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Novel Copper(II) Complexes with N^4,*S*-Diallylisothiosemicarbazones as Potential Antibacterial/Anticancer Drugs

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Abstract: The six new copper(II) coordination compounds $[Cu(HL^1)Cl_2]$ (**1**), $[Cu(HL^1)Br_2]$ (**2**), $[Cu(H_2O)(L^1)(CH_3COO)]\cdot 1.75H_2O$ (**3**), $[Cu(HL^2)Cl_2]$ (**4**), $[Cu(HL^2)Br_2]$ (**5**), $[Cu(H_2O)(L^2)(CH_3COO)]$ (**6**) were synthesized with 2-formyl- and 2-acetylpyridine N^4,*S*-diallylisothiosemicarbazones (HL^1 and HL^2). The new isothiosemicarbazones were characterized by NMR, FTIR spectroscopy, and X-ray crystallography ($[H_2L^2]I$). All copper(II) coordination compounds were characterized by elemental analysis, FTIR spectroscopy, and molar conductivity of their 1mM methanol solutions. Furthermore, the crystal structure of complex **3** was determined using single-crystal X-ray diffraction analysis. The studied complexes manifest antibacterial and antifungal activities, that in many cases are close to the activity of medical drugs used in this area, and in some cases even exceed them. The complexes **4** and **5** showed the highest indexes of selectivity (280 and 154) and high antiproliferative activity against BxPC-3 cell lines that surpass the activity of Doxorubicin. The complexes **1**–**3** also manifest antioxidant activities against cation radicals $ABTS^{\bullet+}$ that are close to that of trolox, the antioxidant agent used in medicine.

Keywords: isothiosemicarbazones; copper complexes; antiproliferative activity; antibacterial activity; antifungal activity; antiradical activity

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1. Introduction

Copper is one of the crucial micronutrients that is located in different amounts in all human body tissues. The highest amount of copper is in the liver [1]. Various metalloproteins depend on copper as their active site, which makes it essential in a range of biochemical processes: electron transfer, oxidation, and oxygen transport. Copper also participates in cellular respiration, antioxidant protection, neurotransmission, connective tissue biosynthesis, and cellular iron metabolism [2]. Over the past few years, copper compounds have been studied as potential therapeutic agents for application as cancer medicine and as diagnostic drugs [3,4]. Many Cu(II) coordination compounds rapidly interact with glutathione in cells to form adducts and as a result the Cu(I) coordination compound is formed. This compound can generate a superoxide anion, which can induce ROS formation in a Fenton-like reaction [5]. However, antiproliferative action is not the only one for copper coordination compounds such as therapeutic agents, because of their high redox activity. For example, the copper(II) coordination compound with indomethacin is widely used as an anti-inflammatory drug in veterinary practice [6].

Cu(II) complexes of thiosemicarbazone are widely described in the literature because they are able to form stable complexes with different metal ions, which are lipophilic, and can easily permeate cell membranes. These complexes exhibit various types of biological activity: anticancer [7–12], antibacterial and antifungal [13–18], and antioxidant [19]. The antioxidant activity of copper(II) complexes is less studied.

There are many reasons why oxidative stress occurs: pollution, smoking, alcohol consumption, obesity etc. Antioxidants can protect us from free radicals that are produced in our body due to oxidative stress. Such free radicals can cause different diseases such as diabetes, cardiac diseases, cancer, and atherosclerosis [20].

In isothiosemicarbazones, alkylation of the sulfur fragment occurs, and they usually coordinate to the central metal atom through azomethine and thioamide nitrogen atoms. Therefore, in contrast to NS donor atoms of thiosemicarbazones, the isothiosemicarbazones have NN donor atoms. Due to the difference in coordination, it becomes possible to obtain coordination compounds of isothiosemicarbazones with a different structure, which will affect their chemical and biological properties. In some cases isothiosemicarbazones and their copper(II) coordination compounds outperform in activity the complexes of corresponding thiosemicarbazones [21]. Copper(II) complexes with isothiosemicarbazones are less often described in the literature [22–24] and there are several references to their biological activity, such as antibacterial [25,26] and anticancer [27,28].

Recently, we have synthesized 2-formylpyridine and 2-acetylpyridine 4-allyl-*S*-methylisothiosemicarbazones and their copper(II) coordination compounds [29,30]. Their biological activities such as anticancer, antibacterial, antifungal, and antioxidant have also been researched. These compounds showed promising results. In this paper we have replaced the *S*-methyl radical with the *S*-allyl one in the structure of isothiosemicarbazone to study how this will affect biological activity.

The aim of the present investigation is the synthesis, characterization, and study of antibacterial, antifungal, anticancer, and antioxidant activities of Cu(II) coordination compounds with 2-formylpyridine and 2-acetylpyridine N^4,*S*-diallylisothiosemicarbazones (HL^1 and HL^2, Figure 1).

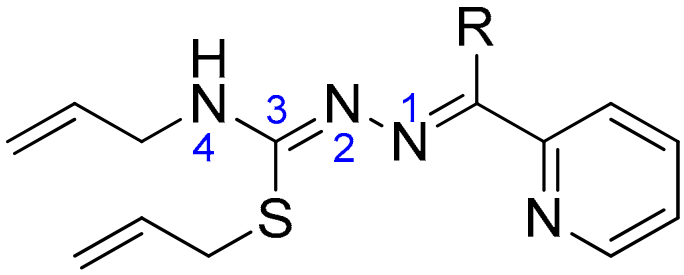
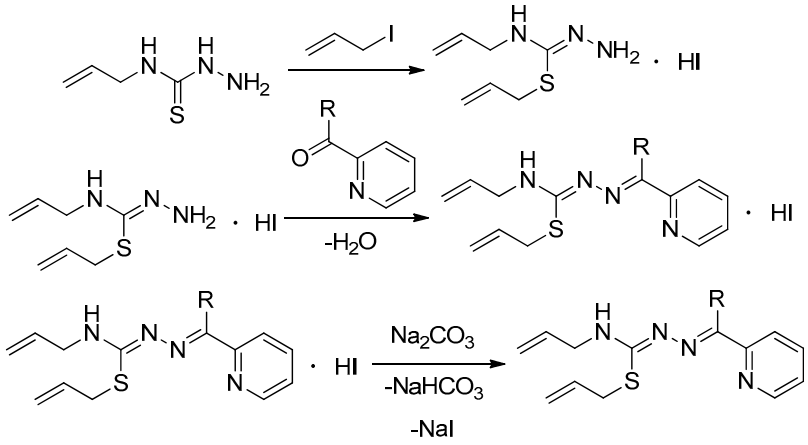


Figure 1. Structural formula of HL^1 (R = H) and HL^2 (R = CH_3).

