applied sciences

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Microwave-Assisted Synthesis, Proton Dissociation Processes, and Anticancer Evaluation of Novel D-Ring-Fused Steroidal 5-Amino-1-Arylpyrazoles

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Received: 4 December 2019; Accepted: 23 December 2019; Published: 27 December 2019

Abstract: Taking into account the pharmacological relevance of heterocycle-fused natural steroids, the objective of the current study was to develop a multistep reaction sequence for the efficient synthesis of novel D-ring-condensed 5-amino-1-arylpyrazoles from dehydroepiandrosterone (DHEA). A condensation reaction of 16-formyl-DHEA with hydroxylamine afforded the corresponding oxime, which was demonstrated to be stable in one of its cyclic isoxazoline forms due to possible ring-chain tautomerism. The subsequent base-induced dehydration to a diastereomeric β-ketonitrile, followed by microwave-assisted heterocyclization with different arylhydrazines led to the desired pyrazoles. The generally good yields of the products depended slightly on the electronic character of the substituent present on the aromatic ring of the reagent. The proton dissociation processes of the DHEA-derived heterocycles were investigated in aqueous solution by UV-visible spectrophotometric titrations to reveal their actual chemical forms at physiological pH. The determined pK_a values attributed to the pyrazole NH^+ moiety were low (1.8–4.0) and varied by the different substituents of the benzene ring. The antiproliferative effects of the structurally similar compounds were screened in vitro on human cancer cells (namely on HeLa, U2Os, MCF-7, PC-3, and A549), along with a noncancerous cell line (MRC-5). The IC_{50} values of the most active derivative were determined on all cell lines.

Keywords: steroid; heterocycles; aminopyrazole; microwave; organic synthesis; proton dissociation; anticancer activity

1. Introduction

Structural modification of natural sex hormones by the introduction of various heterocyclic moieties may alter the physicochemical properties and the pharmacokinetic behavior, as well as the biological activity of the parent compound [1]. Continuous interest in steroid derivatives with nitrogen-containing heterorings condensed with, or connected to ring D of the sterane framework

arises from the significant pharmacological potential of these molecules [2]. Several studies have demonstrated that steroidal aza-heterocyclic derivatives can be effective in the prevention and treatment of hormone-dependent cancers. They can also act as cytotoxic agents through hormone-independent mechanisms of action [3]. Heteroaromatic five-membered pyrazoles containing two adjacent nitrogen atoms have received considerable attention thanks to their facile synthetic availability and their usefulness as intermediates for the preparation of various biologically active compounds, metal complexes, and functional materials [4,5]. Over the past decades, numerous pyrazoles in the androstane series and their partially saturated pyrazoline analogs have been reported to exhibit a more marked antitumor activity rather than their expected hormonal effect (Figure 1) [6–13]. Amongst functionalized pyrazoles, 5-aminopyrazoles have been widely investigated as biologically active agents [14] and as useful binucleophilic precursors for fused pyrazoloazines of medicinal interest [15]. Moreover, 5-amino-1-arylpyrazoles, which are usually obtained by the reaction of β-ketonitriles or malonitrile derivatives with arylhydrazines, constitute the main structural motif of a vast number of bioactive compounds with kinase-inhibitory, anticancer, anti-infective, anti-inflammatory, or other effects [16]. Although β-ketonitriles are also suitable starting materials for the synthesis of other 5-amino-substituted 1,2-azoles, such as isoxazoles [17] or isothiazoles [18], previous research has shown that the incorporation of a *N*,*N*-heterocyclic ring into the sterane backbone is much more beneficial in terms of anticancer activity. As concerns the synthetic methodologies, a number of reactions have been reported so far for the synthesis of different steroid-fused heterocycles under microwave (MW) irradiation due to the advantageous characteristics of this approach, such as enhanced reaction rates, manipulative simplicity, and high product yields [19,20].

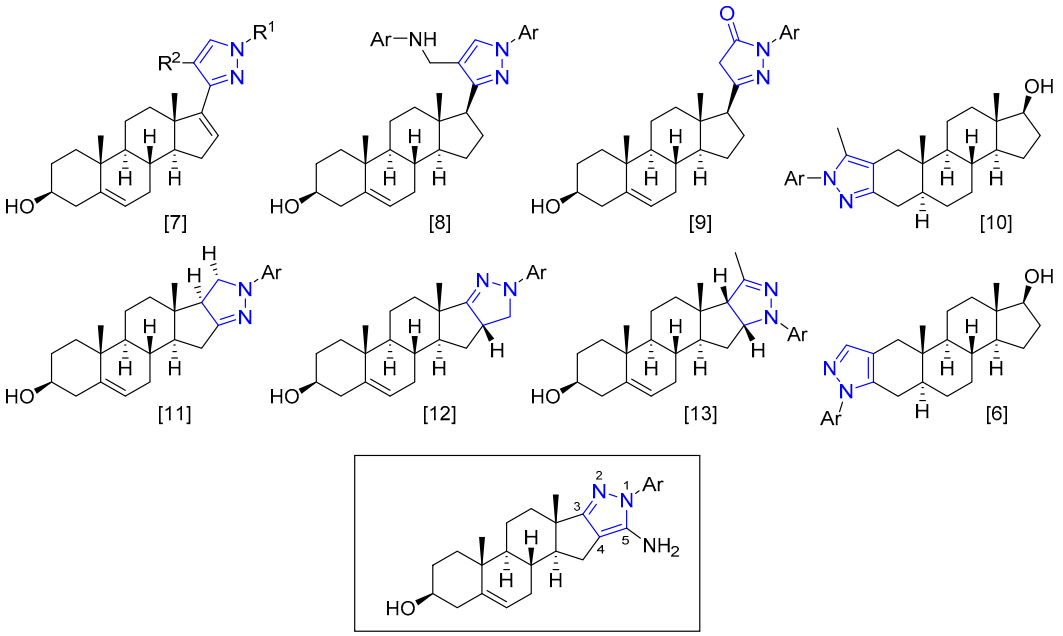


Figure 1. Some previously synthesized androstane-based pyrazol(in)es with anticancer activity [6–13] and 5-amino-1-arylpyrazoles (framed), which are the subjects of the present study.

In view of the marked cytotoxic activity of some androstane-based pyrazol(in)es [6–13], and the pharmacological relevance of drugs containing a 5-amino-1-arylpyrazole moiety [16], the aim of the present study was to introduce such heterocyclic subunit fused to ring D of dehydroepiandrosterone (DHEA) to obtain a set of compounds suitable for further investigations. To our knowledge, steroidal 5-aminopyrazoles have not been synthesized and studied so far. The heterocyclizations were designed and performed from a DHEA-derived β-ketonitrile and various arylhydrazines, respectively, under pre-optimized MW-assisted reaction conditions. Proton dissociation processes of the heterocycles and the dependence of the determined pK_a values on the electron demand

of the aryl substituents were also investigated. The cytotoxicity of the molecules was screened in vitro on different human cancer cell lines, along with a noncancerous cell line by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [21]. In addition, based on the screen, one compound—showing the highest efficacy to inhibit the proliferation of various cancer cells—was selected and subjected to additional in vitro tests in order to determine its IC_{50} values on all tested cell lines.

