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Substituent Effects in Carbon−Nitrogen Cleavage of Thiamin Derivatives. Fragmentation Pathways and Enzymic Avoidance of Cofactor Destruction

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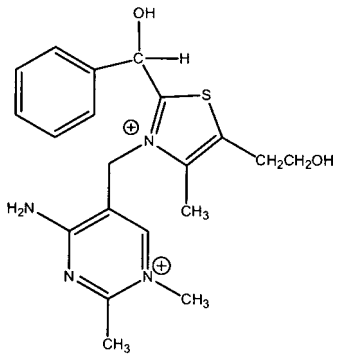
Abstract: The combination of thiamin and benzaldehyde can produce benzoin but also destroys thiamin. The destruction comes from fragmentation of the conjugate of thiamin and benzaldehyde undergoing a process that produces a phenyl thiazole ketone and pyrimidine. The key step in this process is cleavage of the C−N bond between the heterocycles, which occurs by an unknown mechanism. Enzymes that utilize similar intermediates do not fragment the cofactor although fragmentation is inherent to the structure. To analyze the nature of the C−N cleavage step, the rates of fragmentation of a series of phenyl-substituted *N*1′-methyl-2-(1-hydroxybenzyl)thiamin derivatives were determined under two sets of conditions: (1) where proton transfer in the step prior to C−N bond breaking is rate-determining and (2) where C−N bond breaking is rate-determining. The resulting ρ values are 1.6 and 1.8, respectively, leading to the conclusion that C−N cleavage is insensitive to substituent effects. On the basis of these results, we conclude that cleavage occurs by a facile process that resembles the outcome of a [1,5] sigmatropic rearrangement. An enzyme may avoid the fragmentation by holding the intermediate in a conformation that prevents such a process, allowing the normal catalytic process to proceed.

The conjugate base of thiamin adds to aldehydes to form intermediates that can form the equivalent of an acyl carbanion.[1,2] Enzymes catalyze similar reactions by addition of the conjugate base of thiamin diphosphate to carbonyl groups. The formation of benzoin from benzaldehyde that is catalyzed by thiamin involves a stable intermediate, 2-(1-hydroxybenzyl)-thiamin, HBzT, which is analogous to the cyanohydrin in the traditional benzoin condensation (Scheme 1).

The diphosphate of HBzT is an intermediate in the enzyme-catalyzed decarboxylation of benzoylformate,[3−5] so the reaction patterns of HBzT give important information about the role of the covalent intermediate in the enzyme. Mieyal and Sable showed that the C2α proton of HBzT is exchanged for deuterium in alkaline solution, consistent with its role in the thiamin-catalyzed formation of benzoin from benzaldehyde.[6,7] In a later study, Washabaugh reported that buffers promote the decomposition of HBzT to thiamin and benzaldehyde, which competes with the proton exchange reaction.[8] However, unlike the exchange reaction, the role of Brønsted acids and bases in the elimination reaction is not apparent. Further studies revealed that while buffers catalyze the decomposition of HBzT, thiamin and benzaldehyde are not the products.[9] These buffer-promoted products, which form in neutral and acidic solutions, result from cleavage of the thiazole and pyrimidine portions. They are the same as those reported by Oka to result from the reaction of thiamin with benzaldehyde in methanol in the presence of amines (Scheme 2).[10]

Kinetic studies revealed that the fragmentation of HBzT is a consequence of protonation of the pyrimidine, which occurs at N1′ ($HBzTH^+$). The material is a dication. If HBzT is N1′-alkylated, the dicationic form (NMHBzT, **1**) is present even in alkaline solution, so that fragmentation is the only observed process.[11] Our recent report showed that the step in the



NMHBzT (1)