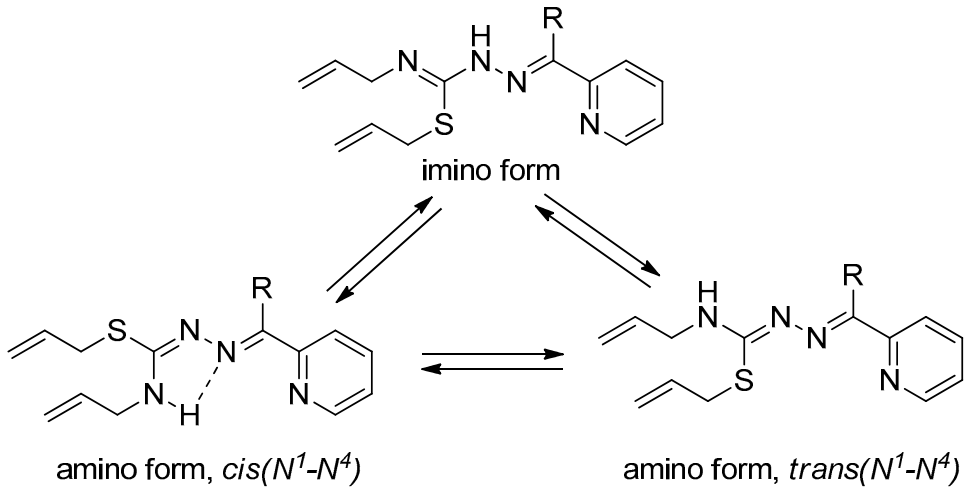
2. Results and Discussion

In this work we have synthesized two new *S*-substituted isothiosemicarbazones, namely 2-formylpyridine N^4,*S*-diallylisothiosemicarbazone (HL^1) and 2-acetylpyridine N^4,*S*-diallylisothiosemicarbazone (HL^2), that were obtained by a three-step method starting with interaction between N^4-allylthiosemicarbazide with allyl iodide, then condensation with 2-formyl-/2-acetil-pyridine, and, finally, neutralization with sodium carbonate (Scheme 1).



Scheme 1. Synthesis of N^4,*S*-diallylisothiosemicarbazones HL^1 and HL^2 (HL^1: R = H; HL^2: R = CH_3).

The structures of the HL^1 and HL^2 were confirmed using ^1H and ^{13}C NMR spectroscopy (Figures S1–S4). The NMR spectra of HL^1 contain peaks of three tautomeric forms that according to the literature [31] presumably are imino form and *cis*(N^1-N^4)/*trans*(N^1-N^4) amino forms (Scheme 2). The NMR spectra of HL^2 contain peaks of two tautomeric forms. Only *cis*(N^1-N^4) and *trans*(N^1-N^4) amino forms of HL^2 can be observed in its spectra.



Scheme 2. The equilibrium in the solutions of HL^1 (R = H) and HL^2 (R = CH_3).

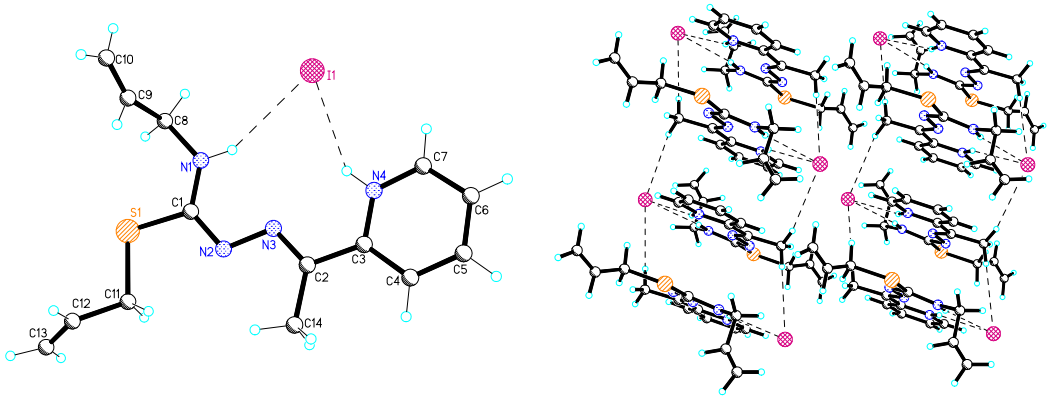
Furthermore, the single crystals of HL^2·HI were obtained by its recrystallization from methanol and their structure has been determined using single-crystal X-ray diffraction analysis. As a result, it was determined that this organic compound crystallizes in the triclinic space group $P^{-}1$ and represents an ionic compound $[H_2L^2]I$ (Table 1, Figure 2a). The organic cation $[H_2L^2]^+$ forms upon the transfer of the proton from HI to HL^2.

The NNCN torsion angle of the isothiosemicarbazide fragment in this cation is 0.1°, which indicates its *cis*(N^1-N^4) form (both terminal nitrogen atoms are on one side of the double C1=N2 bond). The C(1)–N(1) and C(1)–N(2) bonds equal 1.330(7) and 1.312(7) Å (Table 2). This indicates that the isothiosemicarbazide fragment is stabilized in the amino form [31]. The conformation of the $[H_2L^2]^+$ cation is favorable for formation of two intermolecular hydrogen bonds with the iodide anion (Table 3, Figure 2a) and for a tridentate coordination to the transition metal atoms. The survey of the Cambridge Structural Database (CSD) [32] revealed that non-coordinated isothiosemicarbazones are mainly stabilized in the amino form [30,33–36], but in the case of {2-[(2-oxyphenyl)methylidene]hydrazinyl}(methylsulfanyl)-*N*-(prop-2-en-1-yl)methaniminium iodide [37] the imino form is realized. The *cis*(N^1-N^4) conformation similar to that in $[H_2L^2]^+$ cation was found in [30,37] with corresponding torsion angles in the range 0.56–2.31°, while in [33–36] these angles are in

the range of 175.01–178.97°. In the crystal of $[H_2L^2]I$ two intermolecular hydrogen bonds N–H···I link the organic cation to the iodide anion (Table 3). Two weak hydrogen C–H···I bonds unite charged components into chains (Figure 2b).

Table 1. Crystal and Structure Refinement Data for $[H_2L^2]I$ and **3**.

Compound	$[H_2L^2]I$	**3**
Empirical formula	$C_{14}H_{19}I_1N_4S_1$	$C_{15}H_{23.5}Cu_1N_4O_{4.75}S_1$
Formula weight	402.29	431.48
Crystal system	Triclinic	Triclinic
Space group	$P\bar{1}$	$P\bar{1}$
Unit cell dimensions		
a (Å)	7.3553(8)	8.6225(5)
b (Å)	9.0535(9)	10.9536(5)
c (Å)	13.3945(18)	11.3493(8)
α (°)	103.136(10)	89.140(4)
β (°)	91.306(11)	69.700(6)
γ (°)	100.693(9)	81.612(4)
V (Å^3)	851.56(18)	993.85(11)
Z	2	2
ρ_{calc} (g cm^{-3})	1.569	1.442
μ_{Mo} (mm^{-1})	1.999	1.234
$F(000)$	400	449
Crystal size (mm)	0.60 × 0.12 × 0.08	0.48 × 0.40 × 0.21
θ Range (°)	3.12–25.05	3.39–25.25
Index range	$-8 \le h \le 8$, $-10 \le k \le 10$, $-15 \le l \le 15$	$-10 \le h \le 10$, $-12 \le k \le 13$, $-13 \le l \le 11$
Reflections collected/unique	6159/6159 (twin)	6114/3587 (R_{int} = 0.0238)
Completeness (%)	99.8 (θ = 25.05°)	99.6 (θ =25.25°)
Reflections with $I > 2\sigma(I)$	4518	3037
Number of refined parameters	184	240
Goodness-of-fit (GOF)	1.002	1.001
R (for $I > 2\sigma(I)$)	R_1 = 0.0437, wR_2 = 0.0954	R_1 = 0.0403, wR_2 = 0.1226
R (for all reflections)	R_1 = 0.0608, wR_2 = 0.0992	R_1 = 0.0496, wR_2 = 0.1296
$\Delta\rho_{max}/\Delta\rho_{min}$ (e·Å^{-3})	0.988/−0.521	0.687/−0.279



(**a**) (**b**)

Figure 2. (**a**) Molecular structure of $[H_2L^2]I$. (**b**) The formation of chains in the crystal of $[H_2L^2]I$.

