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Introduction

Modifications of the steroid backbone that cause changes in its physiological activity leading to the creation of new, biologically active molecules have become one of the main objectives of today's steroid chemistry. Transformations of the steroidal skeleton involving the introduction of heteroatoms and formation of heterocyclic moieties have received much attention in view of the different and interesting biological activities that have been demonstrated.¹ As a result, different studies of modifications of the steroidal A, B and D rings, including the

Synthesis, characterization and *in vitro* cytotoxic activities of new steroidal thiosemicarbazones and thiadiazolines[†]

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A series of new steroidal mono- and bis(thiosemicarbazones) (2a-e and 3a-e) and corresponding monoand bis(1,3,4-thiadiazolines) (4a-e and 5a-e) was synthesized, characterized and evaluated for their anticancer activity. Detailed NMR analysis of the mono- and bis(thiosemicarbazones) revealed the presence of two stereoisomers (Z and E) with different configurations in the hydrazone moiety at the C-3 position, where the substituents on the C(3)=N double bond in the main isomers adopted the E configuration. The configurations at C-3 and C-17 in thiadiazolines 4a-e and 5a-e were deduced by detailed NMR analysis as well as by the examination of Dreiding molecular models and X-ray analysis of 3-thiadiazoline 4a, which confirmed the structure and absolute configuration at C-3. The synthesized compounds were tested against six cancer cell lines (HeLa, K562, MDA-MB-361, MDA-MB-453, LS174 and A549), the normal human cell line MRC-5 and peripheral blood mononuclear cells (PBMC) isolated from healthy donors. The best activity was exhibited by 3-thiosemicarbazones 2a, 2b, 2c and 2e and 3,17-bis(thiadiazolines) 5a and 5d. Examination of the mechanisms of cytotoxicity on cervical adenocarcinoma HeLa cells revealed the pro-apoptotic action of these compounds, which triggered both extrinsic and intrinsic apoptotic pathways. These compounds also showed the ability to decrease angiogenesis in vitro. In addition, 3,17-bis(thiadiazolines) 5a and 5d showed high selectivity in anticancer activity against all the examined malignant cell lines. Compound 5a displayed prominent anticancer potential. The tested compounds showed poor antimicrobial activity.

> incorporation of heteroatoms or heterocyclic rings, have been reported.2-13 Such systems enhanced the biological activities of these molecules, giving rise to a variety of antiproliferative, antimicrobial, anti-inflammatory, hypotensive, hypocholesterolemic and diuretic activities. On the other hand, substituted thiosemicarbazone derivatives have attracted considerable attention due to their interesting biological activity.14 Compounds containing a thiosemicarbazone moiety are known to possess tranquilizing,¹⁵ muscle relaxing, psychoanaleptic, hypnotic,^{16–19} antidepressant,²⁰ antimicrobial,14,21,22 antiamoebic,²³ analgesic, anti-inflammatory^{24,25} and cytotoxic activities.26 Also, thiosemicarbazones are important compounds as possible ligands for metal complexes and for the synthesis of heterocyclic compounds.²⁷⁻³⁵ Heterocyclic compounds, especially those containing S and N atoms, are of great importance in treating biological systems.²¹ Among these, thiazole, thiazolidinone, and thiadiazoline are of great significance because they constitute the structural frameworks of several naturally occurring alkaloids that show a wide range of pharmaceutical and industrial importance.35

> Our interest in the transformation of steroidal compounds has for quite some time been focused on modified steroids that contain heteroatoms, new functional groups or heterocyclic

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Paper

systems as a part of their skeleton. Within this research area, we have recently reported a few papers on the synthesis and biological activity of steroidal lactams, thiolactams, and several sulfur- and sulfur and phosphorus-containing compounds, including new steroidal dimers, 3-thiones, 3,17-dithiones, 1,2,4-trithiolanes, and phosphorotrithioates, as well as 17-spiro- and 17-substituted-1,3,2-oxathiaphospholane-2-sulfides.^{8,36-39}

The results mentioned above prompted us to prepare new modified A ring- and A and D ring-substituted derivatives from 3-oxo- α , β -unsaturated steroids. Here, we report the synthesis of new mono- and bis(thiosemicarbazones) and mono- and bis(1,3,4-thiadiazolines) derived from several androstene derivatives (androst-4-ene-3,17-dione, 19-norandrost-4-ene-3,17-dione, androsta-4,9(11)-diene-3,17-dione, 11 α -hydroxyandrost-4-ene-3,17-dione and pregn-4-ene-3,20-dione) and their biological activity.

Steroidal thiosemicarbazones are well known and have been prepared for many years, but still there are only a few studies that provide detailed information on steroidal thiosemicarbazone isomers and even fewer about 1,3,4-thiadiazolines. For these reasons, we considered it important to synthesize new examples and provide and discuss all their structural details. The main focus of the chemistry section in the present work is concentrated on detailed configurational analysis, in particular the determination of the Z/E configuration of the C(3)=N double bond in thiosemicarbazones and the absolute configuration at C(3) and C(17) in mono- and bis(1,3,4-thiadiazolines), which was confirmed by X-ray analysis.

In addition, amongst the steroidal thiosemicarbazones and thiadiazolines synthesized so far, a very small number of papers deal with their biological activity and most of these studies describe the antibacterial effect of such compounds (I–VIII) (Fig. 1),^{14,21,40–43} whereas only a few studies have been carried out on steroidal thiosemicarbazones and thiadiazolines as anti-tumor agents (IX–XV) (Fig. 2).^{44–49}

In a similar way, another goal of this work was to perform an extensive investigation of the *in vitro* cytotoxic activity of the newly synthesized steroid derivatives. These compounds were tested against six human malignant cell lines: cervical adenocarcinoma (HeLa), two breast cancer cell lines (MDA-MB-453 and MDA-MB-361), chronic myelogenous leukemia (K562), colon adenocarcinoma (LS174) and lung adenocarcinoma (A549) cells.

Also, their cytotoxic activity was tested against normal human lung fibroblasts MRC-5. Compounds that exerted the most pronounced cytotoxic action were further screened for cytotoxic activity against normal human peripheral blood mononuclear cells (PBMC), which were non-stimulated and stimulated to proliferate with the mitogen phytohaemagglutinin (PHA). The specific aim of the study in this respect was to get an insight into the mechanisms of the cytotoxic effects of the tested compounds.



Fig. 1 Steroidal thiosemicarbazones and thiadiazolines with antibacterial activity.



Fig. 2 Steroidal thiosemicarbazones and thiadiazolines as cytotoxic agents.

Results and discussion

Chemistry

Steroidal thiosemicarbazones 2a-e and 3a-e were prepared starting from steroids 1a-e and thiosemicarbazide in EtOH in the presence of CH₃COOH. The reaction was conducted until complete consumption of 1a-e occurred, leading to thiosemicarbazones 2a-e and 3a-e. Compounds 2a-e with a 3-thiosemicarbazone group were obtained by ensuring an equimolar ratio of **1a-e** to thiosemicarbazide (1:1), because the α,β unsaturated 3-carbonyl group was more active than the 17carbonyl in 1a-d and the 20-carbonyl in 1e, and thiosemicarbazide selectively reacted with the 3-carbonyl. However, even under such conditions bis(thiosemicarbazones) 3a-e were obtained in small amounts (6-13%). In order to synthesize compounds 3a-e, the same reaction was performed with another molar ratio of thiosemicarbazide to 1a-e(2:1), and under these conditions bis(thiosemicarbazones) 3a-e were obtained in much higher yields (48-56%). In this reaction, the formation of 3-thiosemicarbazones 2a-e was observed as well and these products were isolated in yields of 6-11% (Scheme 1). The thiosemicarbazones were further subjected to ring closure by means of acetylating agents^{21,39,50} to obtain the corresponding 1,3,4thiadiazolines **4a–e** and **5a–e** in good yields (56–73%) (Scheme 1). All compounds were characterized by their analytical and spectroscopic data (HRMS, IR, 1D NMR, 2D NMR, HSQC, HMBC, NOESY, and COSY), analysis by which enabled the complete ¹H and ¹³C assignment of each compound and its (*E*)- and (*Z*)-isomers.

The ¹H and ¹³C NMR analysis of compounds **2a–e** and **3a–e** revealed the presence of two pairs of signals, which pointed to the existence of two diastereoisomers, which, despite the fact that the (*E*)-isomers were always obtained in excess (deduced by comparing the peak areas of H-4 and H–N–N=C(3) in the corresponding ¹H NMR spectra), could not be separated. All our efforts to separate the isomers by crystallization or by chromatography on silica failed (no eluent that was tested caused separation of the diastereoisomers). For that reason, all spectra that were taken were of mixtures of **2a–e** and **3a–e**.

Spectral analysis demonstrated that the thiosemicarbazone fragment of **2a** was at the C-3 position and those of **3a** at both C-3 and C-17 positions. The existence of two isomers of both compounds can be attributed to the hydrazone moiety at the C-3 position, where the substituents on the C(3)=N double bond of the main isomers adopted the *E* configuration.



Scheme 1 Synthesis of new steroidal thiosemicarbazones and thiadiazolines 2–5.

The molecular formula of **2a** was deduced to be $C_{19}H_{27}N_3OS$ from ESI-TOF-MS, which showed that the molecular weight of **2a** was 73 Da higher than the molecular weight of the parent 19norandrost-4-ene-3,17-dione (**1a**), which confirmed that compound **2a** contained a thiosemicarbazone moiety.

In the IR spectrum of 2a, the absorption band for carbonyl at C-3 was missing but the C(17)=O absorption band at 1732 cm^{-1} was unchanged. In addition, new absorption bands at 1586 and 1497 cm⁻¹ appeared, which were attributed to ν (C=N) stretching vibrations, as well as additional sharp bands in the region of 3246–3422 cm⁻¹, which were due to ν (N–H) stretching vibrations, and a strong band at 1284 cm⁻¹, which was ascribed to ν (C=S), which all confirmed the formation of the desired thiosemicarbazone compound. The ¹H NMR spectrum of the mixture of isomers of 2a showed two singlets for H-4 at δ 5.92 ppm and 6.19 ppm, respectively, and four broad singlets for the H_2N protons at δ 6.36 and 7.24 ppm for the (*E*)- and at δ 6.31 and 7.22 ppm for the (Z)-isomer. The signals for H–N appeared as singlets at δ 8.71 ppm and 8.90 ppm for the (*E*)- and (*Z*)-isomers, respectively. The ¹³C NMR spectrum also revealed two sets of signals, which confirmed that the obtained compound exists in the form of two isomers. The characteristic signals were: δ 121.4 ppm and 111.2 ppm (C-4), 150.5 ppm and 149.5 ppm (C-3), 153.2 ppm and 158.4 ppm (C-5) and 178.4 ppm and 178.3 ppm (C=S) for the (E)- and (Z)-isomers, respectively.

The absolute configuration was determined by analysis of the NOESY cross-peaks involving the amide proton from the hydrazone moiety. In the major isomer, the proton at δ 8.71 ppm showed NOESY correlations with H α -2 at 2.66 and H β -2 at 2.07 ppm, which indicated the *E* configuration, whereas in the minor isomer the N–H proton at δ 8.90 ppm showed a NOESY cross-peak with H-4 at 6.20 ppm, which corresponds to the *Z* configuration (Fig. 3).

3,17-Bis(thiosemicarbazone) **3a** also occurs as a mixture of two isomers. The molecular formula of **3a** was deduced to be $C_{20}H_{30}N_6S_2$ from ESI-TOF-MS, which showed that the molecular weight of this compound was 73 Da higher than the molecular weight of compound **2a**, which indicated that compound **3a** had one more thiosemicarbazone moiety. Also, in the IR spectrum of **3a** the absorption bands for carbonyls at C-3 and C-17 were missing. Instead, all the characteristic absorption bands for thiosemicarbazones at 3421, 3240, 1583, 1496, and 1282 cm⁻¹ appeared.

From the NMR spectral data it can be seen that the isomers **3a-E** and **3a-Z** differ in the configuration at the C(3)=N double bond, whereas at C-17 only one isomer was obtained.

The ¹³C NMR spectrum of **3a** confirmed the presence of 20 carbon signals and also revealed two sets of signals. The major differences in the spectral data of **3a** compared with those of **2a** were the loss of the carbonyl peak at δ 222.4 ppm, which was previously assigned to C-17 and was replaced by a peak at δ 167.0 ppm (C(17)=N), and the presence of a new peak at δ 178.7 ppm for another C=S group. The other characteristic signals were: δ 121.3 ppm and 111.4 ppm (C-4), 150.7 ppm and 149.7 ppm (C-3), 153.1 ppm and 158.4 ppm (C-5) and 178.3 ppm and 178.2 ppm (C=S) for **3a**-*E* and **3a**-*Z*, respectively.

The ¹H NMR spectrum of 3a also contained two sets of signals: two singlets for H-4 at δ 5.91 for the (*E*)- and 6.22 ppm for the (Z)-isomer, and two broad singlet pairs for the H_2N protons from the hydrazone moiety at C-3 at δ 6.50 and 7.26 ppm for the (E)- and δ 6.45 and 7.23 ppm for the (Z)-isomer. The broad singlet pair for H₂N from the thiosemicarbazone moiety at C-17 appeared at δ 6.53 and 7.18 ppm for both isomers. The signals for the two H–N protons appeared as singlets at δ 8.83 and 8.49 ppm for the (E)-isomer and at δ 9.05 and 8.49 ppm for the (Z)-isomer. The NOESY cross-peaks between NH/H α -2 and NH/H β -2 confirmed the *E* configuration on the C(3)=N double bond in the major isomer of 3a. In both isomers, the hydrazone moiety at C-17 adopted the E configuration, which was confirmed by the NOESY correlations of NH (8.49 ppm) with Ha-16 (2.28 ppm) and H β -16 (2.47 ppm) and the absence of any correlation with CH₃-18. Moreover, inspection of Dreiding models suggests that steric repulsion between CH3-18 from the sterane skeleton and the thiosemicarbazone moiety at C-17 prevents the formation of the (Z)-isomer (Fig. 4).

Almost the same patterns in the ¹H NMR and ¹³C NMR spectra were observed for all the newly synthesized thiosemicarbazones **2a–e** and **3a–e**.

When thiosemicarbazones 2a and 3a as mixtures of both isomers (*E* and *Z*) were treated with acetylating agents in basic media, from each mixture a single new heterocyclic compound was isolated. Thus, thiadiazoline 4a was formed from the mixture of 2a and 3,17-bis(thiadiazoline) 5a from the mixture of 3a.

The molecular formula of **4a** was deduced to be $C_{23}H_{31}N_3O_3S$ from ESI-TOF-MS, which showed a peak at m/z 430.21581 (calculated for $C_{23}H_{32}N_3O_3S [M + H]^+$, 430.21589), and that of **5a** to be $C_{28}H_{38}N_6O_4S_2$ with a peak at m/z 587.24700 (calculated for $C_{28}H_{39}N_6O_4S_2 [M + H]^+$, 587.24687).

In the IR spectra new characteristic absorption bands appeared at 1618 (C=N) and 756 (C-S) cm^{-1} for compound 4a



Fig. 3 Absolute configuration on the C(3)=N double bond in 2a-*E* and 2a-*Z* with the key NOESY correlations.



Fig. 4 Absolute configuration on the C(17)=N double bond in 3a with the key NOESY correlations.

and at 1642, 1615 (C=N) and 722 (C-S) cm^{-1} for compound 5a, due to ring closure.

In the ¹H NMR spectra the absence of signals for the amino groups from the parent compounds **2a** and **3a** was evident. Furthermore, the *N*-acetyl protons of the thiadiazoline rings in both compounds were shown as singlets at δ 2.11 and 2.19 ppm for **4a** and at δ 2.09, 2.12, 2.18 and 2.22 ppm for **5a**. The signal for the alkenyl H-4 proton appeared at about δ 5.60 ppm for both compounds, upfield from the resonance of the corresponding proton in the starting compounds **2a** and **3a**, which also indicated the absence of a conjugated imine moiety, which was present in thiosemicarbazones **2a** and **3a**. In addition, the axial proton at C-16 in compound **5a** (in the α -position to the thiadiazoline ring at C-17) resonates separated downfield from the other ring protons at δ 4.35 ppm, owing to the position of the thiadiazoline ring and the strong anisotropic effect of C==O in the acetyl moiety bound to N-3".

In the ¹³C NMR spectra compound **4a** showed characteristic signals at δ 141.1 ppm for C-5' and compound **5a** at δ 146.4 and 149.4 ppm for C-5' and C-5", respectively, owing to (N=C-S), which indicated the cyclization of the thiocarbamoyl carbon. Also, the signals for C-3 in compound **4a** at δ 79.9 ppm and both C-3 and C-17 in compound **5a** at δ 81.3 and 94.0 ppm, respectively, were situated upfield from the resonances of the same carbon atoms in thiosemicarbazones **2a** and **3a**, which suggested the formation of spiro-thiadiazolines.

The combined use of HSQC, HMBC, COSY and NOESY (ROESY) spectra enabled the complete ¹H and ¹³C assignment of thiadiazolines **4a** and **5a** (see Experimental section). However, that did not help us to determine the absolute configuration at C-3. Luckily, in the case of compound **4a** we were able to obtain crystals that were suitable for X-ray analysis, which confirmed the structure and showed that the absolute configuration at C-3 was *R* (Fig. 5).

