Final report

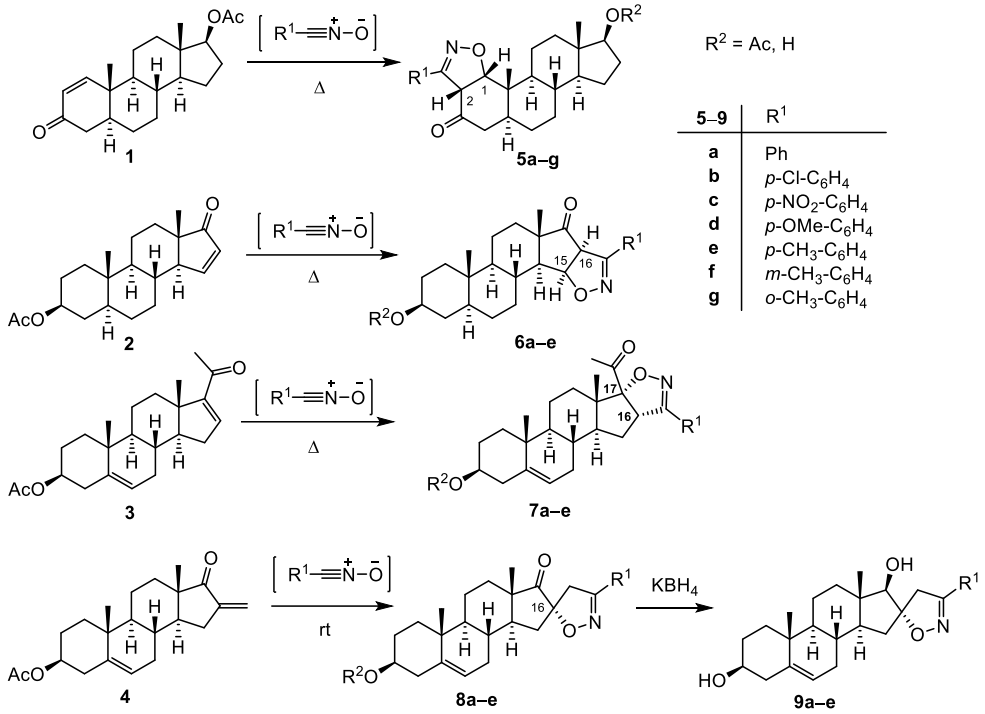
In the frame of the project, our attention was devoted to the design and synthesis of sex-hormone derived novel steroids containing a five- or six-membered heterocyclic moiety condensed to the sterane skeleton. Besides ring-condensed derivatives, some *spiro*- and *exo*-heterocycles were also successfully obtained.

During the supported period, more than 300 novel steroidal heterocyclic derivatives were prepared and completely characterized by different spectroscopic methods. Several compounds exerted noteworthy *in vitro* cell-growth-inhibitory effect on malignant cell lines of diverse origins. Pharmacological studies of the synthesized compounds were accomplished in collaborations (Department of Pharmacodynamics and Biopharmacy, University of Szeged, 1st Department of Medicine, University of Szeged and Department of Biochemistry and Molecular Biology). A number of synthesized derivatives are still under *in vitro* pharmacological investigation.*

1. Synthesis and structure determination of steroidal five-membered *N,O*-heterocycles

The synthesis of *N,O*-heterocycles either condensed to or *spiro*-connected with the sterane skeleton were carried out by intermolecular 1,3-dipolar cycloadditions (1,3-DC).

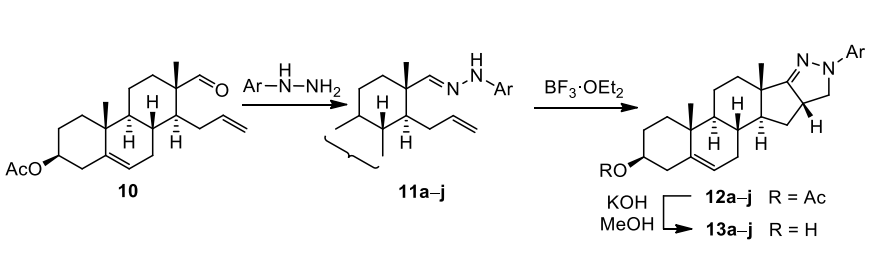
Unsaturated steroidal ketone dipolarophiles (**1** and **2**) were reacted with aromatic nitrile oxide 1,3-dipoles generated *in situ* to furnish novel types of isoxazolines condensed to ring A (**5a**–**g**, R^2 = Ac) or D (**6a**–**e**, R^2 = Ac) [1]. The cyclic enone moiety of the six-membered ring A proved to be less reactive than that of the five-membered ring D, but all the reactions were affected by the substitution pattern of the nitrile oxide significantly. The ring-closures proved to occur regio- and stereoselectively due to steric reasons, and a single isomer containing its heteroring in a 1α,2α-*cis*- (**5**) or 15β,16β-*cis*-connected manner (**6**) was obtained exclusively in moderate to excellent yields. Similar transformations of some pregnane-fused isoxazolines from 16-dehydropregnenolone acetate (**3**) with different arylnitrile oxides were also carried out permitting the formation of 16α,17α-condensed isoxazolines (**7a**–**e**, R^2 = Ac) [2]. Deacetylation of the primary products resulted in the corresponding 3β-OH analogs (**5–7**, R^2 = H). According to the *in vitro* pharmacological results, some compounds exerted moderate cytostatic effects on four different breast cancer cell lines. The efficacy of the intermolecular 1,3-DC for the reactions of a steroidal enone (**4**), containing an *exo*-cyclic double bond at position 16, with aromatic nitrile oxides was also investigated [3]. In view of the higher reactivity of the dipolarophile, these reactions occurred under mild conditions to afford 16-spiroisoxazolines (**8a**–**e**, R^2 = Ac) regio- and stereoselectively in good to excellent yields. The library of compounds was enlarged by further deacetylation reactions (**8a**–**e**, R^2 = H) and subsequent reductions (**9a**–**e**). Two of the structurally related derivatives were found to have a marked inhibitory effect on cell proliferation.



