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Influence of regioisomerism on stability, formation kinetics and ascorbate oxidation preventive properties of Schiff bases derived from pyridinecarboxylic acids hydrazides and pyridoxal 5′-phosphate☆

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1. Introduction

3-Hydroxy-2-methyl-5-[(phosphonoxy)methyl]-4-pyridinecarboxaldehyde (pyridoxal 5′-phosphate, PLP) is one of the most important coenzymes in living organisms. PLP derivatives catalyze a variety of metabolic processes including amino acids, lipids and carbohydrates metabolism, and play the key role in the hormones, neurotransmitters and heme biosynthesis [1,2]. The value of pyridoxal 5′-phosphate for human beings is obvious from the fact that PLP low concentrations in blood serum are associated with a number of pathologies such as systemic inflammation [3], inflammatory bowel disease [4], rheumatoid arthritis [5], epilepsy [6], periphery neuropathy [7], panic attacks [8], depression [9], and anemia [10]. However, PLP level correction in the organism not always leads to the positive health response. Along with PLP or its precursors' malabsorption from food and alcohol abuse [11], pyridoxal 5′-phosphate interaction with drugs may lower significantly its blood content.

An effective antitubercular drug isoniazid (isonicotinic acid hydrazide, INH) [12] is an example of agent decreasing PLP concentration in blood [13]. INH binds either pyridoxal or pyridoxal 5′-phosphate into stable Schiff base [14,15] thus acting as a toxin.

The mechanism of isoniazid bacteriostatic effect differs from its toxicity towards macroorganisms and relates to the free acyl radical formation under the action of KatG catalase-peroxidase enzyme of mycobacteria [16]. This radical then reacts with NADH inactivating it. Since the radical stability depends on the substituent localization in the pyridine ring, the difference between biological activity of 2,4- and 3-isomers should be anticipated. Indeed, 4-isomer and 2-isomer (picolinic acid hydrazide, PH) were found to provide the pronounced antitubercular effect, while 3-isomer (nicotinic acid hydrazide, NH) is inactive [17,18]. However, 6-aminonicotinic acid hydrazide possesses antitubercular properties [18].

Schiff bases formed by pyridoxal/PLP and pyridinecarboxylic acids hydrazides should be noted to have their own biological relevance. For instance, pyridoxal isonicotinoyl hydrazone (PIH) prevents copper-mediated free radicals formation in presence of ascorbate thus providing

☆ Present contribution reports on thermodynamics and kinetics of the reactions between pyridoxal 5′-phosphate and hydrazides of 2-, 3-, and 4-pyridinecarboxylic acids in aqueous solution at pH values of 1.9, 6.6., 7.0, and 7.4. Hydrazones are isolated as solids and studied by means of NMR, IR spectroscopy. The fluorescence of hydrazones dissolved in neutral and alkali media is investigated. The hydrazones capability of inhibiting the copper-mediated ascorbate oxidation is evaluated.

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the antioxidant activity [19]. From the other hand, PIH is capable of Cu^{2+} binding and may show demetalling action useful for treatment of Wilson disease, gene-determined disorder of copper metabolism [19].

Taking all above into consideration, in the present contribution we aim to figure out how —CO—NH—NH_2 group localization in the pyridine ring influences the stability of Schiff bases derived from pyridoxal 5′-phosphate and pyridinecarboxylic acids hydrazides as well as rate constants of Schiff bases formation in aqueous solutions at pH values corresponding to the physiological ones. Hydrazones are also isolated as solids and characterized by means of NMR, IR and fluorimetry. Besides, Schiff bases ability of protecting the ascorbic acid (AA) from copper-mediated oxidation is estimated.

To the date, the reactions between pyridoxal 5′-phosphate and pharmaceutical hydrazides (isoniazid, carbidopa, hydralazine) were described in a few of papers [20–22].

2. Experimental

2.1. Chemicals and apparatuses

Pyridoxal 5′-phosphate (abcr GmbH, Germany), isoniazid (Sigma-Aldrich, USA), ascorbic acid (Hebei Welcome Pharmaceutical Co., Ltd., China), EDTA (Mosreaktiv, Russia), citric acid (Spektr-Khim, Russia), $CuCl_2$ (Reakhim, Russia) were used without additional purification. The purity of reagents claimed by manufacturer was >99% (weight). The hydrazides of 3- and 2-pyridinecarboxylic acids were synthesized using ethyl esters of corresponding pyridinecarboxylic acids [23,24]. The impurities absence was controlled using ^{1}H NMR spectroscopy and GCMS (GCMS-QP2010 Ultra setup, Shimadzu, Germany). Helium was used during GCMS procedure as a carrier gas at the flow-rate of 0.98 ml/min and pressure of 90 kPa. The total run time was 20 min. Hydrazides were injected as solutions in CH_2Cl_2 at T_{inj} = 180 °C. Column oven temperature was 150 °C whilst ion source temperature was 200 °C. Purge flow (He) was 3.0 ml/min. Buffers with pH values of 6.6, 7.0, and 7.4 were prepared using $Na_2HPO_4 \cdot 12H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ (Spektr-Khim, Russia). The pH 1.9 value was set using preliminarily standardized HCl. Acidity of buffers was controlled potentiometrically.

All the solutions were prepared using bidistilled water ($\kappa = 3.6$ μSm/cm, pH = 6.6). The ionic strength value $I = 0.25$ close to that of erythrocytes media [25] was set due to buffer mixture components (at pH values of 6.6, 7.0, and 7.4) or using KNO_3 (pH 1.9).

UV–Vis spectra of PLP, NH, and PH solutions of $5 \cdot 10^{-5}$–$2.0 \cdot 10^{-4}$ mol l^{-1} as well as mixtures of PLP + NH, PLP + PH were recorded on double-beamed Shimadzu UV1800 spectrophotometer (USA) in the wavelength range of 190–500 nm and absorbance range of 0–4. The error of wavelength determination did not exceed 0.5 nm, the maximal inaccuracy of absorbance measurements was of ±0.006 units. The temperature maintained at 298.2 ± 0.1 K using external thermostat. The quartz cells with absorbing layer thickness of 1 cm were used.

The elemental analysis of hydrazones was performed using FLASH EA1112 setup (Termo Quest, Italy). Mass spectra (MALDI TOF) were registered using Shimadzu Biotech Axima Confidence setup (Shimadzu, USA). ^{1}H, ^{13}C and ^{31}P NMR spectra of Schiff bases (D_2O, pD ~ 12) were registered using Avance III Bruker 500 NMR-spectrometer with ^{1}H, ^{13}C, ^{31}P operating frequencies of 500.17, 125.77, and 202.47 MHz respectively. The inaccuracy of chemical shift measurement in respect to the external standard, HMDSO, was evaluated as ±0.005 ppm. The signals in the spectra of studied compounds were assigned using literature data [26] and NMR spectra predicting tools [27]. The IR spectra of Schiff bases were recorded using Tensor 27 FTIR spectrometer (Bruker Optics, Germany) with error of wavenumber determination of ±1 cm^{-1}. Spectral bands were assigned using literature data [26,28].

