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Serine Protease Mechanism-Based Mimics. Direct Evidence for a Transition State Bridge Proton in Stable Potentials

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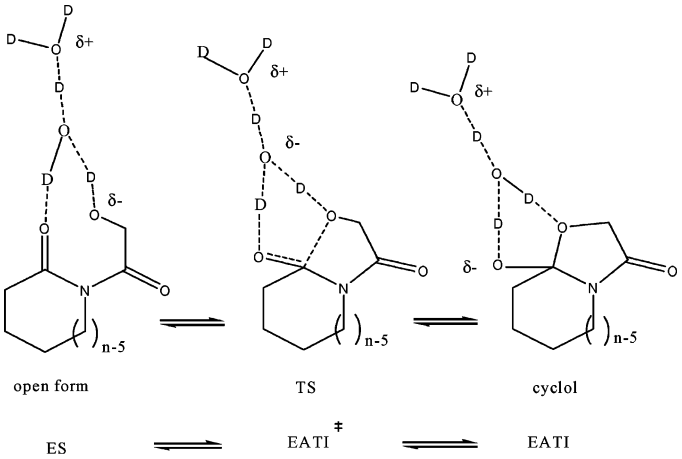

We have synthesized 1-(2-hydroxyacetyl)piperidine-2-one (**2**) and 1-(2-hydroxyacetyl)azepan-2-one (**3**). Equilibrium (K_f) between the free alcohol (open form) and the tetrahedral intermediate (cyclol) is readily established, and both forms are observed in the D_2O ^1H NMR spectra of **2** and **3**. Therefore, their interconversion can be considered as an almost thermoneutral non-identical one. Pseudo-first-order rate constants (k_{obs}) were obtained by simulating the AB ^1H NMR system observed for the cyclol. By best fitting the experimental points of a k_{obs} versus pD profile to the equation $k_{obs} = 0.5k_{0r} + 0.5k_r K_{ac}/(K_{ac} + [D^+]) + 0.5k_f K_{ao}/(K_{ao} + [D^+])$, the parameters involved were obtained: rate constants of rupture and formation (k_{0r} and $k_{0f} = K_f k_{0r}$) catalyzed by water, rate constants of rupture (k_r) and formation (k_f) from the conjugated bases of the cyclol form and the open form, and their acidity equilibrium constants K_{ac} and K_{ao}. The system studied mimics the serine alcohol attack on the peptide bond and its reverse reaction in serine protease enzymes. In fact, the reaction rates are similar or perhaps even faster than the ones obtained for enzymatic reactions. The results also show the participation of water molecules forming catalytic proton bridges in stable potentials with the two interconverted forms. The position change of the bridged proton is sensitive to lactam ring size, and it is manifested by considerable change in the pK_a values of both cyclol and open forms. Other evidence such as kinetics, $\Delta S°$, $\Delta S^‡$, and proton inventory experiments and semiempirical molecular calculations support this proposal.

Introduction

We have been interested in measuring rates of intramolecular transformations in stable tetrahedral intermediates. For instance, we have reported[1,2] results on transannular rates in bislactam macrocycles formed from *N*-(2-aminoacetyl)-2-lactams. We have also reported[3] the rate of formation of the corresponding stable tetrahedral intermediates of *N*-heteroethylphthalimide (hetero: hydroxy, amino, and thioxy) and their rates of rupture

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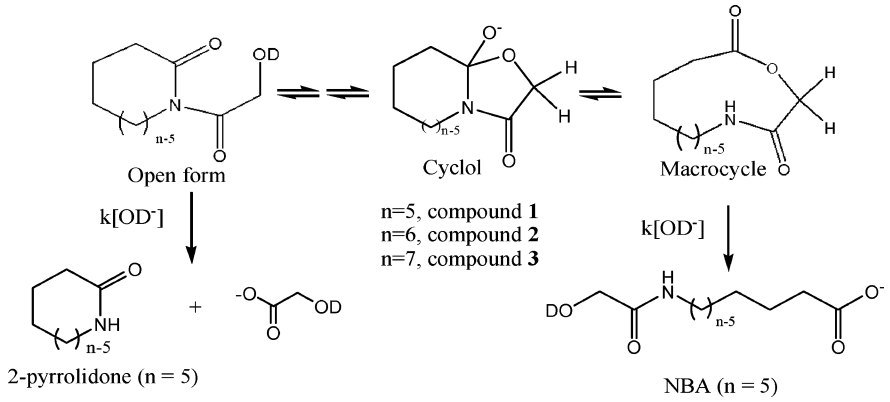
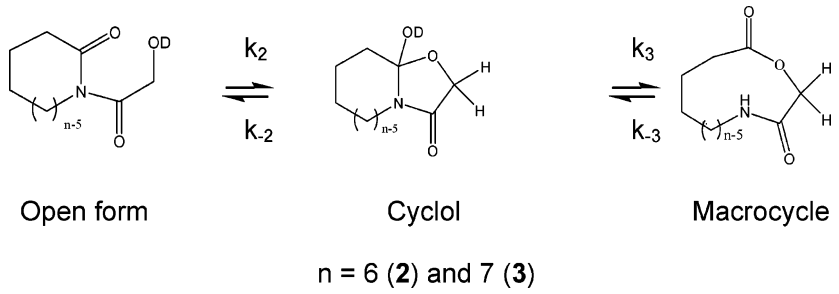
SCHEME 1[a]



[a] Hydrolysis paths for compound **1** ($n = 5$). Only the open form of compound **1** and its hydrolysis products (2-pyrrolidone, hydroxyacetic acid, and NBA) are observed in the ^{1}H NMR at $7 < pD < 11$. The cyclol and macrocycle are barely observed at $pD > 11$. For compounds **3** ($n = 7$), the macrocycle is also observed at any pD, and for **2** ($n = 6$), only the cyclol and open forms are observed. At $pD > 12$, the corresponding hydrolysis products are observed for compounds **2** ($n = 6$) and **3** ($n = 7$).