2. Synthesis and structure determination of steroidal five-membered *N,N*- and *N,N,X*-heterocycles

2.1. Pyrazolines by 1,3-DC

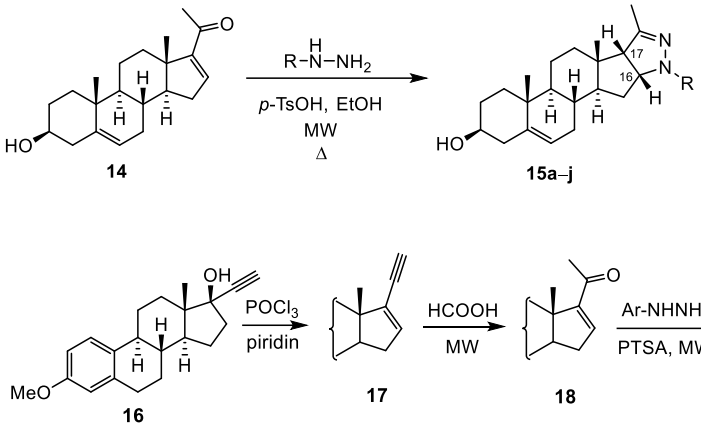
The D-secoaldehyde (**10**) derived from dehydroepiandrosterone via a multistep sequence proved to be a suitable precursor for condensation reactions with arylhydrazine derivatives. The Lewis acid-induced intramolecular 1,3-DC of the arylhydrazone intermediates (**11a**–**j**) furnished novel androsteno-arylpyrazolines (**12a**–**j**) [4]. The stabilities of the hydrazones, the intramolecular ring-closures and the tendency of the newly formed heteroring to undergo oxidation were all observed to depend on the electronic character of the substituent on the aromatic moiety. The reactions proceeded stereoselectively under mild conditions to afford ring D-condensed pyrazoline derivatives in good to excellent yields. Some of the 3-deacetylated cycloadducts (**13a**–**j**) exerted marked cell-growth inhibitory activities.



2.2. Pyrazolines/Pyrazoles from steroidal enones

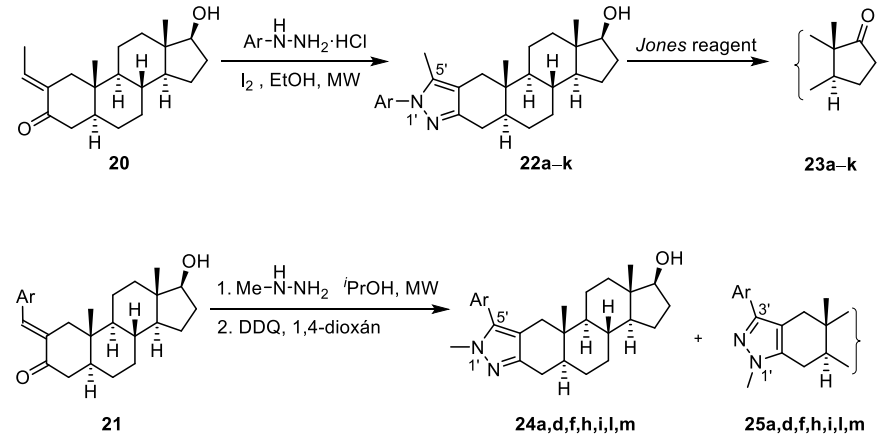
Further ring D-condensed 2-pyrazolines in the Δ^5 androstene series (**15a**–**j**) were efficiently synthesized from 16-dehydropregnenolone (**14**) with methylhydrazine or different arylhydrazines under microwave (MW) irradiation [5]. The reactions are assumed to occur via hydrazone intermediates followed by intramolecular 1,4-addition leading to the fused

heteroring stereoselectively with a 16α,17α-*cis* ring junction. One of the compounds exerted marked *in vitro* antiproliferative effect on human gynaecological cancer cell lines. Similar derivatives in the estrone series (**19a**–**j**) starting from mestranol (**16**) were also synthesized in a multistep pathway and their pharmacological investigations are still in progress.*



