Hydrolysis and Subsequent Cyclization of Etazolate Hydrochloride and Related Compounds in Aqueous Solutions: Application of PMR and Mass Spectrometry in Accelerated Stability Studies

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Abstract □ The hydrolysis of etazolate hydrochloride, an inhibitor of cyclic nucleotide 3′,5′-monophosphate phosphodiesterase that degrades cyclic adenosine 3′,5′-monophosphate (cyclic AMP) to adenosine 5′-monophosphate, and related compounds was studied by PMR and mass spectrometry. The compounds underwent reversible acid-catalyzed hydrolysis in aqueous solutions at 60°, followed by cyclization to a major and a minor product formed by independent pathways. Under the experimental conditions, the minor product was stable. The formation rate of the major product, 6-ethyl-1,6-dihydrodipyrazolo[3,4-*b*:3′,4′-*d*]pyridin-3(2*H*)-one, was considerably greater than that of the minor component, 3-ethoxy-6-ethyl-1,6-dihydrodipyrazolo[3,4-*b*:3′,4′-*d*]pyridine. For the 6-methyl analog of etazolate, the rate of methyl deuteration was considerably slower than the rate of cyclization.

Keyphrases □ Etazolate hydrochloride—hydrolysis in aqueous solutions, PMR and mass spectral stability studies □ Hydrolysis—etazolate hydrochloride in aqueous solutions, PMR and mass spectral stability studies □ Stability—etazolate hydrochloride in aqueous solutions, PMR and mass spectral studies □ Tranquilizers—etazolate hydrochloride, hydrolysis in aqueous solutions, PMR and mass spectral stability studies

NMR and mass spectrometry have been employed extensively to monitor the aqueous stability of pharmacologically active compounds (1). The usual procedure involves monitoring the PMR spectra of acidic, basic, and neutral aqueous solutions kept at 60–65° over 4–5 days. Gross changes in the PMR spectra permit the identification of degradation products.

Information obtained under these drastic conditions is useful in designing bulk stability and preformulation studies. Indeed, many compounds are stable under these conditions and as well in the subsequent bulk and formulation stability studies. The aqueous stability of an inhibitor of cyclic nucleotide 3′,5′-monophosphate phosphodiesterase, etazolate[1] [ethyl 1-ethyl-4-(isopropylidenehydrazino)-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylate] hydrochloride (I), and related 1*H*-pyrazolo[3,4-*b*]pyridines[2] (2) is reported to demonstrate the utility of the approach in establishing the degradation rate and identification of products formed during the aqueous hydrolysis of I.

EXPERIMENTAL

PMR spectra were obtained on a 100-MHz NMR spectrometer[3], which was internally locked to the ^{2}H-frequency of 15.4 MHz. PMR data in aqueous solutions and in dimethyl sulfoxide-d_6 are presented in Tables I and II, respectively. Low- and high-resolution mass spectra were taken on a double-focusing mass spectrometer[4] using a direct insertion probe. Data were acquired *via* frequency-modulated analog tape, which was subsequently processed with the aid of a computer[5] using published programs (6). The pH or pD values were measured with a pH meter[6] with combination pH electrodes.

The calculations were performed by measuring the areas under the respective resonances in the PMR spectra of solutions of known concentrations:

$$\%\ \text{hydrolysis} = \frac{\text{area under acetone resonance} \times 100}{\text{area under acetone resonance} + \text{area under N=C(CH}_3)_2\ \text{group}} \quad \text{(Eq. 1)}$$

or:

$$\%\ \text{hydrolysis} = \frac{\text{area under acetone resonance} \times 100}{\text{area under N=C(CH}_3)_2\ \text{resonance at time 0}} \quad \text{(Eq. 2)}$$

$$\%\ \text{cyclization}^7 = \frac{\text{area under CH}_3\ \text{resonance of C}_2\text{H}_5\text{OD} \times 100}{0.5 \times \text{area of CH}_3\ \text{resonances at time 0}} \quad \text{(Eq. 3)}$$

