01	[a]	[a]	[a]

Fig. 19 *R*-(+)-Warfarin chromone open chain tautomer with side-chain exterior enol. **a** Not calculated

Tautomer I



(3*R*)-3-[(1*S*)-3-hydroxy-1-phenylbut-3-en-1-yl]-2*H*-chromene-2,4(3*H*)-dione
C=C(O)C[C@H](c1ccccc1)[C@@H]2C(=O)c3ccccc3OC2=O

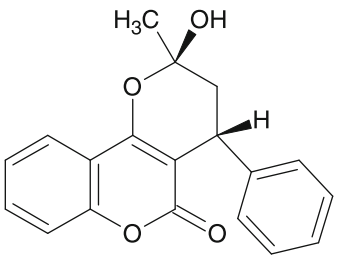


(3*S*)-3-[(1*S*)-3-hydroxy-1-phenylbut-3-en-1-yl]-2*H*-chromene-2,4(3*H*)-dione
C=C(O)C[C@H](c1ccccc1)[C@H]2C(=O)c3ccccc3OC2=O

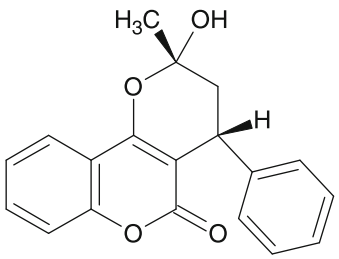
Software	pK_1	pK_2	log*P*	log*D*	Other tautomers?
ClogP	[a]	[a]	2.86	[a]	[a]
SlogP	[a]	[a]	3.92	[a]	[a]
ACD/Labs	9.29	10.90	2.30	2.29	ACI
ADMET Predictor	2.96	10.99	2.67	0.41	[a]
Pipeline Pilot	5.75	[a]	[a]	3.55	[a]
Chemaxon Marvin	6.03	10.19	3.65	2.34	ABCDEFGHI
Epik	8.46	10.01	[a]	[a]	[a]

Fig. 20 *R*-(+)-Warfarin chromandione open chain tautomers with side-chain exterior enol. **a** Not calculated

Tautomer J



(phenyl and hydroxyl *trans*)
(2*R*,4*R*)-2-hydroxy-2-methyl-4-phenyl-3,4-dihydro-2*H*,5*H*-pyrano[3,2-c]chromen-5-one
O=C2Oc1ccccc1C=3O[C@@](C)(O)C[C@@H](C2=3)c4ccccc4



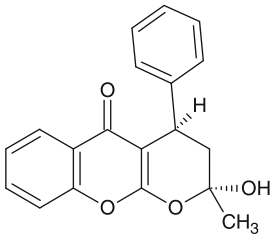
(phenyl and hydroxyl *cis*)
(2*S*,4*R*)-2-hydroxy-2-methyl-4-phenyl-3,4-dihydro-2*H*,5*H*-pyrano[3,2-c]chromen-5-one
O=C2Oc1ccccc1C=3O[C@](C)(O)C[C@@H](C2=3)c4ccccc4

Software	pK_a	log*P*	log*D*	Other tautomers?
ClogP	[a]	3.25	[a]	[a]
SlogP	[a]	2.98	[a]	[a]
ACD/Labs	12.27	3.03	3.03	J
ADMET Predictor	11.96	3.22	3.22	[a]
Pipeline Pilot	[a]	[a]	2.70	[a]
Chemaxon Marvin	12.21	2.59	2.59	J
Epik	13.22	[a]	[a]	[a]

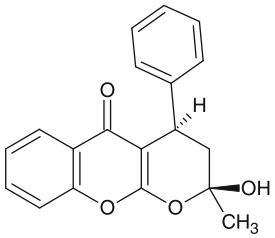
Fig. 21 *R*-(+)-Warfarin coumarin ring tautomers. **a** Not calculated

Fig. 22 *R*-(+)-Warfarin chromone ring tautomers. **a** Not calculated

Tautomer K



(2*R*,4*R*)-2-hydroxy-2-methyl-4-phenyl-3,4-dihydro-2*H*,5*H*-pyrano[2,3-b]chromen-5-one
O=C1c4ccccc4OC=2O[C@@](C)(O)C[C@@H](C1=2)c3ccccc3