15	R
a	CH_3
b	Ph
c	*p*-CH_3-C_6H_4
d	*p*-OMe-C_6H_4
e	*p*-Cl-C_6H_4
f	*p*-NO_2-C_6H_4
g	*p*-CN-C_6H_4
h	*o*,*p*-diCH_3-C_6H_3
i	*p*-F-C_6H_4
j	*p*-Br-C_6H_4

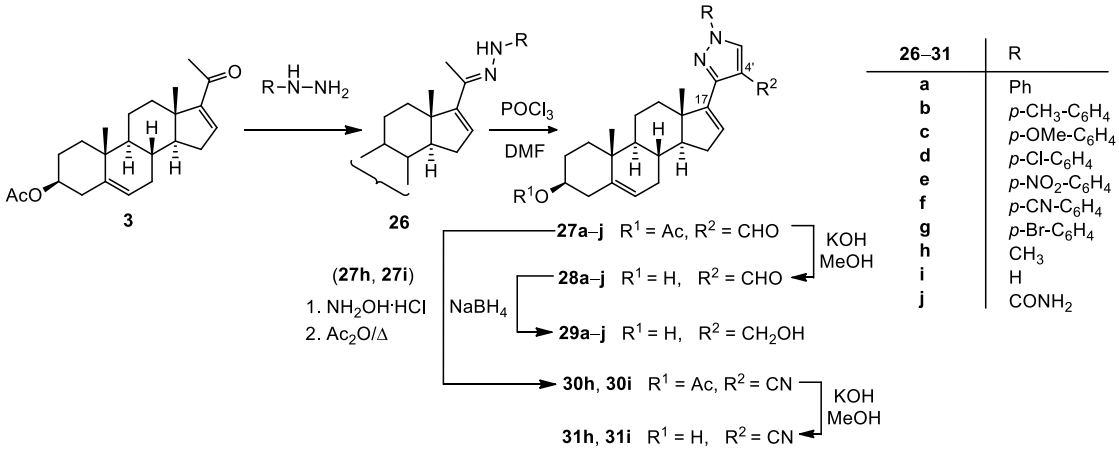
Novel androstano-arylpyrazoles (**22**, **24**, **25**) were also synthesized from steroidal α,β-unsaturated ketones (**20** and **21**), which were obtained from 5α-dihydrotestosterone in good yields by Claisen-Schmidt condensation. A one-pot procedure for the regioselective formation of ring A-fused pyrazoles (**22a**–**k**) was developed, involving the I_2-mediated oxidative cyclization of **20** with different arylhydrazines under MW condition. Subsequent oxidation of the 17β-hydroxy derivatives (**22**) with the *Jones* reagent resulted in the 17-keto analogs (**23**). Similar ring-closure reactions of **21** with methylhydrazine and subsequent oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) furnished a separable regioisomeric mixture of **24** and **25** with **24** as being the main product. Several synthesized derivatives displayed noteworthy antiproliferative activity on breast and prostate cancer cell lines. Further *in vitro* pharmacological studies (cell cycle analysis, caspase inhibition, etc.) are still in progress.*



22–25	Ar
a	Ph
b	*o*-CH_3-C_6H_4
d	*p*-CH_3-C_6H_4
e	o,*p*-diCH_3-C_6H_3
f	*p*-OMe-C_6H_4
g	*p*-F-C_6H_4
h	*p*-Cl-C_6H_4
i	*p*-Br-C_6H_4
j	*p*-CN-C_6H_4
k	*p*-NO_2-C_6H_4
l	2-furyl
m	2-thienyl

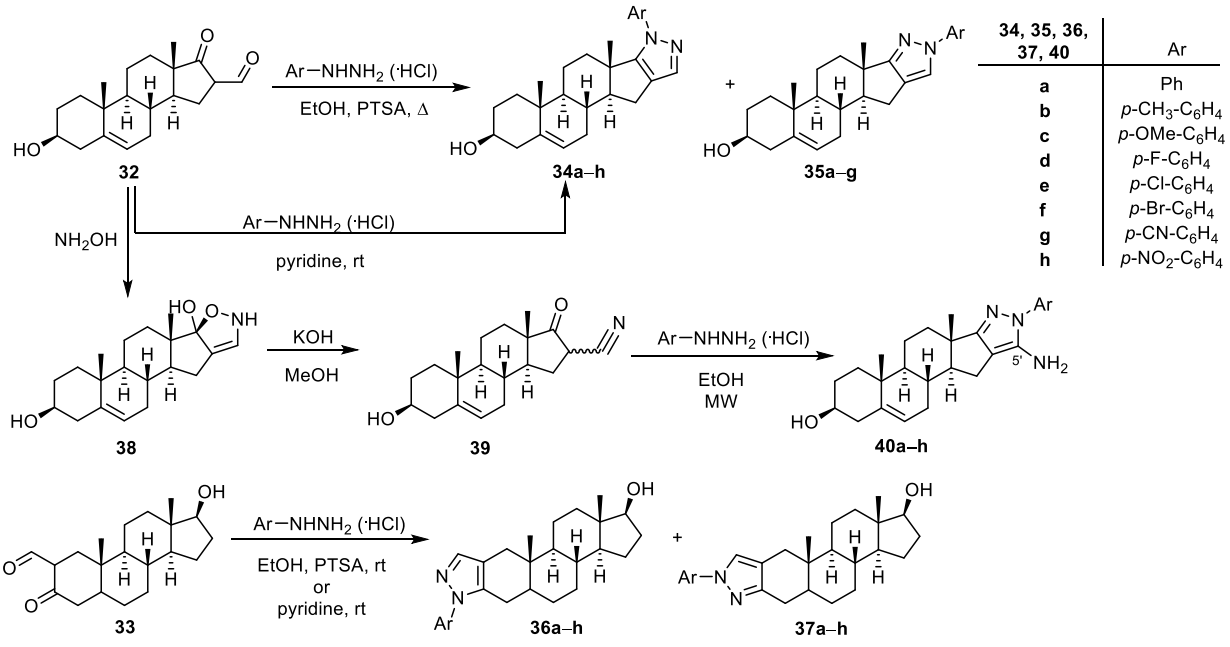
Pregnadienolone-acetate (**3**) proved to be a suitable starting material for the synthesis of some 17-*exo*-heterocyclic compounds (**27a**–**j**) with monosubstituted hydrazines via the cyclization/formylation sequence of the primarily formed hydrazones (**26**) on treatment with the Vilsmeier-Haack reagent [6]. 4′-Formylpyrazoles containing CH_3 (**27h**) or H on the heteroring-*N* (**27i**) were subjected to oxime formation and Ac_2O-induced dehydration to furnish the corresponding 4′-cyano derivatives (**30h**, **30i**) in good yields. Several of the synthesized

