Summary of Ph.D. Thesis

SYNTHESIS OF PHARMACOLOGICALLY ACTIVE 17-*EXO*-HETEROCYCLIC STEROIDS

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Szeged
2015

1. Introduction and aims

Steroids, which are to be found in bioorganisms, are of naturally occurring compounds and play important roles from physiological aspects. In recent decades, considerable attention has been focused on the synthesis of heterocycle-containing steroids, since several such hybrid molecules have been reported to possess a broad spectrum of biological activities. A number of derivatives modified with different heterocycles have been demonstrated to exert significant antiproliferative effects on cancer cell lines of diverse origin without affecting the proliferation of intact cells. Moreover, these compounds have been reported to be specific inhibitors of certain enzymes, and they can therefore be applied in the treatment of hormone-dependent diseases (such as benign prostatic hyperplasia or prostate cancer).

With regard to the previous studies, the course of my doctoral work we envisaged the synthesis of novel, probably pharmacologically active 17-*exo*-heterocyclic steroids. Our aim was the formation of various five-membered heterocycles containing more than one heteroatom by the 1,3-dipolar cycloaddition (1,3-DC) of mestranol in the estrone series, and by different chemical reactions of synthon equivalents synthetized from the transformation of the methyl ketone side-chain of pregnenolone acetate (PA) and pregnadienolone acetate (PDA) in the pregnane series. Besides the optimization of each reaction condition and the investigation of the influence of the quality and quantity of the reagents and the substrates on the yields of the products, the structure determination of all of the novel synthesized compounds was also planned. Furthermore, we set out to subject all products to two different *in vitro* pharmacological studies (antiproliferative and $C_{17,20}$-lyase inhibition measuments).

2. Experimental methods

Most of the reactions were carried out on a millimolar scale and monitored by thin-layer chromatography. The crude products were purified by flash chromatography. The structures of all synthetized compounds were confirmed by ^{1}H NMR, ^{13}C NMR and ESI MS measurements, and in some cases 2D NMR experiments (NOESY) were also performed for the structure determination.

3. Scientific results*

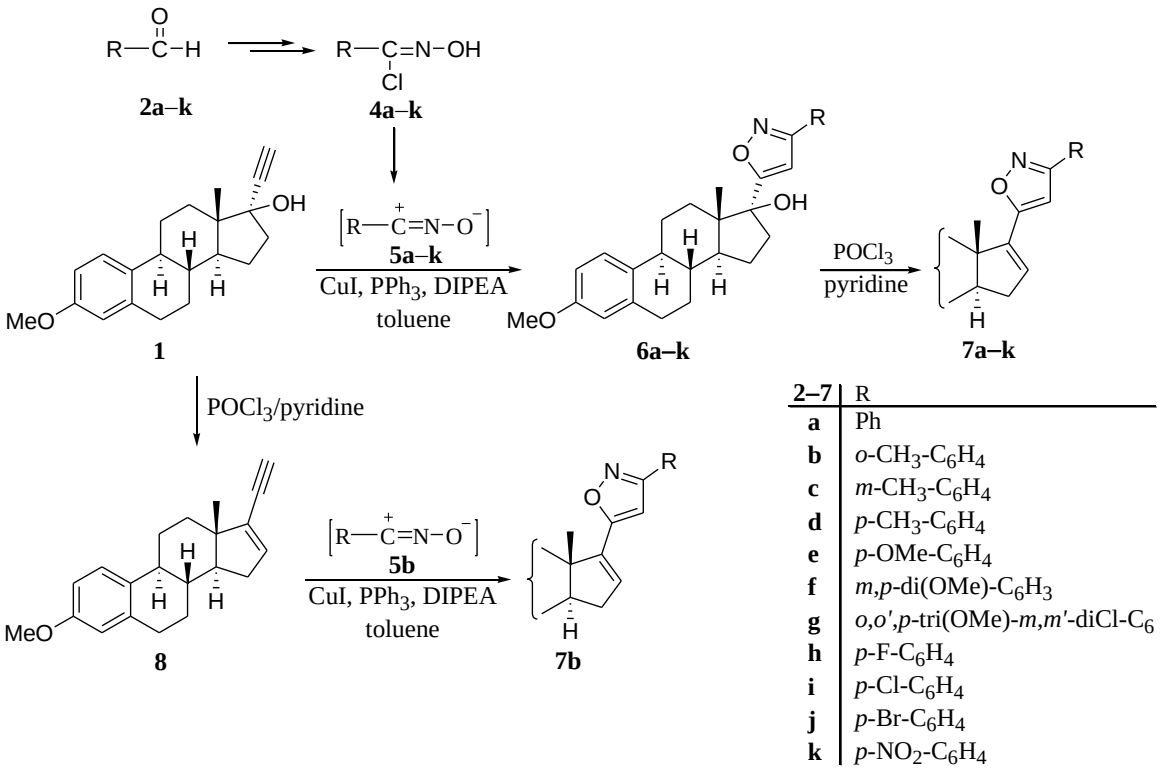
3.1. The copper(I)-catalysed 1,3-DCs of mestranol (**1**) with different aryl nitrile oxides (**5a–k**) under the optimized reaction conditions afforded the corresponding 17β-hydroxy-17α-isoxazolyl derivatives (**6a–k**) in the estrone series in good to excellent yields (*Scheme 1*). The yields of the heteroaromatic products (**6a–k**) were greatly influenced not only by the mode of addition of *N*,*N*-diisopropylethylamine (DIPEA), but by the electronic features of the substituents on the aromatic ring of the 1,3-dipoles (**5a–k**).

3.2. During the halogenation of aldoximes (**3a–k**) with *N*-chlorosuccinimide (NCS), *bis*-chlorination of the aromatic ring was found to occur for 2,4,6-trimethoxybenzaldehyde oxime (**3g**), due to the additive *ortho*-directing effects of the electron-donating methoxy substituents.