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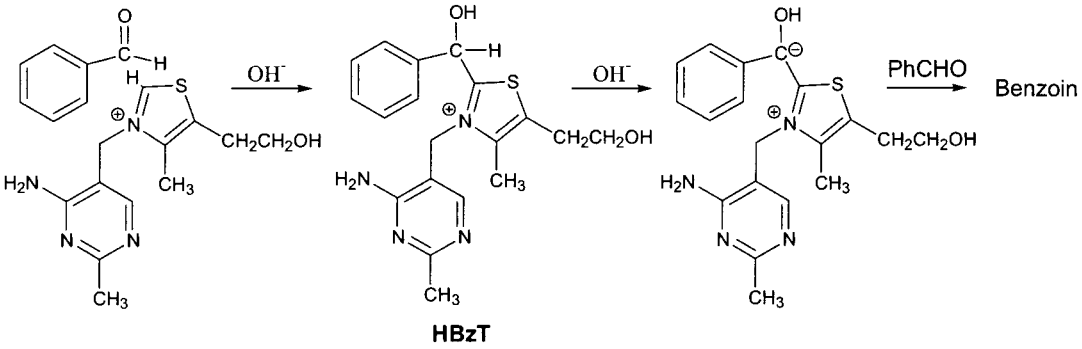
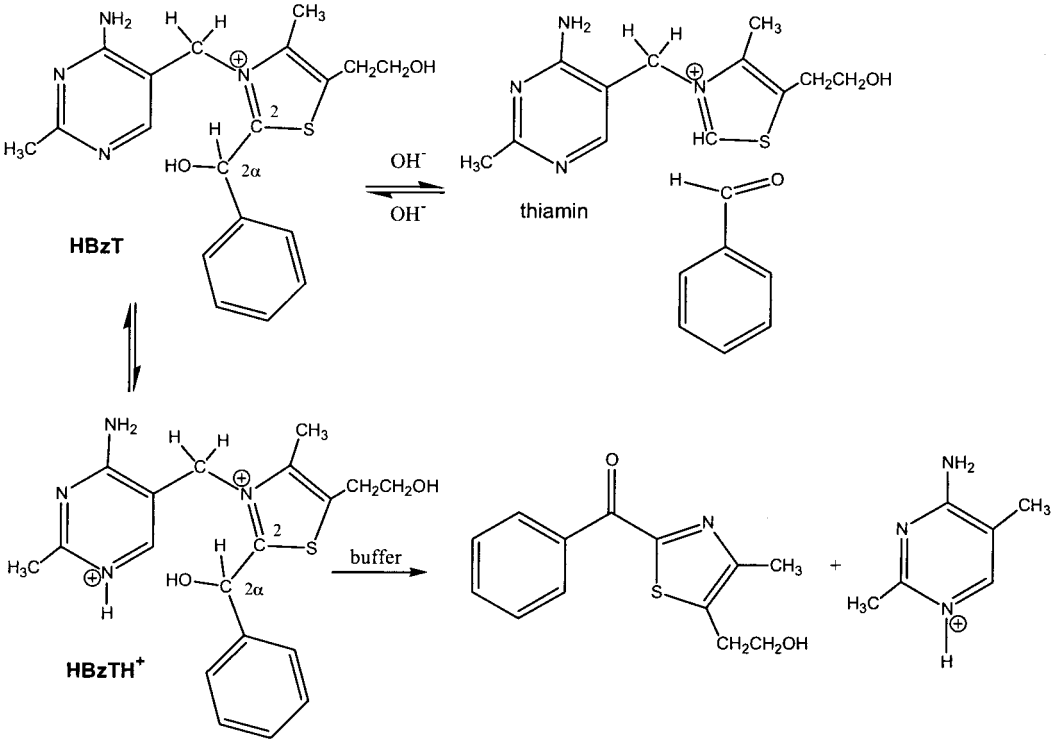
Scheme 1



Scheme 2



fragmentation in which cleavage of the pyrimidine and thiazole occurs from the C2α conjugate base (enamine) intermediate is very fast, comparable in rate to proton transfer.[12,13] In contrast, the enzyme benzoylformate decarboxylase generates the same enamine intermediate from combining thiamin diphosphate and benzoylformate and proceeds without any fragmentation. If the fragmentation process is inherent to the structure, how does the enzyme avoid it?

