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Unexpected isomeric equilibrium in pyridoxamine Schiff bases

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ABSTRACT

Pyridoxamine is a vitamin B_6 derivative involved in biological reactions such as transamination, and can also act as inhibitor in protein glycation. In both cases, it has been reported that Schiff base formation between pyridoxamine and carbonyl compounds is the main step. Nevertheless, few studies on the Schiff base formation have been reported to date. In this work, we conduct a comparative study of the reaction of pyridoxamine and 4-picolylamin (a pyridoxamine analog) with various carbonyl compounds including propanal, formaldehyde and pyruvic acid. Based on the results, 4-picolylamin forms a Schiff base as end-product of its reactions with propanal and pyruvic acid, but a carbinolamine with formaldehyde. On the other hand, pyridoxamine forms a Schiff base with the three reagents, but the end-product is in equilibrium with its hemiaminal form, which results from the attack of the phenolate ion of the pyridine ring on the imine carbon. This isomeric equilibrium should be considered in studying reactions involving amine derivatives of vitamin B_6.

1. Introduction

Pyridoxamine 5′-phosphate (PMP) and pyridoxal 5′-phosphate (PLP) are two B_6 vitamers involved in a number of catalytic processes by virtue of their properties as enzyme cofactors [1–6]. Most enzymes catalyzing the reactions involved in amino acid metabolism (*e.g.* transamination [5,7–9], dealdolation [5,10], β-elimination [2,5,11,12]) are PLP-dependent. In such reactions, PLP initially forms a Schiff base with the amino group of the amino acid that subsequently evolves to the end-products [1–6]. PMP is also a cofactor for enzymes such as *CDP-6-deoxy-*L*-threo-*D*-glycero-4-hexulose-3-dehydrase*, which catalyzes the deoxygenation involved in the biosynthesis of 3,6-dideoxyhexoses [13–15].

In transamination reactions (see Scheme 1), the Schiff base of PLP undergoes deprotonation of its C_α atom to give a carbanionic intermediate that is stabilized by resonance [16]. Protonation of the intermediate gives a ketoimine that is hydrolyzed to PMP and the corresponding ketoacid. The transamination reaction finishes with the condensation of another ketoacid with PMP to form a carbinolamine which undergoes dehydration to a new Schiff base (a ketoimine). Finally, the resulting Schiff base releases a new amino acid and PLP is recovered as a result [1–9].

The Schiff bases of PLP are stabilized by resonance between the imino group and pyridine ring [17]. This influences their UV/vis [18–21] and NMR spectra [22–25] and has enabled their kinetic and thermodynamic elucidation [18,20,21,26–29]. In addition, some authors have examined the influence of the temperature [30–32] and polarity of the medium [33,34], among other factors, on their stability.

Notwithstanding their significance to biological processes, the Schiff bases of ketoacids with PMP (or its unphosphorylated analog pyridoxamine, PM) have scarcely been studied. Because of the absence of imine–pyridine conjugation in these bases, their chemical equilibrium is only slightly displaced and spectral changes associated to their formation are minimal [35]. Kubala and Martell determined the equilibrium formation constants of the Schiff bases of PM with various ketoacids [36]. Other authors used media containing metal ions (Zn^{2+}, Al^{3+} or Cu^{2+}) which displaced the equilibrium to the imine form in order to facilitate its spectroscopic detection [3,37,38].

It has been showed PM to be an inhibitor of pathological glycation and lipoxidation processes [39–43] associated to diabetes mellitus [44–48]. One of their mechanisms of action involves the scavenging of carbonyl compounds capable of modifying proteins. Pyridoxamine forms Schiff bases with such compounds [39,41], therefore, an accurate knowledge of the kinetics and mechanism of formation involved can be highly useful with a view to elucidating their mechanism of pharmacological action. Kinetic studies on this subject are still scant; recently, however, our group determined the kinetic constants of formation of the Schiff bases of PM with compounds involved in glycation processes [49].

In this work, we studied the reactions of PM and 4-picolylamin (4-PAM) with pyruvic acid (PVR), propanal (PNL) and formaldehyde (FA) in 0.5 M phosphate buffer at pH 7.4 at 25 °C. Based on NMR and UV/vis spectra, the reactions of 4-PAM with PVR and PNL yield an imine, whereas that with FA gives a carbinolamine.

