Structure of an α-Keto *β*-Amido Acid, 3-(Phenylacetamido)pyruvic Acid, and Its Methyl Ester in the Solid State and in Organic and Aqueous Solvents

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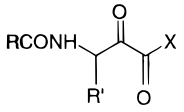
3-(Phenylacetamido)pyruvic acid and its methyl ester both exist in the enol form in the solid state and when dissolved in organic solvents. In aqueous solution, the pK_a's of the enol acid are 3.28 (CO_2H) and 10.14 (enolic OH) and that of the ester is 8.14 (enolic OH). The thermodynamically stable form of both acid and ester in aqueous solution, however, is the keto species. Ketonization rates of 3-(phenylacetamido)pyruvic acid were determined spectrophotometrically in acid and in buffered solution at neutral pH. The dominant reaction at low pH is the pH-independent protonation of the enol carboxylate monoanion with a rate constant of 0.062 s^{-1} M^{-1}. At neutral pH, the ketonization in water alone is very slow but is strongly catalyzed by buffer acids. Rapid, partial (*ca.* 30%) hydration of the keto form also occurs in aqueous solution. The pK_a of the pyruvate as a carbon acid is around 12.5. These results are compared with literature data for pyruvic acid itself. Application of peptidyl pyruvates and their derivatives as protease inhibitors requires careful assessment of the complications illustrated by the behavior of 3-(phenylacetamido)pyruvic acid and its methyl ester in aqueous solution.

A variety of electrophilic ketone derivatives have been found in recent years to inhibit serine and cysteine hydrolases. Their mechanism of action is generally thought to involve addition of the active site nucleophile to the electrophilic carbonyl group to form a tetrahedral anionic adduct that mimics a high-energy tetrahedral intermediate species of normal catalysis by the enzyme.[1] Among the electrophilic ketones that have been employed in this way are the α-keto acids and their derivatives.[2] Crystal structures of specific examples of enzyme-inhibitor complexes support the above-mentioned mechanism of action,[3a,b] although other modes of binding an α-keto acid are also possible.[3c]

Although simple alkyl and aryl α-keto acid derivatives inhibit certain hydrolases,[2a−e] greater specificity and inhibitory power has been sought by incorporation of the α-keto acid moiety into an oligopeptide.[2f−l] Thus, many such inhibitors are 3-(acylamido)pyruvic acid derivatives (**1**), where R, R′, and X can be varied to enhance specificity toward a particular enzyme.



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Compounds of structure **1**, in principle, may exist in aqueous solution in a number of forms (Scheme 1). At low pH, some combination of the neutral enol, keto, and hydrate species will exist, while at higher pH the enolate is likely to dominate. All of these species have been mentioned in the various studies described above,[2] but in no specific case does a systematic quantitative study of the equilibria and rates of Scheme 1 appear to have been made. The issue is of some significance to the evaluation of **1** as inhibitors since the active inhibitor would presumably be the keto form. The effectiveness of **1** as an inhibitor at any time would depend on the proportion present in that form at that time.

We prepared 3-(phenylacetamido)pyruvic acid (**2**) and its methyl ester **3** in order to evaluate them as *β*-lactamase inhibitors.[4] It became clear, however, during the evaluation that the nature of **2** and **3** changed in aqueous solution as a function of time. In order to understand the *β*-lactamase inhibitory activity of these compounds, and in view of the lack of clear guidance from the literature, we studied the behavior of **2** and **3** in some detail and report here the results of our investigation of the structures of **2** and **3** in the solid state, in organic

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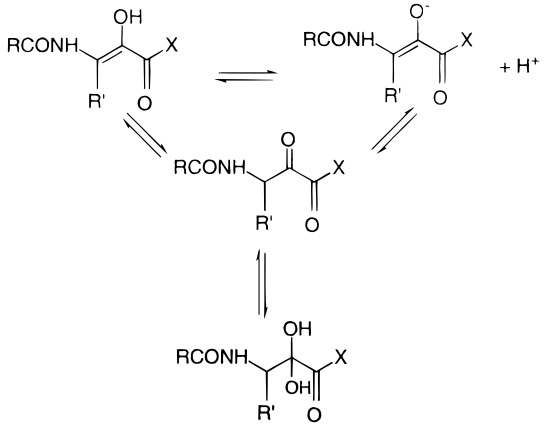
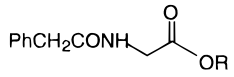
Scheme 1



solvents, and in aqueous solution, the latter within the framework of Scheme 1.



