Summary of Ph.D. Thesis

SYNTHESIS OF 5-MEMBERED *N,O*-HETEROCYCLIC ESTRADIOL DERIVATIVES

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1. Introduction and aims

Steroids are a vital group of natural compounds from a physiological standpoint. Researching their semi-synthetic and synthetic derivatives has become a key area of focus for the modern pharmaceutical industry. In the study of semi-synthetic steroid derivatives, molecular hybridization is a frequently employed technique, wherein a pharmacophore moiety, typically heterocyclic, is formed on the sterane skeleton through condensation or via a linker. In most cases, these modifications are made at position 3, 17, or at adjacent carbon atoms, which can significantly affect hormone receptor binding, leading to a reduction in hormonal effects and the development of a new, desired pharmacological profile.

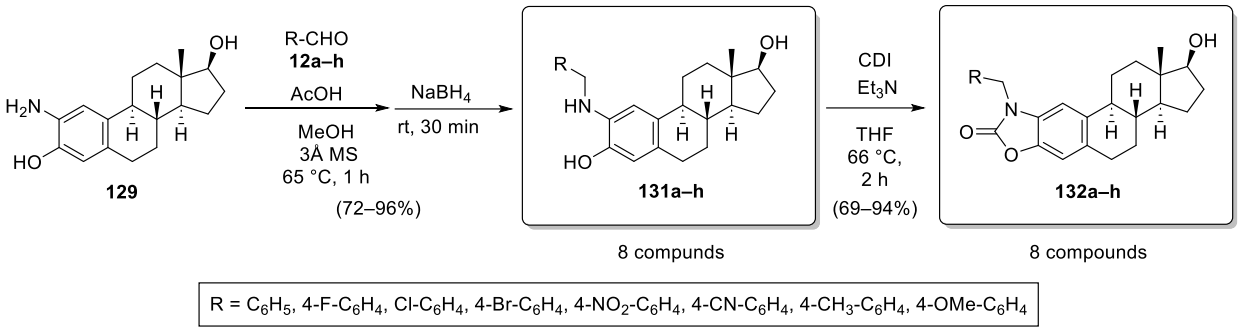
The main objective of my doctoral thesis was to synthesize novel estradiol derivatives that could potentially exhibit anticancer properties. We designed [2,3]-condensed heterocyclic estradiol hybrids, in which a five-membered *N,O*-heterocycle (oxazole, isoxazole, and oxazolone) was condensed to the A-ring of the sterane core. During the molecular design process, our focus was on creating a diverse molecular library that adhered to Lipinski's pharmacokinetic parameters and produced drug-like compounds. We strived to optimize the reaction conditions, including the quality and quantity of the reagents used, temperature, solvent, and purification methods to obtain the target products and confirm their structure. Additionally, we aimed to conduct *in vitro* pharmacological assessments of the synthesized compounds, which was carried out by the biologists of the Department of Biochemistry and Molecular Biology at the University of Szeged.

2. Experimental methods

During the course of our synthetic work, the majority of the reactions were performed in a millimolar scale. Thin layer chromatography (TLC) was employed to monitor the reactions, while normal-phase column chromatography (silica gel), flash chromatography (silica gel) or recrystallization were used to purify the crude products. To confirm the molecular structure of the synthesized compounds, we utilized one-dimensional NMR (^{1}H and ^{13}C) and MS (ESI-MS) techniques. Additionally, in order to design the molecules and estimate their pharmacokinetic parameters, we used Chemaxon's Chemicalize software.

3. Novel scientific results*

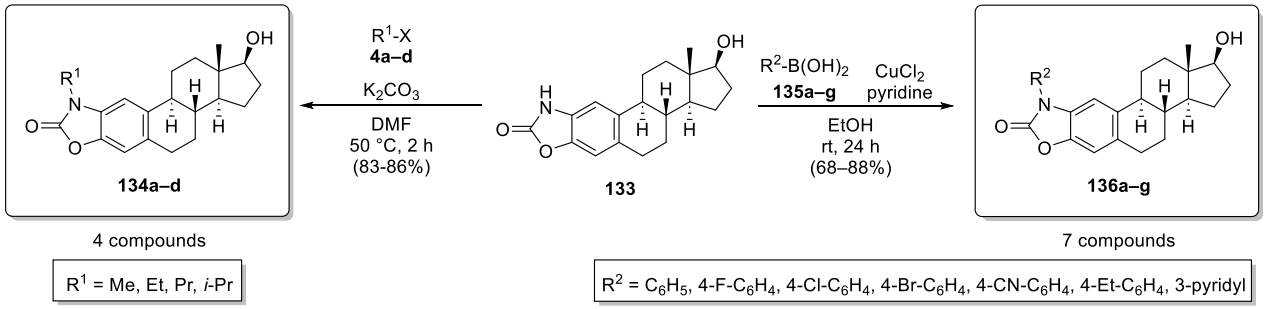
3.1. Secondary amines (**131a–h**) were synthesized by reductive amination of 2-aminoestradiol (**129**) and substituted benzaldehydes (**12a–h**) following the nitration and subsequent reduction of estrone (*Scheme 1*). Using benzaldehyde (**12a**) as the model substrate for the optimization of the reaction conditions, we observed that the reaction time for imine formation can be reduced to 1 hour by adding molecular sieves and acetic acid catalyst to the reaction. After reduction and purification, the target compounds were obtained with good to excellent yields (72–96%), regardless of the electronic nature of the substituent. (**1st publication**)