In the case of 3,17-bis(thiadiazoline) 5a our attempts at growing crystals that were suitable for X-ray analysis failed.



Fig. 5 Molecular structure of 4a with atom numeration. Anisotropic displacement ellipsoids are drawn at the 30% probability level. Hydrogen atoms are depicted as spheres of arbitrary radius. Bond lengths (Å): S1'-C5' = 1.739 (3), S1'-C3 = 1.852 (3), N4'-N3' = 1.397 (3), N3'-C3 = 1.487 (4), N4'-C5' = 1.282 (4), C4-C5 = 1.321 (5), N-C5' = 1.377 (4), N-C20 = 1.342 (4), N3'-C22 = 1.345 (4), O1-C17 = 1.192 (7), O2-C20 = 1.210 (5), O3-C22 = 1.234 (4). Valence angles (°): C5'-S1'-C3 = 89.86 (13), N3'-C3-S1' = 102.01 (17), C4-C3-C2 = 111.2 (3). Torsion angles (°): C5'-N4'-N3'-C22 = -176.3 (4), N3'-N4'-C5'-N = 178.5 (3), C3-C4-C5-C10 = -1.5 (5).

Considering the fact that the configuration at C-3 is already known (by comparison of the spectral data with those of compound 4a and from its X-ray analysis), the configuration at C-17 was determined by detailed analysis of the NMR spectra as well as by the examination of Dreiding molecular models. In the absence of any COSY or NOESY interactions that include the Nacetyl protons of the thiadiazoline ring at C-17, the most helpful were the protons bonded to C-16 (δ 31.0 ppm) at δ 2.06 ppm and especially the other proton, which resonates separated downfield from the other ring protons at δ 4.35 ppm. This signal showed a NOESY correlation with both H α -15 at δ 1.76 and H β -15 at δ 1.42 ppm, as well as with the 18-Me group at δ 0.95 ppm, and, according to the Dreiding models, was attributed to $H\beta$ -16. Because the apparent disparity in the ¹H NMR shifts in the case of H β indicates strong deshielding owing to the anisotropic effect of the C=O group from the acetyl moiety bound to N-3', the configuration at C-17 in 5a must be R (Fig. 6).

The mechanism of heterocyclization that leads to the formation of steroidal 1,3,4-thiadiazolines has already been described.^{21,50,51} It is proposed that the thiadiazoline ring is closed by the attack of the sulfur atom (which is a better nucleophile) of the thiosemicarbazone moiety on the steroidal sp^2 carbon, preferentially from the axial side, which enables the bulky NAc group to occupy the equatorial orientation, giving minimum steric hindrance and maximum stability.⁵¹ Therefore, the formation of the 3*R* and 3*R*,17*R* compounds is preferred over that of their isomers.

In the same way, the structures of thiadiazolines **4a–e** and 3,17-bis(thiadiazolines) **5a–d** were confirmed by their characterization data.

The exception was 3,20-bis(thiadiazoline) 5e derived from 3,20-bis(thiosemicarbazone) 3e, which was obtained as a mixture of two diastereoisomers (20R and 20S, in the ratio of approximately 1.2 : 1, as deduced by comparing the peak areas of H-C(4) and the H₃C-18 and H₃C-19 methyl groups in the corresponding ¹H NMR spectrum) with different configurations at C-20. As we could not separate the isomers, in the absence of any NOESY correlations it was not possible to confirm the complete stereostructure. These difficulties were accompanied by possible rotation around the C(17)-C(20) axis, which was confirmed by inspection of the Dreiding models. In this case, there was no steric repulsion between the bulky NAc group and the sterane skeleton, so closure of the thiadiazoline ring could occur by the attack of the sulfur atom of the thiosemicarbazone moiety on the C-20 sp² carbon atom from either side, which enabled the formation of both isomers.



Fig. 6 3D representation of the proposed structure of 5a and configuration at C-17 on the basis of NOESY correlation.

Molecular and crystal structure of 1,3,4-thiadiazoline 4a

The molecular structure of compound **4a** is depicted in Fig. 5, together with relevant structural data. The compound crystallizes in the non-centrosymmetric space group *I*4, and its absolute configuration was assigned unambiguously by reference to the known chirality of the steroid atoms. The steroidal core has a common structure, with rings B and C in chair conformations and the five-membered ring D in an envelope conformation on C-14. The cyclohexene ring A adopts a half-chair conformation (torsion angle C3–C4–C5–C10 = -1.5 (5)°) induced by the C4–C5 double bond (1.321 (5) Å). To this steroidal core, a thiadia-zoline ring is attached at the C-3 position, thus forming a spiro system. The X-ray structural study revealed that this newly formed stereocenter has *R* configuration, with S1′ in the axial and N3′ in the equatorial position relative to ring A.

There are a few structurally characterized compounds that contain a six-membered carbon ring and a thiadiazoline ring fused in a spiro system,^{34,50,52} but none of these is a steroid derivative. Generally, the thiadiazoline ring in 4a has virtually the same structural parameters as are found in literature data concerning compounds with 1,3,4-thiadiazoline rings.^{34,50,52,53} It slightly deviates from planarity, adopting an envelope conformation with C-3 as a pivot atom. The sulfur atom S-1' is asymmetrically bonded to two carbon atoms in a way that the bond with sp²-hybridized C-5' is shorter (1.739 (3) Å) than the bond with sp³-hybridized C-3 (1.852 (3) Å). The N4'-C5' distance (1.282 (4) Å) corresponds to a localized double bond. The only difference is the fact that in 4a the acetyl group at N-3' and the acetylamino group at C-5' are coplanar with the thiadiazoline fragment (τ (C5'-N4'-N3'-C22) = -176.3 (4)°; τ (N3'-N4'-C5'-N) = 178.5 (4)°), in contrast to other structures in which acetyl groups significantly deviate from coplanarity with the ring (121° $< |\tau(C5'-N4'-N3'-C22)| < 170^{\circ}$). The reason for this is the absence of any steric hindrance in 4a, in contrast to that found in other structures.

From the crystallographic point of view, the crystal packing of the molecules reveals an interesting feature, namely, the acetylamino NH group acts as a hydrogen bond donor to the acetyl oxygen from a neighboring molecule in a geometrical arrangement where four molecules form a molecular ring with crystallographic fourfold symmetry (Fig. 7). In this way, a supramolecular tetramer is formed, which is described by the $R_4^{4}(28)$ graph set.⁵⁴ The central void created by this arrangement is filled with disordered DMSO molecules.

Biological evaluation

Cytotoxic activity. Cytotoxic activity *in vitro* was tested against six human malignant cell lines: cervical adenocarcinoma HeLa, two breast cancer cell lines (originally estrogen receptor-negative and progesterone receptor-negative MDA-MB-453 cells and estrogen receptor-positive and progesterone receptor-positive MDA-MB-361 cells), chronic myelogenous leukemia K562 cells, colon adenocarcinoma LS174 cells and lung adenocarcinoma A549 cells. Also, cytotoxic activity was tested against normal human lung fibroblasts MRC-5 (Table 1). Compounds **2a**, **2b**, **2c**, **2e**, **5a** and **5d**, which exerted the most



Fig. 7 Fourfold-symmetrical hydrogen-bonded molecular ring, a feature of the crystal packing in **4a**. Hydrogen bond data: N-H = 0.78 (4) Å, $H\cdots O3i = 2.05$ (4) Å, $N\cdots O3i = 2.801$ (4) Å, $\angle (N-H\cdots O3i) = 159$ (4)°. Symmetry code (i): -y + 2, *x*, *z*.

pronounced cytotoxic activity, were further screened for cytotoxic activity against normal human peripheral blood mononuclear cells (PBMC), which were non-stimulated and stimulated to proliferate with the mitogen phytohaemagglutinin (PHA).

Against K562 cells, compounds **2a**, **2b**, **2c**, **2e**, **3a** and **5a** showed strong cytotoxic activity, with IC₅₀ values of 11.3, 6.7, 6.7, 10.7, 11.2 and 8.8 μ M, respectively. Compounds **3b**, **3c** and **5d** showed moderate activity, with IC₅₀ values of 28.1, 30.3 and 36.4 μ M, whereas compounds **2d** and **4a** exhibited weak activity against this cell line at 51.1 and 75.7 μ M, respectively. Towards cervical adenocarcinoma HeLa cells, compounds **2b**, **2e** and **5a** exerted strong cytotoxic activity, with IC₅₀ values of 7.6, 8.9 and 7.8 μ M, respectively, whereas compounds **2a**, **2c**, **3c** and **5b** were slightly less active, with IC₅₀ values of 18.1, 17.3, 14.3 and 21.2 μ M, respectively. Compounds **3a**, **3b**, **5c** and **5d** showed moderate activity, with IC₅₀ values from 27.7 to 43.9 μ M, whereas compounds **4a–e** were almost inactive against this cell line.

Compounds 2b, 2c, 2e and 5a exhibited strong activity against MDA-MB-453 cells, with IC50 values of 6.8, 6.9, 6.9 and 11.0 µM, respectively, whereas compounds 2a, 5b, 5c and 5d showed moderate activity, with IC_{50} values from 18.0 to 44.4 μ M. All the other compounds were practically inactive against this cell line. Towards the MDA-MB-361 cell line, compound 2e exerted strong cytotoxic activity, with an IC₅₀ value of 8.7 µM, whereas compounds 2b and 2c were less active, with IC50 values of 20.7 and 18.7 µM, respectively. Compound 5d showed moderate activity, with an IC_{50} value of 54.1 μ M, whereas the other compounds tested were practically inactive. Towards colorectal adenocarcinoma LS174 cells, compounds 2e and 5a showed a strong dose-dependent cytotoxic effect, with IC₅₀ values of 9.2 and 12.6 µM, respectively, compounds 2b, 2c, and 5d moderate and compound 2a weak activity, with IC₅₀ values of 35.2, 26.2, 42.6 and 61.3 µM, respectively.

Against human lung adenocarcinoma A549 cells, compounds 2a, 2b, 2c, 2e and 5a exhibited activity that was

Table 1 In vitro cytotoxic activity of investigated compounds

	$\mathrm{IC}_{50}\pm\mathrm{S.D.}$ ($\mu\mathrm{M}$	$IC_{50} \pm S.D. (\mu M)$								
Comp.	HeLa	MDA-MB-453	MDA-MB-361	K562	LS174	A549	MRC-5			
2a	18.1 ± 3.3	44.4 ± 2.9	144.1 ± 36.6	11.3 ± 2.2	61.3 ± 13.0	10.3 ± 0.7	7.7 ± 1.1			
2b	7.6 ± 0.2	6.8 ± 0.2	20.7 ± 6.0	6.7 ± 0.2	35.2 ± 11.6	8.9 ± 0.5	6.5 ± 0.1			
2 c	17.3 ± 6.8	6.9 ± 0.3	18.7 ± 8.9	6.7 ± 0.3	26.2 ± 6.0	9.5 ± 0.5	7.2 ± 1.3			
2d	≈200	154.3 ± 28.1	183.4 ± 5.2	51.1 ± 15.4	176.7 ± 40.3	178.8 ± 25.5	173.6 ± 45.8			
2d-OAc	43.9 ± 4.9	43.7 ± 5.9	≈200	10.7 ± 1.5	≈200	51.2 ± 7.7	48.1 ± 2.7			
2e	8.9 ± 1.6	6.9 ± 0.3	8.7 ± 3.1	6.6 ± 0.2	9.2 ± 3.9	8.6 ± 0.8	6.7 ± 0.5			
3a	42.5 ± 1.9	193.0 ± 12.1	>200	11.2 ± 0.4	>200	>200	>200			
3b	31.5 ± 3.3	110.8 ± 16.5	≈200	28.1 ± 1.5	>200	≈200	>200			
3c	14.3 ± 7.3	78.5 ± 21.6	>200	30.3 ± 7.5	>200	≈200	>200			
3d	131.4 ± 6.2	183.7 ± 17.1	197.4 ± 3.6	90.5 ± 13.4	194.8 ± 4.5	>200	>200			
3e	182.3 ± 25.0	191.5 ± 25.0	195.5 ± 6.3	158.9 ± 23.5	191.7 ± 11.6	187.4 ± 12.6	186.4 ± 23.6			
4a	199.2 ± 1.3	>200	>200	75.7 ± 17.2	163.8 ± 49.3	>200	>200			
4b	>200	>200	>200	192.8 ± 12.4	>200	>200	>200			
4 c	185.2 ± 20.9	178.2 ± 34.9	>200	181.2 ± 32.6	>200	>200	>200			
4d-OH	>200	>200	>200	195.6 ± 7.7	>200	>200	>200			
4d-OAc	121.6 ± 10.7	141.4 ± 24.4	170.4 ± 41.9	80.5 ± 9.9	>200	195.6 ± 7.7	>200			
4e	108.2 ± 18.3	167.9 ± 34.9	>200	195.8 ± 7.2	>200	187.4 ± 1.0	>200			
5a	7.8 ± 0.3	11.0 ± 1.9	>200	8.8 ± 1.3	12.6 ± 3.8	20.6 ± 1.3	12.7 ± 2.0			
5b	21.2 ± 1.5	18.0 ± 1.7	>200	163.6 ± 51.4	>200	175.3 ± 32.5	145.8 ± 31.5			
5 c	27.7 ± 11.6	29.0 ± 9.3	>200	157.2 ± 60.5	182.5 ± 30.3	121.0 ± 34.3	26.2 ± 8.7			
5d	37.2 ± 3.0	30.4 ± 7.1	54.1 ± 13.1	36.4 ± 2.0	42.6 ± 3.0	35.6 ± 5.0	$\textbf{38.6} \pm \textbf{8.8}$			
5e	151.8 ± 28.7	102.8 ± 2.9	>200	136.5 ± 46.9	200 ± 0.0	132.4 ± 37.2	159.3 ± 40.5			
\mathbf{TSC}^{a}	176.8 ± 9.2	37.7 ± 39.7	23.2 ± 2.6	18.0 ± 0.6	44.5 ± 11.4	176.3 ± 6.2	93.9 ± 16.6			
1a	194.2 ± 8.2	133.2 ± 39.7	>200	190.5 ± 16.5	180.5 ± 27.6	>200	>200			
1b	76.7 ± 7.5	109.2 ± 39.7	188.7 ± 16.0	177.1 ± 6.5	≈200	165.1 ± 1.1	191.0 ± 9.6			
1c	95.5 ± 18.8	128.3 ± 8.1	≈200	≈200	≈200	191.8 ± 14.1	>200			
1d	≈200	199.1 ± 1.6	>200	197.8 ± 3.8	>200	>200	>200			
1e	21.2 ± 1.6	43.7 ± 9.3	124.6 ± 39.4	64.3 ± 7.8	158.8 ± 58.3	186.4 ± 11.8	157.2 ± 15.2			
Cisplatin	2.1 ± 0.2	3.6 ± 0.5	17.1 ± 1.2	5.4 ± 0.3	$\textbf{17.8} \pm \textbf{0.6}$	14.4 ± 0.7	9.2 ± 0.2			
^a Thiosemic	arbazide.									

comparable to, or slightly better than, cisplatin, with IC_{50} values of 10.3, 8.9, 9.5, 8.6 and 20.6 μ M, respectively. Their cytotoxic activity was higher against normal human fetal lung fibroblasts MRC-5, with IC_{50} values of 7.7, 6.5, 7.2, 6.7 and 12.7 μ M. However, cisplatin, a chemotherapy drug that is used to treat different types of cancer, including non-small-cell lung cancer, showed a similar lack of selectivity.

The results mentioned above indicate that of the examined malignant cell lines, K562 cells were the most sensitive to the cytotoxic action of the newly synthesized steroid derivatives, especially 2a–c, 2e, 3a, 5a and 5d. HeLa cells demonstrated high sensitivity to 2a–c, 2e, 3c, 5a, 5b and 5d, and MDA-MB-453 cells to 2b, 2c, 2e, 5a, 5b and 5d. A549 cells were very sensitive to the cytotoxic activity of 2a, 2b, 2c, 2e and 5a, whereas LS174 cells were sensitive to the activity of 2c, 2e and 5a. MDA-MB-361 cells showed the lowest sensitivity to all the investigated compounds except 2b, 2c and 2e (Table 1).

By examining the IC₅₀ values in Table 1, it can be concluded that thiosemicarbazones **2a**, **2b**, **2c** and **2e** (as well as cisplatin, which served as a positive control) exhibited the highest cytotoxic activities against all cell lines, which were much higher than those of the corresponding starting steroids **1a–e** or thiosemicarbazide itself. Bis(thiosemicarbazones) **3a–e** exhibited much lower activities, which suggested that the introduction of another thiosemicarbazone group was not appropriate for cytotoxic activity against almost all the examined cancer cell lines. This pointed to the fact that, in addition to the thiosemicarbazone moiety at C-3, for the activity of these compounds a substituent at C-17 was also responsible. In compounds 2a, 2b and 2c, this was the 17-oxo group and in compound 2e the 17-acetyl group, which both significantly increased the activities of these compounds. However, it is well known that the activity of a new compound does not only depend on its functional groups but also on its threedimensional orientation. Because we are dealing with inseparable mixtures of isomers (Z and E), it is impossible from this perspective to say which one actually had a greater cytotoxic effect upon the cell lines used in this study, especially because there are not many literature data regarding this matter. The only available literature that can be applied to steroidal thiosemicarbazone isomers describes the difference in the cytotoxic activity of E- and Z-steroidal oximes8,49 and, according to these references, there were no significant differences in antiproliferative activity between the isomers.

