Synthesis of *N*-containing oestrone derivatives and *in vitro* evaluation of their antiproliferative activities

The theses of Ph.D. dissertation

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1. Introduction and aims

Recent years have seen an extensive focus of interest in the development of steroids with selective biological activity. A number of non-selective steroids are known in anticancer therapy, which displays a wide range of side-effects, such as hormonal actions and nausea, bone marrow depression or nephrotoxicity. Structural modifications (e.g. the homologization of ring B or D, the synthesis of D-seco derivatives, or the epimerization of naturally occurring steroids) may lead to hormonally inactive and selective therapeutic anticancer agents. Most steroidal anticancer drugs behave as enzyme inhibitors, anti-oestrogens or antimitotic agents. Enzyme inhibitors (e.g. aromatase, steroid sulfatase or 17β-hydroxysteroid dehydrogenase inhibitors) have been developed to reduce the biosynthesis of the endogenous steroids; and therefore hinder the proliferation of hormone-dependent cancer cells. Anti-oestrogens are competitive antagonists of the endogenous oestrogens; they bind to the oestrogen receptor (ER) and inhibit oestrogenic action. The antimitotic agents inhibit the cell cycle in the mitosis phase; they influence the polymerization of tubulin, and hence an anomalous microtubule framework is obtained.

The aim of the present study was the synthesis of new oestrone derivatives by homologization and/or substitution of ring D, or by introduction of a heterocyclic moiety into the 13β- and 13α-oestrone scaffolds.

2. Experimental methods

Most reactions were carried on a millimolar scale, and were monitored by thin-layer chromatography. The crude products were purified by flash chromatography. The structures of the new products were confirmed by NMR (one- and two-dimensional) and MALDI-MS, EI-MS techniques, with C_{70} fullerenes as matrix. The antiproliferative properties of the synthesized compounds were determined on a panel

of human adherent cell lines (Hela – cervical cancer, MCF-7 – breast cancer, A2780 – ovarian cancer, A431 – epidermal skin cancer) and on the intact fibroblast cell lines (HFF-2 and MRC-5) by means of MTT assays. Some of the compounds proved to be more effective (with submicromolar IC_{50} values) than the reference agent cisplatin. The results of the MTT assays led to the selection of some compounds for additional analyses to determine the mechanism of their antiproliferative action. A few of them were subjected to further *in vitro* investigations with MTT assays on a panel of breast cancer cell lines differing in receptorial status, including T-47D (expressing oestrogen, progesterone and androgen receptors), MDA-MB-361 (expressing oestrogen and HER-2 receptors) and triple negative cell line MDA-MB-231 (absence of oestrogen, progesterone and HER-2 receptors). In other cases, cell cycle analyses were performed after 24 and 48 h of exposure. Additionally, the direct effect of the synthesized compounds on tubulin polymerization was examined *in vitro* with the tubulin polymerization assay; the reference agent was paclitaxel.

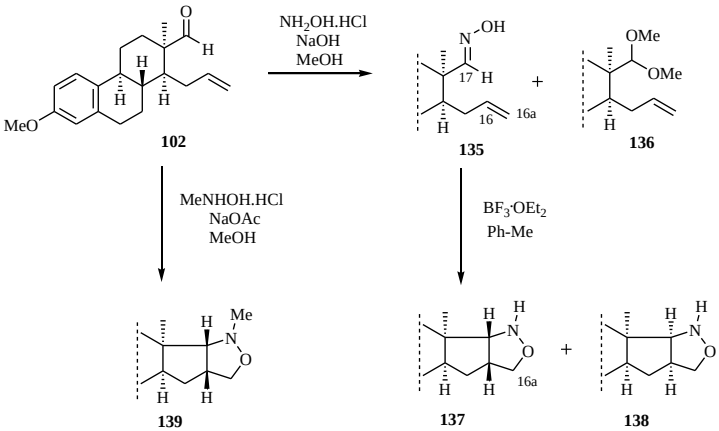
3. Novel scientific results

3.1. The D-secoaldehyde (**102**)[1] of 13α-oestrone was transformed into the appropriate oxime (**135**) in high yield by using hydroxylamine hydrochloride and sodium hydroxide in methanol (Scheme 1). Dimethyl acetal (**136**) was formed as side-product, but this side-reaction could be avoided by using sodium acetate instead of sodium hydroxide.

