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Effect of Substituents on the Reactivity of Ninhydrin with Urea

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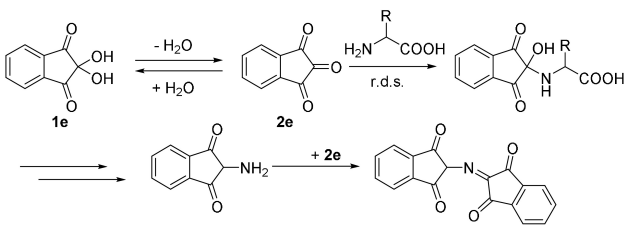
Ninhydrin, i.e. the stable hydrate of the reactive species indanetrione, is a well-known compound used for the quantification of ammonia and amino acids. However, substituent effects on the reactivity of ninhydrin with nucleophiles are not described. In this work, the kinetics of the reaction of C4- and C5- substituted ninhydrins with urea was studied and monitored by ^{13}C-NMR. Surprisingly, the obtained results show that electron donating groups (EDGs) as well as electron withdrawing groups (EWGs) decrease the rate of the reaction. EDGs decrease the electrophilicity of indanetrione, resulting in slower overall kinetics than unsubstituted ninhydrin. The calculated Gibbs free energy differences for the dehydration of unsubstituted and substituted ninhydrins and the subsequent reaction with urea showed that the dehydration of the compounds is more sensitive to electronic effects than the subsequent reaction with urea. Therefore, although EWGs increase the electrophilicity of indanetrione, this is more than counterbalanced by an adverse shift of the hydration equilibrium towards the unreactive hydrate (i.e. ninhydrin), resulting in slower kinetics as well.

Introduction

More than a century ago, Ruhemann reported on the synthesis of ninhydrin (**1 e**) and its remarkable reactivity towards amines and ammonia, yielding colored products.[1,2] He noted that ninhydrin reacts with amines on the central carbon rather than on the adjacent free carbonyls. In the years thereafter, applications such as quantification of ammonia and amino acids and fingerprint detection, using ninhydrin as reagent, were developed.[3–6] A number of publications reported on the kinetics and mechanism of the reaction of ninhydrin with amino acids, leading to the common understanding that the first step is the elimination of water, followed by nucleophilic attack of the non-protonated amine on the central ketone of indanetrione (**2 e**). The latter is considered the rate-determining step (Scheme 1).[7–10] The final compound that is formed, diketohydrindylidenediketo-hydrindamine or Ruhemann's Purple (Scheme 1), adsorbs at 570 nm allowing quantification of e.g. amino acid concentrations in biological samples.



Scheme 1. The mechanism of reaction of ninhydrin with amino acids. R.d.s. = rate-determining step. **1 e** = ninhydrin, Table 1 entry e.

Ninhydrin is a powerful electrophile that reacts not only with amines, enamines, ammonia and amino acids, but also with weak nucleophiles such as anilines, alcohols, ureas and even amides.[11–16] Many studies have corroborated that upon dehydration of ninhydrin the central carbonyl of the resulting indanetrione is the most reactive towards amines. Bhate *et al.* have recently reported on the reaction of ninhydrin with an aniline containing an amide functionality. They calculated, using Density Functional Theory at the B3LYP 6–31G(d,p) level,[17] that the central carbon is the least reactive when hydrated compared to the adjacent carbonyls, but becomes the most electron deficient and therefore the most reactive upon elimination of water, explaining that indanetrione reacts with the amide rather than ninhydrin itself.[14] In 1999 Bowden *et al.* studied the effect of substituents on C4, C5, C6 and combinations thereof on the reactivity of indanetrione with water to form ninhydrin derivatives, and found a slope (ρ-value) of 1.05 in the Hammett Plot. The positive slope indicates that substituents that decrease the electron density in the three carbonyl groups increase the rate of hydration and *vice versa*.[18]

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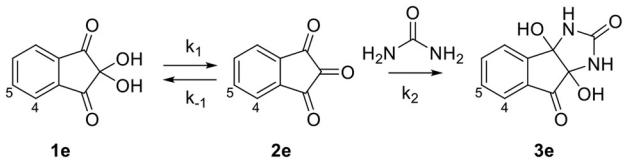
However, substituent effects on the reactivity of ninhydrin with other nucleophiles are still unknown.