A comparison of the derivatives with spiro-1,3,4-thiadiazoline rings indicates that the compounds with two spiro-1,3,4-thiadiazoline rings (5a-d) displayed more pronounced cytotoxicity than those with one (4a-e and 5e),

which were practically inactive against all the tested malignant cell lines. Taking this into account, it can be concluded that the spiro heterocyclic substituent at the C-17 position, as well as the presence of the α , β -unsaturated thiosemicarbazone moiety at C-3, enhanced the activity of the tested compounds.

If the type of steroidal moiety is considered, it is evident that 3-thiosemicarbazones 2a, 2b, 2c and 2e showed similar activities towards the majority of cell lines tested. The absence of the 19-methyl group, the substituent at C-17 (17-oxo or 17-COCH₃) or the degree of unsaturation of the parent steroids were observed not to alter the extent of cytotoxic activity significantly. The exception was 11a-hydroxy-3-thiosemicarbazone 2d, which was practically inactive against all the tested malignant cell lines, which indicated that the substitution of a hydrogen atom by a proton-donating OH group in the 11α position led to a dramatic decrease in activity. This finding was supported by the results obtained for other 11*α*-hydroxy derivatives, compounds 3d and 4d-OH, which were also inactive. In contrast, the introduction of an OCOCH₃ group in the 11a position (compounds 2d-OAc and 4d-OAc) much improved the activity of these compounds. Regarding 3,17-bis(thiadiazolines), from the data presented in Table 1 it can be seen that compounds 5b, with an androstene moiety, and 5c, with Δ^{9-11} unsaturation, showed good cytotoxic activity, but only against HeLa and MDA-MB-453 cell lines. Compound 5d, with an 11a-OCOCH₃ group, showed moderate activity against all the examined cell lines, whereas compound 5a, with a 19-norandrostene moiety, exhibited very good activity against all the cell lines except MDA-MB-361.

The anticancer potential of compounds 2a, 2b, 2c, 2e, 5a and 5d, which exhibited the most pronounced cytotoxic activity against all the examined cancer cell lines, was further examined against normal human non-stimulated and mitogen-stimulated PBMC. The tested compounds showed higher cytotoxic activities against K562, HeLa, MDA-MB-453, LS174 and A549 cells in comparison with normal non-stimulated and PHA-stimulated PBMC. The activity of compounds 2e, 5a and 5d was in most cases less pronounced against non-stimulated than against stimulated PBMC (Table 2).

The selectivity in the anticancer activity of compounds 5a and 5d was very high, especially against HeLa and K562 cell lines (Tables 3 and 4). In addition, compound 2a showed

Table 2	In vitro cytotoxic activity	of 2a,	2b,	2c,	2e,	5a	and	5d	against	t
РВМС										

	$\mathrm{IC}_{50}\pm$ S.D. (μ M)				
Comp.	PBMC^b	$PBMC + PHA^b$			
2a	44.1 ± 13.7	40.1 ± 13.8			
2 b	15.7 ± 3.0	14.3 ± 4.5			
2c	24.9 ± 14.8	26.9 ± 22.7			
2e	20.0 ± 14.3	13.1 ± 6.1			
5a	>200	197.3 ± 3.8			
5 d	>200	159.5 ± 56.3			
CDDP^{a}	60.8 ± 12.6	$\textbf{23.6} \pm \textbf{4.7}$			

^{*a*} Cisplatin. ^{*b*} From four independent experiments.

	PBMC/	PBMC/	PBMC/	PBMC/	PBMC/
Comp.	HeLa	MDA-MB-453	K562	LS174	A549
2a	2.4	1.0	3.9	0.7	4.3
2b	2.1	2.3	2.4	0.5	1.8
2c	1.4	3.6	3.7	1.0	2.6
2e	2.3	2.9	3.0	2.2	2.3
5a	>25.5	>18.1	>22.8	>15.9	>9.7
5 d	>5.4	>6.6	>5.5	>4.7	>5.6
$CDDP^{a}$	28.9	16.9	11.3	3.4	4.2

notably higher cytotoxic activity against K562 and A549 cells than towards normal PBMC.

In order to elucidate the mechanisms of the cytotoxic activity of the steroidal compounds, an examination of changes in the cell cycle of myelogenous leukemia K562 cells and human cervical adenocarcinoma HeLa cells treated with compounds 2a, 2b, 2c, 2e, 5a and 5d for 24 and 48 h was performed. Analysis of the changes in the cell cycle phase distribution of HeLa cells caused by these steroidal compounds applied at concentrations of IC₅₀ and 2IC₅₀ revealed a significant time- and dosedependent increase in cells in the subG1 phase compared with control cells (Fig. 8). At both tested concentrations, compounds 2a, 2b, 2c and 2e exerted to some extent a stronger apoptotic effect with respect to compounds 5a and 5d. Compound 5d applied at a concentration of 2IC₅₀ led to a significant accumulation of HeLa cells in the S phase after treatment for 24 and 48 h. The same effect of compound 5d on HeLa cells was observed after treatment for 48 h at a concentration of IC₅₀. Treatment of K562 cells with concentrations of IC₅₀ of the investigated steroid derivatives induced significant time-dependent increases in cells in the subG1 phase with respect to the control cell sample (with the exception of compounds 2a and 5a after exposure for 24 h). In addition, all the investigated compounds at a concentration of 2IC50 triggered a significant increase in the percentage of K562 cells in the subG1 phase after exposure for 24 h and 48 h (Fig. 9).

To investigate the possible pro-apoptotic activity of the steroidal compounds **2a**, **2b**, **2c**, **2e**, **5a** and **5d** against cervical adenocarcinoma HeLa cells, morphological analysis was performed by fluorescence microscopy of target cells stained with acridine orange-ethidium bromide and exposed for 24 h to these agents.

As can be seen in Fig. 10, the investigated steroid derivatives at concentrations of $2IC_{50}$ induced typical morphological hallmarks of apoptosis in exposed HeLa cells – chromatin condensation, shrinkage of the nucleus and fragmentation of the nucleus, as well as orange-red stained cells in the late stages of apoptosis.

Identification of HeLa cells in different stages of apoptosis induced by the examined steroid derivatives was performed by Table 4 Coefficient of selectivity (Cs) in the anticancer activity of 2a, 2b, 2c, 2e, 5a and 5d as a ratio of the IC₅₀ values for PBMC + PHA and malignant cells

	Cs								
Comp.	PBMC + PHA/HeLa	PBMC + PHA/MDA-MB-453	PBMC + PHA/K562	PBMC + PHA/LS174	PBMC + PHA/A549				
2a	2.2	0.9	3.6	0.7	3.9				
2 b	1.9	2.1	2.2	0.4	1.6				
2c	1.6	3.9	4.0	1.0	2.8				
2e	1.5	1.9	2.0	1.4	1.5				
5a	25.2	17.9	22.5	15.7	9.6				
5 d	4.3	5.3	4.4	3.7	4.5				
$CDDP^{a}$	11.4	6.5	4.4	1.3	1.4				



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Fig. 8 Changes in the cell cycle phase distribution of HeLa cells. Cell cycle analysis of HeLa cells treated with compounds 2a, 2b, 2c, 2e, 5a and 5d at concentrations corresponding to IC_{50} (A, C) and $2IC_{50}$ (B, D) after 24 h (A, B) and 48 h (C, D). The results are presented as the mean \pm S.D. of three independent experiments. ANOVA was performed for statistical analysis. *($\rho < 0.05$).

flow cytometry after staining with annexin V-FITC/PI. In Fig. 11, for each cell sample the percentages of annexin V⁺/PI⁻ early apoptotic cells, annexin V⁺/PI⁺ late apoptotic/secondary necrotic cells and annexin V⁻/PI⁺ dead cells are shown. Increased percentages of early apoptotic and end-stage apoptotic/secondary necrotic HeLa cells were measured in the samples treated for 24 h with concentrations of $2IC_{50}$ of compounds 2a, 2b, 2c, 2e, 5a and 5d in comparison with the control cell sample. In addition, there was an increase in the percentage of dead HeLa cells (annexin V⁻/PI⁺) for each of the tested steroidal compounds.

For further mechanistic studies of the mode of cell death induced by the most active compounds **2a**, **2b**, **2c**, **2e**, **5a** and **5d** in cervical adenocarcinoma HeLa cells, the possible activation of caspase-3, caspase-8 and caspase-9 was investigated by using specific caspase inhibitors. The observed decrease in the percentages of subG1 HeLa cells pretreated with each of the caspase inhibitors and exposed to the tested compounds, in comparison with the percentages of subG1 cells in samples exposed only to the compounds, points to the pro-apoptotic activity of the compounds. All the tested steroidal compounds activate extrinsic and intrinsic apoptotic signaling pathways in HeLa cells (Fig. 12).

To investigate the anti-angiogenic potential of the most active and selective steroid derivatives **2a**, **2b**, **2c**, **2e**, **5a** and **5d**, an endothelial cell tube formation assay was performed. When human endothelial EA.hy926 cells are plated on the surface of a Matrigel matrix, they begin to extend, connect and rearrange in order to form capillary-like tube structures and polygonal structures.⁵⁵ Representative photomicrographs of control EA.hy926 cells and cells exposed to sub-toxic concentrations of IC₂₀ of the tested compounds after 20 h are shown in Fig. 13. As



Fig. 9 Changes in the cell cycle phase distribution of K562 cells. Cell cycle analysis of K562 cells treated with compounds 2a, 2b, 2c, 2e, 5a and 5d at concentrations corresponding to IC_{50} (A, C) and $2IC_{50}$ (B, D) after 24 h (A, B) and 48 h (C, D). The results are expressed as the mean \pm S.D. of three independent experiments. ANOVA was performed for statistical analysis. *($\rho < 0.05$).

can be seen, each of the investigated steroidal compounds showed the ability to decrease angiogenesis *in vitro*. Compound **5a** exerted the strongest anti-angiogenic activity. Compound **2a** also exhibited a remarkable anti-angiogenic effect, whereas compounds **2b**, **2c**, **2e** and **5d** were less effective in inhibiting angiogenesis, especially **5d**, which exerted the weakest antiangiogenic action.

The brine shrimp test. In the brine shrimp test, general toxicity against nauplii of the brine shrimp *Artemia salina* was evaluated. This assay has been established as a safe, practical, and economic method for the determination of the bioactivity of synthetic compounds,⁵⁶ and is considered to show a good correlation with cytotoxicity tests.⁵⁷ Compounds **5a–d**, which displayed high cytotoxicity against HeLa and MDA-MB-453, were also found to be the most active in the brine shrimp assay, whereas compounds **2a–e** and **3a–e**, as well as thiadiazolines **4a–e**, were found to be less active (Table 5).

These results indicate that the bis(thiadiazoline) structure enhances the activity of the newly synthesized compounds, but also that other structural requirements must obviously be satisfied.

Antimicrobial activity. The *in vitro* antimicrobial activity of all given compounds 2–5 was tested against Gram-positive bacteria: *Staphylococcus aureus*, *Kocuria rhizophila*, *Clostridium sporogenes* and *Bacillus subtilis*, Gram-negative bacteria: *Escherichia coli*, *Proteus hauseri*, *Pseudomonas aeruginosa* and *Salmonella enterica*, as well as against fungal species: *Candida albicans*, *Saccharomyces cerevisiae* and *Aspergillus brasiliensis* by an agar well diffusion method.

Almost all the compounds, except **3a–e**, exhibited weak inhibitory activity against the Gram-positive bacteria *C*.

sporogenes and *K. rhizophila* and against the Gram-negative bacterium *P. aeruginosa*. In all cases, the inhibition zones were much smaller than those for amikacin (Table 6). All the novel synthesized compounds, except **3e**, were found to have very weak antifungal activity against *A. brasiliensis*.

Moreover, compounds **2b**, **2e**, **3c**, **4b–e**, **5b**, **5d** and **5e** exhibited weak inhibitory activity against the other two fungi tested, *C. albicans* and *S. cerevisiae* (Table 7).

Experimental

Chemistry

Removal of solvents was carried out under reduced pressure. Thin-layer chromatography (TLC) was performed using aluminium plates coated with silica gel 60 F₂₅₄ (Merck) and flash column chromatography (FCC) was performed on silica gel (0.040-0.063 mm, Merck). TLC spots were detected with 50% aq. H₂SO₄ followed by heating. Melting points were determined on a WRS-1B digital melting point apparatus and are uncorrected. Optical rotations were measured in CHCl₃, CH_3OH , or $CHCl_3/CH_3OH$ (3:1) using a Rudolph Research Analytical Autopol IV automatic polarimeter at 20 °C. IR spectra were recorded on a PerkinElmer 1725X FT-IR spectrophotometer. NMR spectra were recorded on a Varian Gemini-200 spectrometer (¹H at 200 MHz; ¹³C at 50 MHz) or a Bruker Avance 500 MHz spectrometer (¹H at 500 MHz; ¹³C at 125 MHz) in DMSO, CDCl₃ and/or CD₃OD at room temperature using SiMe₄ as internal standard, δ in ppm, J in Hz. HRMS spectra were recorded on an Agilent 6210 LC ESI-MS TOF spectrometer. Androst-4-ene-3,17-dione, 19-norandrost-4-ene-3,17-dione, androsta-4,9(11)-diene-3,17-dione, 11a-hydroxyandrost-4-ene-



Fig. 10 Induction of apoptosis in HeLa cells. Photomicrographs of control HeLa cells and HeLa cells stained with acridine orange/ethidium bromide and exposed for 24 h to cisplatin and the investigated compounds 2a, 2b, 2c, 2e, 5a and 5d, as described in the Materials and methods section. The concentrations applied of the tested compounds corresponded to values of $2IC_{50}$ determined for 72 h.



Fig. 11 Flow cytometry analysis of control and treated HeLa cells stained with annexin V-FITC and propidium iodide. The results are shown as the mean \pm S.D. of two independent experiments. A⁺P⁻ (FITC-annexin V positive, PI negative), A⁺P⁺ (FITC-annexin V positive, PI positive), and A⁻P⁺ (FITC-annexin V negative, PI positive).

3,17-dione and progesterone were purchased from Galenika AD (Beograd) and were recrystallized from a suitable solvent.

General procedure for the synthesis of thiosemicarbazones and bis(thiosemicarbazones)

Thiosemicarbazide (3.16 mmol or 6.3 mmol) was added to a solution of steroid (**1a–e**) (3.16 mmol) in dried ethanol (150 or 300 mL). The reaction mixture was refluxed for 5 h with stirring



Fig. 12 Identification of target caspases. Effects of specific caspase inhibitors (Z-DEVD-FMK – caspase-3 inhibitor; Z-IETD-FMK – caspase-8 inhibitor; Z-LEHD-FMK – caspase-9 inhibitor) on the percentages of subG1 HeLa cells treated with compounds 2a, 2b, 2c, 2e, 5a and 5d at concentrations corresponding to $2IC_{50}$ for 24 h.

and monitored by TLC. The pH of the mixture was adjusted to \sim 4.5 with CH₃COOH (about 5 mL). After completion of the reaction, the solvent was removed under reduced pressure. The residue was crystallized or chromatographed by FCC using different solvents as eluents (the eluents and ratio are mentioned in each experiment). In all cases, the products were obtained as mixtures of two diastereoisomers (*E* and *Z*), which could not be separated. All spectra were recorded using these mixtures.

Mixture of 2a (*E/Z*). Starting with 860 mg 19-norandrost-4ene-3,17-dione (**1a**), elution with toluene/EtOAc (8/2) gave **2a** (727 mg, 66.7%). $R_{\rm f} = 0.66$ (toluene/EtOAc, 6 : 4, double development). Mp > 218 °C (decomp.). IR (ATR/cm⁻¹): 3422 and 3246 (NH), 1732 (C=O), 1586, 1497 (C=N), 1285 (C=S). ESI-TOF-MS: *m*/*z* for C₁₉H₂₇N₃OS [M + H]⁺: calcd 346.19476, found 346.19388.

(*E*)-19-Norandrost-4-ene-3,17-dione 3-thiosemicarbazone (2a-*E*). ¹H-NMR (500 MHz, CDCl₃): 0.82 (m, 1H, H-9), 0.93 (s, 3H, H₃C-18), 1.09 (m, 1H, Hα-7), 1.23–1.41 (m, 4H, Hα-11, Hβ-11, Hα-12, H-14), 1.46 (dq, J = 3, 12 Hz, 1H, H-8), 1.56 (m, 1H, Hβ-15), 1.77–2.14 (m, 7H), 2.19–2.31 (m, 2H), 2.47 (dd, J = 19.0, 9.0 Hz, 1H, Hβ-16, partially overlapped with Hβ-6), 2.50 (m, 1H, Hβ-6), 2.65 (dt, J = 16.0, 4.0 Hz, 1H, Hα-2), 5.92 (s, 1H, H-4), 6.36 and 7.24 (2bs, 2H, NH₂), 8.71 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃): 220.6 (s, C-17), 178.4 (s, C=S), 153.2 (s, C-5), 150.5 (s, C-3), 121.4 (d, C-4), 50.2 (d, C-14), 49.4 (d, C-9), 47.7 (s, C-13), 41.6 (d, C-10), 39.9 (d, C-8), 35.7 (t, C-16), 34.6 (t, C-6), 31.3 (t, C-12), 29.9 (t, C-7), 25.9 (t, C-11), 25.8 (t, C-1), 22.7 (t, C-2), 21.5 (t, C-15), 13.7 (q, C-18).