2. Materials and Methods

2.1. General

General information has been previously reported in detail [22]. In brief, the purchase sources of chemicals, which were used without purification are: Sigma-Aldrich Corporation, St. Louis, MO, USA; TCI, Tokyo, Japan; and Alfa Aesar, Haverhill, MA, USA. MW-reactor: CEM Discover SP (CEM Corporation, Matthews, NC, USA), maximum power (dynamic control program): 200 W. Thin-layer chromatography (TLC) plates (0.25 mm thick): Kieselgel-G (Si 254 F, Merck KGaA, Darmstadt, Germany). Column chromatography: silica gel 60, 40–63 μm (Merck KGaA, Darmstadt, Germany). NMR instrument: Bruker DRX 500 (Bruker, Billerica, MA, USA). NMR spectra (at 298 K) of all compounds were recorded in DMSO-d_6 using the residual solvent signal as reference. Coupling constants (J) are given in Hz and chemical shifts in ppm (δ scale). In the ^{1}H NMR spectra the multiplicities of the signals are reported as a singlet (s), a broad singlet (bs), a doublet (d), a double doublet (dd), or a multiplet (m). ^{1}H-decoupled ^{13}C NMR spectra were recorded. For the determination of multiplicities, the J-MOD pulse sequence was applied. Melting point (Mp) apparatus: SRS Optimelt digital device (Stanford Research Systems Inc., Sunnyvale, CA, USA); Elementary analysis: PerkinElmer CHN analyzer model 2400 (PerkinElmer Inc., Waltham, MA, USA). Automated flow injection analyses: HPLC/MSD system. System accessories: a micro-well plate autoinjector, an Agilent 1100 micro vacuum degasser (Agilent Technologies, Santa Clara, CA, USA), a quaternary pump, and a 1946A MSD with an electrospray ion source (ESI) operated in positive ion mode. ESI parameters: nebulizing gas N_2 (at 35 psi); drying gas N_2 (at 350 °C, 12 L/min); capillary voltage: 3000 V; fragmentor voltage: 70 V. The MSD was operated with a mass range of m/z 60–620 in scan mode. Samples (0.2 μL) were injected directly into the solvent flow (0.3 mL/min) of CH_3CN/H_2O = 70:30 (v/v) with the simultaneous addition of 0.1% formic acid with an automated needle wash. Agilent LC/MSD Chemstation (C.01.08, Agilent Technologies Inc., Santa Clara, CA, USA) was used as software to control the system.

2.2. Synthetic Procedures

2.2.1. Characterization of 2′-Isoxazolino [4′.5′: 16.17] Androst-5-en-3β,17-diol (**2**)

Synthesis of compound **2** was performed from 16-formyl DHEA (**1**) according to literature method [23] with some modifications. Mp 258–260 °C (white solid); R_f = 0.18 (EtOAc/CH_2Cl_2 = 20:80 v/v). Anal. Calcd. for $C_{20}H_{29}NO_3$ (331.46): C, 72.47; H, 8.82. Found: C, 72.60; H, 8.92. ^{1}H NMR (DMSO-$d6$, 500 MHz): δ 0.82–0.85 (4H, m, 1H and s, 3H, 18-H_3), 0.94 (s, 3H, 19-H_3), 0.98 (m, 2H), 1.30–1.68 (overlapping m, 10H), 1.76 (m, 1H), 1.89 (m, 1H), 2.04–2.16 (overlapping m, 2H), 3.12 (d, 1H, J = 10.2 Hz), 3.25 (m, 1H, 3-H), 4.61 (d, 1H, J = 4.5 Hz, 3-OH), 5.24 (d, 1H, J = 4.6 Hz, 6-H), 6.63 (s, 1H, 17-OH), 7.29 (d, 1H, J = 1.7 Hz, 3′-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 15.7 (C-18), 19.1 (C-19), 19.9 (CH_2), 29.0 (CH_2), 30.3 (CH), 31.1 (CH_2), 31.2 (C-16), 31.4 (CH_2), 36.2 (C-10), 36.9 (CH_2), 42.2 (C-13), 46.1 (CH_2), 49.4 (2C, 2 × CH), 54.9 (CH), 69.9 (C-3), 116.5 (C-17), 120.1 (C-6), 141.3 (C-5), 150.4 (C-3′); ESI-MS 332 $[M + H]^+$.

2.2.2. Synthesis of 16-Cyano-3β-Hydroxy-Androst-5-en-17-One (**3**)

To a solution of compound 2 (2 g, 6.03 mmol) in MeOH (20 mL), KOH (1.68 g, 30 mmol), also dissolved in MeOH (10 mL) was added. After stirring the mixture at room temperature for 30 min,

it was poured into water (100 mL) and neutralized with diluted HCl. The precipitate was filtered off and dried. Column chromatographic purification of the crude product with EtOAc/CH_2Cl_2 = 10:90 provided an epimeric mixture (16β-CN (a)/16α-CN (b) = 2:1) of 3 as a white solid (1.64 g, 87%). R_f = 0.48. Anal. Calcd. for $C_{20}H_{27}NO_2$ (313.44): C, 76.64; H, 8.68. Found: C, 76.55; H, 8.59. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.88 (s, 18-H_3, b), 0.90 (s, 18-H_3, a), 0.96 (s, 19-H_3, a + b), 3.26 (m, 3-H, a + b), 3.78 (dd, J = 9.3 Hz, J = 9.3 Hz, 16-H, a), 4.36 (d, J = 8.7 Hz, 16-H, b), 4.63 (d, J = 4.4 Hz 3-OH, a + b), 5.30 (m, 6-H, a + b); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 12.8 (C-18, b), 13.7 (C-18, a), 19.1 (C-19, a + b), 19.7 (CH_2, a + b), 26.9 (CH_2, b), 27.5 (CH_2, a), 29.7 (CH_2, b), 30.1 (CH_2, a), 30.4 (CH, a), 30.9 (CH, b), 30.9 (CH_2, a), 31.1 (CH_2, b), 31.4 (CH_2, a + b), 35.3 (CH, b), 36.2 (C-10, a + b), 36.3 (CH, a), 36.8 (CH_2, a + b), 42.1 (C-13, a + b), 47.0 (CH_2, b), 47.6 (CH_2, a), 48.3 (CH, a + b), 49.2 (CH, b), 49.5 (CH, a), 69.9 (C-3, a + b), 118.4 (CN, a), 118.6 (CN, a), 119.7 (C-6, a), 119.8 (C-6, b), 141.4 (C-5, b), 141.5 (C-5, a), 209.3 (C-17, b), 209.5 (C-17, a); ESI-MS 314 $[M + H]^+$.

2.2.3. General Procedure for The Synthesis of D-Ring-Condensed 5-Amino-1-Arylpyrazoles (**6a**–**h**) Under MW Irradiation

To a solution of 16-cyano-3β-hydroxy-androst-5-en-17-one (**3**, 157 mg, 0.5 mmol) in EtOH (5 mL), arylhydrazine hydrochloride (**5a**–**h**, 1.5 equiv., 0.75 mmol) and NaOAc (61.5 mg, 1.5 equivalent) were added, and the mixture was MW-irradiated at 120 °C for 10 min in a closed vessel. After completion of the reaction, the mixture was poured into water (20 mL), saturated with NH_4Cl and the precipitate formed was filtered off. Column chromatographic purification of the crude product was performed with EtOAc/CH_2Cl_2 = 20:80 as mobile phase.

