Kinetics of C(2α)-Proton Abstraction from 2-Benzylthiazolium Salts Leading to Enamines Relevant to Catalysis by Thiamin-Dependent Enzymes

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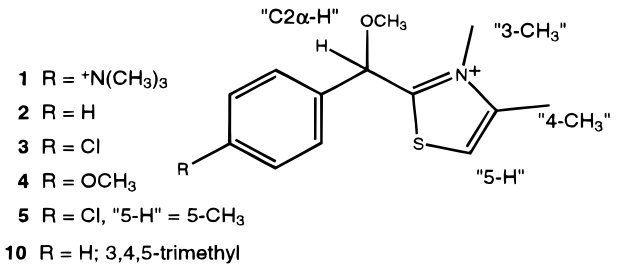
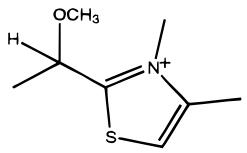
Abstract: The kinetics of proton transfer from the C(2α) position of 2-(1-methoxybenzyl)thiazolium salts was studied for the *p*-H and *p*-$N(CH_3)_3^+$ derivatives as models for the protonation of the enamine/C(2α)-carbanion, a key intermediate in many thiamin diphosphate-dependent enzymatic reactions. The reactions were studied by rapid mixing of the salts with sodium hydroxide in a stopped-flow instrument while monitoring the progress of enamine formation and decomposition in the visible region of the spectrum. It was demonstrated that under the conditions of the experiments, the thiazolium ring opens to give a product with a characteristic ^{1}H-NMR spectrum consistent with both *cis* and *trans* stereochemistry about the amide bond generated in the ring opening. Analysis of the progress curves for the *p*-trimethylammonium-substituted compound (**1**) gave the following rate constants (25 °C): 21 M^{-1} s^{-1} for deprotonation at C(2α), 300−540 s^{-1} for reprotonation, and 3.4 M^{-1} s^{-1} for thiazolium ring opening. For the unsubstituted compound (**2**), the respective rate constants were 0.019 M^{-1} s^{-1}, 1 s^{-1}, and 0.5 M^{-1} s^{-1}. Through comparisons with results for the C(2α)-deuterated form of **1**, the primary hydrogen isotope effect on deprotonation of **1** was found to be 4−6. The C(2α)-H pK_a values were estimated to be 15.0−15.5 for **1** and 15.7 for **2**. When compared with the turnover number for benzoylformate decarboxylase catalysis, the rate constant for reprotonation of the enamine derived from **2** leads to an estimate of 4500 M as a minimum effective molarity for the enzyme.

The bioorganic chemistry of thiamin diphosphate (ThDP, the vitamin B1 coenzyme)[1] is distinguished by the reactivity of two carbanions: the C(2) carbanion which is the conjugate base of the thiazolium ring and the C(2α) carbanion/enamine which is the conjugate base of 2-α-hydroxyethyl(or aryl)thiamin diphosphate. Because these carbanions are widely postulated to be intermediates in reactions catalyzed by enzymes that utilize ThDP, their thermodynamic, kinetic, and structural properties are particularly important. To date, the C(2) carbanion has not been observed directly, although there is evidence from kinetics that the pK_a of the conjugate acid is in the 17−19 range.[2−4] C(2α) carbanions/enamines have been directly observed on pyruvate decarboxylase using conjugated substrate analogs ($XC_6H_4CH{=}CHCOCOOH$)[5−15] and in the absence of enzyme in nonaqueous[16] and aqueous solutions.[17] The enamines derived from C(2)-alkylthiazolium compounds have characteristic UV absorbance maxima near 300 nm, while the C(2)-benzyl compounds give enamines with λ_{max} in the visible spectrum above 380 nm.

Scheme 1. The Counterion in Each Case Was BF_4^-



1 R = $^+N(CH_3)_3$
2 R = H
3 R = Cl
4 R = OCH_3
5 R = Cl, "5-H" = 5-CH_3
10 R = H; 3,4,5-trimethyl



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We now report rate constants obtained by monitoring the enamine absorbance generated in aqueous hydroxide using the thiazolium salts **1** and **2** shown below in Scheme 1. Rate constants for **1** were communicated by us a few years ago.[17]

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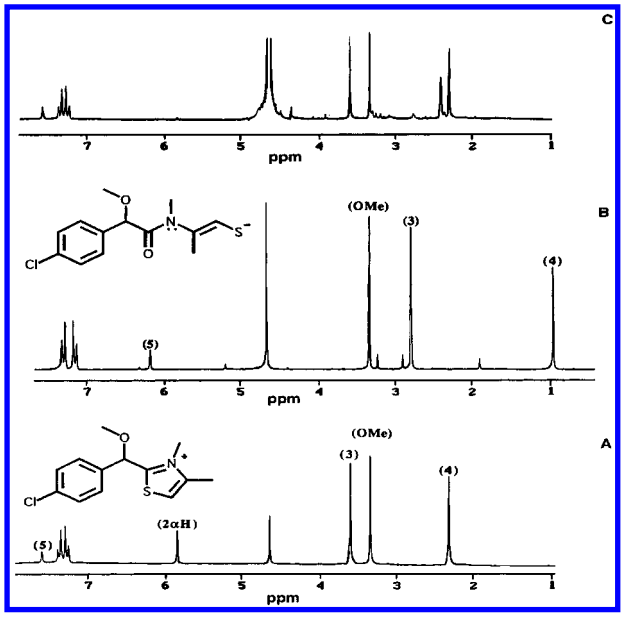


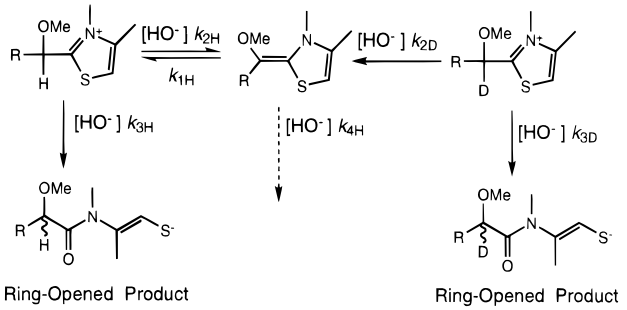
Figure 1. ^{1}H-NMR (200 MHz, D_2O) spectrum of **3** (structure shown in panel A). Panel B shows the spectrum a few minutes after adding enough NaOD/D_2O to make the solution 120 mM in NaOD. Panel C shows the spectrum[64] after the a few drops of DCl was added to the sample used to obtain the spectrum in panel B.

