

MICROBIOLOGIA ȘI BIOTEHNOLOGIA**SYNTHESIS AND BIOLOGICAL ACTIVITY OF
2-FORMYLPYRIDINE N(4)-ALLYL-3-SELENO-SEMICARBAZONE
AND ITS COPPER COORDINATION COMPOUND****Graur Vasilii¹, Garbuz Olga¹, Zariciuc Elena²,
Gudumac Valentin², Gulea Aurelian¹**¹*State University of Moldova*²*State University of Medicine and Pharmacy "Nicolae Testemitanu"***Rezumat**

Această lucrare prezintă sinteza N(4)alil-3-selenosemicarbazonei 2-formilpiridinei, care a fost studiată folosind spectroscopia RMN (¹H și ¹³C). A fost sintetizat compusul complex de clorură de cupru(II) cu acest ligand. Pentru compușii sintetizați a fost studiată activitatea biologică. Aceste substanțe manifestă activitate bacteriostatică și bactericidă împotriva unui șir de bacterii gram-pozitive, gram-negative și fungi în intervalul de concentrații 0.7-500μg/mL. Activitatea compusului complex de cupru împotriva microorganismelor gram-negative este mult mai mare decât cea a ligandului. Proprietățile antioxidante ale selenosemicarbazonei sintetizate depășesc proprietățile antioxidante ale complexului. Compușii sintetizați de asemenea inhibă proliferarea celulelor canceroase MeW-164, HL-60 și HeLa.

Cuvinte cheie: selenosemicarbazone, complecși de cupru, activitate antimicrobiană, activitate antioxidantă, activitate anti-cancer

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Introduction

Despite advances in the sphere of new drug synthesis, the synthesis of new substances with high biological activity remains an actual problem of modern chemistry. In recent years coordination compounds of biometals with thiosemicarbazones have been actively researched for solving this problem [2, 9, 11, 16].

From the literature source it is known that in many cases enhancement of biological activity is observed when going from thiosemicarbazones to the corresponding selenosemicarbazones [1, 3]. Therefore, the aim of this work was the synthesis and study of biological properties of 2-formylpyridine N(4)-allyl-3-seleno-semicarbazone and its copper(II) coordination compound.

Materials and methods**General**

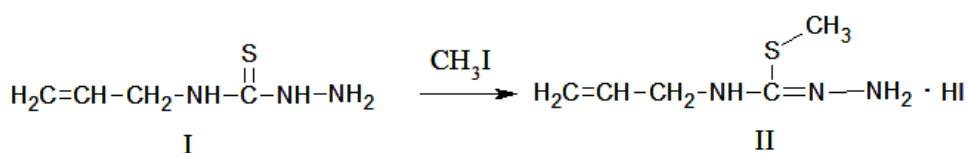
All commercially available reagents and chemicals were of analytical- or reagent-grade purity and used as received. The ¹H and ¹³C NMR spectra of the ligand were determined in DMSO-d₆ at room temperature using Bruker DRX-400 spectrometer. All chemical shifts (¹H, ¹³C) are given in ppm versus SiMe₄.

Quantitative analysis on copper was made using titration method. Classic methods were applied for C, H and N elemental analyses. Melting point of the ligand was measured using capillary method. Molar conductivity value was determined in 10^{-3} mol/L methanol solution using slidewire bridge R-38.

2-formylpyridine N(4)-allyl-3-selenosemicarbazone (H_2L) synthesis

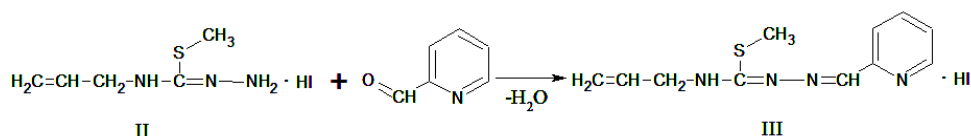
The synthesis of 2-formylpyridine N(4)-allyl-3-selenosemicarbazone was performed starting with N(4)-allyl-3-thiosemicarbazide that was obtained by the reaction between allyl isothiocyanate and hydrazine hydrate as described below [15]. For substitution of sulfur atom by a selenium atom it was used general method [6, 7], but with some modifications.

At first, N(4)-allyl-3-thiosemicarbazide was alkylated using iodomethane. Iodomethane (1.56g, 11mmol) was added to the ethanolic solution of N(4)-allyl-3-thiosemicarbazide (1.31g, 10mmol). The resultant mixture was magnetically stirred at room temperature for 2h.