5′-Amino-1′-Phenylpyrazolo[3′,4′:17,16]Androst-5-en-3β-ol (**6a**)

Phenylhydrazine hydrochloride (**5a**, 108 mg) was used as described in the general procedure. Yield: 182 mg (90%, white solid); Mp 258–260 °C; R_f = 0.23 (EtOAc/CH_2Cl_2 = 20:80 *v*/*v*). Anal. Calcd. for $C_{26}H_{33}N_3O$ (403.57): C, 77.38; H, 8.24. Found: C, 77.27; H, 8.13. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.97 (s, 3H, 18-H_3), 1.01 (s, 3H, 19-H_3 and m, 2H), 1.37 (m, 1H), 1.51–1.81 (overlapping m, 8H), 1.96–2.20 (overlapping m, 5H), 2.42 (dd, 1H, J = 13.3 Hz, J = 5.0 Hz, one of 15-H_2), 3.28 (m, 1H, 3-H), 4.62 (d, 1H, J = 3.0 Hz, 3-OH), 5.09 (s, 2H, 5′-NH_2), 5.33 (d, 1H, J = 4.5 Hz, 6-H), 7.21 (m, 1H, 4″-H), 7.41 (m, 2H, 3″-H and 5″-H), 7.54 (d, 2H, J = 7.7 Hz, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 18.0 (C-18), 19.1 (C-19), 20.2 (CH_2), 23.6 (CH_2), 30.4 (CH), 30.9 (CH_2), 31.5 (CH_2), 33.5 (CH_2), 36.3 (C-10), 36.8 (CH_2), 40.2 (C-13), 42.3 (CH_2), 50.1 (CH), 61.2 (CH), 70.0 (C-3), 104.1 (C-16), 120.2 (C-6), 122.2 (2C, C-2″ and C-6″), 125.0 (C-4″), 128.8 (2C, C-3″ and C-5″), 140.3, 140.6 and 140.7 (C-5, C-5′ and C-1″), 168.4 (C-17); ESI-MS 404 $[M + H]^+$.

5′-Amino-1′-4″-Tolylpyrazolo[3′,4′:17,16]Androst-5-en-3β-ol (**6b**)

4-Tolylhydrazine hydrochloride (**5b**, 119 mg) was used as described in the general procedure. Yield: 186 mg (89%, white solid); Mp 267–269 °C; R_f = 0.26 (EtOAc/CH_2Cl_2 = 20:80 *v*/*v*). Anal. Calcd. for $C_{27}H_{35}N_3O$ (417.60): C, 77.66; H, 8.45. Found: C, 77.47; H, 8.32. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.95 (s, 3H, 18-H_3), 1.01 (s, 3H, 19-H_3 and m, 2H), 1.37 (m, 1H), 1.49-1.80 (overlapping m, 8H), 1.98-2.19 (overlapping m, 5H), 2.31 (s, 3H, 4″-CH_3), 2.40 (dd, 1H, J = 13.3 Hz, J = 4.7 Hz, one of 15-H_2), 3.27 (m, 1H, 3-H), 4.62 (d, 1H, J = 4.5 Hz, 3-OH), 5.03 (s, 2H, 5′-NH_2), 5.32 (d, 1H, J = 4.1 Hz, 6-H), 7.21 (d, 2H, J = 8.2 Hz, 3″-H and 5″-H), 7.40 (d, 2H, J = 8.2 Hz, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 18.0 (C-18), 19.1 (C-19), 20.2 (CH_2), 20.5 (4″-CH_3), 23.6 (CH_2), 30.4 (CH), 30.9 (CH_2), 31.5 (CH_2), 33.6 (CH_2), 36.4 (C-10), 36.9 (CH_2), 40.2 (C-13), 42.3 (CH_2), 50.1 (CH), 61.2 (CH), 70.0 (C-3), 103.8 (C-16), 120.2 (C-6), 122.4 (2C, C-2″ and C-6″), 129.3 (2C, C-3″ and C-5″), 134.3 (C-4″), 137.9 (C-1″), 140.5 (C-5), 141.6 (C-5′), 168.1 (C-17); ESI-MS 418 $[M + H]^+$.

5′-Amino-1′-(4″-Methoxy)phenylpyrazolo[3′,4′:17,16]Androst-5-en-3β-ol (**6c**)

4-Methoxy-phenylhydrazine hydrochloride (**5c**, 131 mg) was used as described in the general procedure. Yield: 199 mg (92%, white solid); Mp 209–211 °C; R_f = 0.17 (EtOAc/CH_2Cl_2 = 20:80 *v*/*v*). Anal. Calcd. for $C_{27}H_{35}N_3O_2$ (433.60): C, 74.79; H, 8.14. Found: C, 74.65; H, 8.02. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.95 (s, 3H, 18-H_3), 1.01 (s, 3H, 19-H_3 and m, 2H), 1.37 (m, 1H), 1.49–1.80 (overlapping m, 8H), 1.97–2.20 (overlapping m, 5H), 2.40 (dd, 1H, *J* = 13.2 Hz, *J* = 4.7 Hz, one of 15-H_2), 3.27 (m, 1H, 3-H), 3.77 (s, 3H, 4″-OMe), 4.63 (d, 1H, *J* = 4.5 Hz, 3-OH), 4.96 (s, 2H, 5′-NH_2), 5.32 (m, 1H, 6-H), 6.96 (d, 2H, *J* = 9,0 Hz, 3″-H and 5″-H), 7.40 (d, 2H, *J* = 9.0 Hz, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 18.1 (C-18), 19.1 (C-19), 20.2 (CH_2), 23.6 (CH_2), 30.4 (CH), 30.9 (CH_2), 31.5 (CH_2), 33.6 (CH_2), 36.4 (C-10), 36.9 (CH_2), 40.2 (C-13), 42.3 (CH_2), 50.1 (CH), 55,3 (4″-OMe), 61.3 (CH), 70.0 (C-3), 103.4 (C-16), 114.0 (2C, C-2″ and C-6″), 120.2 (C-6), 124.4 (2C, C-3″ and C-5″), 133.4 (C-1″), 140.4 (C-5), 141.6 (C-5′), 156.9 (C-4″), 167.8 (C-17); ESI-MS 434 $[M + H]^+$.

