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SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF COPPER(II), NICKEL(II), COBALT(III) AND IRON(III) COORDINATION COMPOUNDS WITH 2-HYDROXY-3-METHOXYBENZALDEHYDE *N*(4)-ALLYL-*S*-METHYLISOTHIOSEMICARBAZONE

Irina USATAIA, Vasilii GRAUR, Victor TSAPKOV, Aurelian GULEA

State University of Moldova

The paper presents the synthesis of the 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone (HL) and seven coordination compounds of copper, nickel, cobalt and iron with this ligand. The new obtained compounds were studied using IR, ^{1}H and ^{13}C NMR spectroscopy, elemental analysis, molar electric conductibility and magnetic susceptibility. The *in vitro* antiproliferative activity of the HL and synthesized coordination compounds was screened on HeLa cancer cells and normal MDCK cells. The isothiosemicarbazone HL shows better anticancer activity towards HeLa cancer cells than doxorubicin that is used in medical practice and it also practically does not affect the growth and proliferation of normal cells. Moreover, it manifests high antioxidant activity towards $ABTS^{\bullet+}$ radical cation that exceeds the activity of trolox.

Keywords: *coordination compounds, 2-hydroxy-3-methoxybenzaldehyde, isothiosemicarbazone, antitumor activity, cancer cells, antioxidant activity.*

SINTEZA, CARACTERIZAREA ȘI ACTIVITATEA BIOLOGICĂ A COMPUȘILOR COORDINATIVI AI CUPRULUI(II), NICHELULUI(II), COBALTULUI(III) ȘI FIERULUI(III) CU *N*(4)-ALIL-*S*-METIL-IZOTIOSEMICARBAZONA 2-HIDROXI-3-METOXIBENZALDEHIDEI

Lucrarea conține descrierea sintezei *N*(4)-alil-*S*-metilizotiosemicarbazonei 2-hidroxi-3-metoxibenzaldehidei (HL) și a șapte compuși coordinativi ai cuprului, nichelului, cobaltului și fierului cu acest ligand. Compușii noi obținuți au fost studiați cu ajutorul spectroscopiilor IR și RMN (^{1}H și ^{13}C), analizei elementale, conductivității molare și susceptibilității magnetice. Cercetarea proprietăților antiproliferative *in vitro* ale ligandului și complecșilor a fost testată pe celule canceroase HeLa și pe celule normale MDCK. Izotiosemicarbazona HL arată o activitate anticancerigenă mai bună față de celulele canceroase HeLa decât doxorubicina care este utilizată în practica medicală și, de asemenea, practic nu afectează creșterea și proliferarea celulelor normale. Mai mult decât atât, HL manifestă o activitate antioxidativă ridicată față de cationul radical $ABTS^{\bullet+}$ care depășește activitatea troloxului.

Cuvinte-cheie: *compuși coordinativi, 2-hidroxi-3-metoxibenzaldehidă, izotiosemicarbazonă, activitate antitumorală, celule canceroase, activitate antioxidativă.*

Introduction

The intensive search of biologically active substances that can be used as medicine is conducted among thiosemicarbazone class of organic and coordination compounds [1-2]. The study of the relationship between the chemical structure of substances and their biological activity allows targeted synthesis of new drugs. Thiosemicarbazone class of organic substances and their coordination compounds attract constant scientific interest due to their antitumor activity [3]. The search for novel anticancer agents with better selectivity and lower toxicity continues to be an area of intensive investigation. The information about *N*(4)-substituted *S*-alkylisothiosemicarbazones is rather scarce, but a recent study showed that they possess biological activity [4-7].

The aim of this work is to find the conditions of synthesis and to determine the composition and physico-chemical properties of the copper, nickel, cobalt and iron coordination compounds with 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone.

Experimental materials and methods

N(4)-allyl-3-thiosemicarbazide was synthesized by the reaction between allyl isothiocyanate and hydrazine hydrate [8]. 2-Hydroxy-3-methoxybenzaldehyde (Sigma-Aldrich), metal salts were used as received.

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The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400, using $CDCl_3$ as a solvent. The chemical shifts (δ) in ppm were measured relative to tetramethylsilane (TMS). Infrared spectra of the compounds were recorded on a Bruker ALPHA FTIR spectrophotometer at room temperature in the range of 4000-400 cm^{-1}.

