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Effect of Molecular Structure on the Relative Hydrogen Peroxide Scavenging Ability of Some α-Keto Carboxylic Acids

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ABSTRACT

The α-keto carboxylic acid, pyruvic acid (**1**) was found to be a very effective peroxide scavenger but is subject to an aldol-like self-condensation/polymerization reaction. The purpose of this study was to evaluate the hydrogen peroxide, H_2O_2, scavenging ability of 3-methyl-2-oxobutanoic acid (**2**), 4-methyl-2-oxopentanoic acid (**3**), and 2-oxo-2-phenylacetic acid (phenylglyoxylic acid, **4**) in the pH range 2-9 at 25°C and the effect of molecular structure on the relative reactivity. The reaction with H_2O_2 was followed by UV spectrophotometry at 220 or 260 nm and high-performance liquid chromatography. Pseudo-first order, buffer-independent decarboxylation kinetics were observed in the presence of molar excess H_2O_2. The second-order rate constants for **2**-**4** followed a sigmoidal shape and mechanism similar to pyruvic acid. Pyruvic acid was a superior H_2O_2 scavenger to **2**-**4** over the pH range 2-9 but **4** was more reactive than **2** and **3** at pH values above 6. There was a qualitative correlation between the degree of keto-group hydration and reactivity of the acids in the pH range 4-6 while the data above pH 7 suggested that the intrinsic decarboxylation step for **4** was faster than for pyruvic acid. Differences in reactivity to molecular structure were analyzed.

Introduction

The aim of this present work was a study of the effect of alkyl and aryl substituents on the second-order rate constants for decarboxylation for a series of α-keto carboxylic acids (**1**-**4**, see Fig. 1 for structures) in the presence of hydrogen peroxide, H_2O_2. Pyruvic acid, while an excellent peroxide scavenger,[1] underwent a limiting aldol-like, self-condensation reaction.[2] The α-keto carboxylic acids, **2**-**4**, were expected to undergo slow or negligible self-condensation due to steric effects for **2** and **3**, and the lack of a nucleophilic carbon, alpha to the keto group, in **4**. A study to confirm the self-condensation reactivity of **1**-**4** will be the subject of a separate paper.

The ability to scavenge peroxides is critical to the stabilization of a number of small and large molecule drugs, especially in the presence of peroxide generating environments and excipients.[3-13] Peroxide oxidation in pharmaceutical and food products can be prevented or minimized by removing peroxides during the manufacturing and packaging processes as well as preserving the stability of formulations during the storage using peroxide scavengers and antioxidants.[14] Finding new, non-toxic excipients, physically and chemically compatible with the drugs, useful as potential antioxidants and peroxide scavengers is a challenge. Pyruvic acid and other α-keto carboxylic acids are H_2O_2 scavengers[1,15] and could be useful in preventing drug/peroxide-driven oxidation.

The use of several techniques, such as proton and carbon nuclear magnetic resonance (NMR) and UV-spectrophotometry, has definitively elucidated the mechanism of reaction of pyruvic acid with hydrogen peroxide.[1,16-18] Based on previous studies on the equilibrium of hydration and dissociation of these same α-keto carboxylic acids, **1**-**4**, and the fast kinetics and mechanistic details of the addition-decarboxylation of pyruvic acid (**1**) in the presence of H_2O_2,[1,19] one could hypothesize that peroxide-driven decarboxylation of α-keto carboxylic acids **2**-**4** proceeded through the reversible intermediate peroxide addition to the α-carbonyl group followed by the irreversible decarboxylation step (Fig. 1).

One might hypothesize that an increase in alkyl branching as well the presence of an aromatic ring in the α-keto acid molecule structure might lead to changes in the reactivity of molecules such as **2**-**4** toward H_2O_2 relative to pyruvic acid. Here, the structure-reactivity relationships and substituent effects in the aqueous