Fluorescence spectra were registered using RF6000 setup (Shimadzu, USA) at the excitation wavelength λ_{ex} = 300 nm in the wavelength range of 310–550 nm. The excitation and emission slit widths were set at 5 nm, scanning rate was 6000 nm min^{-1}. The quartz cell with absorbing layer thickness of 1 cm was used.

2.2. The determination of hydrazones stability constants

The stability constants of Schiff bases PLP-NH, PLP-PH were determined using previously described method [22] using UV–Vis spectra of sets of 8–10 solutions with PLP:NH, PLP:PH initial concentrations ratio varying from 10:1 to 1:1. Before recording the spectra, all the mixtures were allowed to equilibrate during 36 h after preparing. The spectral data were processed using FTMT software (see [29] and Refs. 16–18 within) which calculates the equilibrium constants *via* solving the following equations:

$$A_{\lambda_i} = (\varepsilon_1[R_1] + \varepsilon_2[R_2] + \varepsilon_3[P]) \cdot l, \quad (1)$$

where $A_{\lambda i}$ is the absorbance value at wavelength λ_i; ε_1, ε_2, and ε_3 are the molar extinction coefficients of reagents and product; $[R_1]$, $[R_2]$, $[P]$ are the equilibrium concentrations of reagents and product; l is the thickness of absorbing layer. Eq. (1) expresses both Beer–Lambert–Bouguer law and independent light absorbance principle;

$$C_{Ri}(total) = [R_i] + [P], \quad (2)$$

where C_{Ri}(total) is the total concentration of compound R_i. Eq. (2) expresses material balance for R_i compound;

$$K = \frac{[P]}{[R_1][R_2]}, \quad (3)$$

where K is the equilibrium constant of reaction. Eq. (3) expresses acting masses law.

In order to solve the Eqs. (1)–(3) jointly, one needs to minimize the function:

$$F = \sum_{q=1}^{z} \left(A_{\lambda_i}(\text{ exp}) - A_{\lambda_i}(calc)\right)^2, \quad (4)$$

where $A_{\lambda i}$(exp) and $A_{\lambda i}(calc)$ are the experimental and calculated absorbance values of solution number q; z is the total number of solutions.

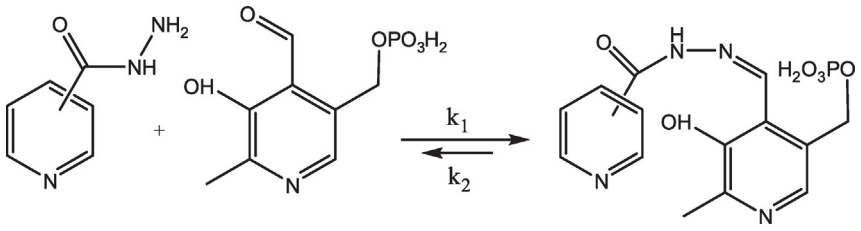
The calculations were performed basing the experimental dependencies of absorbance at 3–5 wavelengths on the reagents initial concentrations ratio (see examples of spectra on Figs. S1–S8). The molar extinction coefficients of PLP, NH, PH required for calculations in FTMT at every pH value and every wavelength were determined preliminarily using calibration plots.

2.3. The determination of rate constants of hydrazones formation

The spectrophotometrical kinetic experiments were performed at the same concentration conditions and spectrophotometer setup as in [22]. At the pH value of 1.9 the reaction proceeds too fast to reliably determine its kinetic characteristics.

2.4. The synthesis of hydrazones

Synthesis was performed in accordance with Scheme 1. The preliminarily heated to 90 °C solution of 0.309 g (1.25 mmol) of pyridoxal 5′-phosphate in 25 ml of water was quickly added to the solution of 1.25 mmol of isoniazid or nicotinoyl hydrazide or picolinoyl hydrazide in the 25 ml of water (at 90 °C). The reaction mixture was cooled during 1 h at room temperature. The precipitated crystalline products were then filtrated, washed with small quantities of icy distilled water and acetone and dried at 90 °C in the air until their weight became constant.



Scheme 1. Formation of hydrazones derived from pyridoxal 5′-phosphate and hydrazides of 2, 3, and 4-pyridinecarboxylic acids.

2.4.1. {3-Hydroxy-4-[(E)-(isonicotinoylhydrazono)methyl]-2-methylpyridin-5-yl}methylphosphate (Schiff base PLP-INH)

Orange solid, T_{decomp} = 276–277 °C. R_f = 0.92 (mobile phase NH_3 25% aq., Polygram Sil G/UV_{254}). Elemental, found (calcd.): C 45.91 (45.91), H 3.85 (4.13), N 15.65 (15.30), O 26.12 (26.21). M_z [Schiff base + H] 367.17. λ_{max}, nm (lg ε) at pH 7.4, H_2O: 294 (4.23). IR: 3313s (ν(N—H) hydrazide residue), 3087s, 3070, 3054s ν(C—H) pyridine rings, 2989s (ν_{asym}(—CH_3—) and ν_{asym}(—CH_2—) PLP residue), 1669s (ν(C═O) hydrazide residue), 1568s, 1490s, 1411s (ν(C═C) and ν(C═N) pyridine rings), 1527m (ν(C═N), —CH═N—NH— group), 1298s (ν(C—N) + δ(N—H) hydrazide residue), 1223s (ν(≥C—OH) PLP residue), 1161m (ν(P═O) PLP residue). NMR (D_2O, pD ~ 12) ^{1}H, δ, ppm: 8.47 s (1H, —CH═), 8.28 d (2H, $H_{2,6}$ INH, J_3 = 5.22 Hz), 7.53 d (2H, $H_{3,5}$ INH, J_3 = 5.87 Hz), 7.43 s (1H, H_6 PLP), 4.66 d (2H, —CH_2— PLP, $J_{2,3}$ = 4.56 Hz), 2.16 s (3H, —CH_3 PLP); ^{13}C, δ, ppm: 169.14 (>C═O), 158.92 (C_3 PLP), 151.43 (—CH═), 149.16 ($C_{2,6}$ INH), 148.86 (C_2 PLP), 144.48 (C_4 INH), 134.01 (C_6 PLP), 130.40 (C_5 PLP), 122.19 ($C_{3,5}$ INH), 120.46 (C_4 PLP), 61.77 (—CH_2— PLP), 18.36 (—CH_3 PLP); ^{31}P, δ, ppm: s 3.76.

