

Synthesis and Biological Activity of the Pinostrobin Oxime Complex Compounds with Some *d*-Metals

A. A. Mashentseva^a, T. S. Seytembetov^a, S. M. Adekenov^b,
B. I. Tuleuov^b, O. P. Loiko^c, and A. I. Khalitova^c

^a Gumilev Eurasian National University, Munaitpasova ul. 5, Astana, 010008 Kazakhstan
e-mail: mashentseva.a@gmail.com

^b International Holding “Phytochemistry,” Karaganda, Kazakhstan

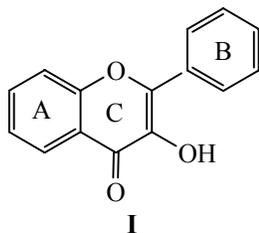
^c Buketov Karaganda State University, Karaganda, Kazakhstan

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Abstract—The process of the complex formation by the natural flavonoid pinostrobin (5-hydroxy-7-methoxyflavone) oxime with the Cu(II) and Fe(III) ions in a water–ethanol medium was investigated. The metal : ligand ratio in the complex was determined by the saturation method. The results of the analysis of IR spectra and the data of mass-spectrometric studies of the structure of the obtained complex compounds are reported. The antiviral activity of the synthesized complexes with respect to the human immunodeficiency virus HIV-1 IIIB and HIV-2 ROD strains was studied. The antioxidant activity of the complexes, as well as the parent pinostrobin oxime ligand, was evaluated with respect to the diphenylpicrylhydrazyl (DPPH) radical and the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical cation.

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In recent years the interest of both scientific community and pharmaceutical companies grew to the polyphenolic compounds of plant origin. Flavonoids that belong to the most widespread group of such compounds show a broad spectrum of biological activity and are widely used as drugs. The flavonoid molecule is underlain by the 2-phenylbenzo- γ -pyrone (**I**). A great variety of molecular structures among the natural flavonoids and a wide range of their biological activity can be attributed to the variety of the types and position of substituents. More than 4000 different biologically available flavonoids have been described [1].



The intensive study of complexation of natural flavonoids with the *d*-metal ions [2–4] indicates the importance of this area as one of the most promising in the development of new drugs based on natural

compounds. The transition metal ions are known to play an important role in the initiation of free radical processes [5], in particular, the Fenton reaction, resulting in the formation of hydroxyl radicals. Thus, the iron(III) ions act as a catalyst in the formation of lipid peroxides in the process of lipid peroxidation. Excess of such compounds causes development of oxidative stress resulting in many serious diseases. Copper is an essential element, a part of many vitamins, hormones, enzymes, and respiratory pigments, and is involved in metabolism, tissue respiration, etc. Copper-containing compounds (copper sulfate, Bordeaux mixture) are widely used to control pathogenic fungi and bacteria in agriculture [6, 7]. However, the high content of copper compounds in the body is very toxic to humans. Chelating the copper excess in the body by natural compounds can significantly reduce its toxicity, while does not remove its useful properties [8].

In addition to their direct antiradical effect, the flavonoids are capable of binding transition metal ions to form chelates. The formation of such complexes leads to inhibition of free radical processes. Due to their chelating properties, the dietary flavonoids can