Magnetochemical research was made at room temperature using Gouy method.

The determination of metal content in the synthesized coordination compounds, using titration methods, was performed similarly to the literature procedures [9-12].

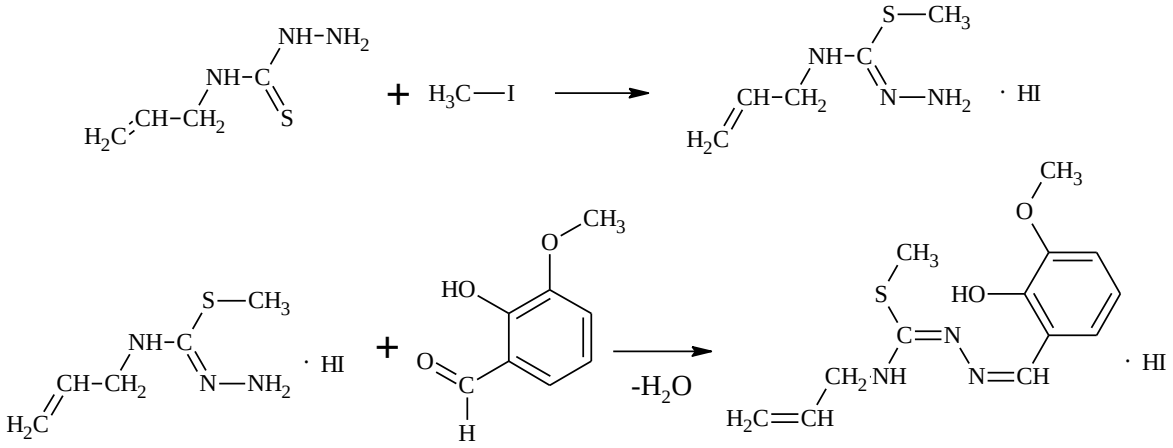
Melting point of the free ligand was measured using capillary method.

Molar conductivity values were determined in 10^{-3} mol/L methanol solutions using slidewire bridge R-38.

Synthesis of the 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone (HL)

2-Hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone (**HL**) **(Scheme 1)** was prepared according to a modification of the procedure described in the literature [13].

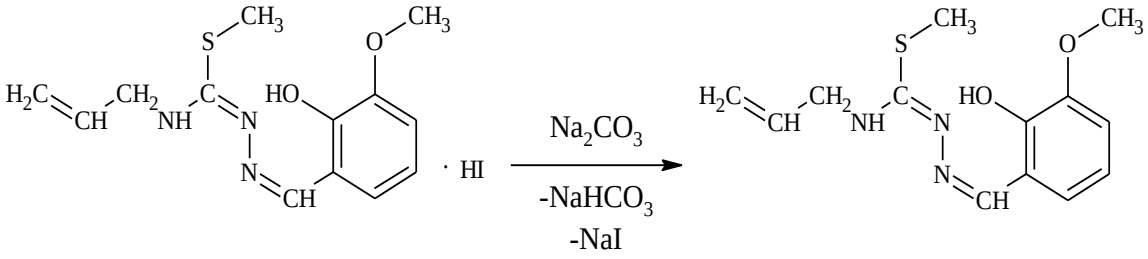
N(4)-Allyl-3-thiosemicarbazide (1.31 g, 10 mmol) was dissolved in 20 mL of ethanol with constant stirring. After that iodomethane (1.56 g, 11 mmol) was added. The mixture was stirred at room temperature for 2 hours and 2-hydroxy-3-methoxybenzaldehyde (1.52 g, 10 mmol) was added. The solution was stirred at 60 ^{0}C for 30 min. After the reaction the mixture was cooled to room temperature, the yellow solid was isolated by filtration, washed with ethanol and dried *in vacuo*.



Scheme 1. Synthesis of 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone hydroiodide.

Sodium carbonate (1.06 g, 10 mmol) was added to the solution of 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone hydroiodide (4.21 g, 10 mmol).

After the reaction mixture was cooled to room temperature, the 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone was extracted with chloroform from the reaction mixture. After evaporation the yellow solid was obtained (**Scheme 2**).



Scheme 2. Neutralization of 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone hydroiodide.

Yellow solid. Yield: 75%; m.p.: 72-74 ^{0}C; FW: 279.36 g/mol;

Main IR peaks (cm^{-1}): ν(OH) 3393, ν(C=C allyl) 1647, ν(C=N^1) 1598, ν(C–O) 1244, ν(CH_3–S) 1072, ν(C–S) 682.