In this paper we investigated the effect of both electron withdrawing groups (EWGs) and electron donating groups (EDGs) on the reactivity of ninhydrin with urea. We expected that EWGs increase and EDGs decrease the reactivity of ninhydrin with urea because ninhydrin is the electrophilic species in the reaction with amines. Urea was chosen as model nucleophile since its reaction with ninhydrin yields tetrahydroindeno-imidazole, a stable adduct (Scheme 2), in contrast to that of ninhydrin with amino acids.[11,12,19–21]



Scheme 2. Reaction of ninhydrin with urea.

Results and Discussion

First, the order and rate constant of the reaction of ninhydrin with urea was determined in phosphate buffered aqueous saline (PBS) at 50 °C by the method of initial rates.[22–24] Various ninhydrin concentrations (5-20 mM) were reacted with a fixed amount of urea (10 mM) and *vice versa*. The ninhydrin concentration was monitored over time by UV spectroscopy at 232 nm and the urea concentration was determined over time with urease, an enzyme that converts urea into CO_2 and ammonia that is subsequently quantified by its reaction with two equivalents of a phenol, yielding a dye that is quantified spectrophotometrically (supporting information, section 2). The graphs of the initial reaction rate (mMh^{-1}) versus the initial concentration of the variable component showed linear correlations with similar rate constant (~0.2 h^{-1}, Figures S3 and S5), and linear regression of the double logarithmic plots gave slopes of 0.95 ± 0.04 for urea and 1.13 ± 0.18 for ninhydrin (Figures S4 and S6). Therefore it was concluded that the reaction of ninhydrin with urea (Scheme 2) is first order in both urea and ninhydrin, and thus second order overall.

The reaction mechanism (Scheme 2) is composed of the equilibrium between elimination of water from **1e** and hydration of **2e** and the subsequent reaction of **2e** with urea to yield tetrahydroindeno-imidazole **3e**. Since the reaction is first order in both ninhydrin and urea, the rate of the formation of reaction product **3e** can be expressed by equation 1 and simplified by equation 2, in which k_{obs} is expressed by equation 3.

$$\frac{d[3e]}{dt} = \frac{k_1 k_2 [ninhydrin][urea]}{k_{-1} + k_2[urea]} \quad (1)$$

$$\frac{d[3e]}{dt} = k_{obs}[urea][ninhydrin] \quad (2)$$

$$k_{obs} = \frac{k_1}{k_{-1} + k_2[urea]} k_2 \quad (3)$$

Because the rate-determining step is the nucleophilic attack of urea on the central carbonyl of indanetrione $k_{-1} \gg k_2[urea]$. Therefore the denominator in equation 3 is dominated by k_{-1}, simplifying equation 3 to equation 4.

$$k_{obs} = \frac{k_1}{k_{-1}} k_2 \quad (4)$$

By using both reactants in stoichiometric amounts, the urea concentration is equal to the ninhydrin concentration thereby simplifying the rate equation 2 to equation 5. The k_{obs}-value can thus be determined by only measuring the urea concentration in time. Solving differential equation 5 yields equation 6, k_{obs} is then obtained from the plot of the inverse of the urea concentration against time (an example is given in supporting information, Figure S7).

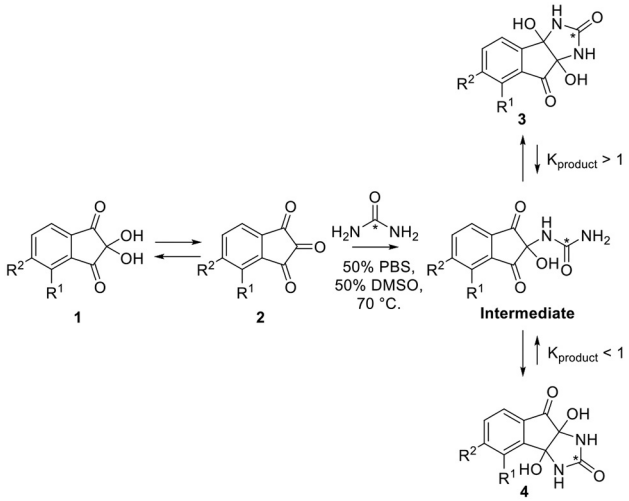
$$\frac{d[3e]}{dt} = k_{obs}[urea]^2 \quad (5)$$

