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FULL-LENGTH PAPER

Synthesis of novel steroidal 16-spiroisoxazolines by 1,3-dipolar cycloaddition, and an evaluation of their antiproliferative activities in vitro

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Abstract Efficient synthesis of novel 16-spiroisoxazolines in the androst-5-ene series was carried out by 1,3-dipolar cycloadditions of different aryl nitrile oxides to 3β-acetoxy-16-methylene-androst-5-en-17-one. During the intermolecular ring closures, the attack of the O terminus of the nitrile oxide dipole from the α side on C-16 predominated for steric reasons permitting the reactions to occur in a regio- and stereoselective manner. The minor isomers in which the angular methyl group on C-13 and the O atom of the isoxazoline heteroring were in the β, β-*cis* orientation were obtained in a yield of only ∼10 %. Moreover, the conversions were influenced to a certain extent by the substituents on the aromatic moiety of the 1,3-dipoles. The stereostructures of the related diastereomers were confirmed by 2D NMR methods. Deacetylation of the primarily formed main products resulted in the corresponding 3β-OH analogs, which were further reduced to furnish 3β, 17β-diols. All of the synthetized compounds were subjected to in vitro pharmacological studies in order to investigate their antiproliferative effects on three malignant human adherent cell lines (HeLa, MCF7, and A431).

Keywords Steroid · Nitrile oxide · Cycloaddition · 16-Spiroisoxazolines · Stereoselective synthesis · Antiproliferative activity

Introduction

Steroids, an important class of naturally occurring regulatory molecules, are well known for their wide range of biological activities and have gained extensive application in the treatment of different diseases and in the improvement of physical and growth performance. Chemical modifications of the steroid nucleus, either by the introduction of heterocyclic moieties or by the replacement of one or more carbon atoms by a heteroatom, thereby giving rise to marked changes in the original bioactivity, have received considerable attention in recent years [1–3]. Considerable synthetic efforts have been devoted to the search for more active compounds untinged by unwanted or toxic side effects and to the recognition of the stereostructural features required for specific receptor binding and therefore selective pharmacological action. Consequently, both structure-based drug design and a more random search for effective derivatives appear to be fruitful routes in the quest for novel steroid-based medicinal agents. The formation of heterocyclic building blocks on the sterane core may alter both the pharmacokinetic and the pharmacodynamic properties of the parent compound, leading to hydrolysis-resistant derivatives with longer half-lives and/or to a better fit to the corresponding target through additional interactions made possible by the presence of a hetero ring. Moreover, the hydrophobic steroid scaffold can facili-

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tate the transportation of the introduced heterocycle through biological membranes.

Among the members of the large steroid family, spirosteroids represent an important class of compounds that are relatively widely found in nature, such as spirostanes and spirosolanes, which include a spiroacetal or spiroaminoacetal moiety and display significant biological effects. The glycoalkaloids solasonine and tomatine, extracted from different plant species, have demonstrated to exert strong antiproliferative effects on various human cancer cell lines of diverse origins [4,5], while the synthetic spiro-type hybrid of estrone and talaromycin B has also been reported to exhibit cytotoxic activity [6,7]. In general, the spiro functionality, in which two rings are connected through merely one carbon atom, is a recurring structural motif in a number of natural products with noteworthy biological activities [8]. For example, coerulescine, horfiline, and elacomine exhibit antitumor effects, whereas rynchophylline and corynoxeine are used in traditional Chinese medicine for the treatment of neurological and cardiovascular diseases [9,10].

The most investigated semi-synthetic spirosteroids are those containing a spiro heteroring at C-17, but much less has been published regarding the synthesis of C-16 spiroheterocyclic compounds [11]. Modifications involving the extant 17-keto functional group or the nearby position of the steroid core with the introduction of a bulky heterocyclic moiety can alter the primary stereostructure of the molecule, which may lead to a change in the substrate-receptor interaction and also greatly affect the biological properties. For this purpose, a number of different heterocyclic systems have been incorporated into the sterane skeleton in a spiro-connected manner, particulary, oxazolidinones [12], pyrazolines [13], pyrrolidines [14], dioxaphosphorinanes [15,16], oxazaphospholes [17], and oxathiaphospholanes [18]. The chemistry of steroidal spiroisoxazolines, however, has not been well investigated, although several 3- and 17-spiro derivatives have been synthetized to date from the corresponding methylene derivatives [19].

The established role of 2-isoxazolines as valuable intermediates in organic synthesis is attributed to their capacity to mask other functionalities within a stable form that allows further substitution of the ring [20]. α,β-Unsaturated ketones [21], β-hydroxycarbonyl compounds [22], and 1,3-aminoalcohols [23] are the most important structural units available from 2-isoxazolines by reductive cleavage of the hetero ring. Although the isoxazoline building block appears to be a rare functionality both in secondary metabolites found in nature [20,24] and among marketed pharmaceutical agents [25], several synthetic derivatives have been reported to exhibit valuable biological activities [25,26]. Several methods have been devised to construct such bio-important compounds, where the 1,3-dipolar cycloaddition of nitrile oxides to an unsaturated substrate has been widely investigated [19]. Nitrile oxides are generally obtained in situ from their relatively stable hydroximidoyl chloride precursors by dehydrohalogenation with a base [27]. In the absence, and even in the presence of the dipolarophile, nitrile oxides often rearrange to form an isocyanate at higher temperature or tend to dimerize to produce furoxan at room temperature, depending on their structure, and these side reactions can reduce the yields of the desired cycloadducts [28]. The Huisgen-type concerted reaction often leads to a regioisomeric isoxazoline mixture and needs elevated temperature and/or a prolonged reaction time for sufficient conversion.

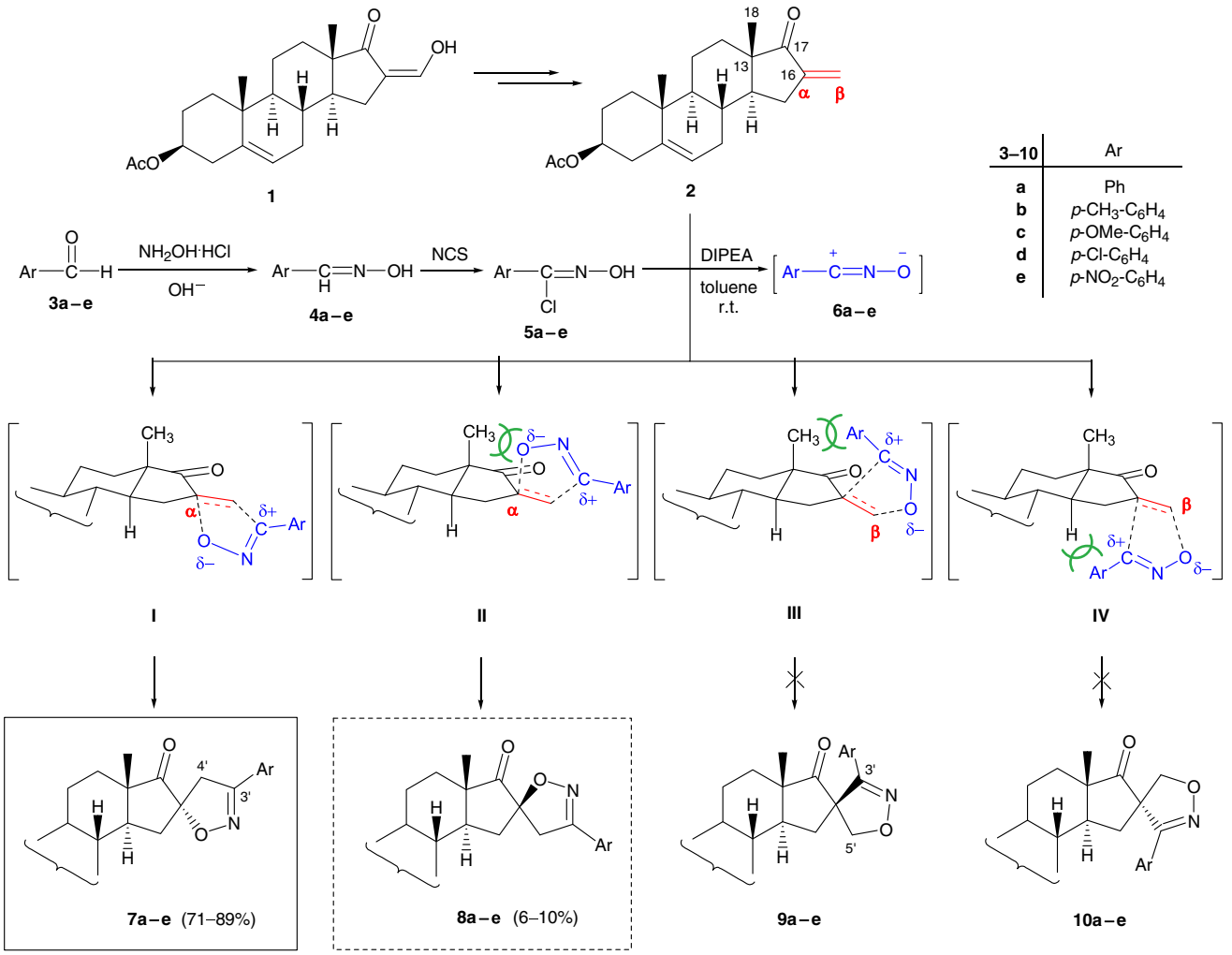
As a continuation of our work for the construction of heterocyclic steroids possessing cell-growth inhibitory effect [29–32], we decided to prepare novel 16-spiroisoxazolinyl androst-5-ene derivatives from an α,β-unsaturated steroidal 17-ketone via 1,3-dipolar cycloaddition. Our goal was to investigate the regio- and stereoselectivity of the process and the influence of steric and electronic factors on the ring-closure reactions. Determination of the stereostructures of the spiro compounds was also an aim of the present study. Moreover, all the synthetized derivatives were screened in vitro for their activities against a panel of three human adherent cancer cell lines (HeLa, MCF7, and A431).

