IRON CHELATORS OF THE PYRIDOXAL 2-PYRIDYL HYDRAZONE CLASS. PART III.[1] IONISATION AND CONFORMATIONAL CHARACTERISTICS OF THE LIGANDS

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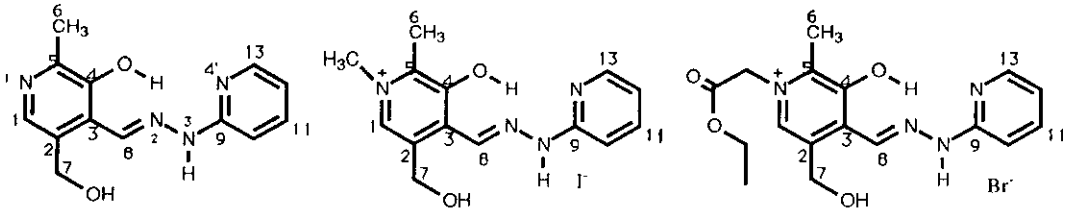
Abstract - pKa values of three biologically active iron chelators: pyridoxal 2-pyridyl hydrazone (PPH), 1-[*N*-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazineiodide (MPH), 1-[*N*-ethoxycarbonylmethylpyridoxylidenium]-2-[2'-pyridyl]hydrazine bromide (EPH) have been determined by a combination of *ab initio* calculations and pH-dependence of ^{13}C nmr spectroscopy. In conformity with pyridoxal isonicotinoyl hydrazone (PIH), all ligands included in this study the pKa values invariably increase in the ordering: pyridinium protonation < pyridoxylidenium protonation < phenolate protonation < amine-hydrazone protonation < alkoxide protonation. Identical ordering was obtained by *ab initio* calculations, based on STO-3G set. Mulliken population analysis indicates that the conformer of the lowest energy of PPH, (I), contains an internal 6-membered-ring H-bond. Rotation about C^3 - C^8 bond in (I), to yield conformer (IV), requires 8.8 kcal / mol, whereas its internal H-bonding (I → II) accounts for 5.8 kcal / mol Protonation of (I) lowers significantly energies both of I → V (6.5 kcal), and I → VI (2.5 kcal) transitions.

Dedicated to Professor Rolf Huisgen on the occasion of his 75th birthday in appreciation for his excellence in Science and devotion to human values

In Parts I[1a] and II[1b] we reported the synthesis, and the single-crystal X-ray analysis, of PPH and MPH, representatives of a new class of orally-active iron chelators capable of removing efficiently toxic accumulation of iron surpluses from iron overloaded erythrocytes (pathological heamoglobins),[2] and inhibiting remarkably the growth *in vitro* of chloroquine-resistant species of Plasmodium falciparum (FCR-3),[3] the main causative agent of human malaria In analogy to pyridoxal[4,5] and PIH analogues,[6,7] the three ligands: PPH, MPH, and EPH (Scheme I), are presumed to exist in aqueous media as at least four distinctly different forms: positively charged (H_4L^{2+}, H_3L^+), neutral (H_2L), and deprotonated (HL^-, HL^{2-}) species (Scheme II). Each of these can react with iron ions differently to yield iron-complexes which differ from each other in composition, stability, passage through membranes, and redox potentials. These associate metal coordination and biological properties with the acidity of the cell compartments in which it occurs. This argument underlines the importance of determining the pKa values of the foregoing biologically-active chelating agents. This aim could be attained by a variety of methods, such as pH-dependence of carbon-13 nuclear magnetic resonance spectroscopy (^{13}C nmr),[8-12] and potentiometric titrations.[6] The pH-dependence ^{13}C nmr technique proved highly useful in identifying chemical shifts which fall in a narrow region of the

spectrum, and in estimating the stability constants, K, hence to assign the pKa values produced in another study using glass electrode potentiometry.[13] However, the assignment of these values to the various ionizable sites in the ligands could not be made with confidence by comparison with its components: pyridoxal and pyridylhydrazine,[14] nor with PIH.[6,7] Caution is necessary since theoretical calculations, given in the sequence, indicate that PPH loses its pyridoxal ring nitrogen proton first, prior to its phenolic proton. To avoid ambiguities, we adopted, in addition, a theoretical approach, using *ab initio* calculations for estimating proton binding-energies, and the Mulliken population analysis[15] for computing distribution of electronic charges at the various ionizable sites in PPH. The pH-dependence ^{13}C nmr technique was applied in spite of difficulties due to low solubilities of the ligands at pH > 9. In addition, the energies of conformational transitions, implying obliteration of internal H-bonding (I → II, and I → IV), and protonation of the pyridoxal ring-nitrogen, were computed (Scheme III).