5′-Amino-1′-(4″-Fluoro)phenylpyrazolo[3′,4′:17,16]Androst-5-en-3β-ol (**6d**)

4-Fluoro-phenylhydrazine hydrochloride (**5d**, 122 mg) was used as described in the general procedure. Yield: 198 mg (94%, white solid); Mp 278–280 °C; R_f = 0.35 (EtOAc/CH_2Cl_2 = 20:80 *v*/*v*). Anal. Calcd. for $C_{26}H_{32}FN_3O$ (421.56): C, 74.08; H, 7.65. Found: C, 73.95; H, 8.73. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.96 (s, 3H, 18-H_3), 1.01 (s, 3H, 19-H_3 and m, 2H), 1.37 (m, 1H), 1.50–1.80 (overlapping m, 8H), 1.97–2.20 (overlapping m, 5H), 2.41 (dd, 1H, *J* = 13.3 Hz, *J* = 4.7 Hz, one of 15-H_2), 3.27 (m, 1H, 3-H), 4.63 (d, 1H, *J* = 4.5 Hz, 3-OH), 5.11 (s, 2H, 5′-NH_2), 5.32 (m, 1H, 6-H), 7.23 (dd, 2H, *J* = 8.8 Hz, *J* = 8.8 Hz, 3″-H and 5″-H), 7.54 (dd, 2H, *J* = 8.8 Hz, *J* = 5.0 Hz, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 18.0 (C-18), 19.1 (C-19), 20.2 (CH_2), 23.6 (CH_2), 30.4 (CH), 30.9 (CH_2), 31.5 (CH_2), 33.5 (CH_2), 36.4 (C-10), 36.9 (CH_2), 40.3 (C-13), 42.3 (CH_2), 50.1 (CH), 61.2 (CH), 70.0 (C-3), 104.1 (C-16), 115.5 (d, 2C, *J* = 22.5 Hz, C-3″ and C-5″), 120.2 (C-6), 124.5 (d, 2C, *J* = 8.4 Hz, C-2″ and C-6″), 136.7 (d, *J* = 2,5 Hz, C-1″), 140.8 (C-5), 141.6 (C-5′), 159.6 (d, *J* = 241.9 Hz, C-4″), 168.4 (C-17); ESI-MS 422 $[M + H]^+$.

5′-Amino-1′-(4″-Chloro)phenylpyrazolo[3′,4′:17,16]Androst-5-en-3β-ol (**6e**)

4-Chloro-phenylhydrazine hydrochloride (**5e**, 134 mg) was used as described in the general procedure. Yield: 175 mg (80%, white solid); Mp 299–301 °C; R_f = 0.32 (EtOAc/CH_2Cl_2 = 20:80 *v*/*v*). Anal. Calcd. for $C_{26}H_{32}ClN_3O$ (438.01): C, 71.30; H, 7.36. Found: C, 71.45; H, 7.22. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.96 (s, 3H, 18-H_3), 1.01 (s, 3H, 19-H_3 and m, 2H), 1,36 (m, 1H), 1,50–1,80 (overlapping m, 8H), 1,98–2,19 (overlapping m, 5H), 2.41 (dd, 1H, *J* = 13.4 Hz, *J* = 4.5 Hz, one of 15-H_2), 3.27 (m, 1H, 3-H), 4.63 (d, 1H, *J* = 4.5 Hz, 3-OH), 5.19 (s, 2H, 5′-NH_2), 5.32 (m, 1H, 6-H), 7.44 (d, 2H, *J* = 8.8 Hz, 3″-H and 5″-H), 7.58 (d, 2H, *J* = 8.8 Hz, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 17.9 (C-18), 19.1 (C-19), 20.2 (CH_2), 23.6 (CH_2), 30.4 (CH), 30.9 (CH_2), 31.5 (CH_2), 33.5 (CH_2), 36.4 (C-10), 36.8 (CH_2), 40.3 (C-13), 42.3 (CH_2), 50.0 (CH), 61.1 (CH), 70.0 (C-3), 104.5 (C-16), 120.2 (C-6), 123.6 (2C, C-2″ and C-6″), 128.8 (2C, C-3″ and C-5″), 128.9 (C-4″), 139.2 (C-1″), 140.9 (C-5), 141.6 (C-5′), 168.8 (C-17); ESI-MS 439 $[M + H]^+$.

5′-Amino-1′-(4″-Bromo)phenylpyrazolo[3′,4′:17,16]Androst-5-en-3β-ol (**6f**)

4-Bromo-phenylhydrazine hydrochloride (**5f**, 168 mg) was used as described in the general procedure. Yield: 202 mg (84%, white solid); Mp > 210 °C (decomp.); R_f = 0.39 (EtOAc/CH_2Cl_2 = 20:80 *v*/*v*). Anal. Calcd. for $C_{26}H_{32}BrN_3O$ (482.47): C, 64.73; H, 6.69. Found: C, 64.84; H, 6.81. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.96 (s, 3H, 18-H_3), 1.01 (s, 3H, 19-H_3 and m, 2H), 1.35 (m, 1H), 1.49–1.79 (overlapping m, 8H), 1.97–2.18 (overlapping m, 5H), 2.41 (dd, 1H, *J* = 13.3 Hz, *J* = 4.5 Hz, one of 15-H_2), 3.26 (m, 1H, 3-H), 4,63 (bs, 1H, 3-OH), 5,22 (bs, 2H, 5′-NH_2), 5.32 (m, 1H, 6-H), 7.52 (d, 2H, *J* = 8.8 Hz) and 7.58 (d, 2H, *J* = 8.8 Hz): 3″-H, 5″-H and 2″-H, 6″-H; ^{13}C NMR (DMSO-d_6, 125 MHz): δ 17.9 (C-18), 19.2 (C-19), 20.2 (CH_2), 23.6 (CH_2), 30.4 (CH), 30.9 (CH_2), 31.5 (CH_2), 33.4 (CH_2), 36.4 (C-10), 36.8 (CH_2), 40.3 (C-13), 42.3 (CH_2), 50.0 (CH), 61.1 (CH), 70.0 (C-3), 104.6 (C-16), 117.2 (C-4″), 120.2 (C-6), 124.0 (2C, C-2″ and C-6″), 131.7 (2C, C-3″ and C-5″), 139.6 (C-1″), 141.0 (C-5), 141.6 (C-5′), 168.9 (C-17); ESI-MS 483 $[M + H]^+$.

5′-Amino-1′-(4″-Cyano)phenylpyrazolo[3′,4′:17,16]Androst-5-en-3β-ol (**6g**)

4-Cyano-phenylhydrazine hydrochloride (**5g**, 127 mg) was used as described in the general procedure. Yield: 182 mg (85%, white solid); Mp 293–295 °C; R_f = 0.34 (EtOAc/CH_2Cl_2 = 20:80 *v*/*v*). Anal. Calcd. for $C_{27}H_{32}N_4O$ (428.58): C, 75.67; H, 7.53. Found: C, 75.75; H, 7.39. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.97 (s, 3H, 18-H_3), 1.01 (s, 3H, 19-H_3 and m, 2H), 1.36 (m, 1H), 1.51–1.79 (overlapping m, 8H), 1.97–2.20 (overlapping m, 5H), 2.43 (dd, 1H, *J* = 13.6 Hz, *J* = 4.7 Hz, one of 15-H_2), 3.27 (m, 1H, 3-H), 4.63 (d, 1H, *J* = 4.5 Hz, 3-OH), 5.32 (m, 1H, 6-H), 5.40 (s, 2H, 5′-NH_2), 7.82 (d, 2H, *J* = 8.7 Hz, 3″-H and 5″-H), 7.85 (d, 2H, *J* = 8.7 Hz, 2″-H and 6″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 17.8 (C-18), 19.1 (C-19), 20.1 (CH_2), 23.5 (CH_2), 30.4 (CH), 30.8 (CH_2), 31.5 (CH_2), 33.3 (CH_2), 36.3 (C-10), 36.8 (CH_2), 40.3 (C-13), 42.3 (CH_2), 50.0 (CH), 61.0 (CH), 70.0 (C-3), 105.7 and 106.2: C-4″ and C-16, 118.9 (4″-CN), 120.1 (C-6), 121.2 (2C, C-2″ and C-6″), 133.2 (2C, C-3″ and C-5″), 141.6 and 141.7: C-1″ and C-5, 144.2 (C-5′), 170.1 (C-17); ESI-MS 429 $[M + H]^+$.