3.3. The subsequent E2-type elimination in the presence of $POCl_3$ and pyridine provided the corresponding Δ^{16}-17-isoxazoles (**7a–k**) in good to excellent yields without any by-product formation.

3.4. The 1,3-DC of **8** obtained from mestranol (**1**) by dehydration was found not to be chemoselective. Thus, construction of the heteroring followed by elimination was demonstrated to be a more efficient pathway for the synthesis of Δ^{16} analogues (**7a–k**).

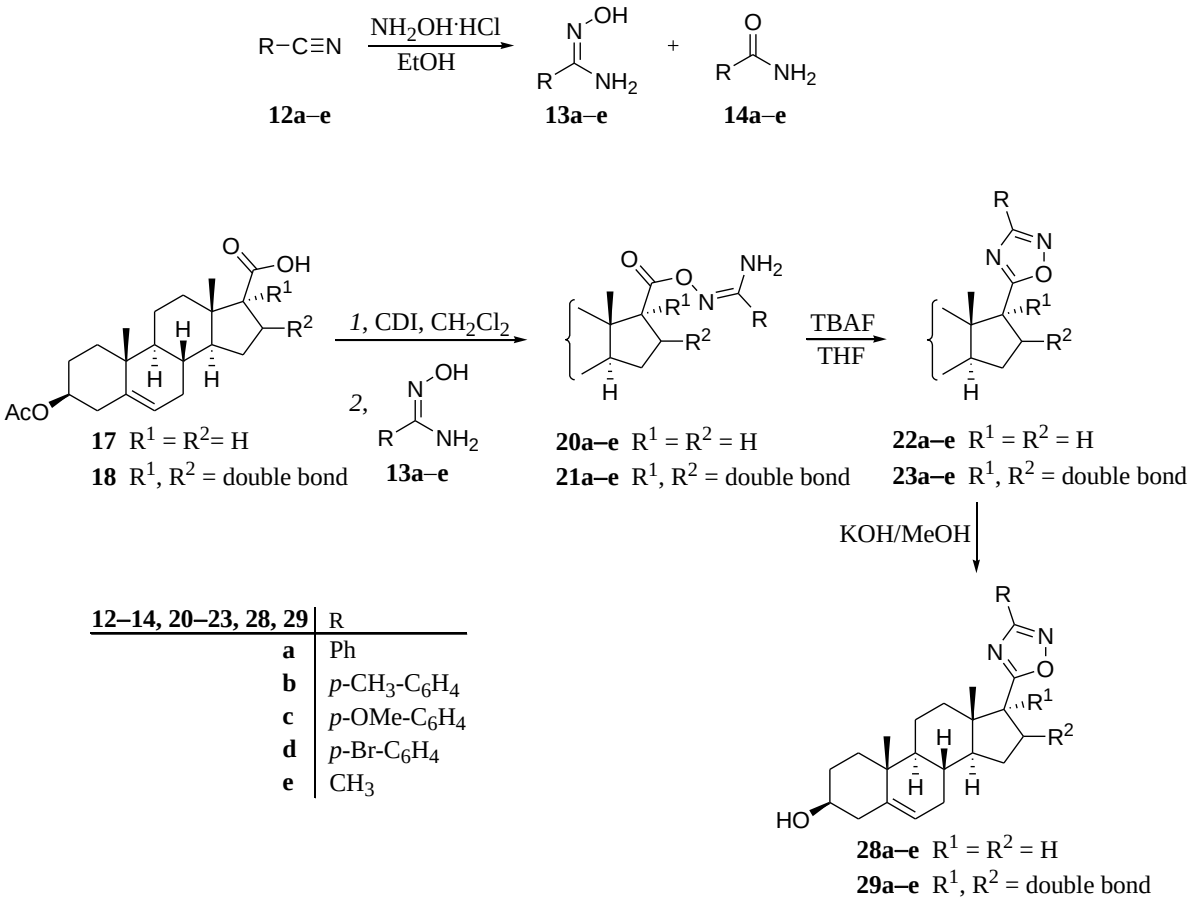
* The numbering of the compounds accords with that in the Ph.D. Thesis



Scheme 1

3.5. In the next step, amidoxime reagents (**13a–e**) were prepared and used for the two-step synthetic procedure to synthetize the 1,2,4-oxadiazolyl derivatives (**22a–e** and **23a–e**) in the androstane series (*Scheme 2*). It was found that the presence of small amounts of carboxylic amide by-products (**14a–e**), formed together with amidoximes (**13a–e**), did not interfere in the subsequent transformations.

3.6. From 3β-acetoxy-17-carboxylic acids (**17** and **18**), obtained from PA (**15**) and PDA (**16**), novel 17-(1',2',4')-oxadiazolyl derivatives (**22a–e** and **23a–e**) were synthetized in a two-step pathway. During the determination of the optimum conditions for the coupling reaction with 1,1'-carbonyldiimidazole (CDI), the best conversions were achieved only in dichloromethane. Furthermore, the application of temperatures higher than 30 °C led to decomposition of the activated carboxylic acids (**26** and **27**). Nucleophilic acyl substitution for both the D-ring saturated and Δ^{16} counterparts resulted in the corresponding *O*-acylated amidoximes (**20a–e** and **21a–e**), and the subsequent cyclocondensation in the presence of tetrabutylammonium fluoride (TBAF) as catalyst afforded the heteroaromatic products (**22a–e** and **23a–e**). Finally, deacetylation of the heterocyclic steroids (**22a–e** and **23a–e**) in basic medium furnished the 3β-hydroxy analogues (**28a–e** and **29a–e**).