$$\frac{1}{[urea]_t} = k_{obs}t + \frac{1}{[urea]_{t=0}} \quad (6)$$

Several ninhydrins substituted at 4- and 5-position with EWGs (4- and 5-Br, 4- and 5-NO_2, 5-CF_3) and EDGs (4- and 5-Me, 5-OMe and 5-*t*Bu) were selected to investigate the electronic effects of the substituents on the reactivity of ninhydrin with urea. The ninhydrin derivatives were synthesized according to a recently published method in a versatile one-step procedure from the corresponding indane-1-ones in similar yields as reported (supporting information, section 5).[25]

Since the pH of the medium influences the reaction rate of ninhydrin (supporting information, section 3), the pH was maintained at 7.4 using phosphate buffer saline (PBS). However, in contrast to unsubstituted ninhydrin (**1e**), the ninhydrin derivatives have low solubility in PBS (< 30 mM). Therefore, the different ninhydrin derivatives were dissolved in a 50/50 mixture of DMSO and PBS to study their reaction kinetics with urea. Since the urease assay is incompatible with DMSO presumably due to oxidation of DMSO rather than ammonia by hypochlorite, urea concentrations were determined by quantitative ^{13}C-NMR of ^{13}C-labeled urea (signal at 162.08 ppm) using Me_2SO_2 as internal standard (IS) at 42.15 ppm relative to the DMSO signal at 39.39 ppm (supporting information, section 4). This new method was validated by comparing k_{obs}-values for the reaction of ninhydrin with urea in PBS obtained by urea concentrations thus measured, with those measured by the urease assay. The new method yielded a k_{obs}-value for this reaction of 6.8 ± 0.3 $M^{-1}h^{-1}$ whereas the urea concentrations determined with the urease assay resulted in a k_{obs}-value of 6.7 ± 0.1 $M^{-1}h^{-1}$, thereby validating the ^{13}C- NMR urea concentration method.

The ninhydrin derivatives were reacted at 70 °C with urea in a 1:1 ratio. Notably, when a substituent is present on ninhydrin, two tetrahydroindeno-imidazole regioisomers **3** and **4** are formed that can be distinguished in the ^{13}C NMR spectra for urea quantification (Scheme 3). The relative amounts of each



Scheme 3. Formation of regioisomers **3** and **4**.

regioisomer in the isolated products were determined by ^{1}H-NMR or 2D Heteronuclear Multiple Bond Correlation (HMBC) ^{13}C- ^{1}H- NMR (Table 2).

Table 1. k_{obs}-Values for the reaction of ninhydrin derivatives with urea determined by quantitative ^{13}C NMR.

Entry 1[a]	R^1 (4-position)	R^2 (5-position)	Sigma-value[26] [a]	k_{obs}- value ($M^{-1}h^{-1}$) [b]
1a	H	OMe	-0.268	3.1
1b	H	tBu	-0.199[27]	13.9
1c	H	Me	-0.170	9.6
1d	Me	H	-0.069	7.4
1e	H	H	0	20.4 ± 2.2[c]
1f	H	Br	0.232	2.0
1 g	Br	H	0.391	2.9
1 h	H	CF_3	0.54[28]	2.4
1i	NO_2	H	0.710	2.1
1j	H	NO_2	0.778	2.4

[a] Substituents in the 4-position are considered meta-substituted and substituents in the 5-position are considered para-substituted. [b] Reactions with urea were performed at 70 °C in 1:1 PBS/DMSO. [c] The error in the measurement was only determined for unsubstituted ninhydrin (n = 3).

Table 1 shows k_{obs}-values of the tested ninhydrin derivatives obtained from the kinetic plots provided in supporting information section 6. Under the experimental conditions (1:1 v/v PBS/DMSO at 70 °C) the reaction equilibrates, therefore the k_{obs} was derived from the initial slope of the plot of the inverse of the urea concentration in time. Unsubstituted ninhydrin gave a k_{obs}-value of 20.4 ± 2.2 $M^{-1}h^{-1}$ (entry e). As expected for

Table 2. Ratios of the isolated regioisomers, corresponding experimental equilibrium constants, and calculated equilibrium constants from ΔG's for the formation of each regioisomer.