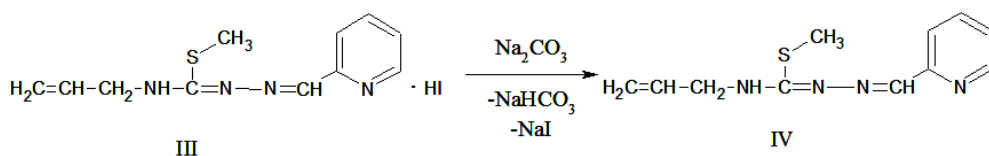
Scheme 1. The reaction of alkylation

After that 2-formylpyridine (1.07g, 10mmol) was added to the reaction mixture. The reaction mixture was heated and stirred for 40min on a magnetic stirrer. After cooling, the yellow precipitate of the synthesized substance appeared. It was filtered out from the solution and dried.



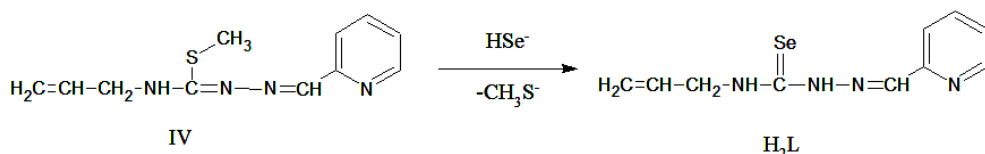
Scheme 2. The reaction of condensation.

The filtered substance (III) was dissolved in ethanol. Aqueous solution of equimolar amount of sodium carbonate was added in order to neutralize hydroiodide.



Scheme 3. The reaction of hydroiodide (III) neutralization.

The resultant substance (IV) was extracted into chloroform. On the last step S-methyl group was substituted by selenium atom.



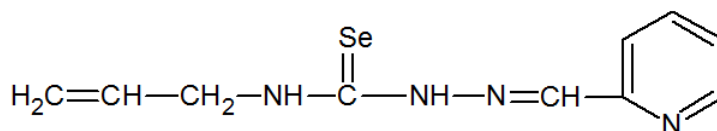
Scheme 4. The reaction of S-methyl group substitution.

The solution of sodium hydrogen selenide was prepared by combining selenium powder (0.119g, 1.5mmol) with sodium borohydride (0.151, 4.0mmol) in ethanol under argon. After that, a single portion of ethanolic solution of substance (IV) (0.234g, 1.0mmol) in ethanol was added to the solution of sodium hydrogen selenide. The resultant solution was stirred and heated for 30 hours. The product was extracted into chloroform. After distillation, the orange powder of 2-formylpyridine N(4)-allyl-3-selenosemicarbazone (H₂L) was obtained. Its purity was proved by melting point and NMR spectroscopy (¹H and ¹³C).

Melting point: 210-212°C

¹H NMR (DMSO-d₆, δ (ppm)): 12.07 (br, 1H, NH); 9.28 (br, 1H, NH); 8.59 (d, CH aromatic); 8.31 (d, CH aromatic); 8.22 (s, 1H, CH=N); 7.90(t, 1H, CH aromatic); 7.44 (t, 1H, CH aromatic); 5.93 (m, 1H, CH from allyl moiety); 5.16 (m, 2H, CH₂=C); 4.33 (m, 2H, CH₂-N).

¹³C NMR (DMSO-d₆, δ (ppm)): 176.35 (C=Se); 153.16, 149.44, 137.70, 124.94, 121.35 (C aromatic); 143.41 (CH=N); 135.04 (CH from allyl moiety); 116.45 (CH₂=); 48.88 (CH₂-N).



Scheme 5. H₂L ligand.

Synthesis of copper(II) chloride coordination compound with H₂L (Cu(HL)Cl)

Coordination compound Cu(HL)Cl was synthesized by reaction between CuCl₂·2H₂O and H₂L. H₂L (0.267g, 1mmol) was dissolved in 10mL of ethanol. After that the solution of CuCl₂·2H₂O (0.171g, 1mmol) in 10mL of ethanol was added to the solution of H₂L. The resultant mixture was heated and stirred on a magnetic stirrer for 40min. After cooling and slow evaporation at room temperature the green precipitate of the synthesized coordination compound appeared. It was filtered out from the solution and dried.

Elemental analysis found for C₁₀H₁₁ClCuN₄Se: C, 32.5; H, 3.2; N, 15.5; Cu, 17.1; calculated: C, 32.9; H, 3.0; N, 15.3; Cu, 17.4%. Molar conductivity: 87 Ω⁻¹·cm²·mol⁻¹.

Antimicrobial and antifungal activities. Antimicrobial and antifungal activities were tested on a series of standard strains of gram-positive, gram-negative microorganisms and fungi. It was studied in vitro using the method of two-fold serial

dilutions in liquid nutrient medium (meat infusion broth, pH = 7.0). [13] The substances were dissolved in DMSO (dimethylsulfoxide) in such amounts that the solutions of concentration 10 mg/mL were obtained. The next dilutions were made using meat infusion broth.