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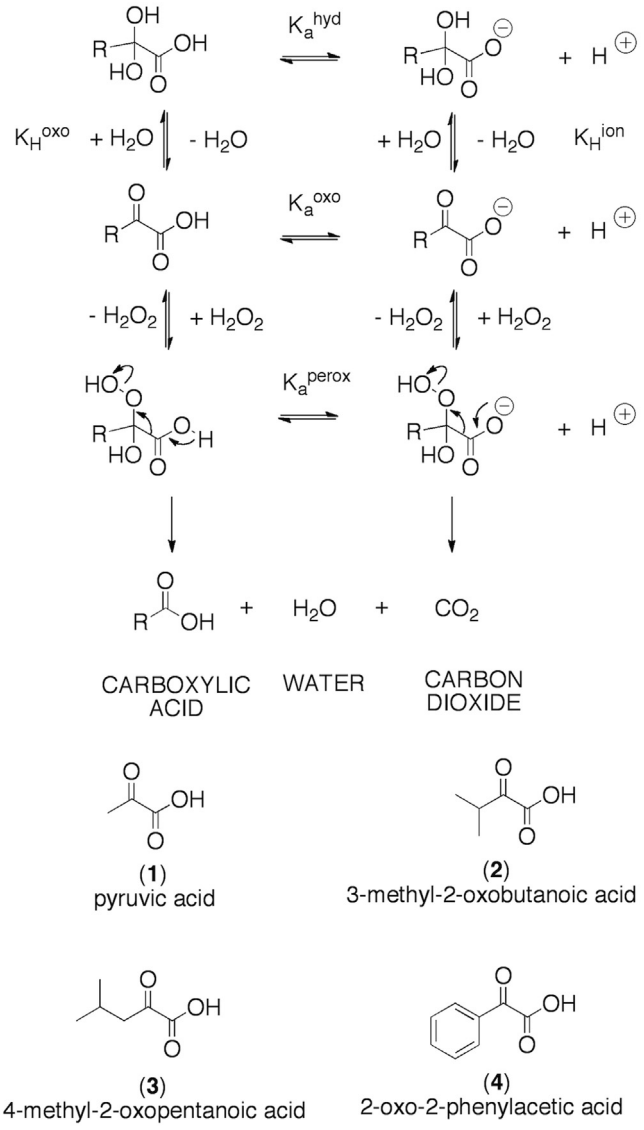

Figure 1. Proposed overall reaction and ionization scheme involving the reaction of various α-keto carboxylic acid species **1**-**4** with hydrogen peroxide.

solution addition-decarboxylation of **1**-**4** by H_2O_2 were investigated and discussed.

Experimental Materials and Methods

Materials

Sodium pyruvate (**1**) ReagentPlus (≥99%), acetic acid American Chemical Society (ACS) reagent (≥99.7%), 3-methyl-2-oxobutanoic acid sodium salt (**2**) (95%), isobutyric acid 99%, sodium 4-methyl-2-oxopentanoate (**3**), isovaleric acid 99%, 2-oxo-2-phenylacetic acid (phenylglyoxylic acid, **4**), benzoic acid 99%, sodium acetate 99%, H_2O_2 50% solution in water, sodium chloride BioXtra ≥99.5%, and HCl 37% ACS reagent were commercially sourced from Sigma-Aldrich (Milwaukee, WI) and used without purification. Monosodium phosphate ($NaH_2PO_4 \cdot H_2O$) and Na_2HPO_4 anhydrous certified ACS were purchased from Fisher Scientific (Fair Lawn, NJ). Tris-HCl +99% was commercially available from Acros Organic (Bridgewater, NJ). Deionized water was used to prepare all the samples for the UV and high-performance liquid chromatography (HPLC) analysis.

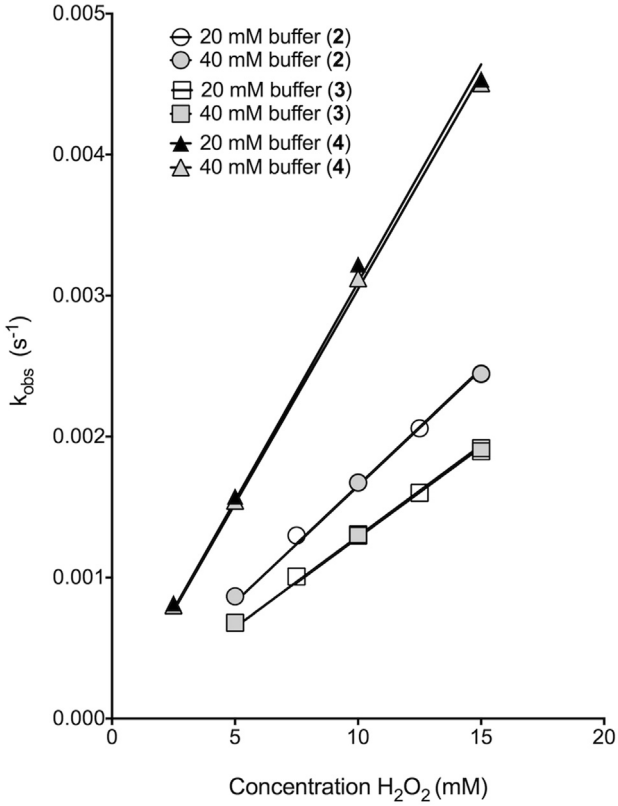

Figure 2. Observed pseudo–first-order rate constants for **2**-**4** plotted against H_2O_2 concentration in 20 and 40 mM phosphate buffers at pH 7.4, ionic strength 0.15 M at 25°C. The slopes of each line represent the second-order rate constant k_{sec}. Standard deviations were within the size of the symbols.