We present a complete study, including kinetic isotope effects for the reaction of **1**, along with ^{1}H NMR evidence for the structure of the product of a reaction, thiazolium ring opening, which competes with C(2α)-proton removal. The results, especially the rate constants for reprotonation of the enamine, have implications for mechanistic studies of ThDP-dependent enzymes such as pyruvate decarboxylase (PDC) and benzoylformate decarboxylase (BFD). A high-resolution X-ray crystallographic structure is available for PDC,[18,19] and for BFD, a high-resolution structure is emerging.[20]

Results

^{1}H-NMR Spectra. The influence of base on the benzylthiazolium salts listed in Scheme 1 was first examined by analyzing the ^{1}H-NMR spectra of the compounds in the presence and absence of base (NaOD). Figure 1 shows spectra for one of the compounds, the *p*-Cl derivative (**3**). The labels in Figure 1 and in Scheme 1 identify several of the peaks in the spectra. Panel A of Figure 1 shows the spectrum of the compound in the absence of base (neat D_2O), panel B shows the spectrum after base was added to give a solution that was 120 mM NaOD, and panel C shows the spectrum of the same solution after it was neutralized by the addition of DCl. The panel B spectrum is consistent with the ring-opened structure shown on the figure and in Scheme 2. Ring-opened products have been reported previously for the reaction of hydroxide ion with other thiazolium-containing compounds.[21,22] On addition of base, the change (panel A to B) in the 5-H chemical shift is in the direction expected if aromaticity in the thiazolium ring is lost. The 3-methyl peak moves as expected if the charge on the nitrogen is lost. The 4-methyl peak in panel B is at a very small chemical shift (0.91 ppm) for allylic hydrogens, perhaps because the *trans* (about the amide bond) form of the ring-opened product can adopt a conformation in which the 4-methyl group is situated under the substituted phenyl group where it can experience ring-current shielding.[23] Minor peaks in the panel B spectrum are consistent with the *cis* (amide) isomer of the ring-opened product.[22]

Scheme 2. Mechanism for the Reaction of **1** ($k_4 = 0$) and **2** ($k_4 \neq 0$) with Hydroxide Ion



Additional support for our explanation of the spectra can be derived from the results shown in Table 1. The spectra for compounds **1** and **4** are qualitatively similar to the spectra for **3**, suggesting that they all undergo a similar thiazolium ring opening. The spectra for **5** are slightly different, because this compound has a methyl group in the 5 position of the thiazolium ring. After adding base, the signal (1.62 ppm) for this 5-methyl group does not show the large shielding experienced by the 4-methyl group (to 0.84 ppm), indicating that only the 4-methyl position can experience the ring-current effect discussed above. For all of the substituted benzyl compounds (**1**−**5**), the intensity of the signal for the C(2α) proton is greatly reduced, or eliminated, upon addition of the base. This finding is in agreement with the mechanism shown in Scheme 2 in which C(2α) proton removal (and exchange for D_2O in the NMR experiments) competes with thiazolium ring opening. The compound with no benzyl group (**6**) shows little or no exchange over the time of the experiment (ca. 30 min). Coupling between the C(2α) proton and the C(2α)-methyl protons is apparent both in neat D_2O and in 100 mM NaOD, providing additional support for our assignments for these signals and for the lack of C(2α) proton exchange.

Kinetics. Reactions were studied by monitoring the absorbance corresponding to the enamine intermediate in a stopped-flow apparatus. Assignment of the absorbance to the enamine is based on the reaction of the thiazolium salt precursor with a non-nucleophilic base in aprotic solvents (DMSO or DMF).[16] For **1h**, it was reported previously that λ_{max} was 410 nm in both DMSO and water.[17] The appearance and loss of the enamine absorbance is presented in Figures 2 and 3 for compounds **1h** and **1d** in the presence of various concentrations of hydroxide ion. Similar results for **2** are shown in Figure 5. These results can be explained using the simple mechanism shown in Scheme 2. For **1h**, the initial rise in the absorbance reflects the increase in enamine concentration as hydroxide ion removes the C(2α)-proton in a step with a pseudo-first-order rate constant k_{2H}. The slow loss of enamine absorbance can be accounted for by a competing decomposition (k_{3H}) of **1h** in the presence of hydroxide ion.

The C(2α)-deuterated substrate **1d** shows a much slower increase in enamine absorbance (Figure 3). The difference in

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Table 1. ^{1}H-NMR Spectra (200 MHz) of Compounds Listed in Scheme 1 in D_2O and in NaOD/D_2O

compd[b]		chemical shift,[a] ppm					
		4-Me (s, 3 H)[c]	OMe (s, 1 H)	3-Me (s, 3 H)	C2αH (s, 1 H)	5H (s, 1 H)	2αCH$_3$
4	D_2O	2.35	3.36	3.62	5.32	7.63	
	95 mM NaOD	0.89, 1.88[d]	3.29, 3.18	2.76, 2.88	5.15	6.17[e]	
1	D_2O	2.37	3.40	3.67	6.00	7.64	
	390 mM NaOD	0.84[e]	3.26[e]	2.70[e]	not seen	6.17[e]	
3	D_2O	2.38	3.41	3.67	5.92	7.68	
	120 mM NaOD	0.91, 1.90	3 .31, 3.22	2.76, 2.79	5.22	6.20, 6.35	
5	D_2O	2.33	3.36	3.61	5.85	2.23[f]	
	95 mM NaOD	0.84, 1.69	3.20, 3.18	2.73, 2.85	not seen	1.62, 1.97	
6	D_2O	2.41	3.40	3.86	5.06	7.62	1.53
					q, $J = 6.0$		d, $J = 6.0$
	100 mM NaOD	1.82, 1.78	3.17, 3.29	2.93, 2.87	4.18, 4.31	6.28	1.31, 1.17
10	D_2O	2.37	3.52	3.75	6.00	2.48[f]	
	100 mM NaOD	0.94, 2.02	3.42, 3.32	2.86, 2.98	not seen	1.95, 2.07	

[a] Chemical shifts are relative to TMS as measured by fixing the water chemical shift at 4.67 ppm. [b] See Scheme 1 for structures and keys for identifying chemical shifts. [c] Integration refers to the major isomer (see note d). [d] The second chemical shift of any pair of values listed in the table refers to the minor isomer of the product. The chemical shift of the major isomer is listed first. [e] In these cases, assignments for the *cis* isomer could not be made. The peaks were either small or obscured by other signals. [f] These entries for compounds **5** and **10** refer to the signal for the 5-methyl group (see Scheme 1).