The mechanism of the process in the fragmentation that cleaves the bond joining the heterocycles remains unknown. The means by which an enzyme would avoid it would be easier to discern if the mechanism were known. The various possible stepwise and concerted mechanisms all require highly energetic species; none of the mechanisms are either ruled out or supported by the patterns resulting from variation of reaction conditions.

The effects of systematic variation of substituents in the reactants on kinetic patterns can provide information beyond that which comes from a single reactant. We therefore have performed kinetic measurements on the fragmentation of a series of phenyl-substituted analogues of HBzT since they can be analyzed by application of linear free energy relationships.

Experimental Section

Methods. Kinetics of Reactions in Water. Reactions were first monitored by recording sequential UV−vis spectra to determine the wavelength where there is a maximum change due to the substituted thiazole−ketone product. Subsequent kinetic runs were done at this wavelength. The rate of fragmentation was monitored using buffer solutions maintained at 40 °C in a jacketed beaker. The pH electrode was standardized against reference buffers at 40 °C. The ionic strength of all reaction solutions was maintained at 0.10 or 1.0 by the addition of potassium chloride. Data were collected with an interfaced computer. First-order rate constants were calculated from nonlinear regression fitting of the data to the integrated first-order rate expression over 5 half-lives. Once the kinetic order was determined for a set of conditions, the data for additional sets of reactions were acquired over a relatively short interval and analyzed by the method of initial rates.

Synthesis. Two general procedures were followed for the synthesis of thiamin−benzaldehyde conjugates. The procedure of Mieyal and

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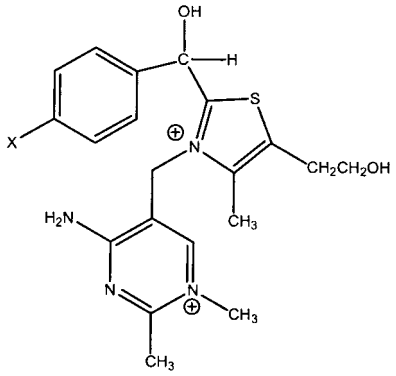
Sable was followed for the synthesis of HBzT, *p*-methyl-HBzT, and *p*-methoxy-HBzT.[14] Doughty's procedure was followed for the synthesis of *p*-chloro, *p*-cyano, and *p*-bromo derivatives.[15] The thiamin–benzaldehyde conjugates were methylated at N1′ with dimethyl sulfate: *p*-methyl-NMHBzT **(2)**, *p*-chloro-NMHBzT **(3)**, *p*-methoxy-NMHBzT **(4)**, *p*-cyano-NMHBzT **(5)**, *p*-bromo-NMHBzT **(6)**. They were isolated and recrystallized as bisperchlorates.[16]



2-6

2 [X = *p*-CH$_3$]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.28 (s, 1H), 8.43 (s, 1H), 7.66 (d, 1H, J = 4.5 Hz), 7.26 (d, 2H, J = 8.0 Hz), 7.08 (d, 2H, J = 8.0 Hz), 6.74 (s, 1H), 6.23 (d, 1H, J = 4.3 Hz), 5.28 (m, 3H), 3.76 (m, 2H), 3.56 (s, 3H), 3.07 (m, 2H), 2.51 (s, 3H), 2.29 (s, 3H), 2.20 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 178.07, 161.38, 159.86, 143.13, 142.65, 138.92, 134.93, 134.13, 129.17, 127.88, 107.94, 70.26, 59.47, 46.10, 41.49, 29.56, 21.29, 20.52, 11.27. FABMS: $[C_{21}H_{28}N_4O_2S]^{2+}$, calcd 399.18547 ($C_{21}H_{27}N_4O_2S$), found 399.18439.