Methods

UV Spectrophotometric Analysis

UV spectrophotometric analysis was performed as described in an earlier study.[1] Briefly, the fast kinetics were monitored by using a UV spectrophotometer, Spectramax® PLUS^{384} (Sunnyvale, CA). Separate α-keto carboxylic acids (1.5 mM for **1**-**3** and 0.15 mM for **4**) and H_2O_2 solutions with concentrations ranging from 5 to 30 mM were prepared using aqueous buffer solutions, tris (pH 9), phosphate (pH 6-8), and acetate (pH 4-5.5) at 20 and 40 mM and HCl 0.01 M, with ionic strength adjusted to 0.15 with NaCl. Equal volume of each compound solution was mixed with each H_2O_2 solutions in a quartz cuvette (Starna Cells, Inc., Atascadero, CA) equilibrated at 25°C in a water bath. The overall drop in absorbance of the mixture during the time was monitored at 220 nm for **1**-**3** and 260 nm for **4** at 25°C.

HPLC Analysis

The HPLC method to analyze **1** and its degradation product, acetic acid, has been described in an earlier study.[1] For the analysis of **2**-**4** a Shimadzu SIL 10-A system including a SIL-10A autoinjector, a SPD-10A UV-VIS detector, a SCL-10A system controller, LC-10AT pumps, and Class-VP version 4.10 software by Shimadzu Scientific Instruments (Columbia, MD) was used. A Phenomenex HyperClone 5 μm dimethyloctylsilane (C8) 120 Å 150 × 4.6 mm column thermostated at 30°C was used. An isocratic elution of 1 mL/min was performed using 25 mM NaH_2PO_4 (pH 2.8)/methanol at a 90/10

ratio for **2** and **3** and 80/20 ratio for **4**. The UV detection of **1**-**3** and their respective carboxylic acids (the degradation products) was carried out at 220 nm. The UV detection of **4** and benzoic acid was carried out at 260 nm. An injection volume of 5 μL was used in all experiments.

Data Fitting

Data fitting to determine various rate constant values was performed using GraphPad/Prism version 6.0 (GraphPad Software, La Jolla, CA) using either non-linear least-squares regression analysis or linear regression analysis. Non-linear least-squares regression analysis with weighting (in order to minimize the weighted sum of squares) was used to obtain best-fit values to the pH rate profile of the second-order rate constant.

Results and Discussion

The decarboxylation of **2**-**4** followed pseudo−first-order kinetics at constant pH in the presence of molar excess H_2O_2 and a linear relationship was seen between these rate constants and H_2O_2 concentrations as described for pyruvic acid (**1**) allowing one to estimate the second-order rate constant, k_{sec}, for the reaction (Fig. 2).[1] The loss of the α-keto-acids was followed using changes in UV spectra over the pH range 2 to 9 at 25°C.[1] The pH rate profile for the second-order rate constants (k_{sec}) are shown in Figure 3.

For all compounds (**1**-**4**) the pH rate profiles were roughly sigmoidal in shape exhibiting 3 apparent phases. First between pH 2 and 4, k_{sec} for **1** and **4** exhibited a relatively pH-independent region, characterized by an apparent minimum rate constant, k_0. As discussed for pyruvic acid, it was believed that this might not be a complete pH-independent region but for description purposes, pH independency was assumed.[1] The pH complexity, a possible bell-shaped region, could be seen more clearly for **2** and **3** in this pH range indicating that the decarboxylation also had a more complex pH dependency than simply describing the kinetics with a k_0 values.

Between pH 4 and pH 7, k_{sec} for **1**-**4** exhibited pH dependency and significantly increased with increasing pH. Above pH 7, k_{sec} of each compound plateaued. The observed pH rate profile for α-keto