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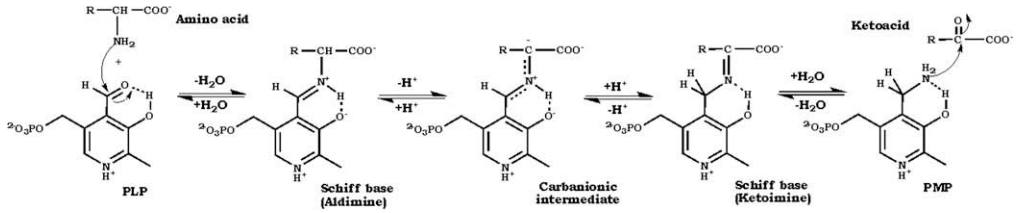


Scheme 1. Overall mechanism for the transamination reaction.

In the other hand our results expose for the first time an isomeric equilibrium of the Schiff bases of PM with a hemiaminal compound formed by attack of the phenolate ion on the imine carbon.

2. Experimental

2.1. Materials

Pyridoxamine (PM), 4-picolylamin (4-PAM), formaldehyde (FA, 30% in water), propanal (PNL), pyruvic acid (PVR), potassium dihydrogen phosphate, sodium cyanoborohydride ($NaCNBH_3$), sodium citrate, isopropylamine, $NiCl_2$ and D_2O (99.9% D) were purchased from Sigma–Aldrich. All solutions were made in Milli-Q water.

2.2. UV/vis spectroscopy

UV/vis absorption spectra were acquired at 25 °C over the wavelength range 220–400 nm, using a *Shimadzu UV-2401* double-beam spectrophotometer furnished with dual-body quartz cells of 8.75 mm path length. The spectrophotometer was used to record the UV/vis spectra for the reaction mixtures of 4-PAM (0.7 mM) or PM (0.1 mM) with PVR, PNL and FA. All mixtures were made in 0.5 M phosphate buffer at pH 7.4 and the nucleophile concentration was kept constant while that of electrophile was gradually increased from 0 to 200 mM.

2.3. NMR spectroscopy

The reaction mixtures of PM and 4-PAM with PVR, PNL and FA were studied from NMR spectra obtained on a *Bruker AMX-300* instrument. Spectra were acquired at 25 °C by using tubes 5 mm in diameter and 3-(trimethylsilyl)-1-propanesulphonic acid (DSS) as internal standard. The reaction mixtures contained a 20 mM concentration of nucleophile (PM, 4-PAM or isopropylamine) and a 200 mM concentration of electrophile (PVR, PNL or FA). All solutions were made in 0.5 M phosphate buffer at pD 7.4 ($pD = -\log[D^+]$) prepared in D_2O; by exception, the mixtures supplied with $NaCNBH_3$ (20 mM) and Ni^{2+} (15 mM) were made in 0.5 M citrate buffer at pD 7.4. Tests were of the one-dimensional (^1H NMR or ^{13}C NMR), polarization transfer (DETP-135) and two-dimensional type (^1H,^{13}C-COSY and ^1H,^{13}C-HMBC [50]).

2.4. Mass spectroscopy (HPLC-MSD)

Mass spectra were obtained by using an *Agilent 1110 Series* HPLC instrument coupled to a mass spectrometer also from the *Agilent 1110 Series*. The mass of each end-product was determined by injecting the reaction mixtures in the flow injection mode in the absence of a chromatographic column. The reaction mixtures contained a 3 mM concentration of nucleophile (PM or 4-PAM) and a 150 mM concentration of electrophile (PNL or FA), and were made in 0.1 M phosphate buffer at pH 7.4. For mass detection, the samples were nebulized and ionized in the electrospray mode, using a nitrogen stream at 350 °C at a flow-rate of 13 ml/min in combination with a pressure of 415.6 kPa and a fragmentation voltage of 100 V. Subsequently, samples were analyzed by using a quadrupole system spanning the *m/z* range 140–800 for positive ions. The mobile phase used consisted of water and 5 mM NH_4OAc buffer at pH 6.0, which as used to trigger the ionization reaction. The analysis flow-rate was 0.3 ml/min.

2.5. Stopped-flow analysis with UV/vis detection (MSFS)

The kinetic study of the reaction between PM and PVR was conducted in 0.5 M phosphate buffer at pH 7.4, using a 1.5 mM solution of PM (a) and a 150 mM solution of PVR (b). Solution (a) was mixed with solution (b) in various ratios ($V_{(a)}$:$V_{(b)}$: 1:1, 1:2, 1:4, 1:10 and 2:1) by using a *Biologic SFM-20* automatic mixer furnished with an *FC-15* quartz cell of 1.5 mm path length that was thermostated at 25 °C. The mixer-cell ensemble had a dead time of 3.7 ms for a flow velocity of 10 ml/ms. The system was coupled to a *J&M Tidas16 256* spectrometer equipped with a 75 W Xe lamp which afforded acquisition of UV/vis spectra (200–700 nm) at each preset reaction time. The irradiation system was furnished with a monochromator in order to avoid photoinduced reactions of PM [51]; this allowed the temporal variation of the absorbance at 321 nm to be monitored without interference.