Results and discussion

Chemistry

For the transformations, the starting material was 3β-acetoxy-16-methyleneandrost-5-en-17-one (**2**) [33], which is readily available from the 16-hydroxymethylene precursor **1** in the presence of an excess of formaldehyde and potassium carbonate via a formal mixed Cannizzaro reaction [34] and subsequent acetylation (Scheme 1). The presence of the exo-methylene group suggested the higher reactivity of the dipolarophile as compared to an endo-located double bond, and improved regioselectivity was expected in view of the monosubstituted character of the alkene moiety [27]. Moreover, conjugation with a C=O bond has been demonstrated to have a strong driving effect on the reactivity of such alkenes [35]. Aromatic hydroximidoyl chlorides (**5a–e**), as relatively stable precursors of nitrile oxide 1,3-dipoles (**6a–e**) [36], were synthetized in a two-step process by the condensation of benzaldehyde (**3a**) or its *p*-substituted derivatives (**3b–e**) with hydroxylamine hydrochloride in alkaline medium and subsequent chlorination of the aldoxime **4a–e** with *N*-chlorosuccinimide (NCS) [37]. Nitrile oxides (**6–e**) can be generated in situ from **5a–e** by dehydrochlorination with a base.

Preliminary ring-closure reactions on **2** with benzonitrile oxide **6a** were first carried out to find the optimal reaction conditions (Scheme 1). 16-Methylene-17-



Scheme 1 Regio- and stereoselective formation of steroidal 16-spiroisoxazolines

ketosteroid (**2**) and *N*-hydroxybenzenecarboximidoyl chloride (**5a**) were dissolved in toluene and 3 equivalents of *N*,*N*-diisopropylethylamine (DIPEA) were added. Since unhindered 1,3-dipoles easily tend to dimerize to furoxanes, which can reduce their quantity available for cycloaddition [38], DIPEA was finally added to the solutions in order to avoid the formation of these unwanted by-products. After stirring of the mixtures for 2 h at room temperature, complete conversion was indicated by TLC, and two products (**7a** and **8a**) were obtained, in yields of 75 and 10 %, respectively, after chromatographic purification. Although the rate of the transformation could be enhanced by refluxing the solution, and the reaction was then completed within 1 h, the application of milder conditions proved to be more favorable for further experiments in order to avoid the rearrangement of other dipoles to isocyanates at the elevated temperature. Similar intermolecular ring closures of **2** with different benzonitrile oxides (**6b–e**), obtained from the appropriate aryl aldehydes (**3b–e**) by the general protocol, were then carried out leading to novel 16-spiroisoxazolines (**7b–e** and **8b–d**) in good yields (Scheme 1). The formation of the possible *E* and *Z* isomers of both the aldoximes (**4a–e**) and their chlorinated analogs (**5a–e**) was detected by TLC; however, the unpurified hydroximidoyl chlorides (**5a–e**) were applied for the subsequent cycloaddition reactions.

In principle, the construction of four isoxazolines (**7–10**) can be conceived in the ring-closure reactions of **2** with aromatic nitrile oxides (**6a–e**), as depicted in Scheme 1. The orientation of the 1,3-dipole relative to the double bond of the dipolarophile can be of two kinds: the negatively charged O terminus may interact with either the α-or the β-carbon of the 16-methylene group of **2**, and the attack can occur from above (β side) or from underneath (α side) the general plane of the sterane molecule. Two regioisomeric pairs (**7**, **8** and **9**, **10**), each involving two diastereomers, may therefore exist as concerns the newly formed stereogenic center on C-16, though only **7** and **8** (at least in most cases) were effectively obtained during the cycloadditions. The formation of regioi-

Table 1 Cycloaddition products of **2** with different aromatic nitrile oxides (**6a–e**)

Entry	Ar—C(+)=N—C(−)	Ar	Products[a]	Overall yields (%)
1	**6a**	Ph	**7a** (75) + **8a** (10)	85
2	**6b**	*p*-CH_3-C_6H_4	**7b** (82) + **8b** (9)	91
3	**6c**	*p*-OMe-C_6H_4	**7c** (89) + **8c** (6)	95
4	**6d**	*p*-Cl-C_6H_4	**7d** (72) + **8d** (8)	80
5	**6e**	*p*-NO_2-C_6H_4	**7e** (71)	71

[a] Yields (%) after purification by column chromatography are given in parenthesis

somers (**9** and **10**) in which the O terminus is attached to the β-carbon of the dipolarophile is considered to be hampered by steric repulsions between the bulky aromatic ring of the nitrile oxide and the steroid portion. The attack of the anionic pole of the nitrile oxide from the β side is also unfavorable due to the C-18 angular methyl group with the same spatial orientation, although it does occur to a certain extent, leading to **8** as a minor by-product. With regard to the presumed transition states (**I–IV**), the most facilitated isomer is undoubtedly **7**, in which the C-O bond of the heteroring is located in the α position opposite the methyl group on C-13. Consequently, both the regio- and the stereoselectivity of the process are influenced by steric factors, in good agreement with earlier observations that the electronic character of the dipolarophile has only a minor effect on such reactions [39].

The overall yields of the epimeric products were affected by the electronic character of the substituents on the aryl moiety of the nitrile oxide **6b–e** (Table 1). The electron-donating CH_3 and OMe groups in **6b** and **6c** (Table 1, entries 2 and 3) facilitated the cycloaddition to **2** due to the lower propensity of these dipoles to dimerize to furoxanes, while the presence of the electron-withdrawing Cl and NO_2 substituents on the aromatic ring in **6d** and **6e** decreased the yields of the corresponding cycloadducts (**7d** and **8d,** or **7e**) (entries 4 and 5). The lowest conversion was found to occur for the reaction of **2** with *p*-nitrobenzonitrile oxide **6e**, which resulted in a single diastereomer (**7e**) in a yield of 71 %.

The ^{13}C NMR spectra recorded for **7a–e** and **8a–d** confirmed the regioselectivity of the process, as the quaternary carbon signal of C-16 appeared at around 89 ppm, revealing the presence of an O atom adjacent to this carbon. In the other regioisomeric pairs (**9** and **10**), C-16 is next to C-5′ of the isoxazolidine ring and its upfield shift would therefore be predicted. The stereostructures of the related epimers were established with the aid of homonuclear 2D NMR (COSY and NOESY) and heteronuclear 2D NMR (HSQC and HMBC) measurements. The two diastereotopic protons of the C-4′ methylene group appear as two doublets at 3.25 and 3.63 ppm for **7b** ($^2J_{H,C,H} = -16.5$ Hz), and at 3.13 and 3.66 ppm for **8b** ($^2J_{H,C,H} = -16.4$ Hz), (Schemes 2 and 3). The NOESY correlations revealed that the C-16 configuration is *S* in **7b** as both signals of the 4′-protons showed cross-peaks with the C-18 methyl protons, while one of the doublets correlated with 15β-H (Scheme 2). The NOESY experiment on cycloadduct **8b**, however, supported the spatial vicinity of 15α-H with one of the 4'-protons, showing a cross-peak between their signals, confirming the *R* configuration of C-16 in this case (Scheme 3).

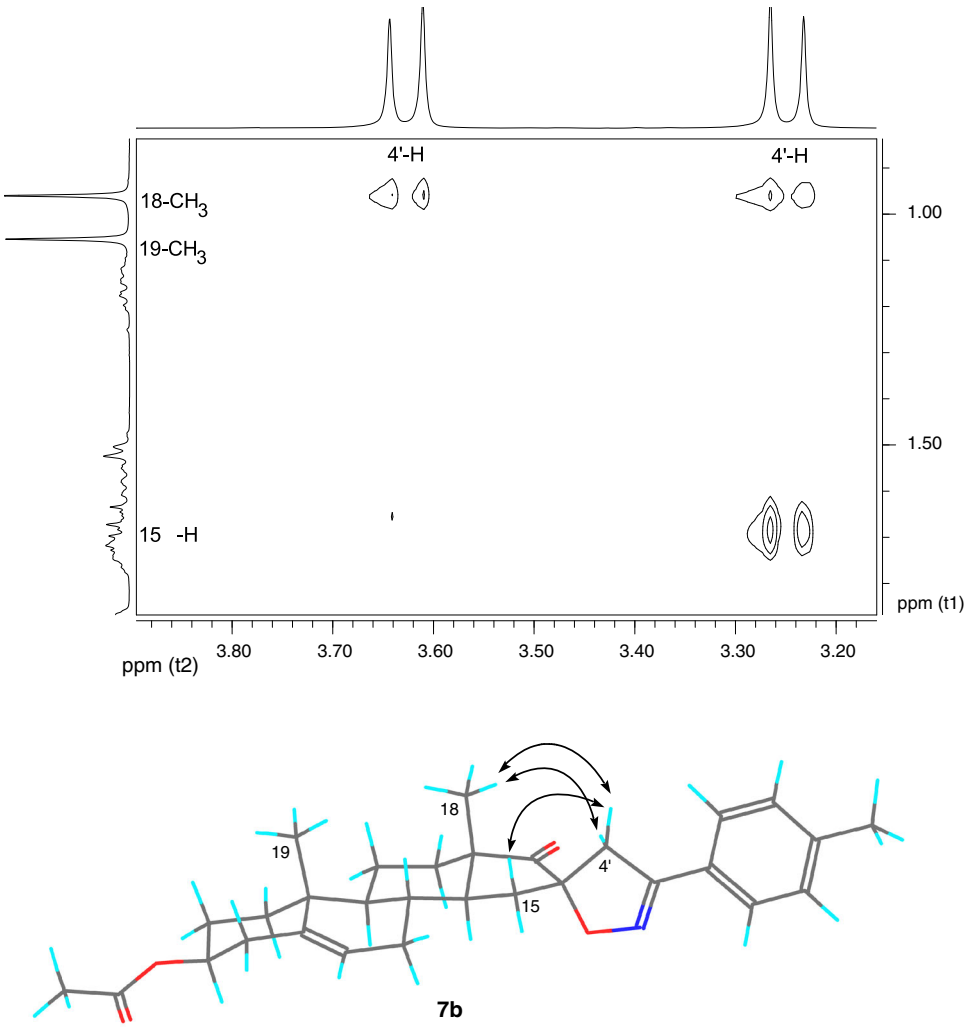
For the enlargement of the compound library suitable for pharmacological studies and in the hope of finding structure-activity relationships, further derivatives of the main products (**7a–e**) were synthetized by simple deacetylations to furnish the corresponding 3β-hydroxy analogs (**11a–e**). Furthermore, 3β,17β-diols (**12a–e**) were obtained by stereoselective reduction of **11a–e** (Scheme 4).