^{1}H-NMR and ^{13}C-NMR (Hydrogen and Carbon Nuclear Magnetic Resonance) Spectra

1st tautomeric form (**HL**(A) on **Scheme 3**): ^{1}H NMR ($CDCl_3$; δ, ppm): 11.99 (s, 1H, OH); 8.38 (s, 1H, CH=N); 6.86 (m, 3H, CH aromatic); 4.46 (br, 1H, NH); 5.97 (m, 1H, CH from allyl moiety); 5.23 (m, 2H, CH_2=C); 4.08 (t, 2H, CH_2-N); 3.90 (s, 3H, OCH_3); 2.40 (s, 3H, CH_3).

^{13}C NMR ($CDCl_3$; δ, ppm): 161.63 (C-S); 155.15, 148.17, 122.58, 119.14, 118.66, 117.31 (C aromatic); 148.53 (CH=N); 133.84 (CH from allyl moiety); 112.79 (CH_2=); 56.06 (OCH_3); 45.97 (CH_2-N); 13.18 (CH_3).

2nd tautomeric form (**HL**(B) on **Scheme 3**): ^{1}H NMR ($CDCl_3$; δ, ppm): 11.75 (s, 1H, OH); 8.44 (s, 1H, CH=N); 6.86 (m, 3H, CH aromatic); 5.84 (m, 1H, CH from allyl moiety); 5.22 (m, 2H, CH_2=C); 5.65 (br, 1H, NH); 3.89 (d, 2H, CH_2-N); 3.91 (s, 3H, OCH_3); 2.48 (s, 3H, CH_3).

^{13}C NMR ($CDCl_3$; δ, ppm): 161.97 (C-S); 156.79, 148.26, 122.83, 118.92, 118.83, 117.18 (C aromatic); 158.41 (CH=N); 133.75 (CH from allyl moiety); 112.99 (CH_2=); 56.06 (OCH_3); 45.93 (CH_2-N); 13.15 (CH_3).

Synthesis of coordination compounds

The complexes **I-II** were obtained by stirring a hot solution of **HL** in ethanol with the corresponding copper in 1:1 molar ratio: $Cu(NO_3)_2{\cdot}3H_2O$ (**I**), $CuCl_2{\cdot}2H_2O$ (**II**). Nickel, cobalt and iron coordination compounds (**III-V**, **VII**) were synthesized similarly, but in 1:2 molar ratio: $Ni(NO_3)_2{\cdot}6H_2O$ (**III**), $Co(NO_3)_2{\cdot}6H_2O$ (**IV**), $CoCl_2{\cdot}6H_2O$ (**V**), $Fe(NO_3)_3{\cdot}6H_2O$ (**VII**). The complex (**VI**) was obtained by stirring a hot solution of **HL**·HI in ethanol with the cobalt acetate salt $Co(CH_3COO)_2{\cdot}4H_2O$ in 1:2 molar ratio. After cooling green (in case of complexes **I-II**) or brown (in case of complexes **III-VII**) precipitates of corresponding coordination compounds were filtered, washed with small amounts of cold ethanol and dried.

Biological studies

***In vitro* antiproliferative activity**

Cell Cultures

Human cervical epithelial cells of line HeLa and Madin Darby Canine Kidney epithelial normal cells of line MDCK were used in this study. Cell lines HeLa and MDCK were cultured in the Dulbecco's Modified Essential Medium (DMEM) with L-glutamine (4 mM), glucose (4.5 g/L), bovine albumin fraction (0,2% v/v), HEPES buffer (N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid) (20 mM), antibiotics penicillin-streptomycin (final concentration 100 U/ml penicillin and 100 μg streptomycin /ml) and supplemented with FBS (10% v/v). Cells were maintained at 37ºC in a 2-5% humidified CO_2 atmosphere in the incubator in 75-cm^2 culture dishes, and used for experiments between passage 5 and 16. The compounds were dissolved at the time of the experiments.

Cell proliferation Resazurin assay

Cells of lines HeLa, MDCK were trypsinized Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) 0.05% (Invitrogen) and counted under an inverted microscope (OLYMPUS). The cell proliferation assay was performed using resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) (SIGMA), which allowed us to measure the number of viable cells.