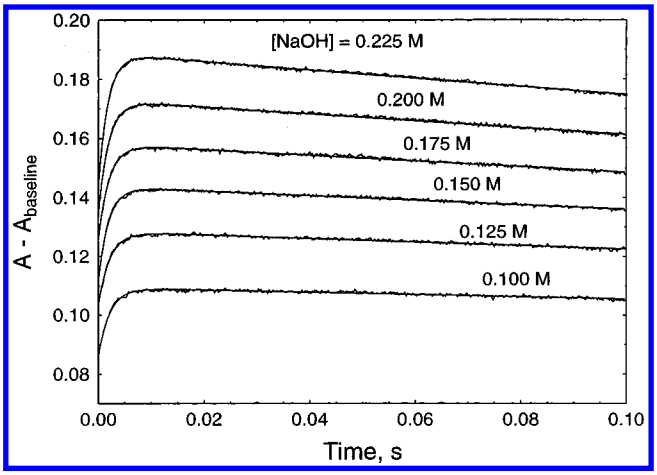

Figure 2. Progress curves observed for the reaction of **1h** (0.00115 M) with aqueous sodium hydroxide (concentrations on the figure) at 25 °C. Concentrations refer to values after mixing in the stopped-flow instrument. The absorbance was monitored at 400 nm. The curves drawn through the data correspond to the fits to eqs 1−5 giving the parameters shown in Table S1 (Supporting Information). The results of the fits are shown in Figure 4. Only half of the runs used to construct Table S1 and Figure 4 are shown in Figure 2.

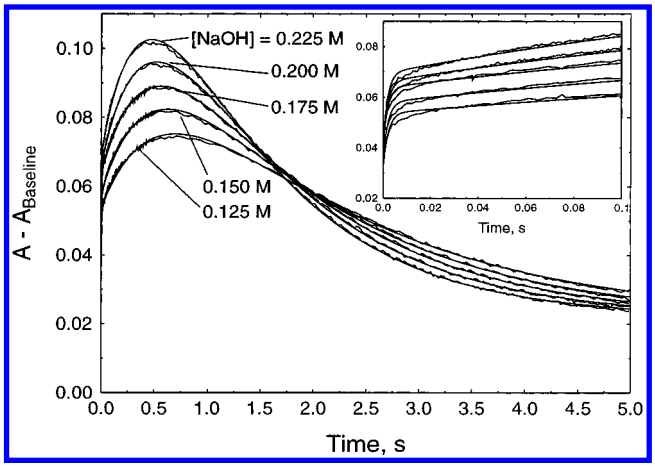

Figure 3. Progress curves observed for the reaction of **1d** (0.00156 M) with aqueous sodium hydroxide (concentrations on the figure) at 25 °C. Concentrations refer to values after mixing in the stopped-flow instrument. The absorbance was monitored at 400 nm. The curves drawn through the data correspond to the fits to eqs 1−5 (with $k_4 = 0$) giving the parameters shown in Table S2 (Supporting Information) and Figure 4.

the results for **1h** and **1d** can be readily explained if there is a significant isotope effect on the rate constant for proton abstraction by hydroxide ion, k_2. The inset on Figure 3 shows that there is also a small, fast component to the absorbance increase, which arises from the reaction of a small amount (0.1−0.2%, based on fitting results) of contaminating **1h** in the sample of **1d**. No return from the enamine to **1d** is shown in Scheme 2. If the HOD produced in the deuteron abstraction from **1d** by hydroxide ion is rapidly lost to the solvent HOH before the enamine can return to **1d**, this is a valid approximation. As is the case with **1h**, the gradual loss of enamine is accounted for in Scheme 2 through the steps identified with the rate constants k_{1H} and k_{3H}.

Analysis of Progress Curves. The progress curves shown in Figures 2, 3, and 5 were fit to the absorbance, *A*, obtained from integration of the rate equations corresponding to Scheme 2 (eqs 1−5). A program was written to replace the function evaluations needed in a standard nonlinear least-squares method[24] with numerical integration.[25] For fits[26] of progress curves for **1h** and **1d**, the extinction coefficient for the enamine (ϵ_E) was fixed at 17 000 M^{-1} cm^{-1} (determined in DMSO; see the next section), and the extinction coefficient for the product (ϵ_P) was fixed at 14 M^{-1} cm^{-1} (determined by comparing the baseline and final absorbances of long runs). The initial concentration of enamine (and the initial concentration of **1d** for the **1d** fits) was included as a parameter. Initial concentration of **1h** was computed from mass conservation assuming that no product had accumulated at the zero time of the instrument. All other initial concentrations were set to 0. These initial concentrations refer to the zero time of the instrument and account for enamine produced during the mixing time. The initial absorbance (zero time of the instrument) was computed in the analysis as $\epsilon_E \times$ [Enamine]$_0$. For fits to the **1h** data, k'_{2D} and k'_{3D} were fixed at

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(26) Selected examples of this approach to fitting can be found in (a) Zimmerle, C. T.; Frieden, C. *Biochem. J.* **1989**, *258*, 381−387 and in (b) Adam, L. C.; Fabian, I.; Susuki, K.; Gordon, G. *Inorg. Chem.* **1992**, *31*, 3534−3541.

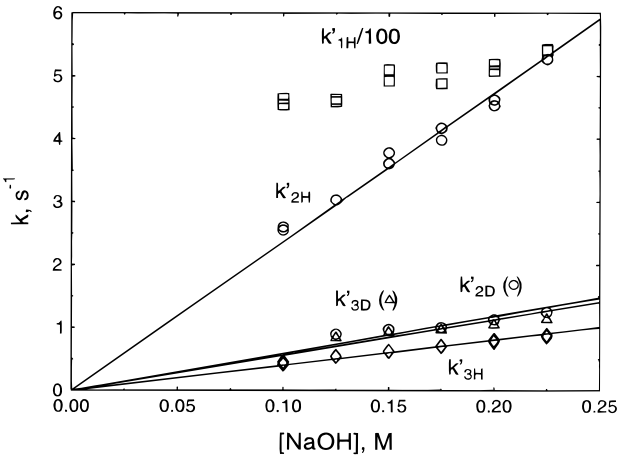

Figure 4. Dependence of first-order rate constants on [NaOH] for **1h** and **1d**. Slopes and intercepts of the least-squares lines drawn through the data are shown at the bottom of Tables S1 and S2. Note that k'_{1H} was divided by 100 for plotting.