Pharmacology

Since a number of compounds of spiroisoxazoline type have been reported to exert noteworthy antiproliferative activities [40–42] and some steroidal derivatives containing similar heterocyclic moieties have also been demonstrated to inhibit cell proliferation [26,29,32], the newly synthetized isoxazolines (**7a–e**, **8a–d**, **11a–e**, **12a–e**) were subjected to in vitro pharmacological studies of their cytotoxic effects on three malignant human adherent cell lines, HeLa, MCF7, and A431 (Table 2). Their antiproliferative activities were determined by a microplate-based MTT colorimetric assay [43], in comparison with cisplatin as reference agent. The cell-proliferation inhibitory potencies, expressed as growth inhibition and/or IC_{50} values, revealed that several of the investigated compounds exhibited marked effects on cell proliferation, especially at 30 μM.

As concerns the structure-activity relationships, the configuration at C-16 of the newly synthetized molecules seems to be the structural feature that mainly determines the antiproliferative properties, since **7a–d** proved to be more potent than their epimeric counterparts **8a–d**. Substitution of the aromatic ring on the isoxazoline moiety tended to increase the antiproliferative capacity in **7b–d**, while the *p*-nitro group on the phenyl ring in **7e** did not have a great impact on the overall efficacy as compared with **7a**. Although the keto function at position 17 of the sterane skeleton is generally favorable, this part of the molecule and also the nature of the

Scheme 2 Partial NOESY spectrum and 3D representation of main product **7b**



substituent OAc or OH at C-3 did not have a crucial effect on the overall antiproliferative activities. The IC_{50} values of the two most potent agents, **7d** and **11d**, both containing a *p*-chloro-phenyl-substituted isoxazoline building block with an *S* configuration on C-16, were lower than or comparable to those of the reference agent cisplatin.

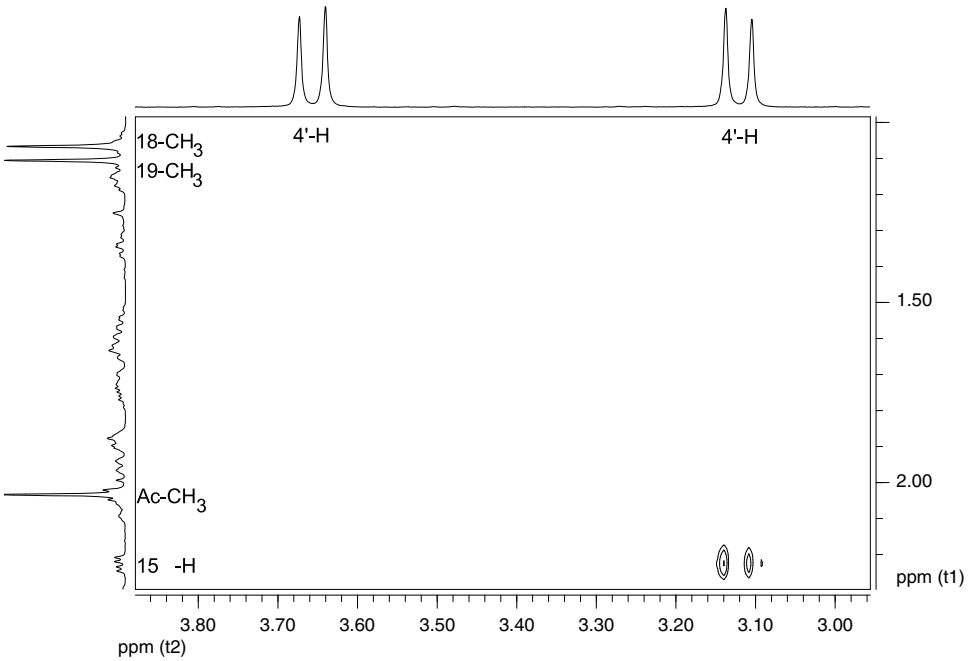
Conclusions

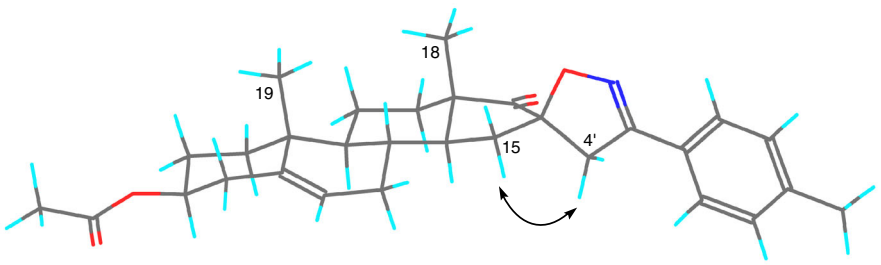
In summary, novel types of spiroisoxazolines in the Δ^5 androstene series were prepared from a 16-methylene-17-ketosteroid with different aromatic nitrile oxides via intermolecular 1,3-dipolar cycloaddition. The ring-closure reactions occurred under mild reaction conditions to afford the heterocyclic products regioselectively and stereoselectively in good to excellent yields. The conversions were observed to increase by the presence of electron-donating groups on the aromatic ring of the nitrile oxide in consequence of the lower tendency of these dipoles to undergo dimerization. The library of compounds was expanded by further deacetylation reactions and subsequent reductions. The cytotoxic efficacy of all compounds was investigated in vitro on three cancer cell lines, and several of the structurally related derivatives were found to have a marked effect on cell division. The pharmacological activities depended mainly on the stereochemistry and functionalization of the incorporated heterocycle rather than on the nature of the substituents on C-3 and/or C-17 of the sterane core. Although only two derivatives of the currently tested agents proved to be specifically potent, the results indicate that steroidal spiroisoxazolines may deserve further attention not only from a synthetic but also from a pharmacological point of view.

Experimental

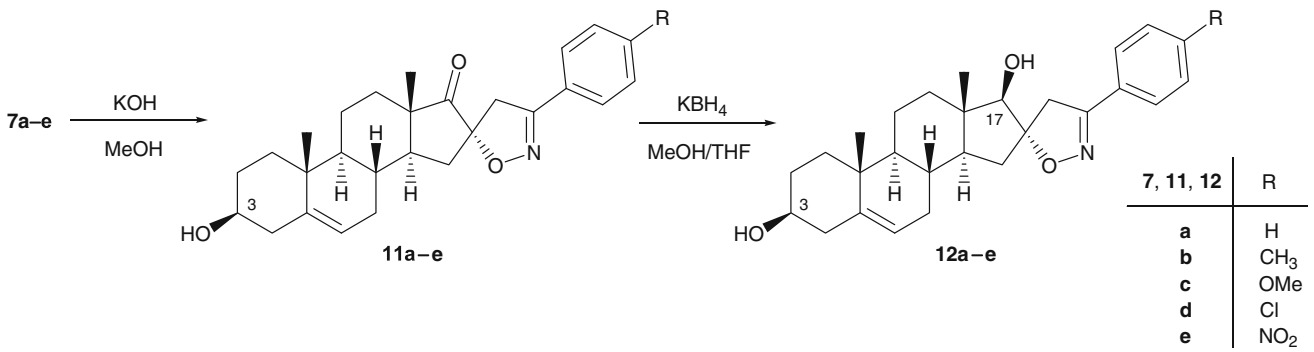
Melting points (mp) were determined on a SMS Optimelt digital apparatus. Elemental analysis data were obtained with a Perkin Elmer CHN analyzer model 2400. NMR spectra were

Scheme 3 Partial NOESY spectrum and 3D representation of by-product **8b**





8b



Scheme 4 Synthesis of 3β-hydroxy- and 3β,17β-dihydroxy-16-spiroisoxazolines

recorded at room temperature on a Bruker DRX 500 instrument. Chemical shifts are reported in ppm (δ scale) and coupling constants (J) in Hz. For the determination of multiplicities, the J-MOD pulse sequence was used. Automated flow injection analyses were performed by using an HPLC/MSD system. The system comprised an Agilent 1100 micro vacuum degasser, a quaternary pump, a micro-well plate autoinjector, and a 1946A MSD equipped with an electrospray ion source (ESI) operated in positive ionization mode. The ESI parameters were nebulizing gas N_2, at 35 psi; drying gas N_2, at 350 °C and 12 L/min; capillary voltage (V_{Cap}) 3,000 V; and fragmentor voltage 70 V. The MSD was operated in scan mode with the mass range m/z 60–620. Samples (0.2 μL) were injected with an automated needle wash directly into the solvent flow (0.3 mL/min) of MeCN/H_2O 70:30 (v/v) supplemented with 0.1 % formic acid. The system was