Scheme 1

3.2. *N*-benzylic oxazolone derivatives (**132a–h**) were synthesized from secondary amines (**131a–h**) in the presence of triethylamine (TEA) and 1,1´-carbonyldiimidazole (CDI) as a phosgene substitute. The reaction was carried out in boiling THF for 2 hours, resulting in the formation of the desired compounds. The unsubstituted analog (**133**) was also obtained through the cyclization of 2-aminoestradiol (**129**). (**1st publication**)

3.3. This cyclic carbamate (**133**) was utilized for base-promoted alkylations using various alkyl halides (**4a–d**) leading to the formation of *N*-alkyl compounds (**134a-d**) (*Scheme 2*).

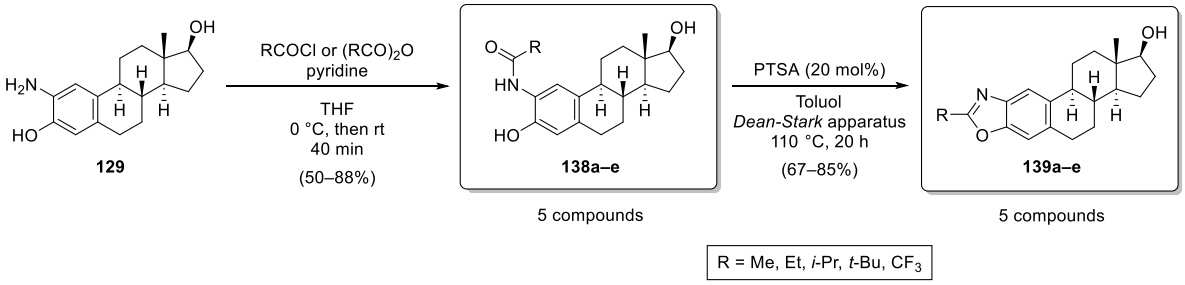


Scheme 2

* The numbering of the compounds follows the numbering in the dissertation.

To acquire additional *N*-(hetero)aryl analogs of the cyclic carbamate (**133**), the *Chan-Evans-Lam* cross-coupling with phenylboronic acid (**135a**) (*Scheme 2*) was investigated. The optimization of the reaction revealed noteworthy solvent effects: in polar aprotic solvents (DMF, DMSO), no conversion was observed. However, when using a polar protic solvent (EtOH), the transformation was complete within 24 hours, leading to the formation of **136a**. Following the optimized protocol, 6 additional *N*-(hetero)aryl analogs (**136b–g**) were synthesized using (hetero)arylboronic acids (**135b–g**) substituted with either electron-donating or electron-withdrawing groups (*Scheme 2*), which after chromatographic purification were obtained in medium-good yields (67–88%).

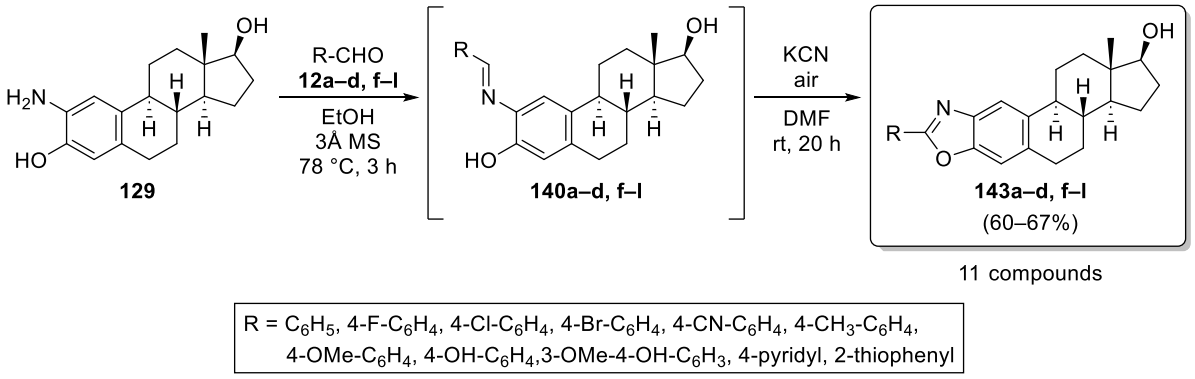
3.4. We observed that under anhydrous conditions, the cyclization of 2-aminoestradiol with triethyl orthoformate yields the unsubstituted oxazole compound (**137**). We also examined the synthesis of analogs that contain alkyl (Me, Et, *i*-Pr, *t*-Bu) and CF_3 substituents in position 2 of the oxazole (*Scheme 3*). By utilizing the appropriate acid chloride/acid anhydride reagent for the amide formations, *N*-acyl estradiols (**138a–e**) were prepared in moderate to good isolated yields (50–88%). During the dehydrocyclizations of the amides, we observed that the ring closure can be driven to completion by removing the water formed during the reaction using *Dean-Stark* apparatus. After chromatographic purification, the target products (**139a–e**) were obtained with good yields (67–85%). (**3rd publication**)