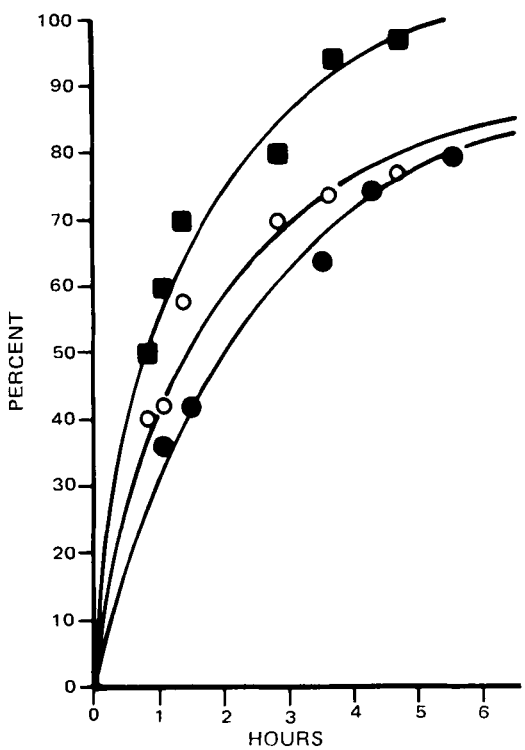


Figure 1—*Rates of hydrolysis [■, loss of N=C(CH$_3$)$_2$ group] and cyclization (○, formation of C$_2$H$_5$OD) of II compared to the rate of cyclization (●, formation of C$_2$H$_5$OD) of V at 65° in aqueous acidic (pH 1.5) solutions.*

[1] SQ20009, E. R. Squibb and Sons, Princeton, N.J.

[2] The compounds, prepared by Dr. H. Höhn, Dr. T. Denzel, and Dr. W. Janssen (3–5), were supplied through the Squibb chemical distribution center and represented typical batches used in clinical and formulation studies.

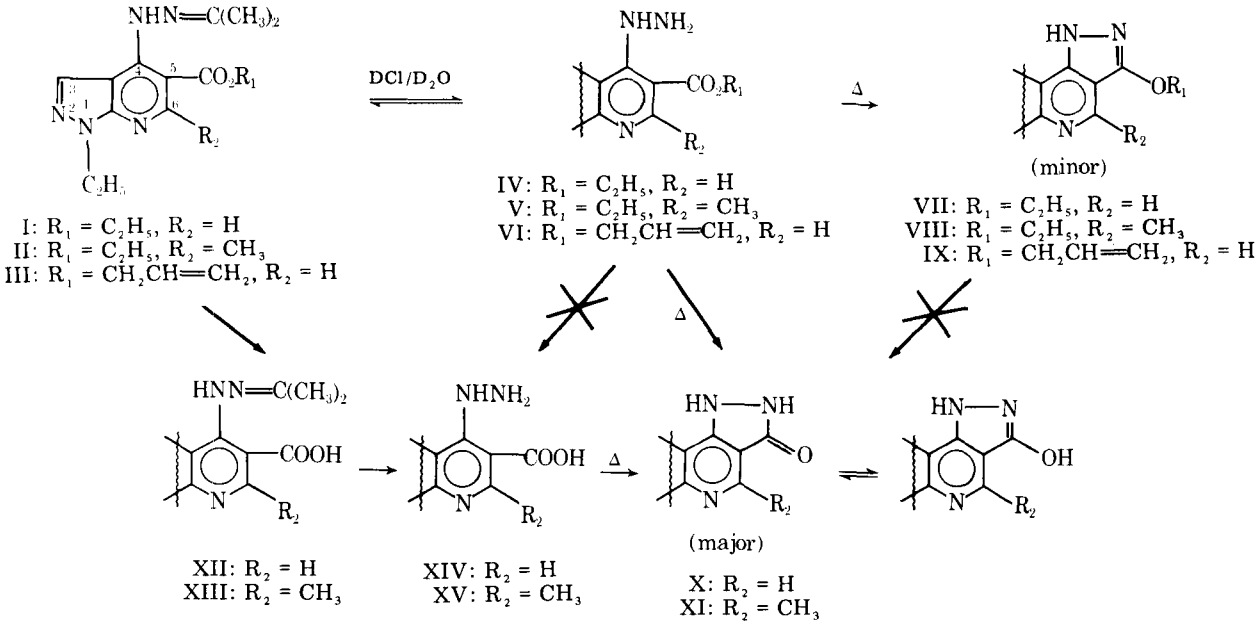
[3] Varian Associates model XL-100-15.

[4] Associated Electrical Industries model M-902.

[5] Digital Equipment Corp. model PDP-11.

[6] Orion Research model 701.