Fig. 13 Effects on *in vitro* angiogenesis of EA.hy926 cells. Photomicrographs of control EA.hy926 cells and cells exposed to sub-toxic concentrations of IC_{20} of compounds 2a, 2b, 2c, 2e, 5a and 5d after 20 h.

(Z)-19-Norandrost-4-ene-3,17-dione 3-thiosemicarbazone (2a-Z). ¹H-NMR (500 MHz, CDCl₃): 0.84 (m, 1H, H-9), 0.93 (s, 3H, H₃C-18), 1.12 (m, 1H, Hα-7), 1.23–1.41 (m, 4H), 1.46 (dq, J = 3.0, 12.0 Hz, 1H, H-8), 1.56 (m, 1H, Hβ-15), 1.77–2.14 (m, 7H), 2.19– 2.31 (m, 2H), 2.40 (dt, J = 15.0, 4.0 Hz, 1H, Hα-2), 2.47 (dd, J =19.0, 9.0 Hz, 1H, Hβ-16), 2.50 (m, 1H, Hβ-6), 6.19 (s, 1H, H-4), 6.31 and 7.22 (2bs, 2H, NH₂), 8.90 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃): 220.5 (s, C-17), 178.3 (s, C=S), 158.6 (s, C-5), 149.5 (s, C-3), 111.2 (d, C-4), 50.1 (d, C-14), 50.0 (d, C-9), 47.7 (s, C-13), 43.2 (d, C-10), 40.0 (d, C-8), 35.8 (t, C-6), 35.7 (t, C-16), 31.3 (t, C-12), 30.4 (t, C-7), 25.9 (t, C-11), 25.5 (t, C-1), 22.7 (t, C-2), 21.5 (t, C-15), 13.7 (q, C-18).

Further elution with toluene/EtOAc (6/4) gave 3a (79.4 mg, 12%).

Mixture of 3a (*E/Z*). Starting with 860 mg 19-norandrost-4ene-3,17-dione (1a), elution with toluene/EtOAc (8/2) gave 2a (115.5 mg, 10.6%). Further elution with toluene/EtOAc (6/4) gave 3a (723 mg, 55.0%). $R_{\rm f} = 0.46$ (toluene/EtOAc, 6 : 4, double development). Mp > 127 °C (decomp.). IR (ATR/cm⁻¹): 3421 and 3240 (NH), 1583, 1496 (C=N), 1282 (C=S). ESI-TOF-MS: m/z for $C_{20}H_{30}N_6S_2$ [M + H]⁺: calcd 419.20461, found 419.20335.

(*E*)-19-Norandrost-4-ene-3,17-dione bis(thiosemicarbazone) (3a-*E*). ¹H-NMR (500 MHz, CDCl₃): 0.81 (m, 1H, H-9), 0.94 (s, 3H, H₃C-18), 1.08 (m, 1H, H α -7), 1.20 (m, 1H, H-14), 1.24–1.46 (m, 4H), 1.51 (m, 1H, H β -15), 1.81–2.12 (m, 7H), 2.17–2.34 (m, 2H), 2.41–2.54 (m, 2H), 2.67 (dt, *J* = 16.0, 4.0 Hz, 1H, H α -2), 5.91 (s, 1H, H-4), 6.50 and 7.26 (2bs, 2H, NH₂), 6.53 and 7.18 (2bs, 2H, NH₂), 8.49 (s, 1H, NH), 8.83 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃): 178.7 (s, C=S), 178.3 (s, C=S), 167.0 (s, C-17), 153.1 (s, C-5), 150.7 (s, C-3), 121.3 (d, C-4), 52.1 (d, C-14), 49.5 (d, C-9), 44.8 (s, C-13), 41.6 (d, C-10), 39.7 (d, C-8), 34.8 (t, C-6), 33.7 (t, C-12), 30.4 (t, C-7), 26.2 (t, C-16), 26.0 (t, C-11), 25.8 (t, C-1), 23.2 (t, C-15), 22.8 (t, C-2), 17.0 (q, C-18).

(Z)-19-Norandrost-4-ene-3,17-dione bis(thiosemicarbazone) (3a-Z). ¹H-NMR (500 MHz, CDCl₃): 0.83 (m, 1H, H-9), 0.94 (s, 3H, H₃C-18), 1.10 (m, 1H, H α -7), 1.20 (m, 1H, H-14), 1.24–1.46 (m, 4H), 1.51 (m, 1H, H β -15), 1.81–2.12 (m, 7H), 2.17–2.34 (m, 2H), 2.40 (dt, J = 14.5, 3.5 Hz, 1H, H α -2), 2.41–2.54 (m, 2H), 6.22 (s, 1H, H-4), 6.45 and 7.23 (2bs, 2H, NH₂), 6.53 and 7.18 (2bs, 2H, NH₂), 8.49 (s, 1H, NH), 9.05 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃): 178.7 (s, C=S), 178.2 (s, C=S), 167.0 (s, C-17), 158.4 (s, C-5), 149.7 (s, C-3), 111.4 (d, C-4), 52.0 (d, C-14), 50.0 (d, C-9), 44.8 (s, C-13), 43.2 (d, C-10), 39.8 (d, C-8), 35.8 (t, C-6), 33.7 (t, C-12), 30.2 (t, C-7), 26.2 (t, C-16), 26.0 (t, C-11), 25.7 (t, C-1), 23.2 (t, C-15), 22.8 (t, C-2), 17.0 (q, C-18).

Mixture of 2b (*E*/*Z*). Starting with 860 mg androst-4-ene-3,17dione (1b), elution with toluene/EtOAc (8/2) gave 2b (727 mg, 67.4%). $R_{\rm f} = 0.60$ (toluene/EtOAc, 6 : 4, double development). Mp > 208 °C (decomp.). IR (ATR/cm⁻¹): 3418 and 3252 (NH), 3149, 2939, 1731 (C=O), 1599, 1504 (C=N), 1266 (C=S), 735.

Table 5 Results of the brine shrimp test for investigated compounds

Comp.	$LC_{50} \left(mM \ \mu M^{-1} ight)$
2a	0.597
2b	0.439
2c	0.663
2d	0.551
2d-OAc	0.386
2e	0.670
3a	0.599
3b	0.465
3c	0.420
3d	0.481
3e	0.300
4a	0.601
4b	0.579
4 c	0.874
4d-OH	0.561
4d-OAc	0.299
4e	0.430
5a	0.213
5b	0.273
5c	0.327
5d	0.224
5e	0.380

Table 6 Antibacterial activity of compounds 2a-e, 3a-e, 4a-e and 5a-e

	Inhibitio	n zone (mm)						
Comp.	E. coli	P. hauseri	P. aeruginosa	S. enterica	K. rhizophila	C. sporogenes	S. aureus	B. subtilis
2a	_	_	10	10	12	10	_	_
2b	_	10	10	_	10	10	_	_
2c	_	10	10	_	_	10	—	—
2d	_	—	—	_	_	_	—	—
2e	10	12	12	12	12	12	10	12
3a	—	—	—	—	—	—	—	—
3b	—	—	—	—	—	—	—	—
3c		—	—	—	—	—	—	—
3d		—	—	—	—	—	—	—
3e	—	—	—	—	_	—	—	—
4a		—	10	—	—	10	—	—
4b	10	10	10	—	12	10	—	—
4c		—	10	—	10	—	10	—
4d-OAc	10	10	12	12	10	12	12	10
4d-OH	_	—	10	—	10	—	_	—
4e		10	—	—	10	—	—	—
5a	—	—	10	10	10	10	—	—
5 b	_	12	10	—	10	—	—	—
5c		10	10	12	—	10	14	12
5 d	10	10	—	10	10	10	—	12
5e		10	10	10	10	—	14	—
Amikacin	42	24	20	24	22	20	32	22

ESI-TOF-MS: m/z for C₂₀H₂₉N₃OS [M + H]⁺: calcd 360.21041, found 360.21148.

(E)-Androst-4-ene-3,17-dione 3-thiosemicarbazone (2b-E). 1 H-NMR (500 MHz, CDCl₃): 0.91 (s, 3H, H₃C-18), 1.05 (m, 1H, Hα-7), 1.10 (s, 3H, H₃C-19), 1.23–1.33 (m, 2H, H-14, Hα-12),

Table 7 Ant	ifungal activity of 2a-e, 3a-e, 4a-e and 5a-e						
	Inhibition zone (mm)						
Comp.	C. albicans	S. cerevisiae	A. brasiliensis				
2a	_	_	10				
2b	10	_	12				
2c	_	_	10				
2d	_	_	10				
2e	12	10	12				
3a	_	_	10				
3b	_	_	12				
3 c	12	12	10				
3d	_	_	10				
3e	_	_	_				
4a	_	_	12				
4b	10	12	10				
4c	10	10	9				
4d-OAc	10	10	10				
4d-OH	9	10	10				
4e	9	9	10				
5a	_	_	12				
5b	12	10	10				
5c	_	—	12				
5 d	10	12	10				
5e	10	10	10				
Nystatin	34	56	32				

1.40–1.50 (m, 2H, H β -11, H α -1), 1.56 (m, 1H, H β -15), 1.62–1.76 (m, 2H, H-8, H α -11), 1.82–2.03 (m, 4H, H β -12, H β -7, H α -15, H β -1), 2.09 (dd, J = 9.0, 19.0 Hz, 1H, H α -16), 2.17 (m, 1H, H β -2), 2.30–2.38 (m, 2H), 2.48 (J = 8.5, 19.0 Hz, 1H, H β -16), 2.62 (dt, J = 16.0, 3.5 Hz, 1H, H α -2), 5.82 (s, 1H, H-4), 6.41 and 7.25 (bs and m, 2H, NH₂), 8.74 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃): 220.5 (s, C-17), 178.3 (s, C=S), 157.9 (s, C-5), 150.1 (s, C-3), 120.5 (d, C-4), 53.6 (d, C-9), 50.9 (d, C-14), 47.4 (s, C-13), 37.9 (s, C-10), 35.7 (t, C-16), 35.2 (d, C-8), 34.5 (t, C-1), 32.2 (t, C-6), 31.3 (t, C-12), 30.8 (t, C-7), 21.7 (t, C-15), 20.6 (2t, C-2 and C-11), 17.7 (q, C-19), 13.6 (q, C-18).

(Z)-Androst-4-ene-3,17-dione 3-thiosemicarbazone (2b-Z). ¹H-NMR (500 MHz, CDCl₃): 0.91 (s, 3H, H₃C-18), 1.05 (m, 1H, H α -7), 1.15 (s, 3H, H₃C-19), 1.23–1.33 (m, 2H, H-14, H α -12), 1.46 (m, 1H, H β -11), 1.50 (m, 1H, H α -1), 1.56 (m, 1H, H β -15), 1.62–1.76 (m, 2H, H-8, H α -11), 1.82–2.03 (m, 4H, H β -1, H β -12, H α -15, H β -7), 2.09 (dd, J = 9.0, 19.0 Hz, 1H, H α -16), 2.17 (m, 1H, H β -2), 2.30–2.38 (m, 2H), 2.48 (J = 8.5, 19.0 Hz, 1H, H β -16), 2.18 (dt, J = 16.0, 3.5 Hz, 1H, H α -2), 6.11 (s, 1H, H-4), 6.35 and 7.17 (bs and m, 2H, NH₂), 8.95 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃): 220.4 (s, C-17), 178.4 (s, C=S), 163.0 (s, C-5), 148.9 (s, C-3), 110.3 (d, C-4), 54.0 (d, C-9), 50.8 (d, C-14), 47.5 (s, C-13), 39.3 (s, C-10), 35.7 (t, C-16), 36.2 (t, C-1), 35.2 (d, C-8), 33.1 (t, C-6), 31.3 (t, C-12), 31.2 (t, C-7), 21.7 (t, C-15), 20.6 (t, C-11), 20.3 (t, C-2), 17.0 (q, C-19), 13.6 (q, C-18).

Further elution with toluene/EtOAc/MeOH (60/40/1) gave 3b (79.4 mg, 6.1%).

Mixture of 3b (E/Z). Starting with 860 mg androst-4-ene-3,17dione (1b), elution with toluene/EtOAc (8/2) gave 2b (115.5 mg, 10.6%). Further elution with toluene/EtOAc/MeOH (60/40/1) afforded **3b** (723 mg, 55.7%). $R_{\rm f} = 0.22$ (toluene/EtOAc, 6 : 4, double development). Mp > 146 °C (decomp.). IR (ATR/cm⁻¹): 3498 and 3272 (NH), 2941, 1565, 1487 (C=N), 1276 (C=S), 1080, 878. ESI-TOF-MS: m/z for $C_{21}H_{32}N_6S_2$ [M + H]⁺: calcd 433.22026, found 433.21908.

(*E*)-Androst-4-ene-3,17-dione bis(thiosemicarbazone) (3b-*E*). ¹H-NMR (500 MHz, DMSO): 0.84 (s, 3H, H₃C-18), 0.95 (m, 1H, H-9), 1.04 (s, 3H, H₃C-19), 1.20–1.32 (m, 2H), 1.33–1.45 (m, 2H), 1.51–1.67 (m, 2H), 1.74–1.96 (m, 4H), 2.17–2.43 (m, 4H), 2.53 (dd, J = 9.0, 19.5 Hz, 1H, H β -16), 2.79 (dt, J = 17.0, 3.0 Hz, 1H, H α -2), 5.76 (s, 1H, H-4), 7.35 and 7.98 (2bs, 2H, NH₂), 7.53 and 8.02 (2bs, 2H, NH₂), 9.81 (s, 1H, NH), 10.03 (s, 1H, NH). ¹³C NMR (125 MHz, DMSO): 178.4 (s, C=S), 178.2 (s, C=S), 167.3 (s, C-17), 156.5 (s, C-5), 150.6 (s, C-3), 120.8 (d, C-4), 53.4 (d, C-9), 52.4 (d, C-14), 44.2 (s, C-13), 37.4 (s, C-10), 34.7 (d, C-8), 34.4 (t, C-1), 33.6 (t, C-6), 31.7 (t, C-7), 31.3 (t, C-12), 26.8 (t, C-16), 23.1 (t, C-15), 21.0 (t, C-11), 20.5 (t, C-2), 17.4 (q, C-19), 16.8 (q, C-18).

(Z)-Androst-4-ene-3,17-dione bis(thiosemicarbazone) (3b-Z). ¹H-NMR (500 MHz, DMSO): 0.84 (s, 3H, H₃C-18), 0.95 (m, 1H, H-9), 1.08 (s, 3H, H₃C-19), 1.20–1.32 (m, 2H), 1.33–1.45 (m, 2H), 1.51–1.67 (m, 2H), 1.74–1.96 (m, 4H), 2.17–2.43 (m, 5H), 2.53 (dd, J = 9.0, 19.5 Hz, 1H, H β -16), 6.61 (s, 1H, H-4), 7.35 and 7.98 (2bs, 2H, NH₂), 7.50 and 7.93 (2bs, 2H, NH₂), 9.81 (s, 1H, NH), 10.34 (s, 1H, NH). ¹³C NMR (125 MHz, DMSO): 178.4 (s, C=S), 178.3 (s, C=S), 167.3 (s, C-17), 160.6 (s, C-5), 148.0 (s, C-3), 112.2 (d, C-4), 53.7 (d, C-14), 52.3 (d, C-9), 44.3 (s, C-13), 38.5 (s, C-10), 36.1 (t, C-1), 34.6 (d, C-8), 33.5 (t, C-6), 32.2 (t, C-7), 31.3 (t, C-12), 26.8 (t, C-16), 23.1 (t, C-15), 21.0 (t, C-11), 20.2 (t, C-2), 17.7 (q, C-19), 16.8 (q, C-18).

Mixture of 2c (*E*/*Z***)**. Starting with 860 mg androsta-4,9(11)diene-3,17-dione (**1c**), elution with toluene/EtOAc (85/15) gave **2c** (430 mg, 39.9%). $R_{\rm f} = 0.44$ (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 C₂₀H₂₇N₃OS [M + H]⁺: calcd 358.19476, found 358.19350.

(*E*)-Androsta-4,9(11)-diene-3,17-dione 3-thiosemicarbazone (2c-*E*). ¹H-NMR (200 MHz, DMSO): 0.79 (s, 3H, H₃C-18), 0.98 (m, 1H, H α -7), 1.21 (s, 3H, H₃C-19), 2.88 (br.d, *J* = 17.4 Hz, 1H, H α -2), 5.49 (br.s, 1H, H-11), 5.81 (s, 1H, H-4), 7.56 and 8.09 (bs and m, 2H, NH₂), 10.11 (s, 1H, NH). ¹³C NMR (50 MHz, DMSO): 220.7 (s, C-17), 178.5 (s, C=S), 154.8 (s, C-5), 150.4 (s, C-3), 146.4 (s, C-9), 121.3 (d, C-4), 116.8 (d, C-11), 47.6 (d, C-14), 45.5 (s, C-13), 40.4 (s, C-10, overlapped by DMSO), 36.7 (t, C-16), 36.1 (d, C-8), 33.3 (t, C-1), 32.8 (t, C-12), 31.8 (t, C-6), 31.3 (t, C-7), 26.4 (q, C-19), 22.5 (t, C-15), 21.5 (t, C-2), 13.8 (q, C-18).