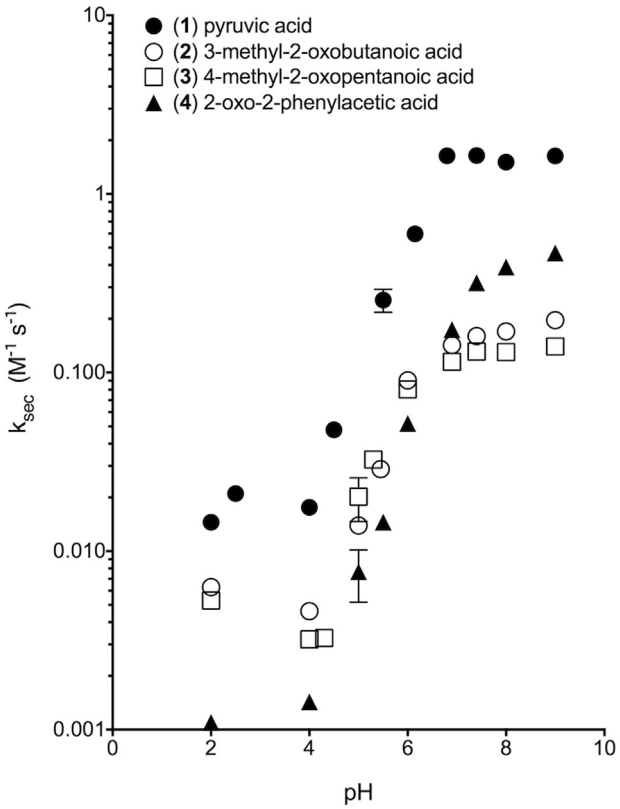

Figure 3. The pH dependence of the k_{sec} for decarboxylation of **1**-**4** by H_2O_2 at 25°C between pH 2 and 9. Experimental values (symbols) were determined at 20 and 40 mM buffer concentration and 0.01 M HCl and reported as mean value at each pH.

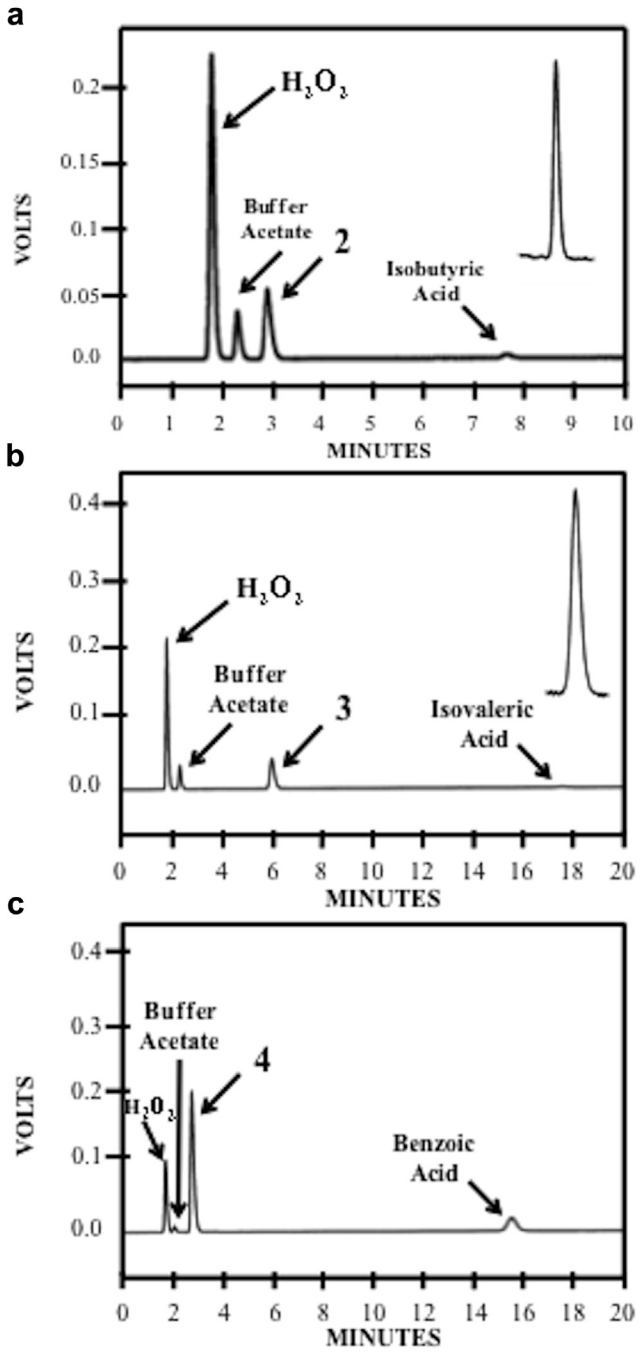

Figure 4. A representative HPLC chromatograms for **2** (a), **3** (b), and **4** (c) (5 mM) upon mixing H_2O_2 (50 mM) in 40 mM acetate buffer, pH 4, ionic strength 0.15 M, at 25°C.