3.2. The intramolecular cyclization of **135** was carried out with Lewis acid ($BF_3 \cdot OEt_2$) in toluene. Two different isoxazolidine stereoisomers (**137**, **138**) with *cis* ring anellations were formed, in similar yields. This can be explained by the flexible molecular framework of the 13α-oestrone derivatives. Under the same reaction

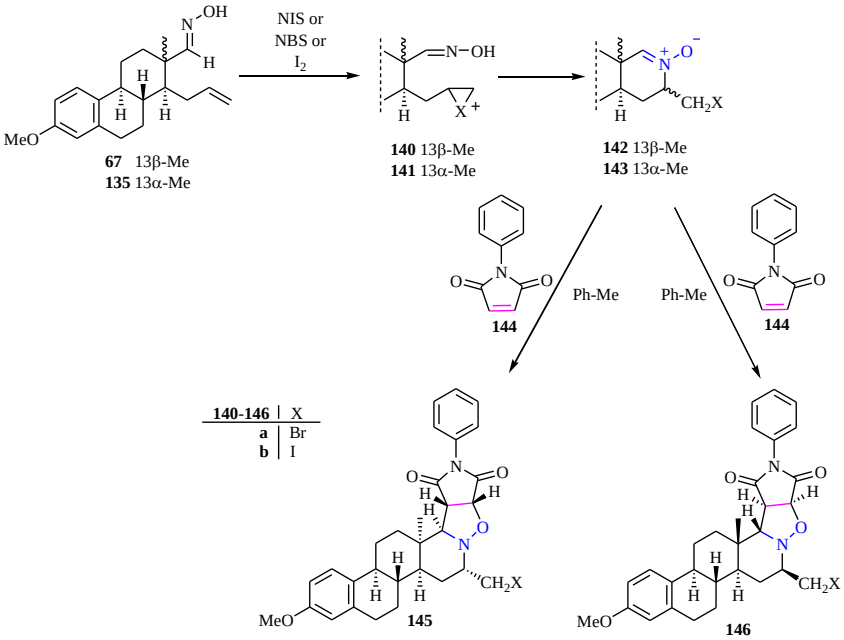
[1]The numbering of the compounds accords with that in the Ph.D. Thesis.

conditions as those used for the oximation, the reaction of **102** with *N*-methylhydroxylamine hydrochloride resulted in one cyclized product (**139**). The *cis*-ring anellations were proved by NOE NMR measurements.