2 R = H
3 R = Me

Experimental Section

General Methods. ^{1}H and ^{13}C NMR spectra were obtained from a Varian Gemini 300 spectrometer. Chemical shifts were determined by reference to tetramethylsilane (^{1}H, 0.00 ppm) and DMSO-d_6 (^{13}C, 39.5 ppm) or acetone-d_6 (^{13}C, 206.6 ppm). IR spectra were obtained in KBr pellets by means of a Perkin-Elmer 1600 FTIR spectrophotometer. Absorption spectra and spectrophotometric rates were obtained from either a Perkin-Elmer Lambda 4B or a Hewlett-Packard HP 8452 spectrophotometer. All spectra were recorded and reactions carried out at 25 °C. Ketonization reactions were initiated by the addition of a small aliquot of a stock solution of **2** or **3** in DMSO to a cuvette containing 1.0 mL of the appropriate buffer. Subsequently, the change in absorption at either 280 or 304 nm with time was monitored, and the rate constants were obtained from nonlinear least-squares fitting of the absorption *vs* time data to a single exponential function. pK_a values were obtained from immediate absorption spectra obtained from solutions prepared as above in different buffers. Buffers (10 mM each, ionic strength 0.1 by KCl addition) were prepared from sodium formate (pH 3.0−4.0), sodium acetate (pH 4.5−5.5), potassium dihydrogen phosphate (pH 6.0−7.5), bicine (pH 8.0−8.5), sodium bicarbonate (pH 9.0−10.5), and dipotassium hydrogen phosphate (pH 11−11.5). Hydrochloric acid solutions were employed for pH 1.0−2.5 and sodium hydroxide for pH 12.0.

3-(Phenylacetamido)pyruvic Acid (2). This compound was prepared exactly as described by Cornforth and Cornforth[5] and recrystallized from *n*-butanol: mp 215 °C dec; (lit.[5] mp 212 °C dec); ^{1}H NMR (DMSO-d_6) δ 3.65 (s, 2H), 7.00 (d, J = 11 Hz, 1H), 7.26−7.32 (m, 5H), 8.45 (s, 1H), 9.95 (d, J = 11 Hz, 1H), 12.6 (s, 1H); ^{13}C NMR (1/1 DMSO-d_6/Me_2CO-d_6) δ 42.0, 111.6, 126.5, 127.3, 128.3, 128.6, 136.1, 166.4, 168.6.

Methyl 3-(Phenylacetamido)pyruvate (3). The α-keto acid **2** in methanol solution at room temperature was methylated by an excess of diazomethane in diethyl ether.[6] After 1 h of reaction the solvents were removed by evaporation, and the residual solid ester was recrystallized from hexane/benzene (3/1): mp 135−8 °C; ^{1}H NMR (DMSO-d_6) δ 3.68 (s, 2H), 3.71 (s, 3H), 7.07 (d, J = 11 Hz, 1H), 7.26−7.32 (m, 5H), 8.66 (s, 1H), 10.07 (d, J = 11 Hz, 1H). MS (EI) m/z (M^+, 3).

Results and Discussion

The Solid Acid 2. A KBr pellet IR spectrum of **2** yielded strong absorptions at 1631, 1674, and 1699 cm^{-1}.

(5) Cornforth, J. W.; Cornforth, R. H. *J. Chem. Soc.* **1953**, 93−98.
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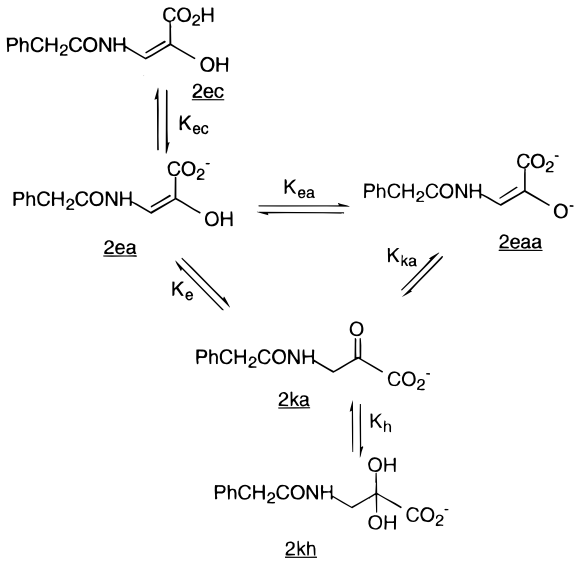
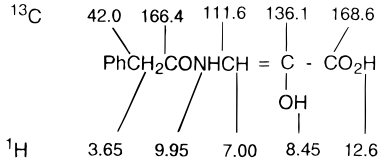
Scheme 2

