

High-resolution mass spectrometric elucidation of electron ionization induced fragmentation pathways of methylated warfarin and selected hydroxylated species

W.J. von Törne^{a,b}, L. Steinhäuser^{a,b}, U.-A. Klyk-Seitz^a, C. Piechotta^{a,b,*}

^a Bundesanstalt für Materialforschung und -prüfung (BAM), Richard-Willstätter-Straße 11, 12489, Berlin, Germany

^b Technischen Universität Berlin, Straße des 17. Juni 135, 10623, Berlin, Germany

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ABSTRACT

The plant secondary metabolite families of coumarin and 4-hydroxy coumarin have a broad pharmacological spectrum ranging from antibacterial to anticancer properties. One prominent member of this substance class is the synthetic but naturally inspired anticoagulant drug and rodenticide warfarin (coumadin). A vast number of publications focus on the identification of warfarin and its major cytochrome P450-mediated phase I metabolites by liquid chromatography (LC) with mass spectrometry (MS) and tandem mass spectrometric (MS/MS) detection techniques. For the first time, electron ionization (EI) induced high-resolution quadrupole time-of-flight mass spectrometric (HR-qToF-MS) data of in-liner derivatized warfarin and selected hydroxylated species is provided in this study as an alternative to LC-MS/MS approaches. Furthermore, the characteristic fragments and fragmentation pathways of the analyzed methyl ethers are concluded. The obtained data of analytical standards, specific deuterated and ¹³C-labeled compounds prove inductive cleavage of the acyl or acetyl side chain, methyl migration, and H-migration, along with consequential inductive cleavage as predominant fragmentation routes. Based on the HR-spectral data, commonalities and differences between the analyzed compounds and fragment groups were evaluated with future applicability in structure elucidation and spectra prediction of related compounds.

1. Introduction

Coumarin and 4-hydroxy derivatives are widely distributed substance classes harboring the 2*H*-chromen-2-one (1,2-benzopyrone) motif. Naturally, these plant-derived secondary metabolites are synthesized via the shikimate biosynthetic pathway [1,2] and have a broad range of pharmacological applications. Reported bioactivities are anti-inflammatory, antibacterial, antifungal, and anticancer [1,3]. Warfarin (Coumadin), the natural product-derived synthetic anticoagulant, was developed by Link et al. in the 1940s as a result of the findings on the ‘hemorrhagic agent’ dicumarol (4-hydroxy-3-[(4-hydroxy-2-oxochromen-3-yl)methyl]chromen-2-one) [4,5]. Warfarin has been one of the most popular therapeutics to prevent thrombosis, atrial fibrillation, and fibrillation since its FDA approval in the 1950s [4,6]. Because of its ability to prevent blood coagulation by inhibiting vitamin K-dependent carboxylation of several blood clotting precursors, it is also used as a rodenticide worldwide [7]. Warfarin has been partially substituted by

far more toxic ‘second generation anticoagulant rodenticides’ [6]. Which have also been found in numerous targeted and untargeted wild-life species. Additionally, there is evidence of anticoagulant rodenticides being present in various environmental compartments. Their presence in aquatic ecosystems and life forms was recently summarized by Regnery et al. [8]. Moreover, anticoagulant rodenticides have been determined in agricultural products [9] and wastewater [10,11]. Numerous publications are reporting the analysis of warfarin and related species by high-performance liquid chromatography with mass spectrometric detection like ultra-high-performance liquid chromatography coupled to high-resolution tandem mass spectrometry (UHPLC-HR-MS/MS) [12], also employing non-targeted approaches using ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight tandem mass spectrometry (UHPLC-qToF-MS/MS) [13], and specific quantification of enantiomeric compounds by UHPLC-triple quadrupole (QqQ)-MS/MS in human plasma [14]. Warfarin’s CYP450-mediated metabolism and hydroxylated phase I metabolites in

* Corresponding author. Bundesanstalt für Materialforschung und -prüfung (BAM), Richard-Willstätter-Straße 11, 12489, Berlin, Germany
E-mail address: christian.piechotta@bam.de (C. Piechotta).