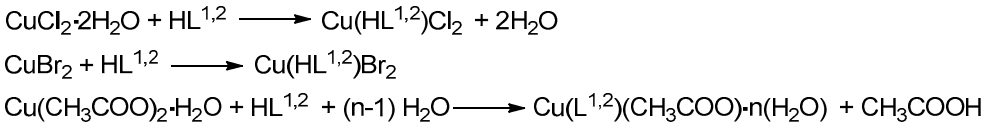
Table 2. Selected Bond Lengths (Å) and Angles (deg) in fragments of isothiosemicarbazones in $[H_2L^2]I$ and **3**.

Bonds	$[H_2L^2]I$	**3**
	(Å)	
N(3)–C(2)	1.292(7)	1.286(4)
N(3)–N(2)	1.374(6)	1.362(3)
C(1)–N(1)	1.330(7)	1.305(4)
C(1)–N(2)	1.312(7)	1.361(4)
C(1)–S(1)	1.760(6)	1.768(3)
S(1)–C(11)	1.821(6)	1.796(4)
N(1)–C(8)	1.463(7)	1.474(4)
Angles	(°)	
C(2)–N(3)–N(2)	112.8(5)	123.1(2)
N(3)–N(2)–C(1)	111.5(5)	107.0(2)
N(2)–C(1)–N(1)	127.1(6)	122.9(3)
N(2)–C(1)–S(1)	115.8(5)	117.0(2)
N(1)–C(1)–S(1)	117.1(5)	120.1(2)
C(1)–S(1)–C(11)	102.5(3)	104.4(2)
C(1)–N(1)–C(8)	126.6(5)	122.1(3)

Table 3. Hydrogen Bond Distances (Å) and Angles (deg) for $[H_2L^2]I$ and **3**.

D–H···A	d(H···A)	d(D···A)	∠(DHA)	Symmetry Transformation for Acceptor
		$[H_2L^2]I$		
N(1)–H(1)···I(1)	2.84	3.622(5)	152	x, y, z
N(4)–H(2)···I(1)	2.75	3.490(5)	146	x, y, z
C(14)–H(2)···I(1)	3.31	4.241(6)	165	$-x+2, -y+1, -z+1$
C(14)–H(3)···I(1)	3.16	4.121(7)	175	$-x+1, -y+1, -z+1$
		3		
O(1W)–H(1)···O(3W)	1.88	2.761(4)	166	$-x, -y+1, -z+2$
O(1W)–H(2)···N(2)	1.94	2.835(3)	176	$-x, -y, -z+2$
O(2W)–H(1)···O(1W)	2.05	2.814(4)	151	x, y, z
O(2W)–H(2)···O(2)	1.95	2.759(4)	158	$x-1, y, z$
O(3W)–H(1)···O(2W)	1.92	2.735(5)	159	x, y, z
O(3W)–H(2)···O(1)	1.99	2.838(3)	174	x, y, z

Six new copper(II) complexes were obtained by the interaction of the corresponding copper(II) salts with isothiosemicarbazones HL^1 and HL^2 (Scheme 3). They have the following compositions: $Cu(HL^1)Cl_2$ (**1**), $Cu(HL^1)Br_2$ (**2**), $Cu(L^1)(CH_3COO)\cdot 2.75H_2O$ (**3**), $Cu(HL^2)Cl_2$ (**4**), $Cu(HL^2)Br_2$ (**5**), $Cu(L^2)(CH_3COO)\cdot H_2O$ (**6**).



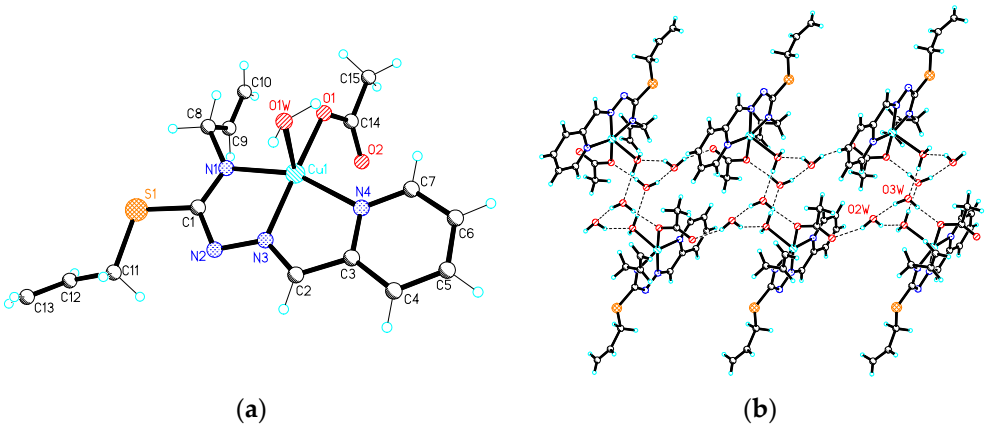
Scheme 3. Synthesis of complexes **1–6** (n = 1, 2.75).

Molar conductivity values of the complexes **1–2** and **4–5** in methanol are in the range of 169–192 $\Omega^{-1}\cdot cm^2\cdot mol^{-1}$ which indicates that they behave like 1:2 electrolytes, while the molar conductivity values of complexes **3** and **6** are in the range of 82–85 $\Omega^{-1}\cdot cm^2\cdot mol^{-1}$ which corresponds to the 1:1 type of electrolyte. The fact that the synthesized complexes **1–6** behave like electrolytes means that the anions of acid residues (Cl^-, Br^-, CH_3COO^-) from the inner sphere are readily substituted with solvent molecules while having been dissolved. It means that complexes **1–2** and **4–5** contain two anions of acid residue (Cl^-/Br^-) in their composition and that in the process of dissolution complex cations and two anions of acid residue are formed. In the case of complexes **3** and **6** only one anion acid residue is present in their composition.

The FTIR spectra of complexes **1–6** were compared with the spectra of corresponding isothiosemicarbazones (HL^1/HL^2) in order to determine the changes that occur during their formation (Figures S5–S12). It was observed that three donor nitrogen atoms of the isothiosemicarbazones HL^1 and HL^2 are involved in the coordination to the copper(II) central atoms. In the spectra of complexes **1–2** and **4–5** the ν(NH) stretching vibration band is shifted by 63–86 cm^{-1} towards lower wavenumbers. Meanwhile, this band disappears in the spectra of complexes **3** and **6**. It means that the NH group of isothiosemicarbazones is deprotonated in the process of coordination to the copper(II) ions in the presence of acetate ions that act like a weak base. The $\nu(C{=}N^1)$ and $\nu(C{=}N_{pyr})$ bands that are observed in the range of 1601–1558 cm^{-1} are shifted by 10–30 cm^{-1} suggesting the coordination of isothiosemicarbazones using azomethine and pyridine nitrogen atoms. Absorption bands of C–S bonds practically are not displaced in the spectra of complexes. Consequently, the sulfur atom is not involved in the coordination to the metal ion in these compounds. Furthermore, the characteristic bands of acetate ions are present in the FTIR spectra of complexes **3** (1620 and 1324 cm^{-1}) and **6** (1614 and 1312 cm^{-1}). According to the literature [38] the difference (Δ) between these two characteristic bands (Δ = 296 cm^{-3} for **3** and Δ = 302 cm^{-3} for **6**) corresponds to monodentate acetate ion in the inner sphere of the coordination compound.