3-OH derivatives (**28**, **29**, **31**) exerted substantial effects on cell proliferation, while three of the tested steroidal pyrazoles displayed noteworthy inhibition on rat testicular $C_{17,20}$-lyase.

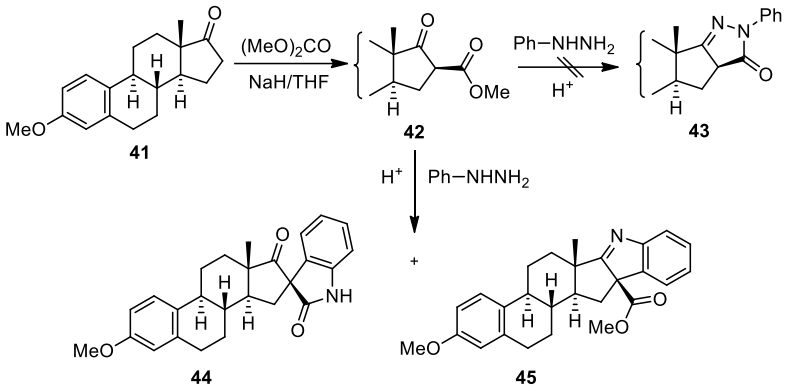


2.3. Pyrazoles by Knorr-type reactions

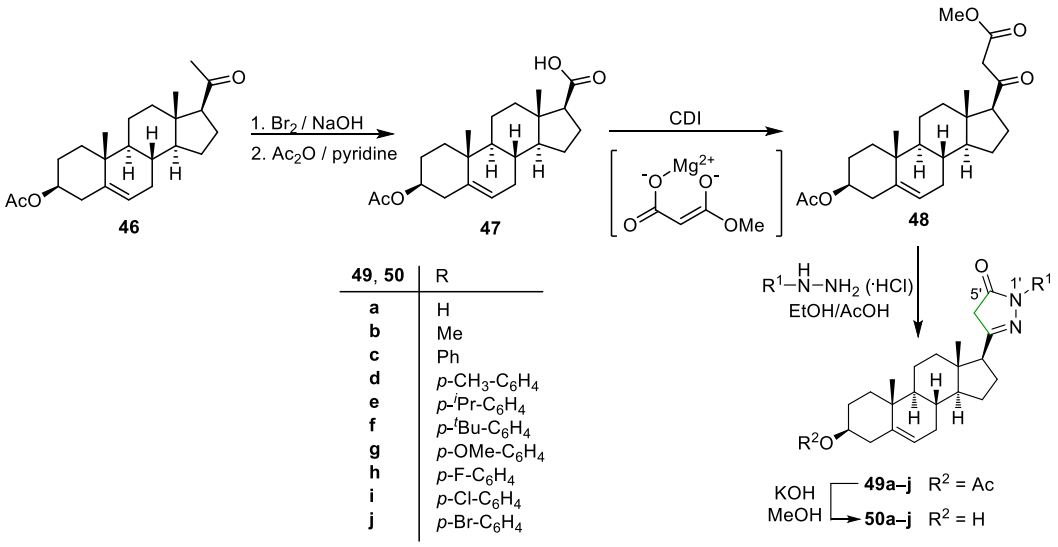
Novel androstanopyrazoles (**34**, **35**, **36**, **37**) were efficiently synthesized from steroidal β-ketoaldehydes (**32** and **33**) with different arylhydrazine hydrochlorides both under acidic and basic conditions [7]. Knorr-type transformations of **32** containing its 1,3-dicarbonyl moiety on ring D, proved to be regioselective (**34**) in pyridine at room temperature, while mixtures of regioisomers (**34** and **35**) were obtained in acidic EtOH under reflux. Contrarily, the cyclocondensation reactions of **33** bearing its reactive functionalities on ring A, led to a mixture of pyrazole regioisomers (**36** and **37**) 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. Some derivatives displayed remarkable antiproliferative action. Knorr-type cyclization reactions of 16-carbonitrile (**39**), easily accessible from **32** via two steps were also carried out to obtain ring D-condensed 5'-aminopyrazoles (**40a**–**h**), which are still under biological evaluation.*