(Z)-Androsta-4,9(11)-diene-3,17-dione 3-thiosemicarbazone (2c-Z). ¹H-NMR (200 MHz, DMSO): 0.79 (s, 3H, H₃C-18), 0.98 (m, 1H, H α -7), 1.26 (s, 3H, H₃C-19), 5.49 (br.s, 1H, H-11), 6.65 (s, 1H, H-4), 7.56 and 8.00 (bs and m, 2H, NH₂), 10.38 (s, 1H, NH). ¹³C NMR (50 MHz, DMSO): 220.7 (s, C-17), 178.3 (s, C=S), 158.9 (s, C-5), 147.8 (s, C-3), 146.4 (s, C-9), 121.3 (d, C-4), 116.8 (d, C-11), 47.6 (d, C-14), 45.5 (s, C-13), 41.3 (s, C-10), 36.7 (t, C-16), 36.5 (d, C-8), 33.4 (t, C-1), 32.8 (t, C-12), 31.8 (t, C-6), 31.3 (t, C-7), 26.7 (q, C-19), 22.5 (t, C-15), 20.4 (t, C-2), 13.8 (q, C-18).

Further elution with toluene/EtOAc/MeOH (60/40/1) gave 3c (77.4 mg, 5.9%).

Mixture of 3c (*E*/*Z*). Starting with 860 mg androsta-4,9(11)diene-3,17-dione (1c), elution with toluene/EtOAc (85/15) gave 2c (115.5 mg, 10.6%). Further elution with toluene/EtOAc/ MeOH (85/15/2) afforded 3c (723 mg, 55%). $R_f = 0.56$ (CHCl₃/ MeOH, 95 : 5, double development). Mp > 146 °C (decomp.). IR (ATR/cm⁻¹): 3503, 3359, 3257, 3148 (NH), 2941, 1587, 1501 (C= N), 1280 (C=S), 1088, 753. ESI-TOF-MS: *m*/*z* for C₂₁H₃₀N₆S₂ [M + H]⁺: calcd 431.20461, found 431.20339.

(*E*)-Androsta-4,9(11)-diene-3,17-dione bis(thiosemicarbazone) (3c-*E*). ¹H-NMR (200 MHz, DMSO): 0.82 (s, 3H, H₃C-18), 1.19 (s, 3H, H₃C-19), 2.88 (d, J = 16.0 Hz, 1H, H α -2), 5.51 (br.s, 1H, H-11), 5.80 (s, 1H, H-4), 7.42 and 8.07 (bs and m, 2H, NH₂), 7.57 and 8.07 (2bs, 2H, NH₂), 9.89 (s, 1H, NH), 10.09 (s, 1H, NH). ¹³C NMR (50 MHz, DMSO): 178.6 (s, C=S), 178.5 (s, C=S), 167.3 (s, C-17), 155.1 (s, C-5), 150.5 (s, C-3), 146.1 (s, C-9), 121.2 (d, C-4), 117.4 (d, C-11), 49.7 (d, C-14), 43.0 (s, C-13), 40.1 (s, C-10), 36.6 (t, C-12), 36.2 (d, C-8), 32.8 (t, C-1), 31.9 (t, C-6), 31.8 (t, C-7), 27.5 (t, C-16), 26.4 (q, C-19), 24.3 (t, C-15), 21.5 (t, C-2), 17.2 (q, C-18).

(Z)-Androsta-4,9(11)-diene-3,17-dione bis(thiosemicarbazone) (3c-Z). ¹H-NMR (200 MHz, DMSO): 0.82 (s, 3H, H₃C-18), 1.25 (s, 3H, H₃C-19), 5.51 (br.s, 1H, H-11), 6.64 (s, 1H, H-4), 7.42 and 8.07 (2bs, 2H, NH₂), 7.57 and 8.00 (2bs, 2H, NH₂), 9.89 (s, 1H, NH), 10.38 (s, 1H, NH). ¹³C NMR (50 MHz, DMSO): 178.6 (s, C=S), 178.3 (s, C=S), 167.3 (s, C-17), 159.1 (s, C-5), 147.9 (s, C-3), 146.1 (s, C-9), 117.4 (d, C-11), 113.0 (d, C-4), 49.7 (d, C-14), 43.0 (s, C-13), 41.3 (s, C-10), 36.6 (t, C-12), 36.3 (d, C-8), 34.4 (t, C-1), 31.9 (t, C-6), 31.8 (t, C-7), 27.5 (t, C-16), 26.5 (q, C-19), 24.3 (t, C-15), 22.5 (t, C-2), 17.2 (q, C-18).

Mixture of 2d (*E*/*Z*). Starting with 910 mg 11α-hydroxyandrost-4ene-3,17-dione (1d), elution with toluene/EtOAc/MeOH (50/50/0.1) gave 2d (876 mg, 78.2%). $R_{\rm f} = 0.53$ (CHCl₃/MeOH, 9 : 1). Mp > 218 °C (decomp.). IR (ATR/cm⁻¹): 3419 and 3257 (NH), 2927, 1736 (C= O), 1587, 1503 (C=N), 1024 (C=S), 737. ESI-TOF-MS: *m/z* for $C_{20}H_{29}N_3O_2S$ [M + H]⁺: calcd 376.20532, found 376.20446; *m/z* for $C_{20}H_{29}N_3O_2S$ [M + Na]⁺: calcd 398.18727, found 398.18694: *m/z* for $C_{20}H_{29}N_3O_2S$ [M + K]⁺: calcd 414.16121, found 414.16159.

(*E*)-11*α*-Hydroxyandrost-4-ene-3,17-dione 3-thiosemicarbazone (2d-*E*). ¹H-NMR (200 MHz, CDCl₃/CD₃OD): 0.95 (s, 3H, H₃C-18), 1.24 (s, 3H, H₃C-19), 3.97 (dt, J = 4.5, 10.2 Hz, 1H, Hβ-11), 5.85 (s, 1H, H-4), 7.24 and 7.39 (2m, 2H, NH₂), 9.31 (s, 1H, NH). ¹³C NMR (50 MHz, CDCl₃/CD₃OD): 220.8 (s, C-17), 177.3 (s, C=S), 158.2 (s, C-3), 151.4 (s, C-5), 120.8 (d, C-4), 67.8 (d, C-11), 58.4 (d, C-9), 49.8 (d, C-14), 47.8 (s, C-13), 41.7 (t, C-12), 38.9 (s, C-10), 35.5 (t, C-16), 35.4 (d, C-8), 34.4 (t, C-1), 32.8 (t, C-6), 30.1 (t, C-7), 21.3 (t, C-15), 20.5 (t, C-2), 18.0 (q, C-19), 14.1 (q, C-18).

(Z)-11 α -Hydroxyandrost-4-ene-3,17-dione 3-thiosemicarbazone (2d-Z). ¹H-NMR (200 MHz, CDCl₃/CD₃OD): 0.95 (s, 3H, H₃C-18), 1.29 (s, 3H, H₃C-19), 3.97 (dt, J = 4.5, 10.2 Hz, 1H, H β -11), 6.22 (s, 1H, H-4), 7.24 and 7.35 (2m, 2H, NH₂), 9.55 (s, 1H, NH). ¹³C NMR (50 MHz, CDCl₃/CD₃OD): 220.4 (s, C-17), 177.2 (s, C=S), 162.8 (s, C-3), 150.3 (s, C-5), 112.2 (d, C-4), 67.8 (d, C-11), 58.4 (d, C-9), 49.7 (d, C-14), 47.1 (s, C-13), 41.7 (t, C-12), 40.2 (s, C-10), 35.5 (t, C-16), 37.4 (d, C-8), 34.2 (t, C-1), 33.5 (t, C-6), 30.5 (t, C-7), 21.3 (t, C-15), 21.3 (t, C-2), 18.3 (q, C-19), 14.1 (q, C-18).

Further elution with toluene/EtOAc/MeOH (50/50/2) gave 3d (88 mg, 10%).

Mixture of 3d (*E/Z*). Starting with 910 mg 11 α -hydroxyandrost-4-ene-3,17-dione (1d), elution with toluene/ EtOAc/MeOH (50/50/0.1) gave 2d (151 mg, 13.3%). Further elution with toluene/EtOAc/MeOH (50/50/2) afforded 3d (640 mg, 47.6%). $R_f = 0.40$ (CHCl₃/MeOH, 9 : 1). Mp > 176 °C (decomp.). IR (ATR/cm⁻¹): 3510 and 3414 (NH), 2925, 1586, 1501 (C=N), 1299, 1086, 867. ESI-TOF-MS: *m/z* for C₂₁H₃₂N₆OS₂: [M + H]⁺: calcd 449.21518, found 449.21447.

(*E*)-11 α -Hydroxyandrost-4-ene-3,17-dione bis(thiosemicarbazone) (3d-*E*). ¹H-NMR (200 MHz, CDCl₃/CD₃OD): 0.96 (s, 3H, H₃C-18), 1.24 (s, 3H, H₃C-19), 3.90 (overlapped with CD₃OD, 1H, H β -11), 5.84 (s, 1H, H-4), 7.19 and 7.38 (2m, 4H, NH₂), 8.89 (s, 1H, NH), 9.28 (s, 1H, NH). ¹³C NMR (50 MHz, CDCl₃/CD₃OD): 177.8 (s, C=S), 177.3 (s, C=S), 166.5 (s, C-17), 158.1 (s, C-3), 151.5 (s, C-5), 120.9 (d, C-4), 68.0 (d, C-11), 58.6 (d, C-9), 51.8 (d, C-14), 44.7 (t, C-12), 44.3 (s, C-13), 38.9 (s, C-10), 35.5 (d, C-8), 34.4 (t, C-1), 32.8 (t, C-6), 30.8 (t, C-7), 25.9 (t, C-16), 23.0 (t, C-15), 20.6 (t, C-2), 18.1 (q, C-19), 17.4 (q, C-18).

(Z)-11 α -Hydroxyandrost-4-ene-3,17-dione bis(thiosemicarbazone) (3d-Z). ¹H-NMR (200 MHz, CDCl₃/CD₃OD): 0.96 (s, 3H, H₃C-18), 1.29 (s, 3H, H₃C-19), 3.70 (m, 1H, H β -11), 6.20 (s, 1H, H-4), 7.19 and 7.38 (2m, 4H, NH₂), 8.89 (s, 1H, NH), 9.51 (s, 1H, NH). ¹³C NMR (50 MHz, CDCl₃/CD₃OD): 177.8 (s, C=S), 177.3 (s, C=S), 166.5 (s, C-17), 162.9 (s, C-3), 150.3 (s, C-5), 111.2 (d, C-4), 68.0 (d, C-11), 59.0 (d, C-9), 51.7 (d, C-14), 44.7 (t, C-12), 44.3 (s, C-13), 40.3 (s, C-10), 37.5 (d, C-8), 34.2 (t, C-1), 33.6 (t, C-6), 31.2 (t, C-7), 25.9 (t, C-16), 23.0 (t, C-15), 21.4 (t, C-2), 18.4 (q, C-19), 17.4 (q, C-18).

Mixture of 2e (*E*/*Z*). Starting with 940 mg progesterone (1e), after filtration the product was recrystallized from ethanol to afford light yellow crystals of 2e (765 mg, 65%). $R_{\rm f} = 0.48$ (toluene/EtOAc, 6 : 4, double development). Mp > 200 °C (decomp.). IR (ATR/cm⁻¹): 3430, 3328 (NH), 2939, 1696 (C=O), 1589, 1503 (C=N), 1291 (C=S), 1048, 735. ESI-TOF-MS: *m*/*z* for C₂₂H₃₃N₃OS [M + H]⁺: calcd 388.24171, found 388.24102; *m*/*z* for C₂₂H₃₃N₃OS [M + Na]⁺: calcd 410.22365, found 410.22379.

(*E*)-Pregn-4-ene-3,20-dione 3-thiosemicarbazone (2e-*E*). ¹H-NMR (200 MHz, $CDCl_3/CD_3OD$): 0.66 (s, 3H, H₃C-18), 1.09 (s, 3H, H₃C-19), 2.14 (s, 3H, H₃C-21), 2.32 (dt, *J* = 15.4, 3.6 Hz, 1H, H α -2), 2.55 (t, *J* = 9.0 Hz, 1H, H α -17), 5.81 (s, 1H, H-4), 6.88 and 7.30 (2m, 2H, NH₂), 9.15 (s, 1H, NH). ¹³C NMR (50 MHz, CDCl₃): 210.3 (s, C-20), 177.4 (s, C=S), 158.3 (s, C-5), 150.8 (s, C-3), 120.1 (d, C-4), 63.4 (d, C-17), 55.9 (d, C-14), 53.2 (d, C-9), 43.8 (s, C-13), 38.5 (s, C-10), 37.6 (t, C-12), 35.5 (d, C-8), 34.4 (t, C-1), 32.2 (t, C-6), 31.8 (t, C-7), 31.3 (q, C-21), 24.2 (t, C-16), 22.6 (t, C-15), 21.1 (t, C-11), 20.4 (t, C-2), 17.6 (q, C-19), 13.1 (q, C-18).

(Z)-Pregn-4-ene-3,20-dione 3-thiosemicarbazone (2e-Z). ¹H-NMR (200 MHz, CDCl₃/CD₃OD): 0.66 (s, 3H, H₃C-18), 1.13 (s, 3H, H₃C-19), 2.14 (s, 3H, H₃C-21), 2.55 (t, J = 9.0 Hz, 1H, H α -17), 6.15 (s, 1H, H-4), 6.82 and 7.19 (2m, 2H, NH₂), 9.40 (s, 1H, NH). ¹³C NMR (50 MHz, CDCl₃): 210.3 (s, C-20), 177.6 (s, C=S), 163.5 (s, C-5), 149.7 (s, C-3), 110.2 (d, C-4), 63.4 (d, C-17), 55.9 (d, C-14), 53.7 (d, C-9), 43.8 (s, C-13), 39.0 (s, C-10), 37.6 (t, C-12), 36.1 (d, C-8), 34.4 (t, C-1), 33.2 (t, C-6), 31.8 (t, C-7), 31.3 (q, C-21), 24.2 (t, C-16), 22.6 (t, C-15), 21.1 (t, C-11), 20.9 (t, C-2), 17.4 (q, C-19), 13.1 (q, C-18).

The residue after crystallization was chromatographed by FCC. Elution with toluene/EtOAc/MeOH (50/50/1) afforded 3e (114 mg, 8.3%).

Mixture of 3e (*E*/*Z*). Starting with 940 mg progesterone (1e), after filtration the product was recrystallized from ethanol. Yellow crystals (808 mg, 59%) were obtained as a mixture of two diastereoisomers, *E* and *Z* (4 : 1), which could not be separated. $R_f = 0.29$ (toluene/EtOAc, 6 : 4, double development). Mp > 201 °C (decomp.). IR (ATR/cm⁻¹): 3427, 3374, 3232, 3146 (NH), 2938, 1590, 1507 (C=N), 1296 (C=S), 1108, 866. Anal. calcd for $C_{23}H_{36}N_6S_2$: C 59.96; H 7.88; N 18.24; S 13.92. Found: C 59.64; H 7.69; N 17.97; S 13.87. ESI-TOF-MS: *m*/*z* for $C_{23}H_{36}N_6S_2$ [M + H]⁺: calcd 461.25156, found 461.25130.

(E)-Pregn-4-ene-3,20-dione bis(thiosemicarbazone) (3e-E). ¹H-NMR (500 MHz, DMSO): 0.55 (s, 3H, H₃C-18), 0.82 (m, 1H, H-9), 0.90 (m, 1H, Ha-7), 1.00 (s, 3H, H₃C-19), 1.08-1.19 (m, 2H, H-14, Hα-15), 1.25-1.39 (m, 3H, Hα-1, Hβ-11, Hβ-12), 1.44 (br.d, J = 10.5 Hz, 1H, H-8), 1.48–1.56 (m, 2H, H α -11, H α -16), 1.60 (t, J =7.0 Hz, 1H, Hβ-15), 1.73 (m, 1H, Hβ-7), 1.79 (m, 1H, Hα-12), 1.88 (s, 3H, H₃C-21), 2.05-2.20 (2m, 2H, Hβ-2, Hα-6), 2.24-2.34 (m, 3H, H β -6, H β -16, H α -17), 2.80 (br.d, J = 17.6 Hz, 1H, H α -2), 5.75 (s, 1H, H-4), 7.38 and 8.07 (2bs, 2H, NH₂), 7.52 and 8.04 (2bs, 2H, NH₂), 9.79 (s, 1H, NH), 10.04 (s, 1H, NH). ¹³C NMR (125 MHz, DMSO): 178.5 (s, C=S), 178.3 (s, C=S), 156.4 (s, C-5), 153.9 (s, C-20), 150.4 (s, C-3), 120.7 (d, C-4), 58.38 (d, C-17), 55.2 (d, C-14), 53.2 (d, C-9), 43.6 (s, C-13), 38.2 (t, C-12), 37.2 (s, C-10), 35.5 (d, C-8), 34.5 (t, C-1), 31.8 (t, C-6), 31.8 (t, C-7), 23.8 (t, C-15), 22.7 (t, C-16), 21.0 (t, C-11), 20.9 (t, C-2), 18.4 (q, C-21), 17.4 (q, C-19), 13.1 (q, C-18).