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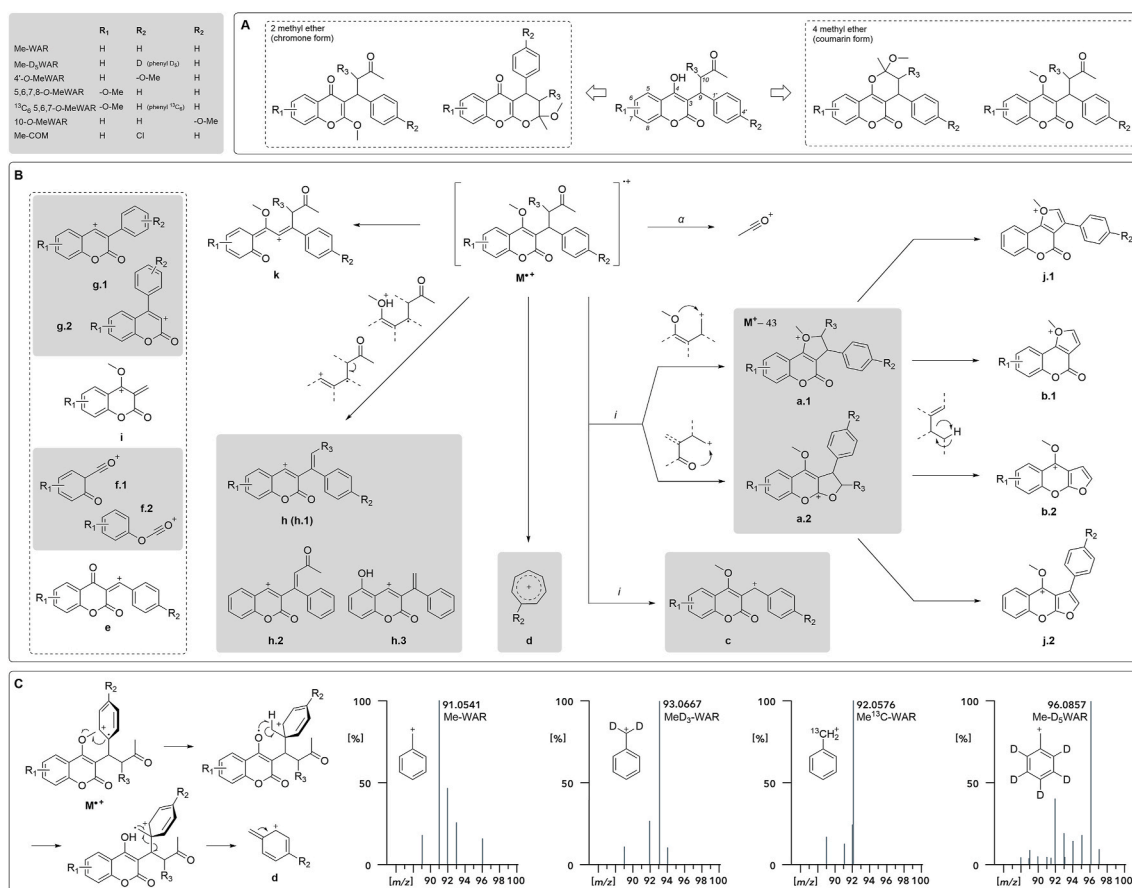
rats, humans, other mammals, and bird species have been extensively studied [15–18]. The major hydroxylated metabolites are 4'-hydroxy warfarin, 6-hydroxy warfarin, 7-hydroxy warfarin, 8-hydroxy warfarin, and 10-hydroxy warfarin [15]. To this day, there is a scarcity of publications found containing literature on gas chromatographic separation of warfarin and its hydroxylated metabolites. Analyses were performed utilizing different types of detectors, mass spectrometric analysis by electron ionization (EI) mass spectrometry being one of them. Most of these publications date back to the 1970s up till the 1990s and do not contain HR-EI data. While resulting EI fragments are described partly [19–23]. Besides the high reproducibility of generated EI-MS spectra, EI-fragmentations usually contain more structural information compared to LC-MS/MS spectra of small molecules. This is highly significant for structural verification and elucidation of known and unknown compounds. Herein, a gas chromatography-electron ionization-high resolution time-of-flight -mass spectrometry (GC-EI-HR-qToF-MS) analysis of in-liner methylated warfarin and selected hydroxylated metabolites is utilized. General fragmentation pathways and common fragment types within the substance family were identified based on high-resolution mass spectrometric data, assisted by isotopic labeled reference compounds (see Scheme 1).

2. Experimental section

Analytical grade warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)chromen-2-one) (PESTANAL®), analytical standard, Fluka, Sigma-Aldrich), phenyl-D₅-warfarin (4-hydroxy-3-(3-oxo-1-(2,3,4,5,6-pentadeuteriophenyl)butyl)chromen-2-one) (Dr. Ehrenstorfer GmbH, Augsburg, Germany), and analytical grade reference standards of 4'-hydroxy warfarin (4-hydroxy-3-(1-(4-hydroxyphenyl)-3-oxobutyl)chromen-2-one) (Sigma-Aldrich) and 10-hydroxy warfarin (4-hydroxy-3-(2-