SCHEME 2[a]



[a] Compound **3** forms observed in D_2O by ^{1}H NMR. For compound **2**, only the open form and cyclol are observed.

to diacylimides. Recently, we have synthesized *N*-(2-hydroxyacetyl)-2-pyrrolidone (**1**), expecting to observe chemical behavior similar to that observed in the *N*-(2-aminoacetyl)-2-lactams. However, we found[4] at $pD > 7.5$ an irreversible cleavage of the exocyclic and endocyclic C−N bond of **1** to yield, in the latter case, *N*-(4-hydroxyacetyl)butanoic acid (NBA) (Scheme 1). This product corresponds to a lactam ring opening, a process of wide interest in bioorganic chemistry. As shown in Scheme 1, the exocyclic cleavage of **1** yields 2-pyrrolidone. Its formation is first-order-catalyzed by OD^-, whereas NBA production is second-order with respect to $[OD^-]$. In fact, NBA is produced from the cyclol form of **1** that cleaves, breaking its amide bond, to produce the macrocycle that finally opens to yield NBA. The cyclol and macrocycle forms are detected only at high pD ($pD > 12$). Therefore, these two forms are not stable enough at neutral pD. However, the pK_a value (11.9) for the cyclol form of **1** could be obtained experimentally[4] from product ratios and estimated ($pK_a = 11.7$) from linear free energy relationships where only inductive effects are taken into account. The low stability of the cyclol and macrocycle forms of **1** has been attributed[4] to the resonance of the planar conformation of the five-membered ring lactam that retards the intramolecular attack of the 2-hydroxyacetyl moiety. Strain in forming the cyclol (tetrahedral intermediate) may also play a role.

Expecting to observe the cyclol and/or macrocycle forms in other less strained 1-(2-hydroxyacetyl)lactams, we have synthesized 1-(2-hydroxyacetyl)piperidin-2-one (**2**) and 1-(2-hydroxyacetyl)azepan-2-one (**3**). In fact, for these compounds, the cyclol (tetrahedral intermediate) is the most stable form at any pH. Therefore, equilibrium constants $K_f = [\text{cyclol}]/[\text{open form}]$ have been measured as well as thermodynamic parameters at neutral pH.

The unusual stability of the tetrahedral intermediate (cyclol form) makes these systems unique since their rates of cleavage and formation, in these almost thermoneutral non-identical reactions, are barely influenced by the difference in stability between reactants and products. In fact, for compounds **2** and **3**, the cyclol and open forms (Scheme 2) are both detected by ^{1}H NMR in an ample pH range, making it possible to measure their interconversion rates via dynamic ^{1}H NMR.

Direct observation and trapping of tetrahedral intermediates have been reported[5,6] previously in the hydrolysis of the *N*,*O*-trimethylenephthalimidium ion in a system similar to the one studied herein. However, two main aspects make these systems chemically different: First, the attacking and leaving groups in the former system are not in the same molecule; that is, the reaction is intermolecular with reactants and products with quite different stability; therefore, thermodynamic enthalpic and entropic factors influence the observed rates. As mentioned above, this is not the case in the system described in this work. Second, in Gravitz's and Jencks's system, there is a weak N

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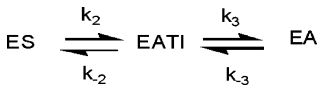
SCHEME 3[a]



[a] The system shown in Scheme 2 can be considered as serine protease mechanism-based mimics of the acyl−enzyme tetrahedral intermediate (EATI) formation and its corresponding partitioning.

(amide) electron pair driving force for the expulsion of the leaving group instead the O electron pair(s) of the cyclol form (Scheme 2).

The almost thermoneutral reactions of this work may also mimic enzyme reactions. For instance, the open form in Scheme 2 could be envisioned as a serine protease active site where peptide (endocyclic amide C═O bond) and serine enzyme residues (exocyclic hydroxymethyl group) coexist in the same molecule. We are then dealing with mechanism-based mimics[7] where the substrate−enzyme binding process has been bypassed. The formation (k_2) and rupture (k_{-2}) of the cyclol form in Scheme 2 mimics the formation (k_2) and cleavage (k_{-2}) of the acyl−enzyme tetrahedral intermediate (Scheme 3), whereas the eventual rupture of the endocyclic C−N bond to yield the macrocycle form mimics its formation (k_3) and its reverse reaction (k_{-3}).

It has been pointed out[8−10] that perfectly evolved Michaelian enzymes should exhibit equilibrium constants close to unity for the interconversion of enzyme-bound substrate and product. Instead of perfection, it has been affirmed[11] that the unity condition corresponds to an optimal state of operation of presently existing enzymes since the corresponding intrinsic barriers are involved. This equilibrium constant close to unity is in fact the one found in the models studied in this work. Therefore, these models may be considered as mimics of an evolved serine protease.

Moreover, recently,[12] it has been reported that tetrahedral intermediates formed in the peptide cleavage in HIV proteases can be trapped at the active site. Therefore, the use of compounds **2** and **3** as tetrahedral intermediate inhibitors of HIV proteases is worth exploring.

The results reported herein indicate that the water molecule plays a special role in the reaction by hydrogen bonding the cyclol and open forms. In agreement with the proposal[13] for the role of hydrogen bonds during proton transfer in general catalytic transition state stabilization in enzyme catalysis, the proton bridges found in this work are strong hydrogen bonds in stable potential. The participation of water at the reactant stage is supported by pK_a, equilibrium entropic values, rate constants, entropic activation parameters, proton inventory experiments, and semiempirical molecular calculations.

Experimental Section

Synthesis. Benzyloxyacetyl Chloride. In a two-neck flask provided with a condenser and a positive pressure of argon, 1.7 mL (12 mmol) of benzyloxyacetic acid and 15 mL of dry toluene were added. After these reactants were mixed, recently distilled thionyl chloride (1.75 mL, 24 mmol) was added dropwise while stirring. The mixture was heated at 70−75 °C for 3 h. After this time, the excess of thionyl chloride was removed by distillation. The remaining product was used directly in the next reaction step without further purification.

1-(2-Benzyloxyacetyl)azepan-2-one and 1-(2-Benzyloxyacetyl)-piperidine-2-one. In a two-neck flask provided with an argon atmosphere, either 1.27 g (10.8 mmol) of azepan-2-one or 1.07 g (10.8 mmol) of piperidin-2-one was mixed with dry pyridine (0.9 mL, 10.8 mmol) and 5 mL of toluene. This mixture was cooled to 0 °C, and the benzyloxyacetyl chloride, previously prepared and maintained at 0 °C, was added slowly. The product solution took on a yellow color. The reaction mixture was stirred at 0 °C for 2 h. After this time, the mixture was refluxed under an argon atmosphere for 12 h. At the end of this time period, the mixture turned a brown color. It was cooled at 0 °C, and 25 mL of ice/water mixture was added. The two phases were separated, and the aqueous one was extracted twice with 20 mL of toluene. The organic phases were combined and treated with sodium bicarbonate and dried with anhydrous magnesium sulfate. Finally, the solvent was evaporated to yield a dark brown oil.