2.4.2. (3-Hydroxy-2-methyl-4-{(E)-[(pyridin-3-ylcarbonyl)hydrazono]methyl}pyridin-5-yl)methylphosphate (Schiff base PLP-NH)

Bright yellow solid, T_{decomp} = 269–270. R_f = 0.91 (mobile phase NH_3 25% aq., Polygram Sil G/UV_{254}). Elemental, found (calcd.): C 46.01 (45.91), 4.08 (4.13), 15.49 (15.30), 26.22 (26.21). M_z [Schiff base + H] 367.17. λ_{max}, nm (lg ε) at pH 7.4, H_2O: 296 (4.23). IR: 3599s (ν(O—H) PLP residue), 3343s (ν(N—H) hydrazide residue), 3113s, 3052m (ν(C—H) pyridine rings), 2989s, 2911s (ν_{asym}(—CH_3—) and ν_{asym}(—CH_2—) PLP residue), 1684s (ν(C═O) hydrazide residue), 1585s, 1487s, 1430s (ν(C═C) and ν(C═N) pyridine rings), 1520w (ν(C═N), —CH═N—NH— group), 1300s (ν(C—N) + δ(N—H) hydrazide residue), 1237m (ν(≥C—OH) PLP residue), 1177s (ν(P═O) PLP residue). NMR (D_2O, pD ~ 12) ^{1}H, δ, ppm: 8.82 dd (1H, H_2 NH, J_4 = 1.30 Hz, J_5 = 0.87 Hz), 8.62 s (1H, —CH═), 8.42 dd (1H, H_6, NH, J_3 = 4.78 Hz, J_4 = 1.52 Hz), 8.12 dq (1H, H_4 NH, J_3 = 7.80 Hz, J_4 = 1.73 Hz), 7.55s (1H, H_6 PLP), 7.35 ddd (1H, H_5 NH, J_3 = 8.04 Hz, J_3 = 5.00 Hz, J_5 = 0.87 Hz), 4.76 d (2H, —CH_2— PLP, $J_{2,3}$ = 4.56 Hz), 2.25s (3H, —CH_3 PLP); ^{13}C, δ, ppm: 169.53 (>C═O), 159.44 (C_3 PLP), 151.67 (—CH═), 150.54 (C_2 NH), 148.96 (C_2 PLP), 148.05 (C_6 NH), 136.55 (C_4, NH), 133.69 (C_6 PLP), 132.25 (C_5 PLP), 130.68 (C_3 NH), 124.13 (C_5 NH), 121.28 (C_4 PLP), 61.95 (—CH_2— PLP), 18.50 (—CH_3 PLP); ^{31}P, δ, ppm: s 3.76.

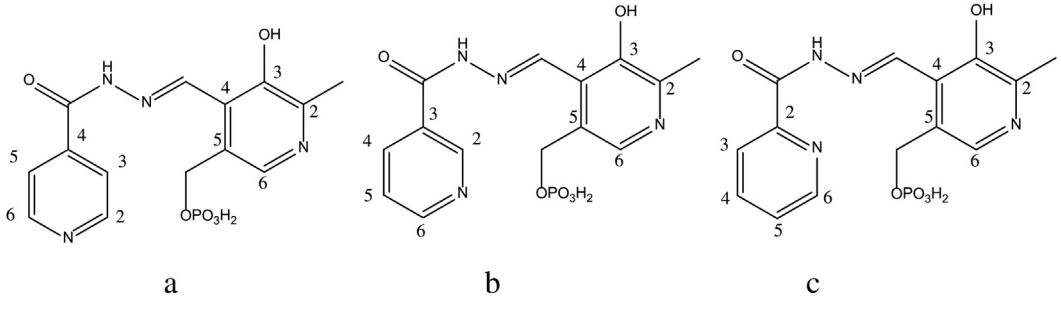
2.4.3. (3-Hydroxy-2-methyl-4-{(E)-[(pyridin-2-ylcarbonyl)hydrazono]methyl}pyridin-5-yl)methylphosphate (Schiff base PLP-PH)

Yellowish orange solid, T_{decomp} = 266–267 °C. R_f = 0.81 (mobile phase NH_3 25% aq., Polygram Sil G/UV_{254}). Elemental, found (calcd.): C 45.86 (45.91), H 4.20 (4.13), N 15.21 (15.30), O 26.18 (26.21). M_z [Schiff base + H] 367.17. λ_{max}, nm (lg ε) at pH 7.4, H_2O: 299 (4.30). IR: 3477s (ν(O—H) PLP residue), 3292w (ν(N—H) hydrazide residue), 3097w, 3066w (ν(C—H) pyridine rings), 2994w, 2920 m (ν_{asym}(—CH_3—) and ν_{asym}(—CH_2—) PLP residue), 1690vs (ν(C═O) hydrazide residue), 1586s, 1466 m, 1407w (ν(C═C) and ν(C═N) pyridine rings), 1305s (ν(C—N) + δ(N—H) hydrazide residue), 1236w (ν(≥C—OH) PLP residue), 1167 m (ν(P═O) PLP residue). NMR (D_2O, pD ~ 12) ^{1}H, δ, ppm: 8.67s (1H, —CH═), 8.47 dq (1H, H_6 PH, J_3 = 4.87 Hz, J_4 = 1.52 Hz, J_5 = 0.87 Hz), 7.87 dt (1H, H_3 PH, J_3 = 8.04 Hz, J_5 = 1.09 Hz), 7.84 td (1H, H_4 PH, J_3 = 7.61 Hz, J_4 = 1.74 Hz), 7.55s (1H, H_6 PLP), 7.43 ddd (1H, H_5 PH, J_3 = 7.39 Hz, J_3 = 5.00 Hz, J_4 = 1.52 Hz), 4.80 d (2H, —CH_2— PLP, $J_{2,3}$ = 4.56 Hz), 2.24 s (3H, —CH_3 PLP); ^{13}C, δ, ppm: 167.75 (>C═O), 160.71 (C_3 PLP), 152.05 (—CH═), 150.08 (C_6 PH), 148.96 (C_2 PLP), 140.49 (C_2 PH), 138.36 (C_4 PH), 132.35 (C_6 PLP), 131.23 (C_5 PLP), 126.58 (C_3 PH), 124.67 (C_5 PH), 123.29 (C_4 PLP), 62.24 (—CH_2— PLP), 18.76 (—CH_3 PLP); ^{31}P, δ, ppm: s 3.86.

The atoms numbering is given (Scheme 2).

2.5. The oxidation of ascorbate

The study was performed analogously with described [19] with some modifications. The 3.5 ml aliquot of the solution of ascorbic acid (0.0001 mol l^{-1}), or ascorbic acid (0.0001 mol l^{-1}) + hydrazone (0.0001 mol l^{-1}) in the buffer with 6.6 pH was sampled into the standard 1 cm quartz cell. 2–3 drops of aqueous solution of $CuCl_2$ (0.01 mol l^{-1}) with preliminarily determined density were added to a solution in the cell. Weighing the syringe containing $CuCl_2$ before and after addition allowed to determine the quantity of Cu^{2+} added. The absorbance of ascorbic acid solutions with/without adding of hydrazones/Cu(II) ions was recorded during 300 s at wavelength of 265 nm. The ascorbate molar coefficient of extinction at this wavelength is known to be ε_{AA} = 14,500 [30] which is close to our own measurements.



Scheme 2. Schiff bases derived from pyridoxal 5′-phosphate and hydrazides of pyridinecarboxylic acids: a) isonicotinic; b) nicotinic; c) picolinic.

Table 1
Stability constants, rate constants of formation and hydrolysis of Schiff bases derived from pyridoxal 5′-phosphate and hydrazides of pyridinecarboxylic acids at different pH.