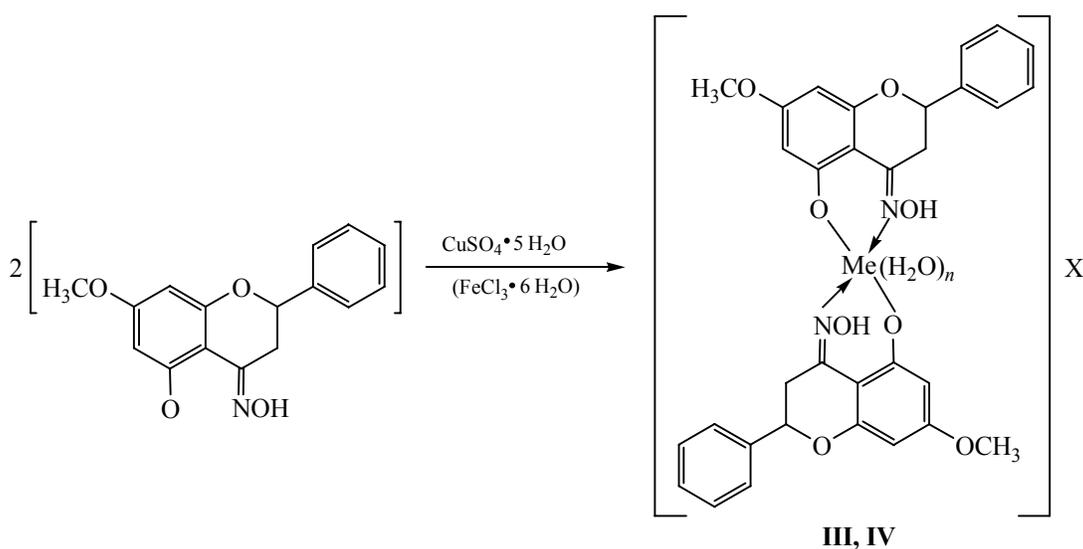
affect ionic balance of metals in the body and oxidative status of cells and tissues. Therefore, chelating of metals can be considered as a possible mechanism of antioxidant protection. A series of studies [9–11] showed that the formation by flavonoids of complex compounds significantly extends the range of their biological activity compared to the original flavonoids. In addition, the low risk of negative side effects, rapid absorption, low toxicity, which are an advantage of flavonoids, suggests the preservation of similar properties in the chelates.

In this study, we investigated a possibility of

complex formation by the flavonone derivative pinostrobin oxime (**II**) with copper(II) and iron(III) ions, and carried out a preliminary screening of biological activity using the PASS program.

As a result of performed reactions we found that iron(III) and copper(II) ions react with pinostrobin oxime in a water–ethanol medium to form complex compounds. The iron complex is purple, and copper complex is green.

Pinostrobin oxime reacts with the studied metal salts by the following scheme.



III, Me = Cu, X = SO₄·(H₂O)_n; **IV**, Me = Fe, X = Cl₃·(H₂O)_n.

Electron absorption spectra of the initial pinostrobin oxime in ethanol and the products of its reaction with iron(III) and copper(II) ions taken relative to the solvent in the wavelength range 300–800 nm differ from each other, which indicates formation of new compounds in the system.

Complex **IV** of iron(III) is characterized by two absorption maxima in the UV and visible ranges of the spectrum, at 360 (ε 355) and 535 nm (ε 17). The spectrum of complex compound **III** with copper(II) also contains two bands, with the absorption maxima at 360 (ε 355) and 610 nm (ε 168). In contrast to the compounds obtained, the organic reagent itself does not absorb in the visible region.

We found that the absorption of the solutions under study varies in time. Optical density decreases sharply in 5 min in the case of the copper(II) complex **III** and

in 10 min in the case of the iron(III) complex **IV**, and then remains almost constant. Taking into account these findings, we performed further studies after 5 and 10 min delay respectively after preparation of solutions.

Our experiments showed that pinostrobin oxime is poorly soluble in water but its solubility increases greatly in the presence of ethanol. The complex compounds of the oxime **II** with the metals also are limitedly soluble in water, so we used their water–ethanol solution. We found that both the studied compounds precipitate at the alcohol content less than 75%. In the case of complex **IV** with iron(III), at the increase in the alcohol concentration the absorption of the solution increases. The complex **IV** solubility at the ethanol content about 75% is 1.87×10⁻³ mol l⁻¹ or 0.30 g l⁻¹.

Table 1. Probability of the promising types of biological activity of compounds **II–IV** according to the PASS program^a

Activity type	II	III	IV
Antivirus	0.885 (0.016)	0.908 (0.009)	0.908 (0.009)
Antivirus (HIV)	0.885 (0.016)	0.908 (0.009)	0.908 (0.009)
Antivirus (Hepatitis B)	–	0.890	–
Apoptosis agonist	–	0.873 (0.004)	–
Antiepileptic	0.885 (0.016)	0.908 (0.009)	0.908 (0.009)
Antiinflammatory	0.885 (0.016)	0.908 (0.009)	0.908 (0.009)
Agonist of membrane integrity	0.885 (0.016)	0.908 (0.009)	0.908 (0.009)
Chemoprotector	0.885 (0.016)	0.908 (0.009)	0.908 (0.009)
Anesthetic	0.885 (0.016)	–	–
Radioprotector	0.885 (0.016)	0.908 (0.009)	0.908 (0.009)
Antipruritic	0.885 (0.016)	0.908 (0.009)	0.908 (0.009)
Anti-neurotoxic	0.783 (0.012)	0.842 (0.005)	0.842 (0.005)
NOS2 expression inhibitor	0.773 (0.008)	0.832 (0.004)	0.832 (0.004)
UGT1A9 substrate	–	0.782 (0.007)	0.782 (0.007)
Chlordecone reductase inhibitor	0.741 (0.038)	0.797 (0.026)	0.797 (0.026)
Ubiquinol–cytochrom reductase inhibitor	–	0.762 (0.056)	0.762 (0.056)
CYP1A inhibitor	–	0.754 (0.005)	0.754 (0.005)
UGT1A7 substrate	–	0.720 (0.004)	0.720 (0.004)