3 [X = *p*-Cl]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.35 (s, 1H), 8.48 (s, 1H), 7.81 (d, 1H, J = 4.7 Hz), 7.42 (d, 2H, J = 8.7 Hz), 7.39 (d, 2H, J = 8.7 Hz), 6.85 (s, 1H), 6.33 (d, 1H, J = 4.7 Hz), 5.29 (m, 3H), 3.73 (m, 2H), 3.59 (s, 3H), 3.08 (m, 2H), 2.53 (s, 3H), 2.30 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 177.12, 161.59, 159.86, 143.35, 142.76, 136.82, 134.41, 133.99, 129.82, 128.74, 108.11, 69.45, 59.45, 46.13, 41.54, 29.55, 21.41, 11.33. FABMS: $[C_{20}H_{25}N_4O_2SCl]^{2+}$, calcd 419.13085 ($C_{20}H_{24}N_4O_2SCl$), found 419.13107.

4 [X = *p*-OCH$_3$]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.30 (s, 1H), 8.45 (s, 1H), 7.61 (d, 1H, J = 4.2 Hz), 7.30 (d, 2H, J = 8.6 Hz), 6.82 (d, 2H, J = 8.6 Hz), 6.78 (s, 1H), 6.22 (d, 1H, J = 3.6 Hz), 5.27 (m, 3H), 3.76 (m, 2H), 3.70 (s, 3H), 3.56, (s, 3H), 3.07 (m, 2H), 2.49 (s, 3H), 2.29 (s, 3H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 178.25, 161.29, 159.90, 159.57, 143.07, 142.68, 133.99, 129.74, 129.30, 113.94, 108.03, 69.95, 59.43, 55.22, 46.01, 41.46, 29.51, 21.14, 11.22. FABMS: $[C_{21}H_{28}N_4O_3S]^{2+}$, calcd 415.18039 ($C_{21}H_{27}N_4O_3S$), found 415.17907.

5 [X = *p*-CN]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.35 (s, 1H), 8.52 (s, 1H), 7.93 (d, 1H, J = 4.9 Hz), 7.86 (d, 2H, J = 8.5 Hz), 7.62 (d, 2H, 8.2 Hz), 6.96 (s, 1H), 6.45 (d, 1H, J = 4.7 Hz), 5.29 (m, 3H), 3.74 (m, 2H), 3.62 (s, 3H), 3.09 (m, 2H), 2.55 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 175.97, 161.78, 159.90, 143.45, 143.23, 142.86, 134.87, 132.78, 128.91, 118.04, 111.86, 108.27, 69.29, 59.44, 46.23, 41.70, 29.60, 21.43, 11.42. FABMS: $[C_{21}H_{25}N_5O_2S]^{2+}$, calcd 410.16507 ($C_{21}H_{24}N_5O_2S$), found 410.16700.

6 [X = *p*-Br]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.35 (s, 1H), 8.52 (s, 1H), 7.86 (d, 1H, J = 4.9 Hz), 7.52 (d, 2H, J = 8.5 Hz), 7.37 (d, 2H, J = 8.5 Hz), 6.87 (s, 1H), 6.33 (d, 1H, J = 4.7 Hz), 5.36 (m, 3H), 3.73 (m, 2H), 3.60 (s, 3H), 3.08 (m, 2H), 2.55 (s, 3H), 2.30 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 177.20, 161.58, 159.87, 143.40, 142.68, 137.25, 134.42, 131.68, 130.15, 122.66, 108.11, 69.57, 59.48, 46.22, 41.54, 29.59, 21.57, 11.34. FABMS: $[C_{20}H_{25}N_4O_2SBr]^{2+}$, calcd 463.08033 ($C_{20}H_{24}N_4O_2SBr$), found 463.08136.