1-(2-Benzyloxyacetyl)azepan-2-one: 2.52 g, 70%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (m, 5H, Ph), 4.64 (s, 2H, $-O-CH_2-$Ph), 4.61 (s, 2H, $-CH_2-O$), 3.92 (t, 2H,CH_2-N, J = 5.13 Hz), 2.67 (t, 2H, lactam $-CH_2-C-(O)$, J = 4.03 Hz), 1.72 (m, 6H, lactam $-(CH_2)_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.8, 173.5, 137.8, 128.5, 128.3, 128.1, 127.9, 125.4, 73.3, 72.9, 43.1, 39.5, 29.2, 28.5, 23.6; MS *m*/*z* (fraction, relative intensity) 261 (M^+, 5), 154 ($M^+ - 107$, 6×10^5), 91 ($M^+ - 170$, 1×10^6).

1-(2-Benzyloxyacetyl)piperidine-2-one: 2.95 g, 99%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (m, 5H, Ph), 4.64 (s, 2H, $-CH_2-O$), 4.63 (s, 2H, $-O-CH_2-$Ph), 3.74 (t, 2H, $-CH_2-N$, J = 6.22 Hz), 2.52 (t, 2H, lactam $-CH_2-C(O)$, J = 5.86 Hz), 1.81 (m, 4H, lactam $-(CH_2)_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.3, 173.3, 137.7, 128.5, 128.1, 127.9, 73.3, 73.0, 44.1, 34.5, 22.3, 20.2; MS *m*/*z* (fraction, relative intensity) 156 ($M^+ - 91$, 5×10^4), 140 ($M^+ - 107$, 2.5×10^5), 91 ($M^+ - 170$, 3×10^5).

1-(2-Hydroxyacetyl)azepan-2-one (3). In a high-pressure resistance flask, 1.53 g (5.86 mmol) of 1-(2-benzyloxyacetyl)azepan-2-one in 125 mL of ethyl acetate was added. To this solution was added 2.18 g of 5% Pd/C catalyst. The mixture was reduced under continuous stirring and hydrogen pressure (40 psi) for 7 h. The mixture was filtered, and the residual solvent was eliminated by vacuum at room temperature.

A white hygroscopic solid (0.68 g, 70% yield) corresponding to 1-(2-hydroxyacetyl)azepan-2-one was obtained: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.61 (s, 2H, $-C(O)-CH_2-O-$), 4.44 (br s, 1H, $-OH$), 3.97 (t, 2H, CH_2-N, J = 5.49 Hz), 2.71 (t, 2H, lactam $CH_2-C(O)$, J = 4.39 Hz), 1.20−1.90 (m, 6H, lactam $-(CH_2)_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.6, 176.8, 65.9, 35.5, 29.1, 27.9, 23.6, 22.1.

1-(2-Hydroxyacetyl)piperidine-2-one (2): Using the same procedure as indicated above for 1-(2-benzyloxyacetyl)azepan-2-one (**3**), 2.37 g (9.6 mmol) of 1-(2-benzyloxyacetyl)piperidine-2-one in 140 mL of ethyl acetate was reduced with 3.05 g of 5% Pd/C. A hygroscopic white solid (1.38 g, 91% yield) was obtained: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.64 (s, 2H, $-C(O)-CH_2-O-$), 3.78 (t, 2H, CH_2N, J = 5.86 Hz), 3.39 (br s, 1H, $-OH$), 2.55 (t, 2H, lactam $-CH_2-C(O)$), 1.86−1.70 (m, 4H, lactam $-(CH_2)_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.5, 173.1, 66.8, 38.4, 24.3, 21.2, 20.0.

Signal Identification. The assignments of the equilibrium forms of compounds **2** and **3** were made based on previous identification[1,2] of *N*-(2-aminoacetyl)-2-lactams, 2D ^{1}H NMR COSY, and NOESY experiments and ^{13}C NMR.

Sample Preparation. Samples of 1-(2-hydroxyacetyl)piperidine-2-one (**2**) and 1-(2-hydroxyacetyl)azepan-2-one (**3**) in D_2O at different pD were prepared using phosphate buffers and at constant

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TABLE 1. Equilibrium Constants, Rate Constants, and Thermodynamic and Activation Parameters Obtained in This Work for Compound 2 ($n = 6$) and Compound 3 ($n = 7$)

n	K_f[a]	pK_a cyclol, open form	k_{0r}[b,c] (s^{-1})	k_{0f} (s^{-1})	k_r[d] (s^{-1})	k_f (s^{-1})	$\Delta G^{\ddagger}_{0r}$ (kJ/mol)	$\Delta G^{\ddagger}_{0f}$ (kJ/mol)	$\Delta G^{\ddagger}_{r}$ (kJ/mol)	$\Delta G^{\ddagger}_{f}$ (kJ/mol)
6	12 ± 0.6	7.3, 10.5	4	48	5.0	20	64	56	63	60
7	36 ± 3	5.1, 9.8	2	72	8.5	64	66 (47)[e] (−40)[f]	55 (30)[e] (−70)[f]	61	57

[a] Neutral pH. [b] Standard deviation from simulations: 5%. [c] Obtained by simulation using eq 1 and equilibrium constants. [d] Obtained by simulation using eq 1. [e] $\Delta H^{\ddagger}$ (kJ/mol). [f] $\Delta S^{\ddagger}$ (J/mol·K).

ionic strength. In a typical sample, 0.2 mol/dm^3 of compounds **2** or **3**, 1 mol/dm^3 of phosphate buffer (H_3PO_4 or KH_2PO_4), and 2 mol/dm^3 of KCl were used. Samples were prepared directly into the NMR tube by adding 25 mg of compounds **2** or **3**, 0.12 g of KCl, 0.7 dm^3 of D_2O, and the corresponding amount of buffer. The sample pH was measured directly into the NMR tube before and after taking the corresponding spectrum. The pH values did not vary more than 0.1 pH units; pD values were obtained using the relation:[14] pD = pH + 0.40.

In order to explore the presence of general catalysis, samples of compound **3** at two different total phosphate buffer concentration and constant pH were prepared. Total buffer concentrations of 0.75, 1.00, 1.50, and 2.00 mol/dm^3 were used while maintaining a pD constant at 6.3 and 9.0. A 400 MHz instrument was used to run the different spectra.

Additionally, a sample of 25 mg of compound **3** in 0.7 cm^3 of D_2O was used to obtain ^{1}H NMR spectra at 18.5, 35, 45, and 55 °C. A similar sample was used to perform COSY and NOESY and EXSY 2D ^{1}H NMR experiments. In the last case, two mixing times of 100 and 300 μs were used. A 500 MHz instrument was used to perform these experiments. For the proton inventory experiments, volume fractions of H_2O in D_2O in the range of 0.02−0.20 were used.