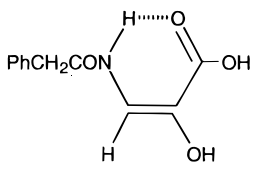


Scheme 3. NMR Assignments in 2



These are best ascribed to amide and α,β-unsaturated carboxylic acid functional groups and therefore strongly suggest that **2** exists in the solid state as the enol **2ec** (Scheme 2). Pyruvic acid[7] and 2-oxobutanoic acid,[8] both thought to exist in the keto form in the pure state, have $\gamma_{C=O}$ values of 1716 and 1720 cm^{-1}, respectively. 3-Arylpyruvic acids also appear to exist largely as enol acids in the solid state.[9,10]

Structure of 2 in Organic Solvents. It is clear from the ^{1}H NMR spectrum of **2** in DMSO-d_6 (Experimental Section) that the enol acid **2ec** was still the stable form (the spectrum was unchanged over several days). Only one methylene resonance was observed. Addition of ^2H_2O left only the 7.00 ppm proton of the downfield resonances (except those of the phenyl hydrogens) as a singlet. This result suggests the assignments of Scheme 3. The rather low-field position of the NH resonance suggests that the enol may be stabilized in organic solvent by intramolecular hydrogen bonding, as in **4**; the ester **3** appears to have a similar structure (see below). The ^{13}C spectrum does



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not contain the resonance of a free carbonyl group, but rather those of alkene carbons at 116.6 and 136.1 ppm.

(7) Bellamy, L. J.; Williams, R. L. *Biochem. J.* **1958**, *68*, 81−84.
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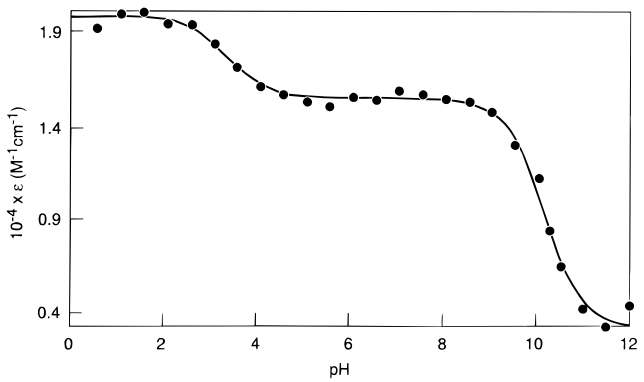

Figure 1. Absorption of **2** at 278 nm as a function of pH. The line represents the best fit to the data of a reaction scheme containing two acid dissociations (pK_a's 3.28 and 10.14).

The ^{1}H NMR spectrum in MeOH-d_4 showed that the enol acid was also the stable (2 days at least) form in that solvent.

UV absorption spectra also indicated the dominance of the enol in organic solvents. For example, in DMSO, a strong absorption (log $\epsilon = 4.27$) maximum was observed at 284 nm. Very similar absorption spectra were obtained from acetonitrile and chloroform solutions.

3-Arylpyruvates show similar spectra.[10]

Observations on 2 in Aqueous Solution. Absorption spectra taken immediately after addition of a small aliquot of a DMSO solution of **2ec** to dilute aqueous acid (λ_{max} 278 nm, log ϵ 4.29) indicate again the presence of **2ec**. An NMR spectrum under these conditions was not possible because of solubility limitations. A similar absorption spectrum was obtained in neutral buffer (λ_{max} 272 nm log ϵ 4.19). In 0.1 M sodium hydroxide, a substantial red shift was observed (λ_{max} 306 nm, log ϵ 4.02), suggestive of conversion of **2ea** to an enolate, presumably **2eaa**. The latter conclusion was supported by a ^{1}H NMR spectrum in 0.1 M NaO^{2}H that showed two singlets at 3.70 ppm (PhCH_2) and 6.35 ppm (CH=).