[7] Conceivably, the rates of ester hydrolysis and cyclization are similar. However, no XIV was found when IV was reacted (Scheme I). Perhaps the nucleophilic attack by the hydrazino group facilitates the cyclization, with a concurrent loss of the ester group. Additionally, the percentage cyclization calculated from the area of the aromatic proton resonances of X, although producing less accurate data because of the errors in measuring a single proton, was comparable to the data obtained from the area measurements of liberated ethanol.



Scheme I

and:

$$\% \text{ deuteration} = \frac{\text{area under C-6 } CH_3 \text{ resonance} \times 100}{\text{area under C-6 } CH_3 \text{ resonance at time 0}} \qquad \text{(Eq. 4)}$$

The kinetic data (60°) are presented in Table III. The plots of log *c versus* time were linear and the slopes were calculated by the method of least squares with an error of ±25%. The pseudo-first-order rate constants for hydrolysis, k_H, cyclization, k_C, and deuteration, k_D, are presented in Table IV.

RESULTS AND DISCUSSION

Semicarbazones, oximes, and phenylhydrazones exist in equilibrium with their constituents in aqueous solutions (7). Similarly, isopropylidenes I–III (Scheme I) establish an acid-catalyzed equilibrium between I, II, or III and their respective hydrazides IV, V, or VI and acetone in deuterium oxide solutions.

The rate of liberation of acetone[8] from I–III was identical at similar concentrations and pH values[9]. Furthermore, the extent of hydrolysis of I–III or their formation from the corresponding hydrazides IV–VI and acetone was temperature dependent. Approximately 50% of I was converted to IV between 32 and 40°, but only 25% was hydrolyzed at 20°. Furthermore, I could be formed from IV and acetone[10]; *e.g.*, with acetone-d_6, 45% of I-d_6 was formed at 32° as determined by PMR and mass spectrometry. When nitrogen was bubbled through a solution of I for 7 hr, to remove liberated acetone from the solution, the PMR spectrum showed the formation of 50% IV.

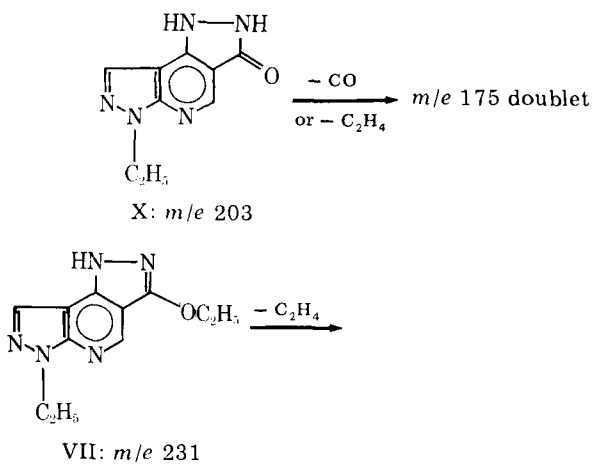
At 65°, the PMR spectrum of a solution of I indicated the presence of secondary products. The structural elucidation of these products was initiated. The extent of the various products present was evaluated by measuring the relative areas under the various resonances. The formation of ethanol-d_1 C_2H_5OD was determined by measuring the areas of resonances at δ 3.58 (quartet) and 1.06 (triplet) ppm, comparing them to the ethyl proton resonances of I and IV occurring at the chemical shifts of δ 4.40 (quartet) and 1.45 (triplet) ppm. With the 6-methyl analog, II, the C-6 methyl proton resonance at δ 2.78 ppm was compared to the resonance at δ 2.7 ppm of a reaction product.

Although acetone generated from the hydrolysis of the isopropylidene group of I and II was present, neither of the respective hydrazides IV and V was observed. The cyclization rates of IV and V were subsequently shown to be reproducible, and IV and V form the same products that result from the heating of acidic solutions of I and II at 65°.

Scheme I represents the chemical transformations taking place at 65°. The pseudo-first-order rate constants are given in Table IV. The PMR spectra of the reaction products of I after drying indicated a major and a minor component. High-resolution mass spectrometry of the major component demonstrated the composition of a molecular ion (M^+) at *m/e* 203 to be C_9H_9NO. The structure of the major component was confirmed as X by comparison of its PMR and mass spectra with those of an authentic sample.

