In vitro inhibition of the estrogen biosynthesis enzyme system with new steroid derivatives

Ph.D. Thesis

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List of publications

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I. Bacsa I, Jójárt R, Schneider G, Wölfling J, Maróti P, **Herman BE**, Szécsi M, Mernyák E: Synthesis of A-ring halogenated 13α-estrone derivatives as potential 17β-HSD1 inhibitors, *Steroids*, **2015**, *104*, 230–236. **IF: 2.513**

II. Szabó J, Bacsa I, Wölfling J, Schneider Gy, Zupkó I, Varga M, **Herman BE**, Kalmár L, Szécsi M, Mernyák E: Synthesis and *in vitro* pharmacological evaluation of *N*-[(1-benzyl-1,2,3-triazol-4-yl)methyl]-carboxamides on d-secoestrone scaffolds, *J Enzym Inhib Med C*, **2016**, *31*, 574–579. **IF: 4.293**

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IV. Szánti-Pintér E, Maksó L, Gömöry Á, Wouters J, **Herman BE**, Szécsi M, Mikle G, Kollár L, Skoda-Földes R: Synthesis of 16α-amino-pregnenolone derivatives *via* ionic liquid-catalyzed aza-Michael addition and their evaluation as $C_{17,20}$-lyase inhibitors, *Steroids*, **2017**, *123*, 61–66. **IF: 2.523**

V. Bacsa I, Jójárt R, Wölfling J, Schneider Gy, **Herman BE**, Szécsi M, Mernyák E: Synthesis of novel 13α-estrone derivatives by Sonogashira coupling as potential 17β-HSD1 inhibitors, *Beilstein J Org Chem*, **2017**, *13*, 1303–1309. **IF: 2.330**

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Publications not related to the thesis

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List of abbreviations

17OHProg	17-hydroxyprogesterone
17β-HSD1	17β-hydroxysteroid dehydrogenase type 1
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone-3-sulfate
EMATE	estrone-3-*O*-sulfamate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
$P450_{17\alpha}$	17α-hydroxylase-$C_{17,20}$-lyase, cytochrome P450 dependent (CYP17A1)
Prog	Progesterone
RC	Relative conversion
RIP	Relative inhibitory potential
STS	Steroid sulfatase (sulfatase)
Test	Testosterone

Code list of test compounds and their numbers in the referred publications

Publication I.
BI-135 – **2**
BI-116AF – **3**
BI-116FF – **4**
BI-113AF – **5**
BI-113FF – **6**
BI-76 – **7**

Publication II.
BI-28 – **10b**
BI-42 – **10c**
BI-25 – **13a**

Publication III.
SZJ-36 – **3a**
JN-14 – **4a**
FG-7 – **5a**
FG-4 – **6a**

Publication IV.
ML-147 – **3j**
ML-208 – **3k**
ML-214 – **3m**
ML-AD-46 – **3p**

Publication VI.
GT-166 – **8o**
GT-70 – **8i**
SCH-1737 – **3b**
GT-101 – **5b**
GT-119 – not published

Publication VII.
EM-1048V – **6a**
EM-1048III – **7a**
BI-341 2/5 – **8a**
EM-1049I – **6b**
EM-1049II – **7b**
BI-340 5/1 – **8b**
BI-347 II 4/4 – **6c**
BI-347 5/4 – **7c**
BI-347 D 5/1 – **8c**
PZ-1 – **9**
BI-113AF – **10a**
BI-113FF – **11a**
BI-76 – **12a**
BI-135 – **10b**
BI-116AF – **11b**
BI-116FF – **12b**
BI-323II – **10c**
BI-123 7/3 – **11c**
BI-123 5/5 – **12c**
PZ-16 – **13**
HK-27II – **16c**

Publication VIII.
SCH-1478 – **11**
SZE-5012 – **17**
SZE-4013 – **18**
SCH-1484 – **20**
SZE-4033 – **22**
SZE-4017 – **25**
SCH-1054 – **28**
SZE-5027 – **33**
SCH-1544 – **35**
SCH-1548 – **40**