hydroxy-3-oxo-1-phenylbutyl)chromen-2-one) (Sigma-Aldrich) were purchased from different suppliers. Reference compounds such as 6-hydroxy warfarin (4,6-dihydroxy-3-(3-oxo-1-phenylbutyl)chromen-2-one), 7-hydroxy warfarin (4,7-dihydroxy-3-(3-oxo-1-phenylbutyl)chromen-2-one), and 8-hydroxy warfarin (4,8-dihydroxy-3-(3-oxo-1-phenylbutyl)chromen-2-one) were purchased from Chemos (Chemos GmbH Co. KG, Altdorf, Germany), while 5-hydroxy warfarin (4,5-dihydroxy-3-(3-oxo-1-phenylbutyl)chromen-2-one), as well as phenyl ¹³C₆-labeled isotopomers of 5-hydroxy warfarin, 6-hydroxy warfarin, 7-hydroxy warfarin (4-trideuteriomethoxy-3-(3-oxo-1-phenylbutyl)chromen-2-one), and (4-(¹³C)methoxy-3-(3-oxo-1-phenylbutyl)chromen-2-one) were kindly synthesized by ASCA GmbH (Angewandte Syntheschemie Adlershof, Berlin, Germany). Coumachlor (Sigma-Aldrich) was included as an additional reference compound.

Stock solutions of the analytes were prepared in MS-grade acetonitrile (Biosolve BV, Valkenswaard, The Netherlands.) and diluted to a final concentration of 10 µg/mL including *m*-TFPTAH, trimethyl-3-trifluoromethyl phenyl ammonium hydroxide 5% in methanol (abr GmbH, Karlsruhe, Germany), for in-liner methylation of hydroxyl groups. Reference compounds were subjected to Agilent's s GC-EI-HR-qToF system (7250 GC/Q-TOF, 8890 GC) equipped with Gerstel's customized MultiPurpose Sampler, while 1 µL of the described standard solution was injected by splitless injection or by split injection employing different split ratios, respectively. A helium flow rate of 1 mL/min was used for chromatographic separation on a DB5 MS-UI 30 m × 0.25 mm × 0.25 µm (Agilent Technologies) fused silica capillary column using the following oven program: 60 °C → 320 °C (10 K/min, 1 min hold 320 °C) and 100 °C → 320 °C (10 K/min, 2 min hold 320 °C). The split/split less injector temperature was set to 270 °C, while the transfer line was heated to 280 °C. Ionization was achieved by standard electron energy of 70 eV and 5.0 µA emission current at 200 °C, relative



Scheme 1. Fragmentation pathways of warfarin and hydroxylated warfarin species.

to the latest used tune file. Mass calibration of the qToF analyzer was performed prior to analysis. Data was recorded in centroid mode in a mass range of 40–500 Da.

3. Results and discussion

Initial experiments showed that underivatized warfarin and related species, including benzylic modifications, are partially detectable by GC-EI-MS, while derivatives harboring additional hydroxylations of the 4-hydroxy coumarin moiety cannot be detected without decomposition. These results indicate the necessity of a derivatization procedure. In literature, reported derivatization procedures for instances of alkylation reactions, resulting in ethyl-derivatives [24] and pentafluorobenzyl-derivatives, respectively [25]. Methylation was achieved by extractive methylation using methyl iodide described by Maurer et al., (trimethylsilyl)diazomethane as demonstrated by Sato, or applying ethereal diazomethane, respectively [26–28]. In this study, the alkylation of hydroxyl groups is performed with *m*-TFPTAH to improve the thermal stability and volatility of the analytes. Etherification processes in the injection port within the liner at high temperatures [26,29,30].

Fig. 1B comprises the chemical structures of the racemic analytical standards used within this study to identify similarities and differences in the fragmentation pathways of hydroxylated species, such as 5-, 6-, 7-, and 8-position, apart from warfarin (WAR). In addition, commercially available phenyl-D₅-warfarin (D₅WAR) was utilized. The resulting mass shifts aid in identifying fragments containing the isotopically labeled moieties. 5-, 6-, and 7-hydroxy warfarin were synthesized by ASCA GmbH as phenyl-¹³C labeled compounds to accomplish this. Furthermore, 10-hydroxy warfarin (10-OH-WAR), 4'-hydroxy warfarin (4'-OH-WAR), and coumachlor (COM) are included to explore the impact of hydroxylation on their fragmentation behavior at different positions in the molecule. Since all compounds undergo methylation during derivatization, isotopically labeled methoxy species, e.g., 4-O-(¹³C) methyl- (Me¹³C-WAR) and 4-O-trideuteromethyl warfarin (MeD₃-WAR) were additionally incorporated to identify the location of the methyl group during fragmentation. Molecular formulas of relevant and characteristic fragment ions are computed based on the exact mass measurements. The theoretical masses and the exact mass measurements are compiled in Table S1 in the supporting information. HR *m/z* values of fragment ions, which are not mentioned in Table S1 (in the supporting information) are discussed within the text.