The pK_a's of the enol acid were determined by absorption measurements at 280 nm as a function of pH. These absorptions were extrapolated to time of mixing because of the ensuing reaction (see below). The data (Figure 1) indicate two dissociation processes. Curve fitting yielded values of pK_{ec} and pK_{ea} of 3.28 ± 0.25 and 10.14 ± 0.07, respectively. These determinations assume, supported by the spectra reported above, that **2** exists primarily as a mixture of **2ec**, **2ea**, and **2eaa** in these solutions directly after addition of the DMSO stock solution. The latter contains **2ec** as described in the previous section. The above pK_a's are in good accord with expectation arising from the analogous pK_a's of pyruvate, 3.91 and 11.55, reported by Kresge.[11]

At all pH values the enol(ate) absorption was observed to decrease with time after mixing, essentially to zero. In order to understand the chemistry of this reaction, a ^{1}H NMR experiment was undertaken. The ^{1}H NMR spectrum of 10 mM **2** in 100 mM $NaHCO_3$ in ^2H_2O (pD *ca.* 8) was followed with time to completion in about 5 h. During this time, singlet peaks initially present at 3.81 and 6.88 ppm and assumed to correspond to **2ea** slowly disappeared and were replaced by two sets of singlets at a constant ratio, *ca.* 7:3. Those at 3.77 ppm and 4.45 ppm were assigned to the keto form **2ka** and those at 3.68 ppm and 3.55 ppm (the minor species) to the hydrate **2kh**. The upfield shift of 0.90 ppm thus proposed for the methylene adjacent to the ketone upon hydration is very similar to that observed (0.86 ppm) for the methyl resonance on hydration of pyruvic acid.[12]

Over the same time scale described above, it was also observed that the new peaks at 4.55 and 3.55 ppm disappeared. This presumably reflects exchange of these protons, adjacent to the carbonyl group in **2ka**, with solvent deuterium, and shows that under these conditions reversal to **2ea** is also facile.

The spectral changes described above thus are in accord with a process of ketonization of the enol and subsequent hydration of the ketone, with a K_h value of 8.5×10^{-3} M^{-1} ($K_h = [\mathbf{2kh}]/[\mathbf{2ka}][H_2O]$, $[H_2O] = 55.5$ M). That this value is larger than that of pyruvate (1.3×10^{-3} M^{-1})[11] is reasonable in view of the electron-withdrawing amido substituent present in **2ka**.

A bicarbonate reaction mixture in H_2O was freeze-dried after completion of the above-described reaction, and NMR spectra of the residue, a colorless solid, were taken in DMSO-d_6 solution. Both ^{1}H and ^{13}C spectra indicated the presence of a single compound with ^{1}H resonances at 3.50 ppm (s, 2H, PhCH_2), 4.13 ppm (d, $J = 6$ Hz, 2H), and 8.10 (br t, 1H). The ^{13}C spectrum had two methylene peaks at 42.0 and 47.0 ppm, a carbonyl resonance at 202.1 ppm, and no peaks in the 80−120 ppm region, indicating the absence of enol and hydrate species. Finally, an IR spectrum (KBr pellet) showed an absorption peak at 1725 cm^{-1} (cf. sodium pyruvate,[13] 1709 cm^{-1}). Together, these spectra suggest that the freeze-dried product contains the pure ketone **2ka**, presumably as the sodium salt. Dissolution of the solid in ^2H_2O gave a solution whose immediate ^{1}H NMR spectrum showed the same mixture of ketone and hydrate as described above. The hydration reaction is therefore rapid ($t_{1/2} < 1$ min), as expected.[12,14]

As described above, the enol absorbance was essentially completely lost when equilibrium had been achieved at pH 1−9, in accord with the ^{1}H NMR observation at pH 8 where only ketone and hydrate were observed at equilibrium. From the residual absorption at equilibrium, it was estimated that ≤1% of the enol species remained; therefore, in aqueous solution $K_e \leq 0.01$ (for pyruvate, p$K_e = 5.11$[11]).