acids **2**-**4** in the pH range 4-9 could be explained by the formation of the peroxide intermediates, as proposed for **1** in an earlier study.[1] In this pH range, the oxo forms of **2**-**4** were more than 99.9% in their deprotonated forms (pK_a^{oxo} <2; Fig. 1) and 5% or less in the hydrated forms (only the oxo form of **4** was detected by proton and carbon NMR at pH values >3).[19] Based on unpublished studies, in water solutions in the absence of added H_2O_2, **1**-**4** was found stable in the time frame of the kinetic studies presented here. NMR studies confirmed that no enol form or dimers, due to an aldol-like self-condensation, of **2**-**4** were observed during the peroxide reaction, kinetic analyses. The aldol-like self-condensation of **1**-**4** will be the subject of a separate study. Therefore, in this pH range, the reactive species involved in the formation of the tetrahedral intermediates were the deprotonated oxo forms of each α-keto carboxylic acid.[19]

The impact of the buffer on the k_{sec} was evaluated performing experiments at 2 different buffer concentrations 20 and 40 mM between pH 4 and 9 (Fig. 2). As for pyruvic acid, the data (Figs. 2 and 3) showed no buffer catalysis at pH values between 4 and 9, indicating no proton transfer was occurring in the rate-determining step (RDS).

The impact of the buffer on the decarboxylation of **2**-**4** was also evaluated performing HPLC experiments at 2 different acetate buffer concentrations, 20 and 40 mM, at pH 4. At this pH, the degradation of each α-keto acid was slower than at higher pH values, thus it was possible to follow the disappearance of the peak for each compound and the appearance of their corresponding carboxylic acids (with a lower response factor) allowing the observed rate constant, k_{obs}, by curve fitting (data not presented) to be estimated. The results were consistent with the UV data. The analysis confirms that the reaction of 5 mM α-keto acid in the presence of 50 mM H_2O_2 was not buffer catalyzed and the only observed degradation products were the corresponding carboxylic acids. Figures 4a-4c show the chromatograms for the degradation products of **2**-**4** at pH 4. That is, the respective carboxylic acids of each decarboxylated α-keto carboxylic acid were the degradation products, along with CO_2, at all pH values studied (also confirmed by HPLC), by comparison of the retention time values of standards for each carboxylic acid.

Based on a previous study on the decarboxylation of **1** in the presence of H_2O_2,[1] the likely mechanism of decarboxylation of **2**-**4** in the presence of H_2O_2 in the pH range 4-9 is shown in Figure 5.

The mechanism of reaction helped explain the reactivity of all the α-keto carboxylic acids evaluated in the pH range 4-9. For the decarboxylation of pyruvic acid,[1] the first step in this pH range was the nucleophilic addition of the mono-deprotonated form of H_2O_2, HOO^-, on the electrophilic carbon of the α-keto group of the carboxylate forms of **2**-**4** leading to the formation of a tetrahedral intermediate, I′, with a formation rate constant, k_1. Such a step accounted for the increase in k_{sec} with increasing pH in this pH range. The reverse reaction was defined by the rate constants, k_{-1}. The oxygen anion of the intermediate I′ of each compound was then instantaneously protonated to the alcohol form, I″. The intermediate I″ could then decarboxylate releasing CO_2, water, and the respective carboxylic acids in the second irreversible step, defined by the rate constant, k_2.

Based on Figure 5, k_{sec} for the reaction of H_2O_2 with **2**-**4** (and **1**), in this pH range, is described by Equation 1[1]:

$$k_{sec} = \frac{k_1 k_2 K_a'}{k_{-1} K_a'' + k_2 [H^+]} \quad (1)$$

where the dissociation constants $K_a'$and K_a'' are also defined in Figure 5. In the pH range 4-6, when the concentrations of hydrogen ions increased, the product $k_2[H^+]$ became bigger than $k_{-1}K_a''$ and Equation 1 could be simplified in Equation 2:

$$k_{sec} = \frac{K_a' k_1}{[H^+]} \quad (2)$$

Thus, the reaction appeared base catalyzed with k_1 representing the RDS. This was consistent with apparent specific base catalysis and lack of buffer effects seen above pH 4 and before the inflection point around pH 6.

When $k_{-1}K_a'' >> k_2[H^+]$, Equation 1 simplified in Equation 3:

$$k_{sec} = \frac{K_a' k_1 k_2}{k_{-1} K_a''} \quad (3)$$

At pH values bigger than 7, therefore, k_{sec} for **1**-**4** became constant and independent of $[H^+]$ and thus, pH. This was explained by assuming the RDS changes because the reversible addition of HOO^- was fast and the decarboxylation became rate-determining.

By curve fitting the experimental data, to Equation 1, the rate constants k_1 and other important kinetics parameters were estimated by assuming that K_a'' had a value of $10^{-11.3}$ (Table1).[20]

At the inflection point, around pH 6.0, the $[H^+]$ corresponded to the ratio of the rate constants for breakdown of the carbonyl-peroxide adduct, I′, and decarboxylation of I″, multiplied by the dissociation constant of the tetrahedral intermediate I′.

$$[H^+] = \frac{k_{-1} K_a''}{k_2} \quad (4)$$

The mechanism of reaction involving the formation of the tetrahedral intermediate was supported from the work by others[1,16,18] as well as the experimental observations made here.