Initial attempts to evaporate the solvent and the derivatization agent separately by utilizing a programmable temperature vaporizing injector

did not show any difference to an injector temperature maintained at 270 °C. EI-MS spectra were recorded at the given mass range with a data threshold of 100 counts and an acquisition rate of 5 Hz. Integration and background subtraction were performed using Agilent's MassHunter Qualitative Analysis 10.0 software. The resulting EI-MS spectra were exported and processed in Origin 2020 for graphical use. Proposed fragments and pathways were compared to literature data on underivatized [19–21] and methylated compounds [22,23]. They are illustrated in Fig. 2, and the recorded spectra of the analytes, including the ratios of the *m/z* values, are summarized in the supporting information in Figs. S1–S14. Fig. 15S (in the supporting information) presents the individual Extracted-Ion Chromatograms (EICs) of the investigated species, with each compound labeled according to its retention time.

Due to the stereocenter in the 9-position, warfarin consists of two enantiomers. However, its tautomerism is even more complex. Fig. 1A summarizes the keto-enol tautomerism, the tautomerism of the keto-lactone moiety, and the ring closure, resulting in 11 distinct tautomers not considering stereochemistry [31,32]. With defined stereochemistry, 40 tautomeric forms can theoretically derive from racemic warfarin [31,32]. Even though the derivatization of hydroxyl groups suppresses certain tautomeric interconversions, alkylation may lead to a mixture of products. Regarding the keto-enol tautomerism, the 4-hydroxy coumarin moiety is converted into the 2-hydroxy chromone moiety via the 2,4-chromandione, further undergoing ring closure and opening [32]. Therefore, methylation results in the 4-methyl ether in the coumarin form and the 2-methyl ether in the chromone form, both in the open-chain and cyclic hemiketal form. Due to the substantial structural differences between the open-chain and the cyclic hemiketal form, EI ionization may result in fragments originating from tautomers. In this study, fragments are postulated from the 4-methyl ether in open-chain form only because the high temperatures in the injector are expected to favor this form for entropic reasons. Combining cheminformatics and quantum chemical investigations, Guasch et al. showed that the cyclic hemiketal form of warfarin is the most stable tautomer in solution [31]. In a vacuum, the open-chain form is preferred [31]. Furthermore, the EI-MS spectra presented here align with the published low-resolution spectra of the open-chain form of warfarin [20,22,26,28].

3.1. Initial fragmentation steps by cleavage of the acyl and acetonil side chain

Most EI-qToF-MS spectra mainly contain low abundance signals but show one characteristic base peak. The radical cation M^+ has a generally low abundance throughout the investigated spectra and is not

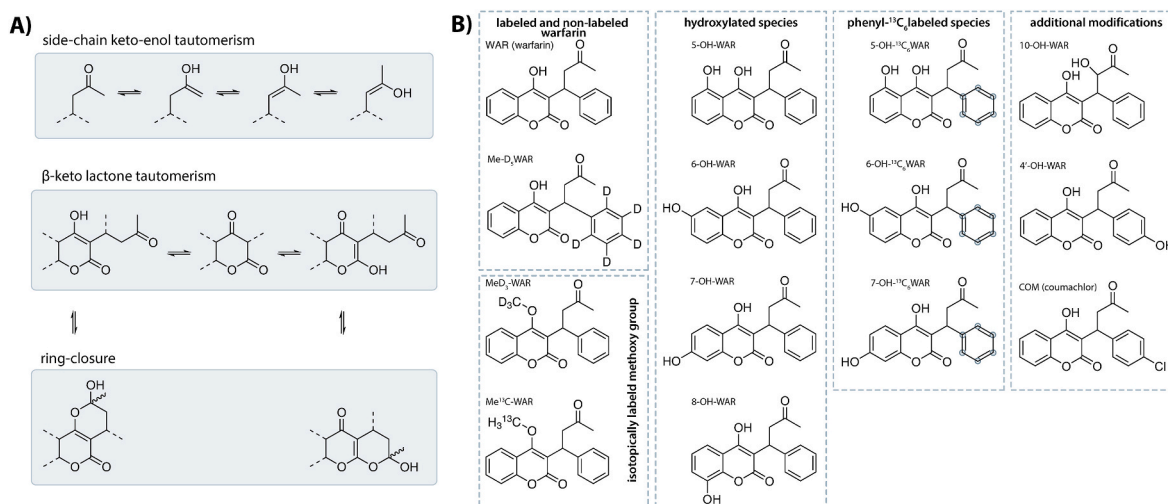


Fig. 1. Scheme of (A) the tautomerism of warfarin and related species and (B) of the compounds and isotopically labeled substances used in this work.