Following the same strategy, β-ketoester in the estrone series (**42**) was efficiently synthesized from estrone 3-methyl ether (**41**) with dimethyl carbonate in THF in the presence of a strong base. Compound **42** was then reacted with phenylhydrazine in acidic medium in order to obtain ring D-condensed pyrazol-5-one (**43**). However, Knorr type condensation did not occur, and two other products (**44** and **45**) were isolated instead. The chemoselectivity of the reaction was observed to be controlled by the applied (Lewis acid-induced or MW) conditions. The scope and limitations of this reaction needs to be further investigated by using different substituted phenylhydrazines.*



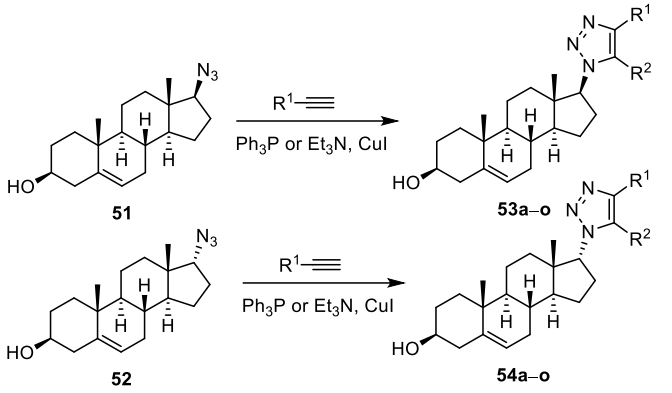
Since our attempts toward the preparation of condensed pyrazolone (**43**) from **42** failed probably due to steric reasons, the synthesis and Knorr-type heterocyclization of another type of steroidal β-ketoester with hydrazine derivatives were accomplished. A steroidal 17-carboxylic acid (**47**) was first synthesized from pregnenolone aetate (**46**) by the bromoform reaction and subsequent acetylation [8]. Its CDI-activated acyl imidazole derivative was then converted to a bifunctional β-ketoester (**48**), which was then reacted with hydrazine and its monosubstituted derivatives under conventional and/or MW heating conditions leading to the regioselective formation of 17-*exo*-heterocycles (**49a**–**j**) in good yields. The yields of 1'-aryl-substituted pyrazol-5'-ones (**49d**–**j**) were found to depend on the electronic character of the substituent of the phenylhydrazine applied. The biological assays revealed that some of the 3-OH derivatives (**50a**–**j**) exerted cell growth-inhibitory effects on some malignant cell lines better than the reference cisplatin.



49, 50	R
a	H
b	Me
c	Ph
d	*p*-CH_3-C_6H_4
e	*p*-^{i}Pr-C_6H_4
f	*p*-^{t}Bu-C_6H_4
g	*p*-OMe-C_6H_4
h	*p*-F-C_6H_4
i	*p*-Cl-C_6H_4
j	*p*-Br-C_6H_4

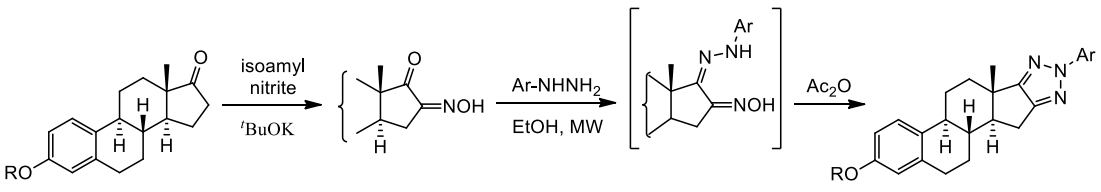
2.4. Triazoles and oxa/thiadiazoles by click reaction and heterocyclization

The regioselective Cu(I)-catalyzed 1,3-DC of steroidal 17β- and 17α-azides (**51** and **52**) with different terminal alkynes afforded novel 1,4-substituted triazolyl derivatives (**53** and **54**, R^1 = cycloalkyl, aryl, R^2 = H) [9]. For the preparation of 5′-iodo-1′,2′,3′-triazoles (**53** and **54**, R^1 = cycloalkyl, aryl, R^2 = I), an improved method was developed directly from steroidal azides and terminal alkynes, in reactions mediated by CuI and ICl as iodinating agents. The *in vitro* pharmacological results revealed that the 17β-azide (**51**) and the 17α-triazolyl compounds (**54**) inhibit the action of $C_{17,20}$-lyase enzyme.