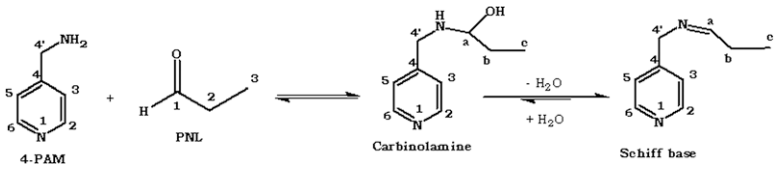
2.6. Kinetic analysis of experimental data

The variation of the absorbance at 321 nm obtained in the reaction between PM and PVR was fitted to its kinetic mechanism by using the software Dynafit (Biokin, Pullman, WA) [52], using a ε_{321} value of 7890 $M^{-1}\,cm^{-1}$ for PM and one of 22.7 $M^{-1}\,cm^{-1}$ for PVR. Additional experiments for the reaction between PM and FA performed by UV/vis spectroscopy at 37 °C, shown that the band at 321 nm disappeared completely at 200 mM of FA. This result led us to assume that the molar absorption coefficient for HE at 321 nm was negligible. The fitting allowed the forward (k_{HE}) and reverse rate constant (k_{-HE}) of formation of HE from the initial reactants at pH 7.4 at 25 °C to be calculated.

2.7. Theoretical methodology

The structures of the Schiff base of PM and FA, and its hemiaminal isomer, were optimized by using the MP2 method [53] in conjunction with doubly diffuse, doubly polarized 6-311++G** basis sets. The effect of solvation was considered by using the continuum method PCM [54]. The UV/vis spectra for the optimized structures were calculated by using the TD-DFT method, which provides an effective way of computing excitation energies by using a time-dependent electrical field [55]. TD-DFT computations were done by using the continuum method PCM and the functional hcth [56] in conjunction with *Dunning's augmented correlation-consistent polarized valence double-zeta* (aug-cc-PVDZ) basis set [57].

All computations were done with the software Gaussian03 [58] as run on a PC and on the HP CP4000 computer of the Supercomputation Center of Catalonia (CESCA).



Scheme 2. Mechanism of the reaction between 4-PAM and PNL.

3. Results and discussion

3.1. Reactions of 4-picolylamin (4-PAM) with PVR, PNL and FA

4-Picolylamin (Scheme 2) is a PM analog with a much more reactive amino group than the parent compound [59,60]. Given the known reactivity between amino groups in aliphatic chains and carbonyl groups, the reactions between 4-PAM and carbonyl compounds should yield a Schiff base.

The reactions of 4-PAM with PVR, PNL and FA were examined by UV/vis spectroscopy. No spectral changes were observed on increasing the concentration of carbonyl compound with respect to 4-PAM.

Scheme 2 shows the mechanism for the reaction between 4-PAM and PNL. The ^{1}H NMR spectrum for the reaction mixture exhibited a singlet at 9.30 ppm not detected in the spectra for the pure reactants (see Fig. S1 in supplementary material). This new signal was assigned to H-C(a) in the resulting Schiff base since its position fell within the typical shift range for imine protons [50,61]. As with H-C(1) in PNL, no splitting of the signal by effect of coupling to the protons in the vicinal C(b) atom was observed. This was a result of the small coupling constants of imine and aldehyde protons with vicinal protons on a saturated carbon [50]. Also, no specific signals for a carbinolamine were detected. The analysis of the reaction mixture by LC-MSD revealed the major signal in the mass spectrum to be that at m/z = 149.3, which is consistent with the value for the $[M+H]^+$ ion in the resulting Schiff base. Based on the experimental results, the major product of the reaction between 4-PAM and PNL is a Schiff base.

The reaction between 4-PAM and PVR, the mechanism of which is depicted in Scheme 3, was also examined by NMR spectroscopy. The ^{1}H NMR spectrum exhibited no salient change with respect to those for the pure reactants. On the other hand, the ^{13}C NMR spectrum exhibited a new, small signal at 171 ppm corresponding to a quaternary carbon; this shift value falls within the typical range for disubstituted imine carbons [50,62], and provides evidence that the reaction product is a Schiff base.

