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Synthesis, characterization and biological activity of Pt(II) complexes with steroidal thiosemicarbazones

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Abstract: In this work, Pt(II) complexes of previously synthesized steroidal thiosemicarbazones were synthesized and characterized. The ligands and their metal complexes were studied by analytical and spectroscopic data (elemental analysis, IR, 1D NMR and 2D NMR, HSQC, HMBC, NOESY, COSY), the analysis of which enabled complete ^{1}H and ^{13}C assignments of each compound including *E* and *Z* isomers. All the synthesized ligands and complexes were screened for their cytotoxic and antimicrobial activity. The results demonstrate that new steroidal thiosemicarbazone complexes were significantly less cytotoxic than corresponding steroidal thiosemicarbazones. Also, complexes show lower antimicrobial activity than the standard drugs, similar to the activity of the starting thiosemicarbazones.

Keywords 3-oxo-α,β-unsaturated steroids; hydrazones; square-planar complexes; cytotoxicity; antimicrobial activity

INTRODUCTION

Steroids are a group of biologically active molecules widespread in nature and play a very important role in biological systems. Also, steroid based chemotherapeutics are widely used in medicine. Therefore, certain functional and structural modifications of the steroid core by the addition of new functional groups or heterocyclic systems can be very useful, giving compounds with new and more pronounced biological activity.[1] Likewise, substituted thiosemicarbazone derivatives have proven to be very useful because of their interesting biological behaviour.[2] These compounds may act as antidepressants,[3] muscle relaxants, psychoeptic, hypnotics,[4–7] but were also shown to have antimicrobial,[2,8,9] antiamoebic,[10] anti-inflammatory,[11,12] and cytotoxic activities.[13] Thiosemicar-

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bazones are compounds that can be used as possible ligands for metal complexes and for the syntheses of heterocyclic compounds.[14–20]

Taking all this into consideration, new steroidal mono- (**2a**,**b**) and bis(thiosemicarbazones) (**3a**,**b**), obtained from 19-norandrost-4-ene-3,17-dione (**1a**) and androsta-4,9(11)-diene-3,17-dione (**1b**), have recently been synthesized by our research group[21] (Scheme 1). All these compounds were fully characterized and their biological activity was examined. It was found that 3-thiosemicarbazones **2a** and **2b** exhibited very high cytotoxic actions against all examined cancer cell lines, much higher than corresponding starting steroids **1a** and **1b** or thiosemicarbazide itself.

In the late 1960s, Rosenberg[22–25] discovered the anticancer activity of cisplatin which began to be used in the treatment of cancer. Since the discovery of cisplatin, to this day, a large number of metal complexes have been synthesized in order to find potential chemotherapeutics with better antitumor potential, higher selectivity in killing cancer cells and fewer side effects.[26]

Motivated by aforementioned issues and as a continuation of our work on the new hetero- steroid derivatives as biologically active molecules,[1,21,27–30] we decided to prepare new steroidal complexes with platinum, in the reaction of previously synthesized 3-thiosemicarbazones **2a** and **2b** with cisplatin and to examine their biological activity and compare it with the activity of those reported earlier.[21] To the best of our knowledge, very few Pt steroidal complexes have been prepared so far.[31,32]

EXPERIMENTAL

Chemistry

Melting points were determined on Digital melting point WRS-1B apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer spectrophotometer FT-IR 1725X. NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer (^{1}H at 500 MHz; ^{13}C at 125 MHz) in DMSO-d_6 at room temperature using $SiMe_4$ as internal standard, δ / ppm, J / Hz. The HRMS spectra were recorded on an Agilent 6210 LC ESI-MS TOF spectrometer. Elemental analyses (C, H, N and S) were performed by standard micro-methods on Vario EL *III* analyzer. Molar conductivities were measured at room temperature (25 °C) on a digital conductivity meter Cond 330i. Thin-layer chromatography (TLC) was performed using aluminium plates coated with Merck silica gel 60 F_{254} and flash column chromatography (FCC) was performed on silica gel Merck 0.040–0.063 mm. TLC spots were detected with 50 % aq. H_2SO_4 followed by heating. 19-Norandrost-4-ene-3,17-dione and androsta-4,9(11)-diene-3,17-dione were purchased from Galenika AD (Belgrade) and recrystallized from a suitable solvent. c*is*-Diamminedichloridoplatinum(II) ([$Pt(NH_3)_2Cl_2$]) was obtained from Sigma–Aldrich.