53, 54	R^1	R^2
a	Ph	H
b	*p*-CH_3-C_6H_4	H
c	*p*-OMe-C_6H_4	H
d	cyclopropyl	H
e	cyclopentyl	H
f	cyclohexyl	H
g	2-pyridyl	H
h	phenoxymethyl	H
i	benzoyloxymethyl	H
j	toluoyloxymethyl	H
k	4-NO_2-benzoyloxymethyl	H
l	hydroxymethyl	H
m	4-NO_2-benzoyloxymethyl	I
n	cyclopropyl	I
o	cyclopropyl	OH

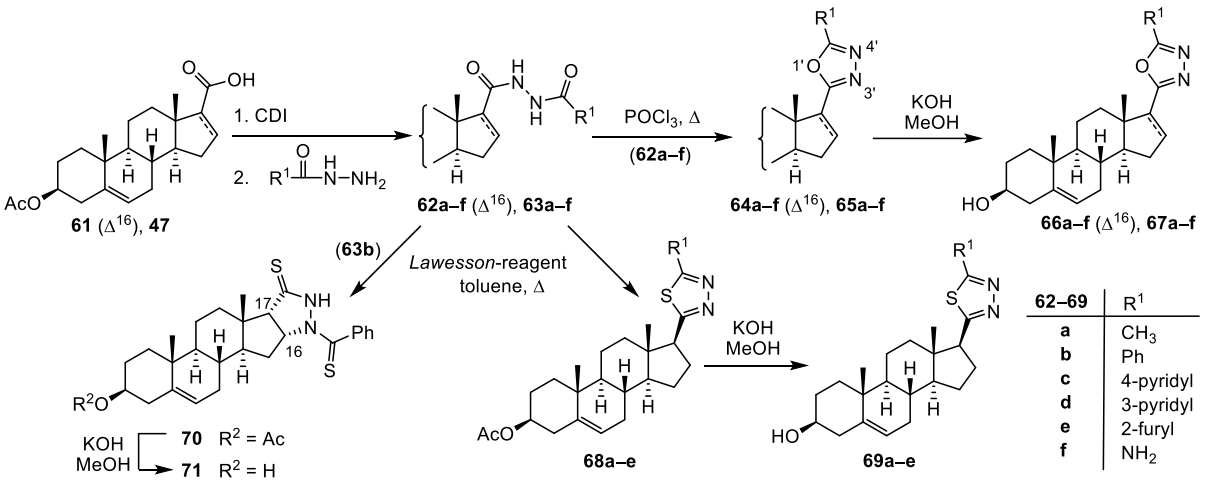
Ring-D condensed *N*(2)-substituted triazoles (**59a**–**e** and **60a**–**e**) in the estrone series were synthesized from estrone (**55**) and its 3-benzyl ether derivative (**56**) via the corresponding 16-oximes (**57** and **58**). The cyclocondensation reactions of **57** and **58** with arylhydrazines under microwave conditions followed by Ac_2O-induced dehydration led to the desired heterocycles in moderate yields. The synthesized derivatives are still under pharmacological evaluation.*



59, 60	Ar
a	Ph
b	*p*-CH_3-C_6H_4
c	*p*-Cl-C_6H_4
d	*p*-F-C_6H_4
e	*p*-OMe-C_6H_4

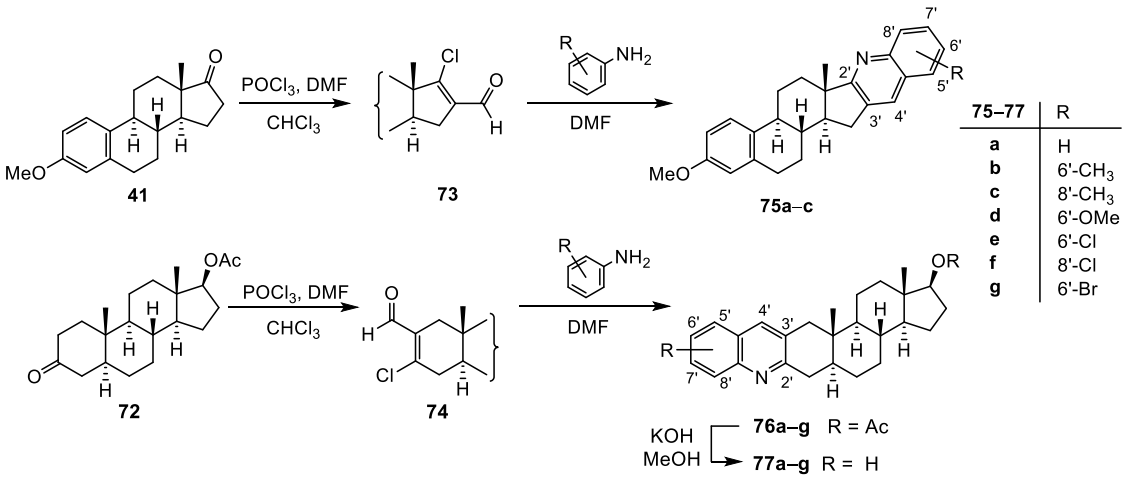
Novel 17-*exo*-(1′,3′,4′)-oxadiazoles in the Δ^5 androstene series (**64**, **65**) were efficiently synthesized *via* $POCl_3$-mediated cyclodehydration of the appropriate *N*,*N*′-disubstituted hydrazine intermediates (**62**, **63**) prepared from steroidal 17-carboxylic acids (**61**, **47**) [10]. Moreover, these latter compounds proved to be appropriate precursors for the approach to novel types of 17β-*exo*-1,3,4-thiadiazoles (**68**) in the presence of the Lawesson reagent. However, unsaturation at position C16–17 of the diacylhydrazine precluded the possibility of thiadiazole formation, and a ring D-condensed pyrazolidine-3-thione (**70**) was formed instead as main product *via* 1,4-Michael addition and *O*→*S* exchange. Most of the heterocyclic products were subjected to deacetylation in alkaline medium in order to obtain the corresponding 3β-hydroxy