The NMR technique was also used to study the reaction between 4-PAM and FA, the mechanism of which is depicted in Scheme 4. Table 1 shows the most salient ^{1}H and ^{13}C NMR shifts for the products. A comparison of NMR signals reveals a slight shift in the peaks for the aromatic protons with respect to 4-PAM. By contrast, the signal for the C(4′) protons was shifted 0.5 ppm upfield. The analysis of the reaction mixture by ^{13}C NMR spectroscopy revealed a downfield shift of 13 ppm in the signal for C(4′) and a new signal at 74.7 ppm that was assigned to a $-CH_2$ group – as determined with the DEPT-135 technique. The NMR spectra contained no signal for the imine protons, which indicates that the reaction between 4-PAM and FA is displaced to the carbinolamine. This result was confirmed by using LC-MSD to monitor the reaction mixture. The resulting spectrum exhibited a major signal at m/z = 121.0, which is consistent with the value for the $[M-H_2O+H]^+$ ion in the carbinolamine.

The previous results are consistent with those of Kallen and coworkers [63,64], who showed the carbinolamines formed between amino groups and FA to be scarcely displaced to the corresponding Schiff bases by effect of their high stability. However, there is evidence of the formation of such bases; thus, the addition of $NaCNBH_3$ (a selective reductant for imino group leaving intact carbonyl groups at neutral pH [65]) to the reaction mixtures leads to the formation of *N*-methyl derivatives [66].

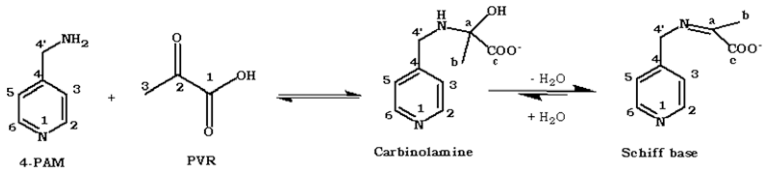
3.2. Reactions of pyridoxamine (PM) with PVR, PNL and FA

The fact that 4-PAM is reactive towards the previous electrophilic compounds suggests that their reactions with PM should involve the initial formation of carbinolamine (one of a high stability with FA, but undergoing dehydration to a Schiff base in the case of PNL and PVR).

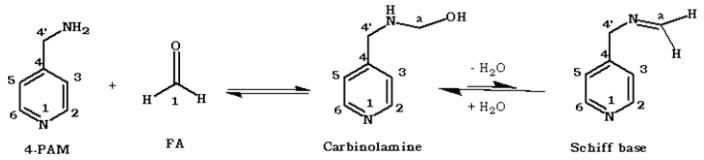
Unlike 4-PAM, the reaction of PM with PNL caused UV/vis spectral changes upon increasing the concentration of the latter relative to the former. The reaction decreased the intensity of the bands at 321 and 249 nm – which are due to the major tautomer of PM at neutral pH [67] – and, simultaneously, increased that of a new band appearing at 280 nm.

The ^{1}H NMR spectrum for the reaction mixture of PM and PNL (Fig. 1) exhibited a decreased area for the peaks associated to H-C(2′), H-C(4′) and H-C(6) in PM. Also, it exhibited new signals at 9.30, 7.95, 4.13 and 2.43 ppm. Gansow and Holm used ^{1}H NMR to study Schiff bases of PM and found the peak for H-C(6) to be that undergoing the greatest changes relative to PM −0.3 ppm downfield, when the shift in the presence of metal ion was only 0.1 ppm [68,69].

Our ^{1}H NMR results for the reaction between PM and PNL indicate that the signal at 9.30 ppm in Fig. 1 corresponds to the imine proton in the resulting Schiff base. The area under this peak is much smaller than that under the peak at 7.95 ppm, which *Gansow* and *Holm* assigned to H-C(6) in the Schiff bases of PM [68,69]. This clearly shows that the signal at 7.95 ppm cannot be associated to a Schiff base. The LC-MSD analysis of the reaction mixture revealed that the major signal, m/z = 209.1, was that for the $[M+H]^+$ ion in a compound of the same molecular weight as the expected Schiff base.



Scheme 3. Mechanism of the reaction between 4-PAM and PVR.



Scheme 4. Mechanism of the reaction between 4-PAM and FA.

Table 1
^{1}H and ^{13}C NMR data of 4-PAM, FA and its reaction mixture (20 mM of 4-PAM and 200 mM of FA) in 0.5 M phosphate buffer at pD 7.4. At 300 MHz; δ in ppm.