General procedure for the synthesis of thiosemicarbazones

Thiosemicarbazones **2a** and **2b** were prepared as described earlier.[21]

To a solution of steroid (**1a**,**b**) (1 mmol) in dried ethanol (50 mL) thiosemicarbazide (1 mmol) was added. The solution was then allowed to reflux for 5 h under stirring. pH of the mixture was adjusted to ~ 4.5 with CH_3COOH (about 3 mL). After completion of the reaction

(monitored by TLC), the solvent was removed under reduced pressure. The residue was chromatographed by FCC using the indicated solvent system. In both cases the products were obtained as inseparable mixtures of *E* and *Z* diastereoisomers.

*19-Norandrost-4-ene-3,17-dione 3-thiosemicarbazone (**2a**) (E/Z=7:3)*

Starting with 270 mg 19-norandrost-4-en-3,17-dione (**la**), elution with toluene/EtOAc (8/2) afforded compound **2a** (217 mg, 63%).

*Androsta-4,9(11)-diene-3,17-dione 3-thiosemicarbazone (**2b**) (E/Z=8:2)*

Starting with 285 mg androsta-4,9(11)-diene-3,17-dione (**1b**) elution with toluene/EtOAc (85/15) afforded compound **2b** (189 mg, 53%).

*Synthesis of complexes **4** and **5***

*Complex **4** (Pt(II) with ligand **2a**).* Into a solution of 19-norandrost-4-ene-3,17-dione 3-thiosemicarbazone (**2a**) (0.1 mmol, 34.5 mg) in dichloromethane (10 mL), cisplatin ($[Pt(NH_3)_2Cl_2]$, 0.1 mmol, 30 mg) was added. The mixture was stirred under reflux for 3 h and filtered. The solution was then allowed to cool slowly to room temperature and then placed in the refrigerator. After six days, a yellow solid precipitate was obtained. The precipitate was filtered off, washed with a small amount of methanol and dried over silica gel to give 16.9 mg (19.4 %) of complex **4** [Pt(**2a**)$_2$]. Mp > 200 °C (decomp.). IR (ATR/cm^{-1}): 3448, 3306, 3116, 2922, 2852, 1736, 1607, 1528, 1447, 1419, 1320, 1006, 879. Anal. Calcd for $C_{38}H_{52}N_6O_2PtS_2$: C 51.63; H 5.93; N 9.51; S 7.25; Found: C 50.96; H 6.16; N 10.24; S 7.52. Λ_M (DMSO) = 3.4 μS cm^{-1}.

^{1}H-NMR (500 MHz, DMSO-d_6): 0.62 (*qd*, *J* = 10, 4 Hz, 1H, H-9), 0.83 (*s*, 3H, CH_3-18), 0.87–0.99 (*m*, 2H, Hα-7, Hα-12), 1.00–1.12 (*m*, 2H, Hα-1, H-14), 1.25 (*qd*, *J* = 13, 2.5 Hz, 1H, Hα-11), 1.49–1.58 (*m*, 2H, H-8, Hβ-15), 1.65 (*dt*, *J* = 12.5, 2.5 Hz, 1H, Hβ-12), 1.73 (*br.d*, *J* = 11.5 Hz, Hβ-11), 1.81–1.90 (m, 3H, Hβ-2, Hβ-7, Hα-15), 1.95 (m, 1H, Hα-16), 2.02 (m, Hβ-1), 2.07 (*m*, 1H, H-10), 2.20–2.30 (*m*, 2H, Hα-6, Hβ-6), 2.39 (*dd*, *J* = 19, 10 Hz, 1H, Hβ-16), 3.40 (*m*, 1H, Hα-2, overlapped with DMSO), 6.52 (*s*, 1H, H-4), 6.70 (*br.s*, 2H, NH_2). ^{13}C NMR (125 MHz, DMSO-d_6): 219.3 (*s*, C-17), 172.3 (*s*, C=S), 161.3 (*s*, C-3), 153.3 (*s*, C-5), 122.1 (*d*, C-4) 50.4 (*d*, C-14), 49.7 (*d*, C-9), 47.1 (*s*, C-13), 41.4 (*d*, C-10), 38.8 (*d*, C-8), 35.1 (*t*, C-16), 34.9 (*t*, C-6), 31.4 (*t*, C-12), 29.8 (*t*, C-7), 26.3 (*t*, C-11), 25.7 (*t*, C-1), 24.8 (*t*, C-2), 21.2 (*t*, C-15), 13.5 (*q*, C-18).