(2*S*,4*R*)-2-hydroxy-2-methyl-4-phenyl-3,4-dihydro-2*H*,5*H*-pyrano[2,3-b]chromen-5-one
O=C1c4ccccc4OC=2O[C@](C)(O)C[C@@H](C1=2)c3ccccc3

Software	pK_a	log*P*	log*D*	Other tautomers?
ClogP		2.918		
SlogP		3.72		
ACD/Labs	12.35	2.05	2.05	K
ADMET Predictor	11.74	3.07	3.07	
Pipeline Pilot			3.168	
Chemaxon Marvin	12.16	3.36	3.36	K
Epik	13.22			

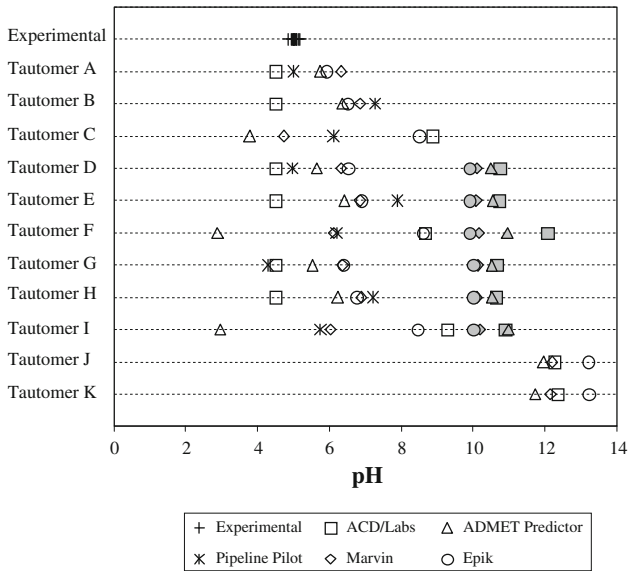


Fig. 23 Experimental ionization constant (pK_a) for warfarin compared to software predictions for each tautomeric form. *Key*: (+) experimental measurements; *open symbols* represent first (or only) pK_a value computed by software; *shaded symbols* represent second pK_a value if one is reported by software

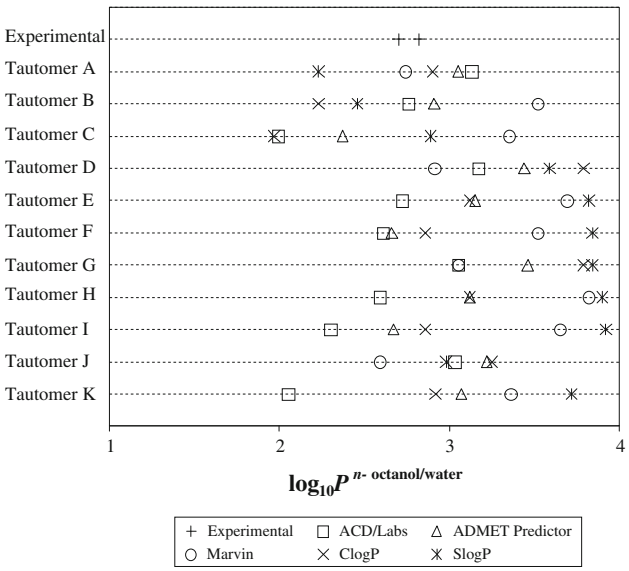


Fig. 24 Experimental octanol–water partition coefficient *P* for warfarin compared to software predictions for each tautomeric form

About all that can be said concerning the results presented in Figs. 23, 24 and 25, realistically, is that reliance on only one computational tool may lead to predictions concerning the chemical behavior of different tautomeric forms that can often be refuted by appeal to a different computational tool. In short, these tools are useless individually as predictors of tautomer behavior, at least in the case of warfarin. The chemist confronted with such a dilemma currently has but one recourse: undertake experimental measurement of the properties in question in vitro.