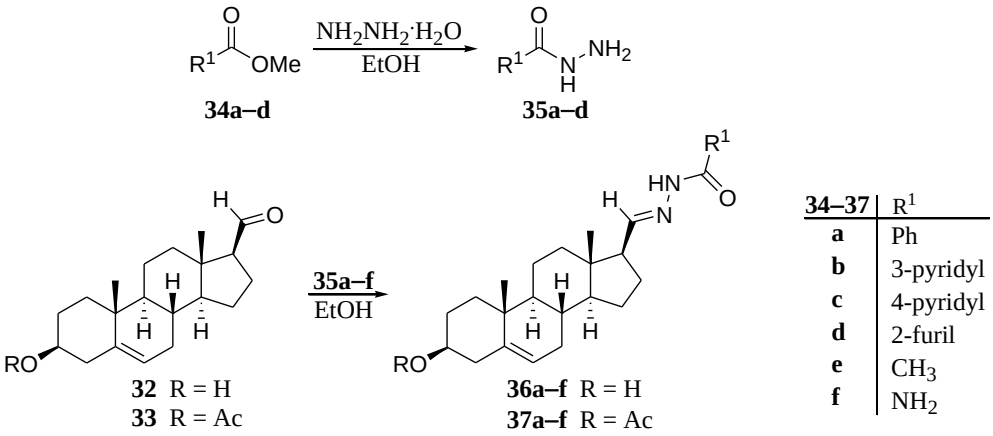


12–14, 20–23, 28, 29	R
a	Ph
b	*p*-CH_3-C_6H_4
c	*p*-OMe-C_6H_4
d	*p*-Br-C_6H_4
e	CH_3

Scheme 2

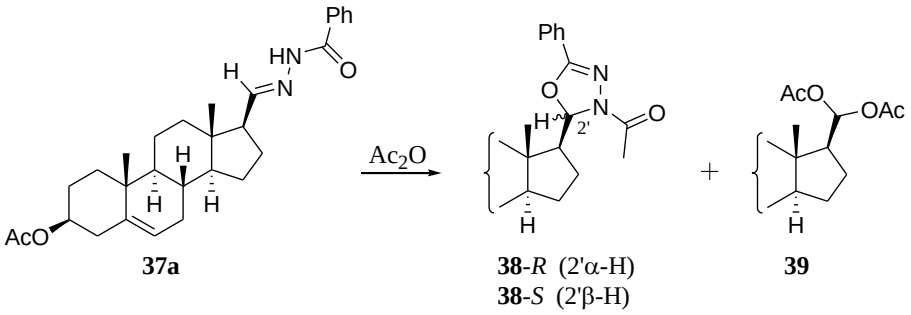
3.7. Most carboxylic acid hydrazides (**35a–d**) were obtained by the nucleophilic acyl substitution of methyl benzoate (**34a**) or heteroaromatic esters (**34b–d**) with hydrazine hydrate under microwave (MW) conditions (*Scheme 3*).

3.8. Condensation reactions of steroidal carbaldehydes (**32** and **33**) with acylhydrazines (**35a–e**) or semicarbazide hydrochloride (**35f**) were performed both by conventional heating and by MW irradiation. Under MW conditions, the yields were only slightly better than those of the thermally-induced method, but the reaction times were significantly shorter. For the 3β-acetoxy derivatives (**37a–f**), somewhat lower yields were achieved, which was attributed to the lower solubility of **33** in the applied solvent (EtOH). The aryl-, heteroaryl- and amino-substituted *N*-acylhydrazones (**36a–d**, **36f**, **37a–d** and **37f**) proved to be quite stable compounds. However, the methyl-substituted analogues (**36e** and **37e**) were subjected to subsequent cyclization directly after chromatographic purification because of their lower stability.



Scheme 3

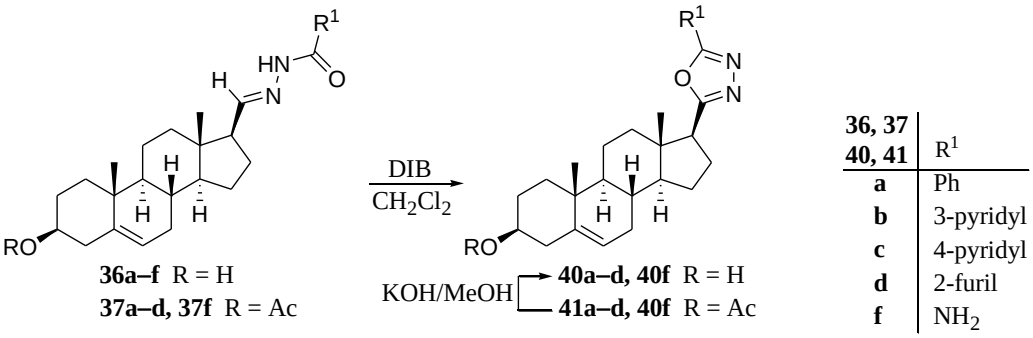
3.9. The following intramolecular ring-closure reaction of *N*′-(3β-acetoxyandrost-5-en-17β-yl-methylidene)benzhydrazide (**37a**) in the presence of Ac_2O (which served as both solvent and reagent) resulted in the 3-acyl-1,3,4-oxadiazoline epimers (**38**-*R* and **38**-*S*) in a moderate overall yield (in a ratio of ~1:1) (*Scheme 4*). The formation of a considerable amount of diacetate by-product (**39**) was explained by regeneration of the parent 3β-acetoxycarbaldehyde (**33**) and the subsequent addition of Ac_2O onto the formyl group at C-17 of the sterane framework under the applied conditions. The absolute configurations of the C-2′ chiral centres of the 3-acyl-1,3,4-oxadiazoline epimers (**38**-*R* and **38**-*S*) were determined through evaluation of their ^{1}H NMR spectra.