	NMR spectroscopic data
4-PAM	^{1}H: 8.58 (d, 1H, $^{3}J_{H2\text{-}H3}$ = 6.2 Hz, H-C(2)); 7.51 (d, 1H, H-C(3)); 4.26 (s, 2H, H-C(4′))
	^{13}C: 151.9 C(2); 146.2 C(4); 126.2 C(3); 44.4 C(4′)
FA	^{1}H: 4.82(s, 2H, H-C(1)) – hydrated form
	^{13}C: 84.4 C(1) – hydrated form
4-PAM + FA	^{1}H: 8.42 (d, 1H, $^{3}J_{H2\text{-}H3}$ = 6.0 Hz, H-C(2)); 7.37 (d, 1H, H-C(3)); 3.74 (s, 2H, H-C(4′)); nd* (s, 2H, H-C(a))
	^{13}C: 151.0 C(2); 146.1 C(4); 127.3 C(3); 74.7 C(a); 57.4 C(4′)

* The spectroscopic data corresponding to H-C(a) could not be determined since two-dimensional NMR experiments showed that it was under D_2O peak.

Fig. 2 illustrates the UV/vis study of the reaction between PM and FA. Raising the FA concentration with respect to PM induced the same spectral changes observed in the reaction between PM and PNL; the changes, however, were even more marked.

The ^{1}H NMR spectrum for the reaction mixture of 20 mM PM and 200 mM FA exhibited markedly decreased signals for PM in addition to new ones. The signal for H-C(6) in PM was strongly reduced, consistent with the appearance of a new, strong signal at 7.95 ppm. The spectrum additionally contained two new, weak signals at 8.45 and 7.53 ppm (see Fig. S2 in supplementary material). The latter was assigned to H-C(6) in the carbinolamine and the former, by analogy with the ^{1}H NMR results for an isopropylamine-FA mixture, to H-C(a) in the resulting Schiff base (see Scheme 5).

Table 2 lists the ^{13}C chemical shifts for PM and its reaction mixture with FA, as well as those for the coupled protons. Carbon signals were assigned on the basis of ^{1}H,^{13}C-COSY, ^{1}H,^{13}C-HMBC and DEPT-135 experiments from corresponding ^{13}C NMR spectra (see Fig S3 in supplementary material).

The ^{13}C NMR spectrum for the reaction mixture exhibited a clear shift in the signals for the pyridine carbons relative to PM in addition to a strong shift in the C(4′) signal with respect to PM and three new signals for $-CH_2$ groups. The signal at 88.4 ppm was assigned to oxydimethanol formed by polymerization of FA [70]. The signal at 83.3 ppm was correlated with those observed at 152.0 and 46.5 ppm, whereas that at 80.0 ppm – which was only observed at an FA concentration above 0.15 M – was correlated to those observed at 83.3 and 46.5 ppm.

Based on the NMR results, the end-product of the reaction of PM with FA results from the formation of a hemiaminal ring condensed with the pyridine via the mechanism depicted in Scheme 5. The nucleophilic attack of the amino group in PM on the carbonyl group of FA produces a carbinolamine. Despite their high stability, FA carbinolamines are in equilibrium with their Schiff bases [71]; this facilitates attack of the phenolate ion on the imine carbon to form its hemiaminal isomer (HE). The obtainment of HE as the major product was confirmed by mass spectrometry; in fact, the major signal in the spectrum was that at m/z = 181.1, which is consistent with the value for the $[M+H]^{+}$ ion in HE. In addition, the NMR results revealed that an FA concentration above 0.15 M in the reaction mixture facilitated attack of the amino group on another FA molecule to form a tertiary amine. This is consistent with previous results of Verardo et al., who found carbinolamines from FA to be able to incorporate a second FA molecule in order to form the corresponding *N*-dihydroxymethyl derivatives [71], the addition leading to the formation of a new carbinolamine (HE-C(a′) in Table 2).