Table 2 Cytotoxic activities of spiroisoxazolines in the androstene series

Compd	Conc. (μM)	HeLa Inhibition % (± SEM)	IC_{50}^{a} (μM)	MCF7 Inhibition % (± SEM)	IC_{50} (μM)	A431 Inhibition % (± SEM)	IC_{50} (μM)
7a	10	59.7 (± 1.2)	–[b]	53.5 (± 1.1)	–	42.1 (± 1.1)	–
	30	81.5 (± 1.9)	–	68.1 (± 1.0)	–	48.0 (± 1.1)	–
7b	10	82.5 (± 0.6)	–	84.7 (± 0.5)	–	65.9 (± 0.7)	–
	30	94.3 (± 1.3)	–	94.0 (± 0.8)	–	83.3 (± 0.5)	–
7c	10	61.4 (± 1.2)	–	66.0 (± 1.1)	–	34.2 (± 2.2)	–
	30	86.7 (± 0.7)	–	74.3 (± 0.6)	–	58.2 (± 1.2)	–
7d	10	97.7 (± 0.2)	7.46	92.0 (± 1.0)	8.07	98.1 (± 0.5)	3.42
	30	98.5 (± 0.1)		95.6 (± 0.2)		98.1 (± 0.4)	
7e	10	55.4 (± 1.2)	–	56.1 (± 2.6)	–	34.2 (± 1.3)	–
	30	55.8 (± 2.7)	–	65.8 (± 1.4)	–	38.3 (± 1.9)	–
8a	10	<20[c]	-	<20	–	<20	–
	30	32.0 (± 1.1)	–	64.7 (± 1.8)	–	<20	–
8b	10	<20	–	26.6 (± 2.9)	–	<20	–
	30	59.8 (± 1.8)	–	49.9 (± 1.3)	–	<20	–
8c	10	<20	–	20.8 (± 2.0)	–	<20	–
	30	<20	–	31.8 (± 1.3)	–	<20	–
8d	10	33.5 (± 1.6)	–	23.0 (± 0.7)	–	<20	–
	30	72.4 (± 2.7)	–	63.8 (± 1.4)	–	30.5 (± 2.1)	–
11a	10	<20	–	35.2 (± 1.2)	–	<20	
	30	96.8 (± 0.3)	–	98.2 (± 0.3)	–	95.8 (± 0.4)	
11b	10	60.3 (± 0.6)	-	51.5 (± 1.7)	–	58.0 (± 1.2)	–
	30	82.5 (± 1.0)	–	88.5 (± 0.7)	–	76.1 (± 0.9)	–
11c	10	42.7 (± 1.3)	–	70.0 (± 1.4)	–	30.6 (± 2.8)	
	30	82.3 (± 1.8)	–	82.8 (± 1.4)	–	41.4 (± 0.7)	
11d	10	88.6 (± 2.7)	7.39	92.9 (± 0.3)	7.13	67.3 (± 2.9)	4.20
	30	98.3 (± 0.2)		93.6 (± 1.1)		98.2 (± 0.1)	
11e	10	<20	–	<20	–	<20	–
	30	33.5 (± 1.3)	–	30.1 (± 1.8)	–	20.6 (± 2.6)	–
12a	10	<20	–	<20	–	<20	–
	30	97.6 (± 0.1)	–	95.9 (± 1.4)	–	95.6 (± 0.3)	–
12b	10	36.4 (± 1.3)	–	<20	–	<20	–
	30	96.4 (± 0.4)	-	98.6 (± 0.1)	–	96.7 (± 0.5)	–
12c	10	<20	–	26.4 (± 2.0)	–	<20	–
	30	50.9 (± 0.7)	–	74.1 (± 1.1)	–	45.2 (± 2.4)	–
12d	10	50.7 (± 0.7)	–	32.4 (± 2.8)	–	44.1 (± 0.8)	–
	30	57.7 (± 1.1)	–	32.6 (± 1.4)	–	46.7 (± 0.6)	–
12e	10	58.8 (± 0.8)	–	51.6 (± 1.6)	–	61.4 (± 1.7)	–
	30	73.7 (± 1.9)	–	73.8 (± 1.2)	–	86.5 (± 0.8)	–
CP[d]	10	42.6 (± 2.3)	12.43	53.0 (± 2.3)	9.63	88.6 (± 0.5)	2.84
	30	99.9 (± 0.3)		86.9 (± 1.3)		90.2 (± 1.8)	

[a] IC_{50} values were determined when the tested compound elicited at least 50 % growth inhibition at 10 μM against any of the cell lines used. The presented values are from two independent determinations with five parallel wells; standard deviation <15 %

[b] Not determined

[c] Inhibition values <20 % are not presented

[d] Cisplatin (reference compound)

controlled by Agilent's LC/MSD Chemstation software. All solvents were distilled immediately prior to use. Reagents and materials were obtained from commercial suppliers and were used without purification. The reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick); solvent systems (ss): (A) EtOAc/CH_2Cl_2 (2:98 v/v), (B) EtOAc/CH_2Cl_2 (10:90 v/v), (C) EtOAc/CH_2Cl_2 (25:75 v/v). The spots were developed by spraying with 5 % phosphomolybdic acid in 50 % aqueous phosphoric acid. The R_f values were determined for the spots observed by illumination at 254 and 365 nm. Flash chromatography: Merck silica gel 60, 40–63 μm.

General procedure for the synthesis of 16-spiroisoxazolyl derivatives (**7** and **8**) in the Δ^5 androstene series

3β-Acetoxy-16-methyleneandrost-5-en-17-one (**2**) (343 mg, 1.00 mmol) and the appropriate aromatic imidoyl chloride [36] (**5a–e**, 1.50 mmol) were dissolved in toluene (15 mL), and DIPEA (0.52 mL, 3.00 mmol) was added dropwise to the reaction mixture, which was subsequently stirred at room temperature for 2 h. The solvent was then evaporated off in vacuo and the resulting crude products were separated by column chromatography.

*Synthesis of 3β-acetoxy-3′-phenyl-spiro [androst-5-ene-16,5′-2′-isoxazolin]-17-one epimers (***7a** *and* **8a***)*

According to Sect. 4.1, *N*-hydroxybenzenecarboximidoyl chloride (**5a**, 233 mg) was used. After purification with CH_2Cl_2 as eluent, **7a** (346 mg, 75 %) and **8a** (46 mg, 10 %) were obtained as white solids (sequence of elution: **8a > 7a**).

7a: mp 245–248 °C, R_f = 0.38 (ss A); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.97 (s, 3H, 18-H_3), 1.06 (s, 3H, 19-H_3), 1.14 (m, 2H), 1.48–1.75 (overlapping m, 8H), 1.86–1.96 (overlapping m, 3H), 2.03 (s, 3H, Ac-CH_3), 2.04 (m, 1H), 2.35 (m, 3H), 3.27 (d, 1H, J = 16.6 Hz, one of 4′-H), 3.65 (d, 1H, J= 16.6 Hz, the other 4′-H), 4.60 (m, 1H, 3-H), 5.40 (m, 1H, 6-H), 7.38 (m, 3H, 3″-H, 4″-H and 5″-H), 7.63 (m, 2H, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 14.3(C-18), 19.3 (C-19), 20.2 (CH_2), 21.4 (Ac-CH_3), 27.6 (CH_2), 30.7 (CH_2), 31.0 (CH), 31.6 (CH_2), 36.7 (C-10), 36.8 (CH_2), 37.5 (CH_2), 38.0 (CH_2), 44.0 (CH_2), 47.1 (C-13), 48.5 (CH), 49.9 (CH), 73.6 (C-3), 88.7 (C-16), 121.7 (C-6), 126.8 (2C, C-3″ and C-5″), 128.7 (2C, C-2″ and C-6″), 129.0 (C-1″), 130.2 (C-4″), 139.9 (C-5), 155.3 (C-3″), 170.4 (Ac-CO), 216.2 (C-17); ESI-MS 485 $[M+Na]^+$; Anal. Calcd. for $C_{29}H_{35}NO_4$ C 75.46; H 7.64. Found C 75.62; H 7.80.

8a: mp 187–190 °C, R_f = 0.44 (ss A); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 1.05 (m, 1H), 1.07 (s, 3H, 19-H_3), 1.11 (s, 3H, 18-H_3), 1.15 (m, 2H), 1.34 (m, 1H), 1.52–1.66 (m, 3H), 1.70–1.80 (m, 2H), 1.88–1.98 (m, 3H), 2.03 (s, 3H, Ac-CH_3), 2.04 (m, 1H), 2.08 (m, 1H), 2.24 (dd, 1H, J = 13.1 Hz, J = 5.8 Hz), 2.35 (m, 2H), 3.14 (d, 1H, J= 16.4 Hz, one of 4′-H), 3.69 (d, 1H, J = 16.4 Hz, the other 4′-H), 4.61 (m, 1H, 3-H), 5.39 (m, 1H, 6-H), 7.39 (m, 3H, 3″-H, 4″-H and 5″-H), 7.64 (m, 2H, 2″-H and 6″-H); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ_C 14.3(C-18), 19.3 (C-19), 20.1 (CH_2), 21.4 (Ac-CH_3), 27.6 (CH_2), 30.8 (CH_2), 30.9 (CH), 31.9 (CH_2), 36.8 (C-10), 36.9 (CH_2), 38.0 (2C, 2 × CH_2), 45.4 (CH_2), 46.4 (CH), 46.8 (C-13), 50.2 (CH), 73.6 (C-3), 88.6 (C-16), 121.4 (C-6), 126.8 (2C, C-3″ and C-5″), 128.7 (2C, C-2″ and C-6″), 129.0 (C-1″), 130.2 (C-4″), 140.0 (C-5), 155.0 (C-3′), 170.5 (Ac-CO), 215.4 (C-17); ESI-MS 485 $[M+Na]^+$; Anal. Calcd. for $C_{29}H_{35}NO_4$ C 75.46; H 7.64. Found C 75.60; H 7.78.