Scheme 4

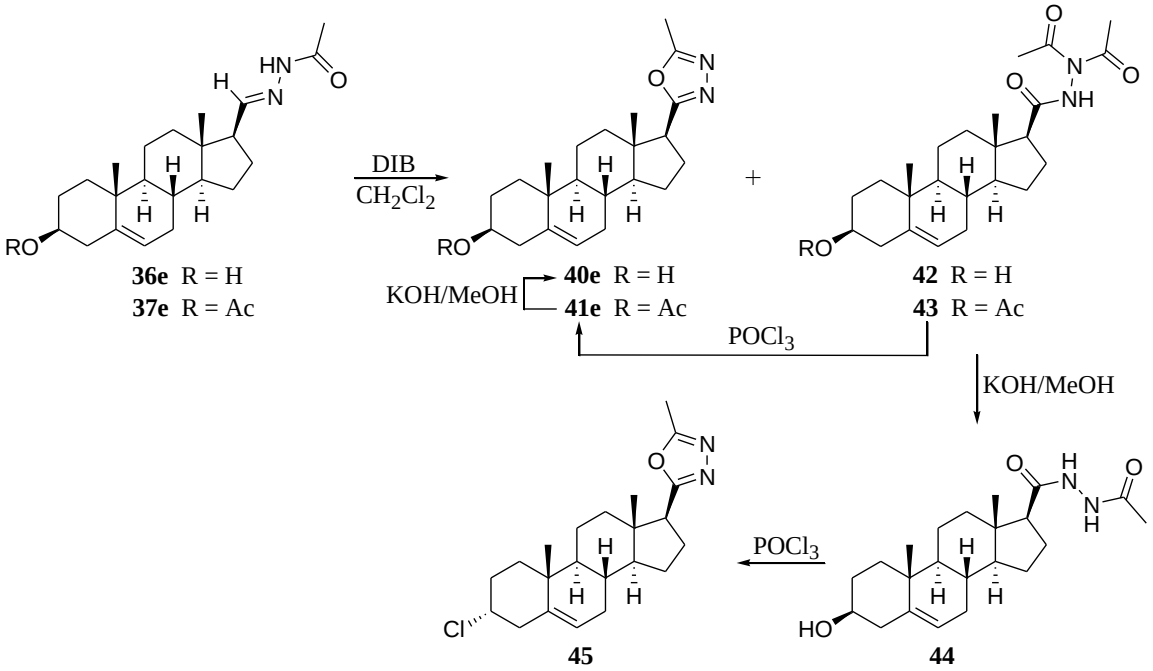
3.10. For the cyclization of *N*-acylhydrazones (**36a–f** and **37a–f**), a hypervalent iodine-containing reagent, [phenyliodonium diacetate (DIB)], was used as oxidizing agent (*Scheme 5*). In dichloromethane at room temperature, the 3β-acetates (**37a–d**, **37f**) furnished the heteroaromatic products (**41a–d**, **41f**) in higher yields than from the 3β-hydroxy derivatives (**36a–d**), which can be attributed to the lower solubility of **36a–d** in the applied solvent. The most polar aminocarbohydrazide (**40f**) could not be synthetized from **36f** by this method.

Nevertheless, all the 3β-hydroxy analogues (**40a–d**, **40f**) could be obtained by the deacetylation of **41a–d** and **41f** in basic media.



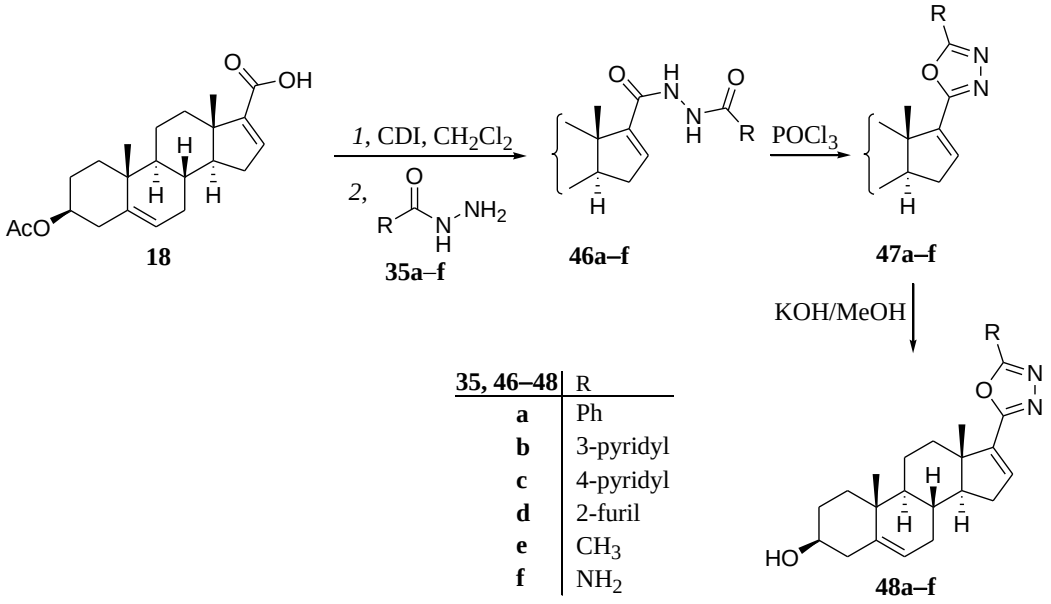
Scheme 5

3.11. Surprisingly, the oxidative cyclization of acetylhydrazones **36e** and **37e** led to two different products; together with the desired heteroaromatic **40e** and **41e**, open-chain *N*,*N*-diacetyl compounds (**42** and **43**) were also formed in a ratio of nearly 1:3 (*Scheme 6*). However, **41e** was also synthetized by the reaction of **43** with $POCl_3$, and was then deacetylated to the heteroaromatic analogue **40e**. Deacetylation of the *N*,*N*-diacyl compounds (**42** and **43**) in basic media furnished *N*-acetyl-3β-hydroxyandrost-5-ene-17β-carbohydrazide (**44**) in excellent yield, and its $POCl_3$-induced cyclodehydration resulted in **45**. In the latter case, the 3β-OH was replaced by a chlorine atom *via* inversion besides the formation of the 1,3,4-oxadiazole heteroring.