Entry	Ninhydrin derivative	Isolated compound ratio **3**:**4**[a]	Experimental $K_{product}$	Calculated $K_{product}$
a	5-OMe	1 : 5.0	0.20	0.037
b	5-*t*Bu	1 : 1.4	0.71	1.375
c	5-Me	1 : 2.0	0.50	0.322
d	4-Me	1.5 : 1	1.5	2.155
f	5-Br	1 : 2.0	0.50	0.958
g	4-Br	1 : 1.5	0.67	0.128
h	5-CF_3	1.2 : 1	1.2	2.011
i	4-NO_2	> 0.01 : 1	> 0.01	0.003
j	5-NO_2	1.4 : 1	1.4	2.688

[a] Ratios were determined by NMR from the mixture of regioisomers after purification. Loss of product was not observed during the purification process (**3 e** was isolated in a 69% yield after 70% conversion was observed in the corresponding kinetic experiment).

an electrophile, EDGs such as methyl and *tert*-butyl (entry b-d) increase the electron density at the carbonyls and decrease the rate of the reaction with urea relative to unsubstituted ninhydrin (7.4 - 13.9 $M^{-1}h^{-1}$). Unexpectedly, EWGs such as bromo-, nitro- and trifluoromethyl (entry f–j) which decrease the electron density at the carbonyls also decreased the rate of the reaction (2.0–2.9 $M^{-1}h^{-1}$) and reacted even slower with urea than ninhydrins bearing an EDG.

To fit the kinetic data of the reaction of urea with substituted ninhydrins in a Hammett plot, the 5-position was taken as *para*-substituted and logically we chose the *meta*-parameter for 4-substituted ninhydrins.[29] Bowden and Rumpal fitted several Hammett parameters in their kinetic studies of the hydration of indanetrione and the ring fission of ninhydrin and found that the 5-position of indanetrione and ninhydrin correlates best with the *para*-parameter. The Hammett plot of the logarithm of the relative *k*-values (k_{obs} for substituted divided by k_{obs} for unsubstituted ninhydrin) as a function of the sigma constant for each substituent is shown in Figure 1. In

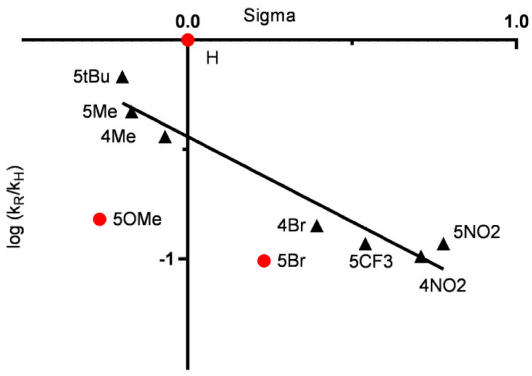


Figure 1. Hammett plot for the reaction of urea with ninhydrin derivatives; k_R and k_H are the k_{obs} for substituted and unsubstituted ninhydrin, respectively. Linear regression (excluding 5-OMe, H and 5-Br) gives y = -0.78x–0.44, R^2 = 0.93.

general the ninhydrin derivatives correlate poorly with the Hammett parameters, resulting in a scattered plot. However, when 5-OMe- and 5-Br-substituted ninhydrin and unsubstituted ninhydrin are left out, the other substituents follow a linear Hammett relationship. A possible explanation for the deviation of the 5-OMe group is that this group can be an EWG or EDG depending whether it is in the *meta*- or *para*-position, respectively. In the investigated reaction, the substituent is in fact in both positions whereas the sigma value that we used only corresponds to the *para*-position. The negative slope in the Hammett plot indicates that the reaction rate decreases with a decreasing electron density on the carbonyls, which is not expected for an electrophile such as ninhydrin.

