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Gas phase retro-Michael reaction resulting from dissociative protonation: fragmentation of protonated warfarin in mass spectrometry

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A mass spectrometric study of protonated warfarin and its derivatives (compounds 1 to 5) has been performed. Losses of a substituted benzylideneacetone and a 4-hydroxycoumarin have been observed as a result of retro-Michael reaction. The added proton is initially localized between the two carbonyl oxygens through hydrogen bonding in the most thermodynamically favorable tautomer. Upon collisional activation, the added proton migrates to the C-3 of 4-hydroxycoumarin, which is called the dissociative protonation site, leading to the formation of the intermediate ion-neutral complex (INC). Within the INC, further proton transfer gives rise to a proton-bound complex. The cleavage of one hydrogen bond of the proton-bound complex produces the protonated 4-hydroxycoumarin, while the separation of the other hydrogen bond gives rise to the protonated benzylideneacetone. Theoretical calculations indicate that the 1, 5-proton transfer pathway is most thermodynamically favorable and support the existence of the INC. Both substituent effect and the kinetic method were utilized for explaining the relative abundances of protonated 4-hydroxycoumarin and protonated benzylideneacetone derivative. For monosubstituted warfarins, the electron-donating substituents favor the generation of protonated substituted benzylideneacetone, whereas the electron-withdrawing groups favor the formation of protonated 4-hydroxycoumarin. Copyright © 2012 John Wiley & Sons, Ltd.

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Keywords: retro-Michael reaction; dissociative proton transfer; ion-neutral complex; warfarin; mass spectrometry

INTRODUCTION

Electrospray ionization mass spectrometry (ESI-MS), which creates solvent-free and unimolecular conditions for gas phase reactions, not only can provide extensive structure information for small organic molecules and biological macromolecules, but also can play an important role in understanding the intrinsic character of the reaction and throwing light on reactivity trends in the condensed phase.[1] In fact, the mass spectrometer has been termed a 'complete chemical laboratory'.[2]

The Michael reaction is one of the most important carbon–carbon and carbon–heteroatom bond-forming process in organic synthesis.[3–5] Over the past decades, a tremendous amount of effort has been devoted to the utilization of the Michael reaction to generate drug-like scaffolds. Warfarin and its derivatives are successful examples of Michael addition of 4-hydroxycoumarin to benzylideneacetone. Warfarin, which has been introduced for clinical use as Marevan, is one of the most widely prescribed anti-thrombotics to help prevent and treat blood clots associated with heart-value replacement or an irregular, rapid heartbeat. As an important chemical pathway of this anti-thrombotic drug, a thorough understanding of the MS/MS and MS^n data of warfarin compounds is essential for bioactivity mechanisms, degradation pathways and metabolism research.

Protonation and proton transfer are of fundamental importance in the explanation of chemical reactions in gas phase.[6–9] The positive charge brought in by protonation is usually the driving force for fragmentation reactions of the protonated molecules, which is termed the dissociative proton attachement.[10–12] However, in some cases, no fragmentation occurs when protonation takes place at the thermodynamically preferred site, but a major reaction is found when the added proton is transferred to a different position, which is described as the dissociative protonation site.[13–16]

Dissociative protonation sometimes does not directly form the final products but result in the non-conventional ion-neutral complex (INC).[13,17] INCs are frequently formed as reactive intermediates in the course of unimolecular dissociation reactions in a mass spectrometer.[18–21] In an INC, the ionic fragment and the neutral molecule are held together by electrostatic interactions. Within INC, various chemical reactions may occur either between the two partners or inside the ion moiety alone prior to the final separation. Recently, more examples on INC-mediated fragmentation reactions in ESI MS have been reported.[22–31] In this paper, we report the results of retro-Michael reaction of warfarin and its derivatives initiated by dissociative proton transfer in MS in conjunction with auxiliary theoretical calculations.

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EXPERIMENTAL

MS

All collision-induced dissociation (CID) experiments were carried out on a Varian 500-MS mass spectrometer equipped with an ESI source in positive ion mode, an ion trap analyzer and the Varian MS Workstation (Varian, America). Nitrogen gas was used as the nebulizing gas and the drying gas. Ionization was performed using the following settings: spray chamber temperature 50 °C, nebulizer pressure 35 psi, drying gas pressure 15 psi, drying gas temperature 350 °C, needle voltage 5000 V, spray shield voltage 600 V, infusion rate 10 μL min^{-1}. The CID mass spectra were obtained with helium as the collision gas after isolation of the desired precursor ion, and the collision energy (resonance mode) was adjusted properly to give suitable energy for the dissociation of all compounds studied.