A line shape analysis program[15] was used to simulate each experimental spectrum and to obtain the pseudo-first-order rate constants. The ^{1}H NMR AB system assigned to the cyclol form was used to evaluate rates. Best match between the experimental and simulated spectrum was obtained by minimizing the line shape difference between the two spectra while changing k_{obs} values. A standard deviation no higher than 5% could be achieved performing simulations.

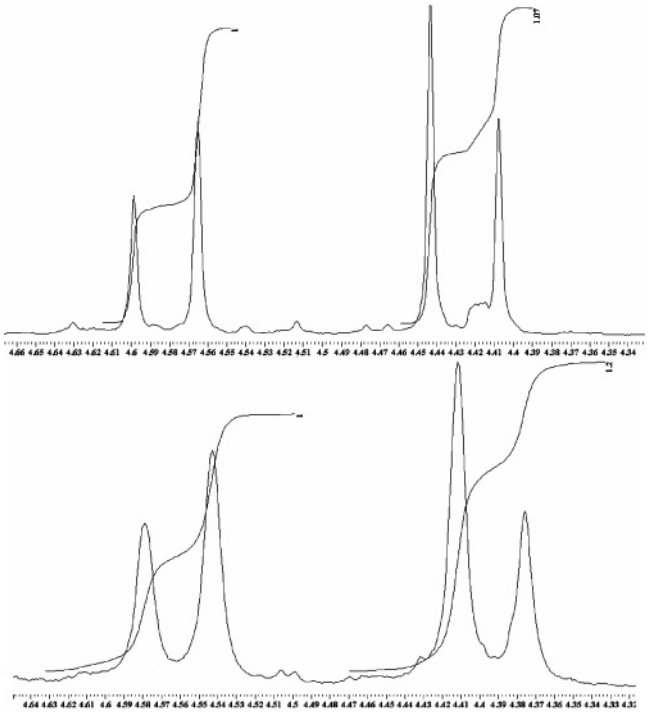

FIGURE 1. D_2O ^{1}H NMR spectra of the AB system of the cyclol form of compound **2** at pD = 6.3 (top) and pD = 9.9 (bottom). The intraconversion between the A and B is catalyzed when increasing pD.

Results

In Figures S1 and S2 (Supporting Information), the ^{1}H NMR spectra of compound **2** in $CDCl_3$ and D_2O are shown. In the same figures, the assignments of the different signals corresponding to the cyclol and open forms are depicted. In Figure S3 (Supporting Information), the assignment of signals in $CDCl_3$ for the three forms observed for compound **3** is shown. From the relative integration of the open form exocyclic methylene protons (ca. 4.6 ppm, see Figure S2 in Supporting Information) and the AB ^{1}H NMR system observed for the same protons in the cyclol (ca. 4.3 ppm, see Figure S2 in Supporting Information), it is found that the equilibrium constants for the cyclol form in D_2O at neutral pD and room temperature for compounds **2** and **3** are 12 and 36, respectively. However, this equilibrium constant decreases considerably in $CDCl_3$ (see Figures S1 and S3 in Supporting Information). For instance, for compound **2** it is equal to 0.9 and for compound **3**, it is equal to 2. However, in D_2O, for compound **3**, the cyclol/open form equilibrium is 3 times the corresponding one for compound **2** at neutral pD and at 19 °C (see Table 1). The equilibrium constant for compound **1** could not be measured[4] since, at neutral pH, only the open form is detectable by ^{1}H NMR. Nevertheless, it can be remarked that for compounds **2** and **3** the relative stability of the three forms in D_2O is cyclol > open form > macrocycle. The macrocycle form of compound **3** is also observable in the ^{1}H NMR. Yet, its contribution in the spectrum is barely detectable as well its participation in the exchange with the cyclol forms. Two-dimensional ^{1}H NMR EXSY experiments at 300 ms confirmed the last observation (not shown). The plot of $\Delta G°$ (kJ/mol) versus T (K) (four points; see Experimental Section) was a straight line ($\Delta G° = 0.0295T - 16.924$; $R^2 = 0.98$). From this plot, $\Delta H° = -16.9$ kJ/mol and $\Delta S° = -29.5$ J/mol·K are obtained.

In Figure 1, the experimental ^{1}H NMR spectrum of the AB system of the cyclol form of **2** in D_2O at two different pD values is shown. A specific base catalysis or a kinetically equivalent one is observed since the AB system changes toward coalescence when pD increases. Similar changes are also observed for compound **3**.

Rate constants for the AB exchange at each pD were obtained by simulating[15] the line shape of each spectrum. In order to

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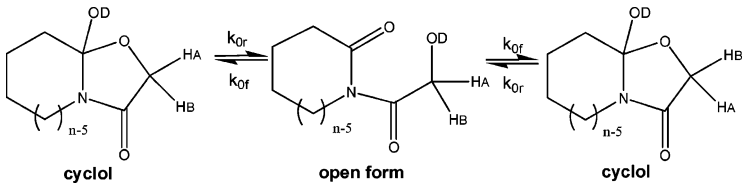
SCHEME 4[a]



[a] Cyclol proton exchange of the observed ^{1}H NMR AB system. As it is shown, the probability of the AB exchange is half the rate of rupture (k_{0r}) and formation (k_{0f}).

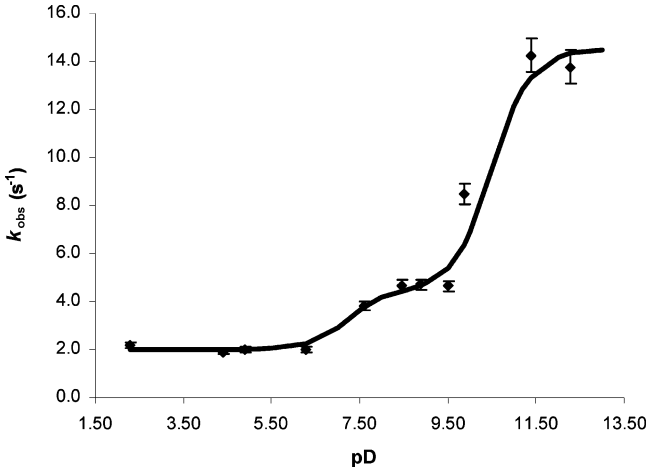


FIGURE 2. Plot of k_{obs} versus pD for the AB ^{1}H NMR system signal exchange of the cyclol form of compound **2**. Dots: experimental points. Solid lines: best fitting of the experimental points to eq 1, from which the following constants were obtained: $k_{0r} = 4$ s^{-1}; pK_a cyclol = 7.3; $k_r = 5$ s^{-1}; pK_a open form = 10.5 and $k_f = 20$ s^{-1}. Error bars: 5% due to error in simulation.