In brief, plate out, in triplicate of $1{\cdot}10^4$ cells in a total of 100 μl medium in 96-well microtiter plates (Becton Dickinson and Company, Lincoln Park, NJ, USA) were incubated at 37 °C, 2% CO_2. Compounds were dissolved in dimethyl sulfoxide to prepare the stock solution of 10mM. These compounds and doxorubicin was diluted at multiple concentrations with culture media, added to each well and incubated for 24 hours. Following each treatment, 20 μL resazurin indicator solution was added to each well and incubated for 4 hours. Subsequently, the absorbance was read with 570 nm and 600 nm filters. The measurement was made by imaging hybrid reader (Synergy H1, Biotek).

The percentage inhibition was calculated according to the formula:

$100-((Abs570nm_{\ sample}-Abs\ 600nm_{\ sample})/(Abs570nm_{\ control}-Abs\ 600nm_{\ control})\times 100)$

The IC_{50} values were evaluated by statistical software.

Antioxidant activity

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The antioxidant activity by the $ABTS^{\bullet+}$ method was assessed according to the method described by Re et al. [14] with modifications. The $ABTS^{\bullet+}$ radical cation was formed through the reaction of ABTS solution 7 mM with potassium persulfate solution 140 mM, incubated at 25 °C in the dark for 12–16 hours. Once formed, the $ABTS^{\bullet+}$ solution was diluted with acetate buffered saline (0,02M, pH 6,5) to give an absorbance of 0.7 ± 0.01 at 734 nm.

Dilutions of trolox and tested substances were prepared in DMSO. After that, 20 µL of each dilution were transferred in a 96 wells microtitre plate and 180 µL of working solution of $ABTS^{\bullet+}$ were dispensed with dispense module of hybrid reader (BioTek), shake 15 s. The decrease in absorbance at 734 nm was measured exactly after 30 min of incubation at 25 °C. Blank samples were run by solvent without $ABTS^{\bullet+}$.

The decrease in absorbance is expressed as % inhibition, which is calculated from the following formula:

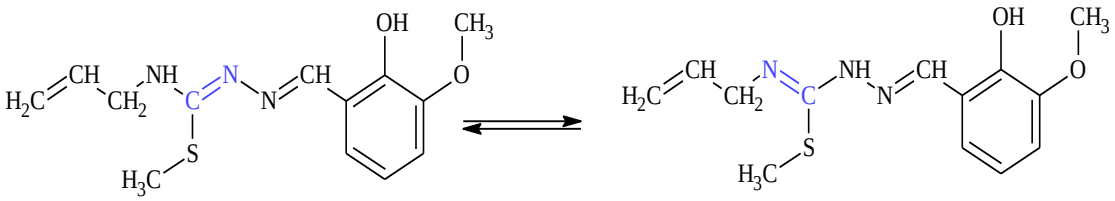
$$((Abs_{control}–Abs_{sample})/Abs_{control})\cdot 100$$

Results and discussion

The free ligand **HL** and seven new metal complexes $Cu(L)NO_3\cdot 2H_2O$ (**I**), $Cu(L)Cl\cdot H_2O$ (**II**), $Ni(L)_2$ (**III**), $Co(L)_2NO_3$ (**IV**), $Co(L)_2Cl$ (**V**), $Co(L)_2I$ (**VI**) and $Fe(L)_2NO_3$ (**VII**) were synthesized in ethanol in good yield.

The structure and purity of **HL** were determined by ^1H and ^{13}C NMR spectroscopy. The alkylation of sulfur atom is proved by the comparative analysis of 2-hydroxy-3-methoxy-benzaldehyde *N*(4)-allylthiosemicarbazone and *N*(4)-allyl-*S*-methylisothiosemicarbazone NMR spectra.

In the NMR spectra all peaks of isothiosemicarbazone **HL** are double [15]. It indicates the presence of tautomeric forms of isothiosemicarbazone in solution. The presence of tautomeric forms can be caused by *syn/anti* isomerism around $C=N^1$ double bond, and *cis/trans (Z/E)* isomerism around $C=N^4$ double bond (**Scheme 4**) [16].



Scheme 3. The tautomeric forms of the isothiosemicarbazone **HL**

The integral ratio between two tautomeric forms is 1:0.4 (**HL**(A):**HL**(B)).