Scheme 6

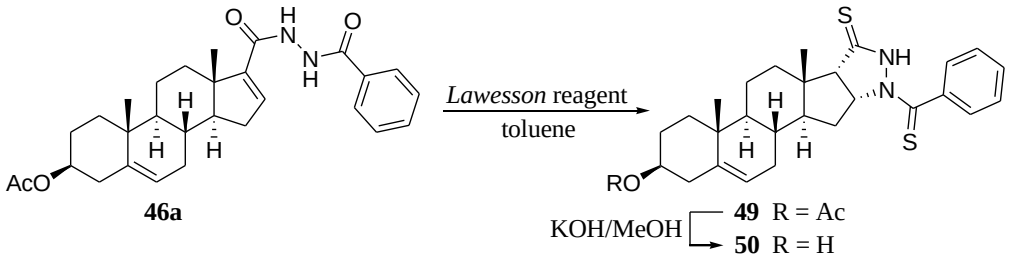
3.12. Under the earlier optimized reaction conditions, coupling of 3β-acetoxyandrosta-5,16-diene-17-carboxylic acid (**18**) with CDI, followed by the reactions with acylhydrazines (**35a–e**) and semicarbazide hydrochloride (**35f**), was also carried out (*Scheme 7*). The subsequent intramolecular cyclodehydration of the *N*,*N*′-disubstituted hydrazines (**46a–f**) with $POCl_3$ furnished the corresponding 1,3,4-oxadiazolyl derivatives (**47a–f**) in good yields. Finally, the 3β-OH analogues (**48a–f**) were prepared in excellent yields by deacetylation of **47a–f** in methanolic KOH solution.



35, 46–48	R
a	Ph
b	3-pyridyl
c	4-pyridyl
d	2-furil
e	CH_3
f	NH_2

Scheme 7

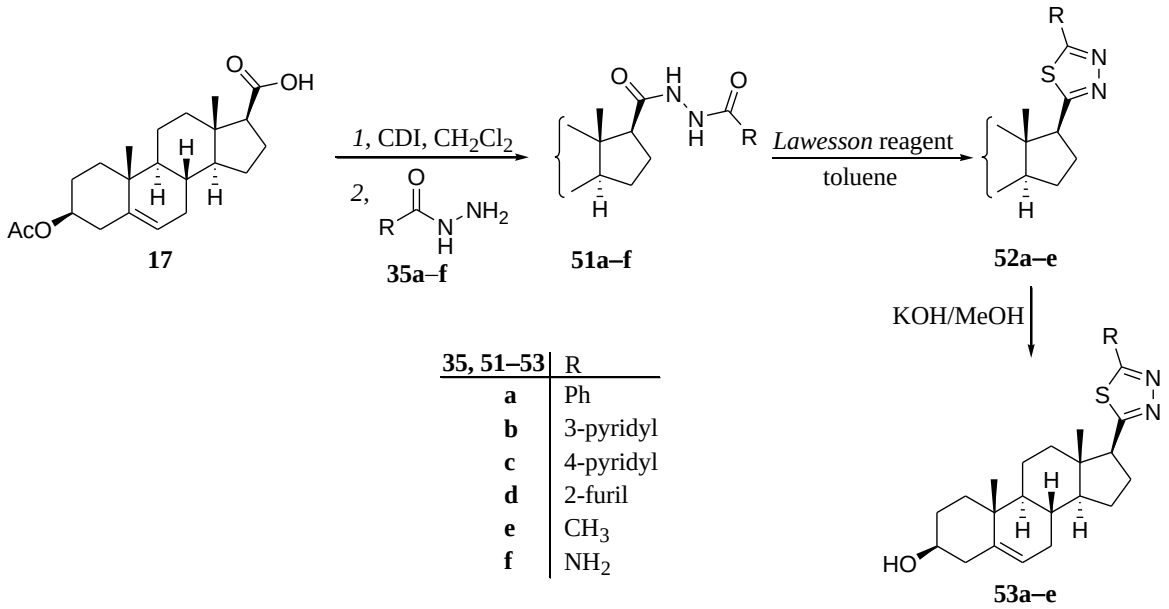
3.13. In the next stage of the work, we set out to introduce a 1,3,4-thiadiazole ring at position 17 of the androstane skeleton, and a preliminary experiment was carried out on **46a** in the Δ^{16} series (*Scheme 8*). In this case, a D-ring condensed pyrazolidine-3-thione (**49**), formed *via* intramolecular 1,4-addition to the C=C bond, was identified as main product, and was subjected to deacetylation in basic medium. The 16α,17α-*cis* junction of the heteroring was established through the NOESY spectrum of **49**, and NMR and MS measurements on **49** and its deacetylated analogue (**50**) confirmed that *O*→*S* exchange occurred on both oxygen atoms of the carbonyl groups directly attached to the nitrogen atoms, but the ester group at C-3 (**49**) remained unchanged.



46a

Scheme 8

3.14. The reactions of 16,17-unsaturated *N*,*N*′-diacylhydrazines (**51a–e**) with the *Lawesson* reagent led to the corresponding 17β-1′,3′,4′-thiadiazoles (**52a–e**), but the attempted synthesis of the amino-substituted analogue (**51f**) was unsuccessful (*Scheme 9*). Although the sulfur-containing reagent was applied in excess, formation of the related 17β-1′,3′,4′-oxadiazoles was also observed in some cases. Deacetylation of the 3β-acetates (**52a–e**) in basic media afforded compounds **53a–e**.