Scheme 3

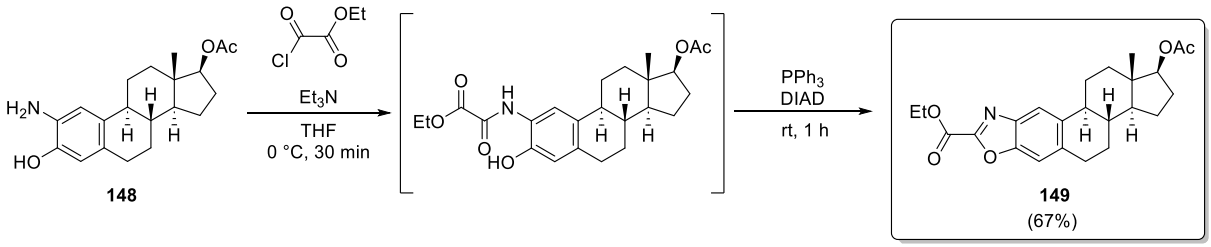
3.5. To expand the molecular library, we prepared 11 additional derivatives (**143a–d, f–l**) substituted with (hetero)aryl groups (*Scheme 4*). Initially, we formed the *Schiff* bases by carrying out a condensation reaction between 2-aminoestradiol and (hetero)aryl aldehydes. The oxidative cyclization was further facilitated by nucleophilic catalysis using potassium cyanide (KCN). Our findings revealed a direct correlation between the speed of the reaction and the amount of KCN utilized. In instances where a catalytic amount of KCN was used, the reaction was sluggish compared to when a stoichiometric amount was employed. The target compounds

were isolated with yields of 60–67% after purification by column chromatography and recrystallization. (**3rd publication**)



Scheme 4

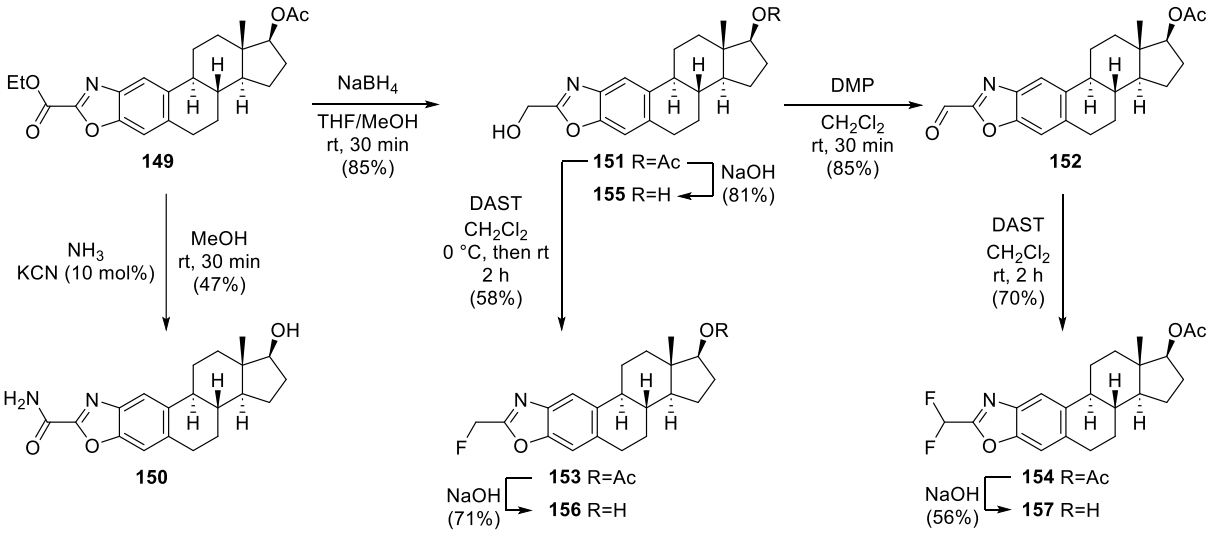
3.6. The cyclization of the amide formed from the reaction of ethyl 2-chloro-2-oxoacetate with 2-aminoestradiol-17-acetate (**148**) was performed to prepare a precursor of 2'-ethylcarboxy oxazole (**149**). This precursor can be used for synthesizing further analogues (*Scheme 5*). We have demonstrated that the *5-exo-trig* cyclization of the amide amide to oxazole can be achieved chemoselectively by an intramolecular *Mitsunobu* reaction. This is in contrast to the previously used ring closure facilitated by *p*-toluenesulfonic acid (PTSA), which only results in the formation of other by-products, such as oxazine-2,3-dione. By further optimizing the reaction conditions, we developed a sequential one-pot method, which enabled us to isolate the precursor **149** with a yield of 67%.