5′-Amino-1′-(4″-Nitro)phenylpyrazolo[3′,4′:17,16]Androst-5-en-3β-ol (**6h**)

4-Nitro-phenylhydrazine hydrochloride (**5h**, 127 mg) was used as described in the general procedure. Yield: 117 mg (52%, Table 1, entry 8^{2a}) or 197 mg (88%, Table 1, entry 8^{2b}), (yellow solid); Mp 242–244 °C; R_f = 0.32 (EtOAc/CH_2Cl_2 = 20:80 *v*/*v*). Anal. Calcd. for $C_{26}H_{32}N_4O_3$ (448.57): C, 69.62; H, 7.19. Found: C, 69.49; H, 7.05. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.98 (s, 3H, 18-H_3), 1.01 (s, 3H, 19-H_3 and m, 2H), 1.37 (m, 1H), 1.51–1.80 (overlapping m, 8H), 1.99–2.20 (overlapping m, 5H), 2.43 (dd, 1H, *J* = 13.6 Hz, *J* = 4.6 Hz, one of 15-H_2), 3.27 (m, 1H, 3-H), 4.63 (d, 1H, *J* = 4.5 Hz, 3-OH), 5.31 (m, 1H, 6-H), 5.49 (s, 2H, 5′-NH_2), 7.92 (d, 2H, *J* = 9.2 Hz, 2″-H and 6″-H), 8.26 (d, 2H, *J* = 9.2 Hz, 3″-H and 5″-H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 17.8 (C-18), 19.1 (C-19), 20.2 (CH_2), 23.5 (CH_2), 30.4 (CH), 30.8 (CH_2), 31.5 (CH_2), 33.2 (CH_2), 36.3 (C-10), 36.8 (CH_2), 40.4 (C-13), 42.3 (CH_2), 50.0 (CH), 60.9 (CH), 70.0 (C-3), 106.1 (C-16), 120.2 (C-6), 120.7 (2C, C-2″ and C-6″), 124.8 (2C, C-3″ and C-5″), 141.6 (C-5), 142.0, 143.0 and 146.0 (C-1″, C-4″ and C-5′), 170.7 (C-17); ESI-MS 449 $[M + H]^+$.

2.3. UV Spectrophotometric Titrations

Stock solutions of the tested compounds were prepared in EtOH at 100 μM concentration. UV-visible spectra were recorded for compounds **6a**-**h** in order to determine proton dissociation constants (pK_a) in the pH range 1.8–10. The compounds had absorption only in the UV range. Measurements were accomplished at a constant ionic strength (0.10 M KCl) and at 25.0 ± 0.1 °C in aqueous phase (with 1% (*v*/*v*) EtOH). For the titrations, a carbonate-free KOH solution (0.10 M) was used, the concentration of which was determined by pH-potentiometric titrations. The initial volume of the samples was 10.0 mL and the spectrophotometric titrations were performed on samples containing 10 μM of tested compounds. Purified argon was bubbled through the samples for deoxygenation circa 10 min prior the measurements. The UV spectra of the individual species in the various protonation states and the proton dissociation constants were calculated with the computer program PSEQUAD [24]. Experimental titration data measured in the absence of any precipitate in the solution (pH = 1.8–5 (or 3.5 in case of **6h**)) were used for the calculations. In the interval 200–800 nm, a diode array spectrophotometer (Hewlett Packard 8452A) was applied to record the spectra. The length of the path was 2 cm.

Attempts to determine the distribution coefficients ($D_{7.4}$) and permeability values have been made in *n*-octanol/buffered aqueous solution by using the traditional shake-flask method at pH 7.40 (20 mM phosphate buffer, 0.10 M KCl), as described earlier [25], and by the PAMPA method using a Corning Gentest pre-coated PAMPA Plate System [26], respectively.

2.4. MTT Cell Viability Assay

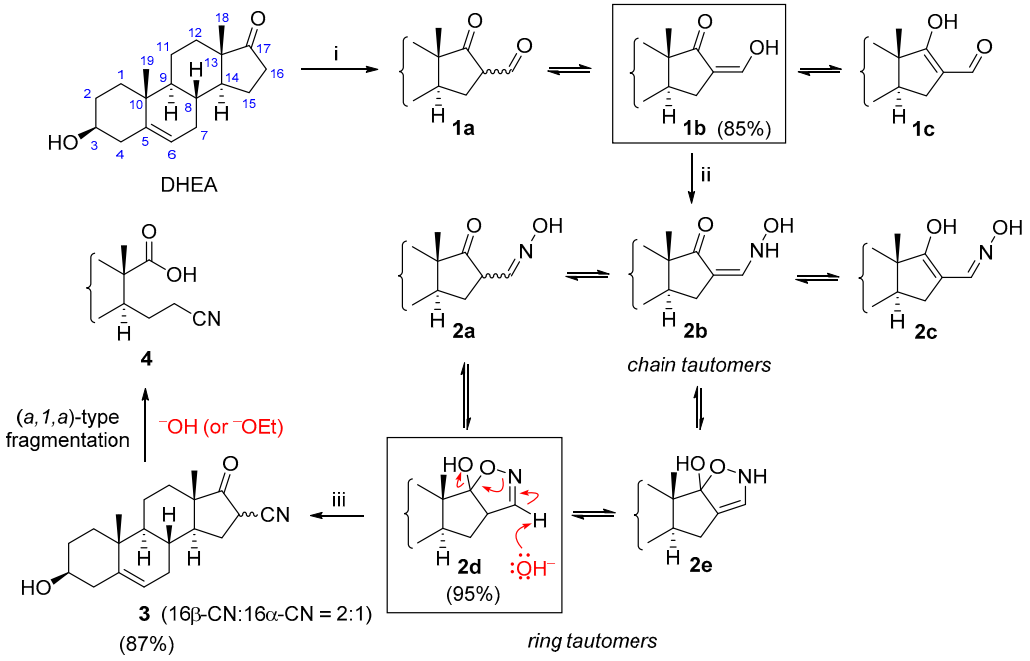
All the test compounds were dissolved in DMSO (cell culture grade, Molar Chemicals, Halásztelek, Hungary) to obtain a stock solution of 10 mM final concentration, and stored at 4 °C, light protected, until further use. Antiproliferative activity of the synthesized steroids was assessed by MTT reduction assay [21] on human MCF-7 breast adenocarcinoma, PC-3 prostate cancer, A549 lung carcinoma,

HeLa cervical adenocarcinoma, U2Os osteosarcoma cell lines, and on MRC-5 human noncancerous fibroblasts. A total 5000 cells/well were seeded into 96 well plates and 24 h later cells were exposed to the test compounds in 5 μM concentration for 72 h. Cells receiving no treatment were used as control throughout the experiments. To obtain IC_{50} values of the selected compound on various cancerous and noncancerous lines, cells were seeded again in 5000 cells/well density in 96 well plates and treated with the compound in 1, 3, 5, 7, and 9 μM concentrations or cisplatin in 20, 40, 80, 160, and 330 nM concentrations for 72 h. After the treatments, cells were washed using phosphate-buffered saline (PBS) and incubated with 0.5 mg/mL MTT reagent (SERVA, GmbH, Heidelberg, Germany) diluted in respective cell culture media without fetal bovine serum (FBS) for 1 h at 37 °C. The formed formazan crystals were dissolved in DMSO and the absorbance was measured using a Synergy HTX plate reader (BioTech-Hungary, Budapest, Hungary) at 570 nm. All the experiments were repeated three times and the data were analyzed by GraphPad Prism 7 software (GraphPad Software; San Diego, CA, USA).