All complexes were prepared by the direct reaction between **HL** and the corresponding metal salts. The syntheses of the coordination compounds are reproducible with good yields. The obtained compounds are microcrystalline solids which are stable in the air.

The elemental analyses on copper, nickel, cobalt and iron suggest the general formulae $Cu(L)X\cdot nH_2O$ ($X=Cl^-$, NO_3^-; n=1-2), $Ni(L)_2$ and $M(L)_2X$ ($M= Co^{3+}$, Fe^{3+}; $X=Cl^-$, I^-, NO_3^-).

Table 1

Physical and analytical data of the metal complexes (I-VII)

No.	Compound	Formula	η[a], %	Found / calculated, metal %	μ_{eff}[b], B.M.	λ[c]
I	$Cu(L)NO_3\cdot 2H_2O$	$C_{13}H_{20}CuN_4O_7S$	81	14.27/14.44	1.90	109
II	$Cu(L)Cl\cdot H_2O$	$C_{13}H_{18}ClCuN_3O_3S$	88	16.18/16.20	1.87	103
III	$Ni(L)_2$	$C_{26}H_{32}N_6NiO_4S_2$	76	9.47/9.54	2.82	7
IV	$Co(L)_2NO_3$	$C_{26}H_{30}CoN_7O_7S_2$	79	7.28/7.40	dia[d]	93
V	$Co(L)_2Cl$	$C_{26}H_{32}ClCoN_6O_4S_2$	87	9.01/9.05	dia[d]	90
VI	$Co(L)_2I$	$C_{26}H_{32}CoIN_6O_4S_2$	85	7.86/7.94	dia[d]	82

VII	$Fe(L)_2NO_3$	$C_{26}H_{30}FeN_7O_7S_2$	78	8.01/8.21	5.71	112

a – yield; b – effective magnetic moments at room temperature (293K); c – molar conductivity in methanol at room temperature, $\Omega^{-1}\cdot cm^2\cdot mol^{-1}$; d – diamagnetic.

Thermal analyses data reveal that compounds **I-II** are hydrated [17]. The molar conductivity values of the copper, cobalt and iron coordination compounds (**I-II, IV-VII**) are in the range 82 – 112 $\Omega^{-1}\cdot cm^2\cdot mol^{-1}$ in methanol that indicates that all these complexes represent 1:1 electrolytes [18]. The molar conductivity value of the nickel coordination compound **III** is very low (7 $\Omega^{-1}\cdot cm^2\cdot mol^{-1}$), suggesting that the complex is non-electrolyte.

The elemental analyses data of the ligand and complexes (reported in Experimental Section) are in agrement with structure of the ligand and with structures of the complexes, respectively. The corresponding anion (Cl^-, I^-, NO_3^-) can be either in the outer sphere or in the inner sphere as it can be easily substituted by the solvent molecule during dissolution process.

The magnetochemical research showed that the synthesized copper coordination compounds **I-II** have monomeric structure because the effective magnetic moments for the synthesized complexes **I-II** vary in the range of 1.87-1.90 B.M. which are close to the spin value for one unpaired electron. The nickel, cobalt and iron coordination compounds have octahedral structure. The cobalt complexes **IV-VI** are diamagnetic that indicates that cobalt(II) is oxidized by oxygen from air to cobalt(III) during the synthesis.

The iron complex **VII** is paramagnetic with effective magnetic moment value 5.71 B.M. The iron ion has d^5-electrons in the t_{2g} and e_g orbitals, that corresponds to high-spin state, while the cobalt complexes **V-VII** have all six electrons in the t_{2g} level, that corresponds to low-spin state. It is also found that **HL** represents an average-field ligand.

The isothiosemicarbazone **HL** coordinates the metal ions as a mononegative tridentate ligand with ONN-set of donor atoms. The type of the ligand coordination with the central ions was elucidated from a comparative analysis of IR spectra of complexes **I-VII** and the isothiosemicarbazone **HL** [19]. It coordinates the central ions by deprotonated phenolic oxygen atom, azomethinic and thiocarbamide nitrogen atoms forming five- and six-membered metallacycles. The proposed distribution of chemical bonds in the coordination compounds is shown in **scheme 4**.