Accurate masses were measured on an Apex III (7.0 Tesla) Fourier transform ion cyclotron resonance (FTICR) MS (Bruker, Billerica, MA, USA) equipped with an ESI source in the positive ion mode. Sodium trifluoroacetate was used as an external calibration compound. Nitrogen was used as nebulizing gas and drying gas. Argon was used as collision gas. The capillary voltage was set at −4448 V, and drying gas temperature was set at 150 °C.

Theoretical calculations

All theoretical calculations were carried out using the Gaussian 03 package of programs. Candidate structures of the reactants, products, intermediates and transition states were optimized at the B3LYP level of theory with the 6-31G (d) basis set. No symmetry constraints were imposed on the optimizations. The reaction pathways were traced forward and backward by the intrinsic reaction coordinate method. All optimized structures were subjected to vibrational frequency analysis to ensure a transition state had only one imaginary vibrational frequency while a local or global minimum had no imaginary vibrational frequency. The energies discussed here are the sum of electronic and thermal energies.

MATERIALS

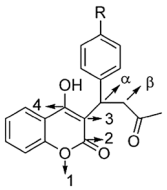
Warfarin and its derivatives bearing substituents in the para position were synthesized in a previous work.[32] Samples were dissolved in methanol for mass spectrometric analysis.

RESULTS AND DISCUSSION

Retro-Michael reaction of protonated warfarin

In the synthesis of warfarin and its derivatives, Michael reaction was utilized for the carbon–carbon bond forming process of 4-hydroxycoumarin and substituted benzylideneacetone. In the CID mass spectra of the $[M+H]^+$ ions of all warfarin compounds studied (Table 1), loss of a benzylideneacetone leading to protonated 4-hydroxycoumarin and loss of a 4-hydroxycoumarin generating protonated benzylideneacetone derivative were observed as retro-Michael reaction, along with other minor reactions. The representative compound **3**, warfarin, was selected as a model to give an explanation of the possible dissociation pathways. The full scan ESI mass spectrum of Compound **3** showed an abundant ion at *m/z* 309. Upon collisional activation, it yielded two product ions at *m/z* 163 and 147, corresponding to the protonated 4-hydroxycoumarin and protonated benzylideneacetone

Table 1. Structures of the compounds studied



Compounds	Molecular mass	R
1	386	Br
2	342	Cl
3	308	H
4	322	CH_3
5	338	OCH_3

respectively, as shown in Fig. 1. The product ion at *m/z* 291 arising from the loss of water and the ion at *m/z* 251 deriving from elimination of acetone are not discussed in this paper. To confirm the proposed structure of product ions, high-resolution FTICR MS was used for the exact mass measurements. The errors between the calculated values and the observed masses are within 1.4 ppm (Table S1 in Electronic supplementary information), indicating the probable elemental compositions of the product ions are of high confidence.

Dissociative proton transfer

Fragmentation reactions of the protonated molecules $[M+H]^+$ are usually triggered by the positive charge formed upon protonation. The warfarin molecules may be protonated at various positions, including the two carbonyl oxygen atoms, the ester oxygen of the 4-hydroxycoumarin moiety, the aromatic rings and the polar substituent. For the identification of the most favorable protonation site, theoretical calculations were carried out using the density functional theory (DFT) at the B3LYP/6-31G (d) level of theory. Overall, the computation results indicate that the carbonyl groups in a chelating conformation are the most thermodynamically favorable protonation sites as shown in Scheme 1. For warfarin and its derivatives, the fragmentation takes place only when the proton is attached to C-3 (Table 1). However, protonation at C-3 is by 17.8 kcal/mol higher than the most thermodynamically favorable protonation involving the two carbonyl groups (Scheme 1).

