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# MODIFICATION OF PREGNENOLONE INTO 5A-ANDROSTANO [17,16-D] PYRAZOLINES: BIOACTIVITY INSIGHT

Nanuli Nadaraia [1], Nana Barbakadze [1,*], Meri Kakhabrishvili [1], Jean Legault [2], Karen Mulkijanyan [3] and Vakhtang Mshvildadze [2,4]

[1] Department of Plant Biopolymers and Chemical Modification of Natural Compounds, Tbilisi State Medical University, I. Kutateladze Institute of Pharmacochemistry, Tbilisi 0159, Georgia.
[2] LASEVE Laboratory, Fundamental Sciences Department, Quebec University at Chicoutimi, Chicoutimi, QC, Canada.
[3] Department of Preclinical Pharmacological Research, Tbilisi State Medical University, I. Kutateladze Institute of Pharmacochemistry, Tbilisi 0159, Georgia.
[4] Department of Phytochemical Research, Tbilisi State Medical University, I. Kutateladze Institute of Pharmacochemistry, Tbilisi 0159, Georgia.
* Corresponding Author

ORCID Details
Nanuli Nadaraia: https://orcid.org/0000-0002-1228-9556
Nana Barbakadze: https://orcid.org/0000-0001-8051-0606
Jean Legault: https://orcid.org/0000-0001-6292-6798
Karen Mulkijanyan: https://orcid.org/0000-0001-9360-3422
Vaktang Mshvildadze: https://orcid.org/0000-0002-4239-4832

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## ABSTRACT

In order to search for new biologically active steroids from pregn-16-en-3β-ol-20-one, some isomeric derivatives of 5α-androstanopyrazolines were synthesized and their cytotoxic, anti-inflammatory activity was studied. The structures of the synthesized compounds are proven by NMR data of 1H, 13C and mass spectra.

**Keywords:** Synthesis, Steroidal Pyrazolines, Cytotoxic and Anti-Inflammatory Activity

## 1 INTRODUCTION

It is well-established that steroid-derived systems offer several advantages including enhanced membrane permeability, greater bioavailability, and lower systemic toxicity [1-3]. Consequently, steroid structures have become a valuable framework for creating bioactive molecules. The incorporation of heterocyclic moieties into steroidal skeletons represents an important strategy in the design of new compounds with improved or modified biological properties. Among various

nitrogen-containing heterocycles, pyrazolines have received considerable attention. Pyrazoline derivatives have been investigated for a broad range of biological activities, including antimicrobial, anti-inflammatory, anti-oxidant, antitumor, analgesic and enzyme-inhibitory effects [4-8].

Several studies have shown that steroid pyrazolines have significant biological potential. In particular, modifications of the steroid ring lead to compounds with known anticancer, anti-inflammatory activity [9-17]. It is noteworthy that several 5α-steroidal pyrazolines previously synthesized by us exhibited significant cytotoxic activity [18-20].

The continuing interest in steroidal pyrazolines is consequently associated with both their synthetic versatility and their potential pharmaceutical applications. Investigation of new derivatives of steroid-pyrazoline hybrids may contribute to the discovery of biologically active molecules with novel mechanisms of action and improved pharmacological profiles. For this reason, the synthesis, structural characterization, and biological evaluation of steroidal pyrazolines remain important areas of research in contemporary organic and medicinal chemistry.

In this study we report the syntheses of several 5α-steroidal pyrazolines and the investigation evaluation of their cytotoxic and anti-inflammatory properties.