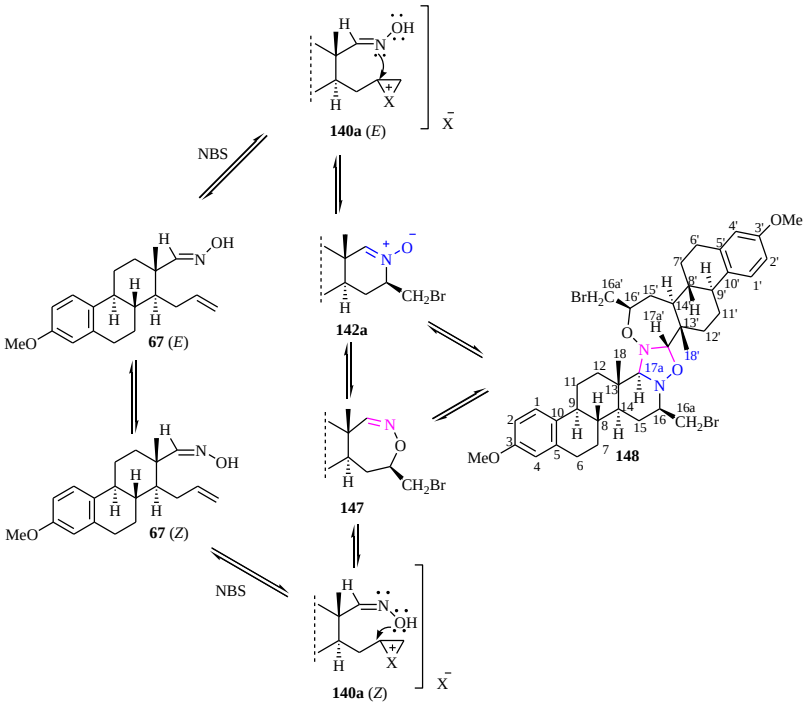
Scheme 1

3.3. The oximes (**67**, **135**) in the 13α and 13β series were transformed into the appropriate nitrones (**142**, **143**) in electrophile-induced reactions with various halogenating agents: *N*-bromosuccinimide (NBS), *N*-iodosuccinimide (NIS) or iodine (I_2) (Scheme 2). The cyclic nitrone dipoles (**142**, **143**) were reacted with a C=C dipolarophile (**144**, *N*-phenylmaleimide, NPM), leading stereoselectively to 16-bromomethyl- (**145a**, **146a**) or 16-iodomethyl-isomers (**145b**, **146b**). The configurations of the newly formed stereogenic centres were identified by means of 2D NMR experiments (COSY, NOESY and HSQC).



Scheme 2

3.4. In the 13β-oestrone series, bromination of the oxime (**67**) led to the formation of a dimeric product (**148**, Scheme 3). The trapping of the intermediate bromonium ion proceeded via the *O*-atom in the case of the *Z*-oxime, and via the *N*-atom in the case of the *E*-oxime. The oxazepine derivative (**147**, as a steroidal C=N dipolarophile) and the cyclic nitrone (**142a**, a 1,3-dipole) reacted with each other in an intermolecular 1,3-dipolar cycloaddition, stereoselectively furnishing a non-symmetrical steroid dimer (**148**). The dimer formation was reversible. The expected cycloadduct (**146a**) was furnished when NPM (**144**) was added to the dimer in acetonitrile. The dimer was also formed in dichloromethane, but did not precipitate from the reaction mixture.



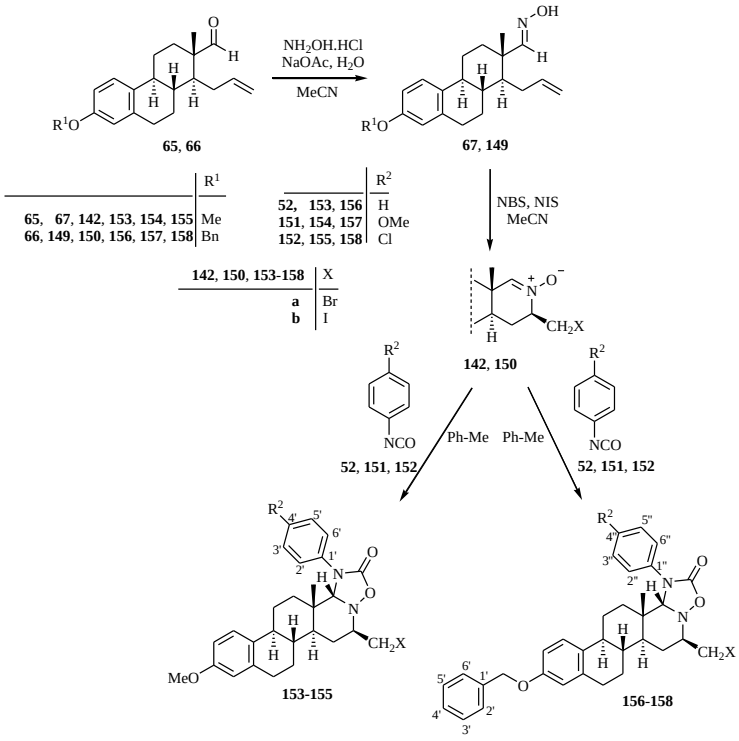
Scheme 3

3.5. Cyclic nitrones of oestrone 3-methyl or 3-benzyl ether (**142**, **150**) were reacted with phenyl isocyanates (**52**, **151**, **152**) as reactive C=N dipolarophiles, chemoselectively yielding condensed homosteroidal oxadiazolidinones (**153**–**158**, Scheme 4). The newly formed stereogenic centres displayed the same configurations as earlier: a 16β-substituent and a 17aβ-hydrogen at the anellation of the piperidine and oxadiazolidinone rings.