The loss of enolate absorption with time at high pH suggested that pK_{ka} must be greater than 13; i.e., even in 0.1 M sodium hydroxide the stable species must be the keto/hydrate mixture. Contrary to the expectation arising from this conclusion, however, it was observed that addition of solid **2ka** to 0.1 M sodium hydroxide solution gave rise to a close to stoichiometric amount of enolate, as judged by its absorption at 306 nm, which then decreased slowly with time. This suggests, on one hand, that the reaction observed at high pH leading to loss of enolate is *not* ketonization and on the other that p$K_{ka} \leq 13$. ^{1}H NMR observation of a sample of **2eaa** in 0.1 M NaO^{2}H with time showed the loss of the enolate and the appearance of a product whose spectrum contained singlet peaks at 3.65 and 3.73 ppm that were shown to be identical to those of phenylacetylglycine. The slow reaction in alkaline solution ($k_{obs} = 3.0 \times 10^{-5}$ s^{-1}

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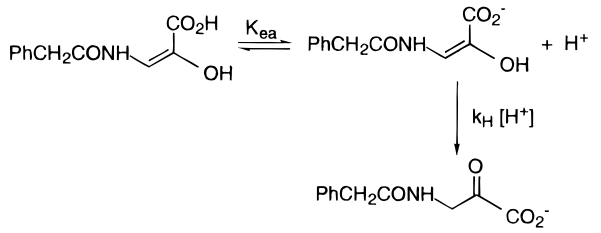
Scheme 4



and [OH^-] independent between 0.02 and 0.1 M) therefore is presumably an oxidative decarboxylation involving reaction of the enolate with molecular oxygen or a derived oxidant. Oxidative decarboxylation of α-keto acids has been shown to occur in their reaction with hydrogen peroxide[15] and singlet oxygen,[16] for example.

A thermodynamic cycle evident in Scheme 2 leads to a lower limit for pK_{ka}: since $pK_{ka} = pK_e$ (≥2) + pK_{ea} (10.1), $pK_{ka} \geq 12.1$, we can conclude therefore that the pK_a of **2ka** as a carbon acid lies between 12 and 13. The quite considerable effect of the amido substituent on the carbon acidity of pyruvate can be seen by comparison of this estimate with the value for pyruvate of 16.65, calculated from the data of Kresge.[11]

Kinetics of Ketonization. Pseudo-first-order rate constants, k_{obs}, of the ketonization reaction were obtained from measurements of enol absorption as a function of time. At low pH, this rate constant [$(3.23 \pm 0.35) \times 10^{-5}$ s^{-1}] did not vary with acid concentration (0.02−1 M HCl). This, in principle, could represent spontaneous or water-catalyzed ketonization of **2ec**, but in view of considerable precedent,[11,17] especially that of acetoacetic acid enol,[18] it is much more likely that the reaction in acid solution involves the kinetically equivalent reaction of the more electrophilic enol carboxylate **2ea** with a proton (Scheme 4). At low pH, from Scheme 4, $k_{obs} = k_H K_{ea}$, and thus, $k_H = 0.062$ s^{-1} M^{-1}. This value is 1 order of magnitude greater than that for enol pyruvate (6.5×10^{-3} s^{-1} M^{-1}).[11] Although enol pyruvate is presumably a stronger carbon base than **2ea**, the greater enol acidity of the latter may be more important in the ketonization transition state—the enol proton must dissociate at some stage along the reaction coordinate.

At pH values above the pK_a of **2ec**, the rate of ketonization in water decreases in a fashion similar to that seen for the enol of acetoacetate.[18] In these solutions, the observed ketonization is largely derived from catalysis by buffer species. For example, the catalysis provided by phosphate at pH 6 and 7.5 is shown in Figure 2. The reaction appears to be catalyzed by the basic form of the catalyst reacting with **2ea**, but again on the basis of precedent,[17,18] the actual reaction is probably the kinetically equivalent protonation of **2eaa** by dihydrogen phosphate (pK_a 6.7). Thus interpreted, the second-order rate constant for the latter reaction, taken from the data of Figure 2, was 160 s^{-1} M^{-1}.

More weakly acidic, but cationic, protonated tertiary amine catalysts were more effective than dihydrogen phosphate—second-order rate constants for protonation

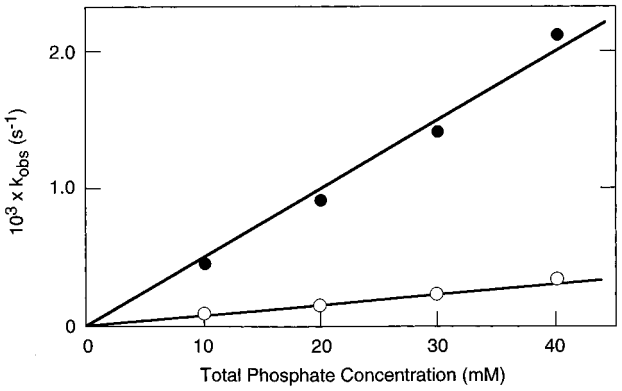
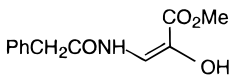