detectable in the spectrum of 10-Me-O-WAR. This compound is unique among the investigated species because of the methoxy substituent in the 10-position. Compared with the other investigated species, 5-Me-O-WAR and 10-Me-O-WAR show more abundant signals in their spectra. The location of the radical-cationic site in nonbonding oxygen orbitals of the ketone rationalizes the formation of the acetylium cation (m/z 43.0178) by radical-initiated α -cleavage (α), which is found without exception in the analyzed data. Whereas charge-initiated α -cleavage, inductive cleavage (l), induces the loss of the acetyl-radical [23], leading to the generation of cation series **c**, and undergoes the loss of the acyl-radical leads to the formation of cation $M^+ - 43$. Except for 5-Me-O-WAR and 10-Me-O-WAR, the base peak is the $M^+ - 43$ signal of the ion type **a**. After inductive cleavage, the positive charge in 10-position induces ring closure via a lone pair of either the methoxy group forming structure **a.1**, or the carbonyl oxygen of the lactone forming structure **a.2**. Ring closure was previously postulated via the methoxy group as well as the secondary alcohol of underivatized warfarin species [20,22,23]. The neutral loss of the benzene ring through homolytic cleavage and subsequent H -abstraction leads to the generation of the resonance-stabilized furano-oxonium-ions **b.1** and **b.2** [20,22,23]. Spectra of 5-Me-O-WAR and 10-Me-O-WAR do not contain ions of this group. In Table S1, in the supporting information, masses of type **b** fragments are given, and an additional signal can be assigned to this group only in the spectra of Me-D₅WAR.

Regarding the expected mass, it is shifted by one Da to m/z 202.0608 ($C_{12}H_8DO_3^+$; theoretical mass 202.0609 Da; Δ ppm -0.49), indicating the exchange of deuterium from the aromatic ring for a hydrogen atom. The migration of aromatic hydrogen or deuterium is plausible, e.g., if the radical cation was located within the benzene ring. But this is unlikely to happen during the formation of ion **b** from cation **a** because both ions show an even number of electrons. Therefore, aromatic hydrogen migration takes place via a transition state immediately after ionization. Cation **j** can be formed in two constitutional isomers undergoing a similar rearrangement, like **a** to **b** undergoing the loss of hydrogen instead of benzene [20,22,23]. Remarkably, ion **j** is not detectable with additional methoxy substituents at the coumarin moiety. This ion type can be rationalized as a dehydro-form of ion type **a**.

Locating the radical cation at the oxygen of the methoxy group in the 4-position leads to H -abstraction and consequent inductive cleavage of this group undergoing the loss of an acyl radical to form fragment type **h**. This ion cannot be assigned correctly in the obtained methylated coumachlor (Me-COM) spectra because of a high mass deviation compared to the theoretical value of 281.0369 Da. However, the recorded signal at 281.0508 clearly shows the isotopic pattern of chlorine. Surprisingly, the $M+2$ signal at m/z 283.0330 shows a mass deviation of -1.77 compared to the expected mass of 283.0335 Da of the $h+2$ ion ($C_{17}H_{10}ClO_2^+$) and can be assigned. In Me-D₅WAR, an additional signal at m/z 251.0999 ($C_{17}H_7D_4O_2^+$; theoretical mass 251.1005 Da; Δ ppm -2.39) proves the exchange of deuterium to hydrogen on the phenyl ring.

Fragment **h** (**h.1**) is the second most intensive peak in the spectrum of 10-Me-O-WAR. It is preferably formed because the methoxy and ketone support inductive cleavage in this position. Notably, the hydroxylated fragment ion **h.1** of 10-Me-O-WAR shows the same theoretical mass as fragment ion **j** of Me-WAR but not with the expected mass of **j** for 10-Me-O-WAR containing the methoxy group. Undergoing the loss of the methoxy group, the expected mass and structure is identical with ion **j** in Me-WAR. It can, therefore, not be excluded that the signal at m/z 277.0860 might comprise ions **h.1** and **j**. The free rotation of the C3–C9 bond places the radical side and the phenyl ring in close vicinity. In the spectrum of Me-D₅WAR, not only was a signal of the D₅-species of fragment **h** exposed ($C_{17}H_6D_5O_{2+}$; theoretical mass 252.1067 Da; Table S1 in the supporting information), an additional D₄-signal ($C_{17}H_7D_4O_2^+$; theoretical mass 251.1005 Da; observed m/z 251.1005; Δ ppm -0.00) further indicates hydrogen-deuterium exchange. The findings suggest that the acyl radical is more likely to be cleaved than

the hydrogen radical, following the described fragmentation route in Fig. 2. In 10Me-O-WAR, the substitution of the hydrogen in 10-position by a methoxy group consequently leads to the formation of the additional cation **h.2** ($C_{18}H_{13}O_3^+$; theoretical mass 289.0859 Da; observed m/z 289.0857; Δ ppm -0.69). The compounds 5-Me-O-WAR, 6-Me-O-WAR, 7-Me-O-WAR, and 8-Me-O-WAR contain the methoxy group at the coumarin moiety. The EI-MS spectra of 6-, 7-, and 8-Me-O-WAR are almost identical.