*Complex **5** (Pt(II) with ligand **2b**).* Into a solution of androsta-4,9(11)-diene-3,17-dione 3-thiosemicarbazone (**2b**) (0.1 mmol, 35.7 mg) in methanol (10 mL), cisplatin ($[Pt(NH_3)_2Cl_2]$, 0.1 mmol, 30 mg) was added. The reaction mixture was stirred with heating for 3 h at 65 °C and filtered. The solution was then allowed to cool slowly to room temperature and then placed in the refrigerator. After slow evaporation of the solvent in the refrigerator (≈ 7 °C) during six days yellow solid precipitate was obtained. The precipitate was filtered off, washed with a small amount of methanol and dried over silica gel to give 14.8 mg (23.9%) of complex **5** [Pt(**2b**)$_2$]. Mp > 200 °C (decomp.). IR (ATR/cm^{-1}): 3455, 3343, 3289, 3197, 2931, 2900, 2843, 1739, 1720, 1608, 1518, 1322, 1017, 826. Anal. Calcd for $C_{40}H_{52}N_6O_2PtS_2$: C 52.91; H 5.77; N 9.25; S 7.06; Found: C 52.83; H 5.80; N 9.21; S 7.01. Λ_M (DMSO) = 1.9 μS cm^{-1}.

^{1}H-NMR (500 MHz, DMSO-d_6): 0.75 (*s*, 3H, H_3C-18), 0.85 (*qd*, *J* = 13, 2.5 Hz, Hα-7), 1.16 (*s*, 3H, H_3C-19), 1.24 (*m*, 1H, H-14), 1.49–1.63 (*m*, 2H, Hβ-1, Hα-15), 1.79 (*br.d*, *J* = 18 Hz, 1H, Hα-12), 1.84–1.92 (*m*, 2H, Hα-1, Hβ-12), 1.95–2.09 (*m*, 4H, Hβ-6, Hβ-7, Hβ-15, Hβ-16), 2.24 (*td*, *J* = 16.5, 4.5 Hz, 1H, Hβ-2), 2.34 (*m*, 1H, H-8), 2.44 (*dd*, *J* = 17.5, 9 Hz, 1H, Hα-16), 2.55 (*m*, 1H, Hα-6, overlapped with DMSO), 3.36 (*m*, 1H, Hα-2, partially overlapped with H_2O from DMSO), 5.31 (*d*, *J* = 5 Hz, H-11), 6.38 (*s*, 1H, H-4), 6.78 (*br.s*, 2H, NH_2). ^{13}C NMR (125 MHz, DMSO-d_6): 220.0 (*s*, C-17), 172.9 (*s*, C=S), 160.6 (*s*, C-3), 156.5 (*s*,

C-5); 146.1 (*s*, C-9), 121.3 (*d*, C-4), 116.1 (*d*, C-11), 47.7 (*d*, C-14), 45.1 (*s*, C-13), 40.4 (*s*, C-10), 36.2 (*d*, C-8), 35.6 (*t*, C-16), 33.5 (*t*, C-12), 32.6 (*t*, C-6), 32.5 (*t*, C-1), 30.9 (*t*, C-7), 25.9 (*q*, C-19), 24.3 (*t*, C-2), 22.1 (*t*, C-15), 13.7 (*q*, C-18).

Biology

Cytotoxicity assay. The cytotoxic activity of the compounds was evaluated against three human malignant cell lines: cervical adenocarcinoma (HeLa), chronic myelogenous leukemia (K562), and acute T-cell leukemia Jurkat cell line. The cytotoxicity assay procedure was described elsewhere.[1,21,30] The positive control was chemotherapy drug cisplatin. Survival of cells was determined by MTT assay after 72 h of continuous action, according to the method of Mosmann,[33] which was modified by Ohno and Abe,[34] and described in detail in our previous studies.[28–30] All tested cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA).

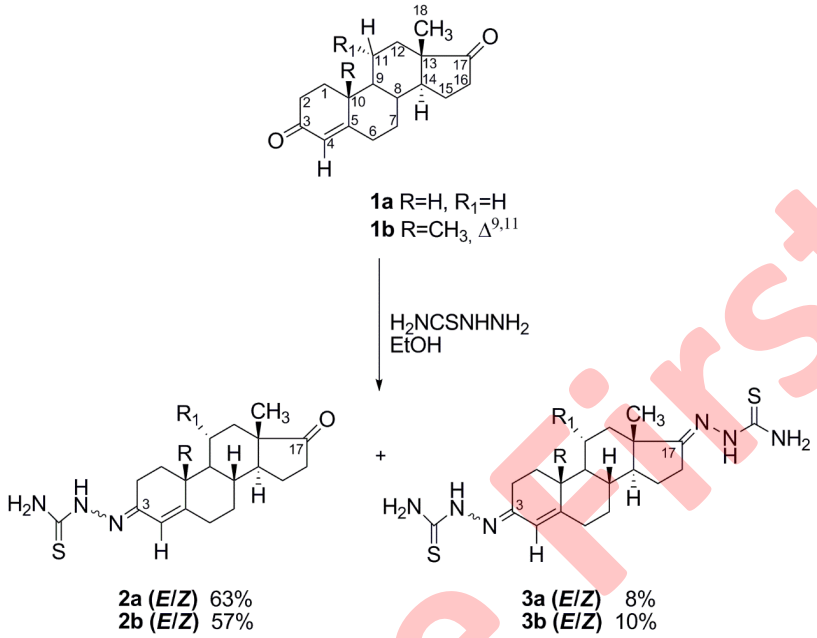
Antimicrobial activity. The antibacterial activity was evaluated using two different strains of bacteria, one Gram-positive bacteria: *Clostridium sporogenes* (ATCC 19404), and one Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 9027). Amikacin (30 μg/100 μL H_2O) was used as positive control, while water and DMSO served as negative controls. Antibacterial activity was determined by well diffusion method,[35] and described in detail in our previous study.[1] The fungus tested was *Aspergillus brasiliensis* (ATCC 16404). Nystatin (30 μg/100 μL DMSO) was used as a positive control, while DMSO served as a negative control. Antifungal activity was determined according to the method described in detail in our previous study.[1]