The diagnostic proton resonances in dimethyl sulfoxide-d_6 are δ 8.29 and 9.1 ppm for the heterocyclic protons, which are within the experimental error of authentic X (δ 8.24 and 9.03 ppm) (*cf.*, Table II). The mass spectrometric analysis of the products derived from IV show that X was the major product. A minor component (~10%) with an M^+ of *m/e* 231 was assigned Structure VII. In the acidic aqueous solution, this component had heterocyclic proton shifts of δ 8.04 and 8.63 ppm. The assignment of the fragmentation of the major product X and the minor product VII is shown in Scheme II.

The percent conversion of II to V and the subsequent cyclization of V to XI are shown as a function of time in Fig. 1. In deuterium oxide–deuterium chloride (pD 1), the isopropylidene group of a 5-mg/ml solution of II was completely hydrolyzed in 21.5 hr at 65°. Compound V subsequently formed two products. The PMR spectrum showed a shift of the heterocyclic proton from δ 8.53 to 8.0 ppm and of the pyridyl methyl from δ 2.84 to 2.62 ppm. The mass spectrum of the evaporated solution demonstrated that the major product was XI (the 6-methyl analog of X) and that the minor product was VIII (the 6-methyl analog of VII) along



Scheme II

[8] The chemical shift of liberated acetone was indistinguishable from that of added acetone.

[9] Dr. D. A. Wadke, Squibb Institute for Medical Research, New Brunswick, N.J., personal communication.

[10] Acetone bound to IV does not exist in any substantial amount.

Table I—PMR Data in Aqueous Solutions [a]

Compound	Concentration, mg/ml	Solvent and pD	Chemical Shift of Proton, ppm					
			H_3	H_6	CH_2	CH_3	N=C-$(CH_3)_2$	CH_3 of Acetone
I	7	0.1 *N* DCl–D_2O, pD = 1.5	8.23	8.56	4.41 m	1.48 t 1.55 t	2.05 2.25	2.31
II	5	pD = 1	8.53	2.84	4.45 m	1.41 t 1.44 t	2.12 2.23	2.21
	10	pD = 1.9	8.39	2.77	4.40 m	1.43 t 1.46 t	2.05 2.22	2.20
III	8	pD = 1.3	8.24	8.58	4.36 q	1.45 t	1.99 2.17	2.20 (6.0, 5.4 CH=CH_2)
IV		0.01 *N* DCl–D_2O, pD = 1.5	8.67	8.73	4.38 q 4.40 q	1.44 t 1.49 t		
V	10	pD = 1.9	8.62	2.70	4.30 q 4.42 q	1.35 t 1.41 t		
VI		pD = 1.3	8.66	8.71	4.36 m	1.45 t		6.0, 5.4 CH=CH_2
VII		pD = 0.9	8.35	8.97	4.45 m	1.43 t 1.46 t		—[b]
X		pD = 2	8.15	8.72	4.43 q	1.45 t		—[c]
		0.1 *N* DCl–D_2O, pD = 2	8.01	8.59	4.48 q	1.45 t		—[b]
XI		pD = 1.9	7.98	2.62	4.36 q	1.45 t		—[d]
XII		0.1 *N* DCl–D_2O, pD = 2.0	8.57	8.75	4.42 q	1.55 t	2.15 2.25	2.31

[a] Parts per million from external 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt. [b] Authentic sample. [c] Chemical shifts are the average of the chemical shifts of the products from I, III, IV, and XII. [d] Chemical shifts are the average of the product obtained from II and V.

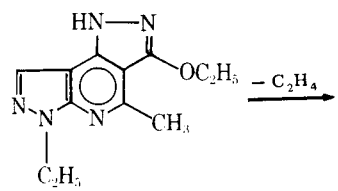
Table II—PMR Data of 1*H*-Pyrazolo[3,4-*b*]pyridines in Dimethyl Sulfoxide-d_6 [a]

Compound	Chemical Shift, ppm					
	CH_2	CH_3	N=C$(CH_3)_2$	H_3	R_2	NH
I	4.35 q 4.49 q	1.37 t 1.42 t	2.04 2.19	8.46	8.65	11.58
II	4.38 q 4.57 q	1.36 t 1.40 t	2.07 2.19	8.53	2.83	11.56
IV	4.35 q 4.59 q	1.35 t 1.40 t		8.52	8.62	8.22
VII[b]	4.54 q	1.43 t		8.16	8.72	10.50
X[b]	4.60 q	1.44 t		8.24	9.03	9.07
X[c]	4.64 q	1.46 t		8.29	9.10	11.06
XI	4.47 q	1.43 t		8.03	2.79	11.52

[a] Parts per million from tetramethylsilane internal reference; q = quartet (J = 7 Hz) and t = triplet (J = 7 Hz). [b] Authentic sample prepared according to U.S. pat. 3,787,430 (Jan. 22, 1974), assigned to E. R. Squibb & Sons. [c] Sample obtained after heating an aqueous solution of I at 65° for 6 days and stripping off water. Traces of I were present.

