Band-shape Analysis of Electronic Spectra and Study of the Hydrolysis of the Schiff Bases of 5′-Deoxypyridoxal and *n*-Hexylamine in Aqueous and Non-aqueous Media

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The bands of the absorption spectra of the Schiff bases formed between 5′-deoxypyridoxal (DPL) and *n*-hexylamine in various aqueous solvents (water–dioxane mixtures) and non-aqueous media (dioxane, pentanol–dioxane mixtures, pentanol, butan-2-ol, propanol, ethanol and methanol) have been analysed at 25 °C. We have also calculated the hydrolysis rate constants of the different tautomers of the Schiff bases. According to our results, the zwitterionic and enol tautomers are more stable on account of the formation of very stable hydrogen bonds. On the other hand, the ion–dipole form is the most reactive owing to the absence of stabilizing intramolecular hydrogen bonds and the protonated state of its pyridine nitrogen.

The results obtained in this work also show that the relative permittivity of the medium does not significantly affect the tautomeric equilibrium of the chemical species of the Schiff bases of DPL and *n*-hexylamine. Marshall plots reveal that the microscopic p*K* most markedly influenced by solvation is that of the phenolic oxygen, whereas that of the pyridine nitrogen is absolutely insensitive to solvation.

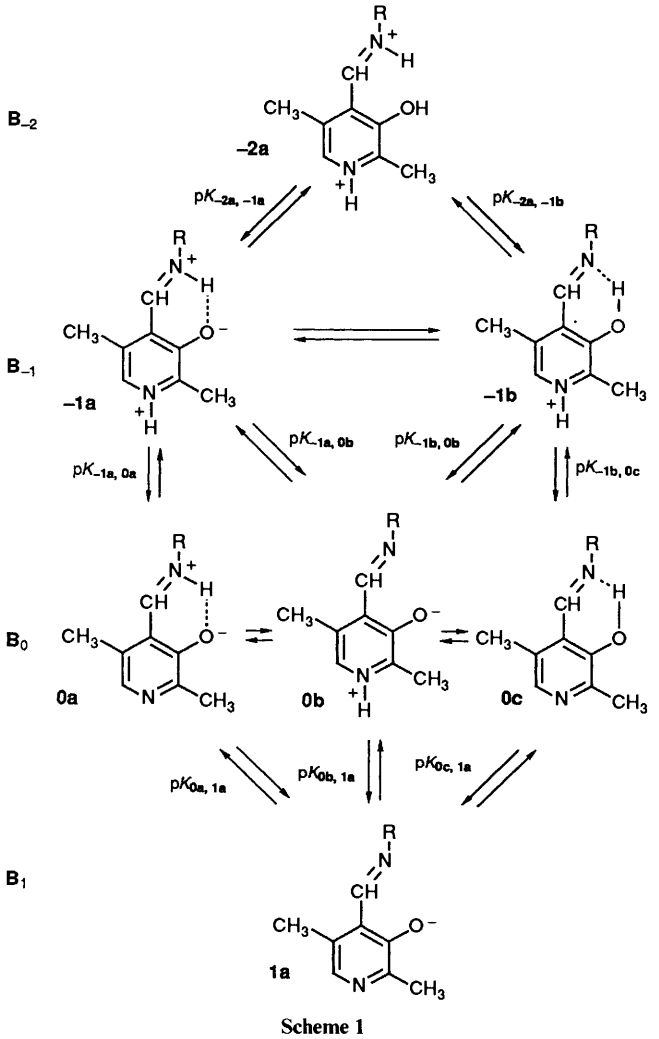
The occurrence of Schiff bases of pyridoxal-5′-phosphate (PLP) as intermediates of a number of biological processes, particularly those involved in amino acid metabolism,[1] has fostered research on the formation and hydrolysis of such Schiff bases.[2–15] Thus, kinetic studies on amines and amino acids in aqueous[2–9] and partly-aqueous media[10–15] have been performed in order to reproduce the PLP environment in some enzymes.[16–18]

A substantial proportion of the information obtained on the state of PLP in enzymes has been gathered by using UV–VIS absorption spectroscopy. Band-shape analysis studies carried out in this area have so far been chiefly concerned with aqueous media[19–28] and only occasionally with non-aqueous media.[15]

5′-Deoxypyridoxal (DPL) is a representative analogue of PLP in as much as it bears the three chemical groups involved in the catalysis of amino acid metabolism (–CH=O, –OH and =N–).[29,30] Also, like the Schiff bases of PLP, those of DPL contain several protonic groups and can occur in different tautomeric forms (see Scheme 1).