Antitumor activity. Antitumor activity was tested on a series of tumor cells: human leukemia HL-60 cells, cervical cancer HeLa cells and melanoma MeW-164 cells.

Cell proliferation assay of HL-60 cells. The cell proliferation assay was performed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Cell Titer 96 Aqueous, Promega, USA) [4, 5, 10]

Cell proliferation assay of HeLa cells. Cells 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, triplicates of 1×10^4 cells in a total of 100 μ l medium in 96-well microtiter plates (Becton Dickinson and Company, Lincoln Park, NJ, USA) were incubated at 37°C, 2% CO₂. Compounds were dissolved in dimethyl sulfoxide (DMSO) 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 μ l resazurin indicator solution was added to each well and incubated for 4 hours. Subsequently, the absorbance was measured at 570 nm and 600nm by imaging hybrid reader (Synergy H1, Biotek).

Cell proliferation assay of MeW-164 cells. Human melanoma cells line MeW-164, passage 45 was used. They were derived from melanoma cell line collection established in culture from melanoma metastases, surgically removed from patients in the Warsaw Cancer Center. Human melanoma cells were routinely grown in suspension in 90% EAGLE (MEM) (Biomed-Lublin) containing L-glutamine and supplemented with 10% foetal bovine serum (FBS, Invitrogen), in culture flasks (CELLSTAR) and incubated at 37°C in a 5% CO₂ humidified atmosphere.

The experimental substances were dissolved in a physiological saline solution of DMSO (dimethylsulfoxide), in which DMSO concentration did not exceed 1%. The experiment was performed according to the method described by Markowicz S.[8]. Cells were counted by flow cytometry using fluorescence microscope (NucleoCounter® NC-100™) and NucleoCassette with fluorescent dye propidium iodide to determine the total number of cells.

ABTS radical cation scavenging activity. The antioxidant activity by the ABTS⁺ method was assessed according to the method described by Re et al. [12] with modifications. The ABTS radical 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 h. Once formed, the radical was diluted with acetate buffered saline (0.02M, pH 6,5). Solutions of tested substances in DMSO were prepared. After that, 20 μ L of tested solution were transferred in a 96 wells microtitre plate and 180 μ L of working solution of ABTS⁺ were dispensed with dispense module of hybrid reader (Synergy H1, Biotek), shake 15 sec. The decrease in absorbance at 734 nm was measured exactly after 30 min of incubation at 25 °C. All the determination was made in triplicate.

DMSO was used as control. Doxorubicin, trolox and ascorbic acid were used for standards. Ascorbic acid (10 mM) prepared in distilled water for use as a stock standard was used as reference at concentrations ranging from 6,25 to 50 μM . Trolox (2 mM methanolic stock solution) was used as reference at dilution concentrations ranging from 0,1 to 100 μM . Doxorubicin hydrochloride (1mM) prepared in DMSO for use as a stock standard was used as reference at concentrations ranging from 0,01 to 100 μM .

The percentage inhibition was calculated according to the formula: $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample.

Results and discussion

The Schiff base H_2L has been prepared by the reaction of substitution of S-methyl group by selenium atom. The structure of H_2L was determined by ^1H and ^{13}C NMR spectroscopy. Coordination compound was prepared by the direct reaction between the ligand H_2L and copper(II) chloride dehydrate in ethanol solution.

In order to find out the biological properties of these substances, their antimicrobial, antifungal, antioxidant, and antitumor activities were studied.

The study of antimicrobial and antifungal activities (table 1) showed that both H_2L and its copper coordination compound possess bacteriostatic and bactericidal activities. The activity of H_2L towards gram-negative microorganisms is less pronounced than towards gram-positive bacteria and fungi. The coordination of H_2L ligand to copper(II) ions results in considerable reduction of minimum inhibitory concentrations and minimum bactericide concentrations towards gram-negative microorganisms.

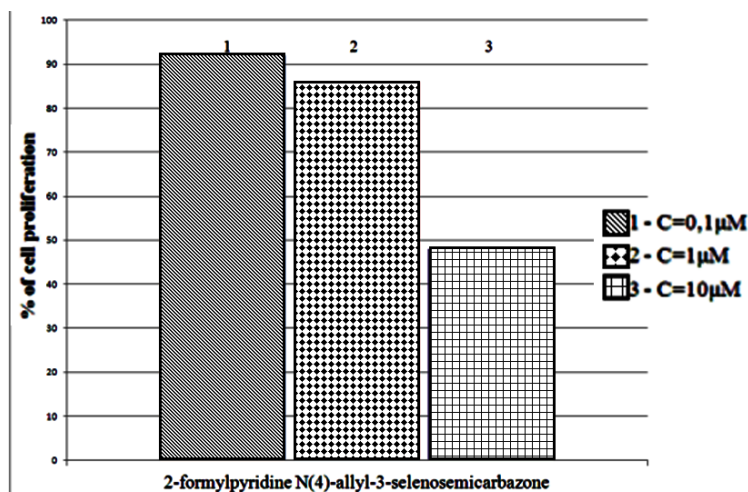
Table 1. The minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) ($\mu\text{g}/\text{mL}$).