The brine shrimp test. The brine shrimp test of toxicity was performed against freshly hatched nauplii of *Artemia salina*.[36] The method was slightly modified by our team and described earlier.[29] The compounds were dissolved in DMSO and diluted by artificial seawater until the concentration range of 0.01–0.50 mg/mL was obtained. The final concentration of DMSO was 1% and did not cause changes of viability of nauplii. The number of nauplii was approximately 20. Surviving nauplii were counted after 24 h, and LC_{50} (concentration lethal to 50 % of the nauplii) were determined after statistical analysis. All the tests were performed in triplicate.

RESULTS AND DISCUSSION

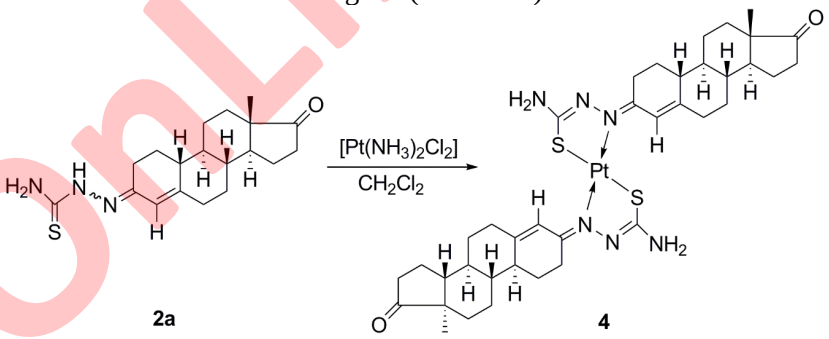
Steroidal thiosemicarbazones **2a** and **2b** were prepared, as described earlier,[21] starting from 19-norandrost-4-ene-3,17-dione (**1a**) or androsta-4,9(11)-diene-3,17-dione (**1b**) and thiosemicarbazide in EtOH in the presence of CH_3COOH. As α,β-unsaturated 3-carbonyl group is more active than 17-carbonyl group, the reaction was conducted by controlling an equimolar ratio of **1a**,**b** and thiosemicarbazide (1:1) to give **2a**,**b** in the yields of 63 and 57 %, respectively. Nevertheless, bis(thiosemicarbazones) **3a**,**b** were obtained as well in a small amount (8 and 10 %), even under such conditions (Scheme 1).

All synthesized compounds were fully characterized by their analytical and spectroscopic data (HRMS, IR, 1D NMR and 2D NMR, HSQC, HMBC, NOESY, COSY). The ^{1}H and ^{13}C NMR analysis revealed the presence of two diastereoisomers, which could not be separated.[21] Therefore, compounds **2a** and **2b** were used as ligands for complexation reactions with $[Pt(NH_3)_2Cl_2]$ in the form of mixtures of both isomers (*E* and *Z*).



Scheme 1. Synthesis of steroidal thiosemicarbazones.

Reaction of 19-norandrost-4-ene-3,17-dione 3-thiosemicarbazone **2a** with $[Pt(NH_3)_2Cl_2]$ (mole ratio 1:1) in dichloromethane (CH_2Cl_2) gave amorphous solid compound **4** soluble in DMSO. Elemental analysis showed that the complex **4** contains two molecules of the ligand (Scheme 2).