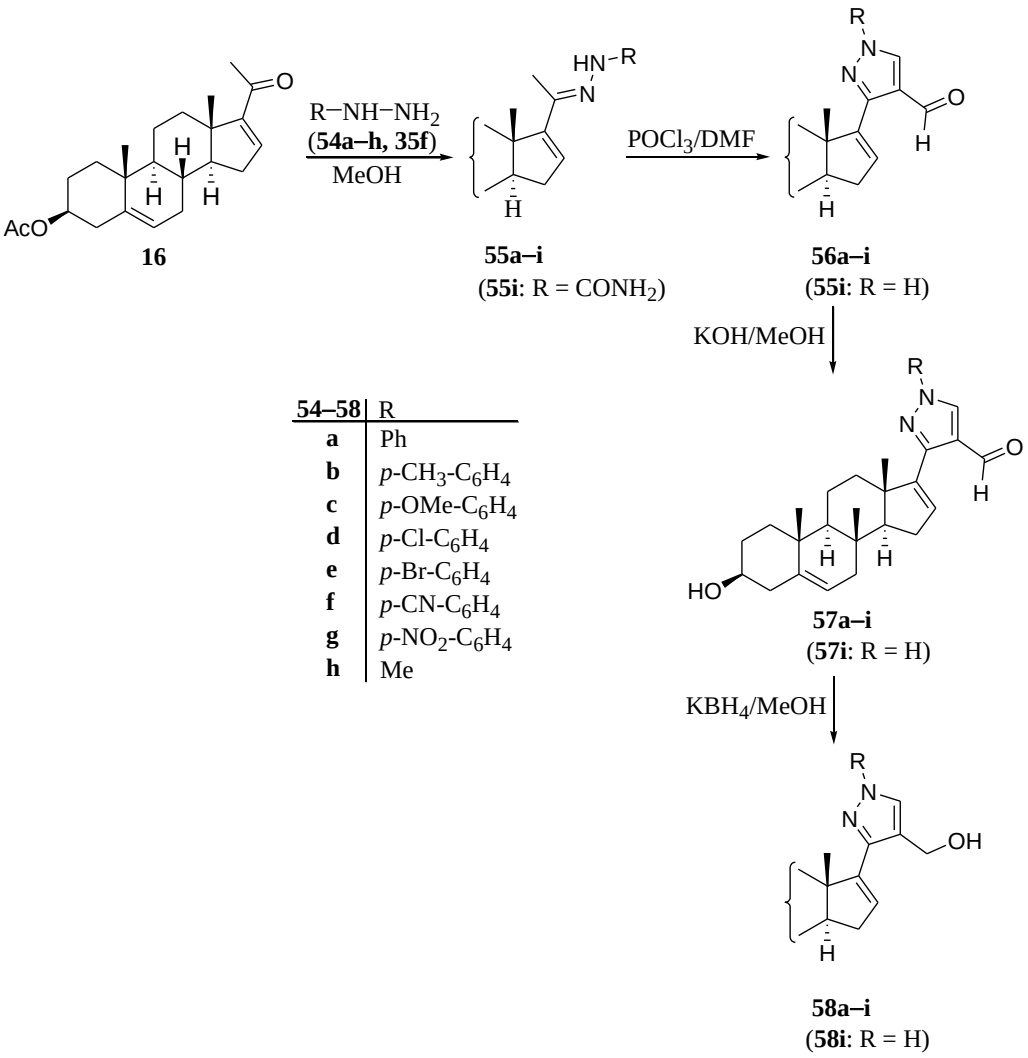
17 **35a–f** **51a–f** **52a–e** **53a–e**

35, 51–53	R
a	Ph
b	3-pyridyl
c	4-pyridyl
d	2-furil
e	CH_3
f	NH_2

Scheme 9

3.15. In the final period of the experimental studies, hydrazones (**55a–h**) and semicarbazone (**55i**), obtained from PDA (**16**), were converted to Δ^{16}-4′-formylpyrazolyl derivatives (**56a–i**) in the presence of the *Vilsmeier-Haack* reagent (*Scheme 10*). The *N*-methyl substituted derivative (**56h**) was isolated in only low yield after chromatographic purification, which could be ascribed to the side-reactions associated with the hydrazone formation and to the low stability of the methylhydrazone (**55h**). In agreement with previous

studies, the *Vilsmeier-Haack* reaction of semicarbazone (**55i**) resulted in the 1′-unsubstituted-4′-formylpyrazole (**56i**) instead of the 1′-aminocarbonyl-4′-formylpyrazole derivative. Subsequent deacetylation of 4′-formylpyrazoles (**56a–i**) in basic medium, followed by reduction with KBH_4, was carried out to furnish the corresponding 3β-hydroxy-4′-hydroxymethylpyrazolyl derivatives (**58a–i**).