Die Theorie leitet, das Experiment entscheidet.
(The theory guides, the experiment decides.)
I. M. Kolthoff

The author thanks Dr. Yvonne Martin for her assistance in computing partition coefficient predictions using ClogP and SlogP software, Dr. Wentao Fu for his assistance in computing pK_a, log *P* and log *D* predictions using ADMET Predictor and Pipeline Pilot software, Dr. James Metz for additional assistance in computing pK_a, log *P* and log *D* predictions using Pipeline Pilot software and Dr. Steven Swann for his assistance in computing pK_a predictions using Epik software. The valuable technical assistance of the support staff at ChemAxon Kft. is greatly appreciated.

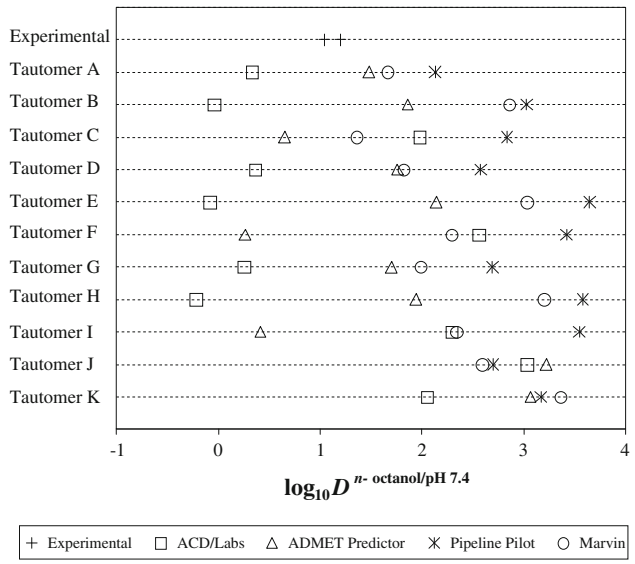


Fig. 25 Experimental octanol-pH 7.4 buffer distribution coefficient *D* for warfarin compared to software predictions for each tautomeric form