In this work we analysed the spectral bands of the Schiff bases of 5′-deoxypryidoxal and *n*-hexylamine at 25 °C in aqueous media (water–dioxane mixtures) and non-aqueous media (dioxane, pentanol–dioxane mixtures, pentanol, butan-2-ol, propanol, ethanol and methanol) in order to elucidate the influence of the polarity and other features of the solvent on the tautomeric equilibrium of the bases.

By determining the tautomeric equilibrium constants and using the already well-known overall hydrolysis constants,[14,15] we obtained the hydrolysis constants of the various tautomers of the Schiff bases studied. The different reactivity of the tautomers may account for the different reactions in which PLP is involved in biological processes. We also calculated the microscopic p*K* values of the Schiff base of DPL and *n*-hexylamine from their macroscopic counterparts and used them to construct Marshall plots,[31] in order to determine the influence of solvation by water molecules.



Scheme 1

Experimental

Materials.—5′-Deoxypyridoxal was synthesized from pyridoxine hydrochloride (Merck) according to the method of Iwata.[32] All other reagents used were Merck reagent-grade chemicals.

Dioxane was purified by distillation over sodium under reflux and subsequent fractional distillation. Freshly distilled dioxane always gave a negative peroxide test (2% KI).

Ethanol was purified over sodium and ethyl succinate under reflux, then distilled and stored after being sieved through 4 Å mesh. Methanol was purified by using the same procedure, and propanol, butan-2-ol and pentanol were dried over calcium hydride and subsequently treated as for ethanol.

Acetate, phosphate and carbonate buffers were used throughout except at high pH values, when NaOH was employed instead.

Methods.—DPL solutions were made in the corresponding buffer or solvent and their exact concentrations were determined by dilution with 0.1 mol dm^{-3} NaOH.[33] The working concentrations used ranged between 1×10^{-5} and 5×10^{-5} mol dm^{-3}.

n-Hexylamine solutions were prepared from *n*-hexylamine hydrochloride or anhydrous *n*-hexylamine by dissolving the appropriate amount of chemical in the corresponding buffer or solvent. The working concentrations used ranged from 5×10^{-5} to 5×10^{-2} mol dm^{-3}.

The ionic strength of the solutions was kept at 0.1 mol dm^{-3} as far as possible. After being mixed, the solutions were allowed to stand for 25 min in order to ensure that equilibrium had been reached. Experimental data were collected over the wavelength range 500–250 nm (ν = 20 000–40 000 cm^{-1}). Absorbances were measured to within 0.001 units. The spectra of the Schiff bases studied in water–dioxane mixtures were recorded at different pH values in the range 4–12.

The pH of the solutions was measured with a Crison pH-meter furnished with Metrohm EA 120 combined electrodes that were pre-calibrated with aqueous buffers at 25 °C. The pH value obtained in every case was corrected by using the following equation:[34]

$$pH_{act} = pH_{meas} - \delta$$

where δ is a pH-independent parameter characteristic of the particular composition of the dioxane–water medium used.

The complete spectra of the solutions were recorded on a Uvikon 940 spectrophotometer furnished with cells of 0.1 cm pathlength. The temperature was kept constant at 25.00 ± 0.05 °C throughout.

The overall reaction between an aldehyde and an amine can be represented by:

$$R^1CHO + R^2NH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R^1\text{–}CH{=}N\text{–}R^2 + H_2O$$

the equilibrium constant of which is given by eqn. (1), where

$$K_{pH} = [B]_e/[P]_e[A]_e \quad (1)$$

$[B]_e$, $[P]_e$ and $[A]_e$ are the equilibrium concentrations of the Schiff base, aldehyde and amine, respectively.

In the wavelength range of interest, the absorption of the mixture formed by the aldehyde and its corresponding aldimine results exclusively from the absorption of these two compounds rather than the free amine present. Therefore, at equilibrium and a given wavelength, the overall absorption of the sample will be given by eqn. (2), where $E_P(\lambda)$ and $E_B(\lambda)$ are the molar

$$A(\lambda) = [P]_e E_P(\lambda) + [B]_e E_B(\lambda) \quad (2)$$

absorptions of the aldehyde and Schiff base, respectively.