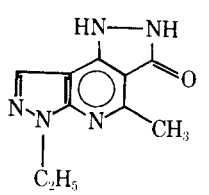
with residual amounts of V. The fragmentation of VIII and XI (Scheme III) is analogous to the respective products VII and X.

The PMR spectra of a deuterium oxide–deuterium chloride (pD 1.3) solution of 9 mg of III/ml heated to 60° for 24 hr showed the complete loss of the isopropylidene group, at a rate six times slower than was observed for I and II. During the next 7 days, the resonances due to the allyl ester group slowly disappeared and the resonances of the major product appeared. The PMR and mass spectra of this degradation product were



VIII: *m/e* 245

m/e 217 $\xrightarrow[\text{or} - C_2H_4]{-CO}$ *m/e* 189 doublet



$\xrightarrow[\text{or} - C_2H_4]{-CO}$ *m/e* 189 doublet

XI: *m/e* 217

Scheme III

identical to those obtained from I and IV and the spectra of authentic X. The rate data indicate that the cyclization rate was somewhat slower for the allyl ester III than for the ethyl ester I, perhaps because the allyl group is somewhat more resistant to hydrolysis. In addition to X, the mass spectrum of the degradation product also indicated the presence of the minor product IX (M^+, *m/e* 243).

Mechanistic Pathways—While both the ester and isopropylidene groups were concurrently hydrolyzed in acidic media, the hydrolysis rate of the latter was much faster. On the other hand, when a large amount of I was hydrolyzed, small amounts of the isopropylidene acid XII (M^+, *m/e* 261) and the hydrazide acid XIV (M^+, *m/e* 221) were detected in the mass spectrum. No XIV was detected, however, when the hydrazide ester IV was reacted under the same conditions. Again, competitive cyclization rates precluded the formation of this compound in any appreciable amount.

To establish the pathway for the formation of X from XII, an authentic sample of XII, the free acid of I, was dissolved in deuterium oxide–deuterium chloride and heated for 2 weeks. The chemical shifts of the product of δ 8.23, 8.80, 4.40, and 1.51 ppm were comparable to those of authentic X. While **X** was the major reaction product, the mass spectra also indicated the presence of small amounts of XIV (M^+, *m/e* 221). The cyclized product, X, was formed after hydrolysis of the isopropylidene groups to XIV and subsequent cyclization according to Scheme I.

Attempts to Hydrolyze VII to X—To establish further the degradation pathway, an authentic sample of the enol ether, VII, was heated in the usual way: 3.3 mg/ml in deuterium oxide–deuterium chloride, pD 0.9, at 65° for 9 days. No change in chemical shifts was noted. Thus, VII is not the precursor of X and both are formed by different and independent pathways. In the hydrolysis of I or IV, X was formed at a faster rate than VII.

Deuteration Rate of C-6 Methyl Group—Prolonged heating of II or V in the deuterated solvent resulted in the loss of the 6-methyl proton

Table III—Kinetic Data [a]

Hydrolysis							
I[b]		I[c]		II[d]		III[d]	
Minutes	Concentration, moles, × 10^{-3}	Minutes	Concentration, moles, × 10^{-3}	Minutes	Concentration, moles, × 10^{-2}	Minutes	Concentration, moles, × 10^{-2}
0	3.46	0	3.46	0	3.4	0	2.2
7	3.39	42	3.29	50	1.7	20	2.0
20	3.32	55	3.36	65	1.4	58	2.0
35	3.04	76	3.22	80	1.0	128	1.6
52	2.66	96	3.01	215	0.2	188	1.5
74	2.35	135	2.70	275	0.1	248	1.2
107	2.21	175	2.67				
145	2.11	212	2.63				