*Synthesis of 3β-acetoxy-3′-4″-tolyl-spiro[androst-5-ene-16,5′-2′-isoxazolin]-17-one epimers (***7b** *and* **8b***)*

According to Sect. 4.1, *N*-hydroxy-4-methylbenzenecarboximidoyl chloride (**5b**, 254 mg) was used. After purification with EtOAc/CH_2Cl_2 = 2:98 as eluent, **7b** (390 mg, 82 %) and **8b** (43 mg, 9 %) were obtained as white solids (sequence of elution: **8b > 7b**).

7b: mp 240–242 °C, R_f = 0.32 (ss A); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.96 (s, 3H, 18-H_3), 1.05 (s, 3H, 19-H_3), 1.50 (m, 2H), 1.48–1.77 (overlapping m, 8H), 1.88 (m, 2H), 1.95 (m, 1H), 2.03 (s, 3H, Ac-CH_3), 2.05 (m, 1H), 2.34 (m, 3H), 2.37 (s, 3H, 4″-CH_3), 3.25 (d, 1H, J = 16.5 Hz, one of 4′-H), 3.63 (d, 1H, J= 16.5 Hz, the other 4′-H), 4.61 (m, 1H, 3-H), 5.40 (m, 1H, 6-H), 7.19 (d, 2H, J = 8.0 Hz, 3″-H and 5″-H), 7.53 (d, 2H, J = 8.0 Hz, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 14.3 (C-18), 19.3 (C-19), 20.2 (CH_2), 21.4 (2C, 4″-CH_3 and Ac-CH_3), 27.6 (CH_2), 30.6 (CH_2), 31.0 (CH), 31.6 (CH_2), 36.7 (C-10), 36.8 (CH_2), 37.6 (CH_2), 38.0 (CH_2), 44.2 (CH_2), 47.1 (C-13), 48.5 (CH), 49.9 (CH), 73.6 (C-3), 88.5 (C-16), 121.7 (C-6), 126.2 (C-1″), 126.8 (2C, C-2″ and C-6″), 129.4 (2C, C-3″ and C-5″), 139.8 (C-4″), 140.4 (C-5), 155.3 (C-3′), 170.4 (Ac-CO), 216.4 (C-17); ESI-MS 477 $[M+H]^+$; Anal. Calcd. for $C_{30}H_{37}NO_4$ C 75.76; H 7.84. Found C 75.92; H 8.00.

8b: mp 193–195 °C, R_f = 0.51 (ss A); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 1.05 (m, 1H), 1.06 (s, 3H, 19-H_3), 1.11 (s, 3H, 18-H_3), 1.15 (m, 2H), 1.34 (m, 1H), 1.51–1.66 (m, 3H), 1.70–1.79 (m, 2H), 1.87-1.99 (m, 3H), 2.02 (m, 1H), 2.03 (s, 3H, Ac-CH_3), 2.08 (m, 1H), 2.23 (dd, 1H, J = 13.1 Hz, J = 5.7 Hz), 2.35 (m, 2H), 2.37 (s, 3H, 4″-CH_3), 3.13 (d, 1H, J= 16.4 Hz, one of 4′-H), 3.66 (d, 1H, J = 16.4 Hz, the other 4′-H), 4.61 (m, 1H, 3-H), 5.39 (m, 1H, 6-H), 7.19 (d, 2H, J = 8.0 Hz, 3″-H and 5″-H), 7.53 (d, 2H, J = 8.0 Hz, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 130.0 (C-18), 19.3 (C-19), 20.1 (CH_2), 21.4 (2C, 4″-CH_3 and Ac-CH_3), 27.6 (CH_2), 30.8 (CH_2), 30.9 (CH), 31.8 (CH_2), 36.8 (C-10), 36.9 (CH_2), 38.0 (2C, 2 × CH_2), 45.6 (CH_2), 46.4

(CH), 46.8 (C-13), 50.2 (CH), 73.6 (C-3), 88.5 (C-16), 121.4 (C-6), 126.1 (C-1″), 126.8 (2C, C-2″ and C-6″), 129.4 (2C, C-3″ and C-5″), 140.0 (C-4″), 140.5 (C-5), 154.9 (C-3′), 170.5 (Ac-CO), 215.5 (C-17); ESI-MS 477 $[M+H]^+$; Anal. Calcd. for $C_{30}H_{37}NO_4$ C 75.76; H 7.84. Found C 75.62; H 7.94.

*Synthesis of 3β-acetoxy-3′-4″-methoxyphenyl-spiro [androst-5-ene-16,5′-2′-isoxazolin]-17-one epimers (***7c** *and* **8c***)*

According to Sect. 4.1, *N*-hydroxy-4-methoxybenzenecarboximidoyl chloride (**5c**, 279 mg) was used. After purification with CH_2Cl_2 as eluent, **7c** (438 mg, 89 %) and **8c** (29 mg, 6 %) were obtained as white solids (sequence of elution: **7c** > **8c**).

7c: mp 248–250 °C, R_f = 0.26 (ss A); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.97 (s, 3H, 18-H_3), 1.05 (s, 3H, 19-H_3), 1.15 (m, 2H), 1.47–1.76 (overlapping m, 8H), 1.88 (m, 2H), 1.94 (m, 1H), 2.03 (s, 3H, Ac-CH_3), 2.04 (m, 1H), 2.35 (m, 3H), 3.24 (d, 1H, *J* = 16.5 Hz, one of 4′-H), 3.62 (d, 1H, *J*= 16.5 Hz, the other 4′-H), 3.83 (s, 3H, 4″-OMe), 4.61 (m, 1H, 3-H), 5.40 (m, 1H, 6-H), 6.90 (d, 2H, *J* = 8.6 Hz, 3″-H and 5″-H), 7.58 (d, 2H, *J* = 8.6 Hz, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C14.3 (C-18), 19.3 (C-19), 20.1(CH_2), 21.4 (Ac-CH_3), 27.6 (CH_2), 30.6 (CH_2), 31.0 (CH), 31.6 (CH_2), 36.7 (C-10), 36.8 (CH_2), 37.5 (CH_2), 38.0 (CH_2), 44.2 (CH_2), 47.1 (C-13), 48.5 (CH), 49.9 (CH), 55.3 (4″-OMe), 73.6 (C-3), 88.4 (C-16), 114.1 (2C, C-3″ and C-5″), 121.5 (C-1″), 121.7 (C-6), 128.4 (2C, C-2″ and C-6″), 139.8 (C-5), 154.9 (C-3′), 161.1 (C-4″), 170.4 (Ac-CO), 216.4 (C-17); ESI-MS 515 $[M+Na]^+$; Anal. Calcd. for $C_{30}H_{37}NO_5$ C 73.29; H 7.59. Found C 73.42; H 7.74.

8c: mp 197–200 °C, R_f = 0.34 (ss A); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.88 (m, 1H), 1.07 (m, 3H, 18-H_3), 1.10 (s, 3H, 19-H_3), 1.15 (m, 2H), 1.34 (m, 1H), 1.52–1.65 (m, 3H), 1.70–1.81 (m, 2H), 1.88–1.99 (m, 3H), 2.03 (s, 3H, Ac-CH_3), 2.04 (m, 1H), 2.09 (m, 1H), 2.23 (dd, 1H, *J* = 13.0 Hz, *J* = 5.6 Hz), 2.34 (m, 2H), 3.11 (d, 1H, *J*= 16.3 Hz, one of 4′-H), 3.65 (d, 1H, *J* = 16.3 Hz, the other 4′-H), 3.83 (s, 3H, 4″-OMe), 4.61 (m, 1H, 3-H), 5.39 (m, 1H, 6-H), 6.90 (d, 2H, *J* = 8.4 Hz, 3″-H and 5″-H), 7.58 (d, 2H, *J* = 8.4 Hz, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C13.0 (C-18), 19.3 (C-19), 20.1 (CH_2), 21.4 (Ac-CH_3), 27.6 (CH_2), 29.7 (CH_2), 30.8 (CH_2), 30.9 (CH), 31.9 (CH_2), 36.8 (C-10), 36.9 (CH_2), 38.0 (CH_2), 45.7 (CH_2), 46.5 (CH), 46.8 (C-13), 50.2 (CH), 55.3 (4″-OMe), 73.6 (C-3), 88.4 (C-16), 114.1 (2C, C-3″ and C-5″), 121.4 (C-6), 121.5 (C-1″), 128.4 (2C, C-2″ and C-6″), 140.0 (C-5), 154.5 (C-3′), 161.1 (C-4″), 170.5 (Ac-CO), 215.6 (C-17); ESI-MS 515 $[M+Na]^+$; Anal. Calcd. for $C_{30}H_{37}NO_5$ C 73.29; H 7.59. Found C 73.39; H 7.78.

*Synthesis of 3β-acetoxy-3′-4″-chlorophenyl-spiro[androst-5-ene-16,5′-2′-isoxazolin]-17-one epimers (***7d** *and* **8d***)*

According to Sect. 4.1, *N*-hydroxy-4-chlorobenzenecarboximidoyl chloride (**5d**, 285 mg) was used. After purification with CH_2Cl_2 as eluent, **7d** (357 mg, 72 %) and **8d** (40 mg, 8 %) were obtained as white solids (sequence of elution: **8d** > **7d**).