To explain the unexpected negative influence of the EWGs on the reactivity of ninhydrin and the high reactivity of unsubstituted ninhydrin, we calculated the energy changes (ΔG's) for the dehydration of ninhydrin **2** and for the subsequent formation of intermediate **I**, for each substituent (supporting information section 8). We used the same DFT method (B3LYP 6–31G(d,p)) as Bhate *et al.* on ninhydrin and indanetrione (calculating the partial atomic (Mulliken) charges), but we performed all calculations using a polarizable continuum model for water instead of the gas phase.[14] Structure optimization was performed (supporting information section 9) and the corresponding frequencies were calculated for the different ninhydrin derivatives, intermediates and products.

Because the reactions converge to an equilibrium instead of reaching full conversion, the reaction products are also expected to be at equilibrium with each other. Hence, the overall rate constant of the reaction (k_{obs}) does not affect the thermodynamic ratio of the two regioisomers. First we considered the isomerization of regioisomers **3** and **4**, by calculating the difference in Gibbs free energy of those products (Equation 7) and calculated the corresponding equilibrium constants $K_{product}$ using Equation 8 (supporting information section 8.3 and Table 2):

$$\Delta G_{isomerization} = G_{Product3} - G_{Product4} \quad (7)$$

$$\Delta G = -RTln(K) \quad (8)$$

A plot of the experimental versus the calculated $K_{product}$ is included in the supporting information section 8.5. The equilibrium constants calculated with the difference in energy of these regioisomers are overall in good agreement with the experimental $K_{product}$ (with exception of entry b), which shows that the chosen model correctly predicts which regioisomer is preferentially formed.

Using the calculated G-values, the differences in Gibbs free energy were determined according to equations 9–11:

Between the intermediate and indanetrione:

$$\Delta G_{Intermediate} = G_{intermediate} - (G_{indanetrione} + G_{urea}) \quad (9)$$

Between indanetrione and ninhydrin:

$$\Delta G_{Dehydration} = (G_{indanetrione} + G_{water}) - G_{ninhydrin} \quad (10)$$

Between the intermediate and ninhydrin:

$$\Delta G_{addition} = (G_{intermediate} + G_{water}) - (G_{ninhydrin} + G_{urea}) \quad (11)$$

In principle, for spontaneous processes like the hydration of indanetrione or its reaction with urea, ΔG values should be negative or close to zero. However, the opposite was found, which can be explained by several aspects of our computational model. First, differential solvation effects between reactant and products might not be accurately represented by a continuum solvent model which does not include specific interactions such as hydrogen bonding. Second, the contribution of translational entropy to the Gibbs free energy may be inaccurately treated in solution. For these reasons the obtained ΔG-values are not absolute values. Of note, these issues are not expected to significantly impact intrinsic substituent effects and trends within a series of analogous compounds can thus be accurately predicted.

The effect of a given substituent on the overall reaction can be determined by subtracting the ΔG of the addition of urea to ninhydrin ($\Delta G_{H\ addition}$) from the ΔG of the addition of urea to substituted ninhydrin ($\Delta G_{R\ addition}$), referred to as $\Delta\Delta G_{R\ addition}$ (equation 12).

$$\Delta\Delta G_R = \Delta G_R - \Delta G_H \quad (12)$$

A positive $\Delta\Delta G_R$ implies that the substituted compound has a higher ΔG than the unsubstituted one, thus the substituent lowers the driving force for the reaction. To a first approximation, effects on the free energy of activation $\Delta G^{\ddagger}$ can be assumed to be proportional to effects on the driving force ΔG_R in a linear free energy relationship. According to the Eyring equation, the logarithm of the rate (log k) is proportional to the negative of the free enthalpy of activation ($-\Delta G^{\ddagger}$), implying that substituent effects on $-\Delta\Delta G_R$ should be proportional to those on log(k). In Figure 2A the $-\Delta\Delta G_{R\ addition.}$ is plotted against the Hammett sigma parameter and, in good agreement with the experimental Hammett plot, a negative slope is observed. To understand why EWGs – somewhat counterintuitively – decrease the reactivity of ninhydrin with urea, the $\Delta\Delta G_R$'s of both the dehydration of ninhydrin and the subsequent reaction of indanetrione with urea were calculated analogously ($-\Delta\Delta G_{R\ dehydration}$ and $-\Delta\Delta G_{R\ intermediate}$) and plotted against the Hammett sigma parameters (Figure 2B and C).