Throughout the investigated spectra, fragment ion **h.1** shows low abundances, but it is the base peak in the spectrum 5-Me-O-WAR. This finding suggests that the proximity of the oxygen atoms in the 4- and 5-position favors H -migration via a six-membered ring transition state. As proposed in Fig. 2, inductive cleavage of the methoxy group in the 4-position realizes the loss of methane. Because of the transition state, the methoxy group in the 5-position can also be inductively cleaved. In that case, two constitutional isomers are formed. That also applies to cation **h.3** (m/z 263.0703). Instead of hydrogen, methyl migration via the six-membered ring transition can be postulated. At the same time, the loss of dimethyl ether leads to fragment ion **h.3** ($C_{17}H_{11}O_3^+$; theoretical mass 263.0703 Da; Δ ppm 0.00). The $^{13}C_6$ -labeled signal at m/z 269.0900 ($C_{17}^{13}C_6H_{11}O_3^+$; theoretical mass 269.0904 Da; Δ ppm -1.49) supports this hypothesis.

3.2. Tropylium ion formation by methyl migration

Throughout the obtained spectra, tropylium and tropylium-like ions (**d**) are observable. These ions fragment into cyclopentadienylium-type cations. Since the benzene ring is linked via a tertiary carbon in warfarin and related compounds, heterolytic bond cleavage results in the loss of a phenylium ion [20,22], which is found only with less intensive signals within the investigated spectra of non-labeled compounds at m/z 77.0387, as well as deuterated ($C_6H_2D_3^+$; theoretical mass 80.0574 Da; observed m/z 80.0577; Δ ppm 3.75), ($C_6H_1D_4^+$; theoretical mass 81.0637 Da; observed m/z 81.0636; Δ ppm -1.23), ($C_6D_5^+$; theoretical mass 82.0700 Da; observed m/z 82.0701; Δ ppm 1.22), and ^{13}C -labeled species ($^{13}C_6H_4^+$; theoretical mass 83.0587 Da; observed m/z 83.0587; Δ ppm 0.00). Since tropylium ions cannot result from heterolytic cleavage, an explanation points to a methyl migration, which was first reported by de Vries et al. in the structurally related compound phenprocoumon (4-hydroxy-3-(1-phenyl propyl)-2H-chromen-2-one) after methylation by low-resolution direct insertion EI-MS [33]. The study used 4- O -(^{13}C)-methyl phenprocoumon and 4- O -trideuteromethyl phenprocoumon, among other isotopically labeled compounds, to verify this hypothesis. The authors stated that methyl migration is one of two possible ways to generate tropylium ions, while the resulting mass shifts proved the incorporation of the isotopically labeled methyl group.

Fig. 3 A illustrates the radical-induced methyl migration and H -abstraction forming tropylium and tropylium-like ions. The radical cation in the aromatic phenyl ring initiates the reaction. In a six-membered transition state, the methyl group migrates in 1'-position, followed by H -abstraction of the C4-oxygen forming, a primary hydroxyl group. By recombination of the radical, the tropylium ion acts as a leaving group. In this study, the proposed methyl migration was further mechanistically investigated utilizing synthesized ^{13}C - and D_3 -labeled methyl ethers in the 4-position of the coumarin moiety and phenyl-D₅-labeled warfarin. Fig. 3 B displays the observed EI-HR mass spectra of Me-WAR, Me-D₅WAR, Me- ^{13}C -WAR, and MeD₃-WAR, including the deviation of the experimental and theoretical m/z values. The GC-EI-HR-MS spectrum of Me- ^{13}C -WAR shows the expected mass shift of +1 Da (Fig. 3 B) non-labeled Me-WAR, confirming the migration of the methyl group of the methyl ether in the 4-position. The observed mass shift of +2 Da (Fig. 3 B) in MeD₃-WAR can be explained by deuterium-abstraction, further corroborating the proposed mechanism. Additionally, the +5 Da (Fig. 3 B) shifted tropylium-like ion in Me-D₅WAR, demonstrating the H -abstraction of the methyl group and not the phenyl ring protons. These findings are also supported by the values from 5-,6,