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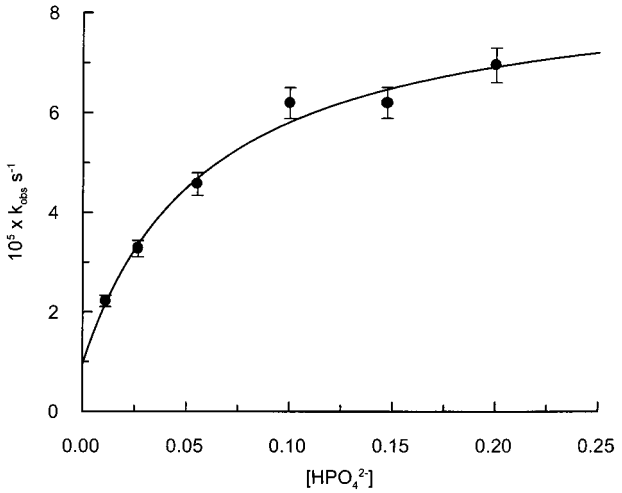
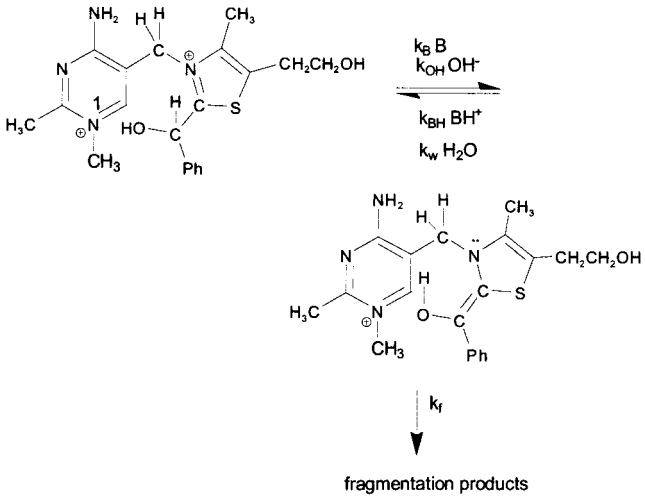


Figure 1. Dependence of the observed first-order rate coefficient for fragmentation of NMHBzT (**1**) on the concentration of hydrogen phosphate ion in water at 40 °C, I = 1.0. The curve is fit to the rate law for a reaction in which a change in rate-determining step occurs with increasing phosphate concentration.

Scheme 3



Results

The observed first-order rate coefficient for fragmentation of **1** was measured with variation of pH 6.1 hydrogen phosphate buffer (Figure 1).

The rate coefficients show a saturating dependence on buffer concentration, as had been seen with the *N*1′-benzyl derivative, resulting from a change in rate-determining step from proton transfer to C–N cleavage. The data are consistent with the steps in Scheme 3. The scheme is similar to that which was presented for the fragmentation of *N*1′-benzyl-HBzT.

Application of the steady-state approximation to Scheme 3 gives eq 1, which describes a rectangular hyperbola. Following

$$k_{obsd} = (k_B[B] + k_{OH}[OH^-])k_f/(k_{BH}[BH] + k_w + k_f) \quad (1)$$

the method outlined by Keefe and Jencks,[17] the kinetic parameters that can be derived from this plot are k_B = 1.5 ×

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Table 1. Pseudo-First-Order Rate Constants for Fragmentation of Compounds **1**−**5** at pH 6.9 and 0.003 M Hydrogen Phosphate

compd	k_{obsd} (s^{-1})	compd	k_{obsd} (s^{-1})
1	$(2.5 \pm 0.1) \times 10^{-4}$	**4**	$(1.58 \pm 0.02) \times 10^{-4}$
2	$(1.5 \pm 0.1) \times 10^{-4}$	**5**	$(5.21 \pm 0.02) \times 10^{-3}$
3	$(4.69 \pm 0.05) \times 10^{-4}$		