^a In parentheses the probability is shown of the absence of this type activity.

As to the complex **III** with copper(II) ions, the absorption of its solution increases with growing ethanol content to 85 vol %, and then decreases. Obviously, at this content of ethanol in the solution the complex is formed most completely. Further studies were performed at the ethanol concentration 85 vol %. Solubility of the complex **III** in such solution is $1.25 \times 10^{-3} \text{ mol l}^{-1}$ or 0.17 g l^{-1} .

Using the method of spectrophotometric saturation [16], we have determined the metal : ligand ratio in the complexes, which has been found equal to 1:2 for both compounds, in consistence with the mass spectrometric data: molar mass of complex **IV** is $640.15 \text{ g mol}^{-1}$, and of complex **III**, $924.38 \text{ g mol}^{-1}$.

The stability constants of the iron(III) and copper (II) complex compounds are $(7.11 \pm 0.01) \times 10^4$ and $(8.31 \pm 0.03) \times 10^6$, respectively, indicating a high stability of the complexes.

Preliminary to the study of the biological activity of the pinostrobin oxime complex compounds with metal ions we carried out a prediction of their biological

activity using the online version of the PASS program (Table 1) [17].

As seen from Table 1, in the case of formation of complex compounds **IV** with iron ions and **III** with copper ions, the probability to find biological activity increases. For the iron complex is found nine, and for the complex with copper six new types of activity of 575 possible, at 70% probability of their occurrence. The results of this screening suggest the presence of a wide spectrum of biological activity of the chelates.

Antiradical activity of these complexes was studied in respect to the DPPH (diphenylpicrylhydrazyl) radicals and ABTS^{•+} [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] radical cations.

Formation of a stable form due to protonation of the radical forms by active antioxidant leads to a change in the color of DPPH radical solution in alcohol from a intense purple to pale yellow. As a reference compound we used butylated hydroxyanisole (BHA) widely used in industry as an antioxidant reference. As shows the graphical dependence in Fig. 1, the anti-

radical activity is of concentration nature. The pinostrobin oxime complex with iron ions (Ox-Pb-Fe) shows a more positive value of antioxidant activity compared to the initial pinostrobin oxime (Ox-Pb) ligand. The complex compound with copper (Ox-Pb-Cu) shows minimal activity toward the studied radical.

The calculated values of effective thermal inhibition index IC_{50} (Table 2) indicates the minimum amount of respective compound required to inhibit 50% of the radical in the reaction mixture.

We also have studied the effect of the exogenous antioxidants (concentration of 0.5 mg ml^{-1}) on the process of inhibition of the radical cation $ABTS^{++}$ in time. The curves in Fig. 2 show that in the case of the reference substance (BHA) within 2 min occurs a sharp decrease in optical density of reaction medium and, consequently, a decrease in the content of the radical active form, and then it falls lineary.

Change in optical density of the pinostrobin oxime iron complex **IV** is more extreme, but after 5 min of the reaction, the absorption reaches the same values as that of the reference. Therewith, the complex **IV** shows much better results compared to the initial pinostrobin oxime ligand **II** and complex **III**.

To determine the effective coefficient of inhibition of the radical cation $ABTS^{++}$, we investigated the concentration dependence of inhibition in the concentration range $0.1\text{--}1.0 \text{ mg ml}^{-1}$. The calculated IC_{50} values are listed in Table 2. As can be seen from these data, the pinostrobin oxime iron complex **IV**

Table 2. The IC_{50} coefficients for the pinostrobin oxime complexes

Compound	DPPH	ABTS
Ox-Pb (II)	2.99	4.19
Ox-Pb-Fe (IV)	3.12	1.54
Ox-Pb-Cu (III)	26.61	110.2
BHA	0.315	0.039

shows lower values of effective thermal inhibition of free radicals compared with the original ligand and with the copper complex **III**, which indicates its higher antioxidant activity.