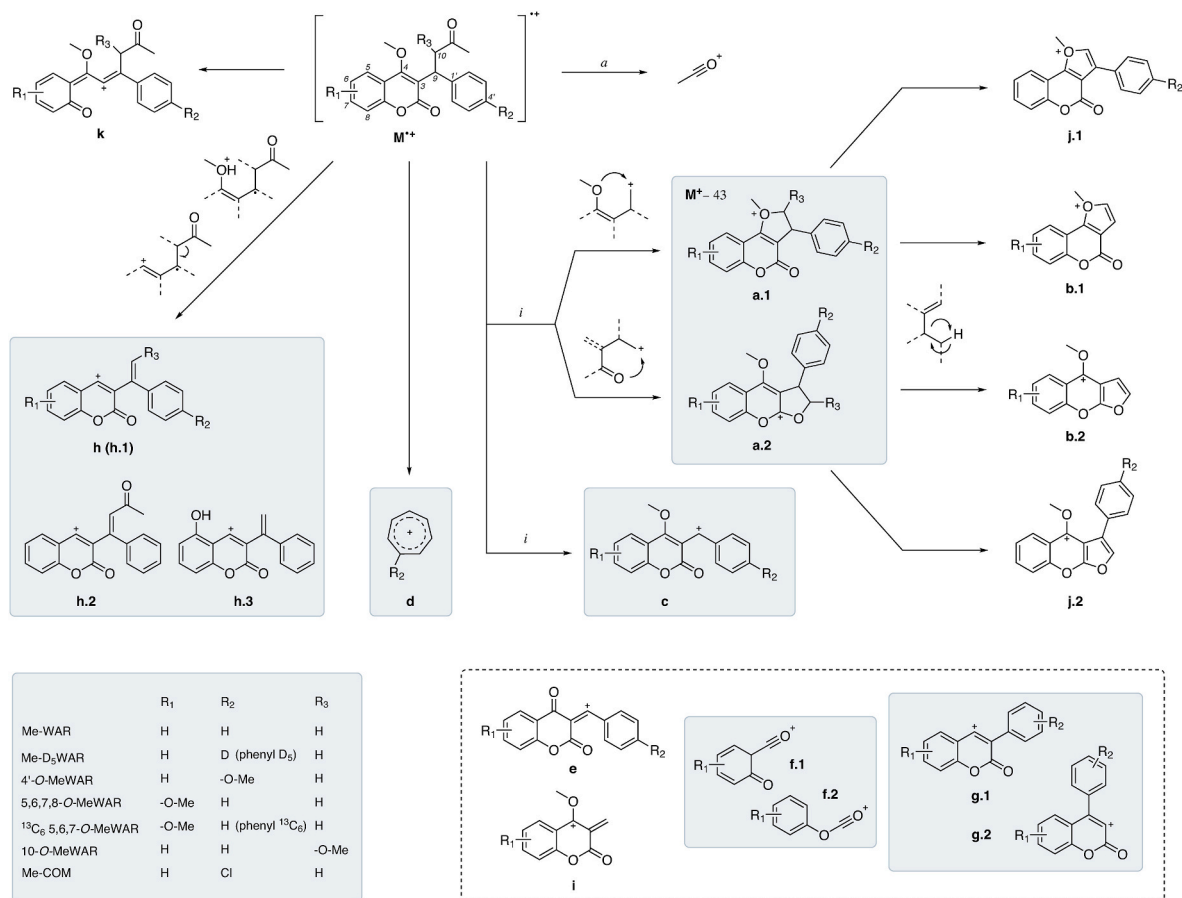


Fig. 2. Overview of the proposed EI-fragmentation pathways. Stereochemistry is intentionally not indicated, as all experiments were performed on racemic compounds. The fragmentation pathways contain lowercase Latin letters indicating common fragment types. Shared fragment types are highlighted in grey boxes.

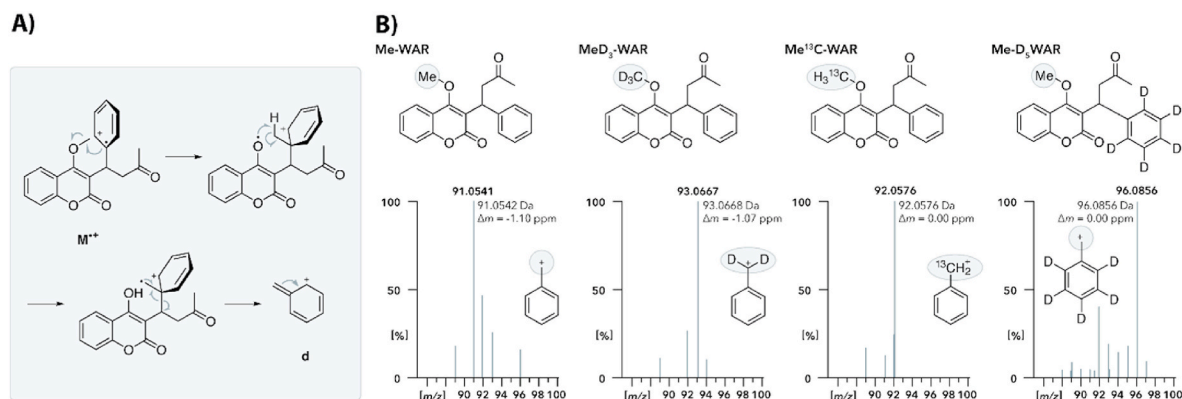


Fig. 3. The proposed methyl migration scheme and the detected tropylium ions of non-labeled and isotopically labeled warfarin species. (A) Proposed pathway of the methyl migration and (B) selected EI-HR-MS spectra of Me-WAR, MeD₅WAR, Me¹³C-WAR, and Me-D₅WAR in the zoomed region from m/z 86 to 100 showing the signals of tropylium ion. In the spectra, interfering signals are found at m/z 92.0256/7, which do not correspond to the isotopic distribution of tropylium ions (C₆H₄O⁺; theoretical mass 92.0262 Da; observed m/z 92.0257 (Δ ppm 0.37) and 92.0256 (Δ ppm -0.72)).

and 7-Me-O-¹³C₆WAR (¹³C₆C₁H⁺; theoretical mass 97.0744 Da; observed m/z 97.0743; Δ ppm -1.03).