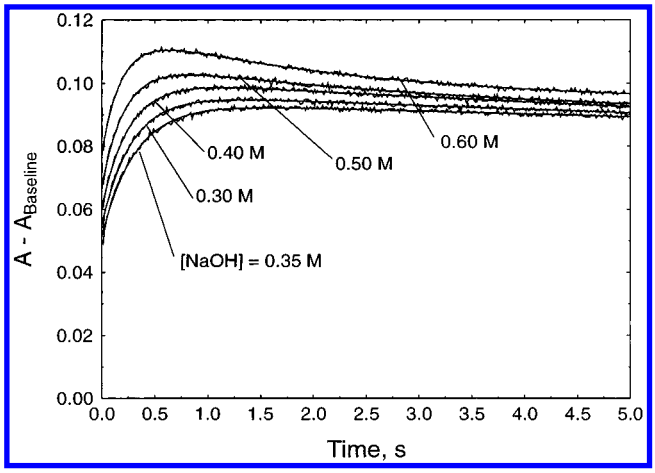

Figure 5. Progress curves for the reaction of **2** (0.00340 M) with aqueous sodium hydroxide at 25 °C. Absorbance was monitored at 380 nm. The curves through the data correspond to the fits to eqs 1−5 with k'_{1H} fixed at 0.1 s^{-1} (see text). Parameters obtained from each fit are shown in Table S3 and Figure 6. Only half of the runs used for Table S3 and Figure 6 are shown above in Figure 5.

0. For **1d** k'_{1H}, k'_{2H}, and k_{3H} were fixed at values obtained from fits for **1h**. Tables S1 and S2 show the results of these least-squares fits. k'_4 was fixed at zero for fits to **1h** and **1d** data. This extra enamine-decomposition pathway was needed only to fit the data for **2** as is described below.

$$d[E]/dt = -k_{1H}[E] + k'_{2H}[SH] + k'_{2D}[SD] - k'_4[E] \quad (1)$$

$$d[SD]/dt = -(k'_{2D} + k'_{3D})[SD] \quad (2)$$

$$d[SH]/dt = -(k'_{2H} + k'_{3H})[SH] + k_{1H}[E] \quad (3)$$

$$d[P]/dt = k'_{3H}[SH] + k'_{3D}[SD] + k'_4[E] \quad (4)$$

$$dA/dt = \epsilon_E\, d[E]/dt + \epsilon_P\, d[P]/dt \quad (5)$$

Fits to the progress curves for **2** in Figure 5 required two modifications to the procedures described for **1**. First, it was necessary to allow the extinction coefficient for the product to be a parameter in the fits. The results (Table S3) showed that ϵ_P varied from about 20 M^{-1} cm^{-1} at 0.30 M NaOH to nearly 28 M^{-1} cm^{-1} (ϵ_E was fixed at 14000 M^{-1} cm^{-1} for all fits of compound **2** runs). Second, it was necessary to allow for a second pathway for decomposition of the enamine (k'_4). With k'_4 fixed at zero, as was done for the fits of **1h** and **1d**, k'_{1H} for compound **2** showed a very strong linear dependence on [NaOH]. According to our model in Scheme 2, there should be no such dependence on hydroxide ion. A second decomposition pathway (k'_4) was therefore included in the model to allow for a hydroxide-dependent loss of enamine. Fitting of the data for **2** to the model with six parameters allowed to float (k'_{1H}, k'_{2H}, k'_{3H}, k'_4, ϵ_P, and initial enamine concentration) gave acceptable results but with very large error estimates. In order to demonstrate the acceptability of our model, the k'_{1H} was fixed at 1.0 M^{-1} s^{-1} for all concentrations of hydroxide. This value is the intercept of a plot against [NaOH] of k'_{1H} obtained from fits in which k'_4 was fixed at zero. The results of the fit are the curves drawn through the data in Figure 5.

Influence of the Enamine Extinction Coefficient. The extinction coefficients fixed for the enamine (ϵ_E) were values determined for the enamine in DMSO. The short lifetime of the enamine made it impossible for us to determine ϵ_E in water, the solvent used for the rate experiments. In order to get a measure of the sensitivity of ϵ_E to solvent, a value (14 000 M^{-1} cm^{-1}, 410 nm) was determined in 90% (volume) dioxane/DMSO. Fortunately, the rate constants are not very sensitive to the choice of extinction coefficient. In a series of fits to the **1h** data, when ϵ_E was varied over the range of 13 000−19 000 M^{-1} cm^{-1} only k_2 showed a significant, but small, dependence on ϵ_E ranging from 27−18 M^{-1} s^{-1}. Instead, the initial enamine concentration is the parameter that is most sensitive to ϵ_E.

Dependence of k' on [NaOH]. Figure 4 shows plots of rate constants corresponding to Scheme 2 against [NaOH] for **1h** and **1d**. The slopes and intercepts of the least-squares lines through these data are shown at the bottom of Tables S1 and S2 (Supporting Information). The resulting second-order rate constants are, for **1h**, k_{2H} = 21.1 ± 0.7 M^{-1} s^{-1} and k_{3H} = 3.41 ± 0.09 M^{-1} s^{-1}, and, for **1d**, k_{2D} = 3.46 ± 0.42 M^{-1} s^{-1} and k_{3D} = 2.84 ± 0.17 M^{-1} s^{-1}. Attempts to control ionic strength were not successful because the large salt concentrations that were needed changed the viscosity of the solutions to such an extent that the stopped-flow experiments could not catch the initial rise in the absorbance. The values reported here for **1h** are not significantly different from our previous report in which a slightly different fitting procedure was used.[27] The slight dependence of k'_{1H} on [NaOH] could be a consequence of fitting to a model that does not incorporate a hydroxide-dependent decomposition of the enamine (k_4). Figure 6 shows the dependence of fitted rate constants vs. [NaOH] for **2**. The slopes of these lines give second-order rate constants for **2**: k_2 = 0.0186 ± 0.0006 M^{-1} s^{-1} and k_4 = 7.11 ± 0.15 M^{-1} s^{-1}. Fitted values of k'_3 for **2** had large estimated errors (Table S3) for the lower hydroxide runs, so the second-order rate constant k_3 is not well determined. The line shown on Figure 6 is a least squares fit forced through a zero intercept (slope = 0.50 ± 0.06). An unconstrained linear fit gave a slope of 1.39 ± 0.10 M^{-1} s^{-1} with a large negative intercept (−0.41 s^{-1}).