Hydrazone	pH	1.94	6.6	7.0	7.4
PLP-INH [22]	lg K	4.51	4.61	4.72	4.21
	k_1, l mol^{-1} min^{-1}	–	49.44	30.31	28.39
	k_2, min^{-1}	–	$1.23 \cdot 10^{-3}$	$5.8 \cdot 10^{-4}$	$1.77 \cdot 10^{-3}$
PLP-NH	lg K	5.33 ± 0.12	4.64 ± 0.28	5.05 ± 0.12	5.16 ± 0.25
	k_1, l mol^{-1} min^{-1}	–	25.20 ± 0.40	22.89 ± 0.46	21.74 ± 0.86
	k_2, min^{-1}	–	$5.8 \cdot 10^{-4} \pm 4.1 \cdot 10^{-4}$	$2.0 \cdot 10^{-4} \pm 6 \cdot 10^{-5}$	$1.5 \cdot 10^{-4} \pm 1.0 \cdot 10^{-4}$
PLP-PH	lg K	5.02 ± 0.13	5.14 ± 0.18	5.04 ± 0.11	4.64 ± 0.13
	k_1, l mol^{-1} min^{-1}	–	57.36 ± 0.76	26.55 ± 1.33	14.80 ± 1.74
	k_2, min^{-1}	–	$4.2 \cdot 10^{-4} \pm 1.8 \cdot 10^{-4}$	$2.4 \cdot 10^{-4} \pm 7 \cdot 10^{-5}$	$3.4 \cdot 10^{-4} \pm 1.4 \cdot 10^{-4}$

In the all cases, we had associated the changes of absorbance with decreasing of ascorbate concentration, which allows estimating it as $\Delta C_{AA} = \Delta A / \varepsilon_{AA}$.

The ascorbate oxidation in presence of EDTA and citric acid (CA) was investigated in the same way.

3. Results and discussion

3.1. Thermodynamics and kinetics of hydrazones formation

The results of spectrophotometric determination of the equilibrium constant as well as rate constants of direct (k_1) and inverse (k_2) reaction of Schiff base formation at different pH are summarized (Table 1).

The k_2 values (Table 1) were calculated using well-known equation:

$$K_r = k_1/k_2 \quad (5)$$

The errors of lg K_r and k_1 values (Table 1) are the half-width of the confidence interval with a confidence probability of 0.95 and sample size of three experiments. The inaccuracies of k_2 values include the determination errors of all values used for k_2 calculations.

Taking into account the peculiarities of distribution of electronic density in pyridine, one could anticipate the similarity between Schiff bases derived from hydrazides of 2- and 4-pyridinecarboxylic acids. However, Table 1 data show no significant differences between hydrazones stability except for 7.4 pH, where PLP-INH Schiff base has the smallest value of lg K. Interestingly, PLP-NH hydrazone is the most stable at pH 1.9.

The stability of each hydrazone changes by ~0.5 log units in the pH range of 6.6–7.4 (Table 1). These variations are caused by the change of equilibrium concentrations of different dissociated species of pyridoxal 5′-phospate with different binding ability towards hydrazides (see *e.g.* [21].).

The hydrazide of nicotinic acid seems to be the most hazardous compound for a healthy tissue, where pH of medium is close to 7.4, since its hydrazone is the most stable and forms readily enough at this pH value (Table 1). The picolinic acid hydrazide could be optimal PLP-binding agent in some tumors with acidic interstitial fluid [31,32].

The rate of PLP-INH and PLP-PH hydrazones formation decreases significantly in the pH range of 6.6–7.4, while k_1 value of PLP-NH remains nearly constant.

3.2. Hydrazones synthesis and fluorescence

The studied Schiff bases form under the relatively mild conditions and require no non-aqueous solvents and high temperatures during the synthesis. When the preliminarily heated solutions of PLP and INH, NH, PH with equal concentrations are mixed, the crystalline insoluble product begins to form quickly and, then, could be filtered easily. Schiff bases formation could be proved by the comparison of UV–Vis, NMR (^{1}H, ^{13}C) and IR spectra of reagents and product.

For example, all the reaction mixtures start to absorb intensively (ε = 17,000–22,500) in the range of 294–303 nm (depending on pH value, see Figs. S1–S8) thus indicating the formation of π-π-p-π conjugated electron system well-known for hydrazones [33,pp. 107–108].

One could observe the aldehyde proton signal in the ^{1}H NMR spectrum of pyridoxal 5′-phosphate at 9.90 ppm. There are no signals in this range in the spectra of synthesized compounds, however, the resonance of —CH═ group neighboring the imine bond appears at 8.47–8.67 ppm. In the ^{13}C NMR spectra, the signal of formyl group of PLP (196.35 ppm.) moves upfield by 40 ppm confirming the —CH═ formation.

Hydrazones are able to exist as a mixture of *E,Z*-isomers due to the —CH═N— group presence in the molecule [33,pp. 48–52]. However, there is the only set of signals in the ^{1}H, ^{13}C NMR spectra of PLP-INH, PLP-NH, PLP-PH hydrazones. Our quantum chemical calculations of neutral and zwitter-ionic species of non-ionized Schiff bases (not shown) have revealed that the *E*-isomers are more stable (G_z-G_E is *ca.* 42 kJ mol^{-1} for zwitter-ions and 8 kJ mol^{-1} for molecular species). Therefore, hydrazones probably precipitate as *E*-isomer.

No frequencies characteristic for —NH_2 groups of INH, NH, PH and CHO-group of PLP could be found in the IR spectra of Schiff bases (see Section 2.4), which confirms hydrazones formation.

Being heated in the argon atmosphere, hydrazones decompose in the temperature range of 267–277 °C (see Section 2.4). IR-analysis of the evolving gases shows the predominant presence of aqueous vapor, CO_2 and NH_3.

The synthesized Schiff bases fluoresce in the solution (Fig. 1).

Fig. 1 data show that the alkali media promote the fluorescent properties of Schiff bases (strongest emission at λ_{em} = 360 nm). Neutral buffer solutions allows only weak fluorescence of PLP-NH and PLP-PH at λ_{em} ~ 480 nm. In the alkali media the emission spectra of PLP-INH and PLP-PH are similar (the latter fluoresce more intensively). The

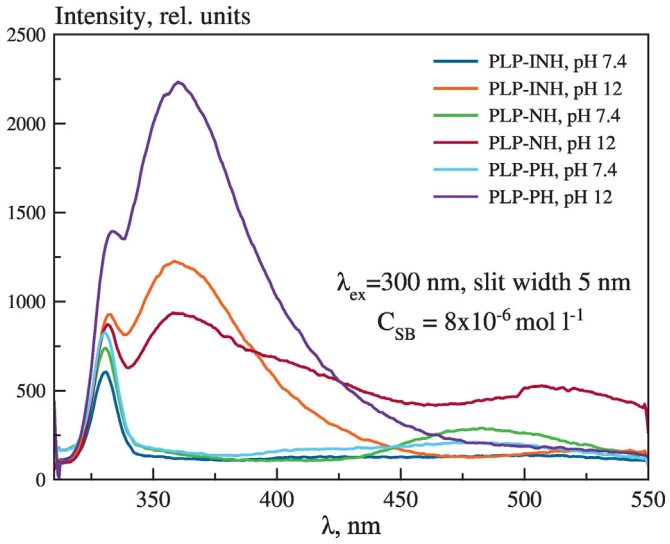


Fig. 1. Fluorescence of hydrazones derived from PLP and hydrazides of 4-, 3- and 2-pyridinecarboxylic acids in the neutral and alkali media (solvent water).

PLP-NH compound emits weaker at 360 nm, but shows the fluorescence in the long-wave range (weak peak at 505 nm).