(Z)-Pregn-4-ene-3,20-dione bis(thiosemicarbazone) (3e-Z). ¹H-NMR (500 MHz, DMSO): 0.55 (s, 3H, H₃C-18), 0.82 (m, 1H, H-9), 0.90 (m, 1H, Hα-7), 1.06 (s, 3H, H₃C-19), 1.13 (m, 2H, H-14, Hα-15), 1.32 (m, 3H, Hα-1, Hβ-11, Hβ-12), 1.44 (br.d, J = 10.5 Hz, 1H, H-8), 1.52 (m, 2H, Hα-11, Hα-16), 1.60 (t, J = 7.0 Hz, 1H, Hβ-15), 1.73 (m, 1H, Hβ-7), 1.79 (m, 1H, Hα-12), 1.88 (s, 3H, H₃C-21), 2.04–2.22 (2m, 2H, Hβ-2, Hα-16), 2.24–2.37 (m, 3H, Hβ-6, Hβ-16, Hα-17), 6.61 (s, 1H, H-4), 7.38 and 8.07 (2bs, 2H, NH₂), 7.50 and 7.94 (2bs, 2H, NH₂), 9.79 (s, 1H, NH), 10.33 (s, 1H, NH). ¹³C NMR (125 MHz, DMSO): 178.5 (s, C=S), 178.0 (s, C=S), 160.7 (s, C-5), 154.9 (s, C-20), 147.8 (s, C-3), 112.1 (d, C-4), 58.34 (d, C-17), 55.1 (d, C-14), 53.6 (d, C-9), 43.5 (s, C-13), 38.1 (t, C-12), 38.3 (s, C-10), 36.0 (t, C-1), 35.4 (d, C-8), 32.3 (t, C-6), 32.2 (t, C-7), 23.8 (t, C-15), 22.7 (t, C-16), 21.0 (t, C-11), 20.8 (t, C-2), 18.4 (q, C-21), 17.6 (q, C-19), 13.1 (q, C-18).

The residue after crystallization was chromatographed by FCC. Elution with toluene/EtOAc (70/30) afforded 2e (86 mg, 7.4%).

General procedure for the synthesis of thiadiazolines

Thiadiazolines **4a–e** and **5a–e** were prepared according to the literature.²¹ To a stirred solution of steroidal thiosemicarbazones **2a–e** and **3a–e** (1 mmol) in chloroform (25 mL), freshly distilled acetic anhydride (2.3 and 4.6 mL, respectively) and pyridine (1.2 and 2.4 mL, respectively) were added. The reaction mixture was stirred for 4 h over an oil bath at 85 °C and monitored by TLC. After completion of the reaction, the solvent

was removed under reduced pressure. The residue was chromatographed by FCC (the eluents and the ratio are mentioned in each experiment).

(3R)-N-(3'-Acetyl-17-oxospiro[19-norandrost-4-ene-3,2'-[1,3,4] thiadiazoline]-5'-yl)acetamide (4a). Starting with 340 mg of the mixture of 2a, elution with toluene/EtOAc (5/5) and crystallization from DMSO gave 4a (285 mg, 67%). $R_{\rm f} = 0.45$ (CHCl₃/ CH₃OH, 95 : 5, double development). Mp. 194.5–195.4 °C; $[\alpha]_{D}$ +58 (c 1.0×10^{-3} in CHCl₃/CH₃OH (3 : 1)); IR (ATR/cm⁻¹): 2928 (C-H), 1736 (C=O), 1618 (C=N), 1410 (C=N), 756 (C=S); ¹H-NMR (500 MHz, CDCl₃/CD₃OD): 0.66 (m, 1H, H-9), 0.91 (s, 3H, $\rm H_{3}C\text{-}18),\,0.99\,(m,\,1H,\,H\alpha\text{-}7),\,1.16\text{-}1.47\,(m,\,5H),\,1.56\,(m,\,1H,\,H\beta\text{-}1.16\,(m,\,1H,\,M\beta))))))))$ 15), 1.74-2.00 (m, 5H), 2.04-2.16 (m, 4H overlapped with C(5')-NHCOCH₃), 2.11 (s, 3H, C(5')-NHCOCH₃), 2.19 (s, 3H, N(3')- $COCH_3$), 2.32 (dt, J = 14.5, 3.0 Hz, 1H, H β -6), 2.46 (dd, J = 19.5, 8.5 Hz, 1H, H β -16), 2.59 (td, J = 13.5, 2.5 Hz, 1H, H α -2), 5.67 (s, 1H, H-4), 10.57 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃): 222.4 (s, C-17), 169.6 (s, C(5')-NHCOCH₃), 169.2 (s, N(3')-COCH₃), 144.5 (s, C-5'), 141.1 (s, C-5), 121.6 (d, C-4), 79.9 (s, C-3), 50.1 (d, C-9), 50.0 (d, C-14), 47.8 (s, C-13), 40.3 (d, C-10), 39.8 (d, C-8), 35.6 (t, C-16), 34.1 (t, C-6), 33.4 (t, C-2), 31.1 (t, C-12), 29.8 (t, C-7), 25.8 (t, C-11), 25.1 (t, C-1), 23.3 (q, COCH₃), 22.4 (q, NHCOCH₃), 21.4 (t, C-15), 13.5 (q, C-18). Anal. calcd for $C_{23}H_{31}N_3O_3S \times 0.5CH_3$ -SOCH3: C 61.51; H 7.31; N 8.97; S 10.26. Found: C 61.18; H 7.16; N 8.99; S 10.85. ESI-TOF-MS: m/z for $C_{23}H_{31}N_3O_3S [M + H]^+$: calcd 430.21589, found 430.21581; m/z for C₂₃H₃₁N₃O₃S [M + Na]⁺: calcd 452.19783, found 452.19790.

(3R)-N-(3'-Acetyl-17-oxospiro[androst-4-ene-3,2'-[1,3,4]thiadiazoline]-5'-yl)acetamide (4b). Starting with 360 mg of the mixture of 2b, elution with toluene/EtOAc (5/5) gave 4b (321 mg, 73%). $R_{\rm f} = 0.58$ (CHCl₃/CH₃OH, 95 : 5, double development). Mp. 222.1–222.8 °C; $[\alpha]_{D}$ +109 (*c* 1.05 × 10⁻³ in CHCl₃/CH₃OH (3:1)); IR (ATR/cm⁻¹): 3233, 2948 (C-H), 2926, 1710 (C=O), 1661 (C=N), 1386 (C=N); ¹H-NMR (500 MHz, CDCl₃/CD₃OD): $0.80 (td, J = 3.5, 12.5 Hz, 1H, H-9), 0.90 (s, 3H, H_3C-18), 0.95 (m, 10.80)$ 1H, Ha-7), 1.12 (s, 3H, H₃C-19), 1.20-1.32 (m, 2H, Ha-12, H-14), 1.39 (dq, J = 13.0, 3.0 Hz, 1H, H α -11), 1.53 (m, 2H, H α -1, H β -15), 1.60-1.68 (m, 2H, H-8, Hα-11), 1.78-1.89 (m, 3H, Hβ-1, Hβ-7, Hβ-12), 1.96 (m, 1H, Hβ-15), 2.03 (br.d, J = 13.5 Hz, 1H, Hβ-2), 2.09-2.12 (m, 2H, Ha-6, Ha-16, overlapped with C(5')-NHCOCH₃), 2.11 (s, 3H, C(5')-NHCOCH₃), 2.19 (s, 3H, N(3')- $COCH_3$), 2.32 (dt, J = 14.5, 3.0 Hz, 1H, H β -6), 2.60 (dd, J = 19.5, 8.5 Hz, 1H, Hβ-16), 2.80 (td, J = 14.0, 2.5 Hz, 1H, Hα-2), 5.54 (s, 1H, H-4), 10.50 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃/CD₃OD): 222.2 (s, C-17), 169.5 (s, C(5')-NHCOCH₃), 169.2 (s, N(3')-COCH₃), 145.4 (s, C-5'), 144.4 (s, C-5), 120.6 (d, C-4), 80.1 (s, C-3), 54.1 (d, C-9), 50.8 (d, C-14), 47.6 (s, C-13), 36.7 (s, C-10), 35.7 (t, C-16), 35.2 (d, C-8), 35.1 (t, C-1), 31.4 (t, C-6), 31.1 (t, C-12), 30.9 (t, C-7), 30.8 (t, C-2), 25.8 (t, C-11), 23.2 (q, COCH₃), 22.5 (q, NHCOCH₃), 21.5 (t, C-15), 17.4 (q, C-19), 13.5 (q, C-18). ESI-TOF-MS: m/z for C₂₄H₃₃N₃O₃S [M + H]⁺: calcd 444.23154, found 444.23112; m/z for C₂₄H₃₃N₃O₃S [M + Na]⁺: calcd 466.21348, found 466.21310.

(3*R*)-*N*-(3'-Acetyl-17-oxospiro[androsta-4,9(11)-diene-3,2'-[1,3,4]thiadiazoline]-5'-yl)acetamide (4c). Starting with 360 mg of the mixture of 2c, elution with toluene/EtOAc (5/5) gave 4c (318 mg, 72%). $R_{\rm f} = 0.35$ (toluene/EtOAc, 6:4, double

development). Mp. 219.1–220.2 °C; $[\alpha]_{\rm D}$ +135 (c 1.01 × 10⁻³ in CHCl₃); IR (ATR/cm⁻¹): 3216, 3075, 2928 (C-H), 1738 (C=O), 1612 (C=N), 1411 (C=N), 1240, 1035, 754; ¹H-NMR (500 MHz, $CDCl_3$): 0.84 (s, 3H, H₃C-18), 1.01 (dq, J = 4.0, 12.5 Hz, 1H, H α -7), 1.25 (s, 3H, H_3C -19), 1.48 (m, 1H, H-14), 1.57 (m, 1H, $H\beta$ -15), 1.87 (td, I = 3.0, 13.5 Hz, 1H, H α -1), 2.15 (s, 3H, C(5')-NHCOCH₃), 2.21 (s, 3H, N(3')-COCH₃), 2.27 (t, J = 11 Hz, 1H, H-8), 2.36 (t, I = 13 Hz, 1H, H β -6), 2.47 (dd, I = 18.5, 9.0 Hz, 1H, Hβ-16), 2.90 (t, J = 13.5 Hz, 1H, Hα-2), 5.44 (d, J = 6.0 Hz, 1H, H-11), 5.56 (s, 1H, H-4), 9.65 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃): 221.9 (s, C-17), 169.2 (s, N(3')-COCH₃), 168.9 (s, C(5')-NHCOCH₃), 144.8 (s, C-5'), 146.4 (s, C-9), 144.0 (s, C-5), 120.9 (d, C-4), 116.9 (d, C-11), 80.3 (s, C-3), 48.0 (d, C-14), 45.9 (s, C-13), 39.4 (d, C-10), 36.9 (t, C-16), 36.2 (d, C-8), 33.2 (t, C-1), 33.0 (t, C-12), 31.8 (t, C-6), 30.9 (t, C-7), 31.5 (t, C-2), 26.4 (q, C-19), 23.7 (q, COCH₃), 23.1 (q, NHCOCH₃), 22.7 (t, C-15), 13.9 (q, C-18). ESI-TOF-MS: m/z for C₂₄H₃₁N₃O₃S [M + H]⁺: calcd 442.21589, found 442.21584; m/z for $C_{24}H_{31}N_3O_3S$ [M + Na]⁺: calcd 464.19783, found 464.19803.

(3R)-N-(11α-Acetoxy-3'-acetyl-17-oxospiro[androst-4-ene-3,2'-[1,3,4]thiadiazoline]-5'-yl)acetamide (4d-OAc). Starting with 375 mg of the mixture of 2d, elution with toluene/EtOAc (65/35) gave **4d-OAc** (335 mg, 67%). $R_{\rm f} = 0.60$ (CHCl₃/CH₃OH, 9 : 1). Mp. 162.3–164.8 °C; $[\alpha]_{\rm D}$ +90 (c 0.97 × 10⁻³ in CHCl₃); IR (ATR/ cm⁻¹): 3235, 3174, 2971, 2935 (C-H), 1730 (C=O), 1703, 1642, 1613 (C=N), 1390 (C=N), 1240, 733; ¹H-NMR (500 MHz, $CDCl_3$): 0.96 (s, 3H, H₃C-18), 1.05 (dq, J = 4.0, 14.0 Hz, 1H, H α -7), 1.17 (s, 3H, H_3 C-19), 1.22–1.31 (m, 2H, H-9, H α -12), 1.40 (m, 1H, H-14), 1.55 (m, 1H, Hβ-15), 1.66-1.74 (m, 2H, Hα-1, H-8), 1.82-1.91 (m, 2H, Hβ-1, Hβ-7), 1.93-1.99 (m, 2H, Hβ-2, Hα-15), 2.01 (s, 3H, OCOCH₃-11), 2.16 (s, 3H, C(5')-NHCOCH₃), 2.21 (s, 3H, N(3')-COCH₃), 2.49 (dd, *J* = 19.0, 9.0 Hz, 1H, Hβ-16), 2.78 $(td, J = 13.5, 2.5 Hz, 1H, H\alpha - 2), 5.22 (m, 1H, H\beta - 11), 5.64 (s, 1H, H\beta - 11)$ H-4), 9.83 (s, 1H, NH). ¹³C NMR (125 MHz, CDCl₃): 218.5 (s, C-17), 169.2 (s, OCOCH₃), 169.2 (2s, N(3')-COCH₃ and C(5')-NHCOCH₃), 144.6 (s, C-5'), 144.1 (s, C-5), 122.5 (d, C-4), 79.5 (s, C-3), 70.5 (d, C-11), 56.4 (d, C-9), 49.6 (d, C-14), 47.4 (s, C-13), 38.2 (d, C-10), 38.1 (t, C-12), 35.7 (t, C-1), 35.6 (t, C-16), 34.8 (d, C-8), 32.3 (t, C-6), 31.0 (t, C-2), 30.7 (t, C-7), 23.6 (q, COCH₃), 23.0 (q, NHCOCH₃), 21.8 (q, OCOCH₃), 21.6 (t, C-15), 18.4 (q, C-19), 14.2 (q, C-18). ESI-TOF-MS: m/z for $C_{26}H_{35}N_3O_5S [M + H]^+$: calcd 502.23702, found 502.23678; m/z for C₂₆H₃₅N₃O₅S [M + Na]⁺: calcd 524.21896, found 524.21936.

Further elution with toluene/EtOAc/MeOH (50/50/4) gave (3*R*)-*N*-(3'-acetyl-11α-hydroxy-17-oxospiro[androst-4-ene-3,2'-[1,3,4]thiadiazoline]-5'-yl)acetamide (**4d-OH**) (38 mg, 8%). $R_f = 0.45$ (CHCl₃/CH₃OH, 9 : 1). Mp. 215.8–220.0 °C; $[\alpha]_D$ +102 (*c* 0.96 × 10⁻³ in CHCl₃/CH₃OH (3 : 1)); IR (ATR/cm⁻¹): 3518, 3320, 2927 (C–H), 1717 (C=O), 1690, 1663, 1605 (C=N), 1377 (C=N), 1238, 1012, 721; ¹H-NMR (500 MHz, CDCl₃/CD₃OD): 0.93 (s, 3H, H₃C-18), 0.97 (m, 1H, H-9), 1.00 (m, 1H, Hα-7), 1.26 (s, 3H, H₃C-19), 1.31 (m, 1H, Hα-12), 1.39 (m, 1H, Hα-1), 1.85 (m, 1H, Hβ-7), 1.97 (m, 2H, Hβ-2, Hα-15), 2.05 (m, 1H, Hβ-12), 2.10 (s, 3H, C(5')-NHCOCH₃), 2.20 (s, 3H, N(3')-COCH₃), 2.26 (m, 1H, H-6), 2.49 (dd, *J* = 19.0, 9.0 Hz, 1H, Hβ-16), 2.55 (dt, *J* = 13.5, 3 Hz, 1H, Hβ-1), 2.84 (td, *J* = 13.5, 2.5 Hz, 1H, Hα-2), 3.95 (td, *J* = 10.5)

5 Hz, 1H, Hβ-11), 5.57 (s, 1H, H-4). ¹³C NMR (125 MHz, CDCl₃/CD₃OD (3 : 1)): 220.7 (s, C-17), 169.1 (2s, N(3')-COCH₃ and C(5')-NHCOCH₃), 145.2 (s, C-5), 144.6 (s, C-5'), 121.5 (d, C-4), 79.8 (s, C-3), 68.1 (d, C-11), 59.5 (d, C-9), 49.9 (d, C-14), 47.8 (s, C-13), 41.9 (t, C-12), 37.9 (d, C-10), 36.6 (t, C-1), 35.6 (t, C-16), 34.8 (d, C-8), 32.2 (t, C-6), 30.8 (t, C-2), 30.3 (t, C-7), 23.1 (q, NHCOCH₃), 22.2 (q, OCOCH₃), 21.4 (t, C-15), 17.7 (q, C-19), 14.2 (q, C-18). ESI-TOF-MS: m/z for C₂₄H₃₃N₃O₅S [M + H]⁺: calcd 460.22645, found 460.22563; m/z for C₂₄H₃₃N₃O₅S [M + Na]⁺: calcd 482.20840, found 482.20780.