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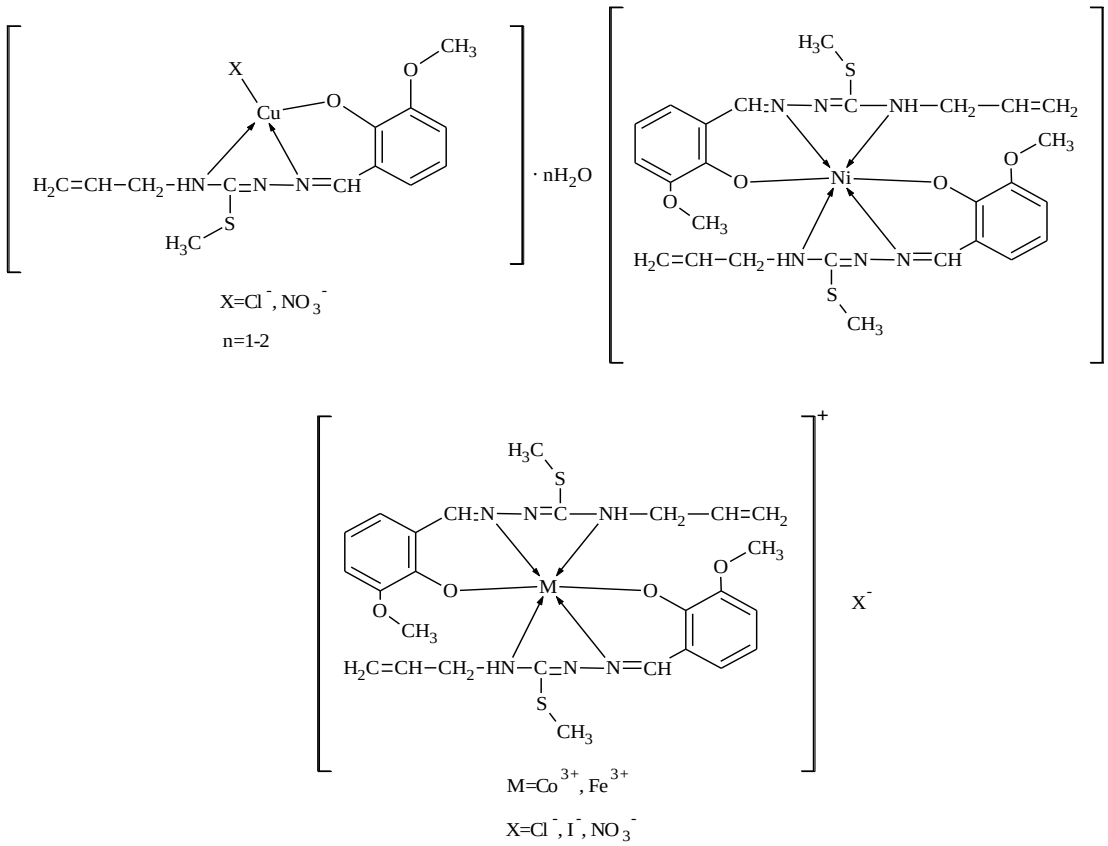

Scheme 4. Proposed distribution of chemical bonds in the metal complexes.

The antitumor activity of the free ligand and complexes on HeLa cancer cells and normal MDCK cells was studied.

In order to find out the biological properties the antitumor activity of the 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone (**HL**) and its coordination compounds were studied. From the obtained data of HeLa cancer cells and normal MDCK cells inhibition the IC_{50} value of tested substances was calculated.

Table 2

Antiproliferative activity of some synthesized compounds on cervical cancer (HeLa) cells at three concentrations and their IC_{50} values

Compound	Inhibition of cell proliferation (%)[a]			IC_{50}, μM
	10 μM	1 μM	0.1 μM	
HL	65.1	16.9	6.7	5.2
HL·HI	69.1	22.4	2.4	4
I	100	13.3	1.3	1.3
II	100	35	0	1.1
III	34.7	9.6	2.2	≥10
IV	21.7	17	3.4	≥100
V	8.7	0	0	≥100
VI	16.9	9.7	0.8	≥100
VII	0	0	0	≥100
Doxorubicin	49.8	12.2	0	10.0

[a]SEM < ±4% of a single experiment in triplicate. The IC_{50} values were calculated using statistical software.