Single crystals of complex **3** were obtained as a result of recrystallization from methanol and their structure was determined using single-crystal X-ray diffraction analysis. The complex **3** crystallizes in the triclinic space group $P^{-}1$ (Table 1). Structural study determined that the formula of **3** is $[Cu(H_2O)(L^1)(CH_3COO)]\cdot 1.75H_2O$. The asymmetric part of the unit cell contains one molecular complex $[Cu(H_2O)(L^1)(CH_3COO)]$ (Figure 3) and 1.75 solvate water molecules. The Cu(II) in **3** is five-coordinated and the coordination polyhedron represents a square pyramid. The tridentate isothiosemicarbazone ligand is coordinated to the central atom in the monodeprotonated form $(L^1)^-$ using an N_3-set of donor atoms (Figure 3a) and forms two fused metallacycles. Such a coordination mode of similar ligands was found in the complexes of various transition metals [27,30,36,37,39]. Nevertheless, the sulfur atom of isothiosemicarbazones can also participate in coordination [30,35,40].

The basal plane of the Cu(II) polyhedron is formed by three donor atoms of the ligand $(L^1)^-$ and an oxygen atom of the acetate ion. The oxygen atom of the coordinated water molecule is at the apex of this polyhedron. The bond distances and angles in coordination surrounding are given in Table 4. The coordination of the $(L^1)^-$ to the Cu(II) ion did not lead to a change in its conformation, but affected the redistribution of bond lengths in the isothiosemicarbazide fragment: C–N interatomic distances, namely C(1)–N(1) and C(1)–N(2) values of 1.305(4) and 1.361(4) Å (Table 2) indicate the stabilization of the imino form.



(**a**) (**b**)

Figure 3. (**a**) The structure of the molecular complex [Cu(H_2O)(L^1)(CH_3COO)] in **3**. (**b**) The six-membered water cluster unites complexes in the chain in **3**.

Table 4. Bond Lengths (Å) and Angles (deg) in Coordination Metal Environment in **3**.

Bonds	Å
Cu(1)–N(1)	1.962(3)
Cu(1)–N(3)	1.948(2)
Cu(1)–N(4)	2.037(3)
Cu(1)–O(1)	1.942(2)
Cu(1)–O(1W)	2.353(2)
Angles	°
N(1)–Cu(1)–N(3)	78.61(10)
N(1)–Cu(1)–N(4)	158.36(11)
N(1)–Cu(1)–O(1)	99.28(10)
N(1)–Cu(1)–O(1W)	98.61(10)
N(3)–Cu(1)–N(4)	80.30(10)
N(3)–M(1)–O(1)	172.76(10)
N(3)–M(1)–O(1W)	99.94(9)
N(4)–M(1)–O(1)	101.04(10)
N(4)–M(1)–O(1W)	89.75(10)
O(1)–M(1)–O(1W)	87.21(9)

The components of the crystal are united in the chain by a system of hydrogen bonds in which two coordinated and four solvate water molecules from two formula units form a six-membered chair-like H-bonded cycle (Table 3, Figure 3b). These chains are associated in the layer parallel to (*ab*) crystallographic plane by intermolecular hydrogen bonds O(W)−H···O(acetate) and O(W)−H···N2.

In order to study the biological properties of the synthesized copper(II) complexes the antibacterial and antifungal properties of the complexes **1**–**6** were tested on Gram-positive (*S. aureus*, *B. cereus*) bacteria, Gram-negative (*E. coli*, *A. baumannii*) bacteria, and fungi (*C. albicans*). The obtained results in form of minimum inhibitory/bactericidal/fungicidal concentrations are shown in Table 5.

First of all, it is seen that copper(II) coordination compounds in most cases show higher activity than the corresponding N^4,*S*-diallylisothiosemicarbazones HL^1 and HL^2. The copper(II) complexes manifest higher antibacterial activity towards Gram-positive microorganisms. Among all synthesized copper(II) complexes, the least active ones were the complexes obtained from copper acetate (**3** and **6**). Other complexes showed approximately the same values of activities. So, the dependence between the activity and acid residue can be seen in these results. The activity decreases in the following order: $Cl^- \approx Br^- > CH_3COO^-$. The ligand also affects the activity: copper(II) complexes with 2-acetylpyridine N^4,*S*-diallylisothiosemicarbazone (HL^2) are more active towards Gram-

positive microorganisms and *A. baumanii* than the complexes with 2-formylpyridine N^4,*S*-diallylisothiosemicarbazone (HL^1). A group of antibiotics (Furacillinum [37,41] and Tetracycline [42–45]) and a group of antifungals (Nystatine [37] and Fluconazole [46]) were used in order to compare the antibacterial and antifungal activities of synthesized complexes with the corresponding activities of medicines. The synthesized complexes **1**, **2**, and **5** manifest greater activity than Furacillinum towards Gram-positive microorganisms and *E. coli*. Complexes **4** and **5** surpass 2–5 times the activity of Furacillinum in the case of *A. baumanii*. Furthermore, complex **5** approximately coincides with the activity of Tetracycline towards Gram-negative microorganism *E. coli*. All the studied copper(II) complexes surpass 4–20 times the activity of standard antifungals (Nystatine and Fluconazole).

Table 5. Minimal inhibitory, bactericidal, and fungicidal concentrations (μg mL^{-1}) of HL^1, HL^2, and copper(II) complexes **1**–**6**.

Compound	*Staphylococcus aureus* ATCC 25923		*Bacillus cereus* ATCC 11778		*Escherichia coli* ATCC 25922		*Acinetobacter baumannii* BAA-747		*Candidaalbicans* ATCC 10231	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MFC**
HL^1	125	250	31.3	62.5	>1000	>1000	-	-	15.6	31.3
1	0.977	1.95	0.977	1.95	15.6	31.3	15.6	31.3	7.81	15.6
2	0.977	1.95	1.95	3.91	15.6	31.3	15.6	31.3	3.91	7.81
3	31.3	62.5	31.3	62.5	250	500	-	-	31.3	62.5
HL^2	31.3	62.5	62.5	62.5	>1000	>1000	>1000	>1000	7.81	62.5
4	0.488	0.488	0.488	0.488	31.3	62.5	1.95	1.95	3.91	15.6
5	0.488	0.488	0.488	0.488	1.95	3.91	1.95	1.95	3.91	15.6
6	3.91	3.91	1.95	3.91	62.5	62.5	31.3	31.3	3.91	31.3
Furacillinum [37,41]	9.3	9.3	4.7	4.7	18.5	37.5	4.7	9.4	-	-
Tetracycline [42–45]	0.25	1.96	0.06	-	0.98	3.91	0.5	-	-	-
Nystatine [37]	-	-	-	-	-	-	-	-	80	80
Fluconazole [46]	-	-	-	-	-	-	-	-	15.6	31.3

Note: MIC—minimum inhibitory concentration; MBC—minimum bactericidal concentration; MFC—minimum fungicidal concentration; «-»—data not available.

The antibacterial activity of the synthesized copper(II) complexes can be compared with compounds with similar structures that were previously described in other articles: copper(II) coordination compounds with 2-formylpyridine and 2-acetylpyridine N^4-allyl-*S*-methylisothiosemicarbazones (*S*-MeT2FP and *S*-MeT2AP, correspondingly) [29,30]. Three types of microorganisms were taken for comparison: Gram-positive *S. aureus*, Gram-negative *E. coli* microorganisms, and fungus *C. albicans*. The copper(II) complexes with 2-formylpyridine N^4,*S*-diallylisothiosemicarbazone (**1**, **2**) showed more modest results towards *S. aureus* than their *S*-methyl substituted analogs (Figure 4a). While the copper(II) complexes with 2-acetylpyridine isothiosemicarbazone HL^2 obtained in this work surpass the activity of Cu(*S*-MeT2AP)Cl_2 and Cu(*S*-MeT2AP)Br_2 described in the literature. In the case of Gram-negative microorganisms *E. coli* complexes **1** and **2** are 4 times more active than the recently described copper(II) complexes (Figure 4b). The complex **5** manifests higher activity than coordination compounds with *S*-MeT2AP.