Cyclization				Deuteration			
II[d]		V[d]		II		V	
Minutes	Concentration, moles, × 10^{-2}	Minutes	Concentration, moles, × 10^{-2}	Hours	Concentration, moles, × 10^{-2}	Hours	Concentration, moles, × 10^{-2}
50	2.0	0	4.0	0	2.48	0	2.85
65	2.0	65	2.6	22.4	1.71	22.5	1.91
85	1.5	93	2.3	67.7	1.14	67.7	1.45
170	1.0	213	1.4	115.1	0.99	115.1	1.17
215	0.9	258	1.0				
275	0.8	333	0.8				

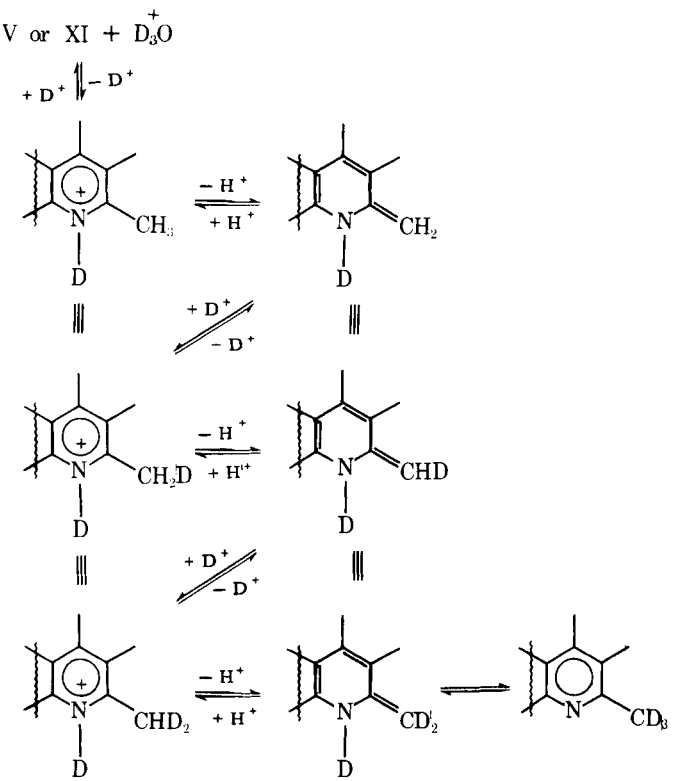
[a] Concentrations are of the starting material. [b] At 40°. [c] At 20°. [d] At 60°.

Table IV—Pseudo-First-Order Rate Constants (Minutes^{-1}) at 60°

Compound	k_H	k_C	k_D
I	1.0×10^{-2} [a]	—	—
II	1.27×10^{-2}	4.33×10^{-3}	1.29×10^{-4}
III	2.28×10^{-3}	—	—
V	—	4.79×10^{-3}	1.20×10^{-4}

[a] Calculated from 20 and 40° data.

resonance, which is attributed to an acid-catalyzed deuterium–protonium exchange. Mass spectral analysis of the products obtained from the hydrolysis of the C-6 methyl compounds, II and V, in the deuterated aqueous medium indicated 33% monodeuteration and 7% dideuteration of the C-6 methyl group (30% overall deuteration by PMR) of II. The



Scheme IV

Table V—Deuteration Rate of the Methyl Group of V and XI [a]

Hours	Percent CH_3 of V Present	Percent CH_3 of XI Present
0	100	100
22.4	67	69
67.7	51	46
115.1	41	40

[a] Measured by disappearance of methyl group, by integration. Concentration of 7.5 mg/ml (pD = 1.9).

extent of deuteration was essentially the same in V.

Whereas the α-methyl protons in 6,7,8-trimethyllumazine (8) and methylpyridine (9) exchange with deuterium, they do not exchange in the presence of mineral acids. A small amount of deuterium was incorporated into the methyl group of 2-picoline when refluxed in deuterium oxide for several days, however. The results of the deuteration[11] rate of the C-6 methyl group of V and XI (Scheme IV) are summarized in Table V. The data demonstrate that the C-6 methyl protons of both compounds exchanged with the deuterium of the solvent at substantially the same rate, which was significantly slower than the rates of hydrolysis (by a factor of 100) and cyclization (by a factor of 30) (Table IV). The mass spectra of the resulting samples confirmed the extent of deuteration determined from their PMR spectra.

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[11] To minimize the dilution of the deuterium content of the solution, the compounds were dissolved in methanol-d_1, which produced immediate exchange of the amine and/or hydroxyl protons. The solution was evaporated and the solid was treated successively twice more in the same manner.