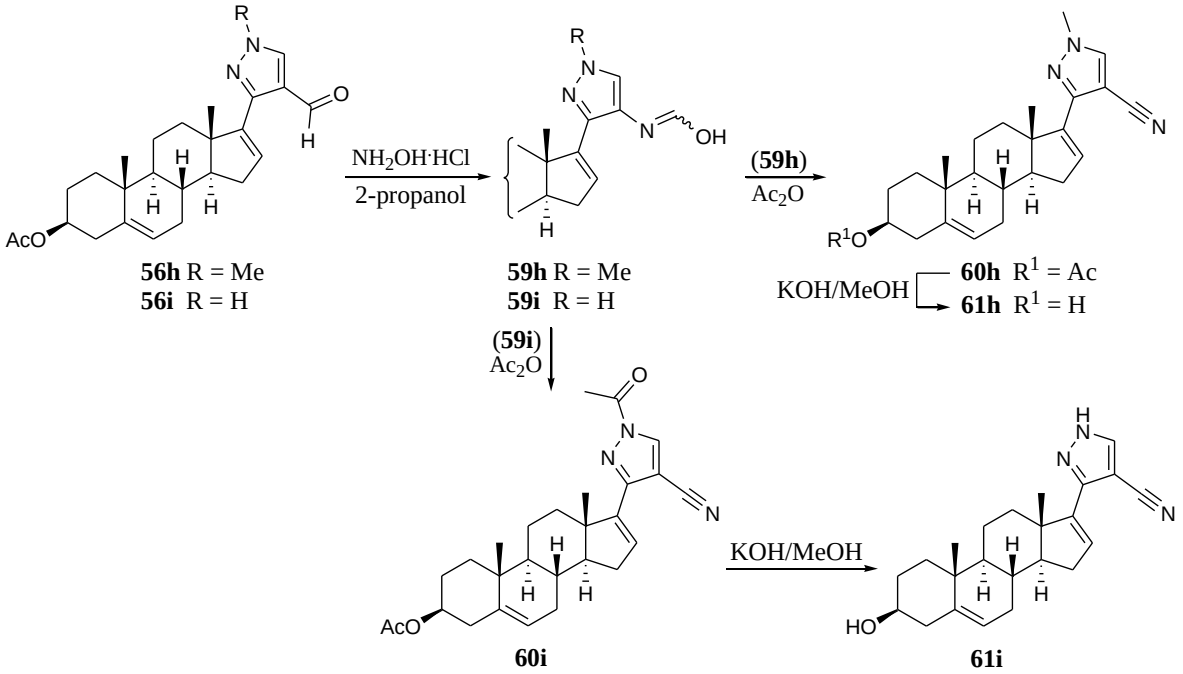


54–58	R
a	Ph
b	*p*-CH_3-C_6H_4
c	*p*-OMe-C_6H_4
d	*p*-Cl-C_6H_4
e	*p*-Br-C_6H_4
f	*p*-CN-C_6H_4
g	*p*-NO_2-C_6H_4
h	Me

Scheme 10

3.16. The 1:1 mixture of *E/Z*-aldoximes (**59h** and **59i**) obtained from **56h** and **56i** was converted without separation to the corresponding 4′-cyanopyrazoles (**60h** and **60i**). In the case of **59i**, containing an unsubstituted heteroring, the 1′-nitrogen of the pyrazolyl ring also underwent *N*-acylation besides the formation of a 4′-cyano group (*Scheme 11*). The 3β-hydroxy derivatives (**61h** and **61i**) of the 4′-cyanopyrazoles (**60h** and **60i**) were prepared by

deacetylation in basic medium. In the case of **60i**, the 3β-acetate underwent hydrolysis immediately after the rapid *N*-deacylation.



56h R = Me
56i R = H

59h R = Me
59i R = H

60h R^1 = Ac
61h R^1 = H

60i

61i

Scheme 11

3.17. The *in vitro* antiproliferative effects of the synthetized compounds were determined by our cooperation partner at the Department of Pharmacodynamics and Biopharmacy (University of Szeged). In measurements on different malignant human adherent cell lines, several derivatives proved to exert significant antiproliferative activities.

3.18. In view of structural relationship of the corresponding 3β-hydroxy-17-*exo*-heterocyclic compounds in the androstane series to abiraterone and galeterone, the inhibitory effects of these derivatives on rat testicular $C_{17,20}$-lyase were tested *in vitro* in collaboration with the 1st Department of Medicine (University of Szeged). Some of the derivatives displayed efficient enzyme inhibition.

4. Scientific publications forming the basis of the thesis

1. **Kovács, D.**; Kádár, Z.; Mótyán, G.; Schneider, Gy.; Wölfling, J.; Zupkó, I.; Frank, É.
Synthesis, characterization and biological evaluation of some novel 17-isoxazoles in the estrone series
Steroids **2012**, *77*, 1075–1085.

IF: 2.803

2. **Kovács, D.**; Wölfling, J.; Szabó, N.; Szécsi, M.; Kovács, I.; Zupkó, I.; Frank, É.
An efficient approach to novel 17-5′-(1′,2′,4′)-oxadiazolyl androstenes *via* the cyclodehydration of cytotoxic *O*-steroidacylamidoximes, and an evaluation of their inhibitory action on 17α-hydroxylase/$C_{17,20}$-lyase
Eur. J. Med. Chem. **2013**, *70*, 649–660.

IF: 3.432

3. **Kovács, D.;** Mótyán, G.; Wölfling, J.; Kovács, I.; Zupkó, I.; Frank, É.
A facile access to novel steroidal 17-2′-(1′,3′,4′)-oxadiazoles, and an evaluation of their cytotoxic activities *in vitro*
Bioorg. Med. Chem. Lett. **2014**, *24*, 1265–1268.

IF: 2.420

4. **Kovács, D.**; Wölfling, J.; Szabó, N.; Szécsi, M.; Minorics, R.; Zupkó, I.; Frank, É.
Efficient access to novel androsteno-17-(1′,3′,4′)-oxadiazoles and 17β-(1′,3′,4′)-thiadiazoles via *N*-substituted hydrazone and *N,N′*-disubstituted hydrazine intermediates, and their pharmacological evaluation *in vitro*
Eur. J. Med. Chem. **2015**, *98*, 13–29.