Scheme 5

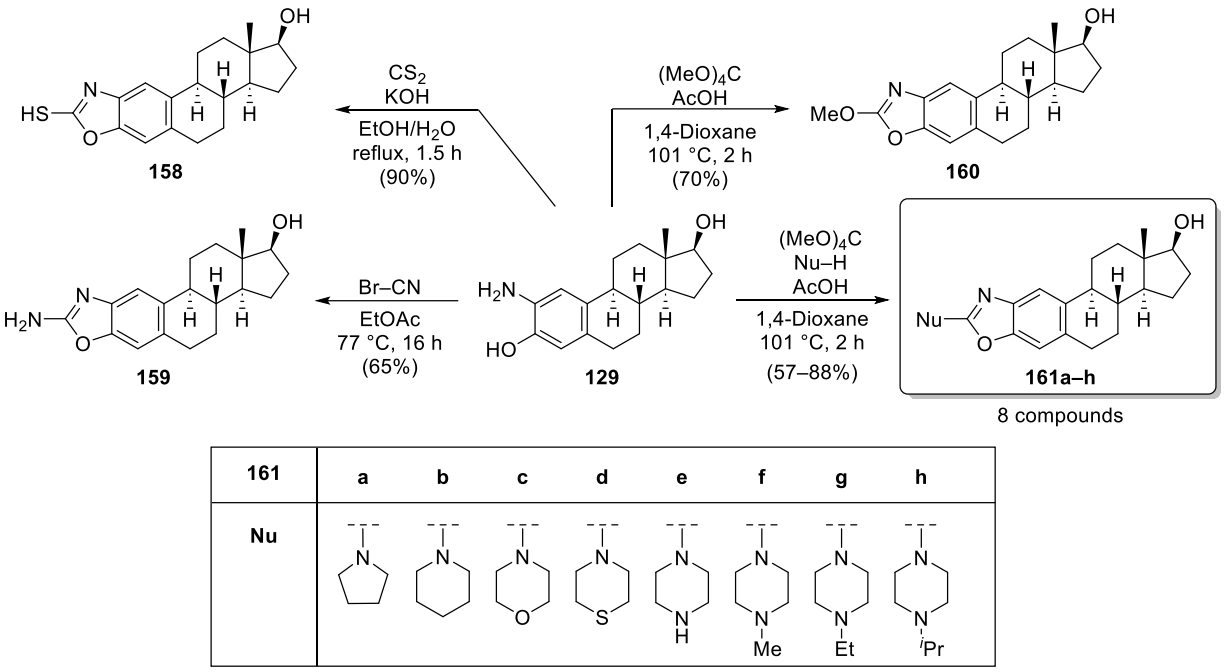
A series of functional group transformations were carried out on the precursor **149** resulting in the synthesis of 8 additional analogs (*Scheme 6*). Upon reaction with ammonia, precursor **149** provided a carboxamide derivative (**150**). It was demonstrated that the ethyl ester functional group of precursor **149** could be selectively reduced with $NaBH_4$ to yield the corresponding

hydroxymethyl analog (**151**), which could then be converted to the formyl oxazole using *Dess-Martin* oxidation. The fluoromethyl (**153**) and difluoromethyl (**154**) derivatives were also synthesized using diethylaminosulfur trifluoride (DAST), a nucleophilic fluorinating agent. In the final step, the removal of the 17-acetate protecting groups was carried out, and NaOH was found to be a suitable reagent for this purpose, as opposed to LiOH, which resulted in the unintended opening of the oxazole ring during initial test reactions. (**3rd publication**)



Scheme 6

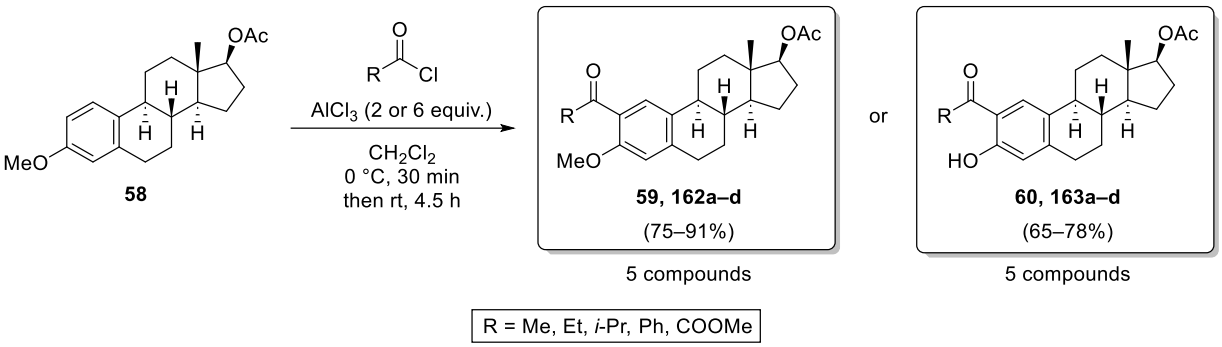
3.7. We obtained 2'-thiol (**158**), 2'-amino (**159**), and 2'-methoxy derivatives (**160**) by cyclizing compound **129** with carbon disulfide (CS_2), cyanogen bromide (BrCN), and tetramethylorthocarbonate electrophilic agents (*Scheme 7*). When studying the aromatic nucleophilic substitution reactions of the latter compound with cyclic amines, a solvent effect was observed. By replacing chloroform with 1,4-dioxane, the reaction time was reduced from 16–20 hours to 1–2 hours. This was due to the better solubility of the starting material in 1,4-dioxane and the solvent's higher boiling point. We explained the reaction rate increase caused by the higher temperature that can be applied. (**3rd publication**)