References

1. Lamb E (2009) Top 200 prescription drugs of 2008. Pharmacy Times, Plainsboro. http://www.pharmacytimes.com/issue/pharmacy/2009/2009-05/RxFocusTop200Drugs-0509. Accessed 08 Mar 2010
2. Ikawa M, Stahman MA, Link KP (1944) 4-Hydroxycoumarins. V. Condensation of α, β-unsaturated ketones with 4-hydroxycoumarin. J Am Chem Soc 66:902–906
3. Overman RS et al (1944) Studies on the hemorrhagic sweet clover disease: XIII. Anticoagulant activity and structure in the 4-hydroxycoumarin group. J Biol Chem 153:5–24
4. Schofield FW (1922) A brief account of a disease of cattle simulating haemorrhagic septicaemia, due to feeding sweet clover. Can Vet Rec 3:74–78
5. Roderick LM (1929) The pathology of sweet clover disease in cattle. J Amer Vet Med Ass 74:314–325
6. Burris RH (1994) Biographical memoir of Karl Paul link, vol 65. Biographical Memoirs, National Academy of Sciences, Washington, DC, pp 176–195
7. https://www.warf.org/about/index.jsp?cid=26&scid=34. Accessed Jan 2010
8. Link KP (1959) The discovery of dicumarol and its sequels. Circulation 19:97–107
9. Campbell HA, Link KP (1941) Studies on the hemorrhagic sweet clover disease. IV. The isolation and crystallization of the hemorrhagic agent. J Biol Chem 138:21–33
10. Stahmann MA, Huebner CF, Link KP (1941) The hemorrhagic sweet clover disease. V. Identification and synthesis of the hemorrhagic agent. J BiolChem 138:513–527
11. Meyer OO (1959) Historical data regarding the experiences with coumarin anticoagulants at the University of Wisconsin Medical School. Circulation 19:114–117
12. Scheel LD (1949) Studies on the anticoagulant dicumarol and other 4-hydroxycoumarins. Sect, II, The anticoagulant activity and toxicity of 3-substituted-4-hydroxycoumarins. PhD Thesis, University of Wisconsin
13. Wines M (2003) New study supports idea that Stalin was poisoned. The New York Times, 5 March
14. Wu DL (1949) Masters Thesis, University of Wisconsin; Scheel LD, Wu D, Link KP (1949) Abstracts of papers, 116th meeting of the American Chemical Society
15. Seidman M (1950) Studies on 3-(α-phenyl-β-acetylethyl)-4-hydroxycoumarin. PhD Thesis, University of Wisconsin
16. Bravic G, Gaultier J, Hauw C (1973) Crystal structure of an antivitamin K, warfarin. C R Acad Sci Paris Ser C 277(22): 1215–1218
17. Valente EJ, Trager WF, Jensen LH (1975) Crystal and molecular structure and absolute configuration of (-)-(S)-warfarin. Acta Cryst B B31(4):954–960
18. Halland N, Hansen T, Jørgensen KA (2003) Organocatalytic asymmetric Michael reaction of cyclic 1, 3-dicarbonyl compounds and alpha, beta-unsaturated ketones—a highly atom-economic catalytic one-step formation of optically active warfarin anticoagulant. Angew Chem Int Ed Engl 42(40):4955–4957
19. Shahzadi S, Ali S, Asif I, Ashraf R, Jin G-X (2006) The mechanism and crystal structure of 2-methoxy-2-methyl-4-phenyl-3, 4, 4a, 10b-tetrahydro-2H, 5H-pyrano[3, 2-c]chromen-5-one. Acetal of warfarin acid. Turkish J Chem 30(6):703–709
20. Porter WR (1976) Synthesis, structure in solution and stereochemical aspects of the microsomal metabolism of warfarin and phenprocoumon. PhD Thesis, University of Washington
21. Link KP (1957) Warfarin-alkali metal derivatives and processes of preparing the same. US patent 2777859
22. Schroeder CH, Link KP (1963) Warfarin sodium. US patent 3077481; Weiner N, Park R, Johnson M, Schoeder CH, Link KP (1966) Preparation of crystalline warfarin sodium-isopropyl alcohol complex. US patent 3246013
23. Hiskey CF, Melnitchenko V (1965) Clathrates of sodium warfarin. J Pharm Sci 54(9):1298–1302
24. Sheth AR, Young VG Jr, Grant DJW (2002) Warfarin sodium 2-propanol solvate. Acta Cryst E58:m197–m199
25. Sheth AR, Brennessel WW, Young VG Jr, Muller FX, Grant DJW (2004) Solid-state properties of warfarin sodium 2-propanol solvate. J Pharm Sci 93(11):2669–2680
26. Huebner CF, Link KP (1945) 4-hydroxycoumarin. VII. Reactions of 4-hydroxycoumarin with cationoid reagents. J Amer Chem Soc 67:99–102
27. Arndt F, Loewe L, Un R, Ayca E (1951) Coumarindiol and coumarin-chromone tautomerism. Chem Ber 84:319–329
28. Klosa J (1953) Possibility of tautomeric forms of 4-hydroxycoumarin. Arch Pharm 286:37–43
29. Chmielewska I, Cieslak J (1958) Vitamins and antivitamins K: tautomerism of dicoumarol. Tetrahedron 4:135–146
30. Jachymczyk W, Cieslak J, Chmielewska I (1960) Tautomerism of dicoumarol. Three isomeric methyl ethyl ethers of dicoumarol. Rocz Chem 34:925–930
31. Knobloch E, Kakac B, Macha F (1952) Anticoagulants. XVIII. A study of the tautomeric equilibria of effective anticoagulant derivatives of 4-hydroxycoumarin. Chem Listy 46:416–419
32. Chmielewska J, Ciecierska D (1952) K vitamins and antivitamins. V. Ultraviolet absorption spectra of biologically active derivatives of 4-hydroxycoumarin. Przemysl Chem 31:253–256
33. Dezelic M, Trkovik M, Zovko M (1963) Absorption spectra of coumarins. Glasnik Hemicara Techol Bosne Hercegovina 12:17–44
34. Perel'son ME, Sheinker YuN (1966) Spectra and structure of hydroxycoumarin and hydroxyfurocoumarin salts. Zh Prikl Spektosk 5(1):104–110
35. Khaikin MS, Rakova NF (1968) Ultraviolet spectra of some 6, 7- and 7, 8-dihydroxycoumarins. Zh Prikl Spektrosk 8(6):1063–1066
36. Masrani KV, Rama HS, Bafna SK (1974) Ultaviolet absorption spectra: substituted coumarins. J Appl Chem Biotechnol 24(6):331–341