IF: 3.447*

Total IF: 12.102*

5. Scientific lectures and posters forming the basis of the thesis

Lectures:

1. **Kovács, D.**; Márton, J.; Baji, Á.; Mótyán, G.; Wölfling, J.; Frank, É.
   Androsztánvázas 17-oxa-, és 17-tiadiazolok szintézise
   12 November 2013, *MTA Szteroid- és Terpenoidkémiai Munkabizottsági ülése*, Budapest

2. **Kovács, D.**; Frank, É.; Wölfling, J.; Zupkó, I.
   Synthesis and *in vitro* pharmacological studies of novel 17β-1′,3′,4′-thiadiazoles in the androstane series
   3–5 July 2015, *5th Meeting of the Paul Ehrlich MedChem Euro-PhD Network*, Kraków (Poland)

Posters:

1. **Kovács, D.**; Kádár, Z.; Schneider, Gy.; Wölfling, J.; Zupkó, I.; Frank, É.
   Efficient approach to novel 17-isoxazolyl steroids by Cu(I)-catalyzed 1,3-dipolar cycloaddition
   26–29 June 2012, *13th Tetrahedron Symposium*, Amsterdam (The Netherlands) (P–1.42)

2. **Kovács, D.**; Mótyán, G.; Baji, Á.; Schneider, Gy.; Frank, É.
   1,2,4-oxadiazolgyűrűvel módosított androsztánvázas vegyületek szintézise
   26–28 June 2013, *Vegyészkonferencia*, Hajdúszoboszló (P–31)

3. **Kovács, D.**; Mótyán, G.; Baji, Á.; Schneider, Gy.; Frank, É.
   Efficient approach to steroidal 1,2,4-oxadiazoles in the androstane series
   30 June – 4 July 2013, *VIIIth Joint Meeting on Medicinal Chemistry*, Lublin (Poland) (P–23)

4. **Kovács, D.**; Frank, É.; Wölfling, J.
   Efficient synthetic pathway to novel steroidal 1,3,4-oxadiazole derivatives
   27–29 September 2013, *3rd Meeting of the Paul Ehrlich MedChem Euro-PhD Network*, Santa Margherita di Pula, Cagliari (Italy) (PO–11)

5. **Kovács, D.**; Frank, É.; Wölfling, J.
   Synthesis of steroidal 17-pyrazolyl derivatives using Vilsmeier-Haack reaction
   7–10 September 2014, *22nd Conference on Isoprenoids*, Prague (Czech Republic) *Chemicke Listy 108*, pg. s133.

6. **Kovács, D.**; Frank, É.; Wölfling, J.; Zupkó, I.
   Efficient synthesis and *in vitro* pharmacological studies of novel 17-pyrazolyl derivatives in the androstane series
   6–10 June 2015, *IXth Joint Meeting on Medicinal Chemistry*, Athens (Greece) (P–54)

7. **Kovács, D.**; Frank, É.; Wölfling, J.; Zupkó, I.
Synthesis and *in vitro* cytotoxic effects of novel steroidal 1,3,4-oxadiazoles
16–19 June 2015, *13th Tetrahedron Symposium*, Berlin (Germany) (P2.061)

6. Scientific publications not forming the basis of the thesis

1. Kádár, Z.; **Kovács, D.**; Frank, É.; Schneider, Gy.; Huber, J.; Zupkó, I.; Bartók, T.; Wölfling, J.
Synthesis and *in vitro* antiproliferative activity of novel androst-5-ene triazolyl and tetrazolyl derivatives
Molecules **2011**, *16*, 4786–4806.

IF: 2.386

2. Horváth, P.; Kósa, P. J.; Wölfling, J.; Balla, B.; **Kovács, D.**; Mátyus, P.; Horváth, E.; Speer, G.; Takács, I.; Nagy, Zs.; Lakatos, P.
D-hormon és CYP24A1-gátlás: Új megközelítés a colorectalis daganatok kezelésében
Magy. Belorv. Arch. **2011**, *64*, 266–272.

IF: −

3. Kósa, P. J.; Horváth, P.; Wölfling, J.; **Kovács, D.**; Balla, B.; Mátyus, P.; Horváth, E.; Speer, G.; Takács, I.; Nagy, Zs.; Horváth, H.; Lakatos, P.
CYP24A1 inhibition facilitates the anti-tumor effect of vitamin D3 on colorectal cancer cells
World J. Gastroenterol. **2013**, *19*, 2621–2628.

IF: 2.433

4. Frank, É.; **Kovács, D.**; Schneider, Gy.; Wölfling, J.; Bartók, T.; Zupkó, I.
Synthesis of novel steroidal 16-spiroisoxazolines by 1,3-dipolar cycloaddition, and an evaluation of their antiproliferative activities *in vitro*
Mol. Div. **2014**, *18*, 521–534.

IF: 1.896

5. Mótyán, G.; Kádár, Z.; **Kovács, D.**; Wölfling, J.; Frank, É.
Regio- and stereoselective access to novel ring-condensed steroidal isoxazolines
Steroids **2014**, *87*, 76–85.

IF: 2.639

Total IF: 9.354

7. Scientific posters not forming the basis of the thesis

1. Kádár, Z.; **Kovács, D.**; Schneider, Gy.; Wölfling, J.; Zupkó, I.; Frank, É.
Synthesis of novel 15β-triazolyl-5α-androstane derivatives as potent antiproliferative agents
26–29 June 2012, *13th Tetrahedron Symposium*, Amsterdam (The Netherlands) (P–1.41)

2. Mótyán G.; **Kovács D.**; Wölfling J.; Frank, É.
Androsztánvázas 16-spiro-izoxazolinek szintézise és szerkezetvizsgálata
26–28 June 2013, *Vegyészkonferencia*, Hajdúszoboszló (P–44)

3. Mótyán G.; **Kovács D.**; Wölfling J.; Frank, É.
Synthesis and structure determination of steroidal 16-spiroisoxazolines
30 June – 4 July 2013, *VIIIth Joint Meeting on Medicinal Chemistry*, Lublin (Poland) (P–34)

4. **Kovács, D.**; Mótyán, G.; Wölfling, J.; Frank, É.
Synthesis, characterization and biological effects of 16-spiro-isoxazolines in the androstane series
20–22 June 2014, *4th Meeting of the Paul Ehrlich MedChem Euro-PhD Network*, Hradec Králové (Czech Republic) (P–12)

5. **Kovács, D.**; Mótyán, G.; Baji, Á.; Frank, É.; Wölfling, J.
Efficient approach to novel ring-condensed steroidal isoxazolines by 1,3-dipolar cycloaddition
29 June – 4 July 2014, *20th International Conference on Organic Synthesis*, Budapest (P–51)