3.3. Further proposed fragmentation pathways

Moreover, there is further evidence of complex rearrangements. One example is the proposed cation **g**. It consists of the 4-methoxy coumarin moiety after a subsequent loss of the methoxy group and the acetyl side chain. The ion still harbors the phenyl ring, verified by mass shifts in

phenyl-d₅ and phenyl-¹³C₆ labeled reference standards. In warfarin and structurally related species, the phenyl ring connects via the tertiary carbon in the 9-position, which is not part of ion **g**. The original position of the benzene moiety must have been altered. Two constitutional isomers (**g.1** and **g.2**), where the phenyl ring is either bound to C3 or C4, are proposed in Fig. 2. Because of a high mass deviation between the theoretical mass and the observed m/z value regarding the spectra of Me-D₅WAR, only the assignment of the D₄-signal (C₁₅H₅D₄O₂⁺; theoretical mass 225.0843 Da; observed m/z 225.0843; Δ ppm -2.22) and

not the D₅-signal (C₁₅H₄D₅O₂⁺; theoretical mass 226.0911 Da; observed *m/z* 226.0885; Δppm −11.50, see Table S1) is determined. This result suggests hydrogen and deuterium exchange at the phenyl ring.

An exception in the spectrum of 5-Me-O-WAR is the relatively high abundance of cation **k**. Mechanistically, it is formed by locating the radical cation at the carbonyl oxygen of the lactone. Undergoing reorientation of the conjugated system and subsequently losing CO results in the proposed structure in Fig. 2. Nevertheless, ring closure of the cationic site with a lone pair of the carbonyl oxygen or enlargement of the conjugated π-system by the carbonyl group are possible reaction pathways. Cation families **e** and **i** were proposed directly from the obtained HR masses, while cation **e** is found in relatively low intensities in all spectra except for 5-Me-O-WAR. In addition to the assigned signal of ion **e** in the spectra of Me-D₅WAR, a signal *m/z* 253.0795 (C₁₆H₅H₄O₃⁺; theoretical mass 253.0797 Da; Δppm −0.79) further proves hydrogen-deuterium exchanges. The fragment ion **i** could not be detected in the spectrum of 10-Me-O-WAR. Cation **f.1** may result from **e** by reorientation of the C=C double bonds within the ring system [20], but it is observed throughout all obtained spectra, including 5-Me-O-WAR where ion **e** is absent, suggesting a different precursor ion and supporting the presence of a cation of the chemical structure **f.2**.

4. Conclusion

In conclusion, the presented study employed gas chromatography coupled with electron ionization high-resolution mass spectrometry (GC-EI-HR-MS) to investigate the derivatization of warfarin and its related compounds. The study demonstrated the necessity of a derivatization procedure and employed the alkylation of hydroxyl groups with *m*-TFPTAH to improve the thermal stability and volatility of the analytes.

By computing the molecular formulas of characteristic and abundant EI-induced fragment ions based on exact mass measurements, the study facilitated identification using characteristic mass shifts of isotopically labeled compounds and additional substituents. The study elucidated common fragmentation pathways, revealing major fragmentation routes involving cleavage of the acyl and acetyl side chains. The investigation also provided insights into the formation of tropylium ions via methyl migration and *H*-abstraction, supported by isotopically labeled compounds. Furthermore, the study proposed complex rearrangements.

The comprehensive analysis presented in this study enhances the understanding of the derivatization and subsequent GC-EI-HR-MS analysis of warfarin and related compounds. The proposed derivatization strategy, along with the elucidation of fragmentation pathways and rearrangements, contributes valuable insights to the analytical characterization of these compounds. Quantum mechanical methods might be advantageous to verify the described fragment ion formation in the future. Precise knowledge of the structure of fragment ions is beneficial when choosing selective MRM-transitions for quantitative analysis or structure elucidation of related species.

CRedit authorship contribution statement

W.J. von Törne: Methodology, Project administration, Supervision, Writing – review & editing, Conceptualization. **L. Steinhäuser:** Investigation, Methodology, Validation, Writing – original draft. **U.-A. Klyk-Seitz:** Data curation, Formal analysis, Investigation, Writing – original draft. **C. Piechotta:** Conceptualization, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijms.2024.117220>.

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