Publication IX.
KL-105 – **1**
KL-86 – **2**
KL-99 – **4**
KL-114 – **5**
EV-74/6 – **6**
EV-98/2 – **8**
E-IL-249/5 – **10**
EV-95/3 – **11**
E-IL-250 – **12**
Etöszba – **13**

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Table of Content

1 Introduction

1.1 Biosynthesis and biological role of estrogens

Estrogens are sexual steroid hormones of females. They are essential to sexual maturation and to function of the reproductive system in women. Estrogens play also important role in male reproduction, as they have regulatory functions in the sperm maturation [Hess et al. 1997]. Additional physiological effects are the influence on bone and lipid metabolism and on the cardiovascular system, as well as regulatory functions in the central nervous system [Cersosimo & Bennaroch 2015]. Estrogen signaling pathway is involved in several cellular processes, among them in regulation of cell proliferation and survival. These hormones control the proliferation and function of the estrogen-sensitive normal tissue and neoplastic, malignant tissue. Estrogens, including the hormonally most active 17β-estradiol exert the hormonal effect *via* binding to the specific membrane receptors or *via* binding to the cytosolic nuclear receptors evoking rapid or long-term genomic estrogen effect in the target tissues, respectively [Björnström & Sjöberg 2005].

In the commencement of biosynthetic cascade of estrogens, C_{21} steroidal precursors are transformed to C_{19} prehormones by the cytochrome P450 dependent 17α-hydroxylase-$C_{17,20}$-lyase enzyme ($P450_{17\alpha}$) (Fig. 1). The enzyme converts C_{21} precursors, pregnenolone and progesterone first to their 17α-hydroxy derivatives 17-hydroxypregnenolone and 17-hydroxyprogesterone (17OHProg). A subsequent side chain cleavage process ($C_{17,20}$-lyase activity) forms C_{19} steroids, dehydroepiandrosterone (DHEA) and androstenedione. Androstenedione then can be converted partially to testosterone (Test). Estrone and 17β-estradiol are synthesised from androstenedione and testosterone, respectively. These transformations are catalysed by the cytochrome P450 dependent aromatase enzyme ($P450_{19A1}$) which performs also the cleavage of the C-19 methyl group beyond the aromatization of the steroidal A ring. Estrone is further metabolized by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) which is activating the estrogenic effect by the stereospecific reduction of the steroidal C-17 oxo function and forms the active hormone 17β-estradiol. Estrogens can be transported and stored in the form of estrone-3-sulfate. This is synthesized from estrone by the action of estrogen sulfotransferase with estrone being regenerated by the steroid sulfatase (STS)-catalysed hydrolysis of estrone-3-sulfate [Miller & Auchus 2011; Thomas & Potter 2013].