3. Results and Discussion

3.1. Synthetic Studies

For the synthesis of D-ring-fused 5-aminopyrazoles, DHEA was used as a starting material. Formylation at C-16 with ethyl formate in the presence of a strong base by the Claisen condensation and the following acidification led to compound **1** [27], which can exist as equilibrating tautomers (**1a**–**c**) in solution (Scheme 1). The predominance of one or the other forms is highly affected by the proticity and polarity of the solvent; however, **1b**—where enolization occurs on the formyl group—was found to prevail in neutral organic medium based on NMR measurements [28]. Condensation reaction of **1** with hydroxylamine hydrochloride in EtOH in the presence of NaOAc resulted in the corresponding oxime capable of ring-chain tauromerism. Thus, two additional cyclic tautomers (**2d** and **2e**) can contribute to the equilibrium besides the three open forms (**2a**–**c**) [29]. According to ^{1}H and ^{13}C NMR (J-MOD) measurements, only one of the possible tautomers (**2d**) is present in dimethyl sulfoxide (DMSO) solution in good agreement with previous studies, where the same form was evidenced by IR and XRD in the solid-state [23].

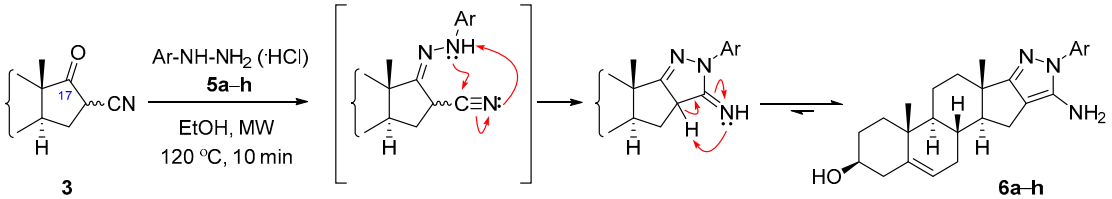


Scheme 1. Multistep synthesis of a steroidal β-ketonitrile (**3**) from dehydroepiandrosterone (DHEA) reagents and conditions: (**i**) HCOOEt, NaOEt, CH_2Cl_2, rt., 5 h; (**ii**) $NH_2OH{\cdot}HCl$, NaOAc, EtOH, reflux, 1 h; (**iii**) KOH, MeOH, rt, 30 min.

Treatment of 2 with KOH in MeOH at room temperature or below resulted in a β-ketonitrile (3) as a mixture of two epimers in excellent yield via base-induced elimination of a water molecule (Scheme 1). It is important to note, however, that (*a*,1,*a*)-type fragmentation can occur leading to a D-seco-carboxylic acid (4) at higher temperatures or by using a stronger and/or excess base [30]. The ^{1}H NMR spectrum of compound **3** showed some duplicated proton peaks (16-H, 18-CH_3) at different chemical shifts and with different integrals, indicating the presence of two diastereomers in a ratio of about 2:1. Furthermore, the peak shapes of the 16-H protons allowed us to identify the epimers; that is, a triplet at 3.78 ppm (J = 9.3 Hz) was assigned for the 16β-CN derivative, while a doublet at 4.36 ppm (J = 8.7 Hz) was assigned for the 16α-CN isomer. The splitting and coupling constants of 16-H signals corresponded to the dihedral torsion angles θ(H16,C16,C15,H15α) and θ(H16,C16,C15,H15β) calculated based on the Karplus equation for the two stereoisomers of **3**.

In the following, β-ketoester (**3**) was used for the synthesis of steroidal heteroaromatic 5-aminopyrazoles without separating the diastereomers, since the asymmetric center disappears during the ring-closure step with arylhydrazines. For preliminary experiments, compound 3 and phenylhydrazine hydrochloride (**5a**, 1.2 equiv.) were reacted in EtOH under MW irradiation at different temperatures (80, 100, and 120 °C) for 5, 10, 15, and 20 min in a closed vessel and the progress of the transformations was monitored by thin-layer chromatography (TLC). Of all the reaction conditions, 120 °C and 10 min were found to be optimal for the complete conversion of the starting material. However, alkaline work-up was needed in this case to neutralize the reaction mixture; otherwise, a certain amount of the desired product was lost with the aqueous phase due to its basic characteristic of forming a HCl salt in the presence of an excess reagent. Alternatively, the MW-assisted transformation could also have been carried out within 10 min in the presence of NaOAc (1.2 equivalent) to liberate the binucleophilic reagent from its HCl salt, and the product (**6a**) was again obtained in excellent yield after purification by column chromatography (Table 1, entry 1). The reaction of 3 with **5a** was also performed in refluxing EtOH for comparison. Conventional heating greatly lengthened the reaction time (12 h), although the yield of the product (**6a**) was only slightly lower (85%) than in the MW-induced method. In the ^{1}H NMR spectrum of **6a** with a phenyl (Ph) group directly attached to the pyrazole-*N*, the signals of the aromatic protons were identified between 7.0 and 7.6 ppm with characteristic multiplicities, while the singlet of 5′-NH_2 appeared at around 5.0 ppm. According to the ^{13}C NMR (J-MOD) spectra of **6a**, the negative signal of C-17 was found at around 170 ppm, consistent with the chemical shift of a carbon in a pyridine-like environment (C = N) for similar pyrazole derivatives [6].

Table 1. Syntheses of D-ring-fused 5-amino-1-arylpyrazoles in the androstane series.



Entry	Ar-NH-NH_2	Ar	Product	Yield [1] (%)
1	5a	Ph	6a	90
2	5b	4-CH_3-C_6H_4	6b	89
3	5c	4-OMe-C_6H_4	6c	92
4	5d	4-F-C_6H_4	6d	94
5	5e	4-Cl-C_6H_4	6e	80
6	5f	4-Br-C_6H_4	6f	84
7	5g	4-CN-C_6H_4	6g	85
8	5h	4-NO_2-C_6H_4	6h	52 [2,a], 88 [2,b]

[1] After purification by column chromatography. [2] Reaction time (**a**) 10 min; (**b**) 20 min.