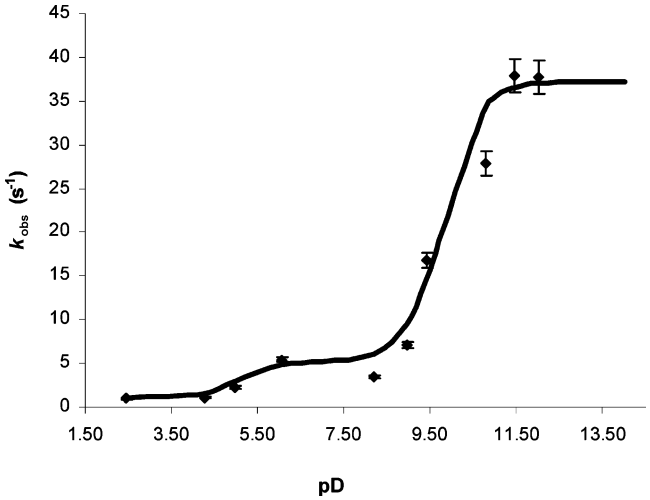


FIGURE 3. Plot of k_{obs} versus pD for the AB ^{1}H NMR system signal exchange of the cyclol form of compound **3**. Dots: experimental points. Solid lines: best fitting of the experimental points to eq 1, from which the following constants were obtained: $k_{0r} = 2$ s^{-1}; pK_a cyclol = 5.1; $k_r = 8.5$ s^{-1}; pK_a open form = 9.8 and $k_f = 64$ s^{-1}. Error bars: 5% due to simulation error.

observe exchange between the A and B protons of the ^{1}H NMR AB system corresponding to the cyclol form, it is required that its C−O bond cleaves to yield the open form and that this form re-closes to form the cyclol with the AB protons exchanged (Scheme 4).

Therefore, the measured rate constants (k_{obs}) are half the rate constants for cleavage and formation. The k_{obs} in the ample pD range studied is then given by

$$k_{obs} = 0.5k_{0r} + \frac{K_{ac}}{K_{ac} + [D^+]} \times 0.5k_r + \frac{K_{ao}}{K_{ao} + [D^+]} \times 0.5k_f \quad (1)$$

where, k_{0r} is the pseudo-first-order rate constant for water-catalyzed rupture of the cyclol; k_r and k_f are the corresponding pseudo-first-order rate constants of rupture and formation of the conjugate bases of the cyclol and open forms; and K_{ac} and K_{ao} are the acidity equilibrium constants for the cyclol and the open form. In Figures 2 and 3, plots of k_{obs} versus pD for compounds **2** and **3** are shown.

The solid line corresponds to the best fitting of the experimental points to eq 1. From the best fit, the rate constants and acidity constants involved in eq 1 have been obtained. These parameters are shown in Table 1. In this table, the corresponding $\Delta G^{\ddagger}$ values are also shown. For the reaction of compound **3** catalyzed by water, the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values are also shown.

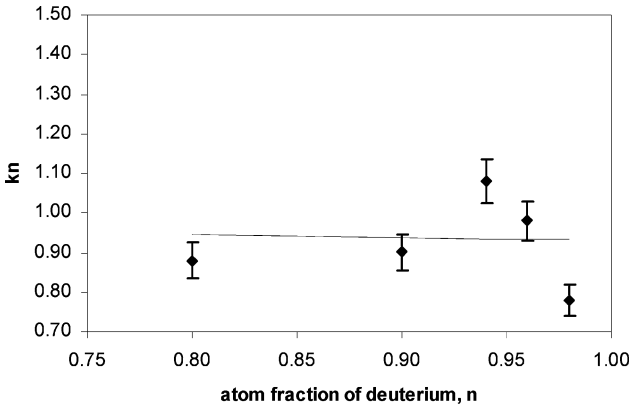
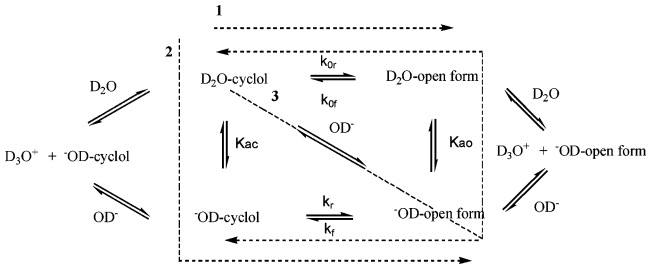


FIGURE 4. Proton inventory experiment performed in the range of 0.8−1 atom fraction of deuterium. Error bars on the experimental points correspond to the maximum deviation (5%) in rate constants according to the line shape simulations. Solid line: $k_n/k_o = (1 + n(\varphi - 1))Z^n$, $\varphi = 0.98$, $Z = 0.95$.

These were obtained using the four temperatures indicated in the Experimental Section. A proton inventory experiment (Figure 4) performed on compound **3** at neutral pH and room temperature shows only a small or no isotope effect ($\varphi = 0.98$, $Z = 0.95$) in the range of the atom fraction of deuterium of 0.80−1.0. General catalysis by phosphate buffer at pH = 5.9 and 8.6 was not observed either.

SCHEME 5[a]



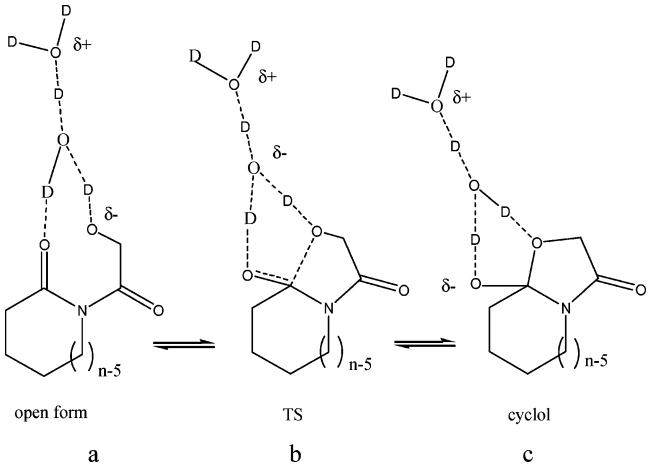
[a] D_2O-cyclol−D_2O-open form interconversion scheme and rate and equilibrium constants involved according to eq 1 and plots of Figures 3 and 4 for compounds **2** and **3**. The three mechanistic routes (**1**, **2**, and **3**), pointed out with dashed lines, correspond, respectively, to the three terms of eq 1. The arrows are placed just after the corresponding rate-limiting steps. To the left and right side of the marked square, participation of a second water molecule is shown. Therefore, the pK_a values obtained correspond to the water hydrogen-bonded complexes.