It is worth noting that the only PLP-PH hydrazide lights with bright green in the crystalline form when being irradiated by mercury UV-lamp ($\lambda_{ex} = 365$ nm).

3.3. Copper-mediated oxidation of ascorbate in the presence of hydrazones

Ascorbic acid, an efficient reductant, oxidizes to the dehydroascorbic acid because of two consecutive one-electron processes [30]. Ascorbate oxidation accelerates in the presence of transition metal ions such as Cu(II) [19] and Fe(III) [34]. The ascorbate was found [30] to be quite sensitive to even traces of metals-catalyzers, and, therefore, it could serve as an indicator of solution contamination by metals. Pyridoxal isoniazid hydrazone has been earlier observed to slow the ascorbate oxidation in the presence of copper(II) ions due to forming the complexes with Cu^{2+} [19].

Authors [19] described a number of the reactions passing in the solution of ascorbate and Cu(II) ions:

$$Cu(II) + \text{ascorbate} \rightarrow Cu(II) - \text{ascorbate} \rightarrow Cu(I) + \text{ascorbyl} \quad (6);$$

$$Cu(I) + O_2 \rightarrow Cu(II) + O_2^- \quad (7);$$

$$Cu(I) + O_2^- + 2H^+ \rightarrow Cu(II) + H_2O_2 \quad (8);$$

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad (9);$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^- + \cdot OH. \quad (10).$$

The Cu(II) capability of accelerating the ascorbate oxidation producing both hydrogen peroxide and hydroxyl radical was confirmed in the recent detailed study [35].

Fig. 2 shows the dependencies of ascorbate (AA) concentration on time in the presence/absence of PLP-INH hydrazone and Cu(II) ions. The Schiff base formed by pyridoxal 5′-phosphate could be assumed to protect ascorbic acid as effectively as pyridoxal isoniazid hydrazine [19] does.

Replacing the buffer with pH value of 6.6 by one with pH 7.4 leads to insignificant differences (Fig. 3). The experimental inaccuracy of

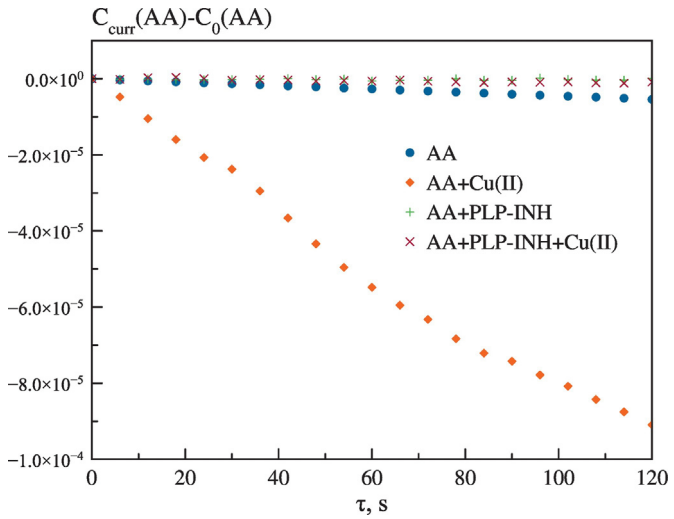


Fig. 2. Time-dependent decreasing of ascorbate concentration in the presence/absence of PLP-INH hydrazone and Cu(II) ions. $C_0(AA) = 0.0001$ mol l^{-1}, $C_0(PLP\text{-}INH) = 0.0001$ mol l^{-1}, $C_0(Cu) = 0.00005$–0.00007 mol l^{-1}.

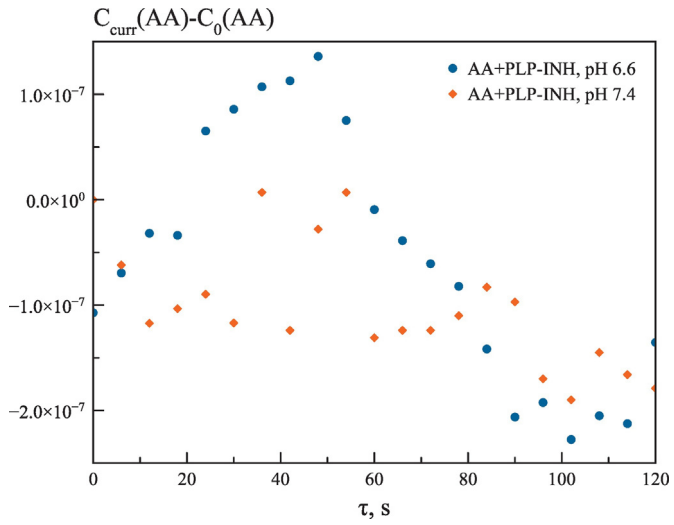


Fig. 3. Time-dependent variation of ascorbate concentration in the presence of PLP-INH hydrazone at 6.6 and 7.4 pH values. $C_0(AA) = 0.0001$ mol l^{-1}, $C_0(PLP\text{-}INH) = 0.0001$ mol l^{-1}.

absorbance measurement determines the variation of ascorbate concentration seen on the plot and the irregular character of observed changes.

All the hydrazones possess similar protecting activity towards ascorbic acid at 6.6 pH in the absence of Cu^{2+} ions (Fig. 4). When $CuCl_2$ is added to the system, PLP-PH hydrazone is the least agent preventing ascorbate oxidation.

Authors [19] had shown the pyridoxal isonicotinoyl hydrazone to form complex with Cu(II) ions thus competing for metal with ascorbate. This complexation probably prevents the reduction of Cu(II) to Cu(I), which could react with dissolved oxygen generating such strong oxidants as O_2^- and H_2O_2. The PLP-INH hydrazone is also capable of forming the coordination compound with Cu^{2+} (see an example on Fig. 5).

Copper ion seems to disturb π-π-p-π conjugated electron system of hydrazone since it interacts with —NH: atom. Therefore, the absorbance at 295 nm becomes less intensive while bathochromic shift is observed (new peak at 410 nm).

The Fig. 5 data allow estimating the complexation equilibrium constant using FTMT software analogously with stability constants of Schiff bases ($\lg K = 4.8 \pm 0.5$).

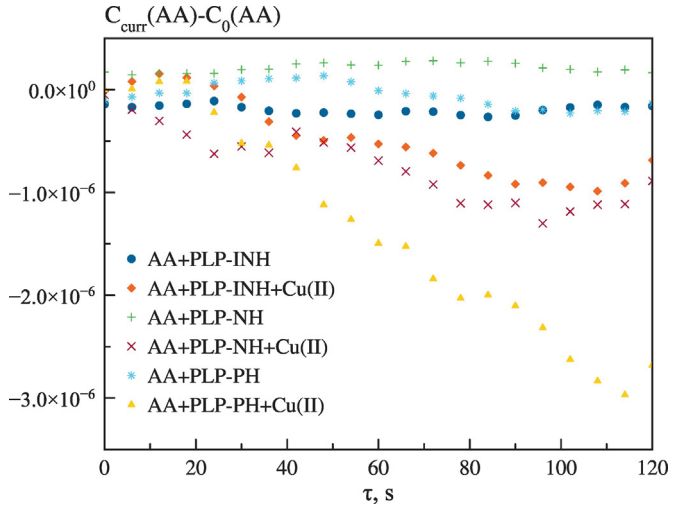


Fig. 4. Time-dependent decreasing of ascorbate concentration in the presence of PLP-INH, PLP-NH, PLP-PH hydrazones and presence/absence of Cu(II) ions. $C_0(AA) = 0.0001$ mol l^{-1}, $C_0(PLP\text{-}INH, PLP\text{-}NH, PLP\text{-}PH) = 0.0001$ mol l^{-1}, $C_0(Cu) = 0.00005$–0.00007 mol l^{-1}.