For comparison of antifungal properties, the activity against *C. albicans* was analyzed (Figure 5). All the synthesized complexes **1**, **2**, **4**, **5** exceed the activity of the corresponding coordination compounds with *S*-MeT2FP and *S*-MeT2AP.

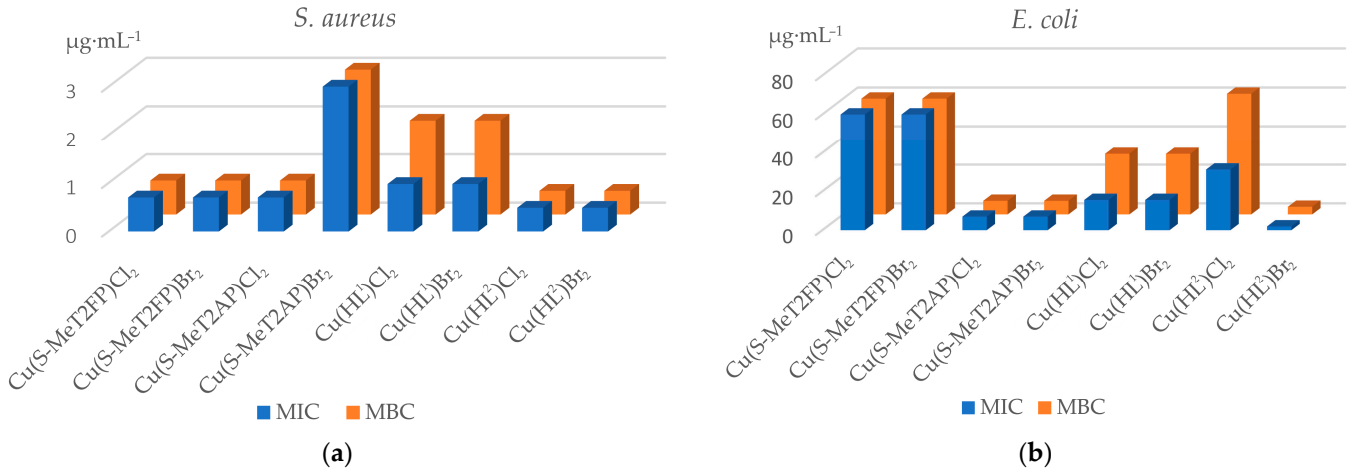


Figure 4. Comparison of the activity of studied complexes with their analogues against *S. aureus* (**a**) and *E. coli* (**b**).

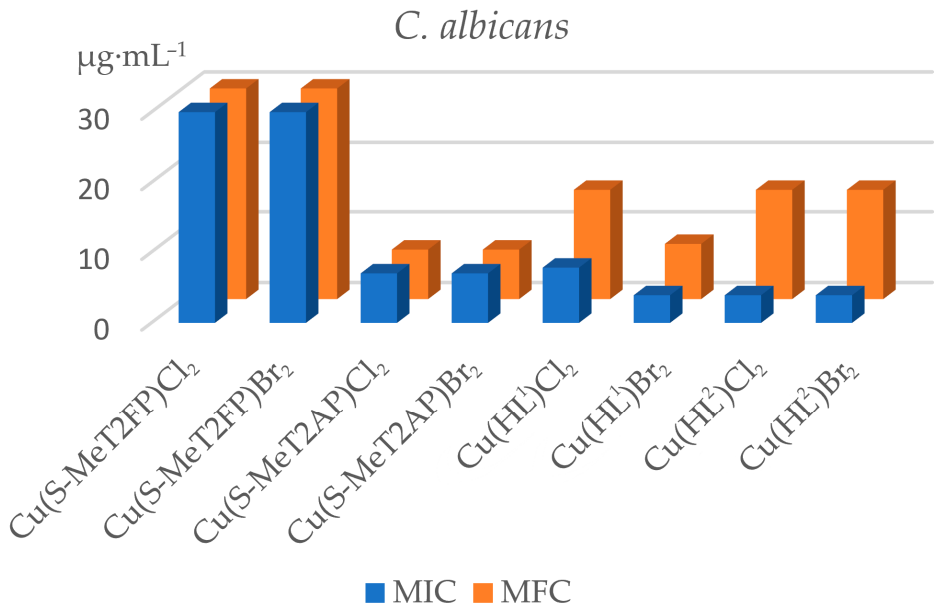


Figure 5. Comparison of the activity of studied complexes with their analogues against *C. albicans*.

Moreover, for the screening of the antiproliferative activity, HL^2 and copper(II) complexes **4** and **5** have been tested towards a series of cancer cell lines (HeLa, BxPC-3, RD) and a normal cell line (MDCK). The obtained results, in the form of semimaximal inhibitory concentrations (IC_{50}) and selectivity indexes (SI), are shown in Table 6 as well as the corresponding values of similar compounds, 2-acetylpyridine N^4-allyl-*S*-methylisothiosemicarbazone and its copper(II) complexes, that are described in [30].

While 2-acetylpyridine N^4,*S*-diallylisothiosemicarbazone (HL^2) does not manifest anticancer activity (only tested on HeLa and BxPC-3 cell lines), copper(II) complexes manifest a strongly marked antiproliferative activity. The complexes **4** and **5** manifest about the same level of activity. They showed the highest selectivity indexes, 280 and 154, towards BxPC-3 which is one of the most aggressive forms of neoplastic diseases [47]. Recently described copper(II) coordination compounds surpass the antiproliferative activity of studied complexes **4** and **5** towards MDCK and RD cell lines. Doxorubicin (DOXO) is a chemotherapy medication used to treat cancer that was used as a standard. Both synthesized complexes showed higher activity and selectivity than DOXO for all of the studied series of cancer cell lines.

Table 6. IC_{50} values of HL^2 and complexes **4** and **5** towards non-cancerous cell line (MDCK), cancer cell lines (HeLa, BxPC-3, RD), and the corresponding selectivity indexes in comparison with doxorubicin and similar compounds described in [30].

Compound	MDCK	HeLa		BxPC-3		RD	
	IC_{50}, μM	IC_{50}, μM	SI	IC_{50}, μM	SI	IC_{50}, μM	SI
DOXO	7.1 ± 0.3	10.0 ± 0.4	0.71	3.7 ± 0.3	1.9	16.2 ± 0.6	0.44
HL^2	-	>100	-	>100	-	-	-
4	1.4 ± 0.1	0.5 ± 0.1	2.80	0.005 ± 0.001	280	0.2 ± 0.1	7.00
5	1.23 ± 0.01	0.39 ± 0.01	3.15	0.008 ± 0.001	154	1.3 ± 0.4	0.95
S-MeT2AP	13.0 ± 1.3	47.6 ± 4.9	0.27	1.5 ± 0.5	8.7	>100	-
[Cu(*S*-MeT2AP)Cl_2]	1.00 ± 0.02	3.0 ± 1.2	0.33	0.09 ± 0.01	11	0.16 ± 0.01	6.3
[Cu(*S*-MeT2AP)Br_2]	0.35 ± 0.01	0.6 ± 0.2	0.58	0.02 ± 0.01	18	0.05 ± 0.01	7.0

Note: *S*-MeT2AP—2-acetylpyridine N^4-allyl-*S*-methylisothiosemicarbazone [30]; SI = IC_{50}(MDCK)/IC_{50}(cancer cell line)—selectivity index.