Since the protonation at C-3 of 4-hydroxycoumarin is the necessary step for C-3–C-α bond cleavage, there may be four pathways (Scheme 2) to generate the dissociative structure. In Route **1**, MH-**1** transforms to the formation of MH-**2** first. Then, the proton migrates

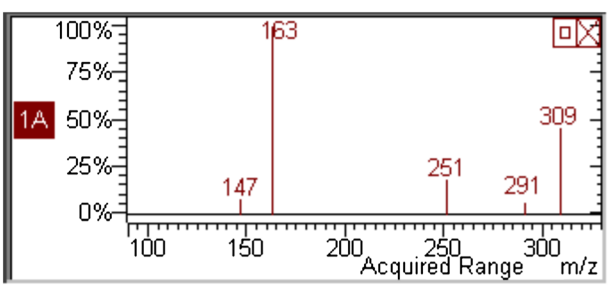
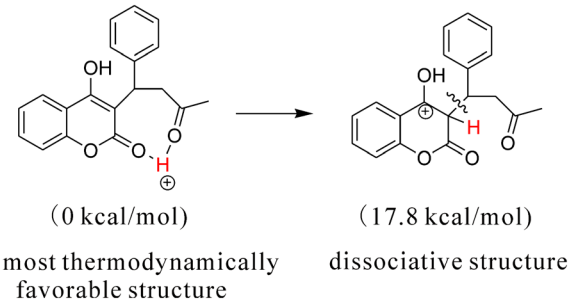
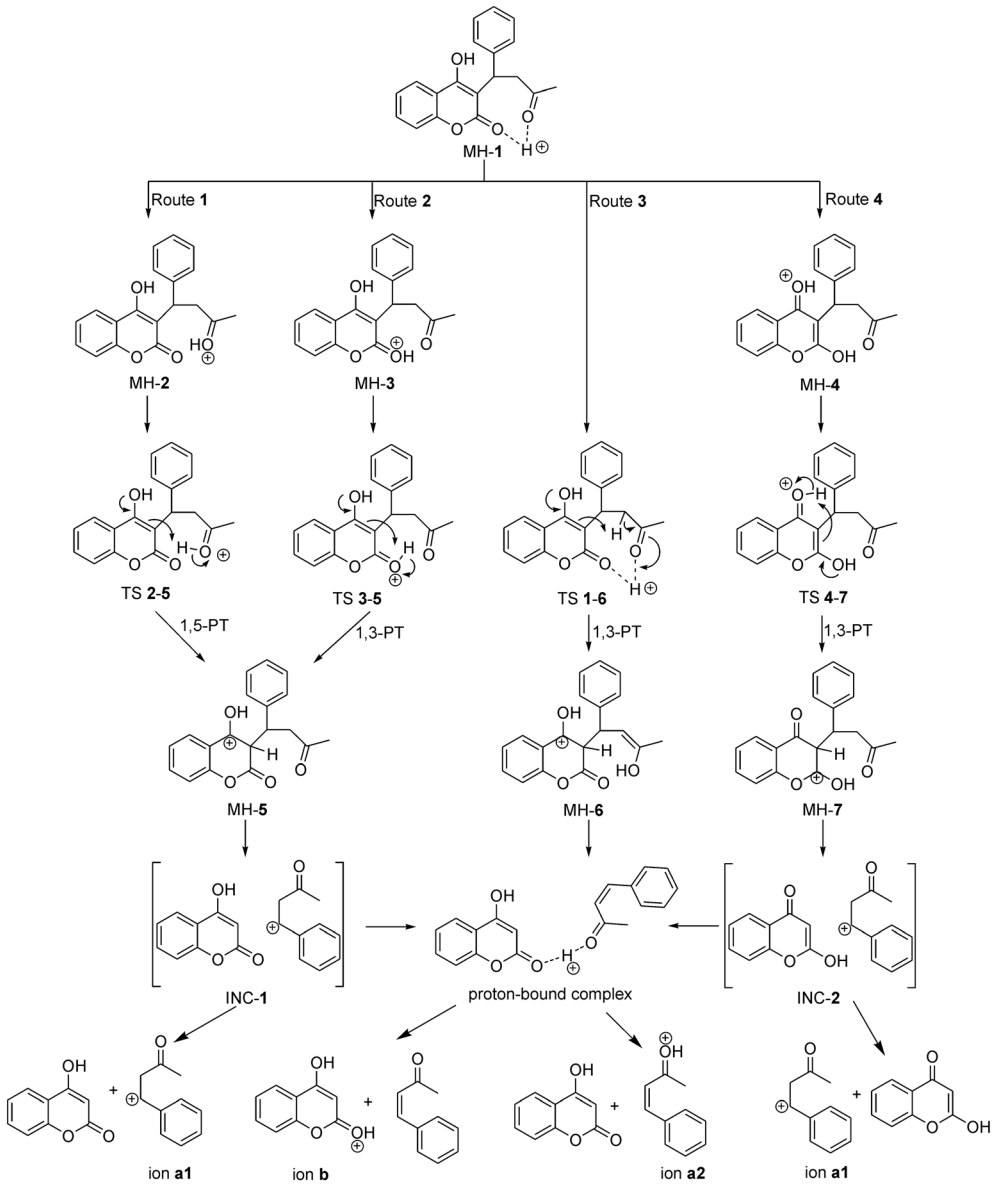