3.6. The condensed homosteroidal oxadiazolidinone derivatives (**153**–**158**) were formed in high yields, the reaction times depending on the nature of the substituents of the phenyl isocyanate. An electron-donating methoxy group on the phenyl ring (**151**) promoted the reaction, and the dual nature of the chloro substituent (**152**) also

surprisingly accelerated the reaction. The lowest extent of reaction was observed with the unsubstituted reagent (**52**).

3.7. The cycloadditions were carried out under reflux or with microwave irradiation. The reaction rates, and the chemo- and stereoselectivities under the two different conditions were compared. The results of the microwave-induced reactions did not depend on the nature of the substituent in the phenyl isocyanates (**52**, **151**, **152**).

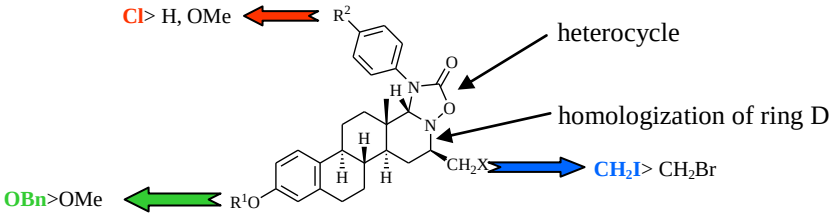


Scheme 4

3.8. The antiproliferative properties of the newly synthesized compounds (**153**–**158**) were characterized (Scheme 5). The oxadiazolidinones (**153**–**158**) bearing different functional groups at positions C-3, C-16a or C-4’ displayed different growth-

inhibitory effects. They were active exclusively against gynaecological cancer cell lines; none of them inhibited the proliferation of A431 human epidermoid cells. It can be concluded that the substituent on C-4' has an impact on the anticancer properties since compounds bearing a 4'-methoxy group (**154a**, **b** and **157a**, **b**) appeared to be totally inactive (IC_{50} > 30 μM). 3-Benzyloxy derivatives were slightly more effective than their 3-methoxy counterparts. The most potent compound was the 16-iodo-*N*-phenyl-3-benzyloxy derivative (**156b**), with IC_{50} = 2.19 μM for A2780 ovarian carcinoma cells. It appeared to be tumour-selective and indicated a cell cycle blockade in the G1–S transition.

3.9. In conclusion, the presence of the benzylic protecting group and the six-membered ring D, the introduction of a heterocyclic moiety into the molecule and the CH_2I substituent at position C-16 were advantageous for the antiproliferative potential (Scheme 5).