37. Knobloch E, Prochzka Z (1953) Studies on anticoagulants. XXV. Infrared spectra of some derivatives of 4-hydroxycoumarin and chromone. Chem Listy 47:1285–1292
38. Abramovich RA, Gear JR (1958) Unsymmetrically substituted 3, 3′-methylene bridged 2, 2′-dihydroxychromones. Can J Chem 36:1501–1510
39. Farmer VC (1959) Spectra and structure of 4-hydroxycoumarins. Spectrochim Acta 10:870–882
40. Hutchinson DW, Tomlinson JA (1969) Structure of dicumarol and related compounds. Tetrahedron 25(12):2531–2537
41. Gaultier J, Hauw C (1965) Structure of an "antivitamin" K, 3-bromo-4-hydroxycoumarin monohydrate. Compt Rend 260(13(Groupe 8)):3666–3667; Gaultier J, Hauw C (1965) Crystalline and molecular structure of 3-bromo-4-hydroxycoumarin monohydrate. Acta Cryst 19(6):927–933
42. Gaultier J, Hauw C (1965) Crystal structure of monohydrated 4-hydroxycoumarin. Compt Rend 260(22(Groupe 8)):5787–5789; Gaultier J, Hauw C (1966) Structure of 4-hydroxycoumarin. Water of hydration and crystal cohesion. Acta Cryst20(5):646–651
43. Bravic G, Gaultier J, Hauw C (1968) Crystalline and molecular structure of dicoumarol [3, 3'-methylenebis(4-hydroxycoumarin)]. C R Acad Sci Paris 267(26):1790–1793
44. Bravic G, Gaultier J, Hauw C (1971) Crystalline and molecular structure of marcoumar. C R Acad Sci Paris 272(12):1112–1114
45. Alcock NW, Hough E (1972) The crystal and molecular structure of 3, 3′-methylene(bis-6-bromo-4-hydroxycoumarin): unusual molecular interactions. Act Cryst B28(6):1957–1960
46. Bravic G, Gaultier J, Geoffre S, Hauw C (1974) Crystal structure of an antivitamin K. 3-(1'α-Naphthyl)-4-hydroxycoumarin. C R Acad Sci Paris C27B:601–603
47. Valente EJ, Trager WF, Lingafelter EC (1976) (–)-3-(1-Phenylpropyl)-4-hydroxycoumarin. Acta Cryst B32(1):277–279
48. Jones PR (1968) Ring-chain tautomerism. Chem Rev 63:461–487
49. Kol'tsov AI, Kheifets GM (1971) Investigation of keto-enol tautomerism by nuclear magnetic resonance spectroscopy. Russ Chem Rev 40(9):773–788
50. Schroeder CH, Titus ED, Link KP (1957) Synthetic approach to some 3-aralkyl-4-hydroxycoumarins. J Amer Chem Soc 79:3291–3292
51. Pohl LR, Haddock R, Garland WA, Trager WF (1975) Synthesis and thin-layer chromatographic, ultraviolet and mass spectral properties of the anticoagulant phenprocoumon and its monohydroxylated derivatives. J Med Chem 18(5):513–519
52. Chan KK, Lewis RJ, Trager WF (1972) Absolute configuration of the four warfarin alcohols. J Med Chem 15(12):1265–1270
53. Robertson DN, Linl KP (1953) Studies of 4-hydroxycoumarins. XII. 3-Substitued-aminoethyl-4-hydroxycoumarin derivatives by the Mannich reaction. J Amer Chem Soc 75:1883–1885
54. Sen K, Bagchi P (1959) Studies on the ultraviolet absorption spectra of coumarins and chromones. II. Hydroxy derivatives. J Org Chem 24:316–319
55. French WN, Wehrli MI (1965) Identification and assay of some coumarin anticoagulants. Can Pharm J 98(5):174–179
56. Mendez J, Lojo MI (1968) Spectral analysis of coumarins. Microchem J 13(3):506–512
57. Mehta MJ, Hegde RS, Bhatt RA, Patel DJ, Bafna SL (1969) Ultraviolet spectra: 4-hydroxycoumarins. J Appl Chem 19(1):29–30
58. Knight AR, McIntyre JS (1968) Deuterated 4-hydroxycoumarin derivatives. Can J Chem 46(11):1949–1951
59. Wheeler CR (1980) Warfarin and phenprocoumon as probes to distinguish inducible forms of cytochrome P-450. PhD Thesis, University of Washington