Discussion

We have been able to measure directly the rate constants for cleavage and formation of a C−O bond in tetrahedral intermediates. As shown in Figures 2 and 3, three plateaus are detected in the k_{obs} versus pD profiles for compounds **2** and **3**. These correspond to the partial contribution to the rate of each term in eq 1. The first one at neutral pD corresponds to the water-catalyzed open form/cyclol interconversion, where the D_2O-cyclol rupture (k_{0r}) is rate-limiting. The rate constants were obtained from the plateau value ($k_{obs} = k_{0r}$) and the equilibrium constant obtained by ^{1}H NMR integration of the cyclol and open form signals: $k_{0f} = K_f k_{0r}$. This mechanism is pointed out in Scheme 5 with a horizontal dashed line. When the pD value is increased, the second term in eq 1 becomes important due to the low pK_a value of the cyclol forms. Therefore, the main contribution to the rate, at this stage, corresponds to the cyclol conjugate base rupture. This mechanism is marked with a vertical dashed line with the arrow just after the rate-limiting step in Scheme 5. When $K_{ac} > [D^+]$, the second level figures are reached. Then, the k_r and pK_a values of the cyclol forms are obtained. At higher pH, the third term in eq 1 predominates. The corresponding mechanism is depicted with a diagonal orientation in Scheme 5. In this mechanism, the cleavage of the D_2O-cyclol form is promoted by OD^-, but this step is fast since the cleavage of the C−O bond is also catalyzed by the water molecule that is forming a hydrogen bond with the cyclol form. Therefore, is the fraction (f) of the $^-$OD-open form complex what becomes relevant in this mechanism. However, this species could bifurcate to the option of returning to the D_2O-cyclol form or go to the $^-$OD-cyclol species. Therefore, the rate law for this step is $k_{obs} = f$ $^-$OD-open form (f D_2O-open form × $k_{0r} + k_f$). At relatively low pD, the first term in parentheses predominates since k_{0f} has a high value; see Table 1. This is then an alternative route to return to the D_2O-cyclol form without involvement of OD^-, but as soon as the pD increases, the second term in the last parentheses predominates since the fraction of the D_2O-open form decreases and the last equation is simplified to the third term in eq 1. Once the $^-$OD-open form fraction becomes equal to 1, the third level in Figures 2 and 3 occurs and the k_f and pK_a values of the open form are obtained. In Scheme 5, the cyclol-open form interconversion in the ample pD range studied is shown.

The rate constants of formation of the cyclol (C−O bond formation) are faster than the corresponding C−O cleavage

SCHEME 6[a]



[a] Water molecules hydrogen-bonded to the cyclol (c), the open form (a), and the transition state (b) of compounds **1** ($n = 5$, p$K_{ac} = 11.9$), **2** ($n = 6$, p$K_{ac} = 7.3$, p$K_{ao} = 10.5$), and **3** ($n = 7$, p$K_{ac} = 5.1$, p$K_{ao} = 9.8$). The pK_a values depend on the relative strain stability introduced by the hydrogen-bonded cycle. For $n = 7$, cyclol, $\delta = 0.4$ (see text), the D is located in the middle of the two donors: the O of the cyclol and the O of water. For $n = 5$, $\delta = 0$ (see text), the hydrogen bond is weak. The difference in pK_a between the two cyclols is ca. 7. A similar situation occurs with the open form −OD alcohol moiety, However, in this case, the hydrogen-bonded cycle is more flexible, and small difference is observed between the two pK_a values (ca. 2). Therefore, the $\Delta\delta$ between them is ca. 0.1. For $n = 6$, there is an intermediate behavior. The reaction at higher pH can be envisioned through the same structures as those shown above, but with full negative charge on alcohol oxygen instead of charge fractions (δ) with a second water participation (mechanism **2** in Scheme 5) or the D_2O-alcohol form D_2O complex interacting with a OD^- molecule (mechanism **3** in Scheme 5).

(Table 1). These results are in agreement with the experimental observation of an equilibrium ([cyclol]/[open form]) slightly shifted toward the cyclol form. The cyclol/open form equilibrium depends on the solvent and the lactam cycle size. For instance, in the case of compound **2** in $CDCl_3$, the equilibrium becomes 1 order of magnitude lower than that in D_2O, and for **3**, it is ca. 20 times lower, indicating a better water stabilization of the cyclol form in this solvent. On the other hand, the equilibrium constant increases when increasing the ring size. For instance, for compound **3** ($n = 7$), $K = 36$, for compound **2** ($n = 6$), $K = 12$, and for compound **1** ($n = 5$), $K \ll 1$. Therefore, the ring strain is an important factor that regulates the relative stabilities of these stable tetrahedral intermediates. We were able to measure the equilibrium (cyclol/open form) thermodynamic parameters for compound **3** at neutral pH. For instance, $\Delta S°$, $\Delta H°$, and $\Delta G°$ values (18.5 °C) are −29.5 J/mol·K, −16.9 kJ/mol, and −8.4 kJ/mol, respectively. Although the $\Delta S°$ is negative, this value is lower than expected since in the open form there are three free rotation modes at the external 2-hydroxyacetyl group worth ca. 21 J/mol·K each that are lost in going to the cyclol form. Therefore, in the open form, there must exist some rotation restriction that increases the entropy value to −29.5 J/mol·K. We attribute this rotation restriction to a water hydrogen bound to the −OH of the 2-hydroxyacetyl pendant substituent (see Scheme 6a). The low pK_a value (5.1) kinetically determined for the cyclol form of **3** is also a result that we have attributed to the water hydrogen bond participation via an intramolecular cyclic interaction, as shown in Scheme 6c. Through this interaction, the cyclol −OH bridges form a strong hydrogen bond that increases the negative charge on the

oxygen and makes the alcohol more acidic (and not more basic since the bridge is established with a water molecule instead of another intramolecular heteroatom). It has been suggested[13] that, in this proton bridge interaction, the proton is in a stable potential and the location of the bridging proton will shift toward the more basic residue. In fact, in Scheme 6c, water becomes the more basic center since its pK_a increases from the hydronium ion one toward the water one since another of its protons is also strongly hydrogen bound to a second water molecule (the third hydrogen forms a weak hydrogen bond since the ether counterpart has a low pK_a). Therefore, the induced change of charge on the active center is drained and balanced at long distance through water hydrogen bonding.

Scheme 6 hydrogen bonds with the water molecule are in a downward potential (perpendicular coordinate in a two-dimensional energy diagram) as predicted by the alternative model[16,17] rather than the canonical[18] one in which the proton transferred in concert with the main reaction coordinate in a upward energy potential (parallel coordinate in a two-dimensional energy diagram).