7d: mp 226–228 °C, R_f = 0.40 (ss A); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.96 (s, 3H, 18-H_3), 1.05 (s, 3H, 19-H_3), 1.14 (m, 2H), 1.47–1.76 (overlapping m, 8H), 1.88 (m, 2H), 1.95 (m, 1H), 2.03 (s, 3H, Ac-CH_3), 2.04 (m, 1H), 2.36 (m, 3H), 3.24 (d, 1H, *J* = 16.6 Hz, one of 4′-H), 3.62 (d, 1H, *J*= 16.6 Hz, the other 4′-H), 4.61 (m, 1H, 3-H), 5.41 (m, 1H, 6-H), 7.37 (d, 2H, *J* = 8.6 Hz, 3″-H and 5″-H), 7.58 (d, 2H, *J* = 8.6 Hz, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 14.3 (C-18), 19.3 (C-19), 20.1 (CH_2), 21.4 (Ac-CH_3), 27.6 (CH_2), 30.6 (CH_2), 31.0 (CH), 31.6 (CH_2), 36.7 (C-10), 36.8 (CH_2), 37.5 (CH_2), 38.0 (CH_2), 43.7 (CH_2), 47.1 (C-13), 48.5 (CH), 49.9 (CH), 73.6 (C-3), 88.9 (C-16), 121.6 (C-6), 127.5 (C-1″), 128.0 (2C, C-3″ and C-5″), 129.0 (2C, C-2″ and C-6″), 136.2 (C-4″), 139.9 (C-5), 154.4 (C-3′), 170.4 (Ac-CO), 216.0 (C-17); ESI-MS 519 $[M+Na]^+$; Anal. Calcd. for $C_{29}H_{34}ClNO_4$ C 70.22; H 6.91. Found C 70.37; H 7.08.

8d: Decomposed above 190 °C, R_f = 0.54 (ss A); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 1.06 (m, 1H), 1.06 (s, 3H, 19-H_3), 1.10 (s, 3H, 18-H_3), 1.14 (m, 2H), 1.33 (m, 1H), 1.52–1.65 (m, 3H), 1.70–1.79 (m, 2H), 1.87-1.97 (m, 3H), 2.03 (s, 3H, Ac-CH_3), 2.05 (m, 1H), 2.08 (m, 1H), 2.23 (dd, 1H, *J* = 12.9 Hz, *J* = 5.3 Hz), 2.34 (m, 2H), 3.10 (d, 1H, *J*= 16.4 Hz, one of 4′-H), 3.64 (d, 1H, *J* = 16.4 Hz, the other 4′-H), 4.60 (m, 1H, 3-H), 5.39 (m, 1H, 6-H), 7.37 (d, 2H, *J* = 8.0 Hz, 3″-H and 5″-H), 7.57 (d, 2H, *J* = 8.0 Hz, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C13.0 (C-18), 19.3 (C-19), 20.1 (CH_2), 21.4 (Ac-CH_3), 27.6 (CH_2), 30.8 (CH_2), 30.9 (CH), 31.8 (CH_2), 36.8 (C-10), 36.9 (CH_2), 37.9 (CH_2), 38.0 (CH_2), 45.2 (CH_2), 46.4 (CH), 46.8 (C-13), 50.2 (CH), 73.6 (C-3), 88.9 (C-16), 121.4 (C-6), 127.5 (C-1″), 128.1 (2C, C-3″ and C-5″), 129.0 (2C, C-2″ and C-6″), 136.2 (C-4″), 140.0 (C-5), 154.1 (C-3′), 170.5 (Ac-CO), 215.2 (C-17); ESI-MS 519 $[M+Na]^+$; Anal. Calcd. for $C_{29}H_{34}ClNO_4$ C 70.22; H 6.91. Found C 70.05; H 7.05.

*Synthesis of 3β-acetoxy-3′-4″-nitrophenyl-spiro[androst-5-ene-16,5′-2′-isoxazolin]-17-one (***7e***)*

According to Sect. 4.1, *N*-hydroxy-4-nitrobenzenecarboximidoyl chloride (**5e**, 300 mg) was used. After purification with EtOAc/CH_2Cl_2 = 1:99 as eluent, **7e** (360 mg, 71 %) was obtained as a pale-yellow solid.

7e: mp 233–235 °C, R_f = 38 (ss A); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.97 (s, 3H, 18-H_3), 1.06 (s, 3H, 19-H_3), 1.15 (m,

2H), 1.48-1.79 (overlapping m, 8H), 1.88 (m, 2H), 1.96 (m, 1H), 2.03 (s, 3H, Ac-CH_3), 2.04 (m, 1H), 2.35 (m, 3H), 3.30 (d, 1H, J = 16.6 Hz, one of 4′-H), 3.65 (d, 1H, J= 16.6 Hz, the other 4′-H), 4.61 (m, 1H, 3-H), 5.40 (m, 1H, 6-H), 7.80 (d, 2H, J = 8.6 Hz, 2″-H and 6″-H), 8.25 (d, 2H, J = 8.6 Hz, 3″-H and 5″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C14.4 (C-18), 19.3 (C-19), 20.1 (CH_2), 21.4 (Ac-CH_3), 27.6 (CH_2), 30.6 (CH_2), 31.0 (CH), 31.6 (CH_2), 36.7 (C-10), 36.8 (CH_2), 37.4 (CH_2), 38.0 (CH_2), 43.1 (CH_2), 47.1 (C-13), 48.6 (CH), 49.9 (CH), 73.6 (C-3), 89.7 (C-16), 121.5 (C-6), 124.0 (2C, C-3″ and C-5″), 127.5 (2C, C-2″ and C-6″), 135.1 (C-1″), 140.0 (C-5), 148.5 (C-4″), 153.8 (C-3′), 170.4 (Ac-CO), 215.5 (C-17); ESI-MS 530 $[M+Na]^+$; Anal. Calcd. for $C_{29}H_{34}N_2O_6$ C 68.76; H 6.76. Found C 68.89; H 6.92.

General procedure for the deacetylation of 16-spiroisoxazolyl derivatives (**7a–e**)

Compound **7a–e** (0.60 mmol) was dissolved in MeOH (10 mL), and KOH (50 mg, 0.89 mmol) was added. The solution was stirred at room temperature for 8 h, then diluted with water and neutralized with dilute HCl. The resulting precipitate was filtered, washed with water, and dried.

*Synthesis of 3β-hydroxy-3′-phenyl-spiro[androst-5-ene-16,5′S-2′-isoxazolin]-17-one (***11a***)*

Substrate: **7a** (277 mg). **11a** was obtained as a white solid (229 mg, 91 %), mp 223–226 °C, R_f = 0.37 (ss B); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.97 (s, 3H, 18-H_3), 1.04 (s, 3H, 19-H_3), 1.11 (m, 2H), 1.52 (m, 3H), 1.65–1.76 (overlapping m, 5H), 1.86 (m, 2H), 1.95 (m, 1H), 2.05 (m, 1H), 2.24 (m, 1H), 2.32 (m, 1H), 2.39 (m, 1H), 3.28 (d, 1H, J = 16.6 Hz, one of 4′-H), 3.54 (m, 1H, 3-H), 3.65 (d, 1H, J = 16.6 Hz, the other 4′-H), 5.37 (m, 1H, 6-H), 7.40 (m, 3H, 3″-H, 4″-H and 5″-H), 7.64 (m, 2H, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C14.3 (C-18), 19.4 (C-19), 20.2 (CH_2), 30.7 CH_2), 31.0 (CH), 31.5 (CH_2), 31.6 (CH_2), 36.6 (C-10), 37.0 (CH_2), 37.5 (CH_2), 42.1 (CH_2), 44.0 (CH_2), 47.1 (C-13), 48.6 (CH), 50.0 (CH), 71.5 (C-3), 88.7 (C-16), 120.7 (C-6), 126.8 (2C, C-3″ and C-5″), 128.7 (2C, C-2″ and C-6″), 129.0 (C-1″), 130.2 (C-4″), 141.0 (C-5), 155.4 (C-3′), 216.3 (C-17); ESI-MS 443 $[M+Na]^+$; Anal. Calcd. for $C_{27}H_{33}NO_3$ C 77.29; H 7.93. Found C 77.43; H 8.15.

*Synthesis of 3β-hydroxy-3′-4″-tolyl-spiro[androst-5-ene-16,5′S-2′-isoxazolin]-17-one (***11b***)*

Substrate: **7b** (285 mg). **11b** was obtained as a white solid (239 mg, 92 %), mp 270–272 °C, R_f = 0.37 (ss B); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.96 (s, 3H, 18-H_3), 1.04 (s, 3H, 19-H_3), 1.10 (m, 2H), 1.51 (m, 3H), 1.65-1.77 (overlapping m, 5H), 1.86 (m, 2H), 1.94 (m, 1H), 2.03 (m, 1H), 2.24 (m, 1H), 2.36 (m, 2H), 2.37 (s, 3H, 4″-CH_3), 3.26 (d, 1H, J = 16.5 Hz, one of 4′-H), 3.54 (m, 1H, 3-H), 3.63 (d, 1H, J = 16.5 Hz, the other 4′-H), 5.37 (m, 1H, 6-H), 7.19 (d, 2H, J = 8.0 Hz, 3″-H and 5″-H), 7.53 (d, 2H, J = 8.0 Hz, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C = 14.3 (C-18), 19.4 (C-19), 20.2 (CH_2), 21.4 (4″-CH_3), 30.7 (CH_2), 31.0 (CH), 31.5 (CH_2), 31.6 (CH_2), 36.6 (C-10), 37.1 (CH_2), 37.6 (CH_2), 42.1 (CH_2), 44.2 (CH_2), 47.1 (C-13), 48.6 (CH), 50.0 (CH), 71.5 (C-3), 88.5 (C-16), 120.8 (C-6), 126.1 (C-1″), 126.8 (2C, C-2″ and C-6″), 129.4 (2C, C-3″ and C-5″), 140.5 (C-4″), 140.9 (C-5), 155.4 (C-3′), 216.4 (C-17); ESI-MS 435 $[M+H]^+$; Anal. Calcd. for $C_{28}H_{35}NO_3$ C 77.56; H 8.14. Found C 77.42; H 8.20.