Scheme I



PPH **MPH** **EPH**

EXPERIMENTAL

Chelating Agents. - PPH (hydrochloride: mp 298°C; free base: mp 288°C),[1a] MPH (1:1-ligand:MeOH: mp 246°C; free ligand: mp 235-6°C),[1b] were prepared according to literature.1-[*N*-ethoxycarbonylmethylpyri - doxylidenium]-2-[2'-pyridyl]hydrazine bromide (EPH), mp 204°C (from MeOH),[16] was prepared (81%) by the method of Sarel *et al.* [16,17] Uv (MeOH, c 5.10^{-6}M) λ_{max} 651 mμ (log ε 1.55), 407.7 (4.41), 242,5 (4.13), 205.4 (4.20). Ms (EI) m/z : 258 {M^+ - ($BrCH_2COOC_2H_5$)}, 108 {$C_6H_8N_2^+$, base-peak}.

Carbon-13 nuclear magnetic resonance spectra were taken on Varian VXR 300S -300 spectrometer operating at 75.43 MHz in the Fourier Transform mode, using a deuterium lock. The spectra were recorded in H_2O + (10% D_2O) at concentrations between 0.2 and 0.5 M in 10 mm tubes. The pH of the samples were adjusted by adding conc. solutions of either HNO_3 or NaOH. Chemical shifts (δ) were recorded in parts per million relative to an external capillary of dioxane; these values were adjusted to the tetramethylsilane (TMS) scale by adding 67.3 ppm to the observed shift. All measurements were carried out at ambient temperature. The results are presented in Tables **1**, **2**, and **3**.

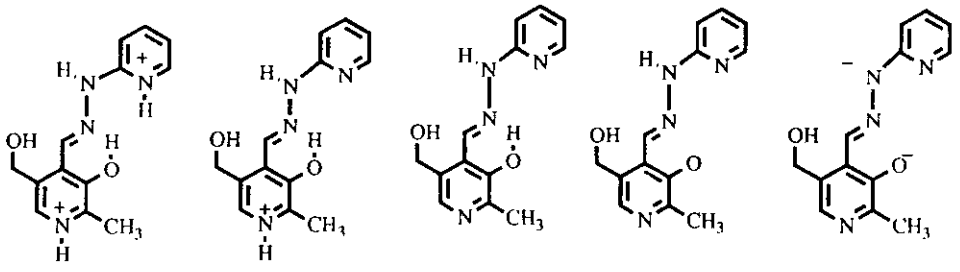
SCF CALCULATIONS

Ab initio calculations were based on STO-3G set [18] Full optimization was performed for the unprotonated species, HL^-, of PPH. For each computation of proton-binding, the optimization was confined to site position of binding. The results of Mulliken population analysis[15] and its protonated species, H_3I^+, were

calculated by CNDO / 2,[19] and validated by comparison with data put out by *ab initio* calculations. The results presented in Table 4 were obtained from two different modes of calculation. Essentially, they are identical. The results from Mulliken population analysis of neutral (H_2L), and positivrly charged (H_3L^+) forms are assembled in Table 5. All calculations were performed by use of IBM RISC / 6000 computer of The Hebrew University of Jerusalem.

Scheme II

pH-Dependence Species of PPH



H_4L^{2+} H_3L^+ H_2L HL^- L^{2-}

RESULTS AND DISCUSSION

The ^{13}C chemical shifts (δ) in PPH, MPH, and EPH were derived from comparisons of spectra of pyridoxal[8,9] and pyridine [12] The identification and assignment of carbon resonances of the pyridoxal and pyridine rings (which fall in a narrow region of the spectrum) was achieved by comparisons of corresponding spectra over the pH range between 1.5 and 12.5, in spite of difficulties due to low solubilities of the neutral forms of the ligands {H_2L} at pH ~ 8. To avoid possible position interchanges in resonance assignments, the additive theory of Harruff[9] and Rainer[10] which will be elaborated in the following, has been invoked. Accordingly, if, for the sake of simplicity, we assume that each of the ligands exists in the aqueous medium as three prototropic species: H_4L^{2+}, H_3L^+, and H_2L, the observed chemical shifts, $\mathbf{S}_{ob}$, can be viewed as a sum of three fractional chemical shifts, s_1, s_2, and s_3 following equation 1:

$$S_{ob} = \chi_1 s_1 + \chi_2 s_2 + \chi_3 s_3 \qquad \text{(equation 1)}$$

Pyridoxal prototropic behaviour has been thoroughly analyzed, and this molecule is known to be present in aqueous media under at least four pH-dependent structures.[4,15] The three ligands, PPH, MPH, and EPH exist, therefore, in aqueous media as several pH-dependent species (see Scheme II). Each of the ligands would, therefore, correspond to a case described in equations **2** - **5**.

$H_4L^{2+} \rightarrow H_3L^+ + H^\pm$	$\mathbf{K}_1 = [H_3L^+][H^+] / [H_4L^{2+}]$	(equation **2**)
$H_3L^+ \rightarrow H_2L + H^+$	$\mathbf{K}_2 = [H_2L][H^+] / [H_3L^+]$	(equation **3**)
$H_2L \rightarrow HL^- + H^+$	$\mathbf{K}_3 = [HL^-][H^+] / [H_2L]$	(equation **4**)
$HL^- \rightarrow L^{2-} + H^+$	$\mathbf{K}_4 = [L^{2-}][H^+] / [HL^-]$	(equation **5**)

$[H_2L]$, $[H_3L^+]$, and $[H_4L^{2+}]$, are the respective concentrations of the neutral, the mono-, and the di-protonated species of the ligand; $[HL^-]$, and $[L^{2-}]$, are the respective concentrations of the mono-, and the di-deprotonated species; $[H^+]$ is the proton concentration which could be estimated experimentally from the pH measurements The equilibrium constants, K_1- K_4, could be estimated correspondingly from equations **2** - **5**.

Mole fractions, χ_1, χ_2, and χ_3, can be replaced by equilibrium constant terms, $\mathbf{K}_{1-4}$, and thus, equations **2 - 5** become: **6**, **7**, and **8**, respectively, as below :

$$\chi_1 = \mathbf{K}_1[H+] / 1 + \mathbf{K}_1[H^+] + \mathbf{K}_2[H^+]^2 \quad \text{(equation 6)}$$

$$\chi_2 = \mathbf{K}_2[H^+]^2 / 1 + \mathbf{K}_1[H^+] + \mathbf{K}_2[H^+]^2 \quad \text{(equation 7)}$$

$$\chi_3 = 1 / 1 + \mathbf{K}_1[H^+] + \mathbf{K}_2[H^+]^2 \quad \text{(equation 8)}$$

Insertion appropriately equations **6 - 8**, into equ. **1**, yields equ. **9**:

$$\mathbf{S}_{ob} = \{s_1\mathbf{K}_1[H^+] + s_2\mathbf{K}_2[H^+]^2 + s_3\} / \{1 + \mathbf{K}_1[H^+] + \mathbf{K}_2[H^+]^2\} \quad \text{(equation 9)}$$

Equation **9** could then be solved with the aid of pH-dependence ^{13}C nmr technique, which, in turn, allows to produce both the desired stability constants, $\mathbf{K}_{1-4}$, and, the chemical shift values, **S**. Moreover, it made it possible to assign with confidence the carbon resonances in PPH, MPH, and EPH, which fall in a narrow range of the spectra (see Tables **1 - 3**).

Study of species distribution over pH range 1.5-12 5 has shown[9] that at pH 1.70, both acylhydrazones (PIH and its analogues), and aryl-hydrazones (PPH, MPH, and EPH) exist in the di-protonated form, H_4L^{2+}. The deprotonated forms, H_3L^+, H_2L, HL^-, and L^{2-}, emerge successively on pH increase, the preponderance of which was observed to be structure-dependent.. Thus, at pH 3.7, PPH exists predominantly in the H_3L^+ form , and in the H_2L form, at pH 5.70.[9] The pyridoxal-betaine analogues, MPH and EPH, by contrast, were found to exist predominantly in the neutral form, H_2L, by 0.7 pH units lower than PIH, or PPH (pH 5.0). Moreover, MPH, and EPH, exist as 35%:65%-H_2L:HL^- mixture, at the physiological pH (7.2), but PPH-PIH present at same pH as a 65%:35%-H_2L:HL^- mixture. This variation is attributed to a 10% reduction in electronic density at pyridoxal ring-nitrogen (see Table **5**)