60. Perel'son ME, Zvolinskii VP, Kagan GI, YuN Sheinker (1973) Investigation of the electronic spectra of a-pyrone derivatives by the Pariser-Parr-Pople method in the variable β approximation. Zh Struckt Khim 14(2):246–254
61. Perel'son ME, Sheinker YuN, Zaitsev BF, Pozdyshev VA (1964) Integrated intensities of the carbonyl bands of a number of pyrones and quinones. Izv Akad Nauk SSSR Ser Khim (5):804–808
62. Perel'son ME, Sheinker YuN (1968) Structure of the alkali metal salts of hydroxycoumarins and hydroxyfurocoumarins and their MO LCAO calculation. Teor Eksp Khim 4(2):184–191
63. Ahuja VK, Kapoor KL, Ray NK (1973) Molecular orbital study of pyrones: part II—chromones. Indian J Chem 11(2):143–145
64. Ray NK, Ahuja VK (1973) Molecular orbital study of the photoreactivity of triplet coumarins. Photochem Photobiol 17(5):347–351
65. Ahuja VK, Kapoor KL, Ray NK (1973) Molecular orbital study of pyrones: part III—coumarins. Indian J Chem 11(5):458–460
66. Linke HAB (1969) Infrared spectra of hydroxychromone. Absorption of the carbonyl valence vibration. Spectrochim Acta 25A(6):1067–1074
67. Porter WR, Kunze K, Valente EJ, Trager WF (1980) The synthesis of C-2 isotopically labeled optically pure warfarin and phenprocoumon. J Label Comp Radiopharm 17(6):763–773
68. Obaseki AO, Porter WR, Trager WF (1982) 4-Hydroxycoumarin/2-hydroxychromone tautomerism: infrared spectra of 2-^{13}C and 3-D labeled 4-hydroxycoumarin and its anion. J Heterocyclic Chem 19:385–390
69. Porter WR, Trager WF (1982) 4-Hydroxycoumarin/2-hydroxychromone tautomerism: Infrared spectra of 3-substituted-2–13C-4-hydroxycoumarins. J Heterocyclic Chem 19:475–480
70. Chan KK, Giannini DD, Cain AH, Roberts JD, Porter WR, Trager WF (1977) Carbon-13 nuclear magnetic resonance studies of coumarin and related compounds. Tetrahedron 33:899–906
71. Valente EJ, Lingafelter EC, Porter WR, Trager WF (1977) The structure of warfarin in solution. J Med Chem 20(11):1489–1493
72. Valente EJ, Porter WR, Trager WF, (1978) Conformations of selected 3-substituted 4-hydroxycoumarins in solution by nuclear magnetic resonance. Warfarin and phenprocoumon. J Med Chem 21(2):231–234
73. Obaseki AO (1982) Improved synthesis, separation and quantitation of warfarin and its metabolites and their use in the study of the tautomerization of 4-hydroxycoumarins and the metabolism of warfarin. Chapter 2, PhD Thesis, University of Wisconsin
74. Obaseki AO, Porter WR (1987) Structure of warfarin analogs in solution. Arch Pharm Chem Sci Ed 5:110–121
75. Heimark LD, Trager WF (1984) The preferred solution conformation of warfarin at the active site of cytochrome P-450 based on the CD spectra in octanol/water model system. J Med Chem 27:1092–1095
76. Holbrook A, Pereira JA, Labiris R, Mcdonald H, Douketis JD, Crowther M, Wells PS (2005) Systematic overview of warfarin and its drug and food interactions. Arch Intern Med 165:1095–1106
77. Mann KG (1999) Biochemistry and physiology of blood coagulation. Thromb Haemost 82(2):165–174
78. Garcia AA, Reitsma PH (2008) VOKORC1 and the vitamin K cycle. Vit Horm 78:23–33
79. Tie J-K, Stafford DW (2008) Structure and function of vitamin K epoxide reductase. Vit Horm 78:103–130
80. Wallin R, Wajih N, Hutson SM (2008) VKORC1: a warfarin-sensitive enzyme in vitamin K metabolism and biosynthesis of vitamin K-dependent blood coagulation factors. Vit Horm 78:227–246
81. Thijssen HHW, Baars LGM, Vervoort-Peters HTM (1988) Vitamin K 2.3-epoxide reductase: the basis for steroselectivity of 4-hydroxycoumarin anticoagulant activity