analogs (**66**, **67**, **69**, **71**). The *in vitro* pharmacological studies of the synthesized compounds on malignant cancer cell lines revealed that several derivatives exerted substantial and selective antiproliferative effects on HeLa cells. The amino-substituted 1,3,4-oxadiazolyl $\Delta^{5,16}$-androstadiene (**66f**) displayed noteworthy inhibition *in vitro* on rat testicular $C_{17,20}$-lyase, with an IC_{50} of the same order of magnitude as that of abiraterone.



3. Synthesis and structure determination of steroidal six-membered *N*-heterocycles

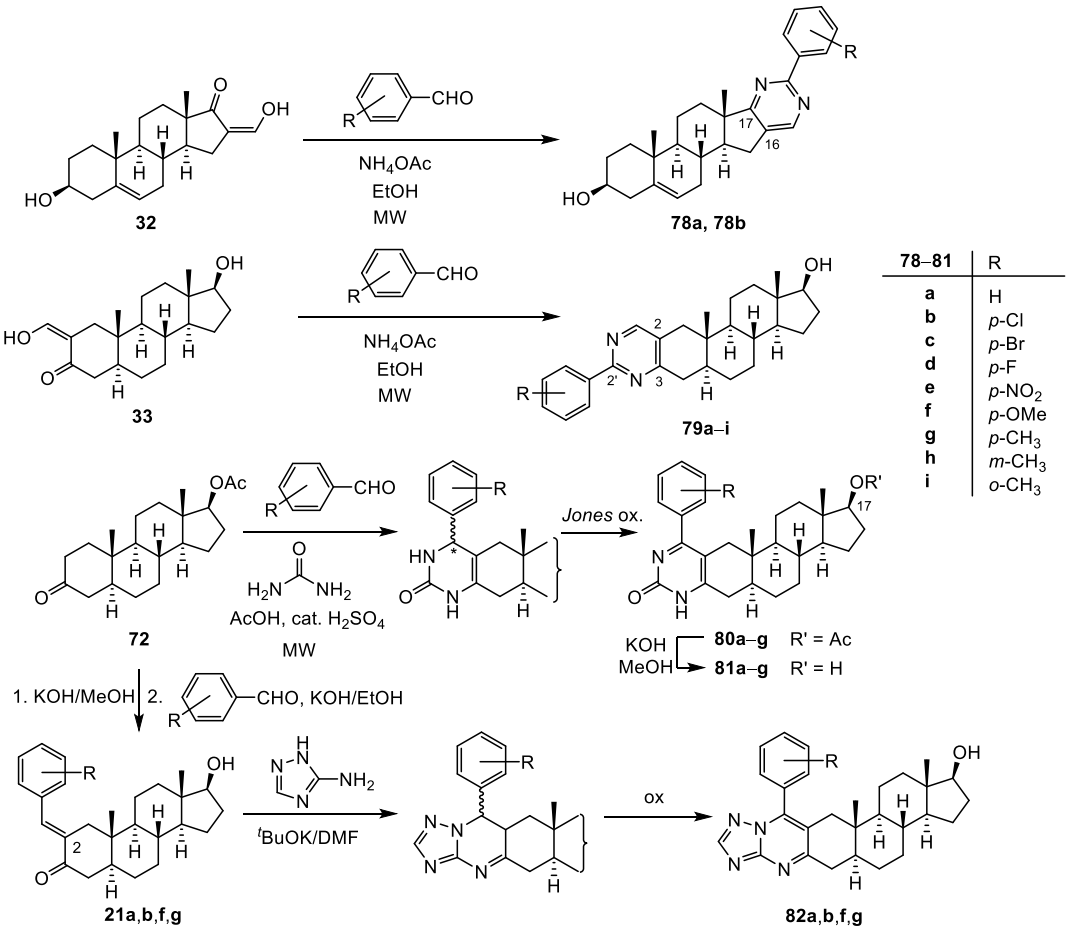
Novel D- and A-ring-fused quinolines in the estrone (**75a**–**c**) and 5α-androstane series (**76a**–**g**) were efficiently synthesized from the corresponding β-chlorovinyl aldehydes (**73** and **74**) with different arylamines in DMF under MW irradiation [11]. The rates of the one-pot catalyst-free syntheses and the yields of the desired products were found to be affected significantly by the electronic and steric character of the substituents on the anilines and the different reactivity of rings D and A of the sterane skeleton. As concerns the pharmacological profile of the presented molecules, most of the deacetylated ring A-fused heterocycles (**77a-g**) exerted weak or modest antiproliferative activities against the utilized panel of human adherent cancer cell lines.