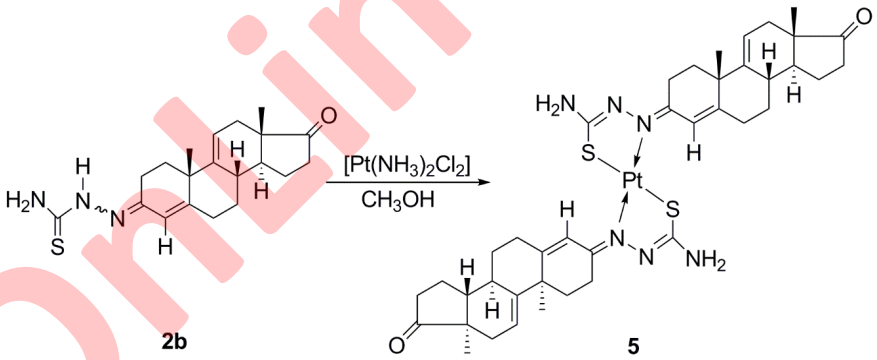
Scheme 2. Synthesis of complex **4**.

In the IR spectrum of **4** the absorption band at 1736 cm^{-1} for C(17)=O carbonyl was unchanged as well as sharp bands in the region 3246–3422 cm^{-1} originating from the *ν*(N-H) stretching. On the other hand, the absorption bands attributed to the *ν*(C=N) stretching vibration appeared at higher frequencies (1528 and 1607 cm^{-1}) compared to the ones from the ligand (1497 and 1586 cm^{-1})

indicating an interaction between the azomethine nitrogen and platinum. The ^{1}H NMR spectrum of the complex **4** contains only one set of signals even though the reaction was performed with a mixture of the diastereomers (*E* and *Z*). The signal for Hα-2 proton has shifted to a lower field and is now at about 3.40 ppm, partially covered by a signal from DMSO in which the spectra were recorded. The singlet for the olefin H-4 proton occurs at δ = 6.52 ppm and the signal for H_2N protons at 6.70 ppm as an extended singlet. Besides, there is a noticeable lack of signal for the H-N proton, which in the ligand was at δ = 10.07 ppm for (*E*)- and at 10.32 ppm for (*Z*)-isomer.

In the ^{13}C NMR spectrum again only one set of signals was observed, indicating that the newly formed complex was symmetric. The characteristic signals were: at δ = 24.8 ppm (C-2), 25.7 ppm (C-1), 41.4 (d, C-10), 122.1 ppm (C-4), 153.3 ppm (C-5), 161.3 ppm (C-3), 172.3 ppm (C=S) and 219.3 ppm (C-17). Furthermore, the values for C-4 and C-10 suggest that the ligand in the complex **4** is (*E*)-isomer.

Complex **5** was obtained in the direct reaction of androsta-4,9(11)-diene-3,17-dione 3-thiosemicarbazone (**2b**) and cisplatin (mole ratio 1:1) in methanol (CH_3OH). The reaction mixture was stirred with heating for 3 h at 65 °C. After filtration and slow evaporation of the solvent in a refrigerator (≈7 °C) during six days, yellow solid precipitate, complex **5**, soluble in DMSO was obtained (Scheme 3).



Scheme 3. Synthesis of complex **5**.

The band at 1739 cm^{-1} corresponding to the carbonyl group at C-17 is still visible in the IR spectrum of the synthesized complex. Also, the sharp bands in the 3289–3455 cm^{-1} region remain unchanged and are attributed to $\nu(NH_2)$ vibrations. In addition, the absorption bands attributed to the ν(C=N) vibrations appeared at higher frequencies (1518 and 1608 cm^{-1}) compared to those in the corresponding ligand (1502 and 1585 cm^{-1}), indicating an interaction between azomethine nitrogen and platinum.

The ^{1}H NMR spectrum of the complex **5** also contains only one set of signals. The spectrum showed doublet for the H-11 olefinic proton at 5.31 ppm (J = 5 Hz), the singlet for H-4 proton at δ 6.38 ppm and an extended singlet at 6.78 ppm for NH_2 protons. The signal for Hα-2 proton has shifted to a lower field and is now at 3.36 ppm, partially covered by a signal from DMSO in which the spectra were recorded. There is also a noticeable lack of signal for the H-N proton, which in the ligand was at δ 10.09 ppm for (*E*)- and 10.36 ppm for (*Z*)- isomer.

In accordance with the above, the ^{13}C NMR spectrum also showed only one set of signals indicating that, regardless of the fact that the reaction was performed with a mixture of isomers, in the complex **5** formation participates only one, probably (*E*)-isomer. The characteristic signals were: δ = 24.3 ppm (C-2), 40.4 ppm (C-10), 121.3 ppm (C-4), 160.6 ppm (C-3), 156.6 (C-5), 172.9 ppm (C=S) and 220.2 ppm (C-17).

The molar conductivity values of **4** and **5** in DMSO are 3.4 and 1.9 μS cm^{-1}, respectively, indicating that both complexes are non-electrolytes and are stable in DMSO. Having all these facts in mind as well as the results of elemental analysis, square-planar complexes **4** and **5** consist of two deprotonated semicarbazone ligands coordinated to metal ion *via* two thiolate sulfur atoms in *trans* position and two azomethine nitrogen atoms.