*Synthesis of 3β-hydroxy-3′-4″-methoxyphenyl-spiro[androst-5-ene-16,5′S-2′-isoxazolin]-17-one (***11c***)*

Substrate: **7c** (295 mg). **11c** was obtained as a white solid (245 mg, 91 %), mp 254–255 °C, R_f = 0.29 (ss B); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.96 (s, 3H, 18-H_3), 1.04 (s, 3H, 19-H_3), 1.10 (m, 2H), 1.52 (m, 3H), 1.65–1.72 (overlapping m, 5H), 1.86 (m, 2H), 1.94 (m, 1H), 2.03 (m, 1H), 2.24 (m, 1H), 2.32 (m, 1H), 2.38 (m, 1H), 3.25 (d, 1H, J = 16.5 Hz, one of 4′-H), 3.54 (m, 1H, 3-H), 3.62 (d, 1H, J = 16.5 Hz, the other 4′-H), 3.83 (s, 3H, 4″-OMe), 5.37 (m, 1H, 6-H), 6.90 (d, 2H, J = 8.5 Hz, 3″-H and 5″-H), 7.58 (d, 2H, J = 8.5 Hz, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 14.3 (C-18), 19.4 (C-19), 20.2 (CH_2), 30.7 (CH_2), 31.0 (CH), 31.5 (CH_2), 31.6 (CH_2), 36.6 (C-10), 37.0 (CH_2), 37.6 (CH_2), 42.1 (CH_2), 44.3 (CH_2), 47.1 (C-13), 48.6 (CH), 50.0 (CH), 55.3 (4″-OMe), 71.5 (C-3), 88.4 (C-16), 114.1 (2C, C-3″ and C-5″), 120.8 (C-6), 121.5 (C-1″), 128.4 (2C, C-2″ and C-6″), 140.9 (C-5), 155.0 (C-3′), 161.1 (C-4″), 216.5 (C-17); ESI-MS 473 $[M+Na]^+$; Anal. Calcd. for $C_{28}H_{35}NO_4$ C 74.80; H 7.85. Found C 74.96; H 8.02.

*Synthesis of 3β-hydroxy-3′-4″-chlorophenyl-spiro [androst-5-ene-16,5′S-2′-isoxazolin]-17-one (***11d***)*

Substrate: **7d** (298 mg). **11d** was obtained as a white solid (259 mg, 95 %), mp 256–259 °C, R_f = 0.38 (ss B); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.95 (s, 3H, 18-H_3), 1.04 (s, 3H, 19-H_3), 1.12 (m, 2H), 1.47-1.78 (overlapping m, 8H), 1.87 (m, 2H), 1.96 (m, 1H), 2.05 (m, 1H), 2.24 (m, 1H), 2.34 (m, 1H), 2.41 (m, 1H), 3.25 (d, 1H, J = 16.6 Hz, one of 4′-H), 3.54 (m, 1H, 3-H), 3.61 (d, 1H, J= 16.6 Hz, the other 4′-H), 5.36 (m, 1H, 6-H), 7.36 (d, 2H, J = 8.6 Hz, 3″-H and 5″-H), 7.57 (d, 2H, J = 8.6 Hz, 2″-H and 6″-H); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ_C = 14.3 (C-18), 19.4 (C-19), 20.2 (CH_2), 30.6 (CH_2), 31.0 (CH), 31.5 (CH_2), 31.6 (CH_2), 36.6 (C-10), 37.0 (CH_2), 37.5 (CH_2), 42.1 (CH_2), 43.8 (CH_2), 47.1 (C-13), 48.6 (CH), 50.0 (CH), 71.4 (C-3), 89.0 (C-16), 120.7 (C-6), 127.5 (C-1″), 128.0 (2C, C-3″ and C-5″), 129.0 (2C, C-2″

and C-6″), 136.2 (C-4″), 141.0 (C-5), 154.5 (C-3′), 216.1 (C-17); ESI-MS 477 $[M+H]^+$; Anal. Calcd. for $C_{27}H_{32}ClNO_3$ C 71.43; H 7.10. Found C 71.52; H 7.29.

*Synthesis of 3β-hydroxy-3′-4″-nitrophenyl-spiro[androst-5-ene-16,5′S-2′-isoxazolin]-17-one(***11e***)*

Substrate: **7e** (304 mg). **11e** was obtained as a yellow solid (262 mg, 94 %), mp 265–268 °C, R_f = 0.37 (ss B); ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H 0.98 (s, 3H, 18-H_3), 1.05 (s, 3H, 19-H_3), 1.12 (m, 2H), 1.48-1.79 (overlapping m, 8H), 1.87 (m, 2H), 1.97 (m, 1H), 2.05 (m, 1H), 2.24 (m, 1H), 2.33 (m, 1H), 2.42 (m, 1H), 3.30 (d, 1H, J = 16.6 Hz, one of 4′-H), 3.54 (m, 1H, 3-H), 3.66 (d, 1H, J= 16.6 Hz, the other 4′-H), 5.40 (m, 1H, 6-H), 7.81 (d, 2H, J = 8.7 Hz, 2″-H és 6″-H), 8.25 (d, 2H, J = 8.6 Hz, 3″-H és 5″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ_C 14.0 (C-18), 19.0 (C-19), 19.8 (CH_2), 30.3 (CH_2), 30.6 (CH), 31.1 (CH_2), 31.2 (CH_2), 36.3 (C-10), 36.7 (CH_2), 37.0 (CH_2), 41.7 (CH_2), 42.8 (CH_2), 46.8 (C-13), 48.2 (CH), 49.6 (CH), 71.1 (C-3), 89.4 (C-16), 120.2 (C-6), 123.6 (2C, C-3″ and C-5″), 127.2 (2C, C-2″ and C-6″), 134.7 (C-1″), 140.6 (C-5), 148.1 (C-4″), 153.5 (C-3′), 215.2 (C-17); ESI-MS 488 $[M+Na]^+$; Anal. Calcd. for $C_{27}H_{32}N_2O_5$ C 69.81; H 6.94. Found C 69.96; H 7.08.

General procedure for the reduction of 16-spiroisoxazolyl derivatives (**11a–e**)

Compound **11a–e** (0.45 mmol) was dissolved in a mixture of MeOH (10 mL) and THF (10 mL), and KBH_4 (50 mg, 0.93 mmol) was added. The solution was stirred at room temperature for 1 h, then diluted with water and neutralized with dilute HCl. The resulting precipitate was filtered, washed with water, and purified by flash chromatography with EtOAc/CH_2Cl_2 = 20:80 as eluent.

*Synthesis of 3β-hydroxy-3′-phenyl-spiro[androst-5-ene-16,5′S-2′-isoxazolin]-17β-ol (***12a***)*

Substrate: **11a** (189 mg). **12a** was obtained as a white solid (186 mg, 98 %), mp 233–236 °C, R_f = 0.44 (ss C); ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 0.67 (s, 3H, 18-H_3), 0.95 (s, 3H, 19-H_3), 0.99 (m, 2H), 1.18 (m, 2H), 1.32–1.42 (overlapping m, 3H), 1.48–1.56 (overlapping m, 3H), 1.69 (m, 1H), 1.76 (m, 2H), 1.88 (m, 1H), 2.00 (m, 1H), 2.06-2.17 (overlapping m, 2H), 3.00 (d, 1H, J = 17.1 Hz, one of 4′-H), 3.27 (m, 1H, 3-H), 3.62 (d, 1H, J = 4.9 Hz, 17-H), 3.78 (d, 1H, J = 17.1 Hz, the other 4′-H), 4.63 (d, 1H, J = 4.3 Hz, 3-OH), 5.26 (m, 1H, 6-H), 5.33 (d, 1H, J = 4.9 Hz, 17-OH), 7.42 (m, 3H, 3″-H, 4″-H and 5″-H), 7.66 (m, 2H, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 11.4 (C-18), 19.1 (C-19), 20.0 (CH_2), 30.8 (CH), 30.9 (CH_2), 31.3 (CH_2), 36.2 (CH_2), 36.3 (C-10), 36.8 (CH_2), 41.1 (CH_2), 41.6 (C-13), 42.1 (CH_2), 42.6 (CH_2), 47.7 (CH), 49.7 (CH), 69.9 (C-3), 85.1 (C-17), 94.9 (C-16), 120.7 (C-6), 126.2 (2C, C-3″ and C-5″), 128.7 (2C, C-2″ and C-6″), 129.6 (C-4″), 129.9 (C-1″), 141.3 (C-5), 156.4 (C-3′); ESI-MS 445 $[M+Na]^+$; Anal. Calcd. for $C_{27}H_{35}NO_3$ C 76.92; H 8.37. Found C 77.06; H 8.55.