Table 1. pH-Dependence of ^{13}C *Nmr* Spectrum of **PPH**

pH	1.5	2.5	Δ^1	3 2	Δ^2	11.4	Δ^3	12.3	Δ^4
C1	143.4	141.4	2 0	142.0	1.4	140 2	3.2	140.7	2.7
C2	129.7	129.3	0 4	128.8	0.9	127.2	2.5	127.4	2.3
C3	137.3	139 8	- 2.5	138.0	- 0.7	140.0	- 2.7	140 0	- 2.7
C4	152.8	153.0	- 0.2	153.1	- 0 3	162.0	- 9.2	162.1	- 9 3
C5	146 3	144 8	1.5	143 9	2.4	152.2	- 5.9	152.3	- 6.0
C6	14.9	14.8	0 1	14 7	0.0	10.7	-4.8	19.8	- 4.9
C7	59.0	59.0	0	58.9	0.1	62.2	- 3.2	62.3	- 3.3
C8	117.6	117.7	- 0.1	118 0	- 0.4	116.8	0.8	116.5	1.1
C9	149.2	150.1	- 0.9	151 5	- 2.3	156.4	- 7.2	156 4	7 2
C10	113.1	111 9	1 2	109.9	3.2	108.2	4.9	108.3	4.8
C11	136.8	136.1	0 7	135.0	1.8	132 5	4.3	132.5	4.3
C12	130.2	129.6	0.6	129.1	1.1	131.4	- 1 2	131.6	- 1.4
C13	145.0	144.5	0.5	143.7	1.3	147.9	- 2.9	147.8	- 2.8

Δ^1 is the difference in carbon-13 chemical shift between pH 1.5 and 2.5; Δ^2 is the difference in ^{13}C chemical shift between pH 1.5 and 3 2; Δ^3 is the difference in ^{13}C chemical shift between pH 1.5 and 11.4; Δ^4 is the difference in carbon-13 chemical shift between pH 1.5 and 12.3

Table 2. pH-Dependence of ^{13}C *Nmr* Spectrum of **MPH**

pH	1.5	2.5	Δ^1		1.5	2.5	Δ^1
C1	143.6	143.0	0.6	C8	118.0	118.4	-0.4
C2	128.5	128.6	-1.0				
C3	137.7	139.4	-1.7	C9	149.2	151.5	-1.9
C4	153.5	154.1	-0.6	C10	113.5	111.1	2.4
C5	147.3	146.8	1.2	C11	136.4	135.4	1.0
C6	13.7	13.6	0.1	C12	135.9	135.0	0.9
C7	59.2	59.3	-0.1	C13	146.6	143.4	3.2

Δ^1 is the difference in carbon-13 chemical shift between pH 1.2 and 2.5

Table 3. pH-Dependence of ^{13}C *Nmr* Spectrum of **EPH**

pH	1.5	2.5	Δ^1	3.2	Δ^2	11.4	Δ^3	12.3	Δ^4
C1	143.7	142.8	0.9	141.2	1.9	140.3	3.4	0.0	---
C2	130.5	129.5	1.0	129.5	1.0	127.8	2.7	126.9	3.6
C3	137.8	138.9	-1.1	137.2	0.6	137.9	-0.1	139.9	-2.1
C4	154.0	153.8	0.2	154.1	-0.1	164.1	-10.1	164.0	-10.0
C5	147.9	146.7	1.2	145.5	2.4	0.0	---	0.0	---
C6	13.7	13.6	0.1	13.7	0.0	17.6	-3.9	17.6	-3.9
C7	59.5	59.3	0.2	59.4	0.1	61.8	-2.3	61.7	-2.2
C8	118.1	118.2	-0.1	118.2	-0.1	117.4	0.7	116.9	1.2
C9	149.4	151.6	-2.2	152.6	-3.2	0.0	---	155.9	-6.5
C10	113.6	110.8	2.8	110.3	3.3	108.9	4.7	108.4	5.2
C11	137.1	135.6	1.5	135.0	2.1	0.0	---	132.5	4.6
C12	136.1	133.7	2.4	132.9	3.2	0.0	---	0.0	---
C13	146.8	143.5	3.3	141.8	5.0	148.0	-1.2	147.6	-0.8

Δ^1 is the difference in carbon-13 chemical shift between pH 1.2 and 2.7; Δ^2 is the difference in ^{13}C chemical shift between pH 1.2 and 3.7; Δ^3 is the difference in ^{13}C chmical shift between pH 1.2 and 8.0; Δ^4 is the difference in carbon-13 chemical shift between pH 1.2 and 12.5.