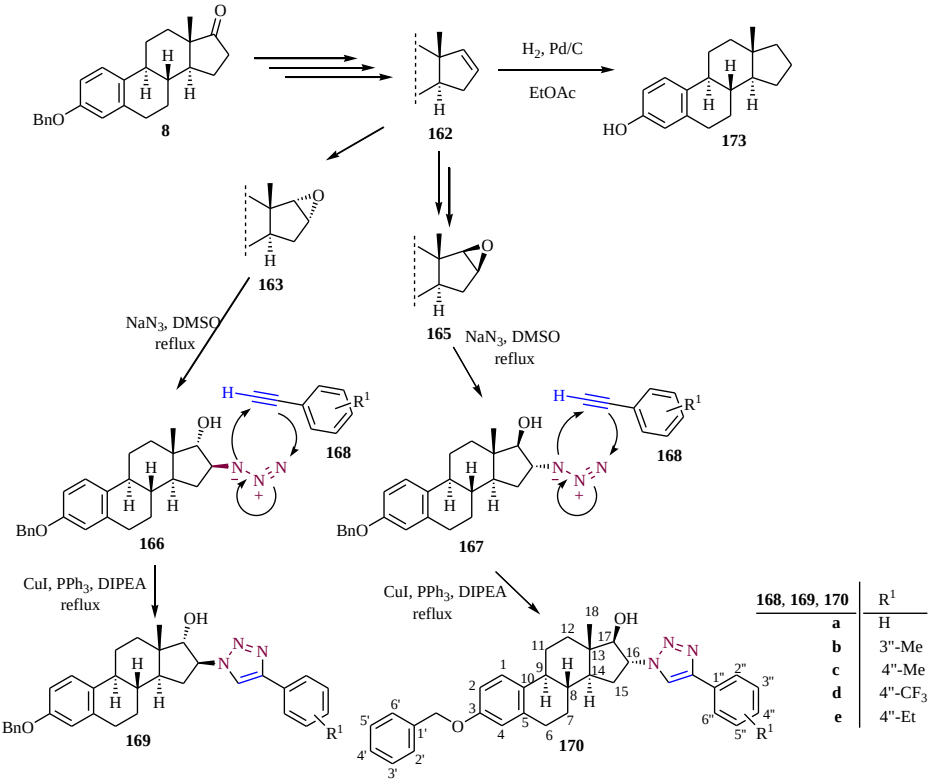


Scheme 5

3.10. The 16α,17α-epoxide (**163**) and the 16β,17β-epoxide (**165**) were synthesized in several reaction steps from the 16-olefin (**162**) of oestrone 3-benzyl ether (**8**, Scheme 6). The cleavage of the epoxides (**163**, **165**) with NaN_3 led to the *trans*-azidoalcohols (**166**, **167**) regioselectively.

3.11. The azidoalcohols (**166**, **167**) were reacted with substituted phenylacetylenes (**168a–e**) in CuAAC reactions (Scheme 6). The cyclizations proceeded

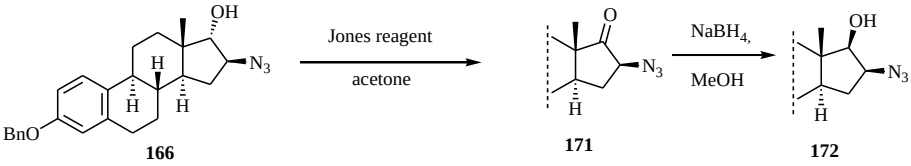
regioselectively, leading to the corresponding 16-triazolyl derivatives (**169**, **170**) in high yields.



Scheme 6

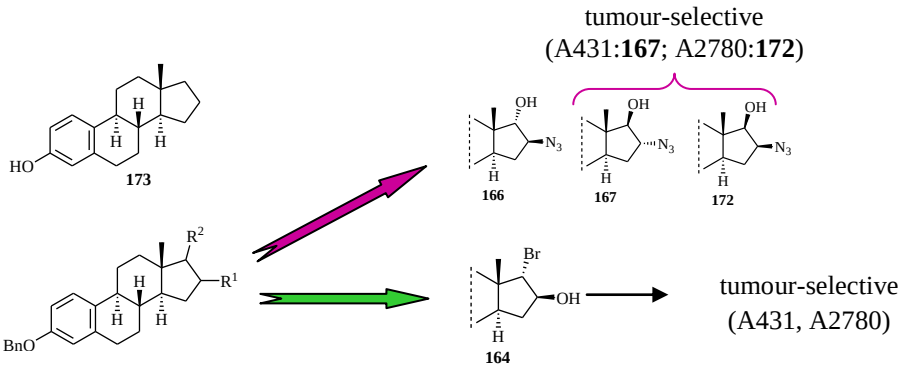
3.12. The benzyl protecting group of the 16-olefin (**162**) was removed by catalytic hydrogenation with Pd/C as catalyst (Scheme 6). Debenzylation and double bond saturation occurred simultaneously, and the 17-desoxy derivative (**173**) was obtained.

3.13. The 16β-azido-17α-hydroxy derivative (**166**) was subjected to Jones oxidation, furnishing the 16β-azido-oestrone-3-benzyl ether (**171**) (Scheme 7). Hydride reduction of **171** led to the *cis*-azidoalcohol (**172**).