According to the alternative proposal,[16,17] bond order will be conserved at unity about the bridging proton so that changes in the proton location will generate shifts in electrical charge within the bonding partners. Furthermore, it has been estimated[13] from cross-correlation plots that the expected sensitivity of the electrical shift charge is 0.06 units of charge/unit change in pK of a bonding partner. This proposal has been used[13] to explain the reported[19,20] change of pK_a of the Asp-His dyad from 7 to 12 when going to a transition state due to long distance proton bridges induced by the serine −OH pK_a change induced by its nucleophilic attack on the peptide carbonyl group. This kind of long distance effect has been used[13] to explain, for instance, the nonlocal character of mutation and to understand enzyme evolution. In our system, the change in pK_a is introduced by molecular strain and not by changing the pK_a of one of the heteroatoms involved. These results represent a new contribution to the understanding of hydrogen bond interactions in the sense that the hydrogen bond is also sensitive to changes in strain introduced, for instance, in these models, by the size of the lactam ring. We predict then that other strains at the enzymatic active site will also produce sensitive changes in the position of the bridged proton perhaps bringing about a change in the enzyme activity.

The alternative model[16,17] is operating in this work since a greater stabilization of the hydrogen bond makes the center more acidic and moves the position of the bridged proton away from the acidic center. This geometric response is expected only if the proton is in a downward potential in which stabilization of products induces a proton movement away from the reactants in a typical "anti-Hammond" sense. Therefore, the more stable this interaction is, the more acidic the cyclol form becomes (the same argument is valid for the open form, Scheme 6a). In fact, for compound **2**, the cyclol pK_a value is 7.5, ca. 2 orders of magnitude less acidic than the cyclol form of compound **3** due to ring strain restriction. The latter is considerably more sensitive to the cyclol acidity than to its stability relative to the open form where only a factor of 3 is involved between compound **3** and compound **2**. This difference in sensitivity is due to the significant change of charge density ($0.06^{13} \times 2 = 0.12$) of positive charge at the O alcohol center ($\Delta\delta = 0.12$ in Scheme 6c, cyclol form). This difference also contributes to the relative stability of the cyclol form with respect to the open form, but the last form is also stabilized by a cyclic hydrogen bond water interaction (Scheme 6a); therefore, the equilibrium constant does not show the same sensitivity as the pK_a values. The sensitivity of the pK_a of compounds **1**, **2**, and **3** is quite related to the stability of the structures arrived at by molecular calculation[21] shown in Scheme 6c. Optimization with molecular mechanics and MOPAC minimization using the AM1 method gives the relative heat of formation of the hydrogen-bonded cyclol forms of compounds **1**, **2**, and **3**. When these heats of formation (−181.7, −190.8, and −193.1 kcal/mol) are plotted versus the experimental pK_a values of these compounds, a good correlation is obtained: $y = 0.57x + 115.69$, $R^2 = 0.98$. The three pK_a values used are the ones shown in Table 1. Notice that the estimated pK_a (11.7) of the cyclol form of **1** is in good agreement with the experimental one[4] (11.9), and it takes into account only inductive effects. This value decreases as predicted by the above correlation due to the third cyclic hydrogen bond strain relief introduced by increasing the lactam ring size. Therefore, in the cyclol form of **1**, the hydrogen bond is weak or does not exist at all due to strain arising from the five-membered ring. In fact, when semiempirical AM1 molecular calculations[21] are performed on the cyclols' conjugate bases interacting with oxonium ion ("cycloxide"-H_3O^+), the stabilities of the three "cycloxide"-H_3O^+ correlate well with the experimental pK_a values. Moreover, in the calculation, an important elongation of the ether C−O bond (ether) is observed that follows the order: $n = 5 \gg n = 6$, $n = 7$. Therefore, for $n = 7$, the stability of the anion may be due to strain, anomeric effect[22] induced by the ether oxygen electron pairs, or both (no change in the C−N bond is observed among the three compounds). In any case, as the experimental data show, the calculation confirms the stability order of the anion-D_3O^+ of $n = 7 > n = 6 \gg n = 5$, so that the pK_a order is $n = 5 \gg n = 6 > n = 7$.

The lack of general base catalysis by phosphate buffer at pH 6.3 for the interconversion of the cyclol and open form of **3** is another piece of evidence for the efficiency of the role of water general catalysis via a strong hydrogen bond. In the alcohol formation from the adducts of *N*,*O*-trimethylenephthalimidium cation, Gravitz and Jencks[6] have found general acid catalysis. However, in this system, the driving force for the C−O cleavage comes from a weak N (amide) electron pair; therefore, general acid catalysis is required. This is not the case for the cyclol forms of this work where the driving force for the C−O cleavage comes from an oxygen (alcohol or alkoxide) electron pair. Furthermore, a water−hydrogen bond as depicted in Scheme 6 is not predicted to be formed in Gravitz's and Jencks's system since there is an entropic factor against the bimolecular expulsion of the associated alcohol. A substitution of water by a general acid−base buffer in the system described in this work is also improbable since the charge dispersion shown in Scheme 6c through a second water molecule makes it possible for the directly attached water molecule to match its pK_a with the cyclol pK_a and form a stronger hydrogen bond. This charge transfer

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efficiency is limited in the case of other bifunctional buffers, such as for instance NaH_2PO_4.

The cyclol/open form increase when going from $CDCl_3$ to D_2O also supports the role of a hydrogen bond with water. In agreement with the strain introduced by the lactam ring, the latter increase is more important for compound **3** ($n = 7$) than for compound **2** ($n = 6$).

For compound **3**, we have also obtained the corresponding activation parameters at neutral pD from a plot of $1/k_{obs}$ versus $1/T$ (see Table 1). As shown in Table 1, the $\Delta S^{\ddagger}$ values for rupture and formation of the cyclol form are both negative. This is not the expected result for the rupture since the transition state is less ordered than the cyclol form. However, if water participates in the rupture, a more ordered transition state (Scheme 6b) as compared to the water-bonded cyclol form is indeed predicted since even if a strong hydrogen bond is formed some rotational movement is allowed in the reactants. In fact, in the case of the ammonium ion[23] and water, the strength of the hydrogen bond only prevents a nearly free rotation. Even where the hydrogen bond appears to be quite strong due to similarities in pK_a between donor and acceptor, there seems to be hardly any barrier to rotation.[24] This movement becomes more restricted at the transition state due to the orientation imposed by the reaction coordinate.

