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Investigation of pH and substituent effects on the distribution ratio of novel steroidal ring D- and A-fused arylpyrazole regioisomers and evaluation of their cell-growth inhibitory effects *in vitro*

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ABSTRACT

Novel androstanopyrazoles have been efficiently synthesized from steroidal β-ketoaldehydes with different arylhydrazine hydrochlorides both under acidic and basic conditions. Knorr-type transformations of 16-hydroxymethylene-dehydroepiandrosterone containing its 1,3-dicarbonyl moiety on ring D, proved to be regioselective in pyridine at room temperature, while mixtures of regioisomers were obtained in acidic EtOH under reflux. Contrarily, the cyclocondensation reactions of 2-hydroxymethylene-dihydrotestosterone bearing its reactive functionalities on ring A, led to a mixture of pyrazole regioisomers in varying ratio depending on the applied medium. The regioisomeric distribution was found to depend on the electronic character of the substituent of the phenylhydrazine applied. After separating the related isomers by column chromatography, they were subjected to *in vitro* pharmacological studies to investigate their antiproliferative activities against three human breast malignant cell lines (MCF7, T47D, MDA-MB-231). Flow cytometry revealed that the most potent agents elicited a cell cycle disturbance on MDA-MB-231 and T47D cells.

1. Introduction

Pyrazoles represent an important class amongst the five-membered *N*-containing heterocycles in view of the potentially high bioactivity profile stemming from their structures. Numerous compounds containing this structural motif are known to display anti-inflammatory, antihypertensive, antibacterial, anticonvulsant, antidepressant, anticancer, or other effects [1,2]. The incorporation of a pyrazole moiety into the relatively rigid and planar backbone of natural sex hormones or their semisynthetic analogs, either connected to or condensed with one of the rings, also deserves attention from pharmacological aspects. Some 17-*exo*-pyrazolylandrostanes based on the structure of natural pregnanes, are potent inhibitors of 17α-hydroxylase-$C_{17,20}$-lyase, a key regulatory enzyme of androgen hormone biosynthesis and therefore can be effective in the treatment of castrate resistant prostate cancer [3,4]. Furthermore, a number of estrone-fused pyrazoles have been reported to exert inhibitory effect against 17β-hydroxysteroid dehydrogenase type I [5], another important steroidogenic enzyme, which can be an attractive target for estrogen suppression in breast cancer. Some 16,17-pyrazolo-annullated androstanes have been found to act as effective vascular endothelial growth factor inhibitors on MDA-MB-231 breast cancer cells [6]. Stanozolol, a modified derivative of dihydrotestosterone with a 2,3-condensed *N*,*N*-heteroaromatic ring, is one of the best-known synthetic anabolic steroids, which has been used widely for both therapeutic and performance enhancing purposes [7]. Its possible application in the treatment of advanced breast cancer had also been raised [8]. However, it failed to enter into medical practice due to its masculinizing side-effects and the development of clinically relevant drugs, such as aromatase inhibitors or estrogen receptor antagonists. The inhibitory action of structurally modified androgens on cell proliferation is known to result, in part, from their suppressive effect on expression of the estrogen receptor [9]. However, some of them have also been reported to exhibit direct antiproliferative effects on breast cancer cell lines in a hormone receptor independent manner by causing a global slowing effect on the duration of the cell cycle [10,11]. The presence of the 17β-hydroxy group of natural or semisynthetic androgens is essential for the ligand–androgen receptor interaction, and the modification of the 3-keto substituent results in a

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significant decrease in the androgenic activity [12,13]. Consequently, the reduction or elimination of hormonal effect can be achieved by the incorporation of bulky heterorings condensed to 2,3 or 16,17 positions.

The oldest procedure for the synthesis of pyrazoles is based on the cyclocondensation of 1,3-dicarbonyl compounds with monosubstituted hydrazines [14]. This Knorr-type reaction of unsymmetrical 1,3-diketones or β-ketoaldehydes often leads to the formation of a mixture of pyrazole regioisomers. The relative ratio of the two products is usually influenced by both the reaction conditions and the structural features of the reactants. The reaction is typically conducted in polar, protic alcohols in acidic or neutral medium or in AcOH, and only a few examples can be found for the application of other solvents [15,16]. Under these conditions, however, longer reaction time or elevated temperature is usually needed for sufficient conversion. The mechanistic details of the condensation are rather complex and the regioisomeric distribution as well as the rate-determining step can be shifted by modulating acidity or varying the substituents of the reaction partners [17]. From a chemical aspect, the control of the condensation to produce a single regioisomer is desirable in order to avoid difficult purification procedures. Nevertheless, the access to both isomers and the comparison of their bioactivities after separation can be of interest from a pharmacological point of view.