Cytotoxic activity in vitro

The cytotoxic activities of steroidal thiosemicarbazones **2a** and **2b**, and their Pt(II) metal complexes **4** and **5** were examined against cervical adenocarcinoma (HeLa), chronic myelogenous leukemia (K562) and acute T-cell leukemia Jurkat cell line with cisplatin used as a positive control. As shown in Table I, steroidal platinum(II) complexes **4** and **5** were almost inactive against HeLa and K562 cells, while both complexes exhibited low cytotoxicity against Jurkat cell line. These results demonstrate that new steroidal thiosemicarbazone complexes were significantly less active than corresponding steroidal thiosemicarbazones **2a** and **2b**.

TABLE I. The *in vitro* cytotoxic activity of compounds **2a**, **2b**, **4**, and **5** (concentration which induced 50 % decrease (IC_{50}) in malignant cell survival).

Compound	IC_{50}±SD, μM		
	HeLa	K562	Jurkat
4	>200	187.99±16.98	139.91±9.48
5	>200	>200	164.60±22.50
2a	18.1±3.3	11.3±2.2	n.a.
2b	17.3±6.8	6.7±0.3	n.a.
*CDDP	4.60±0.07	6.00±0.59	3.44±0.19

*c*is*-Diamminedichloridoplatinum(II)

Antimicrobial activity in vitro

The *in vitro* antimicrobial activity of steroidal thiosemicarbazones and their metal complexes were assayed by agar well diffusion method using cultures of *C.*

sporogenes, P. aeruginosa and A. brasiliensis. The results (Table II) showed that only complex **5** has a very weak antibacterial activity, similar to the activity of the starting thiosemicarbazones, while neither of the complexes has any anti-fungal activity.

TABLE II. Antimicrobial activity of the investigated compounds tested by agar well diffusion method.

Compound	Inhibition zone, mm		
	C. sporogenes	*P. aeruginosa*	*A. brasiliensis*
4	/	/	/
5	10	10	/
2a	10	10	10
2b	10	10	10
Amikacin	22	20	/
Nystatin	/	/	30

The brine shrimp test

The LC_{50} values obtained for the ligands and newly synthesized complexes are shown in Table III. All examined compounds were found to be less toxic when compared to cisplatin. However, complexes were found to be ten times more active in the brine shrimp assay compared to ligands.

TABLE III. Brine shrimp test results of the investigated compounds.

Comp.	LC_{50}, mM
4	0.079
5	0.035
2a	0.597
2b	0.663
*CDDP	0.006

*c*is*-Diamminedichloridoplatinum(II)

CONCLUSION

The reactions of 19-norandrost-4-ene-3,17-dione 3-thiosemicarbazone and androsta-4,9(11)-diene-3,17-dione 3-thiosemicarbazone with cisplatin gave the neutral square-planar Pt(II) complexes which consist of two deprotonated hydrazone ligands coordinated to metal ion *via* two thiolate sulfur and two azomethine nitrogen atoms.

The new steroidal thiosemicarbazone complexes were almost inactive against HeLa and K562 cells, while both complexes exhibited some cytotoxicity against Jurkat cell line. Complex **5** showed a very weak antibacterial activity, similar to the activity of the starting thiosemicarbazones, while neither of the complexes has any antifungal activity.

Platinum complexes mostly exert their activity by covalent modification of DNA. Relatively low activity of the complexes synthesized in this work might be attributed to the absence of a good leaving group and/or high stability of the chelate complexes. Since it is excepted that platinum steroidal complexes can readily pass through cell membranes, bind to cytosolic steroid receptors and migrate to the cell nucleus, further research will be directed to synthesis of platinum steroid complexes with enhanced reactivity with DNA.

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ИЗВОД

СИНТЕЗА, КАРАКТЕРИЗАЦИЈА И БИОЛОШКА АКТИВНОСТ КОМПЛЕКСА Pt(II) СА СТЕРОИДНИМ ТИОСЕМИКАРБАЗОНИМА

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Почевши од претходно синтетисаних стероидних тиосемикарбазона, у овом раду су синтетисани и окарактерисани комплекси платине(II). Лиганди и њихови метални комплекси проучавани су аналитичким и спектроскопским методама (елементална анализа, ИЦ, 1D NMR и 2D NMR, HSQC, HMBC, NOESY, COSY). Анализом добијених података омогућена је потпуна ^{1}H и ^{13}C асигнација свих једињења укључујући *E* и *Z* изомере. За синтетисане лиганде, као и њихове комплексе испитивана је цитотоксична и антимикробна активност. Резултати указују на то да нови стероидни тиосемикарбазонски комплекси испољавају значајно нижу цитотоксичност од одговарајућих стероидних тиосемикарбазона. Поред тога, комплекси поседују антимикробну активност сличну активности полазних тиосемикарбазона, а нижу од стандардних лекова.