From Tables **1**, **2**, and **3**, it can be seen that ^{13}C nmr resonances of ligands included in this study exhibit high sensitivity to pH. Thus, the pyridinic C9 resonance shifts downfield by 2 ppm units on pH increase from 1.5 to 3., whereas the pyridoxal C1 resonance experience 2 ppm upfield shift in the same pH range.This allows to assign with confidence the signal at 143 ppm in the spectrum, to C1, and that at 149 ppm, to C9 atom. Moreover, with the aid of equation **9** it was possible to estimate the pKa values of pyridinium protonation (equation 2) for all ligands, PPH (2.62), MPH (2.39), and EPH (2.31). By the same token it was possible to assign, the 153 ppm signal in the ^{13}C nmr spectra, to pyridoxal-ring C4, which experience downfield shift (10 ppm units) over the pH range 9.0-9.2, due to its proximity to the phenolate anion. The respective pKa values for the phenolate protonations (equation **4**) were: 7.96 (PPH), 7.30 (MPH), and 7.43 (EPH). Around pH 11, all ligands exist essentially as di-anion species (L^{2-}).[9] The ^{13}C nmr signal at 156 ppm was assigned to pyridinic-C9, since at this pH range, it experiences downfield shift by 7 ppm units.The calculated pKa values: 9.84 (PPH), 9.76 (MPH), and 9.70 (EPH), were assigned to amine-hydrazone protonation. Among the ligands, only PPH had an additional pKa = 4.63, assigned to pyridoxylidenium protonation. It is noteworthy that the pH-dependent ^{13}C nmr technique was unable to detect the prototautomeric forms[16,17] of corresponding neutral species, H_2L.

From Table **4** it can be seen that protonation energies increase in the ordering : pyridinium < pyridoxylidenium < phenolate < amine nitrogen-N^3 < alkoxide The highest binding energy (BE) (713.5 kcal / mol) is between hydrogen and alcoholic oxygen ($O^{2'}$) (pKa = 13.35±0.2[1c]), whereas the BE between H and phenolic oxygen ($O^{1'}$) is 140.61 kcal / mol smaller (pKa = 7.96±0.07[1c]). The lowest BE (491.3 kcal / mol) is between H and the pyridinic ring-N^4 (pKa = 2.62±0.09[1c]) which is 31.04 kcal / mol lower than pyridoxylidenium *H*-N^1-bond (pK = 4.63±0.05[1c]), and 120.1 kcal / mol lower than H-N^3-bond (pKa = 9.84±0.07[1c]), in conformity with ionization characteristics of pyridoxal and its analogues.[1b]

Table 4. *The Total and the Relative Energies of Binding of Protons* [18] *at Various Sites of Pyridoxal Pyridyl Hydrazone (PPH)*

Binding Sites	Total Energy (in atomic units)	Binding Energy[a] (in kcal/mol)
HL^-	- 854.33597	---
O^2	- 855.47231	713.1
N^3	- 855.35986	642.5
O^1	- 855 24823	572.4
N^1	- 855 16845	522.4
N^4	- 855.11898	491.3