4. Synthesis and structure determination of steroidal six-membered *N,N*-heterocycles

New ring D- (**78**) and A-fused pyrimidines in the androstane series (**79**, **80**) were also synthesized under MW irradiation *via* two kinds of multicomponent heterocyclization reactions

followed by spontaneous or promoted oxidation [12]. The rates of the one-pot catalyst-free transformations of steroidal β-ketoaldehydes (**32** and **33**), ammonium acetate and substituted benzaldehydes in EtOH were found to be affected slightly by the steric and electronic feature of the substituents on the aromatic ring of the arylaldehyde and the different reactivity of rings D and A of the sterane core. At the same time, the acid-catalyzed Biginelli-type reaction of dihydrotestosterone acetate (**72**), urea and arylaldehydes, and subsequent Jones oxidation of the primarily formed dihydropyrimidinones led to the corresponding ring A-fused 1*H*-pyrimidin-2-ones (**80**) in moderate yields independently of the substituents on the aromatic moiety. As a result of the pharmacological screen, a remarkable structure-function relationship was observed as the acetylated Biginelli products (**80**) exhibited higher toxicity compared to the deacetylated version (**81**) of each compound. Furthermore, in case of three 2′-arylpyrimidine derivatives (**79**) a strong prostate cancer cell specific activity was identified. Further pharmacological investigation of the highly cytotoxic 3-OAc derivatives (**80**) are still in progress.* 2-Arylidene derivatives (**21**) of **72** were reacted with 3-amino-1,2,4-triazole in DMF in the presence of a strong base to furnish ring A-condensed 1,2,4-triazolo[1,5-*a*]pirimidines (**82**) after spontaneous oxidation of the primarily formed heterocyclic product. The *in vitro* antiproliferative assays of these latter derivatives are in progress.*



*Those derivatives, which are still under synthetic or *in vitro* pharmacological investigations will be published later. There will be at least five additional scientific papers based on the application.

Publications related to the project

[1] Mótyán, G.; Kádár, Z.; Kovács, D.; Wölfling, J.; Frank, É. *Steroids* **2014**, *87*, 76–85.

[2] Mótyán, G.; Baji, Á.; Zupkó, I.; Frank É. *J. Mol. Struct*. **2016**, *1110*, 143–149.

[3] Frank, É.; Kovács, D.; Schneider, Gy.; Wölfling, J.; Bartók, T.; Zupkó, I. *Mol. Divers*. **2014**, *18*, 521–534.

[4] Mótyán, G.; Zupkó, I.; Minorics, R.; Schneider, Gy.; Wölfling, J.; Frank, É. *Mol. Divers.* **2015**, *19*, 511–527.

[5] Mótyán G.; Kovács, F.; Wölfling, J.; Gyovai A.; Zupkó, I.; Frank, É. *Steroids* **2016**, *112*, 36–46.

[6] Kovács, D.; Wölfling, J.; Szabó, N.; Szécsi M.; Schelz, Zs.; Zupkó, I.; Frank, É. *Eur. J. Med. Chem.* **2016**, *120*, 284–295.

[7] Baji, Á.; Kovács, F.; Mótyán, G.; Schneider, Gy.; Wölfling, J.; Sinka, I.; Zupkó, I.; Ocsovszki, I.; Frank, É. *Steroids*, **2017**, *126*, 35–49.

[8] Mótyán, G.; Mérai, L.; Kiss, M. A.; Schelz, Zs.; Sinka I.; Zupkó, I.; Frank, É. *Beilstein J. Org. Chem*. **2018** (submitted).

[9] Kiss, A.; Herman, B. E.; Görbe, T.; Mernyák, E.; Molnár, B.; Wölfling, J.; Szécsi, M.; Schneider Gy. *Steroids* **2018**, *135*, 79–91.

[10] Kovács, D.; Wölfling, J.; Szabó, N.; Szécsi, M.; Minorics, R.; Zupkó, I.; Frank, É. *Eur. J. Med. Chem.* **2015**, *98*, 13–29.

[11] Baji Á., Gyovai A., Wölfling J., Minorics R., Ocsovszki I., Zupkó I., Frank É. *RSC Adv.* **2016**, *6*, 27501–27516.

[12] Baji Á., Kiss T., Wölfling J., Kovács D., Igaz N., Gopisetty M. K., Kiricsi M., Frank É. *J. Steroid Biochem. Mol. Biol.* **2017**, *172*, 79–88.

Theses based on or partially based on the project:

Kovács, D. *Synthesis of pharmacologically active 17-exo-heterocyclic steroids*, Ph.D. thesis (**2015**), http://doktori.bibl.u-szeged.hu/2728

Mótyán G. *Synthesis of five-membered heterocycles condensed to the sterane core*, Ph.D. thesis (**2017**), http://doktori.bibl.u-szeged.hu/4070/, doi: 10.14232/phd.4070

Baji, Á. *Synthesis of sterane-condensed nitrogen-containing heterocycles from bifunctional sex-hormone derivatives*, Ph.D. thesis (**2018**), http://doktori.bibl.u-szeged.hu/4210

Frank, É. *Heterociklusokkal módosított nemi hormon származékok szintézise*, D.Sc. thesis (**2018**), http://real-d.mtak.hu/977

Szeged, 28. August, 2018.

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Dr. Frank Éva