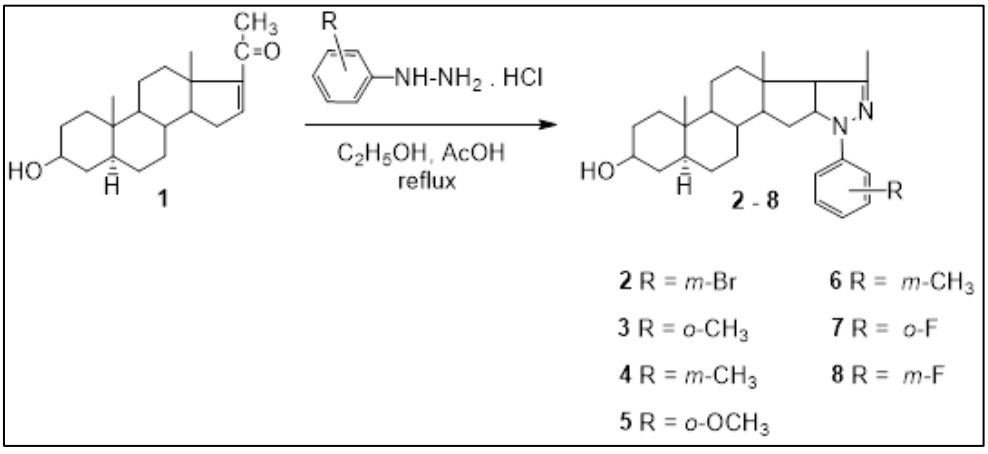
## 2 MATERIALS AND METHODS

Melting points were determined on a NAGEMA apparatus. $^{1}H$ NMR and $^{13}C$ NMR spectra were recorded in $CDCl_3$ with TMS internal standard on a Bruker Avance 400 instrument (400.13 MHz for $^{1}H$ and 100.61 MHz for $^{13}C$). Mass spectra were obtained on an Agilent 1100 series HPLC-APCI-MS (positive-ion mode) using an Inertsil prep-ODS C18 column (4.6 × 250 mm; particle diameter 5 μm) thermostatted at 25°C with elution of steroids by $H_2O$–MeCN (20:80). The course of reactions and purity of products were monitored by TLC on Silica gel plates ($F_{254}$; Merck, Germany) using $C_6H_6$–$Me_2CO$ (10:1) and $C_6H_6$–MeOH (5:0.5) with detection by ethanolic phosphomolybdic acid (10%) and heating at 95–100°C for 2–3 min.

**General Method for Synthesizing 5α-androstanopyrazolines 2–8.** A solution of steroid **1** and the corresponding hydrazine (equal to the steroid weight) in EtOH was refluxed for 4–6 h with a catalytic amount of AcOH, cooled, treated with $H_2O$, and left overnight. The resulting precipitate was filtered off, washed with $H_2O$, dried in air, and crystallized from EtOH.

### 2.1 The synthesis of the 5α-androstano[16,17-d]pyrazolines

Pregnenolone **1** derived from tigogenin [21] was used as the starting material and was transformed into 5α-androstano[16,17-d]pyrazolines **2–8** (Scheme 1) through reactions with various isomeric arylhydrazines, including *m*-bromo-, *o*-methyl, *m*-methyl, *o*-methoxy, *m*-methoxy-, *o*-fluoro and *m*-fluorophenylhydrazine. The reactions were carried out in ethanol in the presence of a catalytic amount of acetic acid. Under these reaction conditions, hydrazone intermediates are initially formed from the unsaturated steroidal ketones. These intermediates subsequently undergo intramolecular cyclocondensation, resulting in the formation of the pyrazoline ring. Cyclization takes place immediately following hydrazone formation and is promoted by the presence of electron-donating substituents attached to the amino nitrogen of the arylhydrazine. The one-pot nature of this transformation provided a convenient approach to the preparation of the target compounds.




**Scheme 1: The synthesis of the 5α-androstano[16,17-d]pyrazolines 2-8**

### 2.2 The in vitro evaluation of biological activity of 5α-androstano[16,17-d]pyrazolines.

Cytotoxic activity of compounds **2–8** was studied against WS1 human skin fibroblasts (ATCC-CRL-1502), A-549 lung adenocarcinoma (ATCC-CCL-185), and DLD-1 colorectal adenocarcinoma (ATCC-CCL-221) cell lines obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultivated in medium containing Earle's salts (Merck KGaA, Darmstadt, Germany), L-glutamine (Cellgro, Mediatech, VA), fetal bovine serum (FBS, 10%, HyClone), vitamins, penicillin (100 U/mL), streptomycin (100 mg/mL), essential amino acids, and sodium pyruvate (Cellgro, Mediatech, VA). Cells were incubated under standard conditions at 37°C, 90% humidity, and 5% $CO_2$.