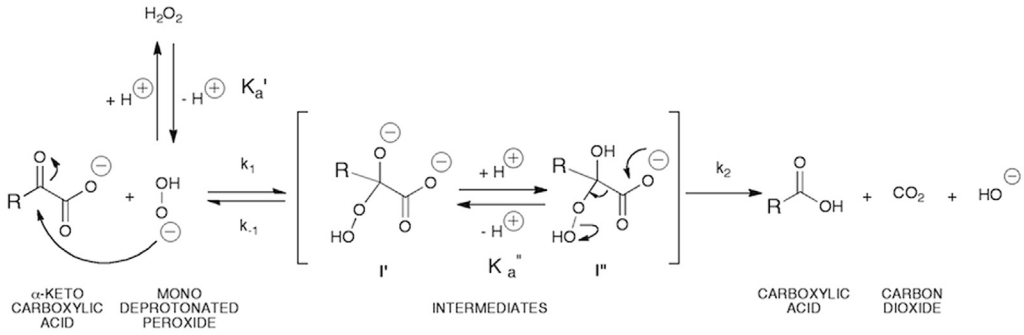

Figure 5. Proposed pathway for the reaction of the deprotonated forms of **1**-**4** with the H_2O_2 between pH 4 and 9 consistent with the pH rate profile and the lack of observed buffer catalysis. The reaction proceeds via the reversible formation of tetrahedral intermediates I′, their protonation to I″, and subsequent irreversible decarboxylation.

Table 1
Rate Constants k_1, Kinetic pK_a Value, k_{sec}, and Equilibrium Constants of Hydration $K_H^{ion} \pm SE$ for **1-4** at 25°C, Ionic Strength 0.15

Compound	k_1 ($M^{-1}s^{-1}$) ± SE	Kinetic pK_a ± SE	k_{sec} ($M^{-1}s^{-1}$) ± SE[a]	K_H^{ion} ± SE[b]	k_{sec}/K_H^{ion}
1	$1.92 \pm 0.22 \times 10^5$[c]	6.25 ± 0.05	1.683 ± 0.131	0.087 ± 0.002	19.34
2	$2.36 \pm 0.20 \times 10^4$	6.17 ± 0.04	0.174 ± 0.01	0.037 ± 0.002	4.70
3	$4.33 \pm 0.36 \times 10^4$	5.78 ± 0.04	0.131 ± 0.011	0.006 ± 0.002	21.83
4	$1.04 \pm 0.11 \times 10^4$	6.91 ± 0.06	0.427 ± 0.038	<0.001[d]	>427

SE, standard error.
[a] The second-order rate constants k_{sec} for **1-4** were determined by fits to Equation 3.
[b] The equilibrium constants of hydration K_H^{ion} of the carboxylate forms of **1-4** were calculated as described by Lopalco et al.[19]
[c] An earlier value[1] was reported to be 3.5×10^5 but this value did not correct for the k_0 term included here.
[d] This value was estimated at pH 3 by ^{1}H-NMR.

Assuming that at pH values below 4 the rate constants of decarboxylation of **1-4** was pH independent and characterized by minimum rate constants k_0, Equation 1 could be written as follows:

$$k_{sec} = \frac{k_1 k_2 K_a'}{k_{-1} K_a'' + k_2 [H^+]} + k_0 \quad (5)$$

By curve fitting the experimental data, to Equation 5, one could estimate the rate constants k_0 (not reported here), the rate constants of nucleophilic addition of HOO^- (k_1), the inflection point, or kinetic pK_a values, and the plateau, second-order rate constants k_{sec} **1-4** at pH values above the kinetic pK_a (Table 1). The fitted pH rate profiles for **1-4** between pH 4 to 9 are seen in Figure 6. It is clear that pyruvic acid, **1**, was a better peroxide scavenger than **2-4** between pH values 2-9.

Above pH 4 and below the inflection point of each compound, k_1, the rate constant for the addition of the nucleophilic specie HOO^- to the α-keto group was rate determining. The variation in relative reactivity for **1-3** was consistent with steric hindrance/interference to the addition reaction. In going from the starting state to I′, the carbon-oxygen bond of the keto-group went from an sp^2 bond to the more crowded sp^3 tetrahedral specie. These types of reactions, such as ester hydrolysis and hydration of ketone groups, tended to be sterically sensitive.[21] The lower k_1 value for **4** was also expected because the electrophilicity of its keto group was moderated by the *pi* bond overlap with the phenyl ring orbitals.