Discussion

The progress curves for reactions of **1h**, **1d**, and **2** with hydroxide are described well by the mechanism of Scheme 2 in which the k_4 pathway for enamine decomposition was needed only to explain the hydroxide dependence of rate constants

(27) In our previous report,[17] rate constants were obtained by fitting to the closed-form integrated expression of the rate equations with a time-shift parameter included to account for the fact that some reaction had occurred before the stopped-flow instrument recorded the first time. For all concentrations of hydroxide, least-squares fits gave a time shift of about 0.003 s.

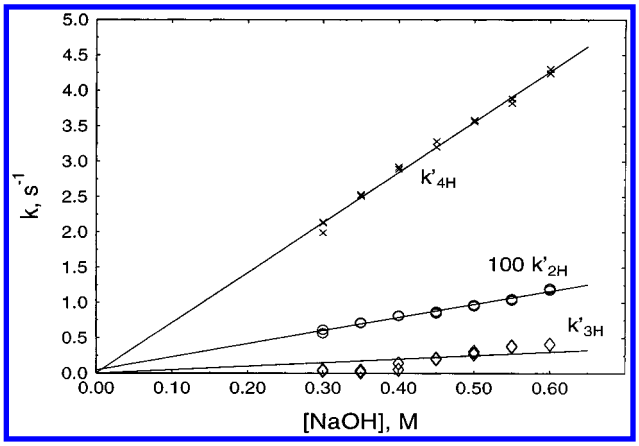

Figure 6. Dependence of first-order rate constants on [NaOH] for **2.** Slopes and intercepts of the least-squares lines are shown at the bottom of Table S3. The line through the k'_{3H} data is a fit constrained to have a zero intercept. Note that the k'_{2H} data was multiplied by 100 for plotting.

obtained for **2**. Simulations of the Scheme 2 mechanism using rate constants obtained from least-squares fits to progress curves predict that the unknown product of the k_4 pathway should be a very minor product. For compound **2** in 0.3 M NaOH, simulations predict 7.4% of the product should be produced from the k_4 pathway, while only 2.2% of the product should be the unknown material in 0.6 M NaOH. We have been unable to characterize what is predicted to be a minor product in the scheme for **2**.[28] The additional decomposition pathway was not needed to explain the results obtained with **1**, which could be studied at lower concentrations of hydroxide ion.

Hydroxide-Dependent Thiazolium Ring Opening. While there have been many studies of the hydrolysis of thiamin-related compounds, only a modest effort has been made to identify the products of hydrolysis. The subject is often complicated by the presence of the 4′-aminopyrimidine ring which can lead to the formation of tricyclic thiamin (bond formation between the nucleophilic amino group and the C(2) carbon),[29,30] and in some cases, the 4′-aminopyrimidine ring can be eliminated from thiamin derivatives.[31−33] Additional complications for product studies are introduced in models with a C(2α)-hydroxyl group. Elimination of the thiazolium ring to produce aldehydes, or as was mentioned above, elimination of the 4′-aminopyrimidine ring are important reactions under specific hydrolytic conditions for compounds containing a C(2α)-hydroxyl group.

The compounds used in our studies do not have these complications: the thiazolium-ring nitrogen is quaternized with a methyl group instead of the 5-(4′-amino-2′-methylpyrimidyl)-methyl group, and in place of a C(2α)-hydroxyl group, our compounds have a methoxy group. Addition of NaOD gave a clean thiazolium ring-opened product containing a mixture of *cis* and *trans* (about the amide bond) isomers as identified by ^{1}H-NMR. Furthermore, the ring-opening appears to be reversible as was demonstrated by the addition of acid after ring opening in the presence of base (spectrum C of Figure 1).

Our rate constants for the ring-opening reaction are in the range of values reported for many related thiazolium-ring hydrolyses. Washabaugh et al.[29,34] and Knoche et al.[35] list rate constants from much of the older literature on thiazolium ring-opening reactions. Their lists, along with rate constants from recent work,[36,37] show that rate constants for the hydroxide-dependent reaction are 3−117 M^{-1} s^{-1} for a broad range of substrates and experimental conditions. For **1h**, we find k_3 = 3.41 ± 09 M^{-1} s^{-1} from the slope of the line through k'_3 in Figure 4 (Table S1). The corresponding rate constant for **2** is not as well determined, because the fits required more parameters (see the Results section).

The mechanism of thiazolium ring opening is widely thought to involve the intermediacy of the pseudobase form of thiazolium ions (the product of hydroxide addition at C2), but there are disparate conclusions about the rate-limiting step for the reaction.[33] Washabaugh et al.[29,34,36] argue that their observations are best explained using a mechanism with rate-limiting decomposition of the pseudobase, while many earlier researchers[35,37−41] favored mechanisms involving rate-limiting formation of the pseudobase. Our observations do not shed significant new light on the controversy; they are consistent with either rate-limiting pseudobase formation or rate-limiting pseudobase decomposition for the ring-opening step.