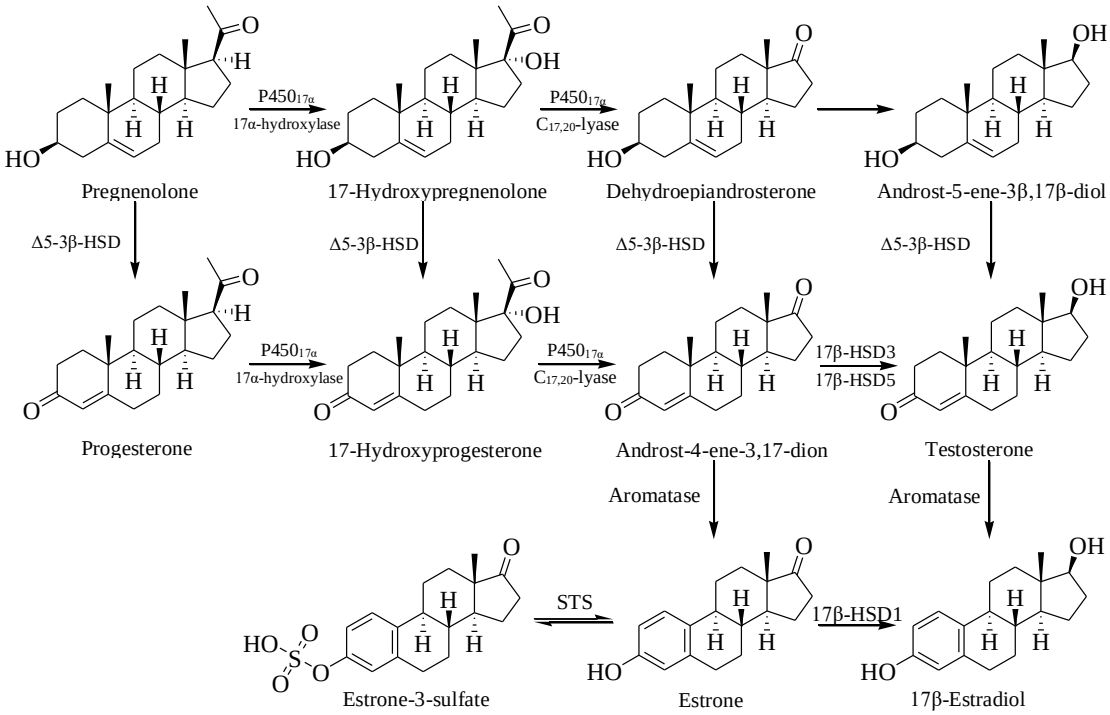


Fig. 1 Major pathways of estrogen biosynthesis

Primary biosynthesis of estrogens is under the endocrine control of the hypothalamo-hypophyseal axis and takes place mainly in functionating ovaries. Substantial production occurs also in extragonadal sites (such as breast and adipose tissue), and this peripheral biotransformation may regulate estrogen action in an intracrine manner [Labrie 1991; Miller & Auchus 2011; Mueller et al. 2015].

Estrogens have major role in development and progression of gynecological pathologies, among them in hormone dependent forms of breast cancer, ovarian tumors, uterine carcinomas, and neoplasms of the endometrium. Breast cancer tissues, for instance, have been shown to express enzymes of the estrogen producing machinery in exaggerated manner [Africander & Storbeck 2018; Chanplakorn et al. 2010; Foster 2008; Harada 1997; Miyoshi et al. 2001]. Increased expression resulted in elevated local 17β-estradiol concentrations, which is believed to promote the pathophysiological cell proliferation. Progression of the hormone dependent tumors can be controlled by withdrawal of the estrogens. Estrogen ablation may be achieved by suppressed estrogen biosynthesis, and the evoked pre-receptorial anti-hormonal effect can be successfully applied in the pharmacotherapy of the hormone dependent tumors [Sasano 2011]. There are further indications for estrogen withdrawal, for example ovulation induction, and the treatment of gynecomastia, male breast cancer and infertility with certain pathologies in men [de Ronde & de Jong 2011].

For an efficient and complete estrogen deprivation concomitant inhibition of the key enzymes in the cascade as well as blockade of the adrenal supply of prehormones *via* inhibition of the $P450_{17\alpha}$ might be necessary. Compounds possessing either specific or multiple inhibitory effect against the $P450_{17\alpha}$, the aromatase, the STS, and the 17β-HSD1 are interesting targets of the drug development [Baston & Leroux 2007; Chumsri et al. 2011; Day et al. 2008; Geisler et al. 2011; Hong & Chen 2011; Hu & Hartmann 2014; Marchais-Oberwinkler et al. 2011; Thomas & Potter 2015].

1.2 Key enzymes of estrogen biosynthesis

1.2.1 17α-hydroxylase-$C_{17,20}$-lyase enzyme ($P450_{17\alpha}$)

$P450_{17\alpha}$ (EC 1.14.14.19) catalyzes the transformation of C_{21} preandrogens to C_{19} androgens. This enzyme is a membrane-bound monooxygenase, product of CYP17A1 gene and the human enzyme consists of 508 amino acids with a molecular mass of 57 kDa. $P450_{17\alpha}$ has a dual-function, the 17α-hydroxylation and the C-17 side chain cleavage. The second process, the $C_{17,20}$-lyase activity of the enzyme is the rate-limiting step in this transformation. It is expressed and active in the adrenal glands, testes, ovaries and placenta, primarily [Moreira et al. 2008].