The effect of the substituent in terms of $-\Delta\Delta G_R$ on the dehydration of ninhydrin shows a negative slope in the Hammett plot, which means that EDGs increase the rate of the dehydration and EWGs decrease the rate of dehydration (Figure 2B). This is in correspondence with the work of Bowden and Rumpal in which they investigated the influence of substituents on the reverse reaction (hydration of indanetrione) and found a positive slope ($\rho = 1.05$) in the Hammett plot.[18] The ninhydrin-indanetrione equilibrium is defined by equation 13 and the equilibrium constant. K is calculated by equation 8,

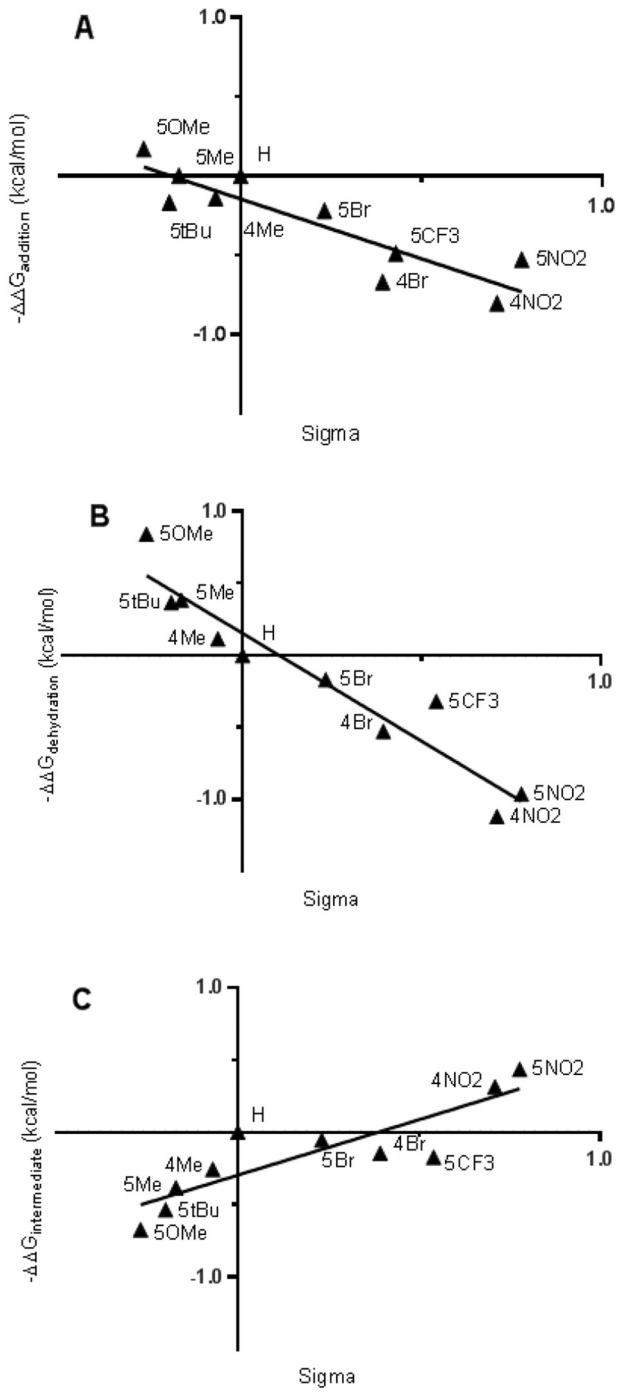


Figure 2. Calculated -ΔΔG's plotted against Hammett sigma constants for A) the reaction of ninhydrin with urea ($y = -0.75x - 0.15$, $R^2 = 0.80$) B) the dehydration of ninhydrin ($y = -1.499x + 0.15$, $R^2 = 0.91$) and C) the reaction of indanetrione with urea ($y = 0.77x - 0.30$, $R^2 = 0.75$). All ΔΔG-values used are found in supporting information section 8.4.

using the calculated ΔG for the formation of ninhydrin from indanetrione and water (Table 3).

Table 3. Calculated equilibrium constants K for the ninhydrin-indanetrione equilibria from calculated ΔG (J), T = 343 K.