The spectra of the Schiff base was obtained by applying a computerized procedure that fits the experimental results to eqn. (2). The concentrations $[P]_e$ and $[B]_e$ were determined from eqn. (1). The E_P and K_{pH} values used for this purpose were either reported elsewhere[4,14,15] or taken from Harris *et al.*[20]

Spectra were deconvoluted into log normal curves by using the method reported by Metzler *et al.*[19] The wavenumber of maximum absorption, the maximum molar absorption, the bandwidth and its skewness are four input parameters required by the computer in each case. The program minimizes the sum of the squares of the deviations and obtains the output data from the best fitting, which allows the area (integrated intensity) of the absorption band of each tautomer to be calculated.

Results and Discussion

Band-shape Analysis.—Fig. 1 shows the spectrum of chemical species $\mathbf{B}_{-1}$ (Scheme 1) of the Schiff base of DPL and *n*-hexylamine, which prevails in moderately acidic aqueous and partly aqueous media. The figure also shows the spectrum broken down into log normal curves by using the method of Metzler.[19] Table 1 lists the values of the characteristic band-shape parameters of the spectra obtained in aqueous and non-aqueous media.

Species $\mathbf{B}_{-1}$ occurs in two tautomeric forms, *viz.* **−1a** and **−1b**, of which the former prevails in polar media, where it yields two absorption bands at 414 and 282 nm. On the other hand, tautomer **−1b** yields a smaller band at 335 nm.[15,21–23,28] As the dioxane content of the medium is increased, the area of the band yielded by the zwitterionic form (**−1a**) decreases while that of the enol form (**−1b**) increases. The decrease in the medium polarity is concomitant with a bathochromic shift in the absorption bands of both tautomers; thus, the band of form **−1a** shifts from 414 to 422 nm and that of form **−1b** from 335 to 344 nm.

Chemical species $\mathbf{B}_0$, which prevails in basic aqueous media and in non-aqueous media, occurs in three tautomeric forms (Scheme 1). The zwitterionic form (**0a**), which is prevalent in aqueous media, yields two absorption bands similar to those of form **−1a**. On the other hand, the enol form (**0c**) prevails in anhydrous dioxane, where it yields an absorption maximum at 340 nm. The tautomeric form **0b** occurs in the lowest proportions in all the solvents assayed and yields an absorption maximum at 353 nm.[23,25,28] As the polarity of the medium is decreased, the area of the band yielded by form **0c** increases and those of forms **0a** and **0b** decrease.

Chemical species $\mathbf{B}_1$, which is prevalent at high pH values, occurs as a single tautomer that yields a band centred at 336 nm, the area of which is independent of the medium polarity.

Tautomerization Ratios and Microscopic Dissociation Constants.—The molar area of each tautomer must be known in order to determine the proportion of each tautomer of the different chemical species. The mole fraction of a tautomer is given by:

$$x_i = a_i/a_i^\circ$$

where a_i is the band area and a_i^0 its molar area. Taking into account that $\Sigma x_i = 1$, then for species $\mathbf{B}_1$ and $\mathbf{B}_0$ we can write eqns. (3) and (4).

$$a_{-1a}/a^\circ_{-1a} + a_{-1b}/a^\circ_{-1b} = 1 \quad (3)$$

$$a_{0a}/a^\circ_{0a} + a_{0b}/a^\circ_{0b} + a_{0c}/a^\circ_{0c} = 1 \quad (4)$$

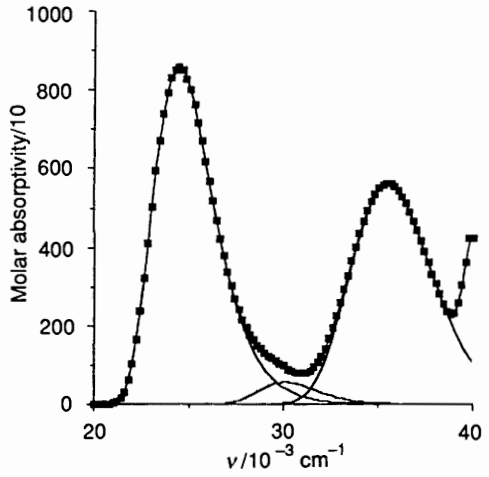


Fig. 1 Spectrum of ionic species $\mathbf{B}_{-1}$ of the Schiff base of n-hexylamine and 5′-deoxypyridoxal fitted with log normal distribution curves

In as much as the molar areas of the tautomeric forms do not depend on the solvent composition,[19,20] they were obtained by applying eqns. (3) and (4) to water–dioxane mixtures (species $\mathbf{B}_{-1}$) and non-aqueous solvents (species $\mathbf{B}_0$), in which only the monoprotonated form $\mathbf{B}_0$ occurs.