161	a	b	c	d	e	f	g	h
Nu								

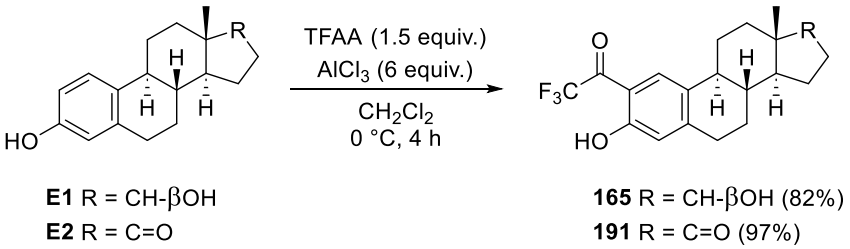
Scheme 7

3.8. We investigated the impact of the solvent on the *Friedel-Craft* acylation and consecutive demethylation reaction of 3-methoxyestradiol-17-acetate (**58**) and acetyl chloride. The reaction was performed using different solvents, including dichloromethane, chloroform, chlorobenzene, nitromethane, and amylene-stabilized dichloromethane. We observed, that the use of ethanol-stabilized solvents did not result in complete conversion of the starting material, even with the excess of acid chloride. The application of nitromethane and chloroform was found to be ineffective in the demethylation of the methyl ether intermediate (**59**). However, amylene-stabilized dichloromethane facilitated both *Friedel-Crafts* acylation and demethylation reactions, which were complete within 4.5 hours. The optimized reaction conditions were utilized to synthesize 2-acyl-estradiol-17-acetate analogs (**163a–d**) in good yield (65–78%). The reaction conditions involved the use of 1.5 equivalents of acid chloride, 6 equivalents of $AlCl_3$, CH_2Cl_2 stabilized with amylene, 0 °C for 30 minutes, followed by room temperature for 4 hours. Additional aliphatic and aromatic acid chlorides were employed alongside the optimized reaction conditions. Similarly, the corresponding methyl ethers (**162a–d**) were prepared with good to excellent yields (75–91%) when the amount of *Lewis* acid was reduced to 2 equivalents.



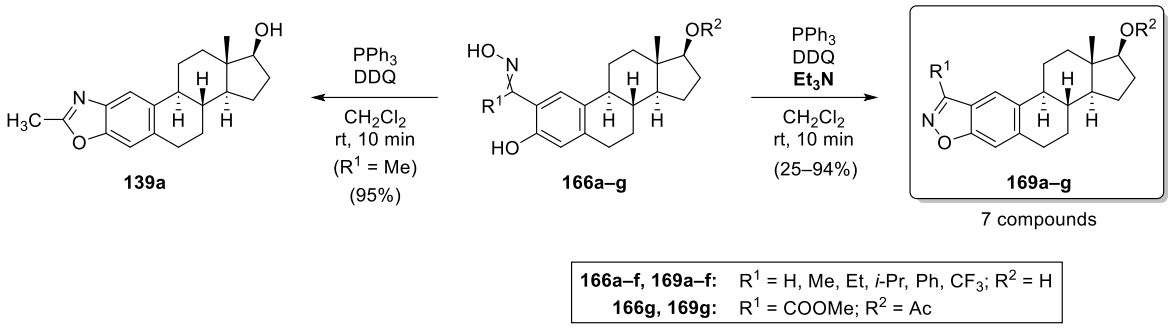
Scheme 8

It has been demonstrated that this method utilizing trifluoroacetic anhydride (TFAA) can successfully be applied to both estradiol (**E2**) and estrone (**E1**) substrates (*Scheme 9*). Through precise control of the temperature, undesirable side reactions such as ester formation and *Wagner-Meerwein* rearrangement can be mitigated, while optimal yields of the desired products (**165** and **191**) can be achieved, ranging from 82% to 97%. (**2nd publication**)