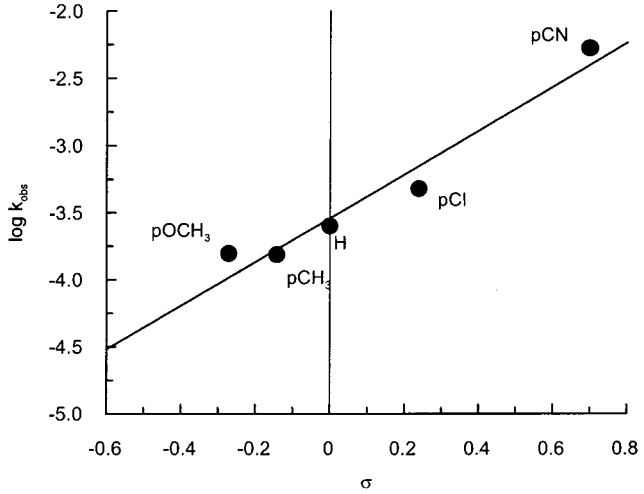

Figure 2. A Hammett plot of observed first-order rate constants for fragmentation of **1**−**5** (indicated by substituent) with rate-determining transfer of the proton from C2α (data from Table 1).

10^{-3} M^{-1} s^{-1} and $k_{BH}/k_f = 17$ M^{-1}. The first rate-determining step is proton transfer from C2α, which is accelerated by buffer. The next rate-determining step, fragmentation, competes with protonation of the conjugate base at C2α but is not facilitated by Brønsted acids and bases.

The reaction rates for the set of *N*1′-methylphenyl-substituted analogues of HBzT yield linear free energy relationships to gauge the electronic requirements of the fragmentation step. We analyzed the substituent effects on both rate-determining steps by determining the Hammett ρ value under conditions where each is rate-determining. Since k_f is rate-determining at high buffer concentrations, the measured value of ρ is the sum of the ρ values for the steps up to and including the fragmentation step. Because the proton-transfer step precedes the fragmentation step, the difference in ρ values obtained under conditions with the differing rate-determining steps gives the net value for ρ for the fragmentation step. The data in Table 1 and Figure 2 were determined in 0.003 M pH 6.9 hydrogen phosphate. Under these conditions loss of the proton from C2α is rate-determining.

Fitting the data to σ_p ($r = 0.975$) gives $\rho = 1.6$. The relatively large positive value of the reaction parameter is consistent with development of negative character in the transition state at C2α (the net charge on the whole species is positive).

We also measured rates of fragmentation for compounds **1**−**6** under conditions where the proton-transfer step is faster than the fragmentation step k_f (high buffer concentration; see Figure 1 and Scheme 3). Thus, the rate data in Figure 3 were determined at pH 6.1 with 0.2 M hydrogen phosphate buffer (Table 2).

The plot fits the data best using σ_p parameters (Figure 3). The plot gives $\rho = 1.8$. Again, the value of ρ is consistent with a transition state that has considerably more localized negative charge than the reactant. The net value of ρ for the fragmentation step (k_f) is very small since the values for ρ under the two sets of conditions sets are similar and differ only by the fragmentation step.

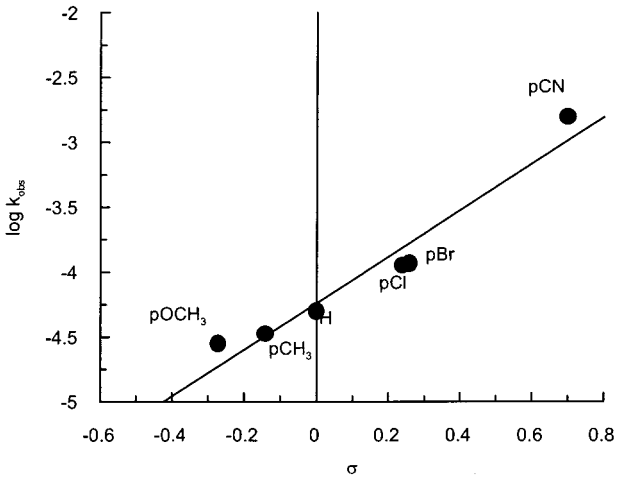

Figure 3. A Hammett plot of the observed first-order rate constants for fragmentation of **1**−**6** under conditions where C−N bond cleavage is rate-determining (data from Table 2).