Figure 1. CID mass spectrum of the $[M+H]^+$ ion of compound **3**.

from the carbonyl oxygen of the benzylideneacetone moiety to the C-3 of 4-hydroxycoumarin, via a 1, 5-proton shift through a six-membered ring transition state (TS **2–5**), to form MH-**5**. In Route **2**, the formation of MH-**3** is derived from the cleavage of one hydrogen bond, followed by proton transfer from the carbonyl oxygen of the 4-hydroxycoumarin moiety to the C-3 of the 4-hydroxycoumarin moiety via a 1, 3-proton shift (TS **3–5**), to form MH-**5**, again. The INC-**1** arising from Route**1** and Route **2** may directly separate leading to ion **a1** or form the proton-bound complex which would give rise to ion **a2** and ion **b**. The migration of the internal proton also probably triggers the fragmentation. In Route **3**, the dissociation is invoked by the internal proton transfer from the C-β to the C-3 of the 4-hydroxycoumarin moiety via a 1, 3-proton shift (TS **1–6**). Direct cleavage induced by the formal positive charge results in the formation of the proton-bound complex, in which the hydrogen bonds bind the 4-hydroxycoumarin moiety and benzylideneacetone moiety together. In Route **4**, the migration of the hydroxyl hydrogen to the C-3 via a 1, 3-proton shift (TS **4–7**) triggers the cleavage of the C-3–C-α bond, forming the INC-**2**. The proton-bound complex may again be generated from INC-**2**.The following proton transfers within proton-bound complex are similar with Route**1** and Route **2**, leading to ion **a2** and ion **b**.



Scheme 1. Protonation sites and dissociative protonation site for warfarin.



Scheme 2. Proposed fragmentation mechanisms for the protonated warfarin.

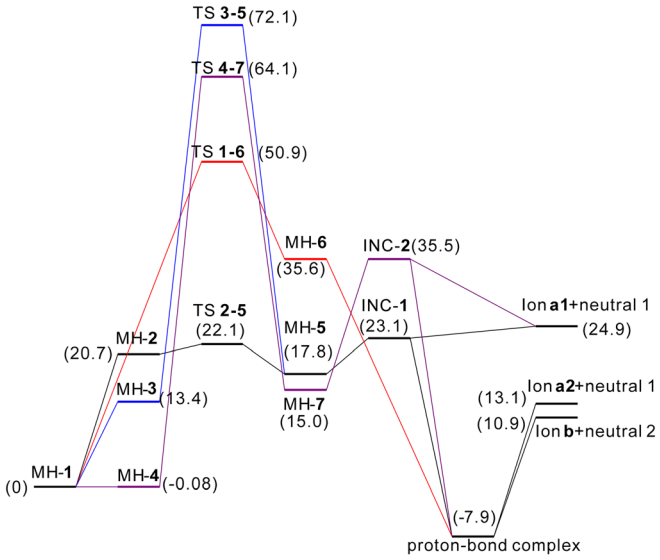


Figure 2. Potential energy diagram for the formation of the product ions **a1**, **a2** and **b** from protonated warfarin using DFT at the B3LYP level of theory with the 6–31 G (d) basis set. Relative energies are given in kcal/mol.