The antiradical activity against $ABTS^{\bullet+}$ cation radicals was studied for HL^1, HL^2, and copper(II) complexes **1**–**6**. The obtained results in form of semimaximal inhibitory concentrations (IC_{50}) are shown in Table 7. The HL^1 and its copper(II) complexes **1**–**3** manifest the highest antiradical activity that is close to the activity of trolox, which is used in medicine as standard antioxidant agent. Complexes **4** and **5** are practically inactive towards $ABTS^{\bullet+}$ cation radicals.

Table 7. Antiradical activity of complexes **1**–**6** against $ABTS^{\bullet+}$.

Compound	IC_{50}, μM
HL^1	28.5 ± 4.0
1	28.9 ± 6.1
2	32.7 ± 0.9
3	30.1 ± 1.3
HL^2	80.8 ± 13.4
4	>100
5	>100
6	95.0 ± 7.3
Trolox	33.3 ± 0.2

3. Experimental Section

3.1. Materials and Instrumentation

All the reagents used were chemically pure. Copper(II) salts $CuCl_2 \cdot 2H_2O$, $CuBr_2$, $Cu(CH_3COO)_2 \cdot H_2O$ (Merck) were used as supplied. Allyl isothiocyanate, 50–60% (*w*/*w*) aqueous solution of hydrazine, allyl iodide, 2-formylpyridine, 2-acetylpyridine, and sodium carbonate were used as received (Sigma-Aldrich). N^4-Allyl-3-thiosemicarbazide was synthesized by reaction of fourfold excess of 50–60% (*w*/*w*) aqueous solution of hydrazine and allyl isothiocyanate [48]. The solvents were purified and dried according to standard procedures [49].

Bruker DRX-400 was used to record the ^1H and ^{13}C NMR spectra. Acetone-d_6 was used as a solvent to prepare probes for the NMR study. Bruker ALPHA FTIR spectrophotometer was used to record FTIR spectra of studied substances in the range of 4000–400 cm^{-1} at rt. The elemental analysis was performed similarly to the literature procedures [50] and on the automatic Perkin Elmer 2400 elemental analyzer. R-38 rheochord bridge was used to measure the resistance of 1 mM methanol solutions of complexes **1**–**6** at 20 °C.

3.2. Synthesis

3.2.1. Synthesis of N^4,*S*-Diallylisothiosemicarbazones

2-Formylpyridine N^4,*S*-Diallylisothiosemicarbazone (HL^1)

At the first step, the allyl iodide (1.68 g, 10.0 mmol) has been added to the solution of N^4-allylthiosemicarbazide (1.31 g, 10.0 mmol) in ethanol [51]. After 2 h of stirring at room temperature, 2-formylpyridine (1.07 g, 10.0 mmol) was added. The solution was stirred at 70 °C for 30 min. After cooling to room temperature, a yellow precipitate formed from the solution, which was filtered off, washed with ethanol, and dried in air. The obtained precipitate was dissolved in ethanol, and aqua solution of sodium carbonate was added dropwise to the obtained solution until the pH reached value 7–8. After that, the 2-formylpyridine N^4,*S*-diallylisothiosemicarbazone was extracted by chloroform and dried in vacuo.

Pale yellow solid. Yield: 75%; mp 62–63 °C. FW: 260.36 g/mol; Anal Calc. for $C_{13}H_{16}N_4S$: C, 59.97; H, 6.19; N, 21.52; S, 12.32; found: C, 60.28; H, 6.03; N, 21.48; S, 12.49%. FTIR data (cm^{-1}): ν(N-H) 3219; ν (C=N) 1599, 1575, 1560; ν(CH_2–S) 1096; ν (C–S) 766.

Form A (amino form, *cis*(N^1-N^4)). ^{1}H NMR (acetone-d_6): 8.59 (d, 1H, CH aromatic); 8.33 (s, 1H, CH=N); 8.04 (d, 1H, CH aromatic); 7.79 (t, 1H, CH aromatic); 7.33 (t, 1H, CH aromatic); 7.47 (br, 1H, NH); 6.12–5.88 (m, 2H, CH allyl); 5.44–4.96 (m, 4H, 2×CH_2=C); 3.96 (t, 2H, CH_2-N); 3.72 (d, 2H, CH_2-S). ^{13}C NMR (acetone-d_6): 163.61 (C-S); 154.91, 152.51, 135.38, 123.35, 119.92 (C aromatic); 149.46 (CH=N); 136.04, 134.17 (CH allyl); 117.24, 115.37, (CH_2=); 45.34 (CH_2-N); 32.24 (CH_2-S).

Form B (imino form). ^{1}H NMR (acetone-d_6): 8.57 (d, 1H, CH aromatic); 8.24 (s, 1H, CH=N); 8.13 (d, 1H, CH aromatic); 7.77 (t, 1H, CH aromatic); 7.31 (t, 1H, CH aromatic); 5.13 (br, 1H, NH); 6.12–5.88 (m, 2H, CH allyl); 5.44–4.96 (m, 4H, 2×CH_2=C); 4.09 (d, 2H, CH_2-N); 3.83 (d, 2H, CH_2-S). ^{13}C NMR (acetone-d_6): 163.56 (C-S); 155.21, 151.73, 134.91, 123.62, 120.46 (C aromatic); 149.37 (CH=N); 136.05, 133.75 (CH allyl); 117.83, 115.26, (CH_2=); 45.58 (CH_2-N); 32.49 (CH_2-S).

Form C (amino form, *trans*(N^1-N^4)). ^{1}H NMR (acetone-d_6): 8.69 (d, 1H, CH aromatic); 8.23 (s, 1H, CH=N); 8.24 (d, 1H, CH aromatic); 7.97 (t, 1H, CH aromatic); 7.48 (t, 1H, CH aromatic); 6.12–5.88 (m, 2H, CH allyl); 5.44–4.96 (m, 4H, 2×CH_2=C); 3.95 (t, 2H, CH_2-N); 3.93 (d, 2H, CH_2-S). ^{13}C NMR (acetone-d_6): 163.13 (C-S); 154.49, 152.53, 133.42, 124.18, 122.77 (C aromatic); 148.96 (CH=N); 137.25, 133.01 (CH allyl); 118.01, 116.87 (CH_2=); 47.50 (CH_2-N); 35.78 (CH_2-S).

2-Acetylpyridine N^4,*S*-Diallylisothiosemicarbazone (HL^2)

The isothiosemicarbazone HL^2 was synthesized similarly to HL^1 using 2-acetylpyridine (1.21 g, 10.0 mmol) instead of 2-formylpyridine.

Pale yellow solid. Yield: 80%; mp 96–97 °C. FW: 274.38 g/mol; Anal Calc. for $C_{14}H_{18}N_4S$: C, 61.28; H, 6.61; N, 20.42; S, 11.69; found: C, 61.07; H, 6.48; N, 20.37; S, 11.48%. FTIR data (cm^{-1}): ν(N-H) 3215; ν (C=N) 1601, 1583, 1558; ν(CH_2–S) 1044; ν (C–S) 743.