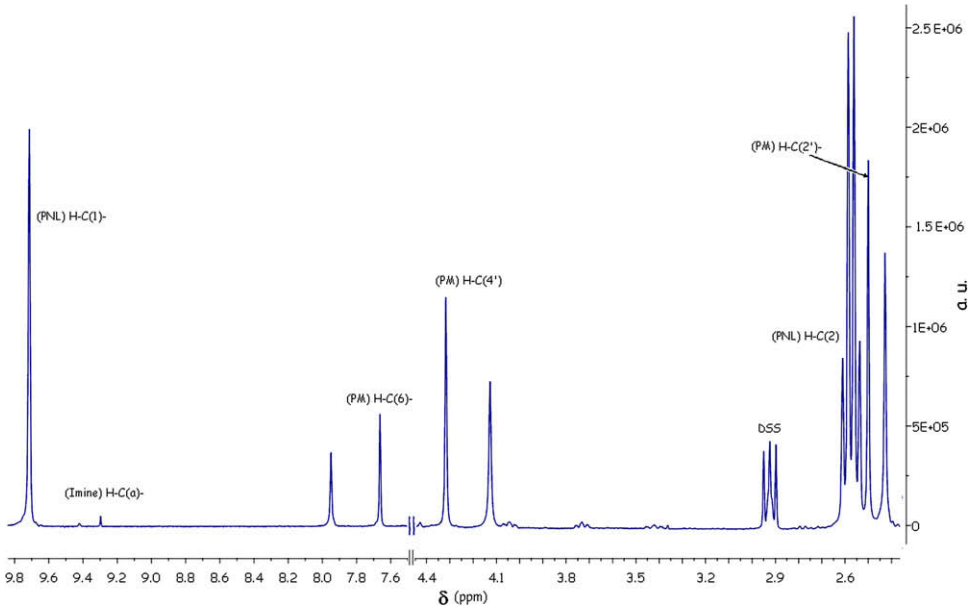


Fig. 1. ^{1}H NMR spectrum for a reaction mixture containing PM (20 mM) and PNL (200 mM) at pD 7.4 at 25 °C.

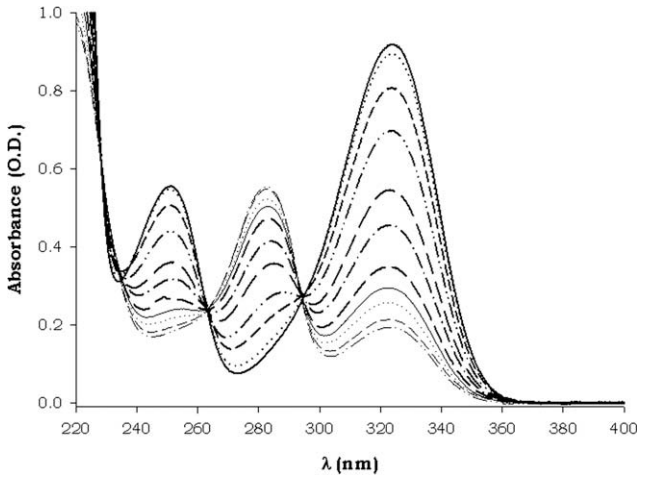


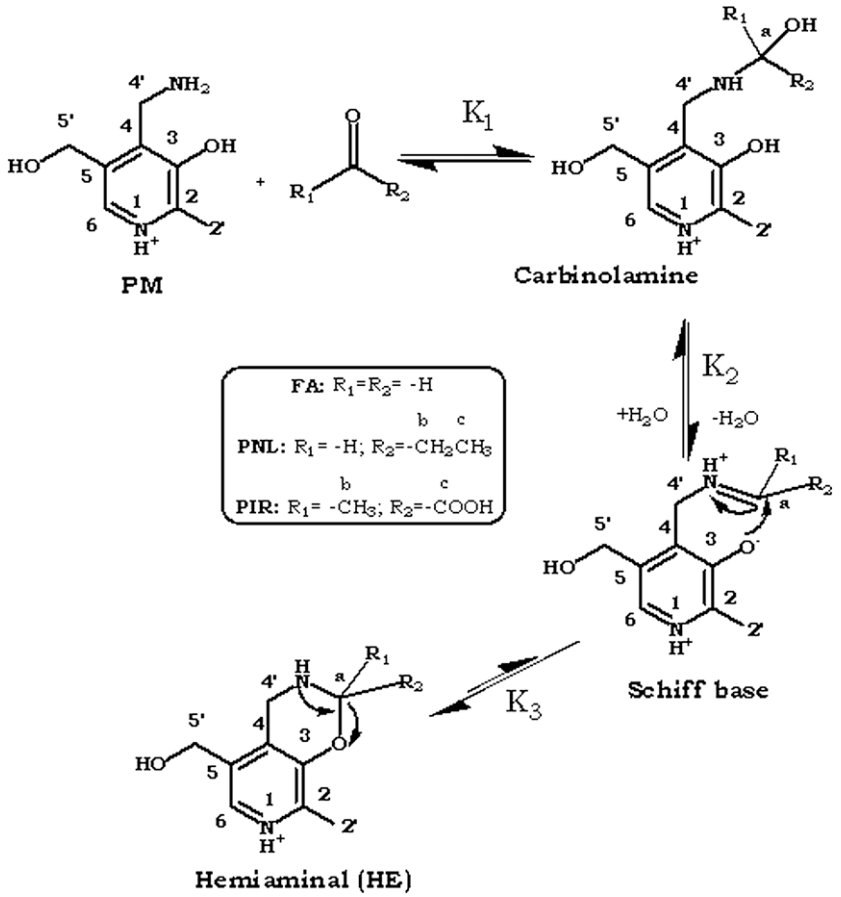
Fig. 2. Variation of the UV/vis spectrum for PM (0.1 mM) with the concentration of FA in the medium (pH 7.4 at 25 °C): [FA] = 0 mM (—); 0.5 mM (·····); 2.5 mM (- - - -); 5 mM (··–··–); 10 mM (– – –); 15 mM (–·–·–); 25 mM (— — —); 35 mM (—); 50 mM (·····); 100 mM (-----); 200 mM (··–··–).