82. Fasco MJ, Principe LM, Walsh WA, Friedman PA (1983) Warfarin inihibition of vitamin K 2, 3-epoxide reductase in rat liver. Biochem 22:5655–5660
83. Park BK (1988) Warafin: metabolismand mode of action. Biochem Pharmacol 37(1):19–27
84. Gebauer M (2007) Synthesis and structure-activity relationships of novel warfarin derivatives
85. He M, Korzekwa KR, Jones JP, Rettie AE, Trager WF (1999) Structural forms of phenprocoumon and warfarin that are metabolized at the active site of CYP2C9. Arch Biochem Biophys 372(1):16–28
86. Porter RS, Sawyer WT, Lowenthal DT (1986) Warfarin. In: Evans WE, Schentag JJ, Jusko WJ (eds) Applied pharmacokinetics, 2nd edn. Applied Therapeutics, Spokane, pp 1057–1104
87. Larsen FG, Larsen CG, Jakobsen P, Brodersen R (1985) Interaction of warfarin with human serum albumin: a stoichiometric description. Mol Pharmacol 27:263–270
88. Petitpas I, Bhattacharya AA, Twine S, East M, Curry S (2001) Crystal structure analysis of warfarin binding to human serum albumin: Anatomy of drug site I. J Biol Chem 276(25):22804–22809
89. Kaminsky LS, Zhang Z-Y (1997) Human P450 metabolism of warfarin. Pharmacol Ther 73(1):67–74
90. Black DJ, Kunze KL, Wienkers LC, Gidal BE, Seation TL, McDonnell AP, Evans SJ, Bauwens JE, Trager WF (1996) Warfarin-fluconazole. II. A metabolically based drug interaction: in vivo studies. Drug Metab Dispos 24:414–421
91. Aquilante CL, Langaee TY, Lopez LM, Yarandi HN, Tromberg JS, Mohuczy D, Gaston KL, Waddell CD, Chirico MJ, Johnson JA (2006) Influence of coagulation factor, vitamin K epoxide reductase complex sububnit 1, and cytochrome P450 2C9 gene polymorphisms on warfarin dose requirements. Clin Pharmacol Thera 79(4):291–302
92. Yin T, Miyata Y (2007) Warfarin dos and the pharmacogenomics of *CYP2C9* and *VKORC1*—rationale and perspectives. Thromb Res 120:1–10
93. Williams PA, Cosme J, Ward A, Angove HC, Vinkoviĉ DM, Jhotl H (2003) Crystal structure of human cytochrome P450 @C9 with bound warfarin. Nature 424(24):464–468
94. Jones JP, He M, Trager WF, Rettie AE (1996) Three-dimensional quantitative structure-activity relationship for inhibitors of cytochrome P4502C9. Drug Metab Dispos 24(1):1–6
95. Haining RL, Jones JP, Henne KR, Fisher MB, Koop DR, Trager WF, Rettie AE (1999) Enzymic determinants of the substrate specificity of CYP2C9: Role of B'-C loop residues in providing the π-stacking anchor site for warfarin binding. Biochem 38(11):3285–3292
96. Rao S, Aoyama R, Schrag M, Trager WF, Rettie A, Jones JP (2000) A refined 3-dimensional QSAR of cytochrome P450 2C9: computational predictions of drug interactions. J Med Chem 43(15):2789–2796
97. Kazuya Y, Noriyuki Y, Hiroaki G, Hideki T, Shuichi H (2009) Structure-based CoMFA as a predictive model - CYP2C9 inhibitors as a test case. J Chem Info Modeling 49(4):853–864
98. Robertson AO (1953) PhD Thesis, University of Wisconsin, Madison, WI
99. Ross Montgomery E, Taylor S, Segretario J, Engler E, Sebastian D (1996) Development and validation of a reverse-phase liquid chromatographic method for analysis of aspirin and warfarin in a combinarion tablet formulation. J Pharm Biomed Anal 15:73–82