The molar areas and mole fractions obtained for the different tautomers are listed in Tables 2–4. The molar areas (Table 2) are similar to those reported by Blazquez *et al.*[35] for the Schiff bases of PLP and *n*-hexylamine and those of PLP bound to the enzyme cytosolic aspartate aminotransferase from pig heart (AATase).[24] As a rule, the values obtained for the tautomers of species $\mathbf{B}_0$ and $\mathbf{B}_1$ are comparable. The differences found, chiefly for species $\mathbf{B}_{-1}$, can be attributed to the presence of a charged phosphate group in the PLP molecule which may alter the intensities of the electron jumps of this species.

The proportion of the zwitterionic tautomer **−1a** decreases from 90% in water to 13% in 70:30 dioxane–water mixtures as a result of the decrease in the medium polarity. The behaviour of species $\mathbf{B}_0$ in partly aqueous media is similar to that of species $\mathbf{B}_{-1}$. The enol tautomer of species $\mathbf{B}_0$ prevails in non-aqueous media (Table 4). We should note that the tautomer proportions obtained in 70:30 dioxane–water mixtures ($\varepsilon = 16$) are similar to those obtained in methanol ($\varepsilon = 34$). Hence the relative permittivity of the medium is not correlated with the stability of the tautomers, which rather depends on other major factors such as the solvation power, acidity and viscosity of the medium. It is worth noting here that tautomer **0b**, the occurrence of which is somewhat more favourable in water, is the one present in the lowest proportions in all the assayed media, so its stability must be determined by the possibility of interacting with the medium in some other way. The occurrence of this form has been confirmed by resonance Raman spectroscopy,[36] and enzyme bound 6-fluoro-PLP presents this dipolar ionic ring structure as well.[37]

The macroscopic pK values of the Schiff base in water–dioxane mixtures (Table 5) were determined according to the method reported by Metzler *et al.*[38,39] On the other hand, the microscopic pK values (Table 5) were readily obtained from their macroscopic counterparts and the mole fractions of the different tautomers.

The macroscopic pK values obtained (pK_{-1B} and pK_{-0B}) are consistent with the microscopic pK values of the prevalent tautomers irrespective of the medium polarity. Thus, pK_{-1B} and pK_{0B} are 6.23 and 11.81, respectively, for aqueous media, while the microscopic $pK_{-1a,0a}$ and $pK_{0a,1a}$ values are 6.13 and 11.71. On the other hand, in 70:30 dioxane–water, pK_{1B} and pK_{0B} are 6.52 and 12.20, and $pK_{-1a,0a}$ and $pK_{0a,1a}$ are 6.52 and 12.03, respectively.

The microscopic $pK_{-1a,0a}$ and $pK_{0a,1a}$ values are formally consistent with those obtained by kinetic methods[3,14] as the wavelengths at which the kinetic experiments were performed coincide with the absorption maxima of the tautomers concerned (415–280 nm).

The behaviour of the microscopic pK values can be assessed on the basis of Marshall's theory.[31] Thus, by plotting the microscopic pK values as a function of the logarithm of the molar concentration of water in the medium one obtains a curve, the plot of which coincides with the difference in the number of solvation water molecules between the protonated and unprotonated tautomeric form. In our case, the deprotonation of the pyridine nitrogen results in a very small change in the number of solvation molecules ($n = 0 \pm 0.5$). On the other hand, the deprotonation of the imine nitrogen results in an increase by *ca.* 1 solvating molecule. However, the deprotonation of the phenolic oxygen increases the number of solvation molecules by as much as 3 ± 1 as a result of the transformation of an uncharged site into a charged one. The above results indicate that the site next to the imine double bond is the one most markedly influenced by solvation, whereas the pyridine ring is scarcely affected by polarity changes and hence by solvation.

Reactivity of the Schiff Base.—Tautomers **−1a** and **0a** prevail in aqueous media. In earlier work we determined the hydrolysis rate constants for the Schiff base of DPL and *n*-hexylamine in an aqueous medium at a wavelength corresponding to the absorption maxima of the two forms (Table 6). By fitting these kinetic constants to a kinetic scheme we calculated the corresponding specific rate constants for each chemical species, which must be assigned to tautomers **−1a** and **0a**.