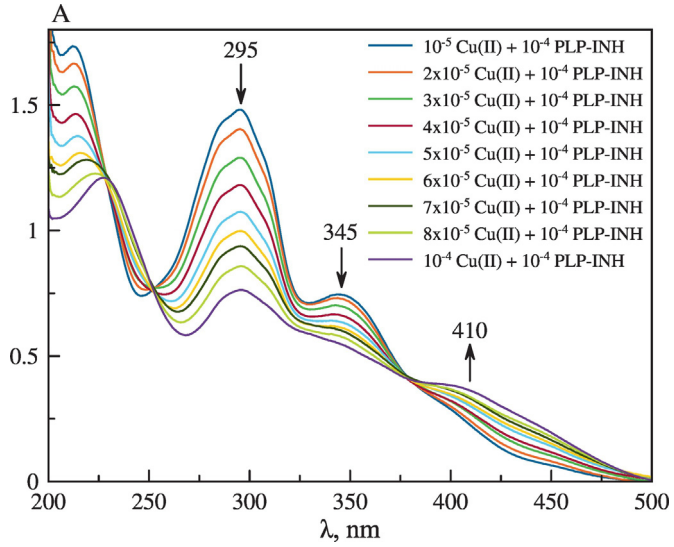


Fig. 5. Complex formation between Cu(II) and PLP-INH hydrazone in aqueous solution at 6.6 pH. The values in plot description stand for initial molar concentrations.

If it is the thermodynamic stability of complex with metal which determines the protecting action of hydrazones towards ascorbate in the mixture with Cu(II), it would be reasonable to assume the ligands binding the copper ions more effectively to exhibit more oxidation preventing action. However, there is no direct link between lg K and protecting effect (Fig. 6).

The inhibition of copper-mediated ascorbate oxidation by the strong chelator EDTA is slightly less pronounced than that by PLP-INH hydrazone. This observation in in correspondence with previous findings for pyridoxal isonicotinoyl hydrazone [19]. Citric acid forming at pH > 5.5 the copper(II) complexes of $Cu_2(CA)_2^{4-}$ composition (lg K = 5.5–6.0 [36,37]) protects the ascorbic acid even less. (Fig. 6). Ascorbate oxidation preventive action of citric acid along with a number of other organic acids is studied in details in [38].

Free radical scavenging and competing for oxidizer are other probable explanations of oxidation preventive action of Schiff bases towards

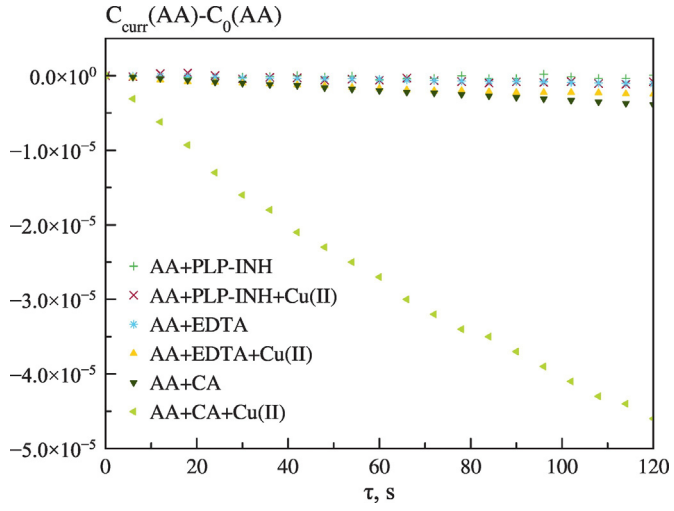


Fig. 6. Time-dependent decreasing of ascorbate concentration in the presence of PLP-INH/EDTA/citric acid (CA) and presence/absence of Cu(II) ions. C_0(AA) = 0.0001 mol l^{-1}, C_0(PLP-INH, EDTA, CA) = 0.0001 mol l^{-1}, C_0(Cu) = 0.00005–0.00007 mol l^{-1}.

ascorbate. Though authors [19] had found it is unlikely that ligands bind the forming free radicals, Schiff bases should be noted to be oxidized by H_2O_2 as it follows from weeklong NMR control of PLP-PH + H_2O_2 mixture at pH 12 we have performed. The number of signals in the low-field range of spectra decreased from 6 to 5. It could be a consequence of the process:

$$R_1 — CO — NH — N{=}CH — R_2 \rightarrow R_1 — CO — NH — NH — CO — R_2, \quad (11)$$

where —CH= group transforms into >C=O and its ^{1}H NMR signal disappears.

The oxidation of analogous compound, 2-pyridylcarboxaldehyde isonicotinoylhydrazone, by the Scheme (11) was found to be catalyzed by Fe(III) ions [39]. Thereby, the PLP-INH, PLP-NH, PLP-PH hydrazones may also react readily with H_2O_2 evolving in the presence of Cu(II). In our opinion, these suggestions require some further investigations.

4. Conclusions

The reaction between pyridoxal 5′-phosphate and hydrazides of 2-, 3-, and 4-pyridinecarboxylic acids were studied in aqueous solution. The stability constants as well as the rate constants of formation of hydrazones derived from nicotinic and picolinic acids hydrazides were determined experimentally at pH values of 1.9; 6.6; 7.0; and 7.4. Isoniazid was found to form the least stable Schiff bases with PLP at every studied pH value. In the acidic medium, nicotinic acid derivative forms the most stable hydrazone. In the pH range of 6.6–7.4, the stability of PLP-NH and PLP-PH hydrazones vary in the opposite way. The rate of PLP-INH and PLP-PH Schiff bases formation decreases significantly in the pH range of 6.6–7.4, while the k_1 value of PLP-NH remains nearly constant.

The PLP-INH, PLP-NH, and PLP-PH were isolated as solids and characterized by means of NMR, IR-spectroscopy and fluorimetry. The PLP-PH compound was found to fluoresce stronger than two other Schiff bases either in the solid phase and the solution (λ_{em} = 360 nm). The luminescence spectrum of PLP-INH hydrazone is similar with that of 2-isomer, however, it emits much weaker. The peculiarity of nicotinic acid hydrazide derivative is the additional weak emission in the long-wave range (505 nm).

The PLP-INH, PLP-NH, and PLP-PH hydrazones ability of preventing the copper-mediated ascorbate oxidation was studied. All the Schiff bases are efficient antioxidants, however the derivative of 2-isomer is the least protective agent. The protective activity of hydrazones does not change significantly in the physiological range of pH values (6.6–7.4).

The PLP-INH hydrazone forms the 1:1 coordination compound with copper(II) ions, which stability is lg K ~ 5. Despite the stability of Cu(II)-PLP-INH complex lower than that of Cu(II)-EDTA, Cu(II)-CA complexes, sodium ethylenediaminetetraacetate and citric acid are less effective in preventing the copper-mediated oxidation of ascorbate.