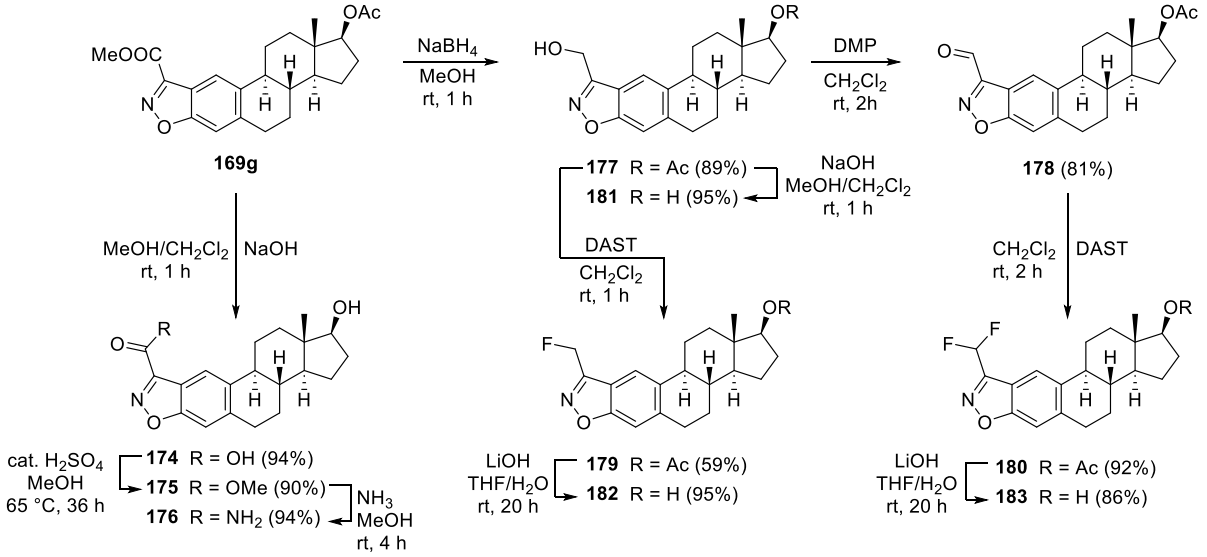
Scheme 9

3.9. Oxime formation reactions were conducted with 2-formyl- and 2-acyl-estradiol compounds (**55**, **163d**, **164a–c**, **165**) and hydroxylamine hydrochloride. It was observed that in the case of the more sterically hindered compounds **164b**, **164c,** and **165**, complete conversion of the starting material could only be achieved by substituting the NaOAc base with pyridine, using an excess of the hydroxylamine hydrochloride reagent and boiling the reaction mixture. We investigated the ring closure reactions of oximes **166a–g** using the PPh_3/DDQ reagent combination (*Scheme 10*). Our observations revealed that the chemoselectivity of the reaction can be controlled for compounds containing the electron-donating group (R = Me, Et, *i*-Pr). Specifically, without the use of a base, the formation of the oxazole **139a** was observed in the case of **166b** cyclization, whereas the presence of TEA resulted in the formation of only the isoxazole **169b**. However, in the case of the aldoxime **166a** and the compounds **166f** and **166g** containing electron-withdrawing groups (R = CF_3 or COOMe), the base did not influence the chemoselectivity of the reaction, but it increased the reaction rate. (**2nd publication**)