(3R)-N-(3'-Acetyl-20-oxospiro[pregn-4-ene-3,2'-[1,3,4]thiadiazoline]-5'-yl)acetamide (4e). Starting with 387 mg of the mixture of 2e, after evaporation of the solvent the product was crystallized from a mixture of CHCl₃/MeOH to give pure 4e (363 mg, 77%). $R_f = 0.40$ (toluene/EtOAc, 6 : 4, double development). Mp. 213.4–214.9 °C; $[\alpha]_{\rm D}$ +108 (c 1.03 × 10⁻³ in CHCl₃/CH₃OH (3 : 1)). IR (ATR/cm⁻¹): 3229, 3169, 2929, 2846 (C-H), 1693 (C= O), 1657, 1612 (C=N), 1379 (C=N), 1237, 721. ¹H-NMR (200 MHz, CDCl₃/CD₃OD): 0.44 (s, 3H, H₃C-18), 0.90 (s, 3H, H₃C-19), 1.92 (s, 3H, H₃C-21), 1.95 (s, 3H, C(5')-NHCOCH₃), 2.00 (s, 3H, N(3')-COCH₃), 2.39 (t, J = 9 Hz, 1H, H α -17), 2.62 (td, J = 13.8, 2.2Hz, 1H, Hα-2), 5.32 (s, 1H, H-4). ¹³C NMR (50 MHz, CDCl₃/ CD₃OD): 211.0 (s, C-20), 169.7 (s, C(5')-NHCOCH₃), 169.2 (s, N(3')-COCH₃), 145.6 (s, C-5'), 144.4 (s, C-5), 120.0 (d, C-4), 79.9 (s, C-3), 63.3 (d, C-17), 55.8 (d, C-14), 53.7 (d, C-9), 43.8 (s, C-13), 38.4 (s, C-10), 36.4 (t, C-12), 35.4 (d, C-8), 34.9 (t, C-1), 31.8 (t, C-6), 31.4 (q, C-21), 30.9 (t, C-7), 30.7 (t, C-2), 23.9 (t, C-16), 23.0 (q, COCH₃), 22.3 (q, NHCOCH₃), 22.0 (t, C-15), 20.7 (t, C-11), 17.1 (q, C-19), 12.8 (q, C-18). ESI-TOF-MS: *m*/*z* for C₂₆H₃₇N₃O₃S [M + H^{+} : calcd 472.26284, found 472.26186; *m*/*z* for C₂₆H₃₇N₃O₃S [M + Na]⁺: calcd 494.24478, found 494.24438; *m*/*z* for C₂₆H₃₇N₃O₃S $[M + K]^+$: calcd 510.21872, found 510.21919.

(3R,17R)-N,N'-(3,3"-Diacetyldispiro[[1,3,4]thiadiazoline-2,3'-19-norandrost-4-ene-17',2"-[1,3,4]thiadiazoline]-5,5"-diyl)bis [acetamide] (5a). Starting with 420 mg of the mixture of 3a, elution with toluene/EtOAc/MeOH (50/50/1) and crystallization from MeOH gave 5a (410 mg, 70%). $R_{\rm f} = 0.24$ (CHCl₃/MeOH, 95 : 5, double development). Mp. 197.5–202.6 °C; $[\alpha]_{\rm D}$ +290 (c 1.0×10^{-3} in CH₃OH); IR (ATR/cm⁻¹): 2939 (C-H), 1642, 1615 (C=N), 1401, 722 (C=S); ¹H-NMR (500 MHz, CD₃OD): 0.59 (m, 1H, H-9), 0.94 (m, 1H, Ha-7 overlapped with H₃C-18), 0.95 (s, 3H, H₃C-18), 1.16–1.34 (m, 4H), 1.37–1.50 (m, 2H), 1.68–1.90 (m, 5H), 2.01-2.16 (m, 4H overlapped with two CH₃ groups), 2.09 (s, 3H, C(5')-NHCOCH₃), 2.12 (s, 3H, C(5")-NHCOCH₃), 2.18 (s, 3H, N(3')-COCH₃), 2.22 (s, 3H, N(3'')-COCH₃), 2.28 (dt, J = 14, 3 Hz, 1H, H β -6), 2.54 (td, J = 13.5, 3.0 Hz, 1H, H α -2), 4.35 (dtd, J =13.5, 13.25, 3.0 Hz, 1H, Hβ-16), 5.61 (s, 1H, H-4). ¹³C NMR (125 MHz, CDCl₃): 173.5 (s, N(3")-COCH₃), 171.9 (s, C(5")-NHCOCH₃), 171.7 (s, C(5')-NHCOCH₃), 171.2 (s, N(3')-COCH₃), 149.4 (s, C-5"), 146.4 (s, C-5'), 143.1 (s, C-5), 122.8 (d, C-4), 94.0 (s, C-17), 81.3 (s, C-3), 53.3 (s, C-13), 51.4 (d, C-9), 47.8 (d, C-14), 42.2 (d, C-8), 41.9 (d, C-10), 35.6 (t, C-6), 34.9 (t, C-2), 33.1 (t, C-12), 32.3 (t, C-7), 31.0 (t, C-16), 27.4 (t, C-11), 27.0 (t, C-1), 25.1 (q, C(5")-NHCOCH₃), 24.3 (t, C-15), 24.0 (q, N(3')-COCH₃), 23.1 (q, N(3")-COCH₃), 23.0 (q, C(5')-NHCOCH₃), 16.0 (q, C-18). Anal. calcd for $C_{28}H_{38}N_6O_4S_2\times 2$ CH_3OH: C 55.36; H 7.12; N 12.91; S 9.85. Found: C 55.64; H 7.32; N 13.31; S 9.63. ESI-TOF-MS: m/z for $C_{28}H_{38}N_6O_4S_2 [M + 2H]^{2+}$: calcd 294.12707, found 294.12704; m/z for $C_{28}H_{38}N_6O_4S_2 [M + H]^+$: calcd 587.24687, found 587.24700; m/z for $C_{28}H_{38}N_6O_4S_2 [M + Na]^+$: calcd 609.22882, found 609.22878.

(3R,17R)-N,N'-(3,3"-Diacetyldispiro[[1,3,4]thiadiazoline-2,3'androst-4-ene-17',2"-[1,3,4]thiadiazoline]-5,5"-diyl)bis[acetamide] (5b). Starting with 432 mg of the mixture of 3b, elution with toluene/EtOAc/MeOH (50/50/1) and crystallization from MeOH gave 5b (336 mg, 56%). $R_{\rm f} = 0.24$ (CHCl₃/MeOH, 95 : 5, double development). Mp. 200.2–202.3 °C; $[\alpha]_{\rm D}$ +62 (c 1.02 × 10⁻³ in CH₃OH); IR (ATR/cm⁻¹): 2938 (C-H), 2853, 1665, 1639, 1610 (C=N), 1380, 1254; ¹H-NMR (500 MHz, CD₃OD): 0.72 (td, *J* = 11, 3.5 Hz, 1H, H-9), 0.91 (m, 1H, Hα-7 overlapped with H₃C-18), 0.94 (s, 3H, H₃C-18), 1.10 (s, 3H, H₃C-19), 1.23 (m, 1H, H-14), 1.31-1.60 (m, 6H, Hβ-11, Hα-15, Hβ-12, H-8, Hβ-1, Hα-11), 1.69–1.90 (m, 4H, H β -15, H α -1, H α -12, H β -7), 1.98 (d, J = 12.5Hz, H β -2), 2.02–2.08 (m, 2H overlapped with two CH₃ groups, Ha-6, Ha-16), 2.09 (s, 3H, C(5')-NHCOCH₃), 2.12 (s, 3H, C(5")-NHCOCH₃), 2.18 (s, 3H, N(3')-COCH₃), 2.22 (s, 3H, N(3")- $COCH_3$), 2.78 (td, J = 13.3, 2.5 Hz, 1H, H α -2), 4.35 (dtd, J = 2.5, 13.25 Hz, 1H, Hβ-16), 5.48 (s, 1H, H-4). ¹³C NMR (125 MHz, CD₃OD): 173.5 (s, N(3")-COCH₃), 171.9 (s, C(5")-NHCOCH₃), 171.7 (s, C(5')-NHCOCH₃), 171.3 (s, N(3')-COCH₃), 149.3 (s, C-5"), 147.3 (s, C-5), 146.2 (s, C-5'), 121.9 (d, C-4), 93.9 (s, C-17), 81.3 (s, C-3), 55.5 (d, C-9), 53.0 (s, C-13), 48.5 (d, C-14), 38.0 (s, C-10), 37.6 (d, C-8), 36.7 (t, C-2), 33.4 (t, C-7), 33.1 (t, C-12), 33.0 (t, C-1), 32.9 (t, C-6), 31.0 (t, C-16), 25.1 (q, N(3")-COCH₃), 24.4 (t, C-15), 24.0 (q, N(3')-COCH₃), 23.1 (q, C(5")-NHCOCH₃), 23.0 (q, C(5')-NHCOCH₃), 21.9 (t, C-11), 18.3 (q, C-19), 15.8 (q, C-18). ESI-TOF-MS: m/z for $C_{29}H_{40}N_6O_4S_2$ [M + 2H]⁺: calcd 301.13490, found 301.13535; m/z for $C_{29}H_{40}N_6O_4S_2$ [M + H]⁺: calcd 601.26252, found 601.26266; m/z for $C_{29}H_{40}N_6O_4S_2$ [M + Na]⁺: calcd 623.24447, found 623.24458.

(3R,17R)-N,N'-(3,3"-Diacetyldispiro[[1,3,4]thiadiazoline-2,3'androsta-4,9(11)-diene-17',2"-[1,3,4]thiadiazoline]-5,5"-diyl)bis [acetamide] (5c). Starting with 430 mg of the mixture of 3c, elution with toluene/EtOAc/MeOH (50/50/1) gave 5c (371 mg, 62%). $R_{\rm f} = 0.19$ (CHCl₃/MeOH, 95 : 5, double development). Mp. 199.2–201.1 °C; $[\alpha]_D$ +178 (c 1.05 × 10⁻³ in CH₃OH); IR (ATR/cm⁻¹): 3240, 3071, 2931 (C-H), 1670, 1644, 1613 (C=N), 1378, 1293, 1237, 1030, 724; ¹H-NMR (500 MHz, CD₃OD): 0.85 (s, 3H, H₃C-18), 0.94 (m, 1H, Hα-7), 1.25 (s, 3H, H₃C-19), 1.39 $(br.q, J = 9.5 Hz, 1H, H-14), 1.53 (m, 1H, H\alpha-15), 1.78 (m, 1H, 1)$ Hβ-12), 1.86-1.93 (m, 2H, Hα-1, Hβ-15), 1.97 (m, 1H, Hβ-7), 2.06-2.16 (4H, Hβ-2, Hα-6, H-8, Hα-16, overlapped with two CH₃ groups), 2.09 (s, 3H, C(5')-NHCOCH₃), 2.12 (s, 3H, C(5")-NHCOCH₃), 2.19 (s, 3H, N(3')-COCH₃), 2.23 (s, 3H, N(3")- $COCH_3$, 2.58 (m, 1H, H α -12), 2.89 (td, J = 12.5, 5 Hz, 1H, H α -2), 4.32 (dtd, J = 4.0, 13.0 Hz, 1H, H β -16), 5.46 (d, J = 6.0 Hz, 1H, H-11), 5.48 (s, 1H, H-4). ¹³C NMR (125 MHz, CD₃OD): 173.4 (s, N(3")-COCH₃), 172.0 (s, C(5")-NHCOCH₃), 171.9 (s, C(5')-NHCOCH₃), 171.3 (s, N(3')-COCH₃), 149.1 (s, C-5"), 147.1 (s, C-9), 146.2 (s, C-5'), 146.0 (s, C-5), 122.0 (d, C-4), 118.9 (d, C-11), 93.5 (s, C-17), 81.4 (s, C-3), 51.4 (s, C-13), 46.1 (d, C-14), 40.6 (d, C-10), 39.6 (d, C-8), 35.5 (t, C-12), 34.7 (t, C-1), 33.7 (t, C-7), 33.1 (t, C-6), 32.6 (t, C-2), 31.4 (t, C-16), 26.9 (s, C-19), 25.2 (t, C-15), 24.9 (q, N(3")-COCH₃), 23.9 (q, N(3')-COCH₃), 23.0 (q, C(5")-NHCOCH₃), 22.9 (q, C(5')-NHCOCH₃), 15.8 (q, C-18). ESI-TOF-MS: m/z for C₂₉H₃₈N₆O₄S₂ [M + 2H]²⁺: calcd 300.12707, found 300.12731; m/z for C₂₉H₃₈N₆O₄S₂ [M + H]⁺: calcd 599.24687, found 599.24660; m/z for C₂₉H₃₈N₆O₄S₂ [M + Na]⁺: calcd 621.22882, found 621.22859.

(3R,17R)-N,N'-(11a-Acetoxy-3,3"-diacetyldispiro[[1,3,4]thiadiazoline-2,3'-androst-4-ene-17',2"-[1,3,4]thiadiazoline]-5,5"divl)bis[acetamide] (5d). Starting with 450 mg of the mixture of 3d, elution with toluene/EtOAc/MeOH (50/50/0.5) gave 5d (411 mg, 62%). $R_{\rm f} = 0.44$ (CHCl₃/MeOH, 9 : 1). Mp. 199.2–201.8 °C; $[\alpha]_{\rm D}$ +215 (c 1.03 × 10⁻³ in CHCl₃/CH₃OH (3 : 1)); IR (ATR/ cm⁻¹): 2963 (C-H), 2931, 1703, 1669, 1606 (C=N), 1371, 1240, 1012, 723; ¹H-NMR (500 MHz, CDCl₃/CD₃OD): 1.00 (s, 3H, H₃C-18), 1.02 (m, 1H, Ha-7 overlapped with H₃C-18), 1.16 (s, 3H, $H_{3}C-19$, 1.26 (t, I = 11.0 Hz, 1H, H-9), 1.34–1.47 (m, 2H, H-14, H α -15), 1.55 (dq, J = 3, 11 Hz, 1H, H-8), 1.68–1.87 (m, 5H, H α -1, H β -15, H β -7, H β -12, H β -1), 1.97 (br.d, J = 13.5 Hz, 1H, H β -2), 2.02 (s, 3H, OCOCH₃), 2.11 (s, 3H, C(5')-NHCOCH₃), 2.14 (s, 3H, C(5")-NHCOCH₃), 2.19 (s, 3H, N(3')-COCH₃), 2.22 (s, 3H, N(3")- $COCH_3$), 2.80 (td, J = 14, 3 Hz, 1H, H α -2), 4.37 (dtd, J = 3.5, 13.0 Hz, 1H, Hβ-16), 5.16 (br.q, J = 10 Hz, 1H, Hβ-11), 5.58 (s, 1H, H-4). ¹³C NMR (125 MHz, CDCl₃/CD₃OD): 171.6 (s, N(3")-COCH₃), 170.5 (s, OCOCH₃), 169.7 (s, C(5')-NHCOCH₃), 169.6 (s, C(5")-NHCOCH₃), 169.2 (s, N(3')-COCH₃), 146.7 (s, C-5"), 144.3 (s, C-5'), 144.2 (s, C-5), 121.9 (d, C-4), 91.5 (s, C-17), 79.3 (s, C-3), 71.4 (d, C-11), 55.5 (d, C-9), 51.5 (s, C-13), 45.9 (d, C-14), 38.2 (t, C-12), 37.7 (s, C-10), 35.9 (t, C-1), 35.5 (d, C-8), 32.1 (t, C-6), 31.2 (t, C-7), 30.8 (t, C-2), 29.9 (t, C-16), 24.2 (q, N(3")-COCH₃), 23.1 (t, C-15), 22.9 (q, N(3')-COCH₃), 22.3 (q, C(5")-NHCOCH₃), 22.2 (q, C(5')-NHCOCH₃), 21.5 (q, OCOCH₃), 17.9 (q, C-19), 15.5 (q, C-18). ESI-TOF-MS: m/z for $C_{31}H_{42}N_6O_4S_2$ [M + 2H]²⁺: calcd 330.13764, found 330.13822; m/z for $C_{31}H_{42}N_6O_4S_2$ [M + H]⁺: calcd 659.26800, found 659.26750; m/z for $C_{31}H_{42}N_6O_4S_2$ [M + Na]⁺: calcd 681.24995, found 681.24989.