The hydrazones derived from hydrazides of 2-, 3-, and 4-pyridinecarbopylic acids and PLP could be considered as analogues of known biologically active compound, pyridoxal isonicotinoyl hydrazone. The latter is known as low-toxic, membranotropic [40], demetalling towards copper [19,41] (but not calcium/magnesium [42]), antioxidant [43]. They are easy to synthesize, sufficiently stable, capable of fluorescing (especially, PLP-PH), bind the copper ions efficiently, which make it possible to use them in medicine, *e.g.*, Wilson disease treatment. PLP-PH Schiff base could be also used *in vivo* as luminescent sensor.

The ability of PLP-INH, PLP-NH, and PLP-PH hydrazones to protect the ascorbic acid from oxidation is also valuable, since ascorbic acid, but not its oxidized form, dehydroascorbic acid, reduces the malignant potential of human melanoma [44]. Thus, the hydrazones could be

further studied as additives increasing the effectiveness of the intake of ascorbic acid at the treatment of cancer.

Conflict of interests

Authors declare none.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.molliq.2017.07.106.

References

[1] McCormick D.B. Biochemistry of coenzymes (in: Encyclopedia of Molecular Biology and Molecular Medicine/Ed. Meyers R.A.). Weinheim: VCH, 1996. T. 1. pp. 396–406.
[2] J.E. Leklem, Vitamin B-6, in: L. Machlin (Ed.), Handbook of Vitamins, Marcel Decker Inc., New York 1991, pp. 341–378.
[3] A. Ulvik, Ø. Midttun, E.R. Pedersen, S.J. Eussen, O. Nygård, P.M. Ueland, Evidence for increased catabolism of vitamin B-6 during systemic inflammation, Am. J. Clin. Nutr. 100 (1) (2014) 250–255, http://dx.doi.org/10.3945/ajcn.114.083196.
[4] J. Selhub, A. Byun, Z. Liu, J.B. Mason, R.T. Bronson, J.W. Crott, Dietary vitamin B6 intake modulates colonic inflammation in the IL10$^{-/-}$ model of inflammatory bowel disease, J. Nutr. Biochem. 24 (12) (2013) 2138–2143, http://dx.doi.org/10.1016/j.jnutbio.2013.08.005.
[5] E.-P. Chiang, J. Selhub, P.J. Bagley, G. Dallal, R. Roubenoff, Pyridoxine supplementation corrects vitamin B6 deficiency but does not improve inflammation in patients with rheumatoid arthritis, Arthritis Res. Ther. 7 (6) (2005) R1404–R1411, http://dx.doi.org/10.1186/ar1839.
[6] P.T. Clayton, B6-responsive disorders: a model of vitamin dependency, J. Inherit. Metab. Dis. 29 (2–3) (2006) 317–326, http://dx.doi.org/10.1007/s10545-005-0243-2.
[7] K. Moriwaki, Y. Kanno, H. Nakamoto, H. Okada, H. Suzuki, Vitamin B6 deficiency in elderly patients on chronic peritoneal dialysis, Adv. Perit. Dial. 16 (2000) 308–312 11045317.
[8] Y. Mikawa, S. Mizobuchi, M. Egi, K. Morita, Low serum concentrations of vitamin B6 and iron are related to panic attack and hyperventilation attack, Acta Med. Okayama 67 (2) (2013) 99–104 23603926.
[9] A.M. Hvas, S. Juul, P. Bech, E. Nexø, Vitamin B6 level is associated with symptoms of depression, Psychother. Psychosom. 73 (6) (2004) 340–343, http://dx.doi.org/10.1159/000080386.
[10] W.H. Pan, Y.P. Chang, W.T. Yeh, Y.S. Guei, B.F. Lin, I.L. Wei, F.L. Yang, Y.P. Liaw, K.J. Chen, W.J. Chen, Co-occurrence of anemia, marginal vitamin B6, and folate status and depressive symptoms in older adults, J. Geriatr. Psychiatry Neurol. 25 (3) (2012) 170–178, http://dx.doi.org/10.1177/0891988712458365.
[11] R.L. Vech, L. Lumeng, T.K. Li, Vitamin B6 metabolism in chronic alcohol abuse the effect of ethanol oxidation on hepatic pyridoxal 5′-phosphate metabolism, J. Clin. Invest. 55 (5) (1975) 1026–1032, http://dx.doi.org/10.1172/JCI108003.
[12] Centers for Disease Control and Prevention, Treatment of Tuberculosis, 52, American Thoracic Society, CDC, and Infectious Diseases Society of America, 2003 3–6 (No. RR-11). MMWR.
[13] M.E. Visser, C. Texeira-Swiegelaar, G. Maartens, The short-term effects of anti-tuberculosis therapy on plasma pyridoxine levels in patients with pulmonary tuberculosis, Int. J. Tuberc. Lung Dis. 8 (2) (2004) 260–262 15139457.
[14] P. Lainé-Cessac, A. Cailleux, P. Allain, Mechanisms of the inhibition of human erythrocyte pyridoxal kinase by drugs, Biochem. Pharmacol. 54 (8) (1997) 863–870, http://dx.doi.org/10.1016/S0006-2952(97)00252-9.
[15] P. Preziosi, Isoniazid: metabolic aspects and toxicological correlates, Curr. Drug Metab. 8 (2007) 839–851, http://dx.doi.org/10.2174/138920007782798216.
[16] J. Suarez, K. Ranguelova, A.A. Jarzecki, J. Manzerova, V. Krymov, X. Zhao, S. Yu, L. Metlitsky, G.J. Gerfen, R.S. Magliozzo, An oxyferrous heme/protein-based radical intermediate is catalytically competent in the catalase reaction of *Mycobacterium tuberculosis* catalase-peroxidase (KatG), J. Biol. Chem. 284 (2009) 7017–7029, http://dx.doi.org/10.1074/jbc.M808106200.
[17] H.C. Beyerman, J.S. Bontekoe, On the tuberculostatic activity of the thiazole carboxylic acid hydrazides, Recl. Trav. Chim. 72 (3) (1953) 262–268, http://dx.doi.org/10.1002/recl.19530720314.
[18] E. Kingsberg, The Chemistry of Heterocyclic Compounds, Pyridine and Its Derivatives, Part Three, Interscience Publishers, New York-London, 1962 227, http://dx.doi.org/10.1002/9780470186671.
[19] M. Hermes-Lima, M.S. Gonçalves, R.G. Andrade Jr., Pyridoxal isonicotinoyl hydrazone (PIH) prevents copper-mediated *in vitro* free radical formation, Mol. Cell. Biochem. 228 (2001) 73–82, http://dx.doi.org/10.1023/A:1013348005312.
[20] G.R. Echevarria-Gorostidi, A. Basagoitia, E. Pizarro, R. Goldsmid, J.G.S. Blanco, F.G. Blanco, Kinetic study of the reaction of pyridoxal 5′-phosphate with hydrazino compounds of pharmacological activity, Helv. Chim. Acta 81 (5) (1998) 837–844, http://dx.doi.org/10.1002/hlca.19980810505.