1.2.2 Aromatase

Aromatase (also called $P450_{19A1}$, EC 1.14.14.1) is a cytochrome P450 dependent enzyme which catalyzes the multi-step conversion of androgens to estrogens [Hong & Chen 2011]. The human microsomal enzymatic complex is the product of the CYP19A1 gene (EC: 1.14.14.1) and consists of a haem group and a polypeptide chain of 503 amino-acid residues with a molecular mass of 58 kDa [Ghosh et al. 2009]. This enzyme is present in multiple organs including adipose tissue, brain, blood vessels, skin, bone, endometrium and breast tissue [Chumsri et al. 2011]. Aromatase is the rate-limiting process in estrogen biosynthesis.

1.2.3 Steroid sulfatase (STS)

STS (EC 3.1.6.2.) hydrolyzes estrone-3-sulfate to estrone, as well as cholesterol sulfate, pregnenolone sulfate and dehydroepiandrosterone sulfate (DHEA-S) to their corresponding unconjugated forms [Lanisnik Rizner 2016; Shah et al. 2016]. The human enzyme protein contains 562 amino acids with a molecular mass of 63 kDa, and it is associated with the membrane of the endoplasmic reticulum (ER) [Hernandez-Guzman

et al. 2003]. STS is ubiquitously expressed, with its highest protein levels in placenta, and lower levels in breast, skin, liver, lung, ovary, adrenal gland, endometrium, brain, and some other tissues [Lanisnik Rizner 2016].

1.2.4 17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1)

The human 17β-HSD1 (EC 1.1.1.62) protein is comprised of 328 amino acids and exists as a cytosolic functional homodimer with a subunit molecular mass of 35 kDa [Marchais-Oberwinkler et al. 2011; Moeller & Adamski 2009]. The 17β-HSD1 is a pluripotent enzyme in terms of substrate, cofactor, and the oxidative and reductive direction of the 17β-hydroxy – 17-oxo interconversion. Even though, this isozyme is enable for 3β-hydroxy reduction of substrates bound in reverse mode [Moeller & Adamski 2006]. Under *in vivo* conditions, as in living cells, however, the isoenzyme functions unidirectionally [Jin & Lin 1999; Luu-The et al. 1995; Negri et al. 2010] and it catalyzes predominantly the NADPH promoted stereospecific reduction of estrone to 17β-estradiol, the final, hormone activating process in the estrogen biosynthesis [Jin & Lin 1999; Puranen et al. 1997]. The highest expression and activity of the isozyme can be detected in the female steroidogenic reproductive tissues such as the ovaries and the placenta [Martel et al. 1992]. This isozyme has a major contribution to the general gonadal supply and to the circulating level of 17β-estradiol in the blood. 17β-HSD1 is also expressed and active in peripheral tissues, where it regulates the intracellular accumulation of 17β-estradiol and consequently the intracrine estrogen effect [Moeller & Adamski 2006; Poirier 2003].

1.3 Biomedical relevance of inhibition of enzymes in the estrogen biosynthesis

Suppression of estrogenic effects is a major therapeutic approach of estrogen dependent pathologies. GnRH analogs, SERMs (selective estrogen receptor modulators) and estrogen receptor antagonists have been included in the routine endocrine therapies for a long time. However, all these therapeutics have certain drawbacks and may develop side effects [Montagna et al. 2013].

Suppressed estrogen production achieved *via* inhibition of steroidogenic enzymes offer a further way for treatment of estrogen dependent diseases. Blockade of the estrogen biosynthesis in earlier steps of the biosynthetic cascade, and biotransformation taking place in the adrenal glands and in the gonads evolves systemic estrogen ablation (aromatase, $P450_{17\alpha}$). Inhibition of the final hormone activating steps of the estrogens and

which are carried out in the peripheral tissues induce an intracrine effect and targeted antiestrogenic action (17β-HSD1, STS).

Inhibition of enzymes in the estrogen biosynthesis may occur unintentionally, too. Certain xenobiotics either from natural, or from synthetic origin might be capable of the inhibition of $P450_{17\alpha}$, aromatase, STS and 17β-HSD1 and evolve consequently estrogen deprivation and endocrine disrupting effect [Brozic et al. 2008; Patisaul & Belcher 2017].