Several researchers [18, 19] indicate a high potential of chelate complexes of flavonoids as antiviral drugs and as the agents possessing anticancer activity. The obtained complex compounds of pinostrobin oxime with ions of iron and copper were also tested for antiviral activity. Table 3 lists the results of studies of the antiviral activity against strains of the human immunodeficiency viruses HIV-1 IIIB and HIV-2 ROD performed in accordance with [20].

The selectivity index SI ($av.IC_{50}/CC_{50}$) given in the Table 3 points to the activity of the compound with respect to the virus strain. The higher the value of this index, the more effective is the drug impact on the cells infected with MT-4. A comparative analysis of the pinostrobin oxime **II** and its complexes **III** and **IV** with iron and copper with the reference drugs

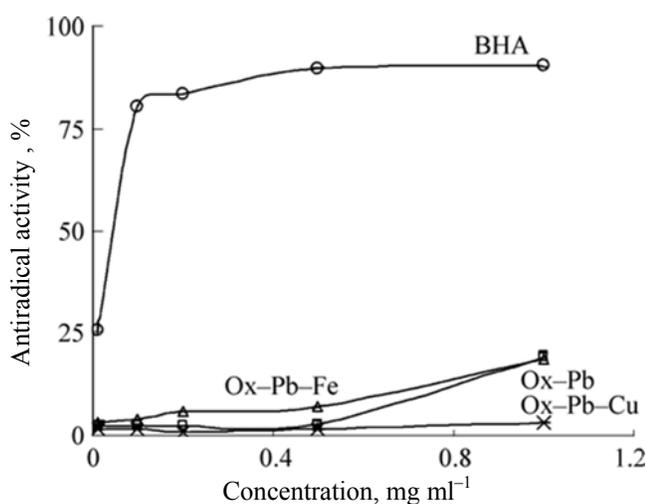


Fig. 1. Dynamics of the antiradical activity change of the pinostrobin oxime complexes.

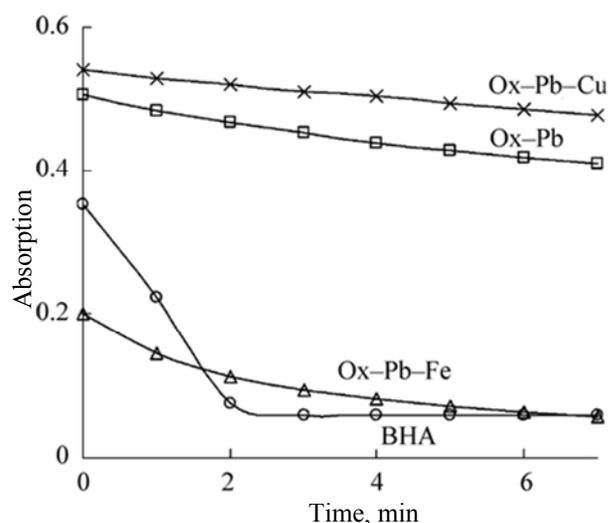


Fig. 2. Dynamics of inhibition of the $ABTS^{++}$ radical cation in time.

Table 3. Biological activity of the complexes against some HIV strains

Compound	Strain	av.IC ₅₀ ^a , Ug ml ⁻¹	av.CC ₅₀ ^b , Ug ml ⁻¹	SI
Ox–Pb (II)	IIIB	>0.25	0.25	<1
	ROD	>0.25	0.25	<1
Ox–Pb–Fe (IV)	IIIB	>0.35	0.35	<1
	ROD	>0.35	0.35	<1
Ox–Pb–Cu (III)	IIIB	>51.25	51.25	<1
	ROD	>51.25	51.25	<1
Nevirapin (NVP)	IIIB	0.050	>4.00	>80
	ROD	>4.00	>4.00	<1
Retrovir (AZT)	IIIB	0.0022	>25.00	>11587
	ROD	0.00094	>25.00	>26731

^a The average concentration at which the compound protects 50% of cells from exposure to the virus strain. ^b The average concentration at which the compound kills 50% of uninfected cells.

nevirapin and retrovir showed that the studied pinostrobin oxime complex compounds with metal ions do not possess antiviral activity against the studied HIV infection strains.