On the basis of the aforementioned literature background and our interest in obtaining novel steroidal ring A- and D-fused heterocycles with remarkable antiproliferative effects, we now report a facile synthesis of novel arylpyrazolo[16,17]dehydroepiandrosterone and arylpyrazolo[2,3]dihydrotestosterone regioisomers from steroidal β-ketoaldehydes and arylhydrazines. The isomeric distribution was examined both under acidic and mildly basic conditions at different temperatures. Our further goal was to investigate the electronic effect of the substituent of the aromatic ring of hydrazines on the reaction rate and the regioisomeric ratio. The cell-growth-inhibitory effects of all synthesized compounds were screened *in vitro* against three breast cancer cell lines (MCF7, T47D and MDA-MB-231) by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [18]. The most potent derivatives were further investigated by flow cytometric cell cycle analysis.

2. Experimental

2.1. General

All solvents were distilled shortly prior to use. Reagents and materials were obtained from commercial suppliers and used without purification. Reactions were monitored by TLC on Kieselgel-G (Merck Si 254F) layers (0.25 mm thick); solvent systems (ss): (A) EtOAc/CH_2Cl_2 = (5:95 v/v), (B) EtOAc/CH_2Cl_2 (10:90 v/v) and (C) EtOAc/CH_2Cl_2 (20:80 v/v). The spots were detected by spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid. Flash chromatography: Merck silica gel 60, 40–63 μm. Melting points (Mps) were determined on an SRS Optimelt digital apparatus and are uncorrected. Elementary analysis data were determined with a PerkinElmer CHN analyzer model 2400 and FT-IR spectra were recorded on a FT/IR-4700 spectrometer (Jasco) using ATR. Infrared absorbance is reported in cm^{-1}. NMR spectra were obtained at room temperature with a Bruker DRX 500 instrument. Chemical shifts are reported in ppm (δ scale), and coupling constants (J) in Hz. The multiplicities of the ^1H resonance peaks are indicated as a singlet (s), a doublet (d), a double doublet (dd), a triplet (t) or a multiplet (m). ^{13}C NMR spectra are ^1H-decoupled. 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 ion mode. The ESI parameters were as follows: nebulizing gas N_2, at 35 psi; drying gas N_2, at 350 °C and 12 L/min; capillary voltage 3000 V; fragmentor voltage 70 V. The MSD was operated in scan mode with a mass range of m/z 60–620. Samples (0.2 μL) with automated needle wash were injected directly into the solvent flow (0.3 mL/min) of CH_3CN/H_2O 70:30 (v/v) supplemented with 0.1% formic acid. The system was controlled by Agilent LC/MSD Chemstation software.

2.2. General procdure for the reactions of 2 with arylhydrazine hydrochlorides (3a–h·HCl) or 7 with phenylhydrazine hydrochloride (3a) in acidic EtOH

16-Hydroxymethylene-androst-5-en-3β-ol-17-one (**2**, 1.00 mmol, 316 mg) or 2-hydroxymethylene-5α-androst-17β-ol-3-one (**7**, 1.00 mmol, 318 mg) and *p*-toluenesulfonic acid (PTSA, 57 mg, 0.3 equiv.) were dissolved in EtOH (10 mL) and (substituted) phenylhydrazine hydrochloride (**3a–h**, 1.1 equiv.) was added. The reaction mixture was kept at reflux temperature (in case of **2**) or stirred at room temperature (in case of **7**) for 10 min. During work-up, the reaction mixture was poured into water, neutralised with $NaHCO_3$ and extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4. After evaporation, the crude product was purified by column chromatography with EtOAc/CH_2Cl_2 = 10:90 to give **4a–h** and **5a–h** or **8a** and **9a**. In all cases, compounds **5a–h** or **9a** were the first-eluting, less polar isomers.

2.2.1. 3β-Hydroxy-1′-phenylpyrazolo[4′,5′:16,17]androst-5-ene (4a) and 3β-hydroxy-1′-phenylpyrazolo[4′,3′:16,17]androst-5-ene (5a)

Compound **2** and phenylhydrazine hydrochloride (159 mg) were used for the synthesis. Yields: **4a** (171 mg, 44%) and **5a** (160 mg, 41%).