Form A (amino form, *cis*(N^1-N^4)). ^{1}H NMR (acetone-d_6): 8.58 (d, 1H, CH aromatic); 8.26 (d, 1H, CH aromatic); 7.71 (t, 1H, CH aromatic); 7.29 (t, 1H, CH aromatic); 7.27 (br, 1H, NH); 5.99 (m, 2H, CH allyl); 5.20 (m, 4H, 2×CH_2=C); 3.96 (t, 2H, CH_2-N); 3.86 (d, 2H, CH_2-S); 2.51 (s, 3H, CH_3). ^{13}C NMR (acetone-d_6): 161.53 (C-S); 157.19, 156.99, 135.66, 123.00, 120.16 (C aromatic); 148.46 (C=N); 135.55, 134.41 (CH allyl); 116.98, 115.15, (CH_2=); 45.31 (CH_2-N); 32.35 (CH_2-S); 12.30 (CH_3).

Form B (amino form, *trans*(N^1-N^4)). ^{1}H NMR (acetone-d_6): 8.56 (d, 1H, CH aromatic); 8.20 (d, 1H, CH aromatic); 7.75 (т, 1H, CH aromatic); 7.31 (t, 1H, CH aromatic); 5.98 (m, 2H, CH allyl); 5.29 (m, 4H, 2×CH_2=C); 5.10 (br, 1H, NH); 4.11 (t, 2H, CH_2-N); 3.69 (d, 2H, CH_2-S); 2.43 (s, 3H, CH_3). ^{13}C NMR (acetone-d_6): 161.26 (C-S); 158.23, 156.79, 135.60, 123.25, 120.35 (C aromatic); 148.53 (C=N); 134.99, 133.99 (CH allyl); 117.67, 115.38 (CH_2=); 45.88 (CH_2-N); 32.28 (CH_2-S); 12.02 (CH_3).

3.2.2. Synthesis of Copper(II) Complexes

$[Cu(HL^1)Cl_2]$ (**1**)

Copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$) (0.170 g, 1 mmol) was added to a hot (55° C) ethanolic solution (25 mL) of 2-formylpyridine N^4,*S*-diallylisothiosemicarbazone HL^1 (0.260 g, 1 mmol). The mixture was stirred for 30 min at 55 °C. By cooling to room temperature, a green precipitate was obtained. It was filtered out, washed with cold ethanol, and dried in vacuo.

Green solid. Yield: 80%. Anal. Calc. for $C_{13}H_{16}Cl_2CuN_4S$ (394.81 g mol^{-1}): C, 39.55; H, 4.08; Cl, 17.96; Cu, 16.10; N, 14.19; S, 8.12. Found: C, 39.38; H, 4.05; Cl, 17.91; Cu, 15.89; N, 14.02; S, 7.95. Main FTIR peaks (cm^{-1}): ν(NH) 3156, ν(C=N) 1591, 1567, 1534, ν(CH_2–S) 1095, ν(C–S) 768. χ(CH_3OH): 169 Ω^{-1} cm^{-2} mol^{-1}.

$[Cu(HL^1)Br_2]$ (**2**)

The coordination compound **2** was synthesized similarly to compound **1** using $CuBr_2$ (0.223 g; 1 mmol) and HL^1 (0.260 g; 1 mmol).

Green solid. Yield: 85%. Anal. Calc. for $C_{13}H_{16}Br_2CuN_4S$ (483.71 g mol^{-1}): C, 32.28; H, 3.33; Br, 33.04; Cu, 13.14; N, 11.58; S, 6.63. Found: C, 32.05; H, 3.20; Br, 33.17; Cu, 13.45; N, 11.71; S, 6.72. Main FTIR peaks (cm^{-1}): ν(NH) 3139, ν(C=N) 1593, 1567, 1538, ν(CH_2–S) 1098, ν(C–S) 765. χ(CH_3OH): 178 Ω^{-1} cm^{-2} mol^{-1}.

$[Cu(H_2O)(L^1)(CH_3COO)] \cdot 1.75H_2O$ (**3**)

The coordination compound **3** was synthesized similarly to compound **1** using $Cu(CH_3COO)_2 \cdot H_2O$ (0.200 g; 1 mmol) and HL^1 (0.260 g; 1 mmol).

Brown solid. Yield: 82%. Anal. Calc. for $C_{15}H_{23.5}CuN_4O_{4.75}S$ (431.48 g mol^{-1}): C, 41.75; H, 5.49; Cu, 14.73; N, 12.98; S, 7.43. Found: C, 41.62; H, 5.58; Cu, 14.79; N, 12.81; S, 7.29. Main FTIR peaks (cm^{-1}): ν(C=O) 1620, ν(C=N) 1596, 1558, 1532, ν(C–O) 1324, ν(CH_2–S) 1091, ν(C–S) 766. χ(CH_3OH): 85 Ω^{-1} cm^{-2} mol^{-1}.

$[Cu(HL^2)Cl_2]$ (**4**)

The coordination compound **4** was synthesized similarly to compound **1** using $CuCl_2 \cdot 2H_2O$ (0.170 g; 1 mmol) and HL^2 (0.274 g; 1 mmol).

Green solid. Yield: 78%. Anal. Calc. for $C_{14}H_{18}Cl_2CuN_4S$ (408.84 g mol^{-1}): C, 41.13; H, 4.44; Cl, 17.34; Cu, 15.54; N, 13.70; S, 7.84. Found: C, 41.23; H, 4.56; Cl, 17.51; Cu, 15.72; N, 13.57; S, 7.93. Main FTIR peaks (cm^{-1}): ν(N–H) 3129, ν(C=N) 1591, 1571, 1544, ν(CH_2–S) 1044, ν(C–S) 746. χ(CH_3OH): 192 Ω^{-1} cm^{-2} mol^{-1}.

$[Cu(HL^2)Br_2]$ (**5**)

The coordination compound **5** was synthesized similarly to compound **1** using $CuBr_2$ (0.223 g; 1 mmol) and HL^2 (0.274 g; 1 mmol).

Green solid. Yield: 72%. Anal. Calc. for $C_{14}H_{18}Br_2CuN_4S$ (497.74 g mol^{-1}): C, 33.78; H, 3.65; Br, 32.11; Cu, 12.77; N, 11.26; S, 6.44. Found: C, 33.95; H, 3.82; Br, 32.29; Cu, 12.65; N, 11.10; S, 6.26. Main FTIR peaks (cm^{-1}): ν(NH) 3143, ν(C=N) 1591, 1569, 1542, ν(CH_2–S) 1043, ν(C–S) 747. χ(CH_3OH): 178 Ω^{-1} cm^{-2} mol^{-1}.

$[Cu(H_2O)(L^2)(CH_3COO)]$ (**6**)

The coordination compound **6** was synthesized similarly to compound **1** using $Cu(CH_3COO)_2 \cdot H_2O$ (0.200 g; 1 mmol) and HL^2 (0.274 g; 1 mmol).

Brown solid. Yield: 81%. Anal. Calc. for $C_{16}H_{22}CuN_4O_3S$ (413.98 g mol^{-1}): C, 46.42; H, 5.36; Cu, 15.35; N, 13.53; S, 7.75. Found: C, 46.19; H, 5.42; Cu, 15.12; N, 13.59; S, 7.49. Main FTIR peaks (cm^{-1}): ν(C=O) 1614, ν(C=N) 1595, 1561, 1543, ν(C–O) 1312, ν(CH_2–S) 1041, ν(C–S) 741. χ(CH_3OH): 82 Ω^{-1} cm^{-2} mol^{-1}.