Scheme 7

3.14. The antiproliferative actions of the newly synthesized compounds (**162**–**173**) were investigated on a panel of human adherent cell lines (Scheme 8). The epoxides (**163**, **165**) proved to exert moderate cell-growth inhibition in contrast with the azidoalcohols (**166**, **167**). Azidoalcohols containing a 17β-hydroxy group (**167**, **172**) appeared to be tumour-selective (A431: **167**, IC_{50}= 8.1 μM; A2780: **172**, IC_{50} = 10.7 μM). The oxidation of the 17-OH group (**171**) reduced the cell-growth inhibition, suggesting that the 17-OH function was necessary for the antitumour effect. The bromohydrin (**164**, 16-Br) behaved similarly to the 17β-hydroxyazidoalcohols (**167**, **172**). The triazoles exerted moderate cell-growth inhibition on the cell lines. The unsubstituted 17-desoxy derivative (**173**) exhibited high antiproliferative activity, which reached 90% cell-growth inhibition at 30 μM.

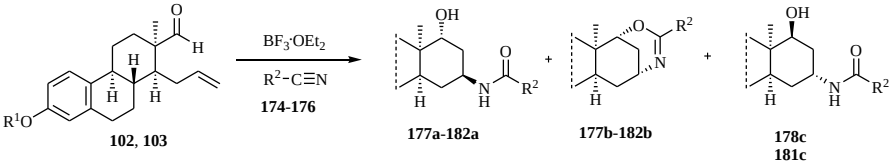


Scheme 8

3.15. The one-pot Prins-Ritter reactions of the δ-alkenyl-D-seco-oestrone-3-benzyl or 3-methyl ether (**102**, **103**) in the 13α-oestrone series were carried out with $BF_3{\cdot}OEt_2$ as catalyst and different nitriles (aceto-, chloraceto- and benzonitrile) as reagents and solvents (Scheme 9). The first reaction step was the Lewis acid-induced Prins cyclization of the δ-alkenyl-aldehyde (**102**, **103**), leading to the carbocation at C-16. This was followed by the Ritter reaction step with nitriles (**174**–**176**) acting as nucleophilic reagents.

3.16. All the reactions proceeded in a similar manner, yielding two products in a ratio of 4:1, a substituted 16-*N*-acyl-17a-hydroxy- (**177a**, **c** – **182a**, **c**) and a cyclized (**177b**–**182b**) derivative. With chloroacetonitrile (**175**) as reagent and solvent, three products (**178a**–**c** and **181a**–**c**) were formed in a ratio of 7:2:1 in both the 3-benzyl and the 3-methyl ether series.

3.17. The structures of the Prins-Ritter products (**177**–**182**) were identified by means of NMR spectroscopy. The main isomers (**177a**–**182a**) formed in each reaction were the 16β,17aα compounds; the bridged derivatives (**177b**–**182b**) have 16α,17aα orientations. When chloroacetonitrile (**175**) was used, the third compound formed (**178c**, **181c**) was the diastereomer of the main product (**178a**, **181a**).



102, **177-179**: R^1 = Me
103, **180-182**: R^1 = Bn

174, **177**, **180**: R^2 = Me
175, **178**, **181**: R^2 = CH_2Cl
176, **179**, **182**: R^2 = Ph

Scheme 9

3.18. In order to acquire more information on the stereochemistry of **180b**, a compound structurally related to the earlier synthesized 17a-ethoxy-16-iodo derivative

(**117**) was synthesized by the acetylation of **180b**. Under the conditions used for the acetylation, the bridged structure of **180b** opened, yielding the 16-acylamino-17a-acetoxy derivative (**184**). The NMR spectra of the two similarly substituted compounds (**117**, **184**) led to the conclusion that the bridged compounds are *cis*-16α,17aα isomers.

3.19. The tumour cell proliferation inhibition caused by the homosteroids (**177**–**182**) was greatly affected by the structures of the compounds (**177**–**182**). **177a** and **180a** proved to be less potent than the bridged products (**177b**, **180b**). **180b** displayed outstanding antiproliferative properties against all the tested cell lines, with IC_{50} values of ~1 μM. A benzylic protecting group at position C-3 generally improved the growth-inhibitory potential. *N*-Chloroacetyl derivatives (**178**, **181**) displayed a broader spectrum of activities, since all three products (**181a**–**c**) in the 3-benzyl ether series exhibited > 90% inhibition on all cells except MCF-7 (at 30 μM). The *N*-benzoyl compounds (**179**, **182**) were less potent derivatives.