To quantitatively describe the energy requirements for the migration of the proton, theoretical calculations were carried out using the DFT at B3LYP/6-31G (d) level of theory. A schematic potential energy diagram for the four possible routes of fragmentation of protonated warfarin is given in Fig. 2. The activation barriers of the 1, 5-proton shift in Route **1**, 1, 3-proton shift in Route **2**, 1, 3-proton shift in Route **3** and 1, 3-proton shift in Route **4** are 22.1, 72.1, 50.9, 64.1 kcal/mol in energy, respectively. This indicates that the activation energy of 1, 5-proton shift through a six-membered ring is the lowest of the four routes, that is to say, the Route **1** is most reasonable in terms of energy.

Proton transfer via the INC

In Route **1**, to generate the product ion **b** (*m/z* 163), i.e. the protonated 4-hydroxycoumarin, there must be at least three steps: the addition of the external proton at C-3, the cleavage of C-3–C-α bond and the migration of a proton from the benzylideneacetone moiety to the 4-hydroxycoumarin moiety. The INC-mediated reaction mechanism can provide a convincing explanation of these crucial steps. The cleavage of the carbon–carbon bond linking the two moieties forms the intermediate INC-**1** (Scheme 2). Within the INC-**1**, the hydrogen at the C-β of benzylideneacetone moiety is transferred to the 4-hydroxycoumarin, generating the proton-bound complex, while direct dissociation generates the ion **a1**. The cleavage of one hydrogen bond of proton-bound complex produces the ion **b**, and the separation of the other hydrogen bond gives rise to the ion **a2** which is 11.8 kcal/mol lower than that of its isomer ion **a1**.The isomerization of **a1** to **a2** via intramolecular 1, 3-proton shift needs to surmount a considerable energy barrier of 34.6 kcal/mol (not shown in Fig. 2). However, this isomerization can be promoted by the 4-hydroxycoumarin within INC-**1**, which is known as a proton-transport catalysis process.[16,33–35]

Investigation of the substituent effects

To study the influence of the substituents on the formation of the two product ions, the spectra of a series of compounds with different substituents at *para* position of the phenyl ring were measured. Generally, all the compounds underwent similar fragmentation in the CID experiments, whereas the nature of the substituents merely affected the relative abundances (RAs) of the product ions. The RAs of the product ions are listed in Table 2 for all the compounds studied. A plot of abundance ratios of these two ions, $\ln[(C_9H_7O_3^+)/(RC_{10}H_{10}O^+)]$ *versus* the substituent constants,[36] σ_p^+,was obtained as shown in Fig. 3. The trend indicates that the electron-donating substituents favor the loss of 4-hydroxycoumarin, whereas the electron-withdrawing groups favor the loss of substituted benzylideneacetone. It seems that the substituents have no effect on the transition state for there is no conjugative effect. The reason for this trend is probably that for the compounds with electron-donating groups, the positive charge can be delocalized into substituent, resulting in more stable product ions.

The formation of the protonated 4-hydroxycoumarin and protonated benzylideneacetone derivative can also be explained by the kinetic method.[37,38] For $[M+H]^+$ ions producing two individual protonated molecules $[A+H]^+$ and $[B+H]^+$ through intermediate $[A\cdots H^+\cdots B]$, the two neutral species **A** and **B** compete for the shared proton to form the corresponding final fragment ions. The fragmentation is governed by the proton affinities (PAs) of the molecules **A** and **B**. The PA for the following reaction is defined as the negative of reaction enthalpy at 298.15K.

$$A + H^+ = [A+H]^+ \quad (1)$$

Table 2. Major product ions observed in the CID spectra of the $[M+H]^+$ ions of the compounds studied

Compounds	$[M+H]^+$	Ion **a**	Ion **b**
1	387	225 (4.6)[a]	163 (100)
2	343	181 (5.9)	163 (100)
3	309	147 (8.9)	163 (100)
4	323	161 (100)	163 (94.1)
5	339	177 (100)	163 (5.8)

[a]m/z (Relative abundance %).