Compounds **1-4** all underwent rapid reversible hydration both in their acidic or neutral state, as well as their anionic forms at pH values above their pK_a.[19] Water addition to the carbonyl was similar to peroxide or peroxide anion addition to the carbonyl. Equilibrium hydration of **1-4** at pH >4 is best defined by K_H^{ion} in Figure 1. Values for K_H^{ion} from an earlier study are reproduced here in Table 1.[19] A plot of log k_1 versus log K_H^{ion} is shown in Figure 7. Although not a perfect correlation, because K_H^{ion} represented an equilibrium process and thus a ratio of the forward rate constant for the hydration step over the reverse, dehydration reaction, these results do support the idea that the kinetics of peroxide anion attack defined by k_1 were determined by steric factors (**2** and **3** relative to **1**) and the electrophilicity of the carbonyl carbon (mainly with **4**).

In an earlier study, the pH rate profile for pyruvic acid (Fig. 6) was described by an apparent pK_a value corresponding to the inflection point around pH 6-7.[1] This also appeared to be the case for **2-4**. Based on the mechanism for the decarboxylation discussed above, this apparent pK_a value could be defined by the $-\log [H^+]$ value defined in Equation 4. Such inflection points do not correspond to known ionization steps but represented what was often called kinetic pK_a values. The values for **1-4** are also reported in Table 1.

At pH values above the kinetic pK_a values, the rate equation for the decarboxylation is defined by Equation 3 where the plateau value for k_{sec} is a complex function of K_a', K_a'', the ratio of k_1/k_{-1} (K_{eq1}), and k_2, where k_2 is said to be rate determining because K_a', K_a'', and K_{eq1} all represented rapid, non-RDS processes at these pH values. That is, the decarboxylation step was the RDS. Unlike k_1 values, one cannot actually determine the values for k_2 because a number of the equilibrium constants could not be estimated.

Assuming the equilibrium for peroxide anion adduct, defined by $k_1/k_{-1} = K_{eq1}$, and that K_a'/K_a'' is defined by a constant, Q, and that these constants are approximately the same for **1-4**, Equation 3 could be written as Equation 6:

$$k_{sec} \cong Q K_{eq1} k_2 \quad (6)$$

Therefore, differences in K_{eq1} values have the same weight as a change in k_2 in defining the numerical value of k_{sec}. That is, the relative plateau k_{sec} values could not be directly related back to only changes in k_2 values. In an effort to understand why **4** was more reactive than **2** and **3** in this pH region compared to pH values less

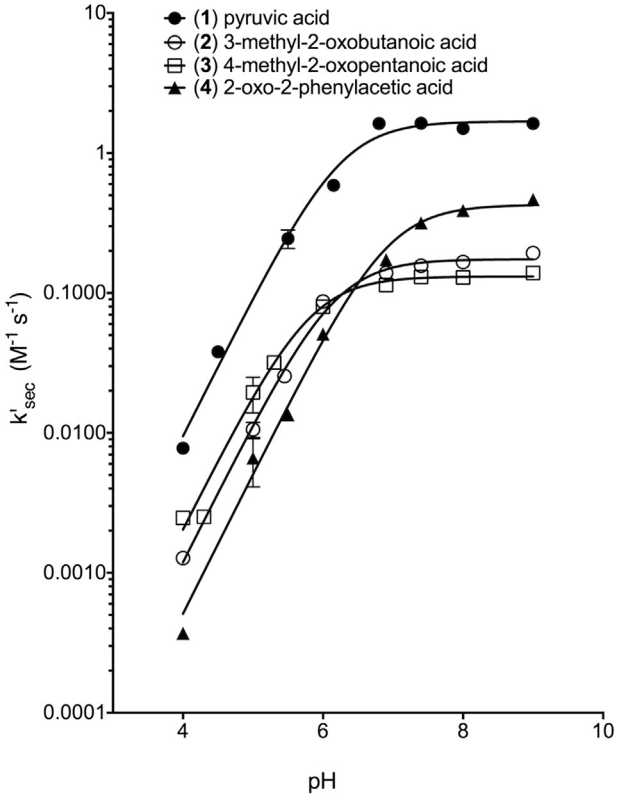

Figure 6. The pH dependence of the k'_{sec} for decarboxylation of **1-4** by H_2O_2 at 25°C between pH 4 and 9. The k'_{sec} on the y-axis was the k_{sec} values from which the k_0 values were subtracted each of the respective α-keto acids, **1-4**. Experimental values (symbols) were determined at 20 and 40 mM buffer concentration and reported as mean value at each pH value. The solid line was obtained by fitting the resultant constants to Equation 5.