3.20. The cancer selectivity of the selected potent products (**180b**) was tested by means of the MTT assay, using non-cancerous human fibroblast cells MRC-5. **180b** proved to be tumour-specific, in contrast with the generally toxic *N*-chloroacetyl compound (**181a**). The most effective Prins-Ritter derivative (**180b**) was subjected to additional investigations in order to describe its antiproliferative properties against a panel of human breast cancer cell lines differing in receptorial status. Since no substantial differences were detected in the activities of **180b**, its oestrogenic effect can be excluded. The direct effect of **180b** on tubulin polymerization was tested *in vitro*. The results indicate that **180b** caused a concentration-dependent increase in the rate of polymerization, similar to that of the reference agent paclitaxel.

4. Publications directly related to the dissertation

1. **J. Huber**, J. Wölfling, Gy. Schneider, I. Ocsovszki, M. Varga, I. Zupkó, E. Mernyák. Synthesis of antiproliferative 13α-D-homoestrones via Lewis acid-promoted one-pot Prins-Ritter reactions of D-secosteroida δ-alkenyl-aldehydes. *Steroids* **2015**, *102*, 76. **Impact factor: 2,639 (2014)**
2. E. Mernyák, **J. Huber**, J. Szabó, Gy. Schneider, A. Hetényi, L. Márk, G. Maász, Á. Berényi, I. Kovács, R. Minorics, I. Zupkó, J. Wölfling Cycloadditon of Steroidal Cyclic Nitrones to C=N Dipolarophiles: Stereoselective Synthesis and Antiproliferative Effects of Oxadiazolidinones in the Estrone Series. *Steroids* **2013**, *78*, 1021. **Impact factor: 2,716**
3. E. Mernyák, **J. Huber**, G. Benedek, R. Pfoh, S. Rühl, G. Schneider, J. Wölfling. Electrophile- and Lewis acid-induced nitrone formation and1,3-dipolar cycloaddition reactions in the 13α- and 13β-estroneseries. *Arkivoc* **2010**, *xi*, 101. **Impact factor: 1,096**

Total impact factor: 6,451

5. Other publications

1. N. Szabó, Z. Iványi, M. Szécsi, J. Julesz, E. Mernyák, **J. Huber**, J. Wölfling, R. Minorics, I. Zupkó, Gy. Schneider. Synthesis of methoxycarbonylpyrazolylandrostene derivatives, and their potential inhibitory effect on androgen biosynthesis and cell proliferation. *Steroids* **2015**, *98*, 143. **Impact factor: 2,639**
2. E. Mernyák, J. Szabó, I. Bacsa, **J. Huber**, Gy. Schneider, R. Minorics, N. Bózsity, I. Zupkó, M. Varga, Zs. Bikádi, E. Hazai, J. Wölfling. Syntheses and antiproliferative effects of D-homo- and D-secoestrones. *Steroids* **2014**, *87*,128. **Impact factor: 2,639**

3. Z. Iványi, N. Szabó, **J. Huber**, J. Wölfling, I. Zupkó, M. Szécsi, T. Wittmann, Gy. Schneider. Synthesis of D-ring-substituted (5'R)-and(5'S)-17β-pyrazolinylandrostene epimers and comparison of their potential anticancer activities. *Steroids* **2012**, *77*, 566. **Impact factor: 2,803**
4. Z. Kádár, D. Kovács, É. Frank, Gy. Schneider, **J. Huber**, I Zupkó, T. Bartók, J. Wölfling. Synthesis and In Vitro Antiproliferative Activity of Novel Androst-5-ene Triazolyl and Tetrazolyl Derivatives. *Molecules* **2011**, *16*, 4786. **Impact factor: 2,386**