Proton Transfer from C(2α). Our results are consistent with the classification of the 2-benzylthiazolium salts as typical carbon acids characterized by rate-limiting proton transfer in their acid/base behavior. It is clear from the vast qualitative differences in the progress curves for **1h** (Figure 2) and **1d** (Figure 3) that there must be significant isotope effects on one or more of the rate constants of Scheme 2. Using the least-squares slopes of k' vs. [NaOH] isotope effects (H/D) of 6.1 ± 0.8 and 1.2 ± 0.1 can be estimated for k_2 and k_3, respectively. Given our uncertainty about ionic strength effects on the rate constants, the linear fits through the data shown in Figure 4 may not be entirely appropriate for evaluating the second-order rate constants k_2 and k_3. Ratios of rate constants as a function of [NaOH] offer another measure of the isotope effects on our system. From this analysis, we find that k'_{2H}/k'_{2D} is about 4 while k'_{3H}/k'_{3D} is about 0.7. We conclude that the isotope effect on the second-order rate constant k_2 is 4−6, while the isotope effect on k_3 is near unity. A kinetic isotope effect of 4−6 is typical of isotope effects for proton transfers from carbon acids to hydroxide ion.[42]

(28) Compound **10** (Scheme 1 and Table 1) was prepared for use in attempts to identify unknown products generated in the presence of sodium hydroxide. The only difference between **10** and **2**, the compound for which the k_4 pathway was needed, is the 5-methyl group on the thiazolium ion ring. The spectrum of **10** after adding enough NaOH to give a 0.1 M solution was consistent with the ring-open product (last entry in Table 1). When acid was added the spectrum was again consistent with **10**. Based on inspection of peak heights, we estimate that ca. 5% of the products were unknowns. Some unknown side products formed when base was added, and some remained or resulted from the addition of acid. We were unable to make structural assignments for these minor side products. We observed that in generating the enamine with base, cleaner spectra were obtained when the parent compound has an electron withdrawing group on the phenyl ring. This finding lends qualitative support to the conclusion that a hydroxide-dependent pathway (k_4) for enamine decomposition is needed to explain the kinetics for **2** but not for **1**.

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Our results compare favorably with results obtained by Washabaugh, Stivers, and Hickey[43] using iodination initial rate assays and a substrate similar in structure to ours. These authors found $k = 2.9\ M^{-1}\ s^{-1}$ for C(2α) proton removal from 2-(1-hydroxy-1-phenylmethyl)oxythiamin by hydroxde ion; our corresponding rate constants are $k_2 = 21\ M^{-1}\ s^{-1}$ for **1h** and 0.019 $M^{-1}\ s^{-1}$ for **2.** Washabaugh, Stivers, and Hickey concluded that their reactions were rate-limited by proton transfer based largely on their observation of $k_H/k_D = 1.5$ and $k_H/k_T = 1.8$ for removal of the C(2α) by cacodylate ion (conjugate acid $pK_a = 6.27$[44]). Our isotope effect is larger ($k_H/k_D = 4-6$) suggesting a more symmetrical transition-state structure for the proton transfer to hydroxide ion (conjugate acid $pK_a = 15.7$[44]). It is interesting to note that, according to Washabaugh and Stivers,[45,46] the rate-limiting step for C(2α) proton removal from 2-(1-hydroxy*ethyl*)thiazolium ions by hydroxide ion or buffer bases (isotope effects are near unity) is diffusional separation of product rather than proton transfer. In the yeast pyruvate decarboxylase catalyzed formation of acetoin, however, proton transfer from 2-(1-hydroxyethyl)thiamin is rate limiting with $k_H/k_D \approx 4$[47,48] and coupled to carbonyl-addition (there is evidence suggesting a large ^{14}C isotope effect) according to the mechanism of Stivers and Washabaugh.[48]

Additional insights into the nature of the proton transfer reaction can be obtained though a comparison of compounds **1** and **2**. The benzyl compound (**2**) reacted much more slowly than did the *p*-trimethylammoniumbenzyl compound (**1**), but our analysis shows that the apparent pK_a's of the two compounds are very similar. Allowing for uncertainty in ionic strength effects and ϵ_E we used $k'_{1H} = 300-540\ s^{-1}$ (the intercept and the highest hydroxide point in Figure 4) and $k_{2H} = 18-28\ M^{-1}\ s^{-1}$ (the range of k_{2H} observed when ϵ_E was varied (see the Results section)) to estimate a pK_a for **1h** of 15.0−15.5 (using $K_w = 10^{-14}\ M^2$).[49] A similar analysis of the data in Figure 6 gives an estimate of 15.7 for the pK_a of **2**, in agreement with an estimate of 15 ± 1 reported for 2-(1-hydroxybenzyl)-oxythiamin.[43] Thus the equilibrium constants for proton removal to generate the enamines are very similar (they may differ by about one p*K* unit—one order of magnitude in *K*). The rate constants k_2 differ by three orders of magnitude. The slope of k'_{2H} vs. [NaOH] in Figure 4 for compound **1h** is 21 $M^{-1}\ s^{-1}$, while the same slope for **2** is only 0.019 $M^{-1}\ s^{-1}$. The difference in sensitivity of the equilibrium and rate constants to substitution in the benzyl ring may reflect another feature of carbon acids with resonance-stabilized conjugate bases—transition-state "imbalance" in accord with Bernasconi's principle of imperfect synchronization.[50−53] The rate-limiting transition-state may experience very little resonance stabilization from the thiazolium nitrogen and thereby be subject to substantial substituent effects. The relative insensitivity of the equilibrium constants to substitution may reflect a very enamine-like (as opposed to carbanion-like) conjugate base.

Protonation of the Enamine. Among the rate constants determined by us, k_1 (Scheme 2), for the protonation of the enamine by water is the rate constant most relevant to catalysis by benzoylformate decarboxylase. Protonation of the enamine is very likely an essential step in the enzymatic reaction following decarboxylation of the intermediate 2-(1-carboxy-1-hydroxy-1-phenylmethyl)thiamin. We found k_1 to be 1 s^{-1} for the enamine of 2-(1-hydroxy-1-phenylmethyl)-3,4-dimethylthiazolium ion (**2**). Dividing by 55.5 M, to represent the concentration of the proton donor water gives an estimated second-order rate constant of 0.018 $M^{-1}\ s^{-1}$. The enzyme must protonate the enamine with a rate constant greater than k_{cat} (81 s^{-1}).[54] The ratio of these two numbers gives a minimum effective molarity[55] of 4500 M for the enzyme in this proton-transfer process. Effective molarities have been estimated to be greater than 60 000 M in carefully-designed systems involving intramolecular proton transfers to carbon,[56] but values of 1−10 M are typical of less sophisticated nonenzymic intramolecular systems involving general acid−base catalysis.[57] Our estimate of a minimum value of 4500 M for the proton transfer in benzoylformate decarboxylase catalysis is sufficiently large to suggest that the enzyme does not allow the enamine to be protonated by bulk solvent. Instead, an acidic residue must be poised to effect catalysis of this proton-transfer step.