Regarding the symmetry and mobility of the hydrogen bond, due to the asymmetry of the involved donors, a double-well potential hydrogen jumping between donors is expected. Recently, it has been shown[25] that, even in the cases of symmetric donors and counterion, the H bond is asymmetric and there is an equilibrium between solvatomers. If it is the case, the change in pK_a observed in this work may also be interpreted as the average contribution of the two donors' pK_a, for instance, in the case of the cyclol form: H_3O^+ (pK_a = ca. −1) and cyclol−OH (pK_a = ca. 12), that for compound **3**, assuming 50% contribution of each donor, the final pK_a would be $0.5 \times (-1) + 0.5 \times 12 = 5.5$ (experimental pK_a = 5.1).

The proton inventory results are particularly germane to the water participation hypothesis. As shown in Figure 4, there is a k_H/k_D isotope effect close to 1 for compound **3**. This is, in fact, what should be expected if the proton transfer does not occur along the reaction coordinate since the transfer has been prearranged at the reactants through strong hydrogen bond proton bridges. Hence, the protons were already bonded at the reactants, and therefore, there is no isotopic contribution at the transition state (Scheme 6b).

The kinetic rate constants measured in this work establish a rate constant range for the serine hydroxyl attack on the peptide bond in serine proteases: from water-catalyzed activation of the serine alcohol group to fully unprotonated alkoxide attack. For instance, the k_{0f} values in these models are 72 and 48 s^{-1} for $n = 7$ and 6, respectively. These rate constants mimic the minimum value for serine −OH attack to the peptide bond (k_2 values) in enzyme proteases since water is acting as the general base catalyst instead of histidine. Meanwhile, k_f (ranging from 64 s^{-1} ($n = 7$) to 20 s^{-1} ($n = 6$)), represents the value that should be obtained in enzyme proteases in the absence of an acid catalyst since the attacking group is the alkoxide group of the open form that does not require any base catalysis assistance, but the formed alkoxide group is assisted by water and not by oxonium ion. When both are present, as is the case of the first step of the mechanism **3** in Scheme 5, the rupture and formation of the C−O bond are very fast compared to the rates measured in this work and probably even higher than the reported enzymatic values. Although the intramolecular distances may resemble those at the enzyme active site, neither the orientation between the serine hydroxyl group and the protein carbonyl group nor the inductive effect may be the same. Therefore, changes in these maximum k_2 and k_{-2} values may be observed. For instance, k_2 values[26] for trypsin and modified Cys191−Cys220 trypsin hydrolysis of D-ValLeuLys-AMC are 280 and 1.4 s^{-1}, respectively.

Finally, it is important to point out that the rate of water exchange of the proton forming hydrogen bond should be quite fast. For instance, according to the Swain−Grunwald mechanism[27] for the cyclol form of compound **3** (pK_a = ca. 5), this rate is ca. 10^5 s^{-1} since the rate of dissociation of the hydrogen-bonded form is ca. 10^9 s^{-1}, so that the rate-limiting step for the exchange is the proton transfer to the water molecule. Therefore, the proton-bridged signal is averaged with the water one in the ^1H NMR spectrum. As has been established,[28] this averaging makes the direct detection of the hydrogen bond not possible. More chances to observe the hydrogen bond by ^1H NMR are for the bridge −OH of the open form where the pK_a is ca. 9−10. In this case, the exchange rate will be ca. 1−10 s^{-1} and non-coalescence with the dominant water signal might be observed in the H_2O spectrum; we did not observe any −OH signal when using a 20% H_2O/D_2O mixture. However, in $CDCl_3$ (Figure S1, Supporting Information), both −OH signals for the cyclol form (ca. 4.15 ppm) and the open form (ca. 3.4 ppm) are observed but the open form signal exchanges (with traces of water) slower than the cyclol one.

Conclusions

The relative stability between the cyclol (tetrahedral intermediate) and open form (peptide−enzyme complex) in this serine protease mechanism-based mimic depends on the ring size of the lactam cycle. The cyclol stability increases when increasing the cycle size ($n = 5-7$). Water is highly associated with the cyclol and open form through strong hydrogen bonds in stable potential proton bridges. This suggestion is supported by thermodynamic and kinetic entropic values, the strong sensitivity of pK_a values to the lactam ring size (strain effect), the lack of an observable solvent isotope effect and phosphate buffer catalysis, and semiempirical molecular calculations. The general acid−base catalysis by water does not occur in the reaction coordinate, and the strength of the hydrogen bond is sensitive to the strain arising from the size of the lactam ring. This result can be extrapolated to the enzyme active site where, in general, any strain could cause a significant change in the stability of the hydrogen bond, reducing the enzymic activity even at long distance since the effect could be transmitted through a hydrogen bond.

Evidence for the existence of hydrogen bonds in the active sites of enzymes with energies in the range of 10−20 kcal/mol, using intramolecular proton transfer (general acid−base)

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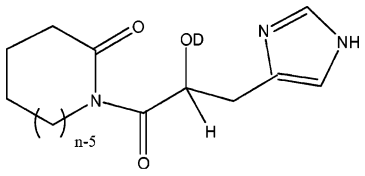
catalysis in model systems, has been previously presented[29] but at the transition state level. The results presented in this work would be the first case, to our knowledge, where evidence is presented of the existence of an asymmetric (double potential) strong hydrogen bond with water and at the reactants level.

The system studied mimics the serine alcohol attack to the peptide bond and its reverse reaction in serine protease enzymes. A rate constant range for the serine attack can be readily established: from serine −OH attack without base catalysis and $-O^-$ attack. However, k_2 values higher than those shown in this work have been reported, for instance, for trypsin. It is quite possible that the five-membered ring formation involved in the cyclol form of these models introduces a strain that is not involved in the enzyme reaction. It is also important to remark that, in the systems studied, the cleavage to the acyl alcohol (C−N bond) is slower than the reverse cyclol formation (C−O bond cleavage), making the acyl formation rate-limiting. This observation can also be specific for these models since opening the cyclol brings about a strain release when the C−O bond is broken and the open form is formed. Although, in the formation of the macrocycle (peptide bond cleavage), there will also be strain relief, a cyclic peptide is formed. Nevertheless, the kinetics obtained for these models (ca. 2−70 s^{-1}) are of the same order of magnitude as those in the enzymatic reactions. The lactonization of 2-hydroxymethylbenzoic acid and the corresponding benzamide as models for the acylation step in the chymotrypsin-catalyzed hydrolysis has been previously reported;[30] however, the rates reported are at least 60 times slower than the values reported in this work. We are now involved in the synthesis and kinetic study of compound **4** in which a pendant imidazole group has been attached to the exocyclic methylene group of the open form. Therefore, we will have in the same molecule the substrate peptide bond, the hydroxymethyl group that mimics the serine residue at the active site, and an imidazole group that mimics the enzymic histidine role.



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Supporting Information Available: Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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