The reaction is considered to involve a nucleophilic addition of the terminal nitrogen of the arylhydrazine (**5**) to the carbonyl-C17 of **3** and subsequent dehydration to afford hydrazones, which then undergo intramolecular ring-closure by the attack of the internal nitrogen on the nitrile carbon to provide 5-aminopyrazole **6** (Table 1) [14]. According to the proposed mechanism, the rate of both consecutive reaction steps depends on the nucleophilicity of the reagent, which may be affected by the electron demand of the substituent of the aromatic ring. Therefore, similar reactions of 3 were also performed with para-substituted phenylhydrazine hydrochlorides (**5b–h**) in order to investigate the possible substituent effect on the heterocyclizations. All MW-assisted reactions were carried out in a closed vessel under identical conditions at 120 °C for 10 min. However, due to the elevated temperature and efficient heat transfer provided by the MW conditions, a marked substituent effect could not be observed, except for the reaction of 3 with para-nitrophenylhydrazine (**5h**) where the strong electron-withdrawing effect of the NO_2 group hampered the ring-closure to occur, and substantially decreased the yield of the desired product **6h** (Table 1, entry 8). However, complete conversion was also achieved in this case by prolonging the reaction time to 20 min. The novel D-ring-condensed arylpyrazoles (**6b–h**) were purified by column chromatography and characterized by NMR spectroscopy similarly to **6a**.

3.2. Proton Dissociation Processes

As expected, compounds **6a–h** have a poor aqueous solubility, according to UV-visible spectrophotometric titrations. This fact means that lower concentrations had to be used to monitor their proton dissociation processes, thus titrations were done under highly diluted conditions (10 μM) in aqueous solution (containing 1% (*v*/*v*) EtOH). Compounds displayed absorption bands in the UV region mostly due to π-π* transitions of the benzene ring and the pyrazole moiety. Representative UV spectra for **6a** are shown in Figure 2a. Deprotonation of the compounds resulted in characteristic spectral changes, namely the λ_{max} was increased (Table 2) and the absorbance decreased upon this process. Notably, precipitate appeared while increasing the pH (from pH > 5) in all cases except the nitro derivative **6h** (pH > 3.5). Thus, spectra recorded in the pH range 1.8–5 (or 3.5) were used for the calculations. The changes revealed one deprotonation step in the studied pH range. Computed molar spectra for the HL^+ and L ligand species of 6a are shown in Figure 2b.

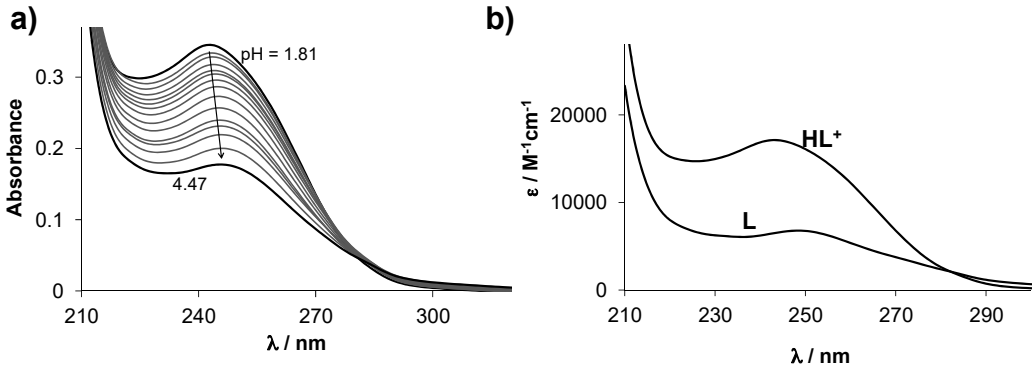


Figure 2. (**a**) UV spectra recorded for **6a** at various pH values; (**b**) Calculated molar absorbance spectra for the HL^+ and L species of **6a**. {c = 10 μM; l = 2 cm; t = 25 °C; I = 0.1 M (KCl); 1% (*v*/*v*) EtOH/H_2O}.

Proton dissociation constants (pK_a) were determined by the deconvolution of the measured spectra and are collected in Table 2. The determined pK_a values are attributed to the pyrazole N_2H^+ moiety, and its deprotonation results in the formation of the neutral L form with fairly limited solubility. The pK_a values vary by the different substituents of the benzene ring. The methoxy (**6b**), methyl (**6c**), and fluorine groups (**6d**) resulted in similar or somewhat lower pK_a value of the pyrazole nitrogen (Table 2, entries 2–4) compared with that of the reference compound **6a** (entry 1). On the other hand, the presence of the chloro (**6e**), bromo (**6f**), cyano (**6g**) and nitro substituents (**6h**) decreased the pK_a value significantly due to their strong electron-withdrawing effect (Table 2, entries 5–8).

In summary, all steroidal heterocycles (**6a**–**h**) are neutral (L) at pH 7.4 based on the determined pK_a values. Moreover, they are highly lipophilic and characterized by a poor water solubility. The lipophilic character and membrane permeability are important drug properties as they strongly affect the passage through biological membranes. Thus, we attempted to determine the $logD_{7.4}$ and permeability values by the traditional shake-flask method in *n*-octanol/H_2O (buffered aqueous solution, pH 7.40) and by the parallel artificial membrane permeability assay (PAMPA), respectively. All these compounds were found to be so lipophilic that $logD_{7.4}$ and permeability data could not be obtained experimentally, since practically the total quantity of the compounds remained in the *n*-octanol phase after the partitioning, which hindered the concentration determination in the aqueous phase. Therefore, $logD_{7.4}$ values were estimated for the compounds by the means of the MarvinSketch software [31] (Table 2). The predicted $logD_{7.4}$ values indicated that the aforementioned compounds are strongly lipophilic, which should be accompanied by very good membrane permeability.

Table 2. Proton dissociation constants (pK_a) of the compounds determined by UV-visible titrations, λ_{max} values (nm) of the ligand species in HL^+ and L forms and $logD_{7.4}$ (= logP) values predicted by the MarvinSketch software [31]. {t = 25 °C; I = 0.1 M (KCl); 1% (*v*/*v*) EtOH/water}.

Entry	Compd.	R	pK_a	λ_{max} (nm) of HL^+	λ_{max} (nm) of L	$logD_{7.4}$ (Predicted)
1	**6a**	H	3.97 ± 0.03	243	249	+4.31
2	**6b**	CH_3	3.37 ± 0.03	243	252	+4.77
3	**6c**	OMe	3.78 ± 0.06	240	244	+4.05
4	**6d**	F	3.56 ± 0.04	242	2212 [1]	+4.45
5	**6e**	Cl	2.00 ± 0.03	245	- [1]	+4.83
6	**6f**	Br	2.07 ± 0.06	247	- [1]	+5.10
7	**6g**	CN	<1.8	~260	~276	+4.12
8	**6h**	NO_2	2.13 ± 0.09	248, 289	236, 334	+4.26

[1] The spectrum of L has no characteristic absorption band in the studied wavelength range.