Experimental Section

Most chemicals were purchased from Aldrich Chemicals, Inc. and were used without further purification, unless specified otherwise below. 4-Methylthiazole was purchased from Pyrazine Specialties. Syntheses of compounds similar to **1**−**6** have been previously reported.[16,58,59] In brief, the compounds used in the present report were prepared by addition of a thiazole to an aldehyde, followed by methylation using of the thiazole nitrogen and the C(2α) hydroxyl group to yield fluoroborate salts of substituted thiazolium ions. The syntheses and analyses of **2h**, **4**, and **6** used in this study have been published.[60]

2-[1-Hydroxy-1-(*p*-dimethylaminophenyl)methyl]-4-methylthiazole (7). Compound **7** was prepared using *p*-dimethylaminobenzaldehyde in place of benzaldehyde according to the procedure published for 2-(1-hydroxy-1-phenylmethyl)-4-methylthiazole.[60] ^1H NMR (200 MHz, DMSO-d_6−DMSO): d 7.17 (d, 2H, J = 8.8 Hz, Ar), 6.65 (d, 2H, J = 8.8 Hz, Ar), 7.07 (s, 1H, C5-H), 6.39(s, 1H, C2a-H), 5.72 (br, 1H, OH), 2.83 (s, 6H, Ar-N-Me_2), 2.25 (s, 3H, C4-CH_3).

2-[1-Methoxy-1-(*p*-dimethylaminophenyl)methyl]-4-methylthiazole (8). To a stirred solution of **7** (10.9 mmol) in 35 mL of dry THF at 0 °C under Ar was added NaH (0.43 g, 14.2 mmol) in one portion. The mixture was stirred for 30 min, and then a solution of CH_3I (6.2 g, 43.7 mmol) in THF was added in two portions (half was added 1 h after the first portion). After 2 h, the reaction was quenched with 20 mL of water:ethanol (1:1) and concentrated using a rotary evaporator to afford an oil which was extracted with diethyl ether and water. The organic layers were dried (Na_2SO_4) and concentrated to yield. The crude product was purified using a column of silica gel eluted with ether−hexanes (2:1). The product oil (1.0 g) was obtained in 64% yield. ^1H NMR (200 MHz, $CDCl_3-CHCl_3$): δ 7.29 (d, 2H, J

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= 8.9 Hz, Ar), 6.69 (d, 2H, J = 8.9 Hz, Ar), 6.78 (s, 1H, C5-H, 5.42 (s, 1H, C2α-H), 3.39 (s, 3H, OCH_3), 2.91 (s, 6H, Ar-N-Me_2), 2.38 (s, 3H, C4-CH_3).

2-[1-Methoxy-1-(*p*-trimethylammoniumphenyl)methyl-3,4-dimethylthiazolium Fluoroborate (1h). Using the procedure described for the synthesis of **2h**,[60] compound **8** was converted into a fluoroborate salt using 2 equiv of $(CH_3)_3OBF_4$. The salt was obtained as a white powder (0.95 g, 60%) which was purified on a cellulose plate eluted with CH_3CN. ^{1}H NMR (200 MHz, D_2O-H_2O): δ 7.80 (d, 2H, J = 8.0 Hz, Ar), 7.62 (s, 1H, C5-CH), 7.57 (d, 2H, J = 8.0 Hz, Ar), 5.94 (s, 1H, C2α-H), 3.62 (s, 3H, N-CH_3), 3.48 (s, 9H, ArN^+-Me_3), 3.35 (s, 3H, OCH_3), 2.31 (s, 3H, C4-CH_3). Anal. Calcd for $C_{16}H_{24}N_2OS\cdot[BF_4]_2\cdot 0.75H_2O$: C, 40.07; H, 5.30; N, 5.85; S, 6.68. Found: C, 39.99; H, 5.27; N, 5.87; S, 6.96.

2-(1-Deutero-1-methoxy-1-phenylmethyl)-3,4-dimethylthiazolium Fluoroborate (2d). The same series of reactions used to prepare **2h**[60] was repeated using [C7-^{2}H]benzaldehyde in place of benzaldehyde. In 38% overall yield, 1.14 g of **2d** was obtained as a yellow oil. ^{1}H NMR (200 MHz, D_2O-H_2O): δ 7.62 (s, 1H, C5-H), 7.41−7.35 (m, 5H, Ar), 3.61 (s, 3H, N-CH_3), 3.37 (s, 3H, OCH_3), 2.33 (s, 3H, C4-CH_3). Anal. Calcd for $C_{13}H_{15}NOS\cdot BF_4D\cdot 0.5H_2O$: C, 47.15; H, 4.87; N, 4.23; S, 9.68. Found: C, 47.32; H, 5.00; N, 4.15; S, 9.89.

***p*-Dimethylamino-[C7-^{2}H]benzaldehyde (9)**. Compound **9** was prepared from the reduction of an acid chloride based on related literature methods.[61,62] To a 0.023 mol (3.8 g) solution of *p*-dimethylaminobenzoic acid in 50 mL of anhydrous CH_2Cl_2, ClCOCOCl (5.84 g, 0.046 mol) was added dropwise under argon gas over a 30 min period. After the solution was allowed to reflux for 2 h, the solvent was removed leaving a green residue containing the acid chloride product. The acid chloride was reduced using LiAl(*t*-BuO)$_3$D prepared, according to standard methods,[63] from $LiAlD_4$ and *t*-BuOH. Over a 1 h period, a 100 mL diglyme solution containing 0.023 mol of LiAl(*t*-BuO)$_3$ was added to a diglyme solution of the acid chloride (4.2 g, 0.023 mol) at −78 °C. ^{1}H NMR (200 MHz, $CDCl_3-CHCl_3$): δ 7.72 (d, 2H, J = 8.9 Hz, Ar), 6.68 (d, 2H, J = 8.8 Hz, Ar), 3.07 (s, 6H, $N(CH_3)_2$).