Analysis of the computer prediction data of biological activity of test substances using the PASS program in comparison with the data obtained from *in vitro* and *in vivo* studies revealed some discrepancy between theoretical calculations and experimental data, which may be due to the structural features of the compounds. It is noteworthy that the dependence between the increased probability of manifestation of biological activity going from the structure **II** to the structures of complexes **III** and **IV** seen in the table is partially confirmed in the study of the antiradical activity. Thus, the complex compound with iron ions shows better results in the inhibition of free radicals than the original pinostrobin oxime ligand. The very low activity of the complex with copper ions possibly relates to both the structural features of the ion chelator and a synergistic effect of the interaction with the flavonoid chelate.

EXPERIMENTAL

IR spectra were recorded on a Vector-22 instrument. Electron absorption spectra were recorded on a Specord UV-VIZ spectrophotometer. The optical

density of solutions was measured on a KFK-3 device.

Preparation of pinostrobin oxime II. A mixture of 200 mg of pinostrobin and 77 mg of hydroxylamine hydrochloride was dissolved in ethanol and 100 mg of NaHCO₃ was added to the mixture. The reaction was carried out at 60°C. After 4 h the mixture was treated with 1% HCl solution and water [2]. Colorless rhombic crystals were obtained, yield 84%, mp 182–184°C. IR spectrum (KBr), ν , cm⁻¹: 3431 (OH), 1647 (C=N), 1617, 1578 (C=C). UV spectrum, λ_{\max} , nm (ϵ , EtOH): 205 (23 442), 251 (4074), 279 (20 417).

Iron(III) solution. The solution of the concentration 5×10^{-2} mol l⁻¹ was prepared by dissolving accurately weighed FeCl₃·6H₂O in water [12]. The exact concentration of the resulting solution was determined using permanganatometric method [13].

Pinostrobin oxime complex with iron(III). Iron(III) chloride (0.2 ml of 5×10^{-2} M solution) was mixed with 2 ml of 1×10^{-2} M solution of compound **I** and 1.3 ml of 96% ethanol was added. The resulting solution is of violet color. After stirring electronic spectra of the solution were recorded relative to water in the wavelength range 300–800 nm. To isolate the substance in solid form, the solution was evaporated at room temperature. The resulting purple reaction product had mp 234–236°C. Yield 94%. IR spectrum (KBr), ν , cm⁻¹: 621.93, 485.15 (C–Fe). UV spectrum, λ_{\max} , nm (ϵ): 360 (355), 535 (17). Mass spectrum: C 61.89, H 5.04, Fe 8.72, N 4.37, O 19.98.

Pinostrobin oxime complex with copper(II). Copper(II) sulfate (0.2 ml of 5×10^{-2} M solution) was mixed with 2 ml of 1×10^{-2} M solution of compound **I** and 1.3 ml of 96% ethanol was added. The resulting solution is of green color. After stirring electronic spectra of the solution were recorded relative to water in the wavelength range 300–800 nm. To isolate the substance in solid form, the solution was evaporated at room temperature. The resulting green reaction product had mp 240–242°C. Yield 92%. IR spectrum (KBr), ν , cm⁻¹: 521.93, 485.15 (C–Cu). UV spectrum, λ_{\max} , nm (ϵ): 360 (355), 610 (168). Mass spectrum: C 42.88, H 5.67, Cu 6.87, N 3.03, O 38.08, S 3.47.

Inhibition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was carried out in accordance with the procedure [14]. An aliquot of the sample (0.1 ml) was added to 3 ml of 6×10^{-5} M solution of the radical in methanol. After vigorous stirring, the solution was left in the dark for 30 min. The change in the optical density was measured at 520 nm.

Interaction with 2,2'-azinobis-(3-ethylthiazolin-6-sulfonate cation radical (ABTS^{•+})) was investigated according to [15]. To generate the radical, 5 ml of 14 mM ABTS solution was mixed with 5 ml of potassium persulfate (0.0066 g in 5 ml of deionized water). The mixture was left for 16 h in a dark place. To determine the antiradical activity, a 10 μ l sample was added to 990 ml of working solution of the radical previously diluted with ethanol until the optical density of 0.700 ± 0.020 at 734 nm. The reduction in the optical density was measured at 734 nm after 1 min. The antiradical activity value (ARA) was calculated with the following formula:

$$\text{APA (\%)} = [(A_0 - A_\tau)/A_0] \times 100\%.$$

Here A_0 is optical density at $\tau = 0$, A_τ is optical density measured after 1 min keeping.

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