#### 2.2.1 Evaluation of cytotoxic activity.

Exponentially growing cells were inoculated into 96-well microplates (BD Falcon) with $5 \times 10^3$ cells per well in culture medium (100 mL, DMEM with 10% FBS). Before the 48 h incubation the cells, the increasing concentrations of each compound in DMSO (100 µL/well) were added. The DMSO concentration in the culture medium was held at 0.25% to avoid its toxic effect. A Fluoroskan Ascent F1TM automated 96-well plate reader (Labsystems, Milford, MA, USA) was used to measure cytotoxicity based on the decrease in resazurin fluorescence at excitation and emission wavelengths of 530 and 590 nm, respectively [22]. Cell viability percent was determined by comparing the fluorescence in wells treated with the tested compounds and doxorubicin, respectively. Further, the cellular DNA was determined by the fluorescence of Hoechst 33342 stain (excitation and emission wavelengths 352 and 461 nm, respectively). Each experiment was performed in triplicate. The final results were expressed as mean $IC_{50}$ values (concentration causing 50% growth inhibition of cells) ± standard deviation.

#### 2.2.2 Evaluation of anti-inflammatory activity.

Anti-inflammatory activity was assessed from inhibition of NO production [22]. RAW 264.7 murine macrophages (ATCC« TIB-71; Manassas, VA, USA) were incubated with increasing steroids concentrations before being activated by 100 ng/mL of lipopolysaccharide (LPS). N-ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) at two distinct concentrations of 250 µM (67 µg/mL) and 1 mM (270 µg/mL) served as positive control. Cell-free supernatants were collected 24 h after LPS stimulation. The NO concentration was determined using the Griess reaction. Aliquots (100 mL) of cell supernatants were incubated with a mixture (100 mL) of sulfanilamide (1%) in $H_3PO_4$ (2.5%) and N-1-naphthylethylenediamine hydrochloride (0.1%) in $H_2O$ at room temperature for 20 min. Absorption was measured at 540 nm by a Multiskan GO plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Nitrite was quantitatively determined using a standard $NaNO_2$ curve. Inactivated (subjected to the action of only medium) and LPS stimulated cells were used as a negative and a positive control, respectively.

## 3 RESULTS AND DISCUSSION

### 3.1 Structural analysis of 5α-androstano[16,17-d]pyrazolines 2–8

Structural characterization of the synthesized 5α-androstano[16,17-d]pyrazolines **2–8** was performed using $^1H$ and $^{13}C$ NMR and mass spectrometry data. The formation of the pyrazoline ring was supported by the disappearance or significant alteration of the characteristic signals associated with the starting carbonyl functionality and the appearance of new signals attributable to the newly formed heterocyclic system.

In the $^1H$ NMR spectra of pyrazolines **2-8**, signals for the 18-$CH_3$, 19-$CH_3$, and 21-$CH_3$ methyl groups appeared at δ 0.81–0.78, 0.95–0.90, and 2.05–2.04 ppm, respectively. The multiplet signals of the 3α-protons in the 3β-alcohols were observed at δ 3.58–3.56 ppm. Signals for the hydrogen atoms at C-17 were noted in the 3.19–3.13 ppm range, while signals for the hydrogen atoms at C-16 appeared at 4.87–4.43 ppm. Aromatic protons appeared in the 7.34–6.31 ppm range. Signals for the remaining protons were consistent with the proposed structures.

In the $^{13}C$ NMR spectra of steroidal pyrazolines **2–8**, peaks corresponding to aromatic carbons were present in the 160.6–97.8 ppm range. Signals for the C=N bond of the heterocycle appeared in the 150.6–149.3 ppm range. Signals for C-3 were observed at δ 71.2 ppm, while peaks for C-16 and C-17 appeared at 65.8–63.8 ppm and 66.8–66.3 ppm, respectively.

Mass spectra confirm the empirical formulas of compounds **2–8**.