The study of cervical cancer HeLa cells proliferation in the presence of 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone showed that this isothiosemicarbazone inhibits the proliferation of these cells by 65.1-69.1 % at the concentration 10 μM, but at the concentration 1 μM activity practically disappears. The nickel and cobalt complexes **III-VI** has insignificant inhibitor activity. The iron complex **VII** do not inhibit HeLa cancer cells at studied concentrations 10-0.1 μM.

The complexes **I-II** and isothiosemicarbazone **HL** manifest better activity than doxorubicin that is used in medical practice.

Since the main drawback of substances with anti-cancer properties is their toxicity, then for synthesized substances it is necessary to determine the selectivity of their action on cancer cells. For this purpose, the cytostatic effect of these substances was studied on normal MDCK cells.

The experiment showed that **HL** and **HL**·HI do not inhibit proliferation of these cells (Table 3). The free ligand, cobalt and iron coordination compounds do not represent a danger to normal MDCK cells, their IC_{50} values are more than 100 μM.

Table 3

Antiproliferative activity of some synthesized compounds on normal MDCK cells at three concentrations and their IC_{50} values

Compound	Inhibition of cell proliferation (%)[a]			IC_{50}, μM
	10 μM	1 μM	0.1 μM	
HL	0	0	0	≥100
HL·HI	0	0	0	≥100
I	67.5	21.7	0	4
III	23	16.7	0	30
IV	0	0	0	≥100
V	8.3	2.5	0	≥100
VI	0	9.8	10.3	≥100
VII	10.7	0	0	≥100
Doxorubicin	-	56.0	25.1	19.1

[a]SEM < ±4% of a single experiment in triplicate. The IC_{50} values were calculated using statistical software.

The study of the influence of synthesized compounds on MDCK cells, showed that they have lower cytotoxic effect on normal cells. The isothiosemicarbazone **HL** showed promising antiproliferative activity and low toxicity.

Moreover, the antioxidant properties of the synthesized compounds were studied using the $ABTS^{\bullet+}$ method. Almost all studied substances manifest higher antioxiant activity than trolox, which is used in medical practice as standard antioxidant. The isothiosemicarbazone **HL** and its salt **HL**·HI show better antioxidant activity than the corresponding coordination compounds. The activity of coordination compounds depends on the nature of the central atom and acid residue and increases in the following way: Cu<Co<Ni<Fe. The antioxidant activity of the most active substance from this series exceeds 4.3 times the activity of trolox (Table 4).

Table 4

IC_{50} values of some of the synthesized substances towards $ABTS^{\bullet+}$ radical cation

Compound	IC_{50}, μM
HL	8.7
HL·HI	7.7
I	38.8
II	20.7
III	15.4
IV	20.0
V	23.1
VI	19.1

VII	9.6

Conclusion

In this work 2-hydroxy-3-methoxybenzaldehyde *N*(4)-allyl-*S*-methylisothiosemicarbazone was synthesized and studied using NMR spectroscopy. This free ligand was used for synthesis of seven coordination compounds of copper, nickel, cobalt and iron. The new obtained compounds were investigated by IR, ^{1}H and ^{13}C NMR spectroscopy, elemental analysis, molar electric conductibility and magnetic susceptibility. It was determined, that the **HL**, **HL**·HI and coordination compounds show antiproliferative activity.

The synthesized compounds selectively inhibit the growth of HeLa cancer cells in the range of concentrations 10^{-5}-10^{-7} mol/L. The free ligand and copper coordination compounds **I-II** manifest higher activity than doxorubicin. The study of the influence of these compounds on MDCK cells, showed that they have lower cytotoxic effect on normal cells, therefore are of interest in further study. Moreover, the isothiosemicarbazone **HL** and corresponding coordination compounds manifests high antioxidant activity towards $ABTS^{\bullet+}$ radical cation that exceeds the activity of trolox.

Acknowledgements

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Date despre autori:

Irina USATAIA, doctorandă, Şcoala doctorală *Ştiinţe Chimice*, USM.
E-mail: departamentchimie@mail.ru

Vasilii GRAUR, doctor în ştiinţe chimice, cercetător ştiinţific în Laboratorul de cercetări ştiinţifice *Materiale Avansate în Biofarmaceutică şi Tehnică*, USM.

Victor TSAPKOV, doctor în ştiinţe chimice, conferenţiar universitar, Facultatea de Chimie și Tehnologie Chimică, USM.

Aurelian GULEA, doctor habilitat, profesor universitar, academician al AŞM.

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