2-[1-Deutero-1-methoxy-1-(*p*-trimethylammoniumphenyl)methyl]-3,4-dimethylthiazolium fluoroborate (1d). Using *p*-dimethylamino-[C7-^{2}H]-benzaldehyde (**9**), compound **1d** was prepared using the procedures described above for **1h**. The product was obtained as an oil which was purified by thin-layer chromatography using a cellulose plate eluted with EtAc−CH_3CN (9:1). The product was washed out of the cellulose with CH_3CN. After removing the solvent using rotary evaporation, 0.9 g of **1d** was obtained in 51% overall yield from **11**. ^{1}H NMR (200 MHz, D_2O-H_2O): δ 7.82 (d, 2H, J = 8.9 Hz, Ar), 7.64 (s, 1H, C5-H), 7.60 (d, 2H, J = 8.8 Hz, Ar), 3.65 (s, 3H, N-CH_3), 3.51 (s, 9H, Ar-N-$(CH_3)_2$), 3.37 (s, 3H, OCH_3), 2.34 (s, 3H, C4-CH_3). Anal. Calcd for $C_{16}H_{23}N_2OSB_2F_8D\cdot 2H_2O$: C, 38.20; H, 5.41; N, 5.57; S, 6.37. Found: C, 38.31; H, 5.42; N, 5.76; S, 6.65.

2-[1-Methoxy-1-(*p*-chlorophenyl)methyl-3,4-dimethylthiazolium Fluoroborate (3). Following the same procedures described for the preparation of **7**, **8**, and **2h**, 0.80 g (70%) of **3** was prepared from *p*-chlorobenzaldehyde. ^{1}H NMR (200 MHz, D_2O-H_2O): δ 7.55 (s, 1H, C5-H), 7.35 (d, 2H, J = 8 Hz, Ar), 7.26 (d, 2H, J = 8 Hz, Ar), 5.82 (s, 1H, C(2αH)), 3.58 (s, 3 H, N-CH_3), 3.32 (s, 3H, OCH_3), 2.29 (s, 3 H, C4-CH_3).

2-[1-Methoxy-1-(*p*-chlorophenyl)methyl-3,4,5-trimethylthiazolium Fluoroborate (5). Following the same procedures described for the preparation of **7**, **8**, and **2h**, 1.14 g (80%) of **5** was prepared from *p*-chlorobenzaldehyde and 4,5-dimethylthiazole. ^{1}H NMR (200 MHz, D_2O-H_2O): δ 7.40 (d, 2H, J = 8.6 Hz, Ar), 7.30 (d, 2H, J = 8.6 Hz, Ar), 5.86 (s, 1H, C(2αH)), 3.62 (s, 3H, N-CH_3), 3.37 (s, 3H, OCH_3), 2.33 (s, 3H, C4-CH_3), 2.23 (s, 3H, C5-CH_3).

2-(1-Hydroxy-1-phenylmethyl)-4,5-dimethylthiazole (11). To a stirred solution of 4,5-dimethylthiazole (3.5 g, 13.2 mmol) in 50 mL of dry THF at −78 °C *n*-BuLi (16.5 mmol) was added dropwise under N_2. The mixture was stirred for 1 h, and then a solution of benzaldehyde (40 mmol) in THF was added. After stirring the mixture at −78 °C for 30 min, the reaction temperature was allowed to rise to room temperature, and the reaction proceeded for another 45 min. The reaction was then quenched with water (40 mL) and extracted with ethyl ether (3 × 20 mL). The organic layer was dried ($MgSO_4$) and concentrated. The crude product was purified using a silica gel column eluted with ethyl acetate−petroleum ether (1:3). After crystallization, the product was obtained as a pale yellow solid in 41% yield.

2-(1-Methoxy-1-phenylmethyl)-4,5-dimethylthiazole (12). To a stirred solution of **11** (1.5 g, 6.8 mmol) in 30 mL of dry THF was added NaH (0.43 g, 18 mmol) at 0 °C under N_2. The mixture was stirred for 30 min at 0 °C, and then CH_3I (2.0 g, 13.9 mmol) was added dropwise at room temperature. After 3−4 h, the reaction was quenched with 20 mL water and extracted with ethyl ether (3 × 15 mL). The organic layer was dried ($MgSO_4$) and concentrated. The crude product was purified on a silica gel column eluted with ethyl acetate−petroleum ether (3:7). A crystalline product was obtained in 83% yield. ^{1}H NMR (500 MHz, $CDCl_3$/TMS): δ 7.45 (d, 2H, Ar), 7.35 (t, 2H, Ar), 7.30 (m, 1H, Ar), 5.5 (s, 1H, C(2αH)), 3.42 (s, 3 H, O-CH_3), 2.30 (s, 3H, 2.29 C_4-CH_3 or C_5-CH_3) 2.29 (s, 3H, C_4-CH_3 or C_5-CH_3).

2-(1-Methoxy-1-phenylmethyl)-3,4,5-trimethylthiazolium Trifluoromethanesulfonate (10). To a stirred solution of **12** (0.22 g, 0.94 mmol) in 10 mL of dry CH_2Cl_2 was added methyl trifluoromethanesulfonate (0.15 g, 0.94 mmol) at 0 °C under N_2. The mixture was stirred for 0 °C for 30 min, then the temperature was allowed to rise, and the reaction was stirred for another 3 h at room temperature. The reaction mixture was concentrated and then purified on a silica gel column with methanol−methylene chloride (1:9). The product was obtained as an oil (0.32 g) in 86% yield. ^{1}H NMR (500 MHz, D_2O): δ 7.55 (m, 3H, Ar), 7.45 (m, 2H, Ar), 5.98 (s, 1H, C(2αH)), 3.72 (s, 3H, N-CH_3), 3.48 (s, 3H, O-CH_3), 2.46 (s, 3H, C_5-CH_3), 2.31, 3H, C_4-CH_3).

Kinetics. A Hi-Tech model stopped-flow instrument was used to monitor the enamine absorbance produced in the reaction of **1** (410 nm) or **2** (380 nm) with hydroxide ion at 25 °C. The stopping syringe was adjusted to collect 250 mL (125 mL each of substrate and NaOH solutions). A 1 cm path-length configuration was used. Distilled, deionized, and degassed water was used to prepare reaction solutions. Concentrations of NaOH solutions were determined by titration to phenolphthalein end points using potassium hydrogen phthalate. Syringe concentrations of the substrate solutions were 2.3 mM (**1h**), 3.1 mM (**1d**), 6.8 mM (**2h**), and 2.2 mM (**2d**). Progress curves were analyzed as described in the Results section.

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Supporting Information Available: Tables S1, S2, and S3 containing parameters obtained from the least-squares fits of each of the progress curves obtained at various [NaOH] for compounds **1h**, **1d**, and **2**. Also included are Figures S1a, S1b, S1c, and S2 containing ^{1}H NMR spectra of compound **10** in D_2O, NaOD, and DCl (6 pages). See any current masthead page for ordering and Internet access instructions.

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