Tautomer **0a** is the most stable in water ($\log k_2^0 = -0.905$) since the addition of water to the imine double bond must cleave the hydrogen bond to the phenoxide group at position 3, which is very strong. Addition of a proton to form **0a** yields **−1a** ($\log k_2^{-1} = -0.485$), which is considerably more reactive than **0a**, even though it also has a hydrogen bond arising from the protonation of the pyridine nitrogen, which deactivates the aromatic ring to a great extent and hence increases the electrophilic character of the carbon atom in the imine double bond. Only form **1a** occurs in strongly basic media, where it has a very large hydrolysis constant ($\log k_2^1 = 1.93$) resulting from the absence of a hydrogen bond that might stabilize the imine bond as in forms **−1a** and **0a**. The stabilizing effect of the addition of the first proton to the Schiff base has been observed over a wide temperature range in aqueous and low-polarity, partly-aqueous media.[2–14]

In order to determine the reactivity of tautomers **0b** and **0c** of the Schiff base of DPL and *n*-hexylamine we calculated its hydrolysis rate constants for non-aqueous media (Table 6).[15] The experiments involved were performed at a wavelength where both tautomers absorb (345 nm) and the calculation procedure used is described in detail elsewhere.[2,12,15] The non-aqueous media used were chemically similar with the exception of anhydrous dioxane. As a hydrolysis reaction, this is a unimolecular reaction involving the attack of a water molecule to an uncharged imine double bond; hence the reactivity of forms **0b** and **0c** must be virtually independent of the medium polarity. Thus, the hydrolysis constants obtained in aqueous media can be expressed as eqn. (5), where X_{0b} and X_{0c} are the

$$k_2 = X_{0b}k_{0b} + X_{0c}k_{0c} \quad (5)$$

mole fractions of tautomers **0b** and **0c**, and k_{0b} and k_{0c} are their corresponding hydrolysis rate constants.

Table 1 Positions and shapes of absorption bands of Schiff bases (**B**) resolved with log normal distribution curves

Schiff base	Band maximum λ/nm	Height/dm^3 mol^{-1} cm^{-1}	Area/10^{-6} mmol^{-1}
Hex + DPL (water)			
$\mathbf{B}_{-1}$	414	8.58	347.0
	335	0.56	25.1
	282	5.61	301.5
$\mathbf{B}_0$	415	6.62	296.0
	353	0.43	20.0
	340	1.01	49.8
	281	7.77	426.2
Hex + DPL (50:50 dioxane–water, v/v)			
$\mathbf{B}_{-1}$	420	3.05	123.8
	339	4.37	173.4
	281	2.51	134.9
$\mathbf{B}_0$	418	2.41	106.2
	353	0.25	11.7
	339	3.10	150.5
	282	2.71	149.5
Hex + DPL (70:30 dioxane–water, v/v)			
$\mathbf{B}_{-1}$	422	1.23	50.4
	344	5.56	221.8
	280	1.33	71.9
$\mathbf{B}_0$	420	1.03	45.4
	353	0.11	5.1
	339	3.92	190.2
	283	1.77	98.4
Hex + DPL (methanol)			
$\mathbf{B}_0$	418	0.98	42.9
	353	0.16	7.4
	340	3.85	189.5
	282	2.07	114.2
Hex + DPL (ethanol)			
$\mathbf{B}_0$	418	0.69	30.2
	353	0.12	5.6
	340	4.09	201.3
	282	1.41	49.1
Hex + DPL (propanol)			
$\mathbf{B}_0$	419	0.51	22.4
	353	0.10	4.9
	340	4.16	204.6
	283	0.46	57.9
Hex + DPL (isobutyl alcohol)			
$\mathbf{B}_0$	419	0.41	17.9
	353	0.08	3.7
	340	4.21	207.2
	283	0.89	49.1
Hex + DPL (pentanol)			
$\mathbf{B}_0$	419	0.34	14.9
	353	0.07	3.4
	340	4.25	209.0
	283	0.80	41.9
Hex + DPL (25:75 dioxane–pentanol, v/v)			
$\mathbf{B}_0$	420	0.31	13.8
	353	0.05	2.7
	340	4.33	212.3
	283	0.76	41.9
Hex + DPL (50:50 dioxane–pentanol, v/v)			
$\mathbf{B}_0$	420	0.19	8.6
	353	0.04	2.7
	340	4.35	213.4
	283	0.61	33.6
Hex + DPL (75:25 dioxane–pentanol, v/v)			
$\mathbf{B}_0$	420	0.16	7.5
	353	0.03	1.7
	340	4.38	214.9
	283	0.46	25.4
Hex + DPL (anhydrous dioxane)			
$\mathbf{B}_0$	420	0.09	3.9
	340	4.46	219.6
	283	0.10	5.5