Entry	Ninhydrin derivative	$K_{dehydration}$
a	5-OMe	0.08
b	5-*t*Bu	0.17
c	5-Me	0.16
d	4-Me	0.24
e	H	0.29
f	5-Br	0.37
g	4-Br	0.62
h	5-CF_3	0.46
i	4-NO_2	1.48
j	5-NO_2	1.18

$$K = \frac{[ninhydrin]}{[indanetrione][H_2O]} \quad (13)$$

The higher calculated equilibrium constants for the EWGs relative to unsubstituted ninhydrin suggest that the indanetrione concentrations are indeed lower in the presence of an EWG, and higher in the presence of an EDG.

Using $-\Delta\Delta G_{R\ intermediate}$, Figure 2C illustrates the substituent effect in a Hammett plot. In contrast to the dehydration of ninhydrin, a positive slope is observed, which indicates that EWGs increase the rate of the reaction of indanetrione with urea, and EDGs decrease this rate. Interestingly, the magnitude of the slope of the Hammett plot for the dehydration of ninhydrin (Figure 2B) shows that this reaction is more affected by the substituent than the reaction of indanetrione with urea (Figure 2C). In other words, the change in enthalpy for the dehydration of ninhydrin, and therefore the activation energy,[30] is more sensitive for electronic substituent effects than the reaction with urea.

The computational results suggest that the introduction of an EWG on ninhydrin shifts the ninhydrin-indanetrione equilibrium towards ninhydrin, resulting in a decrease of the indanetrione concentration and slower steady state kinetics. Because the dehydration of ninhydrin is affected by the EWGs to a greater extent than the reaction of indanetrione with urea, the overall rate of the reaction is decreased, thereby explaining why the reactivity of electron-deficient ninhydrins towards urea is not increased compared to unsubstituted ninhydrin. The other way around, Figure 2B shows that for EDGs the $\Delta\Delta G_{R\ dehydration}$ are negative and thus the indanetrione concentrations are higher. However, the experimental results (Figure 1) show that also EDGs do not increase the reactivity of ninhydrin towards urea in water. The reason for this is that EDGs make the triketonesystem of indanetrione more electron-rich and thus less electrophilic than unsubstituted ninhydrin.

In addition, the experimental result that the electrophilicity of ninhydrin decreases with the introduction of an EWG is consistent with the mechanism in which the initial attack of the nucleophile on ninhydrin takes place at the C2 position, since an initial attack at the C1 position would be promoted by an EWG.

Conclusions

A kinetic study is performed on the reaction of substituted ninhydrins with urea in water, which is first order in both ninhydrin and urea and second order overall. Two regioisomers are formed in the rate determining step, which is the reaction between the dehydrated form of ninhydrin (indanetrione) and urea. Because this reaction is a (pH dependent) equilibrium, the major regioisomer formed corresponds to the compound with the lowest calculated energy (G). The reactivity of the electrophilic indanetrione with urea in water showed surprising results. Both electron withdrawing and donating groups significantly decreased the rate of the reaction, EWGs even more than EDGs.

Computational studies on this system provided us with the suitable explanation that the dehydration of ninhydrin is more affected by the substituent than the reactivity towards urea. An EWG decreases rate of the dehydration of ninhydrin, thereby decreasing the indanetrione concentration, resulting in slower kinetics. Although EDGs do promote the dehydration of ninhydrin towards indanetrione, they decrease the electrophilicity of indanetrione towards urea, resulting in slower overall kinetics than unsubstituted ninhydrin but faster than EWG-substituted ninhydrins. This study shows that unsubstituted ninhydrin is the most reactive towards urea in water and that this result is an outlier in the Hammett plot.

Supporting Information Summary

SI contains experimental procedures, raw data for the order-determination experiments, effect of the pH of the medium on the rate of the reacton of ninhydrin with urea, validation and error analysis of the urea concentration determination method with ^{13}C- NMR, synthesis and characterization of ninhydrins and new compounds, calculated G vales for all ninhydrins, products and intermediates, ΔG values for all ninhydrins and intermediates, coordinates for optimized structures of all products and intermediates and ^{1}H- and ^{13}C NMR spectra of all new compounds.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: computational chemistry · ninhydrin · quantitative ^{13}C -NMR · substituent effects · urea

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