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SUPPLEMENTARY MATERIAL TO

Synthesis, characterization and biological activity of Pt(II) complexes with steroidal thiosemicarbazones

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ISOLATED YIELDS AND SPECTROSCOPIC DATA OF SYNTHESIZED COMPOUNDS

*19-Norandrost-4-ene-3,17-dione 3-thiosemicarbazone (**2a**) (E/Z=7:3)*

Yield 63 %. R_f = 0.66 (toluene/EtOAc, 6 : 4, double development). Mp > 219 °C (decomp.). IR (ATR/cm^{-1}): 3422 and 3246 (NH), 1732 (C=O), 1586, 1497 (C=N), 1285 (C=S), 754 (C–S). ESI-TOF-MS: *m/z* for $C_{19}H_{27}N_3OS$ $[M + H]^+$: Calcd. 346.19476, found 346.19388.

(2a-*E*). ^{1}H-NMR (500 MHz, DMSO-d_6): 0.72 (*m*, 1H, H-9), 0.86 (*s*, 3H, H_3C-18), 0.98 (*qd*, *J* = 12, 4 Hz, 1H, Hα-7), 1.12–1.33 (*m*, 4H, Hα-1, Hα-11, Hα-12, H-14), 1.42–1.56 (*m*, 2H, H-8, Hβ-15), 1.64 (*d*, *J* = 11.5 Hz, 1H, Hβ-12), 1.76–1.92 (*m*, 3H, Hβ-1, Hβ-7, Hα-15), 1.96–2.09 (*m*, 3H, H-10, Hβ-11, Hα-16), 2.21 (*td*, *J* = 11, 4 Hz, 1H, Hα-6), 2.25 (*m*, 1H, Hβ-2), 2.37–2.45 (*m*, 2H, Hβ-6, Hβ-16), 2.82 (*dt*, *J* = 16.5, 3.5 Hz, 1H, Hα-2), 5.87 (*s*, 1H, H-4), 7.51 and 8.03 (2*br.s*, 2H, NH_2), 10.07 (*s*, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): 219.7 (*s*, C-17), 178.2 (*s*, C=S), 152.0 (*s*, C-5), 150.9 (*s*, C-3), 121.6 (*s*, C-4), 49.5 (*d*, C-14), 49.1 (*d*, C-9), 47.2 (*s*, C-13), 40.9 (*d*, C-10), 39.2 (*d*, C-8, overlapped with DMSO), 35.3 (*t*, C-16), 34.3 (*t*, C-6), 31.2 (*t*, C-12), 29.7 (*t*, C-7), 25.8 (*t*, C-11), 25.3 (*t*, C-1), 23.3 (*t*, C-2), 21.3 (*t*, C-15), 13.5 (*q*, C-18).

(2a-*Z*). ^{1}H-NMR (500 MHz, DMSO-d_6): 0.72 (*m*, 1H, H-9), 0.86 (*s*, 3H, H_3C-18), 0.98 (*qd*, *J* = 12, 4 Hz, 1H, Hα-7), 1.12–1.33 (*m*, 4H, Hα-1, Hα-11, Hα-12, H-14), 1.42–1.56 (*m*, 2H, H-8, Hβ-15), 1.64 (*d*, *J* = 11.5 Hz, 1H, Hβ-12), 1.76–1.92 (*m*, 3H, Hβ-1, Hβ-7, Hα-15), 1.96–2.09 (*m*, 3H, H-10, Hβ-11, Hα-16), 2.21 (*td*, *J* = 11, 4 Hz, 1H, Hα-6), 2.25 (*m*, 1H, Hβ-2), 2.31 (*dt*, *J* = 15, 3.5 Hz, 1H, Hα-2), 2.37–2.45 (*m*, 2H, Hβ-6, Hβ-16), 6.70 (*s*, 1H, H-4), 7.51 and 7.95 (2*br.s*, 2H, NH_2), 10.32 (*s*, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): 219.7 (*s*, C-17), 178.0 (*s*, C=S), 156.2 (*s*, C-5), 148.2 (*s*, C-3), 113.2 (*s*, C-4), 49.4 (*d*,

C-14), 48.9 (*d*, C-9), 47.2 (*s*, C-13), 42.1 (*d*, C-10), 39.2 (*d*, C-8, overlapped with DMSO), 35.3 (*t*, C-16), 34.9 (*t*, C-6), 31.2 (*t*, C-12), 30.0 (*t*, C-7), 27.1 (*t*, C-2), 25.8 (*t*, C-11), 25.1 (*t*, C-1), 21.3 (*t*, C-15), 13.5 (*q*, C-18).