Table 2 Molar areas (10^{-6} mmol^{-1}) of the DPL Schiff bases and the PLP Schiff bases (*n*-hexylamine and AATase)[b]

	DPL	PLP[a]	PLP[b]
a°_{-1a}	385	490	400
a°_{-1b}	255	211	280
a°_{0a}	373	353	354
a°_{0b}	335	323	—
a°_{0c}	220	226	—

[a] Taken from ref. 35. [b] Taken from ref. 24.

Table 3 Molar fraction of DPL Schiff bases in dioxane–water mixtures

(v/v)	0%	50%	70%
X_{-1a}	0.90	0.32	0.13
X_{-1b}	0.10	0.68	0.87
X_{0a}	0.79	0.29	0.12
X_{0b}	0.06	0.03	0.01
X_{0c}	0.15	0.68	0.87

Table 4 Molar fraction of DPL Schiff bases in non-aqueous media

	X_{0a}	X_{0b}	X_{0c}
Dioxane	0.005	—	0.995
75:25 Dioxane–water, v/v	0.02	0.005	0.975
50:50 Dioxane–pentanol, v/v	0.025	0.007	0.968
25:75 Dioxane–pentanol, v/v	0.037	0.008	0.955
Pentanol	0.04	0.01	0.95
Butan-2-ol	0.05	0.012	0.938
Propanol	0.06	0.014	0.92
Ethanol	0.08	0.017	0.875
Methanol	0.12	0.022	0.858

Table 5 Macroscopic and microscopic protonation constants of DPL Schiff bases in dioxane–water mixtures

v/v)	0%	50%	70%
pK_{-1b}	6.23	6.39	6.52
pK_{-1b}	11.81	11.95	12.10
$pK_{-1a,0a}$	6.28	6.43	6.55
$pK_{-1a,0b}$	7.40	7.41	7.63
$pK_{-1b,0c}$	6.13	6.39	6.52
$pK_{-1b,0b}$	6.53	7.75	8.46
$pK_{0a,1a}$	11.71	11.41	11.18
$pK_{0b,1a}$	10.59	10.43	10.27
$pK_{0c,1a}$	10.99	10.43	10.27
pK_{1B}[a]	6.23	6.40	6.48
pK_{2B}[a]	11.69	11.38	11.31

[a] Taken from refs. 3 and 14 and obtained from kinetic experiments

Table 6 Rate constants of hydrolysis of DPL and *n*-hexylamine Schiff bases in different media

Medium[a]	log k_2[b]
Dioxane	0.0690
75:25 Dioxane–pentanol	0.1698
50:50 Dioxane–pentanol	0.1698
25:25 Dioxane–pentanol	0.2575
Pentanol	0.2944
Butan-2-ol	0.3327
Propanol	0.3532
Ethanol	0.3892
Methanol	0.5101

[a] Medium: water, $\log k_2^1 = 1.93$; $\log k_2^0 = -0.905$; $\log k_2^{-1} = -0.485$. [b] k_2 and k_2^1 in min^{-1}.

By fitting eqn. (5) we achieved a very good correlation ($r = 0.994$) and the following values: $k_{0b} = 19.9 \pm 0.8$ min^{-1} and $k_{0c} = 0.08 \pm 0.01$ min^{-1}. Accordingly, tautomer **0b** is much more reactive than **0c** since, as noted earlier, the deactivation of the pyridine ring increases the electrophilic character of the carbon atom in the imine double bond and no hydrogen bond that might stabilize such a bond is present. In summary, the different reactivity of the tautomers of the Schiff base of DPL and *n*-hexylamine can be accounted for as follows.

(*a*) Those tautomers stabilized by forming a hydrogen bond between the imine nitrogen and the hydroxy group at position 3 (**0a** and **0c**) will be more stable, depending on whether charge separation is also involved.

(*b*) The protonation of the pyridine nitrogen destabilizes the molecule by increasing the electrophilic character of the imine bond (tautomer **0b**).

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