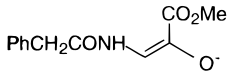
Figure 2. Variation of pseudo-first-order rate constants for ketonization of **2ea** with phosphate buffer concentration. The closed circles represent data obtained at pH 7.5 and the open circles that at pH 6.0.

of **2eaa** by the protonated form of MOPS (pK_a 7.2) and triethanolamine (pK_a 7.8) were 1700 s^{-1} M^{-1} and 200 s^{-1} M^{-1}, respectively. This difference presumably reflects electrostatic effects that are well-known in the catalysis of keto−enol transformations.[17] The converse case with cationic ketones has been described.[19] In the present case, the interaction of catalysts with the pyruvate carboxylate in the transition state must be stronger than the interaction of the former with the developing positive charge on the enol.

Structure and Reactions of Methyl 3-(Phenylacetamido)pyruvate (3). The methyl ester of **2** also appeared to exist in the solid state as the enol **3e**—the solid-state infrared spectrum showed only one carbonyl stretching vibration above 1700 cm^{-1}, the ester carbonyl at 1741 cm^{-1}. The enol structure also obtained in organic solvents as shown by the ^1H NMR spectrum in DMSO-d_6 for example (see Experimental Section), which includes an enol CH resonance at 7.07 ppm, and UV absorption spectra which show strong absorption maxima around 280 nm (e.g., in DMSO, $\lambda_{max} = 286$ nm, $\log \epsilon = 4.18$).



3e



3ea

In aqueous solution, the enol was initially observed at low pH (λ_{max} 280 nm, $\log \epsilon$ 4.15), and another species, presumably the enolate **3ea**, at high pH (λ_{max} 314 nm, $\log \epsilon$ 4.09). Absorption measurements (extrapolated to zero time) as a function of pH yielded a value of 8.14 ± 0.07 for the enol dissociation. In acidic and neutral solution, the enol absorption decreased as a function of time. Buffer catalysis was again important at pH values around neutral. Presumably, a thermodynamically favored ketonization reaction occurred here as with the acid. Detailed kinetic measurements were not made in this case, however, since ^1H NMR observations indicated that methyl ester hydrolysis competed with ketonization/hydration.

Conclusions

The α-keto β-amido acid **2** and its methyl ester **3** exist in the enol form both in the solid state and when

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dissolved in organic solvents, but are largely ketonized and partly hydrated at equilibrium in aqueous solution. The latter is also true of the monoanion **2ea** of **2**. Ketonization at neutral pH is slow but is markedly accelerated by buffer acids, particularly those of tertiary amines, e.g., the half-time of ketonization of **2ea** in 20 mM MOPS buffer, pH 7.5, is *ca.* 20 s. At pH values above neutral, significant amounts of the enolates **2eaa** and **3ea** are present in solution. The behavior of these compounds is therefore very similar to that of 3-arylpyruvates.[9,10]

Many compounds analogous to **2** and **3** have been employed as enzyme inhibitors.[2] In many of the cited papers more than one of the species shown in Schemes 1 and 2 has been observed and mentioned. Many of the peptidyl α-keto acyl compounds as prepared are not crystalline; some do appear to contain enol[2b] and hydrate[2l] as well as keto forms (enols appear to be common in cases where, as in **2** and **3**, there is no alkyl substituent on C-3), and it is usually not clear whether the isolated material represents a sample at tautomeric equilibrium. Geometric isomerism is also possible in the enol form. The consequence of all of this, as illustrated by the present work, is that a quite complicated series of reactions may occur on introduction of the sample to the aqueous medium employed for enzyme inhibition measurements, including reestablishment of the position of tautomeric equilibrium, hydration of the α-keto group, exchange of hydrogens (potential epimerization[2i,k]) adjacent to the α-keto group, and dissociation of acidic carboxyl and possibly enolic protons. α-Keto esters may also hydrolyze. The apparent inhibitory power of the compound therefore may well be observed to change, conceivably in either direction, with time, with pH and with buffer composition. These problems have been addressed in an empirical way by several groups and have been described in some detail by Brady et al. in their study of thrombin inhibitors.[2l] The actual K_i of the keto species itself can only be obtained after the effects of these reactions have been taken into account. Apparent slow-binding enzyme inhibition[2h,i,k] may either reflect these external reactions, slow intereactions of the keto species with the enzyme, or both.

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