*Androsta-4,9(11)-diene-3,17-dione 3-thiosemicarbazone (**2b**) (E/Z=8:2)*

Yield 53 %. R_f = 0.44 (toluene/EtOAc, 6 : 4, double development). Mp > 198 °C (decomp.). IR (ATR/cm^{-1}): 3425 and 3256 (NH), 3142, 2928, 1737 (C=O), 1585, 1502 (C=N), 1297 (C=S), 1087, 877. ESI-TOF-MS: *m/z* for $C_{20}H_{27}N_3OS$ $[M + H]^+$: Calcd. 358.19476, found 358.19350.

(2b-*E*). ^{1}H-NMR (500 MHz, DMSO-d_6): 0.79 (*s*, 3H, H_3C-18), 0.98 (*qd*, *J* = 13, 3 Hz, 1H, Hα-7), 1.20 (*s*, 3H, H_3C-19), 1.45 (*m*, 1H, H-14), 1.58 (*d*, *br.s*, *d*, *J* = 11 Hz, 1H, Hα-15), 1.69 (*td*, *J* = 13.5, 4.5 Hz, 1H, Hβ-1), 1.91 (*dd*, *J* = 17, 5 Hz, 1H, Hα-12), 1.96–2.05 (*m*, 4H, Hα-1, Hβ-7, Hβ-12, Hβ-15), 2.11 (*dd*, *J* = 19, 9.5 Hz, 1H, Hβ-16), 2.20–2.37 (*m*, 3H, Hβ-2, Hβ-6, H-8), 2.41 (*dd*, *J* = 20, 11 Hz, 1H, Hα-16), 2.51 (*m*, 1H, Hα-6), 2.88 (*dt*, *J* = 17.4, 3.5 Hz, 1H, Hα-2), 5.49 (*d*, *J* = 5.5 Hz, 1H, H-11), 5.81 (*s*, 1H, H-4), 7.54 and 8.07 (*br.s* and *m*, 2H, NH_2), 10.09 (s, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): 220.3 (*s*, C-17), 178.3 (*s*, C=S), 154.5 (*s*, C-5), 150.0 (*s*, C-3), 146.1 (*s*, C-9), 121.0 (*d*, C-4), 116.5 (*d*, C-11), 47.3 (*d*, C-14), 45.2 (*s*, C-13), 39.9 (*s*, C-10, overlapped with DMSO), 36.5 (*d*, C-8), 35.8 (*t*, C-16), 33.1 (*t*, C-12), 32.6 (*t*, C-1), 31.6 (*t*, C-6), 31.1 (*t*, C-7), 26.2 (*q*, C-19), 22.3 (*t*, C-15), 21.2 (*t*, C-2), 13.6 (*q*, C-18).

(2b-*Z*). (500 MHz, DMSO-d_6): 0.79 (*s*, 3H, H_3C-18), 0.98 (*qd*, *J* = 13, 3 Hz, 1H, Hα-7), 1.20 (*s*, 3H, H_3C-19), 1.56 (*m*, 1H, H-14), 1.58 (*d*, *br.s*, *d*, *J* = 11Hz, 1H, Hα-15), 1.80 (*td*, *J* = 13.5, 4 Hz, 1H, Hβ-1), 1.91 (*dd*, *J* = 17, 5 Hz, 1H, Hα-12), 1.96–2.05 (*m*, 4H, Hα-1, Hβ-7, Hβ-12, Hβ-15), 2.11 (*dd*, *J* = 19, 9.5 Hz, 1H, Hβ-16), 2.20–2.35 (*m*, 3H, Hβ-2, Hβ-6, H-8), 2.41 (*dd*, *J* = 20, 11 Hz, 1H, Hα-16), 2.47 (*m*, 1H, Hα-2), 2.51 (*m*, 1H, Hα-6), 5.47 (*m*, 1H, H-11), 6.65 (*s*, 1H, H-4), 7.53 and 7.98 (*br.s* and *s*, 2H, NH_2), 10.36 (*s*, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): 220.3 (*s*, C-17), 178.3 (*s*, C=S), 158.7 (*s*, C-5), 147.5 (*s*, C-3), 146.1 (*s*, C-9), 116.6 (*d*, C-11), 112.6 (*d*, C-4), 47.3 (*d*, C-14), 45.2 (*s*, C-13), 41.1 (*s*, C-10), 36.2 (*d*, C-8), 35.8 (*t*, C-16), 34.2 (*t*, C-1), 33.1 (*t*, C-12), 32.0 (*t*, C-6), 31.5 (*t*, C-7), 27.5 (*t*, C-2), 26.5 (*q*, C-19), 22.3 (*t*, C-15), 13.8 (*q*, C-18).