3.3. X-ray Crystallography

The single-crystal X-ray analysis of $[H_2L^2]I$ and complex **3** were carried out at room temperature (293 K) on an Xcalibur E CCD diffractometer equipped with a CCD area detector and a graphite monochromator, Mo$K\alpha$ radiation (0.71073 Å). CrysAlis PRO software was used for data collection and reduction, and unit cell determination. The structures were solved and refined using the SHELXS97 and SHELXL2014 software packages [52,53]. The non-hydrogen atoms were treated anisotropically (full-matrix least squares method on F^2). The hydrogen atoms were placed in calculated positions and were treated using riding model approximations with $U_{iso}(H) = 1.2U_{eq}(C)$, while the oxygen-bounded H atoms were found from differential Fourier maps at an intermediate stage of the structure refinement. These hydrogen atoms were refined with the isotropic displacement parameter $U_{iso}(H) = 1.5U_{eq}(O)$.

The crystallographic data were deposited with the Cambridge Crystallographic Data Center, CCDC nos. 2253067 and 2253068 for $[H_2L^2]I$ and **3**, respectively. Copies of this information may be obtained free of charge from the Director, CCDC, 12 Union Road, Cambridge CHB2 1EZ, UK (Fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk or www.ccdc.cam.ac.uk (accessed on 26 April 2023)).

3.4. Antibacterial and Antifungal Activity

Antibacterial and antifungal activities of the isothiosemicarbazones HL^1, HL^2, and copper(II) coordination compounds **1**–**6** were studied on a series of standard strains: *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Acinetobacter baumannii* (BAA-747), *Escherichia coli* (ATCC 25922), and *Candida albicans* (ATCC 10231). The minimum inhibitory concentrations (MICs, μg mL^{-1}), minimum bactericidal concentrations (MBCs, μg mL^{-1}), and minimum fungicidal concentrations (MFCs, μg mL^{-1}) were determined using the method of serial dilutions in liquid broth. The solutions of the tested substances were prepared in DMSO with a 10 mg mL^{-1} concentration. Subsequent dilutions were prepared by incorporating 2% peptonate bullion.

3.5. Antiproliferative Activity

3.5.1. Cell Cultures

The BxPC-3 (ATCC CRL-1687) cells were grown as a monolayer in Roswell Park Memorial Institute 1640 medium to which penicillin–streptomycin (final concentration of penicillin 100 U mL^{-1}; final concentration of streptomycin 100 μg mL^{-1}) was added. Furthermore, fetal bovine serum (FBS) was added to the medium at a concentration of 10% v/v.

The HeLa (ATCC CCL-2), RD (ATCC CCL-136), and MDCK (ATCC CCL-34) cell lines were grown in Dulbecco's modified essential medium. The medium contained glucose (4.5 g L^{-1}), L-glutamine (4 mM), HEPES buffer (20 mM), bovine albumin fraction (0.2% v/v), and penicillin-streptomycin (final concentration of penicillin 100 U mL^{-1}; final concentration of streptomycin 100 μg mL^{-1}). Moreover, the medium was supplemented with FBS at a concentration of 10% v/v.

The cells were cultured in 75-cm^2 dishes in a 5% humidified CO_2 environment at 37 °C.

3.5.2. Resazurin Test

The viability of cancer cells (BxPC-3, HeLa, RD) and normal cells (MDCK) was determined by using resazurin as a reagent.

Stock solutions (1×10^{-2} M) of the tested compounds (HL^1, HL^2, and complexes **1**–**6**) were prepared by dissolving 10^{-5} mol of each substance in 1 mL DMSO. These stock solutions were then used to prepare diluted solutions with final concentrations of 0.1, 1, 10, 100, and 1000 μM. Corresponding media were used for the dilution process.

To perform the assay, 90 μL of corresponding culture medium containing 1×10^4 cells were placed in the wells of a 96-well microtiter plate and incubated at 37 °C, 5% CO_2 for a 2–3 h period to allow the attachment of cells. Next, 10 μL of diluted solutions (0.1–1000 μM) of the tested compounds were added to the wells with culture medium. The incubation

continued for 24 h, after which resazurin indicator solution (20 µL) was added to each well. After 4 h of incubation in presence of resazurin, the absorbance was measured at two wavelengths (570 nm and 600 nm).

3.6. Antiradical Activity

The $ABTS^{\bullet+}$ method [54] with modifications was used to study the antiradical activity of HL^1, HL^2, and complexes **1**–**6**.

The reaction of 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS, 7 nM) and potassium persulfate (140 mM) gave the $ABTS^{\bullet+}$ radical cations. The reaction was performed in the dark at 25 °C for 12 h. The acetate-buffered saline (0.02 M, pH 6.5) was used for dilution of the obtained solution up to a concentration at which its absorbance at 734 nm was 0.70 ± 0.01 AU.

Stock solutions (1×10^{-2} M) of the tested compounds (HL^1, HL^2, and complexes **1**–**6**) in DMSO were diluted to obtain final concentrations of 10, 100, and 1000 µM. After that, 180 µL of $ABTS^{\bullet+}$ working solution and 20 µL of each tested compound solution were mixed and homogenized in the wells of a 96-well microtiter plate. After 30 min of incubation at 25 °C, the absorbance of the solutions was measured at 734 nm. The experiment was conducted three times to ensure accuracy.

4. Conclusions

Two new N^4,*S*-diallylisothiosemicarbazones and six new copper(II) coordination compounds have been synthesized. The structure of isothiosemicarbazones HL^1 and HL^2 was determined using NMR spectroscopy. Isothiosemicarabzones exist in different tautomeric forms in the solution. Crystal structures of $[H_2L^2]I$ and complex **3** ($[Cu(H_2O)(L^1)(CH_3CO1O)]\cdot 1.75H_2O$) were proved using X-ray diffraction analysis. The studied isothiosemicarbazones behave as tridentate ligands with N_3-set of donor atoms. All the studied complexes (**1**–**6**) are electrolytes, which indicates the process of substitution of acidic residues (Cl^-, Br^-, CH_3COO^-) by solvent molecules in the process of dissolution of these complexes.

Biological evaluation showed that the synthesized complexes manifest promising antibacterial, antifungal, and anticancer activity. Their antibacterial/antifungal activity in many cases is close to the activity of some drugs that are used in medicine for these purposes and, in some cases, surpass them. Complexes **4** and **5** selectively inhibit proliferation of BxPC-3 cancer cell line with IC_{50} values 5–8 nM. Thus, these complexes exceed 400–700 times the corresponding activity of doxorubicin and 2.5–18 times the activity of the corresponding copper(II) complexes with 2-acetylpyridine N^4-allyl-*S*-methylisothiosemicarbazone. Moreover, their selectivity indexes are in the range of 150–280 which confirms their strongly marked selectivity.

In addition, HL^1 and complexes **1**–**3** exhibit antiradical activity that exceeds that of trolox. Therefore, copper(II) complexes with *S*-substituted N^4-allylisothiosemicarbazones manifest promising biological properties, which are also affected by the nature of *S*-substituent.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/inorganics11050195/s1, Figure S1: ^1H NMR spectrum of 2-formylpyridine N^4,*S*-diallylisothiosemicarbazone (HL^1); Figure S2: ^{13}C NMR spectrum of 2-formylpyridine N^4,*S*-diallylisothiosemicarbazone (HL^1); Figure S3: ^1H NMR spectrum of 2-acetylpyridine N^4,*S*-diallylisothiosemicarbazone (HL^2); Figure S4: ^{13}C NMR spectrum of 2-acetylpyridine N^4,*S*-diallylisothiosemicarbazone (HL^2); Figure S5: FTIR spectrum of HL^1; Figure S6: FTIR spectrum of **1**; Figure S7: FTIR spectrum of **2**; Figure S8: FTIR spectrum of **3**; Figure S9: FTIR spectrum of HL^2; Figure S10: FTIR spectrum of **4**; Figure S11: FTIR spectrum of **5**; Figure S12: FTIR spectrum of **6**.

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