(3R,20R/S)-N,N'-(3,3"-Diacetyldispiro[[1,3,4]thiadiazoline-2,3'-pregn-4-ene-20',2"-[1,3,4]thiadiazoline]-5,5"-diyl)bis[acetamide] (5e) (R/S). Starting with 460 mg of the mixture of 3e, elution with CH₂Cl₂/MeOH (80/20) and crystallization from CH₂Cl₂ gave 5e (221 mg, 35%) as a mixture of two isomers (ratio, 1.2:1). $R_{\rm f} = 0.32$ (CH₂Cl₂/MeOH, 20:1), $R_{\rm f} = 0.16$ (toluene/ EtOAc, 6:4, double development). Mp. 191.9–193.2 °C; $[\alpha]_D$ +14 (c 0.97×10^{-3} in DMSO); IR (ATR/cm⁻¹): 3325 (C-H), 2933, 1664, 1644, 1615 (C=N), 1387, 1233. Anal. calcd for $C_{31}H_{44}N_6O_4S_2 \times CH_2Cl_2$: C 53.85; H 6.50; N 11.77; S 8.97. Found: C 53.76; H 6.46; N 11.93; S 8.97. ESI-TOF-MS: m/z for $C_{31}H_{44}N_6O_4S_2[M + 2H]^{2+}$: calcd 315.15055, found 315.15006; *m*/ z for C₃₁H₄₄N₆O₄S₂ [M + H]⁺: calcd 629.29382, found 629.29187; m/z for C₃₁H₄₄N₆O₄S₂ [M + Na]⁺: calcd 651.27577, found 651.27484; m/z for C₃₁H₄₄N₆O₄S₂ [2M + H]⁺: calcd 1257.58037, found 1257.57804.

(5e major). ¹H-NMR (500 MHz, DMSO): 0.66 (m, 1H, H-9), 0.80 (m, 1H, H α -7), 0.85 (s, 3H, H₃C-18), 0.94 (m, 1H, H-14), 0.99 (s, 3H, H₃C-19), 1.91 (s, 3H, H₃C-21), 2.008 (s, 3H, C(5')-NHCOCH₃), 2.013 (s, 3H, C(5'')-NHCOCH₃), 2.07 (s, 3H, N(3'')-COCH₃), 2.09 (s, 3H, N(3')-COCH₃), 2.69 (m, 2H, H α -2, H α -17 from the minor product), 3.13 (t, *J* = 8.0 Hz, 1H, H α -17), 5.40 (s, 1H, H-4), 11.42 (s, 1H, NH), 11.44 (s, 1H, NH). ¹³C NMR (125 MHz, DMSO): 169.2 (s, C(5')-NHCOCH₃), 169.1 (s, C(5'')-NHCOCH₃), 168.2 (s, N(3'')-COCH₃), 167.3 (s, N(3')-COCH₃), 144.6 (s, C-5), 143.4 (s, C-5''), 143.1 (s, C-5'), 120.7 (d, C-4), 83.8 (s, C-20), 78.8 (s, C-3), 54.9 (d, C-14), 54.1 (d, C-17), 53.6 (d, C-9), 42.0 (s, C-13), 36.6 (t, C-12), 36.3 (s, C-10), 34.8 (t, C-1), 34.4 (d, C-8), 30.5 (t, C-2), 31.9 (t, C-7), 31.3 (t, C-6), 30.9 (q, C-21), 24.6 (t, C-16), 24.6 (q, N(3'')-COCH₃), 23.6 (q, N(3')-COCH₃), 23.1 (t, C-11), 22.5 (q, C(5'')-NHCOCH₃), 22.5 (q, C(5')-NHCOCH₃), 20.2 (t, C-15), 17.3 (q, C-19), 13.0 (q, C-18).

(5e minor). ¹H-NMR (500 MHz, DMSO): 0.68 (m, 1H, H-9), 0.73 (s, 3H, H₃C-18), 0.80 (m, 1H, H α -7), 1.01 (s, 3H, H₃C-19), 1.05 (m, 1H, H-14, overlapped with H₃C-19 signal), 1.94 (s, 3H, H₃C-21), 2.01 (2s, 6H, C(5')-NHCOCH₃ and C(5'')-NHCOCH₃), 2.05 (s, 3H, N(3'')-COCH₃), 2.09 (s, 3H, N(3')-COCH₃), 2.69 (m, 2H, H α -2, H α -17), 5.41 (s, 1H, H-4), 11.35 (s, 1H, NH), 11.45 (s, 1H, NH). ¹³C NMR (125 MHz, DMSO): 169.2 (s, C(5'')-NHCOCH₃), 169.2 (s, C(5')-NHCOCH₃), 168.1 (s, N(3'')-COCH₃), 167.3 (s, N(3')-COCH₃), 144.6 (s, C-5), 143.5 (s, C-5''), 143.1 (s, C-5'), 120.7 (d, C-4), 82.0 (s, C-20), 78.8 (s, C-3), 55.3 (d, C-14), 54.6 (d, C-17), 53.6 (d, C-9), 42.9 (s, C-13), 38.7 (t, C-12), 36.3 (s, C-10), 34.8 (t, C-1), 34.7 (d, C-8), 30.5 (t, C-2), 31.9 (t, C-7), 31.3 (t, C-6), 29.1 (q, C-21), 25.4 (t, C-16), 24.5 (q, N(3'')-COCH₃), 22.5 (q, C(5'')-NHCOCH₃), 20.4 (t, C-15), 17.3 (q, C-19), 13.3 (q, C-18).

Single-crystal X-ray crystallography

Crystals suitable for structural analysis were obtained by slow recrystallization from DMSO. Single-crystal X-ray diffraction data for **4a** were collected at room temperature on an Agilent Technologies Gemini S diffractometer with Mo K α radiation ($\lambda = 0.71069$ Å). Data collection, unit cell finding, intensity integration and scaling of the reflections were performed with *CrysAlisPro* software.⁵⁸ An empirical absorption correction using spherical harmonics, which was implemented in the *SCALE3 ABSPACK* scaling algorithm within *CrysAlisPro*, was applied. The crystal structure was solved by the dual space method using *SHELXT*⁵⁹ and refined using *SHELXL-2014* (ref. 59) with *SHELXLE*⁶⁰ as a graphical interface.

All non-hydrogen atoms were refined anisotropically. The hydrogen atoms that were attached to C atoms were placed at geometrically idealized positions, with the C–H distances fixed at 0.93 and 0.96 Å, and their isotropic displacement parameters were set at equal to $1.2U_{eq}$ and $1.5U_{eq}$ from sp² and sp³ C atoms, respectively. The orientation of methyl group hydrogens was refined to best fit the observed electron density. The hydrogen atom that was attached to a nitrogen atom was located in a difference Fourier map and refined isotropically. An absolute structure was assigned to match the known absolute configuration of the steroid molecule.

Inspection of the 3D difference Fourier map revealed voids in the crystal structure, which were occupied by disordered solvent molecules. The total volume of solvent-accessible voids calculated using *PLATON*⁶¹ was found to be 22.4% of the unit cell volume. Because the disorder could not be modeled reasonably, the contribution of the disordered solvent region to the observed structural factors was removed by the *SQUEEZE* procedure⁶² implemented in *PLATON*. The estimated number of electrons in void regions was calculated to be 120 per unit cell, which corresponds to approximately 3 DMSO molecules per unit cell. Analytical data on the chemical composition point to 4 DMSO molecules per unit cell.

The final results of the crystal structure determination are summarized as follows. Molecular formula: $C_{23}H_{31}N_3O_3S$; $M_r =$ 429.57; crystal system: tetragonal; space group: *I*4; unit cell parameters: a = 21.9810(3) Å, c = 10.9904(3) Å, V = 5310.2(2) Å³; Z = 8; $\mu = 0.146$ mm⁻¹; 15 692 reflections measured, 6161 unique reflections ($R_{int} = 0.047$); 99.7% data completeness up to $2\theta = 52.64^\circ$, 90.3% data completeness up to $2\theta_{max} = 58.24^\circ$; 4874 reflections with $I > 2\sigma(I)$; number of refined parameters: 278; $R_1 = 0.055$ for data with $I > 2\sigma(I)$; w $R_2 = 0.149$ for all data; goodness-of-fit S = 1.066; Flack x = 0.05(12) determined by conventional TWIN/BASF refinement;⁶³ Flack x = 0.03(3)determined using the post-refinement Parsons quotient method⁶⁴ (1931 quotients); Hooft y = 0.04(3) determined using 2769 Bijvoet pairs (9 outliers)⁶⁵ using an optimized Student's *t*probability plot ($\nu = 99$, corr. coeff. = 0.999).⁶⁶†

Biology

Cytotoxicity assay. For the evaluation of cytotoxicity, six human cancer cell lines were used: cervical adenocarcinoma HeLa, breast carcinoma MDA-MB-453, breast adenocarcinoma MDA-MB-361, chronic myelogenous leukemia K562, colon adenocarcinoma LS174, and lung carcinoma A549, and one normal human cell line, lung fibroblasts MRC-5. Experiments were performed according to previously described methods.³⁹ HeLa (2000 cells per well), MDA-MB-453 (3000 cells per well), MDA-MB-361 (10 000 cells per well), LS174 (7000 cells per well), A549 (5000 cells per well) and MRC-5 (5000 cells per well) were seeded into 96-well microtiter plates and 20 h later, after cell adhesion, five different concentrations of the tested compounds were added to the cells. Only nutrient medium was added to the cells in the control wells. K562 cells (5000 cells per well) were seeded 2 h before the addition of the compounds. Stock solutions of the compounds, prepared according to a previously described procedure,37-39 were diluted with complete nutrient medium and applied to the target cells at various final concentrations ranging from 6.25 µM to 100 µM or 12.50 µM to 200 µM. The final DMSO concentration did not affect cell viability. Survival of the target cells was determined by an MTT test after 72 h of continuous activity of the agents, according to the method of Mosmann,67 which was modified by Ohno and Abe68 and used in our previous studies.37-39 Cisplatin was used as a positive control.

The isolation and treatment of peripheral blood mononuclear cells (PBMC) were carried out according to a previously reported method.⁴⁸

Live subject statement

The authors state that all experiments were performed in compliance with the relevant laws and institutional guidelines. The cytotoxicity assays using PBMC were approved by the Ethics Committee of the Institute of Oncology and Radiology of Serbia. Written informed consent was obtained from each healthy blood donor.

Cell cycle analysis. K562 and HeLa cells were treated with two concentrations of compounds 2a, 2b, 2c, 2e, 5a and 5d (at values of IC₅₀ and 2IC₅₀ determined after treatment for 72 h for each cell line) for 24 and 48 h. After the mentioned incubation times, the cells were collected, washed and fixed in 70% ethanol, according to standard protocol.^{39,69,70} Cell cycle distribution data are presented as Xav \pm S.D. of three independent experiments. The statistical significance of differences between the control and treated cell samples was evaluated using one-way repeated-measures ANOVA with Dunnett's post test. Values of *p* below 0.05 were considered to be statistically significant.

Morphological assessment of mode of cell death of HeLa. To examine the mode of cell death of HeLa induced by compounds **2a, 2b, 2c, 2e, 5a** and **5d**, morphological analysis was undertaken, as described elsewhere.^{39,69}

FITC-annexin V/propidium iodide double staining assay. Determination of human cervical adenocarcinoma HeLa cells in the early and late stages of apoptosis was performed by flow cytometry using FITC-annexin V/propidium iodide staining. FITC-annexin V was a product of BD Biosciences PharmingenTM. In brief, HeLa cells (200 000 cells per well) were exposed to concentrations of double IC₅₀ of the tested steroidal compounds for 24 h. After treatment, the cells were harvested, stained with FITC-annexin V and propidium iodide according to the manufacturer's instructions, and analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data are presented as mean \pm S.D. of two independent experiments.

Identification of target caspases. To identify the caspases involved in the apoptotic pathways induced by compounds 2a, 2b, 2c, 2e, 5a and 5d in HeLa cells, the percentages of cells in the subG1 phase pretreated with caspase inhibitors were determined, as previously described by Matić *et al.*⁶⁹ The specific caspase inhibitors applied at a concentration of 40 μ M were: Z-DEVD-FMK, a caspase-3 inhibitor, Z-IETD-FMK, a caspase-8 inhibitor and Z-LEHD-FMK, a caspase-9 inhibitor. The caspase inhibitors were purchased from R&D Systems (Minneapolis, USA). The compounds were applied to HeLa cells at concentrations that corresponded to double the values of IC₅₀ obtained after treatment for 72 h.

Endothelial cell tube formation assay. Possible effects of compounds 2a, 2b, 2c, 2e, 5a and 5d on the inhibition of angiogenesis in vitro were examined by the tube formation assay on a human umbilical vein endothelial EA.hy926 cell line.55 The assay was performed according to a previously described procedure.71 In brief, 24-well plates were coated with 200 µL Corning® Matrigel® basement membrane matrix (Corning cat. number 356234). Suspensions of EA.hy926 cells (40 000 cells per 400 µL) were plated on top of each Matrigel-coated well. Then, 200 µL complete nutrient medium (Dulbecco's modified Eagle's medium with high glucose and 4 mM L-glutamine supplemented with 10% heat-inactivated FBS and 25 mM HEPES) was added to the control cell sample, while solutions in nutrient medium of the tested compounds were added to the other cell samples. The plates were incubated for 20 h at 37 °C in an atmosphere of 5% CO₂ and humidified air. Photomicrographs

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of EA.hy926 cells were captured using an inverted phasecontrast microscope after treatment for 20 h. In our experiment, the EA.hy926 cells were exposed to low sub-toxic concentrations of the investigated compounds (concentrations of IC₂₀ obtained by an MTT test after treatment that lasted for 24 h): for compound **2a**, the applied concentration was 3.5 μ M, for compounds **2b**, **2c** and **2e** the concentration was 10 μ M, for compound **5a** the concentration was 4.5 μ M and for compound **5d** the concentration was 60 μ M.

The brine shrimp test. The brine shrimp test was performed against freshly hatched nauplii of *Artemia salina*.⁵⁷ The method was slightly modified by our team.³⁹ The compounds were dissolved in MeOH/CHCl₃, then in various amounts (1.0–0.05 mg) applied to filter paper discs (8 mm diameter) and the solvent was evaporated. All samples were tested in triplicate. LC_{50} was defined as the concentration of compounds that caused the death of 50% of nauplii.

Antimicrobial activity. Antibacterial activity was evaluated using four different strains of Gram-positive bacteria: Staphylococcus aureus (ATCC 6538), Kocuria rhizophila (ATCC 9341), Clostridium sporogenes (ATCC 19404), and Bacillus subtilis (ATCC 6633), and four different strains of Gram-negative bacteria: Escherichia coli (ATCC 25922), Salmonella enterica subsp. enterica serovar Enteritidis (ATCC 13076), Proteus hauseri (ATCC 13315) and Pseudomonas aeruginosa (ATCC 9027). Antibacterial activity was determined by the well diffusion method.72 To each Petri dish (90 mm diameter), 22 mL nutrient agar (HiMedia, Mumbai, India) and 100 μ L bacterial suspension (10⁶ cells per dish) were added. A well with a diameter of 8 mm was then punched carefully using a sterile cork borer and 100 µL test substance (1 mg/100 µL DMSO) was added to each labeled well. Amikacin (30 μ g/100 μ L H₂O) was used as a positive control, whereas 100 µL water and DMSO served as negative controls. The same procedure was repeated for different microorganisms. After the inoculation of the organisms, compounds and controls, the plates were incubated for 24 h at 37 °C. Zones of inhibition were recorded in millimeters.

The fungi tested were: *Candida albicans* (ATCC 10231), *Saccharomyces cerevisiae* (ATCC 9763) and *Aspergillus brasiliensis* (ATCC 16404). Sabouraud dextrose agar (Torlak, Belgrade, Serbia) was prepared according to the manufacturer's instructions. Into each sterile Petri dish (90 mm diameter), 22 mL previously prepared agar suspension was poured and 100 μ L fungi (10⁵ spores per dish) was added. A well with a diameter of 8 mm was punched using a sterile cork borer. Into each well 100 μ L test substance (1 mg/100 μ L DMSO) was added. Nystatin (30 μ g/100 μ L DMSO) was used as a positive control, whereas 100 μ L DMSO served as a negative control. The plates were incubated for 48 h at 24 °C. Antifungal activity was determined by measuring the diameter of the inhibition zone.

Conclusions

In this study, a series of new steroidal mono- and bis(thiosemicarbazones) **2a–e** and **3a–e** were prepared and further subjected to ring closure by means of acetylating agents to afford the corresponding 1,3,4-thiadiazolines **4a–e** and **5a–e** in good yields.

All compounds were biologically evaluated. The results of our *in vitro* study may indicate the significant anticancer potential of steroidal compounds 2a, 2b, 2c, 2e, 5a and 5d, owing to their strong antiproliferative and pro-apoptotic effects against malignant cell lines and the specificity to cancer cells of their activity. Among these novel steroid derivatives, the best anticancer properties were exhibited by compound 5a. The spiro heterocyclic substituent at the C-17 position, as well as the presence of an α , β -unsaturated thiosemicarbazone moiety at C-3, enhanced the activity of the tested compounds. Moreover, their low antimicrobial activities indicate the selective activity of these new substituted and heterocyclic steroid derivatives.

Further research is needed to investigate the molecular mechanisms of their anticancer activity, in addition to *in vivo* evaluation of their antitumor efficacy.

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