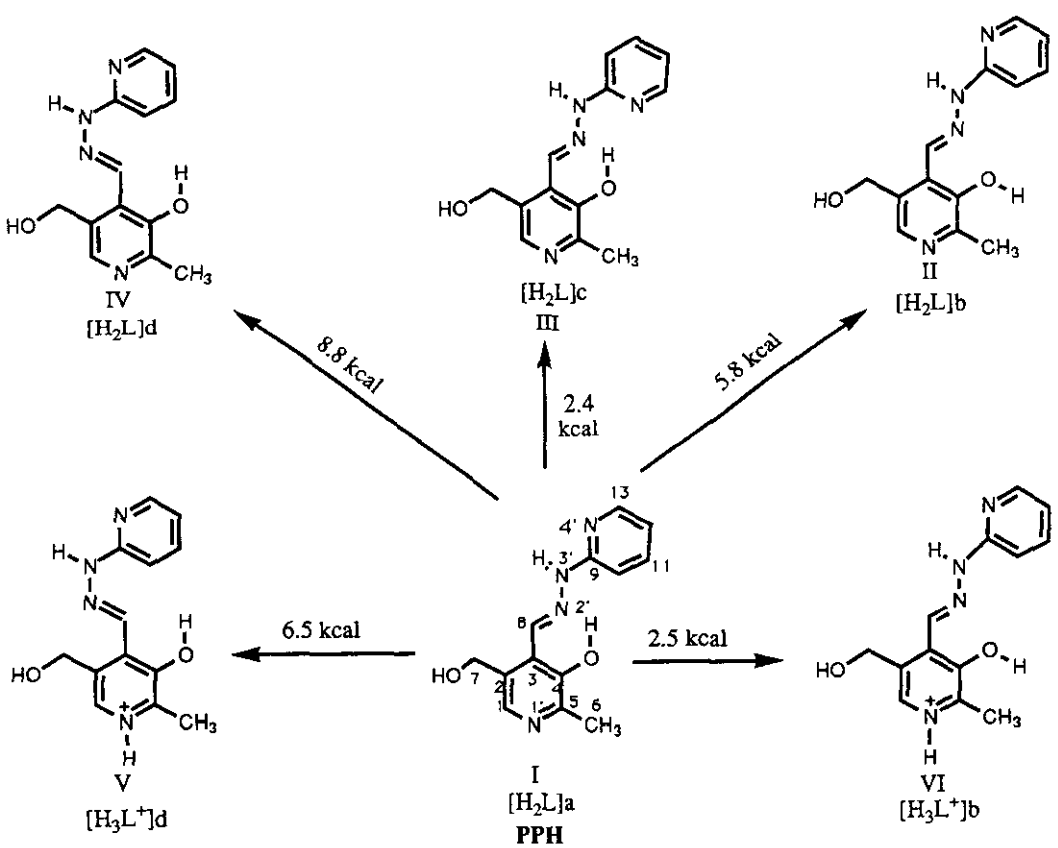
a) The binding energy relative to the mono-deprotonated species, HL^-

Scheme III delineates structures of four neutral conformational species of PPH, $[H_2L]_{a-d}$, and in addition two protonated species, $[H_3L^+]_b$ and $[H_3L^+]_d$, which arise from rotations about respective C^4 - O^1, C^3 - C^8, and N^3 - C^9 bonds. Conformation $[H_2L]_a$ appears to be the lowest in energy, whereas that of structure $[H_2L]_d$ is the highest in energy (loss of internal H-bonding). Protonation at pyridoxal ring-N^1 lowers the conformational $[H_2L]_a \rightarrow [H_3L^+]_d$ interconversion by a quantity of 2.3 kcal / mol. This is also evident from the data assembled in Table **5**, clearly indicating that N^1-protonation implys decrease in the electron density on the azomethinic nitrogen, N^2, from 0.1399 to 0.0163 electrons and hence weakening the internal H-bonding.

Table 5. *Mulliken Population Ananlysis [15,19] of Neutral and Protonated Pyridoxal Pyridyl Hydrazone*

Atom	H_2L	H_3L^+	Atom	H_2L	H_3L^+
N1	-0.2643	-0.1511	C3	0.0481	-0.0797
N2	-0.1399	-0.0163	C4	-0.0884	-0.0797
N3	-0.2536	-0.2205	C5	0.2174	0.1515
N4	-0.2409	-0.2000	C7	-0.0364	-0.0008
O1	-0.3119	- 0.2607	C8	0.1463	0.1269
O2	-0.3192	-0.2578	C9	0.0928	0.1551
C1	-0.0455	-0.1462	C10	0.0060	-0.0608
C2	-0.0867	-0.1866	C11	-0.0051	-0.0013

Scheme III



Conclusion

The data presented here permits to assign with confidence the pKa values of ionizable protons in PPH, MPH, and EPH, which follow the ordering:
pyridinium protonation < pyridoxylidenium protonation < phenolate protonation < amine hydrazone protonation < alkoxide protonation, in remarkable conformity with the literature.[6,7,20-23]

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