#### 3.1.1 Experimental part

- **3β-Hydroxy-1′-*m*-bromophenyl-3′-methyl-5α-androstano[17,16-d]pyrazoline (2).** Yield 80%, m.p. 221-223°C. $^1H$ NMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.81 (3H, s, 18-$CH_3$), 0.93 (3H, s, 19-$CH_3$), 2.04 (3H, s, 21-$CH_3$), 3.18 (1H, d, J= 10.2, H-17), 3.57 (1H, m, H-3), 4.43 (1H, dd, J=10.2, 5.8, H-16), 6.83-7.11 (4H, H-Ar). $^{13}C$ NMR

spectrum ($CDCl_3$, δ, ppm): 63.8 (C-16), 66.5 (C-17), 71.2 (C-3), 110.3 (C-2//), 114.6 (C-6//), 120.3 (C-4//), 123.2 (C-3//), 130.3 (C-5//), 146.0 (C-1//), 150.6 (C=N). LC-MS m/z 486 $[M+H]^+$, $C_{27}H_{37}BrN_2O$. MM 485.

- **3β-Hydroxy-1/-*o*-methylphenyl-3/-methyl-5α-androstano[17,16-d]pyrazoline (3).** Yield 63%, m.p. 105-107°C. $^1H$ NMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.78 (3H, s, 18-$CH_3$), 0.90 (3H, s, 19-$CH_3$), 2.04 (3H, s, 21-$CH_3$), 2.28 (3H, s 2//-$CH_3$), 3.13 (1H, d, J = 10.1, H-17), 3.56 (1H, m, H-3), 4.69 (1H, dd, J=10.2, 5.8, H-16), 6.90-7.12 (4H, H-Ar). $^{13}C$ NMR spectrum ($CDCl_3$, δ, ppm): 65.3 (C-16), 66.3 (C-17), 71.2 (C-3), 120.1 (C-6//), 122.4 (C-4//), 126.2 (C-2//), 129.3 (5//), 131.0 (C-3//), 144.8 (C-1//), 149.7 (C=N). LC-MS m/z 421$[M+H]^+$, $C_{28}H_{40}N_2O$. MM 420.
- **3β-Hydroxy-1/-*m*-methylphenyl-3/-methyl-5α-androstano[17,16-d]pyrazoline (4).** Yield 70%, m.p. 194-196°C. $^1H$ NMR spectrum ($CDCl_3$, , ppm, J/Hz): 0.81 (3H, s, 18-$CH_3$), 0.94 (3H, s, 19-$CH_3$), 2.05 (3H, s, 21-$CH_3$), 2.32 (3H, s, 3//-$CH_3$), 3.18 (1H, d, J = 10.2, H-17), 3.57 (1H, m, H-3), 4.46 (1H, dd, J=10.2, 5.8, H-16), 6.57-7.13 (4H, H-Ar). $^{13}C$ NMR spectrum ($CDCl_3$, δ, ppm): 64.0 (C-16), 66.3 (C-17), 71.2 (C-3), 108.8 (C-2//), 112.9 (C-6//), 118.6 (C-4//), 128.9 (5//), 138.9 (3//), 145.0 (1//), 149.3 (C=N). LC-MS m/z 421$[M+H]^+$, $C_{28}H_{40}N_2O$. MM 420.
- **3β-Hydroxy-1/-*o*-methoxyphenyl-3/-methyl-5α-androstano[17,16-d]pyrazoline (5).** Yield 73%, m.p. 129-131°C. $^1H$ NMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.78 (3H, s, 18-$CH_3$), 0.95 (3H, s, 19-$CH_3$), 2.05 (3H, s, 21-$CH_3$), 3.16 (1H, d, J = 10.1, H-17), 3.56 (1H, d, H-3), 3.81 (3H, d, 3//-$OCH_3$), 4.87 (1H, dd, J=10.1, 5.7, H-16), 6.83-7.23 (4H, H-Ar). $^{13}C$ NMR spectrum ($CDCl_3$, δ, ppm): 55.7 ($OCH_3$), 65.7 (C-16), 66.6 (C-17), 71.2 (C-3), 111.5 (C-3//), 119.9 (C-6//), 121.2 (C-4//), 121.8 (C-5//), 135.6 (C-1//), 149.1 (C=N), 150.0 (C-2//). LC-MS m/z 437$[M+H]^+$, $C_{28}H_{40}N_2O_2$. MM 436.