The attack of the phenolate ion on the imine carbon and its subsequent methylation alters the electron distribution on the pyridine ring. This accounts for the strong UV/vis and NMR spectral changes observed in the reactions of PM with PNL and FA. Also, the absence of a phenolate group on pyridine ring of 4-PAM accounts for the lack of spectral changes in its two reactions.

The fact that the UV/vis spectral changes were due to the formation of HE was confirmed in theoretical terms. To this end, we used the MP2 method with the 6-311++G** basis set to optimize the structure of HE showed in Scheme 5. The effect of solvation was simulated by including the continuum method PCM in the optimization process. Then, the UV/vis spectrum for HE was computed by using the TD-DFT method in conjunction with the functional hcth and the aug-cc-PVDZ basis set (see Section 2). Based on the results, HE exhibits a single electron transition over the range 220–450 nm; the resulting band is centered at 280 nm and has a molar absorption coefficient of 6500 M^{-1} cm^{-1}, both of which are consistent with experimental evidence (λ_{max} = 280 nm, ε = 6300 ± 250 M^{-1} cm^{-1}, determined at 37 °C where the reaction was completely displaced).

The addition of $NaCNBH_3$ – which selectively reduces imino groups at neutral pH [65] – and Ni^{2+} – which prevents the formation of cyanomethyl derivatives [66] – to the reaction mixtures of PM with PNL and FA revealed the presence of an equilibrium between HE and the Schiff base. Addition of the reductant led to a spectrum similar to that for PM which was assigned to the reduced form of the Schiff base. The reaction mixtures of PM with FA and PNL in the presence of the reductant were also examined by NMR and mass spectroscopies, which revealed the formation of an *N*-dimethyl and *N*-propyl derivative of PM in its reaction with FA and PNL, respectively.

The UV/vis spectroscopic study of the reaction between PM and PVR exposed the same spectral changes – albeit to a much lesser extent – on increasing the concentration of PVR with respect to PM as those previously found in the reactions with PNL and FA. These results are consistent with those of Kubala and Martell in

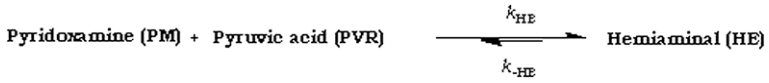


Scheme 5. Mechanism of the reactions of PM with FA, PNL and PVR.

Table 2
^{1}H and ^{13}C NMR data of PM, FA and its reaction mixture (20 mM of PM and 200 mM of FA) in 0.5 M phosphate buffer at pD 7.4. At 300 MHz; δ in ppm.

	NMR spectroscopic data
PM	^{1}H: 7.64 (s, 1H, H-C(6)); 4.73 (s, 2H, H-C(5′)); 4.31 (s, 2H, H-C(4′)); 2.48 (s, 3H, H-C(2′)) ^{13}C: 165.5 C(3); 147.5 C(5); 138.5 C(4); 135.2 C(2); 126.1 C(6); 61.7 C(5′); 38.9 C(4′); 17.8 C(2′)
FA	^{1}H: 4.82(s, 2H, H-C(1)) – hydrated form ^{13}C: 84.4 C(1) – hydrated form
PM + FA	^{1}H: 7.95 (s, 1H, H-C(6)); 5.08 (s, 2H, H-C(a)); 4.62 (s, 2H, H-C(5′)); 4.59 (s, 2H, H-C(a′))*; 4.25 (s, 2H, H-C(4′)); 2.40 (s, 3H, H-C(2′)) ^{13}C: 152.0 C(3); 148.7 C(5); 139.6 C(6); 134.2 C(2); 133.2 C(4); 83.3 C(a); 80.0 C(a′)*; 61.0 C(5′); 46.5 C(4′); 19.2 C(2′)

* Only was detected when [FA] >0.15 M.



Scheme 6. Mechanism of formation of HE by reaction of PM with PVR.

their determination of the equilibrium constants of formation of ketoimines between PM and various ketoacids [36]. ^{1}H NMR study of the reaction mixture clearly exposed the presence of a singlet at 7.95 ppm typical of the hemiaminal isomer of the Schiff base. Accordingly, the Schiff bases of PM with ketoacids must also be in isomeric equilibrium with their HE forms as shown in Scheme 5.