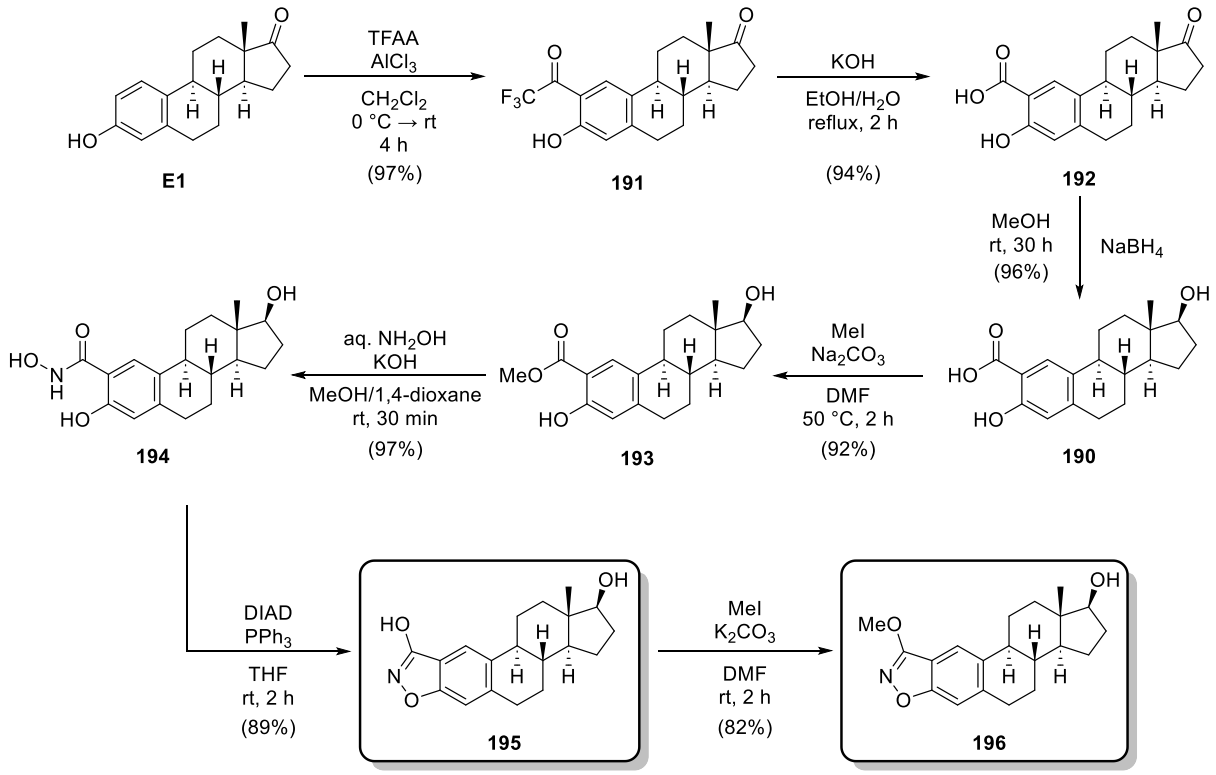
Scheme 10

3.10. Starting from the precursor **169g**, we have successfully demonstrated the synthesis of 10 additional 3-substituted isoxazole analogs through a series of functional group transformations, such as amide formation, reduction, oxidation, and deoxofluorination (*Scheme 11*). (**2nd publication**)



Scheme 11

3.11. A multistep linear sequence starting from estrone for the synthesis of compounds **195** and **196** (*Scheme 12*) was also developed. It was demonstrated that the synthesis of 2-carboxyestrone (**192**), which is the key intermediate of the reaction sequence, from 2-trifluoroacetylestrone (**191**) can be achieved with an exceptional isolated yield of 94%. Following optimization of the steps of the reaction sequence, including reduction, esterification, and hydroxamic acid formation, compound **195** was prepared by intramolecular *Mitsunobu* reaction. The methoxy analog **196** was also synthesized through *Williamson* ether synthesis from compound **195**. (**2nd publication**)



Scheme 12

4. Pharmacological results

Several compounds demonstrated activity during the *in vitro* pharmacological evaluation of the produced compounds. Halogenated compounds **131c** and **132b** from the benzoxazolone compound family showed IC_{50} values around 1 μM on the DU-145 prostate cancer cell line, while the unsubstituted carbamate **133** demonstrated promise on the HeLa cell line. Among the benzoxazoles, the 2′-ethyl derivative (**139b**) exhibited the most effectiveness on the MCF-7 breast cancer cell line with an IC_{50} value of 2.04 μM, while the pyridyl (**143k**), the amino (**159**), and the *N*-ethylpiperazine derivative (**161g**) demonstrated IC_{50} values around 5 μM on A549, DU-145, HeLa, and MCF-7 cell lines. The alkyl-substituted compounds **169b**, **169c**, and **169d** were particularly notable from the benzisoxazole molecular family, of which derivative **169c**, containing an ethyl substituent, showed IC_{50} values below 1 μM on both DU-145, HeLa, and MCF-7 cell lines while maintaining excellent selectivity.

5. Scientific publications directly related to the Ph.D. Thesis (MTMT identifier: 10069242)

1. **Ferenc Kovács**, Mohana K. Gopisetty, Dóra I. Adamecz, Mónika Kiricsi, Éva A. Enyedy, Éva Frank
   Synthesis and Conversion of Primary and Secondary 2-Aminoestradiols into A-Ring-Integrated Benzoxazolone Hybrids and Their in Vitro Anticancer Activity
   RSC Advances, **2021**, 11 (23), 13885–13896.

   IF = 4.036

2. **Ferenc Kovács**, Dóra I. Adamecz, Ferenc I. Nagy, Benedek Papp, Mónika Kiricsi, Éva Frank
   Diversity-Oriented Synthesis and In Vitro Anticancer Activity of Framework-Integrated Estradiol-Benzisoxazole Chimeras
   Molecules, **2022**, 27 (21), 7456.

   IF = 4.600

3. **Ferenc Kovács**, Ildikó Huliák, Hédi Árva, Mónika Kiricsi, Dóra Erdős, Marianna Kocsis, Gergely Takács, György T. Balogh, Éva Frank
   Medicinal-Chemistry-Driven Approach to 2-Substituted Benzoxazole–Estradiol Chimeras: Synthesis, Anticancer Activity, and Early ADME Profile
   ChemMedChem, **2023**, e202300352.

   IF = 3.400

Total IF = 12.036

6. Other scientific publications

1. Éva A. Enyedy, Anett Giricz, Tatsiana V. Petrasheuskaya, János P. Mészáros, Nóra V. May, Gabriella Spengler, **Ferenc Kovács**, Barnabás Molnár, Éva Frank
   Comparative Solutions Equilibrium Studies on Anticancer Estradiol-Based Conjugates and Their Copper Complexes
   Inorganics, **2024**, 12(2), 49.

   IF = 2,900*

2. János P. Mészáros, Hilda Kovács, Gabriella Spengler, **Ferenc Kovács**, Éva Frank, Éva A. Enyedy
   A comparative study on the metal complexes of an anticancer estradiol-hydroxamate conjugate and salicylhydroxamic acid
   Journal of Inorganic Biochemistry, **2023**, 244, 112223.