- **3β-Hydroxy-1/-*m*-methoxyphenyl-3/-methyl-5α-androstano[17,16-d]pyrazoline (6).** Yield 68%, m.p. 125-127°C. $^1H$ NMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.81 (3H, s, 18-$CH_3$), 0.95 (3H, s, 19-$CH_3$), 2.04 (3H, s, 21-$CH_3$), 3.18 (1H, d, J = 10.1, H-17), 3.57 (1H, d, H-3), 3.80 (3H, s, 3//-$OCH_3$), 4.46 (1H, dd, J=10.1, 5.7, H-16), 6.31-7.14 (4H, H-Ar). $^{13}C$ NMR spectrum ($CDCl_3$, δ, ppm): 55.2 ($OCH_3$), 64.1 (C-16), 66.4 (C-17), 71.2 (C-3), 97.8 (C-2//), 103.3 (C-6//), 104.6 (C-4//), 129.8 (C-5//), 146.3 (C-1//), 149.6 (C=N), 160.6 (C-3//). LC-MS m/z 437$[M+H]^+$, $C_{28}H_{40}N_2O_2$. MM 436.
- **3β-Hydroxy-1/-*o*-fluorophenyl-3/-methyl-5α-androstano[17,16-d]pyrazoline (7).** Yield 69%, m.p. 145-147°C. $^1H$ NMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.80 (3H, s, 18-$CH_3$), 0.91 (3H, s, 19-$CH_3$), 2.06 (3H, s, 21-$CH_3$), 3.19 (1H, d, J = 10.1, H-17), 3.57 (1H, m, H-3), 4.81(1H, dd, J=10.1, 5.8, H-16), 6.76-7.34 (4H, H-Ar). $^{13}C$ NMR spectrum ($CDCl_3$, δ, ppm): 65.8 (C-16), 66.8 (C-17), 71.2 (C-3), 116.2 (d, J=7.2, C-6//), 118.3 (d, J = 22.2, C-3//), 119.6 (d, J=7.1, C-4//), 124.5 (d, J = 3.0, C-5//), 129.4 (d, J = 10.2, C-1//), 149.4 (C=N), 150.4 (d, J = 240, C-2//). LC-MS m/z 425 $[M+H]^+$, $C_{27}H_{37}FN_2O$. MM 424.
- **3β-Hydroxy-1/-*m*-fluorophenyl-3/-methyl-5α-androstano[17,16-d]pyrazoline (8).** Yield 72%, m.p. 242-244°C. $^1H$ NMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.81 (3H, s, 18-$CH_3$), 0.94 (3H, s, 19-$CH_3$), 2.05 (3H, s, 21-$CH_3$), 3.19 (1H, d, J = 10.1, H-17), 3.58 (1H, m, H-3), 4.44 (1H, dd, J=10.1, 5.8, H-16), 6.40-7.17 (4H, H-Ar). $^{13}C$ NMR spectrum ($CDCl_3$, δ, ppm): 64.0 (C-16), 66.5 (C-17), 71.2 (C-3), 99.0 (d, C-2//, J=22.1), 104.1 (d, C-4//, J = 22.3), 107.3 (d, C-6//, J = 2.9), 130.1 (d, C-5//, J = 7.3), 146.5 (d, C-1//, J = 7.2), 150.4 (C=N), 163.9 (d, C-3//, J=245). LC-MS m/z 425$[M+H]^+$, $C_{27}H_{37}FN_2O$. MM 424.