The attack of the phenolate ion on the imine carbon of the Schiff base was previously suggested by other authors. In 2002, Voziyan et al. isolated the end-products of the reactions of PM with glyoxal and glycolaldehyde, which formed via a mechanism involving the attack of the phenolate ion on the imine carbon [41]. In previous work, our group sought to elucidate whether the Schiff base formed between PM and glucose evolved via a glucosamine – by cyclization of a glucidic Schiff base [72] – or by attack of the phenolate ion on the imine carbon. The UV/vis spectrum obtained 70 s after the reaction product was isolated contained a band at 280 nm that disappeared as one centered at 321 nm appeared [73]. This result confirms that the isomerization of the Schiff bases of PM takes place via a faster equilibrium than that of glucidic Schiff bases with their glucosamine forms.

3.3. Thermodynamic study of the reactions of pyridoxamine (PM) with PVR, PNL and FA. Kinetic study of the reaction with PVR

Integrating the signals at 8.45, 7.95, 7.64 and 7.53 ppm in the ^{1}H NMR spectrum for the reaction mixture of PM and FA allowed the equilibrium constants shown in Scheme 5 to be determined, namely: $K_1 = (27 \pm 3)\ M^{-1}$, $K_2 = 0.063 \pm 0.005$ *y* $K_3 = 78 \pm 8$, all at pD 7.4 at 25 °C.

Also, integrating the peaks in the ^{1}H NMR spectrum for the reaction mixture of PM with PNL (Fig. 1) allowed us to determine the isomerization constant between the imine and HE (K_3 in Scheme 5): $K_3 = 17 \pm 2$ at pD 7.4 at 25 °C. Also, integrating the signal for proton H-C(6) in residual PM provided the equilibrium formation constant for the Schiff base between PM and PNL: $K_{BS} = K_1 \cdot K_2 = (0.26 \pm 0.03)\ M^{-1}$.

The ^{1}H NMR study of the reaction between PM and PVR allowed us to calculate the equilibrium constant between the reactants and HE: $K_{HE} = K_1 \cdot K_2 \cdot K_3 = (0.32 \pm 0.06)\ M^{-1}$ at pD 7.4 at 25 °C.

The increased stability of the hemiaminal isomer relative to the imine isomer apparent from the thermodynamic results was confirmed by theoretical calculations on both FA structures (see Scheme 5) as optimized by using the MP2 method in conjunction with 6-311++G** basis sets. The effect of solvation was simulated by including the continuum method PCM in the optimization process. Based on the results, the hemiaminal isomer was 8.3 kcal/mol more stable than the imine isomer.

The kinetics of the reaction between PM and PVR was studied at pH 7.4 at 25 °C. The variation of the absorbance at 321 nm of the reaction mixture as a function of time (see Fig. S4) was fitted to Scheme 6 by using the software Dynafit [52]. The results were fitted by using the molar absorption coefficient for each species at pH 7.4 (see under Experimental). The fitting provided a k_{HE} value of $(2.4 \pm 0.3) \times 10^2\ M^{-1}\ h^{-1}$ and a k_{-HE} value of $(3.0 \pm 0.2) \times 10^2\ h^{-1}$, both at pH 7.4 at 25 °C. These values allowed the equilibrium constant, K_{HE}, to be determined: $(0.80 \pm 0.15)\ M^{-1}$, similar to that determined by ^{1}H NMR spectroscopy. The K_{HE} value provided by the spectroscopic measurements is consistent with that obtained by Kubala and Martell for the reaction between PM and PVR, which was ascribed to the formation of a Schiff base [36].

4. Conclusions

The results obtained in this work clearly show that the reaction between 4-PAM and FA yields a carbinolamine as major end-product. In the reactions with PNL and PVR, however, the carbinolamine undergoes dehydration to a Schiff base as end-product. The reactions of PM with the carbonyl compounds studied lead to a hemiaminal condensed to the pyridine ring. This compound is the result of the attack of the phenolate at position 3 on the pyridine ring in PM on the imino group in the Schiff base. The formation of the hemiaminal is favoured over that of the Schiff base; therefore, the presence of this isomeric equilibrium should be considered in studying reactions involving amine derivatives of vitamin B_6. The significance of the formation of HE in biological system is supported by the results of Metzler et al., who found methylation of the phenolate group in B_6 vitamers to completely inhibit their non-enzymatic transamination [7].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2008.11.002.

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