100. Krishnamurthy K, Ramasivan T, Singh DP (1971) Rodents and their control. VII. Effect of impurities in warfarin on its acceptability and mortality to black rats (*Rattus rattus*). Bull Grain Technol 9(4):252–256
101. Norinder U, Österberg T (2000) The applicability of computational chemistry in the evaluation and prediction of drug transport properties. Perspec Drug Discov Design 19:1–18
102. Ertl P, Rohde B, Selzer P (2000) Fast calculation of molecular polar surface area as a sum of fragment-based contributions and its application to the prediction of drug transport properties. J MedChem 43(20):3714–3717
103. Bravi G, Wikel JH (2000) Application of MS-WHIM descriptors: 3. Prediction of molecular properties. Quant Struct-Activ Relat 19(1):39–49
104. Emoto C, Murayama N, Rostami-Hodjegan A, Yamazaki H (2009) Utilization of estimated physicochemical properties as an integrated part of predicting hepatic clearance in the early drug-discovery stage: impact of plasma and microsomal binding. Xenobiotica 39(3):227–235
105. Liao C, Nicklaus MC (2009) Comparison of nine programs predicting pKa values of pharmaceutical substances. J Chem Inf Model 49:2801–2812
106. Box KJ, Völgyi G, Baka E, Stuart M, Takács-Nowák T, Comer JEA (2006) Equilibrium versus kinetic measurements of aqueous solubility, and the ability of compounds to supersaturate in solution—a validation study. J Pharm Sci 95(6):1298–1307
107. Otagiri M, Fokkens JG, Hardee GE, Perrin JH (1978) The interaction of some coumarin anticoagulants with β-cyclodextrin in phosphate buffers. Pharm Acta Helv 53(8):241–247
108. Illum L, Bundgaard H, Davis SS (1983) A constant partition model for examining the sorption of drugs by plastic infusion bags. Int J Pharm 17:183–192
109. Stella VJ, Mooney KG, Pipkin JD (1984) Dissolution and ionization of warfarin. J Pharm Sci 73(7):946–948
110. Opong-Mensah K, Woller TW, Obaseki AO, Porter WR (1984) Chemical and statistical considerations in the determination of partition coefficients of weakly ionizable drugs and poisons. J Pharm Biomed Anal 2(3/4):381–394
111. Walter K, Kurz H (1988) Binding of drugs to human skin: Influencing factors and the role of tissue lipids. J Pharm Pharmacol 40:689–693
112. Baars LGM, Schepers MT, Hermans JJR, Dahlmans HJJ, Thijssen HHW (1990) Enantioselective structure-pharmacokinetic relationships of ring substituted warfarin analogues in the rat. J Pharm Pharmacol 42:861–866
113. Ishihama Y, Oda Y, Asakaw N (1994) Microscale determination of dissociation constants of multivalent pharmaceuticals by capillary electrophoresis. J Pharm Sci 83(10):1500–1507
114. Ishihama Y, Oda Y, Asakaw N (2005) Review report for the active substance warfarin. ec.europa.eu/food/plant/protection/evaluation/existactive/warfarin_en.pdf Downloaded 25Jan2010
115. Völgyi G, Ruiz R, Box K, Comer J, Bosch E, Takács-Novák K (2007) Anal Chim Acta 583:418–428
116. Box K, Comer J, Gravestock T, Mole J (2009) Fast pK_a screening using 3μL of 10 mM DMSO stock. Presented at: LogP2009—the 4th LogP symposium, Feb. 8–11, 2009, ETH-Zurich, Switzerland