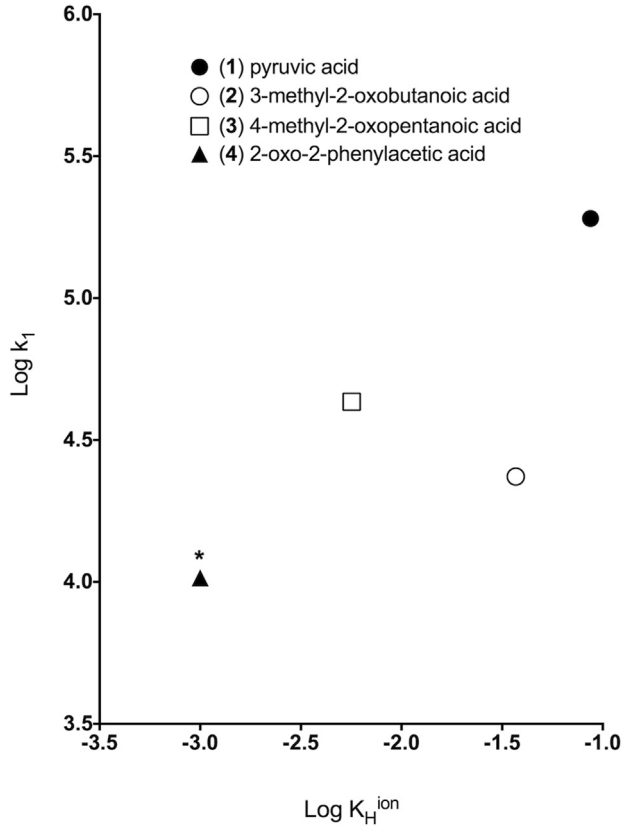

Figure 7. Log k_1 plotted versus log K_H^{ion} for **1**-**4**. The log K_H^{ion} for compound **4** (*) was plotted assuming that the value K_H^{ion} was equal to 0.001.

than 6, the following approximation was attempted. If one assumed that K_{eq1} values were proportional to K_H^{ion} values, the 2 constants for peroxide addition and water addition, as then Equation 7 resulted:

$$K_{eq1} \cong CK_H^{ion} \tag{7}$$

where C was a proportional constant that took into account the nucleophilicity and basicity of hydroxide versus hydroperoxide species in solutions. From the proton NMR experiments with ethylpyruvate in the presence of hydrogen peroxide, it was found that C had a value about 800.[1]

Placing Equation 7 into Equations 6, Equation 8 results:

$$k_{sec} \cong QCK_H^{ion}\ k_2 \tag{8}$$

and by rearrangement, Equation 9 is derived:

$$\frac{k_{sec}}{K_H^{ion}} \cong Q' k_2 \tag{9}$$

where Q' is a constant and a product of Q and C. The values for k_{sec}/K_H^{ion} in the plateau region of the pH rate profile, displayed in Table 1, were very telling. Although there were differences between **1** and **3**, the value for **4** was dramatically different from **1** to **3**. That is, while **4** was still a poorer peroxide scavenger than for **1** (Figs. 3 and 6), the numerical value for k_2 must be actually faster than for **1**, but the plateau k_{sec} value was inferior to **1** because the peroxide addition reaction was less favorable for **4** compared to **1**.

Although α-keto acids, **2**-**4**, were kinetically slower at scavenging H_2O_2 compared to pyruvic acid, none of the 3 underwent significant loss due to the aldol-like self-condensation seen with pyruvic acid, the subject of a forthcoming paper. The reactive nature of **4** at pH values greater than 6 was actually quite promising because it compared favorably to pyruvic acid and while the safety of **4** itself was unknown, its decarboxylation product, benzoic acid, is quite safe.

Ongoing kinetic studies comparing a range of classical peroxide scavenger and antioxidant molecules, including methionine, to α-keto carboxylic acids will allow one to set up a useful peroxide scavenging scale. This may provide a formulator the ability to tailor the choice of scavenger to their need.

Conclusions

UV spectrophometry and HPLC was used here to study the fast decarboxylation kinetics of a series of α-keto carboxylic acids, **1-4**, in the presence of H_2O_2. HPLC analyses also confirmed the formation of the expected degradation products. Based on a previous study on the mechanism for the decarboxylation of pyruvic acid, **1**, in the presence of H_2O_2, the mechanism of reaction for **2**-**4** involved a change in rate-determining formation of tetrahedral intermediate species to rate-determining decarboxylation between pH values 4 and 9. There was a correlation between the decarboxylation of α-keto acids **1**-**4** in the presence of H_2O_2 to structure with steric and electronic factors affecting the reaction in unfavorable ways. Although inferior to pyruvic acid, phenyl glyoxylic acid, **4**, was quite promising as a peroxide scavenger.

Acknowledgments

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