Total impact factor: 10,467

6. Lectures and posters related to the dissertation

Lectures

1. **Huber Judit**, Mernyák Erzsébet, Schneider Gyula, Zupkó István, Wölfling János. Újszerű, N-tartalmú ösztronszármazékok szintézise és hatástani vizsgálata. MTA Szteroid és Terpenoidkémiai Munkabizottsági ülés, Richter Gedeon Nyrt. Budapest, **2013**.
2. **Huber Judit**, Zupkó István, Mernyák Erzsébet, Wölfling János, Schneider Gyula. Ösztránvázas oxadiazolidinonok szintézise és antiproliferációs vizsgálata. Kémiai Előadói Napok, Szeged, **2011**. The book of abstracts (Pg. 174.).
3. **Huber Judit**, Mernyák Erzsébet, Wölfling János. Ösztánvázas gyűrűs nitron dipólusok cikloaddíciós reakciói C=N dipolarofilekkel. Országos Tudományos Diákköri Konferencia, Debrecen, **2009**. The book of abstracts (Pg. 219.).
4. **Huber Judit**, Mernyák Erzsébet. Ösztránvázas nitron dipólusok cikloaddíciója fenilizocianátokkal. Kémiai Előadói Napok, Szeged, **2008**.

The book of abstracts (Pg. 91.).

5. **Huber Judit**, Mernyák Erzsébet. Nitrogéntartalmú D-homoszteroidok új szintézise. MTA Szteroidkémiai Munkabizottsági ülés, Szeged, **2008**.
6. **Huber Judit**, Mernyák Erzsébet. Ösztránvázas gyűrűs nitron dipólusok cikloaddíciója fenilizocianát C-N dipolarofillel. A Szegedi Ifjú Szerves Kémikusok Támogatásáért Alapítvány tudományos előadóülése, Szeged, **2008**.

Posters

1. **Huber Judit**, Wölfling János, Schneider Gyula, Zupkó István, Mernyák Erzsébet. Syntheses of 16-acylamino derivatives in the 13α-estrone-series and their antiproliferative activities. 20th International Conference on Organic Synthesis, Budapest, **2014**. www.icos20.hu (P-41)
2. Mernyák Erzsébet, **Huber Judit**, Farkas Nóra Johanna, Schneider Gyula, Wölfling János, Zupkó István. Ösztránvázas savamidok Prins-Ritter reakcióval történő előállítása és *in vitro* hatástani vizsgálata a 13α-ösztron sorban. MKE Vegyészkonferencia, Hajdúszoboszló, **2013**. The book of abstracts (Pg. 100.).
3. **Huber Judit**, Mernyák Erzsébet, Schneider Gyula,Wölfling János, Zupkó István. 16β-triazolil-17α-hidroxiösztron származékok előállítása és antiproliferatív aktivitásuk meghatározása. MKE Vegyészkonferencia, Hajdúszoboszló, **2013**. The book of abstracts (Pg. 82.).
4. Mernyák Erzsébet, **Huber Judit**, Wölfling János, Schneider Gyula Ösztránvázas gyűrűs nitron dipólusok cikloaddíciója fenilizocianát C-N dipolarofilekkel. MKE Vegyészkonferencia, Hajdúszoboszló, **2008**. (P-37)
5. E. Mernyák, E. Kozma, **J. Huber**, J. Wölfling, Gy. Schneider Synthesis of steroidal dipolarophiles and dipoles and their 1,3-dipolar cycloadditions. 2nd European Chemistry Congress, Torino, **2008**. DVD ROM

7. Other lectures and posters

Lectures

1. Wölfling János, Schneider Gyula, Frank Éva, Kádár Zalán, Iványi Zoltán, Kovács Dóra, Mótyán Gergő, Görbe Tamás, Juracsek Beáta, Sárközy Anita, Fodor Katalin, **Huber Judit**, Molnár Judit, Zupkó István. Triazolil-szteroidok, új, potenciális gyógyszerhatóanyagok. Magyar Tudomány Ünnepe, Szeged, **2010**.

Posters

1. **Huber Judit**, Mernyák Erzsébet, Schneider Gyula, Schönecker Bruno, Wölfling János. Újszerű nitrogéntartalmú, kétfogú, királis ligandumok szintézise a 13α-ösztron sorban. MKE 1. Nemzeti Konferencia, Sopron, **2011**. The book of abstracts (Pg. 185.).