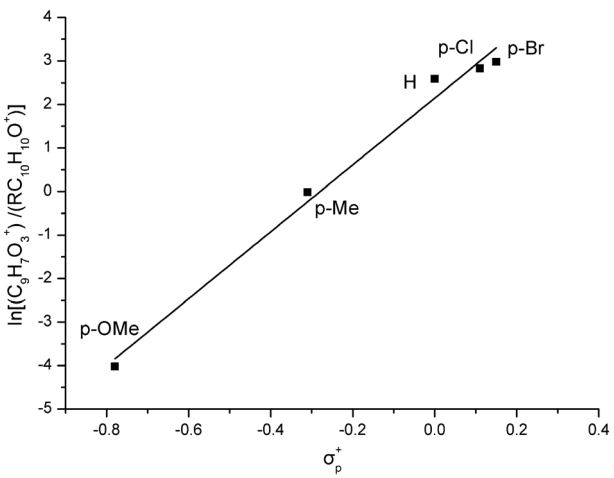


Figure 3. Plot of $\ln[(C_9H_7O_3^+)/(RC_{10}H_{10}O^+)]$ *versus* the substituent constants, σ_p^+ for the fragmentation of protonated warfarin being mono-substituted at the *para* position of the phenyl ring.

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Table 3. Computed proton affinities (PAs) of substituted benzylideneacetone (**A**) and 4-hydroxycoumarin (**B**)[a]

Compounds	PA(**A**)[b]	PA(**B**)	PA(**A**)- PA(**B**)
1	215.2	217.8	2.3
2	216.9	217.8	0.9
3	218.6	217.8	−0.8
4	221.8	217.8	−4.0
5	226.2	217.8	−8.4

[a]B3LYP/6-31 G (d)
[b]kcal/mol

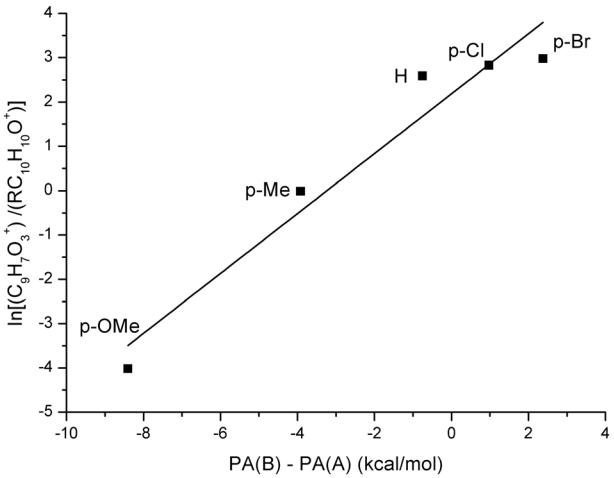

Figure 4. Plot of $\ln[(C_9H_7O_3^+)/(RC_{10}H_{10}O^+)]$ *versus* the differences in proton affinity of 4-hydroxycoumarin (**B**) and substituted benzylideneacetone (**A**) for the fragmentation of protonated warfarin being monosubstituted at the *para* position of the phenyl ring.

$$PA(A) = -\Delta H^{\Theta} \quad (2)$$

The PAs of protonated benzylideneacetone derivative (ion **a2**) and protonated 4-hydroxycoumarin (ion **b**) are calculated by the method introduced above using DFT (Table 3).

The RAs of the final product ions $[\mathbf{A}+\mathrm{H}]^+$ and $[\mathbf{B}+\mathrm{H}]^+$ follow a relationship with the PAs of **A** and **B** (Eqn.(3)).[39,40]

$$\ln\left([A+H]^+/[B+H]^+\right) = \{PA(A) - PA(B)\}/RT_{eff} \quad (3)$$

Where R is the ideal gas constant, T_{eff} is an effective temperature. In this case, the intensity ratios of the two individual protonated monomers, on the logarithmic scale, yield a linear relationship with the differences in PA of substituted benzylideneacetone (**A**) and 4-hydroxycoumarin (**B**) as shown in Fig. 4. These can be used as evidence to support the intermediacy of INC-**1** and the proton-bound complex in the fragmentation reactions of protonated warfarin and its derivatives discussed here.[41]

CONCLUSION

In the fragmentation of protonated warfarin and its derivatives, retro-Michael reaction was observed. On the basis of the theoretical calculations and experimental studies, it was found that this retro-Michael reaction only takes place when the external proton migrates from the most thermodynamically favorable protonation site to the dissociative protonation site C-3 of the coumarin moiety. And of all these proton transfers, energy barrier of the proton transfer from the carbonyl oxygen of benzylideneacetone to the C-3 is the lowest. The kinetic method was successfully applied, and a linear free-energy relationship was found between the RAs of the final product ions **a** and **b** and the PA of the two aromatic moieties of the warfarin scaffold.

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Supporting Information

Supporting information may be found in the online version of this article.

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