4a [12]: White solid; Mp 229–231 °C (lit. [12] 232–235 °C); R_f 0.34 (ss C); IR (cm^{-1}): 3194 (OH), 1600 (Ph C═C), 1538 (C═N), 1505 (Ph C═C), 1066, 1056 (C—O). ^1H and ^{13}C NMR (solvent: $CDCl_3$) spectral data correlate well with the literature values [12].

5a: White solid; Mp 178–181 °C; R_f 0.46 (ss C); IR (cm^{-1}): 3287 (OH), 1599 (Ph C═C), 1581 (C═N), 1507 (Ph C═C), 1054, 1036 (C—O). Anal. Calcd. for $C_{26}H_{32}N_2O$ (388.251): C, 80.37; H, 8.30. Found: C, 80.24; H, 8.16. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.09 (s, 3H) and 1.10 (s, 3H): 18-H_3 and 19-H_3, 1.12–2.43 (overlapping m, 16H), 2.62 (dd, 1H, J = 14.1 Hz, J = 5.8 Hz, one of 15-H_2), 3.54 (m, 1H, 3-H), 5.39 (m, 1H, 6-H), 7.20 (m, 1H, 4″-H), 7.39 (m, 2H, 3″-H and 5″-H), 7.51 (s, 1H, 5′-H), 7.61 (d, 2H, J = 7.6 Hz, 2″-H and 6″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 18.0 (C-18), 19.4 (C-19), 20.6 (CH_2), 24.2 (CH_2), 30.9 (CH), 31.4 (CH_2), 31.6 (CH_2), 33.9 (CH_2), 36.8 (C-10), 37.2 (CH_2), 40.7 (C-13), 42.3 (CH_2), 50.5 (CH), 62.0 (CH), 71.6 (C-3), 119.0 (2 C, C-2″ and C-6″), 121.0 (2 C, C-6 and C-5′), 124.4 (C-16), 125.4 (C-4″), 129.3 (2 C, C-3″ and C-5″), 140.8 (C-1″), 141.2 (C-5), 170.8 (C-17); ESI-MS 389 $[M+H]^+$.

2.2.2. 3β-Hydroxy-1′-(4″-tolyl)-pyrazolo[4′,5′:16,17]androst-5-ene (4b) and 3β-hydroxy-1′-(4″-tolyl)-pyrazolo[4′,3′:16,17]androst-5-ene (5b)

Compound **2** and 4-tolylhydrazine hydrochloride (174 mg) were used for the synthesis. Yields: **4b** (129 mg, 32%) and **5b** (222 mg, 55%).

4b: White solid; Mp 248–251 °C; R_f 0.28 (ss B); IR (cm^{-1}): 3485 (OH), 1601 (Ph C═C), 1533 (C═N), 1520 (Ph C═C), 1055, 1040 (C—O). Anal. Calcd. for $C_{27}H_{34}N_2O$ (402.267): C, 80.55; H, 8.51. Found: C, 80.73; H, 8.42. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.04 (s, 3H, 18-CH_3), 1.06 (s, 3H, 19-CH_3), 1.10 (m, 2H), 1.45–2.31 (overlapping m, 14H), 2.38 (s, 3H, 4″-CH_3), 2.56 (dd, 1H, J = 13.6 Hz, J = 6.1 Hz, one of 15-H_2), 3.51 (m, 1H, 3-H), 5.38 (m, 1H, 6-H), 7.22 (d, 2H, J = 7.9 Hz, 2″-H and 6″-H), 7.37 (s, 1H, 3′-H), 7.38 (d, 2H, J = 7.9 Hz, 3″- and 5″-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 17.5 (C-18), 19.3 (C-19), 20.3 (CH_2), 21.0 (4″-CH_3), 24.2 (CH_2), 30.7 (CH), 31.2 (CH_2), 31.5 (CH_2), 34.7 (CH_2), 36.6 (C-10), 37.0 (CH_2), 42.2 (CH_2), 42.3 (C-13), 50.2 (CH), 63.0 (CH), 71.5 (C-3), 121.0 (C-6), 123.5 (2 C, C-2″ and C-6″), 125.8 (C-16), 129.4 (2 C, C-3″ and C-5″), 135.0 (C-3′), 137.2 and 137.7: C-1″ and C-4″, 141.0 (C-5), 157.2 (C-17); ESI-MS 403 $[M+H]^+$.

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