Compound	Escherichia coli		Klebsiella pneumoniae		Staphylococcus aureus		Candida albicans	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
H_2L	500	500	500	500	0.7	1.5	15	15
$\text{Cu}(\text{HL})\text{Cl}$	3	3	15	15	0.7	0.7	7	7

The study of antioxidant activity showed that both free ligand and its coordination compound possess antioxidant activities towards ABTS radical cation. The obtained IC_{50} values are 9.1 μM for 2-formylpyridine N(4)-allyl-3-selenosemicarbazone and 51 μM for its copper(II) chloride coordination compound. The ligand shows much better antioxidant activity that exceeds the activity of Trolox that is used in medical practice as a standard antioxidant [14].

The antitumor activity of synthesized compounds was studied on a series of tumor cells: human leukemia HL-60 cells, cervical cancer HeLa cells and melanoma MeW-164 cells. The proliferation of melanoma MeW-164 cells in presence of H_2L is shown in scheme 6.

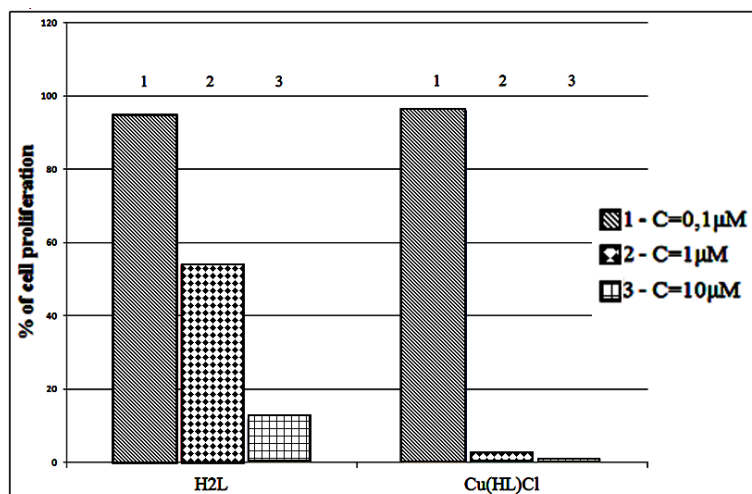
Both free ligand and its copper(II) coordination compound manifest antitumor activity towards HL-60 cells at concentrations 10 μM and 1 μM . The activity at lower concentration (0.1 μM) practically disappears. The activity of copper(II) coordination compound surpasses the activity of the ligand H_2L at concentrations 1 μM and 10 μM .



Scheme 6. Proliferation of MeW-164 cells in presence of H₂L.

The IC₅₀ value of 2-formylpyridine N(4)-allyl-3-selenosemicarbazone is 9.7 μM.

The proliferation of human leukemia HL-60 cells in presence of H₂L and Cu(HL)Cl is shown in scheme 7.



Scheme 7. Proliferation of HL-60 cells in presence of tested substances.

Both free ligand and its copper(II) coordination compound manifest antitumor activity towards HL-60 cells at concentrations 10 μM and 1 μM. The activity at lower concentration (0.1 μM) practically disappears. The activity of copper(II) coordination compound surpasses the activity of the ligand H₂L at concentrations 1 μM and 10 μM.

The study of cervical cancer HeLa cells proliferation in the presence of 2-formylpyridine N(4)-allyl-3-selenosemicarbazone showed that this selenosemicarbazone inhibits the proliferation of these cells by 17% at the concentration 100 μM. So the activity of this selenosemicarbazone towards HeLa cells is less pronounced than towards MeW-164 and HL-60 cells

Conclusions

In this work 2-formylpyridine N(4)-allyl-3-selenosemicarbazone was synthesized and studied using NMR spectroscopy. This ligand was used for synthesis of the

copper(II) chloride coordination compound. It was determined that both free ligand and copper coordination compound show antibacterial and antifungal activities. The copper coordination compound manifests much better antimicrobial activity towards gram-negative microorganisms than free ligand. The study of antitumor activity showed that synthesized substances inhibit proliferation of the melanoma MeW-164, human leukemia HL-60, and cervical cancer HeLa cells.

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