Table 2. Observed First-Order Rate Constants for Fragmentation of Compounds **1**−**6** at pH 6.1 and 0.2 M Hydrogen Phosphate

compd	k_{obsd} (s^{-1})	compd	k_{obsd} (s^{-1})
1	$(4.9 \pm 0.3) \times 10^{-5}$	**4**	$(2.8 \pm 0.6) \times 10^{-5}$
2	$(3.36 \pm 0.07) \times 10^{-5}$	**5**	$(1.56 \pm 0.01) \times 10^{-3}$
3	$(1.11 \pm 0.07) \times 10^{-4}$	**6**	$(1.15 \pm 0.02) \times 10^{-4}$

Discussion

The rates of fragmentation of **1**−**6** are proportional to the concentrations of buffer in dilute solution but become independent of buffer concentrations at higher levels. This change in dependence is consistent with a change in rate-determining step with respect to the availability of Brønsted acids and bases. The kinetic expressions for both regions of the plot are given in eqs 2 and 3.

$$k_{obsd} = k_B[B^-] + k_{OH}{}^{18} \quad (2)$$

$$k_{obsd} = R(k_B/k_{BH})(k_f) \quad (3)$$

The computed value of ρ for conditions where proton removal from C2α is rate-limiting is 1.6. This is a reasonable value for such a process. In the case of ionization of phenols in water, $\rho = 2.2$, where stabilization of the negative charge is provided solely by the aromatic substituents, while in **1**−**6** delocalization into the thiazolium ring is significant in stabilizing the charge.

Using Jordan's measured $pK_a = 15.4$[19] for a related compound as a reasonable estimate for the loss of a proton from C2α, we calculate the rate coefficient for conversion of the enamine into the fragments as $k_f = 8 \times 10^4$ s^{-1}. Therefore, the initial intermediate formed by loss of a proton from C2α has to be relatively close in energy to the transition state for cleavage of the C−N bond.

The difference in the values obtained for ρ under the two sets of rate-limiting conditions indicates that the value of ρ

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associated with k_f is approximately 0.2 if we assume that the structure of the transition state for the endothermic proton transfer resembles the conjugate base. The scatter in the plot is sufficient to make this value indistinguishable from zero. Thus, there is probably little if any increase or decrease of polar character in the transition state in which the C−N bond is broken, after the initial intermediate has lost a proton from carbon. The ketonic product results from the hydroxyl group at C2α. If the proton were removed prior to the transition state, negative character would develop and ρ would increase significantly since the substituent is on the portion of the molecule that becomes part of the ketone. This would occur in a step that would lead to formation of the conjugate base of the hydroxyl. If the hydroxyl proton were retained in the transition state of the rate-determining step, the appearance of the protonated ketone would have an inverse substituent dependence and we would expect to see a large decrease in ρ. Since neither corresponds to what we observe, an alternative mechanism is necessary.