[21] G.R. Echevarría, A. Basagoitia, J.G. Santos, Blanco F. García, Determination of the rates of formation and hydrolysis of the Schiff bases formed by pyridoxal 5′-phosphate and hydrazinic compounds, J. Mol. Catal. A Chem. 160 (2) (2000) 209–215, http://dx.doi.org/10.1016/S1381-1169(00)00266-1.
[22] G.A. Gamov, M.N. Zavalishin, T.R. Usacheva, V.A. Sharnin, Effect of medium acidity on the thermodynamics and kinetics of the reaction of pyridoxal 5′-phosphate with isoniazid in an aqueous solution, Russ. J. Phys. Chem. A 91 (5) (2017) 843–849, http://dx.doi.org/10.1134/S0036024417050107.
[23] B.C. Revanasiddappa, E.V.S. Subrahmanyam, D. Satyanarayana, Synthesis and biological studies of some novel 2-azetidinones, Int. J. ChemTech Res. 2 (1) (2010) 129–132.
[24] M.J. Taghizadeh, H. Karimi, H.S. Abandansari, Vanadium–Schiff base complex-functionalized SBA-15 as a heterogeneous catalyst: synthesis, characterization and application in pharmaceutical sulfoxidation of sulfids, Res. Chem. Intermed. 42 (12) (2016) 8201–8215, http://dx.doi.org/10.1007/s11164-016-2589-5.
[25] M.F. Mouat, K.L. Manchester, The intracellular ionic strength of red cells and the influence of complex formation, Comp. Haematol. Int. 8 (1) (1998) 58–60, http://dx.doi.org/10.1007/BF02628107.
[26] T.J. Bruno, P.D.N. Svoronos, Handbook of Basic Tables for Chemical Analysis, 3rd ed. CRC Press, Boca Raton, 2011 441–470.
[27] http://www.nmrdb.org (accessed 20.04.2017).
[28] http://webbook.nist.gov/chemistry/vib-ser.html (accessed 20.04.2017).
[29] S.N. Gridchin, L.A. Kochergina, P.G. Konovalov, Stability constants of Cu(II) hydroxypropylenediaminetetraacetates, Russ. J. Coord. Chem. 29 (12) (2003) 868–870, http://dx.doi.org/10.1023/B:RUCO.0000008399.53700.43.
[30] G.R. Buettner, B.A. Jurkiewicz, Ascorbate radical: a valuable marker of oxidative stress, in: A.E. Favier, J. Cadet, B. Kalyanaraman, M. Fontecave, J.-L. Pierre (Eds.), Analysis of Free Radicals in Biological Systems, Birkhauser Verlag, Basel, Boston, Berlin 1995, pp. 145–164.
[31] Kato Y., Ozawa S., Miyamoto C., Maehata Y., Suzuki A., Maeda T., Baba Y. Acidic extracellular microenvironment and cancer. Cancer Cell Int. 2013, 13, Number of article 89, DOI: http://dx.doi.org/10.1186/1475-2867-13-89.
[32] V. Estrella, T. Chen, M. Lloyd, J. Wojtkowiak, H.H. Cornnell, A. Ibrahim-Hashim, K. Bailey, Y. Balagurunathan, J.M. Rothberg, B.F. Sloane, J. Johnson, R.A. Gatenby, R.J. Gillies, Acidity generated by the tumor microenvironment drives local invasion, Cancer Res. 73 (5) (2013) 1524–1535, http://dx.doi.org/10.1158/0008-5472.CAN-12-2796.
[33] Yu.P. Kitaev, B.I. Buzykin, Hydrazones, Nauka, Moscow, 1974 416 (In Russian).
[34] H.M. Schulman, M. Hermes-Lima, E.M. Wang, P. Ponka, In vitro antioxidant properties of the iron chelator pyridoxal isonicotinoyl hydrazone and some of its analogs, Redox Rep. 1 (1995) 373–378, http://dx.doi.org/10.1080/13510002.1995.11747014.
[35] P. Zhou, J. Zhang, Y. Zhang, Y. Liu, J. Liang, B. Liu, W. Zhang, Generation of hydrogen peroxide and hydroxyl radical resulting from oxygen-dependent oxidation of L-ascorbic acid via copper redox-catalyzed reactions, RCS Adv. 6 (2016) 38541–38547, http://dx.doi.org/10.1039/c6ra02843h.
[36] J. Piispanen, L.H.J. Lajunen, Complex formation equilibria of some aliphatic alpha-hydroxycarboxylic acids. 2. The study of copper(II) complexes, Acta Chem. Scand. 49 (1995) 241–247, http://dx.doi.org/10.3891/acta.chem.scand.49-0241.
[37] E.E. Kiss, M. Jezowska-Bojczuk, T. Kiss, Complexes of aminophosphonates part 9. Copper (II) complexes of citric acid derivatives, J. Coord. Chem. 40 (1–2) (1996) 157–166, http://dx.doi.org/10.1080/00958979608022854.
[38] T. Akbyk, I. Sönmezoğlu, K. Güçlü, I. Tor, R. Apak, Protection of ascorbic acid from copper(II)catalyzed oxidative degradation in the presence of fruit acids: citric, oxalic, tartaric, malic, malonic, and fumaric acids, Int. J. Food Prop. 15 (2) (2012) 398–411, http://dx.doi.org/10.1080/10942912.2010.487630.
[39] P.V. Bernhardt, P. Chin, D.R. Richarson, Unprecedented oxidation of a biologically active aroyldyrazone chelator catalyzed by iron(III): serendipitous identification of diacylhydrazine ligands with high iron chelation efficacy, J. Biol. Inorg. Chem. 6 (2001) 801–809, http://dx.doi.org/10.1007/s007750100258.
[40] G.M. Brittenham, Pyridoxal isonicotinoyl hydrazone. Effective iron chelation after oral administration, Ann. N. Y. Acad. Sci. 612 (1990) 315–326, http://dx.doi.org/10.1111/j.1749-6632.1990.tb24319.x.
[41] T.R. Rao, G. Singh, X-ray diffraction study of copper(II) complexes of pyridoxal isonicotinoyl hydrazone, Cryst. Res. Technol. 24 (10) (1989) 169–172, http://dx.doi.org/10.1002/crat.2170241019.
[42] D.R. Richardson, P. Ponka, Pyridoxal isonicotinoyl hydrazone and its analogs: potential orally effective iron-chelating agents for the treatment of iron overload disease, J. Lab. Clin. Med. 131 (4) (1998) 306–315, http://dx.doi.org/10.1016/S0022-2143(98)90180-9.

[43] M. Bhattacharya, P. Ponka, P. Hardy, N. Hanna, D.R. Varma, P. Lachapelle, S. Chemtob, Prevention of postasphyxia electroretinal dysfunction with a pyridoxal hydrazone, Free Radic. Biol. Med. 22 (1–2) (1997) http://dx.doi.org/10.1016/S0891-5849(96)00274-2.

[44] A.P. Fischer, S.L. Miles, Ascorbic acid, but not dehydroascorbic acid increases intracellular vitamin C content to decrease hypoxia inducible factor-1 alpha activity and reduce malignant potential in human melanoma, Biomed Pharmacother 86 (2017) 502–513, http://dx.doi.org/10.1016/j.biopha.2016.12.056.