### 3.2 Biological profile of 5α-androstano[16,17-d]pyrazolines 2–8

Cytotoxic activity of compounds **2-8** was studied against W-1 human skin fibroblasts (ATCC-CRL-1502 line), A-549 lung adenocarcinoma (ATCC-CCL-185), and DLD-1 colorectal adenocarcinoma (ATCC-CCL-221). All tested compounds revealed no meaningful cytotoxic activity comparable to that of doxorubicin (DOX). (Table 1)

**Table 1: Cytotoxic activity of compounds 2–8.**

| **Comp№** | **Resazurine** | | | **Hoechst** | | |
|---|---|---|---|---|---|---|
| | A-549 | DLD-1 | WS-1 | A-549 | DLD-1 | WS-1 |
| 2 | 87 ± 9 μM | 45 ± 4 μM | >100 μM | 98 ± 7 μM | 45 ± 7 μM | >100 μM |
| 3 | 47 ± 3 μM | 50,6 ± 0,2 μM | 41 ± 6 mM | 44 ± 4 μM | 37 ± 4 μM | 35 ± 1 μM |
| 4 | 48 ± 3 μM | 36,8 ± 0,9 μM | 47 ± 4 μg/ml | 57 ± 2 μM | 30 ± 4 μM | 60 ± 3 μM |
| 5 | 43 ± 5 μM | 51 ± 1 μM | 31 ± 3 μM | 44 ± 1 μM | 37 ± 3 μM | 36 ± 2 μM |
| 6 | 67 ± 2 μM | >100 μM | 47 ± 4 μM | 95 ± 7 μM | >100 μM | 62 ± 4 μM |
| 7 | 17,8 ± 0,4 μM | 49 ± 3 μM | 13,4 ± 0,7 μM | 24 ± 1 μM | 33 ± 3 μM | 14,5 ± 0,2 μM |
| 8 | 38 ± 2 μM | 26 ± 1 μM | 45 ± 1 μM | 44,1 ± 0,8 μM | 28 ± 1 μM | 46,8 ± 0,4 μM |
| DOX | 5,1 ± 0,4 μM | 1,04 ± 0,06 μM | 2,0 ± 0,2 μM | 3,9 ± 0,8 μM | 1,07 ± 0,09 μM | 2,0 ± 0,2 μM |

Anti-inflammatory activity of steroids **2-8** *in vitro* was assessed by inhibition of nitric oxide (NO) by LPS-stimulated RAW 264.7 murine macrophages (ATCC-TIB-71) as compared to the nonselective NO-synthase inhibitor L-NAME. All of the investigated compounds revealed strong anti-inflammatory effect, and among them hydrazones **3, 7** and **8** (99, 95 and 83%, respectively) appeared to be most active (Table 2).

**Table 2: Anti-inflammatory activity of compounds 2–8.**

| Compound № | IC50 | % inhibition of NO |
|---|---|---|
| 2 | >80 μM | 48% |
| 3 | 27 ± 2 μM | 99% |
| 4 | 41 ± 2 μM | 68% |
| 5 | 35 ± 3 μM | 60% |
| 6 | >80 μM | 18% |
| 7 | 41 ± 5 μM | 95% |
| 8 | 29 ± 2 μM | 83% |
| L-NAME (250 μM) | | 42 % |
| L-NAME (1 mM) | | 78 % |

**Synthesized compounds 3 and 4, 5 and 6, 7 and 8 represent structural *o*- and *m*-isomers of each other with respect to the $CH_3$-, $OCH_3$- and F-groups substituted in the aromatic ring of pyrazoline.** As shown by the biological experiment, based on the structure-activity relationship, the *o*-isomer is significantly more active than the *m*-isomer in all three cases. Additionally, it is noteworthy that both isomers of the F-substituted pyrazolines, 7 and 8, exhibit high anti-inflammatory activity (**95% and 83%**, respectively).

## 4 CONCLUSION

**Seven novel steroid pyrazolines** were synthesized and evaluated for their cytotoxic and anti-inflammatory activities. The anti-inflammatory activity of these compounds depended on both the nature of the substituents ($CH_3$-, $OCH_3$-, and F-groups) and their positions (*o*- or *m*-) on the aromatic region of the pyrazoline ring. Specifically, the presence of *m*-substituents decreased anti-inflammatory activity, whereas *o*-substituents enhanced it. None of the tested compounds exhibited significant cytotoxic activity.

Herewith it should be noted that both isomers of fluorine-substituted pyrazolines exhibit high anti-inflammatory activity.

## STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

*Disclosure of conflict of interest*

The authors declare no conflicts of interest.

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