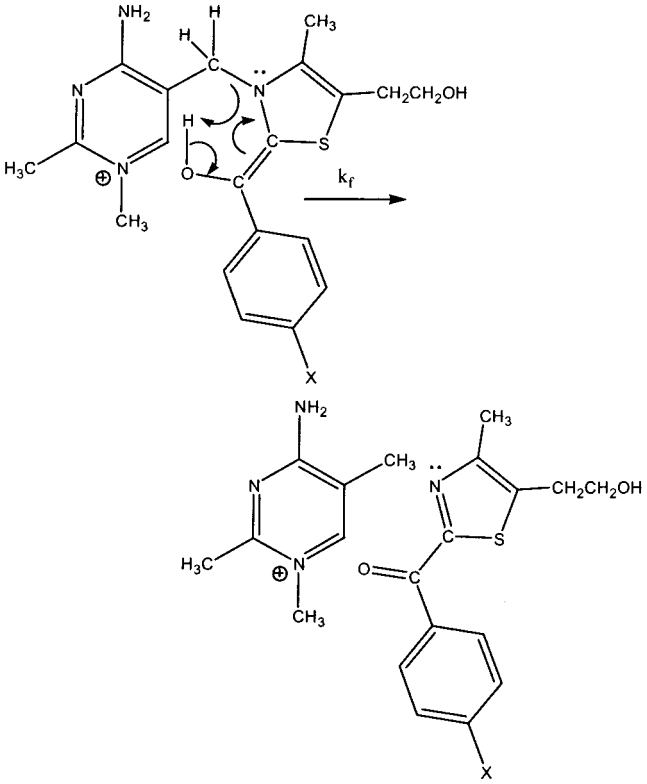
The other required process in transforming the intermediate to the products is breaking the bond connecting the methylene bridge and the thiazole nitrogen. If this were the initial process, the intermediate would become locally seriously deficient in electron density. Therefore, ρ for that step would be significantly negative, reducing the net slope. Again, this is not what we observe, and the scenario is unlikely. Furthermore, any stepwise process will generate what must be high-energy species, yet the rate constants associated with each value of k_f are large. Such small barriers do not permit development of even higher energy species.

The remaining alternatives to these heterolytic processes are a homolytic process (via high-energy free radicals that must recombine) and a concerted process in which C−N cleavage is accompanied by transfer of the hydroxyl hydrogen to the bridging methylene group that is exocyclic to the pyrimidine. We see no route that generates radicals readily that will lead to the products. A concerted six-electron mechanism that resembles a suprafacial [1,5] sigmatropic reaction, which is allowed according to the Woodward−Hoffmann rules, requires front side substitution at the saturated carbon. The process resembles the Alder ene reaction in some aspects but differs in the substitution at the saturated carbon (Scheme 4). The hydroxyl group must rotate out of the plane to accommodate the necessary overlaps. Molecular modeling suggests that a chairlike transition-state structure can be achieved. We do note that we have observed that the reaction requires that the pyrimidine possess a positive charge, while the sigmatropic reaction as drawn is not subject to polar effects. However, weakening of the C−N bond may well be induced by the strongly electron withdrawing effect of the positively charged ring. It is well established that polarization by Lewis acids, which adds charged character, promotes other electrocyclic processes.

The structure in Scheme 4 is drawn as the enamine derivative resonance structure. However, the thiazolium form may be more significant since it retains aromaticity in the heterocycle. This permits rotation about the C2 bond, which leads to the chairlike conformation.

The facility of the fragmentation of these thiamin derivatives helps to explain why thiamin catalysis of the benzoin condensation is subject to breakdown after several turnovers. In contrast,

Scheme 4



the enzymic generation of the C2α conjugate base of thiamin diphosphate of HBzT in benzoylformate decarboxylase[4,5,20] does not lead to fragmentation of the cofactor, although the intermediate should be prone to this process. The rate constant for the fragmentation step from the C2α conjugate base of the N1′-protonated species is as fast as the normal enzymic process. Suppression of the extent of protonation of the pyrimidine could slow the rate of the fragmentation, but this dynamic process cannot be avoided completely. On the other hand, the enzyme might hold the intermediate in a conformation that would not allow the proposed sigmatropic-like process. By hydrogen-bonding of the critical OH in a way that holds it remote from the methylene bridge, this pathway would be blocked. This is a form of stereoelectronic control that ensures specificity and productive catalysis by preventing a competing process, which can be as important as promoting a desirable process.[21,22] If the fragmentation from the conjugate base were stepwise, such a restraint would not be effective. In contrast, the catalytic process, benzaldehyde and thiamin diphosphate separating, is not subject to this stereoelectronic limitation.

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