   IF = 3.900*

3. Tatsiana V. Petrasheuskaya, **Ferenc Kovács**, Nóra Igaz, Andrea Rónavári, Bálint Hajdú, Laura Bereczki, Nóra V. May, Gabriella Spengler, Béla Gyurcsik, Mónika Kiricsi, Éva Frank, Éva A. Enyedy
   Estradiol-Based Salicylaldehyde (Thio)semicarbazones and Their Copper Complexes with Anticancer, Antibacterial and Antioxidant Activities
   Molecules, **2023**, 28, 54.

IF = 4.600*

4. Tatsiana V. Petrasheuskaya, **Ferenc Kovács**, Gabriella Spengler, Nóra V. May, Éva Frank, Éva A. Enyedy
   A comparative study on the complex formation of 2-aminoestradiol and 2-aminophenol with divalent metal ions: solution chemistry and anticancer activity
   Journal of Molecular Structure, **2022**, 1261, 132858.
   IF = 3.800

5. Ádám Baji, **Ferenc Kovács**, Gergő Mótyán, Gyula Schneider, János Wölfling, Izabella Sinka, István Zupkó, Imre Ocsovszki, Éva Frank
   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
   Steroids, **2017**, 126, 35–49.
   IF = 2.523

6. Gergő Mótyán, **Ferenc Kovács**, János Wölfling, András Gyóvai, István Zupkó, Éva Frank
   Microwave-assisted stereoselective approach to novel steroidal ring D-fused 2-pyrazolines and an evaluation of their cell-growth inhibitory effects in vitro
   Steroids, **2016**, 112, 36–46.
   IF = 2.282

Total IF = 20.005

7. Lectures and posters related to the Ph.D. Thesis

1. **Kovács Ferenc**, Éva Frank
   Ösztránváz A-gyűrűjéhez kondenzált oxazolok és izoxazolok régiószelektív szintézise
   MTA Szteroid- és Terpenoidkémiai Munkabizottsági ülés, online, 28 November 2022.

2. **Kovács Ferenc**, Éva Frank
   Ösztránvázas vegyületek A-gyűrűjének módosítása öttagú, *N,O*-heterociklusokkal
   MTA Szteroid- és Terpenoidkémiai Munkabizottsági ülés, online, 6 December 2021.

3. **Kovács Ferenc**, Éva Frank
   Ösztránváz A-gyűrűjéhez kondenzált *N,O*-heterociklusok szintézise
   A Szegedi Ifjú Szerves Kémikusok Támogatásáért Alapítvány előadóülése, online, 18 May 2020.

8. Other lectures and posters

1. Tamás Pivarcsik, **Ferenc Kovács**, Gabriella Spengler, Éva Frank, Bernhard K. Keppler, Wolfgang Kandioller, Éva A. Enyedy
   Half-sandwich Ru(II) and Rh(III) organometallic complexes with sterane-based bidentate ligands bearing (N,N) donor set

16th International Symposium on Applied Bioinorganic Chemistry, ISABC, Ioannina, Greece, 11–14 June 2023. (poster presentation)

2. Tatsiana V. Petrasheuskaya, **Ferenc Kovács,** Nóra V. May, Andrea Rónavári, Mónika Kiricsi, Gabriella Spengler, Éva Frank, Éva A. Enyedy
   Estradiol-based salicylaldehyde (thio)semicarbazones and their copper complexes with anticancer, antibacterial and antioxidant activities
   International Symposium on Metal Complexes, ISMEC, Valencia, Spain, 5–8 June 2022. (poster presentation)

3. Tamás Pivarcsik, **Ferenc Kovács**, Gabriella Spengler, Éva Frank, Éva A. Enyedy
   Sterane-based bidentate ligands with (N,N) donor set: synthesis, biological activity, solution chemistry and interaction with half-sandwich Ru and Rh cations
   International Symposium on Metal Complexes, ISMEC, Valencia, Spain, 5–8 June 2022. (poster presentation)

4. János P. Mészáros, Hilda Kovács, Gabriella Spengler, **Ferenc Kovács**, Éva Frank, Éva A. Enyedy
   Aqueous solution behaviour of half-sandwich Ru and Rh complexes of an salicylhydroxamic acid derivative
   International Symposium on Metal Complexes, ISMEC, Valencia, Spain, 5–8 June 2022. (poster presentation)

5. Gergő Mótyán, **Ferenc Kovács**, János Wölfling, Éva Frank
   Application of microwave irradiation for the stereoselective synthesis of androstenefused 2-pyrazolines
   16th Tetrahedron Symposium - Challenges in Bioorganic & Organic Chemistry, Berlin, Germany, 16–19 June 2015. (poster presentation)

6. Gergő Mótyán, Dóra Kovács, **Ferenc Kovács**, Gyula Schneider, Éva Frank
   Stereoselective synthesis of new androstene-fused arylpyrazolines as potent antiproliferative agents
   20th International Conference on Organic Chemistry, Budapest, Hungary, 29 June – 4 July 2014.

Cumulative IF = 32.041