*Synthesis of 3β-hydroxy-3′-4″-tolyl-spiro [androst-5-ene-16,5′S-2′-isoxazolin]-17β-ol (***12b***)*

Substrate: **11b** (195 mg). **12b** was obtained as a white solid (186 mg, 95 %), mp 227–230 °C, R_f = 0.44 (ss C); ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 0.66 (s, 3H, 18-H_3), 0.95 (s, 3H, 19-H_3), 0.99 (m, 2H), 1.17 (m, 2H), 1.32–1.40 (overlapping m, 3H), 1.47–1.55 (overlapping m, 3H), 1.67 (m, 1H), 1.76 (m, 2H), 1.89 (m, 1H), 1.98 (m, 1H), 2.07-2.16 (overlapping m, 2H), 2.33 (s, 3H, 4″-CH_3), 2.96 (d, 1H, J = 17.1 Hz, one of 4′-H), 3.27 (m, 1H, 3-H), 3.61 (d, 1H, J = 4.8 Hz, 17-H), 3.74 (d, 1H, J = 17.1 Hz, the other 4′-H), 4.60 (d, 1H, J = 4.4 Hz, 3-OH), 5.26 (m, 1H, 6-H), 5.30 (d, 1H, J = 4.9 Hz, 17-OH), 7.23 (d, 2H, J = 7.8 Hz, 3″-H and 5″-H), 7.55 (d, 2H, J = 7.8 Hz, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 11.4 (C-18), 19.1 (C-19), 19.9 (CH_2), 20.9 (4″-CH_3), 30.7 (CH), 30.9 (CH_2), 31.3 (CH_2), 36.1 (CH_2), 36.3 (C-10), 36.7 (CH_2), 41.2 (CH_2), 41.6 (C-13), 42.1 (CH_2), 42.6 (CH_2), 47.7 (CH), 49.6 (CH), 69.9 (C-3), 85.0 (C-17), 94.6 (C-16), 120.1 (C-6), 126.2 (2C, C-2″ and C-6″), 127.1 (C-1″), 129.2 (2C, C-3″ and C-5″), 139.2 (C-4″), 141.2 (C-5), 156.2 (C-3′); ESI-MS 437 $[M+H]^+$; Anal. Calcd. for $C_{28}H_{37}NO_3$ C 77.20; H 8.56. Found C 77.34; H 8.74.

*Synthesis of 3β-hydroxy-3′-4″-methoxyphenyl-spiro [androst-5-ene-16,5′S-2′-isoxazolin]-17β-ol (***12c***)*

Substrate: **11c** (202 mg). **12c** was obtained as a white solid (197 mg, 97 %), mp 235–237 °C, R_f = 0.44 (ss C); ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 0.66 (s, 3H, 18-H_3), 0.95 (s, 3H, 19-H_3), 0.97 (m, 1H), 1.14–1.27 (overlapping m, 3H), 1.31–1.43 (overlapping m, 3H), 1.45–1.55 (overlapping m, 3H), 1.67–1.78 (m, 3H), 1.88 (m, 1H), 1.97 (m, 1H), 2.06–2.16 (overlapping m, 2H), 2.96 (d, 1H, J = 17.1 Hz, one of 4′-H), 3.26 (m, 1H, 3-H), 3.61 (d, 1H, J = 4.9 Hz, 17-H), 3.72 (d, 1H, J = 17.1 Hz, the other 4′-H), 3.78 (s, 3H, 4″-OMe), 4.64 (d, 1H, J = 4.2 Hz, 3-OH), 5.26 (m, 1H, 6-H), 5.31 (d, 1H, J = 4.9 Hz, 17-OH), 6.98 (d, 2H, J = 8.6 Hz, 3″-H and 5″-H), 7.59 (d, 2H, J = 8.6 Hz, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 11.4 (C-18), 19.1 (C-19), 20.0 (CH_2), 30.8 (CH), 30.9 (CH_2), 31.4 (CH_2), 36.2 (CH_2), 36.4 (C-10), 36.8 (CH_2), 41.5 (CH_2), 41.7 (C-13), 42.2 (CH_2), 42.6 (CH_2), 47.7 (CH), 49.7 (CH), 55.2 (4″-OMe), 69.9 (C-3), 85.0 (C-17), 94.5 (C-16), 114.1 (2C, C-3″ and C-5″), 120.1 (C-6), 122.4 (C-1″), 127.8 (2C, C-2″ and C-6″), 141.3 (C-5), 156.0

(C-3′), 160.3 (C-4″); ESI-MS 475 $[M+Na]^+$; Anal. Calcd. for $C_{28}H_{37}NO_4$ C 74.47; H 8.26. Found C 74.61; H 8.36.

*Synthesis of 3β-hydroxy-3′-4″-chlorophenyl-spiro[androst-5-ene-16,5′S-2′-isoxazolin]-17β-ol (***12d***)*

Substrate: **11d** (204 mg). **12d** was obtained as a white solid (195 mg, 95 %), decomposed above 190°C, R_f = 0.48 (ss C); ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 0.66 (s, 3H, 18-H_3), 0.95 (s, 3H, 19-H_3), 1.00 (m, 2H), 1.19 (m, 2H), 1.31–1.43 (overlapping m, 3H), 1.49–1.55 (overlapping m, 3H), 1.68 (m, 1H), 1.76 (m, 2H), 1.88 (m, 1H), 1.99 (m, 1H), 2.06–2.17 (overlapping m, 2H), 3.01 (d, 1H, J = 17.2 Hz, one of 4′-H), 3.26 (m, 1H, 3-H), 3.62 (d, 1H, J = 4.9 Hz, 17-H), 3.76 (d, 1H, J = 17.2 Hz, the other 4′-H), 4.60 (d, 1H, J = 4.4 Hz, 3-OH), 5.25 (m, 1H, 6-H), 5.33 (d, 1H, J = 5.0 Hz, 17-OH), 7.49 (d, 2H, J = 8.4 Hz, 3″-H and 5″-H), 7.68 (d, 2H, J = 8.4 Hz, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C11.3 (C-18), 19.1 (C-19), 19.9 (CH_2), 30.7 (CH), 30.9 (CH_2), 31.3 (CH_2), 36.1 (CH_2), 36.3 (C-10), 36.7 (CH_2), 40.9 (CH_2), 41.5 (C-13), 42.1 (CH_2), 42.6 (CH_2), 47.6 (CH), 49.6 (CH), 69.8 (C-3), 85.0 (C-17), 95.3 (C-16), 120.0 (C-6), 127.7 (C-1″), 127.9 (2C, C-3″ and C-5″), 128.7 (2C, C-2″ and C-6″), 134.1 (C-4″), 141.3 (C-5), 155.6 (C-3′); ESI-MS 479 $[M+Na]^+$; Anal. Calcd. for $C_{27}H_{34}ClNO_3$ C 71.11; H 7.52. Found C 71.01; H 7.64.

*Synthesis of 3β-hydroxy-3′-4″-nitrophenyl-spiro[androst-5-ene-16,5′S-2′-isoxazolin]-17β-ol (***12e***)*

Substrate: **11e** (209 mg). **12e** was obtained as a pale-yellow solid (204 mg, 97 %), mp 271–274 °C, R_f = 0.49 (ss C); ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H 0.67 (s, 3H, 18-H_3), 0.95 (s, 3H, 19-H_3), 0.99 (m, 2H), 1.20 (m, 2H), 1.31–1.43 (overlapping m, 3H), 1.50–1.58 (overlapping m, 3H), 1.68 (m, 1H), 1.76 (m, 2H), 1.88 (m, 1H), 2.01-2.17 (overlapping m, 3H), 3.11 (d, 1H, J = 17.3 Hz, one of 4′-H), 3.27 (m, 1H, 3-H), 3.64 (d, 1H, J = 4.8 Hz, 17-H), 3.82 (d, 1H, J = 17.3 Hz, the other 4′-H), 4.60 (d, 1H, J = 4.5 Hz, 3-OH), 5.26 (m, 1H, 6-H), 5.38 (d, 1H, J = 4.8 Hz, 17-OH), 7.92 (d, 2H, J = 8.7 Hz, 2″-H and 6″-H), 8.27 (d, 2H, J = 8.6 Hz, 3″-H and 5″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 11.3 (C-18), 19.1 (C-19), 19.9 (CH_2), 30.7 (CH), 30.8 (CH_2), 31.3 (CH_2), 36.1 (CH_2), 36.2 (C-10), 36.7 (CH_2), 40.5 (CH_2), 41.5 (C-13), 42.1 (CH_2), 42.6 (CH_2), 47.6 (CH), 49.6 (CH), 69.8 (C-3), 85.0 (C-17), 96.4 (C-16), 120.0 (C-6), 123.9 (2C, C-3″ and C-5″), 127.3 (2C, C-2″ and C-6″), 136.0 (C-1″), 141.3 (C-5), 147.6 (C-4″), 155.4 (C-3′); ESI-MS 490 $[M+Na]^+$; Anal. Calcd. for $C_{27}H_{34}N_2O_5$ C 69.50; H 7.35. Found C 69.64; H 7.54.

Determination of antiproliferative activities

Antiproliferative effects were measured in vitro on three human cancer cell lines (ECACC; Salisbury, UK): HeLa (cervix adenocarcinoma), MCF7 (breast adenocarcinoma), and A431 (skin epidermoid carcinoma). The cells were cultivated in minimal essential medium (Sigma-Aldrich, Budapest, Hungary) supplemented with 10 % fetal bovine serum, 1 % non-essential amino acids, and an antibiotic-antimycotic mixture. Near-confluent cells were seeded into a 96-well plate (5,000 cells/well) and, after overnight standing, the medium (200 μL) containing the tested compound (at 10 or 30 μM) was added. Following a 72-h incubation period in a humidified atmosphere of 5 % CO_2 at 37 °C, the living cells were assayed by the addition of 20 μL of 5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution [44]. During a 4-h contact period, the MTT was converted by intact mitochondrial reductase and precipitated as blue crystals. The medium was then removed, the precipitated formazan crystals were solubilized in DMSO (100 μL) during a 60-min period of shaking at 25 °C, and the absorbance was read at 545 nm with a microplate reader. Wells with untreated cells were utilized as controls. All in vitro experiments were carried out on two microplates with at least five parallel wells. Stock solutions of the tested substances (10 mM) were prepared with DMSO. The DMSO concentration (0.3 %) of the medium did not have any significant effect on cell proliferation. Cisplatin was used as a reference compound. A set of dilutions were additionally applied in order to determine the IC_{50}values of the most active compounds. Sigmoidal dose–response curves were fitted to the measured points, and the values were calculated by means of GraphPad Prism 4.0 (GraphPad Software; San Diego, CA, USA).

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