3.3. Pharmacological Studies

In order to screen the cancer cell-specific antiproliferative effect of the synthesized D-ring-fused steroidal 5-aminopyrazoles (**6a**–**h**), various human cancerous cell lines, namely MCF-7, PC-3, A549, HeLa and U2Os cells and MRC-5 noncancerous fibroblasts were treated with the test compounds in 5 μM concentration for 72 h. A heat map (Figure 3) was constructed based on the obtained viability data (Supplementary Material, Table S1). It was found that compound **6g** showed the most prominent antiproliferative activity and proved to be efficient on all the tested cancer cells. The other compounds exerted minor or fair cytotoxic effects and their cancer-specific antiproliferative features were much less pronounced than that of **6g**.

Based on the primary screen, compound **6g** was selected from the compound library. Then its cytotoxicity was evaluated more precisely on all aforementioned noncancerous and cancerous cell lines. The IC_{50} values of **6g,** especially those obtained on PC-3, HeLa, and U2Os cells are notably lower compared to that on MRC-5 noncancerous fibroblasts, despite falling in the same order of magnitude (Table 3, Supplementary Material, Figure S1a). Among all cancer cell lines, A549 showed the lowest, whereas U2Os displayed the highest sensitivity to **6g** treatment. As a positive control, the same set of cell lines was treated with the 'gold standard' cisplatin for 72 h, and the IC_{50} values were determined likewise (Table 3, Supplementary Material, Figure S1b). As expected, cisplatin exhibited strong antiproliferative activity at fairly low concentrations on the tested cell types, whereas compound **6g** was effective at concentrations in the μM range. It is well-known that a number of cancer cells exhibit resistance towards cisplatin treatment. In line with previous reports [32], we also observed that PC-3 cell lines displayed resistance to the applied cisplatin, indicated by the high IC_{50} value. Interestingly, compound **6g** effectively inhibited the proliferation of PC-3 cells, and the IC_{50} value of compound **6g** obtained on this particular cell line was comparable to its IC_{50} values on other tested cancer cells. No resistance toward molecule **6g** could be observed upon our experiments.

Apart from cancer cell resistance, the sensitivity of noncancerous cells can also cause complications. In fact, noncancerous MRC-5 cells were more sensitive to cisplatin treatment than cancerous cells. This was clearly demonstrated by the IC_{50} value for cisplatin on fibroblasts, being one magnitude smaller (37 nM) than any of its IC_{50} values on cancerous cells. However, noncancerous fibroblasts did not manifest higher sensitivity toward compound **6g**, yielding an advantageous feature for this test molecule over cisplatin.

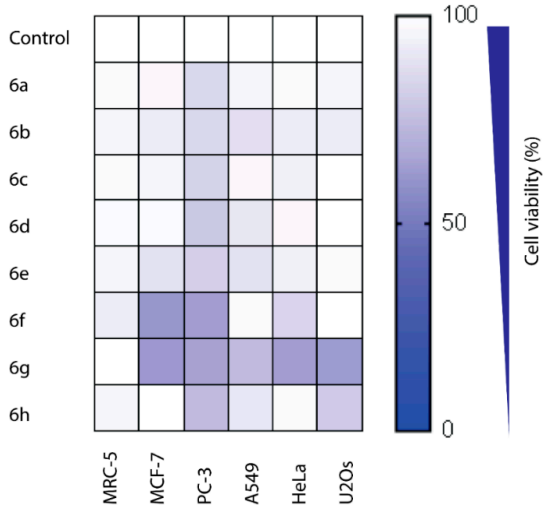


Figure 3. Primary antiproliferative effect of the steroidal D-ring-fused 5-aminopyrazoles on different cancerous cell lines and on MRC-5 fibroblasts represented as heat map {compound = 5 μM; 72 h incubation time}. Control represents the viability of cells receiving no treatment.

Table 3. IC_{50} (±SD) values of compound **6g** and of cisplatin assessed on noncancerous MRC-5 cells and on various cancer cell lines.

Compd.	IC_{50}					
	MRC-5	**MCF-7**	**PC-3**	**A549**	**HeLa**	**U2Os**
6g (μM)	8.9 ± 1.1	6.2 ± 1.0	4.9 ± 1.1	7.9 ± 1.0	4.0 ± 1.0	3.5 ± 1.0
Cisplatin (nM)	37.72 ± 1.2	338.5 ± 1.1	902 ± 1.6	369.8 ± 1.2	254.5 ± 1.2	342.8 ± 1.2

4. Conclusions

The main aim of the present study was to develop a multistep synthetic route for the preparation of novel D-ring-fused 5-aminopyrazoles (**6a**–**h**) in the androstane series involving a MW-assisted heterocyclization of a steroidal β-ketonitrile (3) with different arylhydrazines (**5a**–**h**) as the key step. Although the cyclization of β-ketonitriles with monosubstituted hydrazines usually needs a long reaction time (12–72 h), the applied MW-induced protocol allowed the high yield of the desired products within 10–20 min. The ring-closures were found not to be significantly influenced by the electron demand of the substituent R present on the aromatic ring of the reagents, with the only exception of the strong electron-withdrawing NO_2-group. Proton dissociation processes of the heterocycles were investigated by UV-visible spectrophotometric titrations in aqueous solution, as pK_a of a bioactive compound is a key physicochemical parameter affecting the pharmacokinetic properties. Moreover, based on its value, the actual protonation state can be established at physiological pH. Furthermore, the antiproliferative activity of the 5-aminopyrazoles was screened in vitro on different human cancer cell lines, along with a noncancerous cell line. Compound **6g** was selected for further studies as among the tested molecules this was the most potent in inhibiting the proliferation of the tested cancer cells, including cisplatin-resistant PC-3 prostate cancer cells. These results indicate that D-ring-fused 5-amino-1-arylpyrazoles can be considered as promising synthetic platforms for obtaining new active steroidal derivatives. Additionally, their biological significance, in particular those of the

CN-group-containing compounds, can be broadened to novel cancer therapy approaches. It is worth noting that the syntheses of similar derivatives with improved water solubility are in progress and the results will be published in the near future.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/1/229/s1, ^{1}H NMR and ^{13}C NMR spectra of the synthesized compounds, Table S1: Mean ± SD values of primary growth inhibitory screen (given as cell viability) used for heat map construction, Figure S1: Representative cell viability curves to determine growth inhibition and IC_{50} values following treatments with (a) compounds **6g** or (b) cisplatin on different cell lines.

Author Contributions: Conceptualization, É.F.; M.K. and É.A.E.; Chemical synthesis and optimization experiments, G.M. and Á.B.; Pharmacological studies, M.K.G. and D.I.A.; UV-visible spectrophotometric titrations, M.A.M.; Structural analysis, G.M.; Formal analysis and interpretation of data, Á.B., D.I.A. and M.A.M.; Methodology, resources, supervision, É.F.; M.K. and É.A.E.; Writing—original draft preparation, G.M. and M.K.G.; Writing—review and editing, É.F.; M.K. and É.A.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: This work was supported by the National Research, Development and Innovation Office-NKFIA through projects GINOP-2.3.2-15-2016-00038 and FIKP program TUDFO/47138-1/2019-ITM. M.A.M. thanks the support of Visegrád Scholarship Program. The research of G.M. was supported by the National Talent Program of the Hungarian Ministry of Human Capacities.

Conflicts of Interest: The authors declare no conflict of interest.

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