1.3.1 Inhibitors of $P450_{17\alpha}$

A non-specific P450 inhibitor, the nonsteroidal imidazolyl compound ketoconazole was applied in the beginnings for the inhibition of $P450_{17\alpha}$ mainly in the treatment of androgen dependent prostatic carcinoma. The first-in-class specific $P450_{17\alpha}$ inhibitor, a 17-pyridyl-androstene derivative Abiraterone (17-(3-pyridinyl)androsta-5,16-dien-3β-ol acetate, Zytiga) was introduced into the medical practice in 2011 [Hu & Hartmann 2014]. Another C-17 heterocyclic steroid compound Galeterone (17-(1H-benzimidazol-1-yl)androsta-5,16-dien-3β-ol, TOK-001, VN/124-1) is seemed to be a promising P450 inhibitor candidate in several clinical trials [Njar & Brodie 2015]. The $P450_{17\alpha}$ meanwhile became an important target for the treatment of breast cancers [Baston & Leroux 2007; Yap et al. 2008].

1.3.2 Inhibitors of aromatase

Aromatase inhibitors have been applied in the medical practice for three decades. By now an effective armament was developed, in which the inhibitors are categorized by generations from I to IV, according to the order of their clinical introduction. The use of the currently available aromatase inhibitors, however, may lead to increased risk of stroke, endometrial cancer and blood clots, moreover breast cancer can become resistant to them. Aim to eliminate these side effects still propels this research area. The steroidal (type 1) aromatase inhibitors offer higher selectivity and prolonged effect, therefore compounds with steroid skeleton remain a promising area of the development [Yadav et al. 2015].

1.3.3 Inhibitors of STS

The development of STS inhibitors started in the 1990s. STS inhibitors known to date can be divided into steroidal and non-steroidal compounds, and further into sulfamoylated and non-sulfamoylated compounds [Geisler et al. 2011; Maltais & Poirier 2011; Shah et al. 2016; Thomas & Potter 2015]. Clinical studies have already been performed with STS

inhibitors Irosustat (also known as, i.e., 667 Coumate or STX64 or BN83495) and E_2MATE [Thomas & Potter 2015] but these pharmacons were not introduced into the medical practice up to now.

1.3.4 Inhibitors of 17β-HSD1

The 17β-HSD1 has been studied for more than half a century and numerous steroidal and non-steroidal inhibitor candidates have been developed [Brozic et al. 2008; Day et al. 2008; Marchais-Oberwinkler et al. 2011; Poirier 2003; Poirier 2010; Poirier 2011]. The most promising inhibitor candidates (e. g. a bisubstrate analog 2-ethyl-16β-m-pyridylmethylamidomethyl-estrone, Sterix 1040) have undergone extensive preclinical evaluation, but none of them yet entered clinical trials [Penning 2011].

1.3.5 Dual aromatase/STS inhibitors (DASIs)

Inhibition of two or more enzymes in the biosynthetic cascade may evolve a more complete estrogen withdrawal. If the two effects are combined in one molecule, the negative interactions could be avoided and resistance is not likely to develop simultaneously in the different enzymes. A series of compounds that can inhibit both aromatase and STS have been developed based on the typical STS inhibitor EMATE, and also by the incorporation of the sulfamate pharmacophore into established nonsteroidal triazole aromatase inhibitors [Geisler et al. 2011]. Compounds prepared by this approach were promising in pre-clinical investigations, but they were finally not probed clinically [Lanisnik Rizner 2016].

1.3.6 Dual STS/HSD1 inhibitors

Simultaneous inhibition of STS and 17β-HSD1 became a novel treatment approach. The aim of this idea could be achieved by inhibition of both enzymes with a single inhibitor whose structure is derived for this dual mode of action. Transferring the main pharmacophore of STS inhibitors, an unsubstituted aryl sulfamate to an appropriate position of highly potent 17β-HSD1 inhibitor can be adopted for the design of dual inhibitors [Salah et al. 2017].

1.3.7 Irreversible and reversible inhibitors

When an irreversible inhibitor is used as a drug, effect can be maintained after its clearance from the system, and new biosynthesis of the enzyme protein is required for restoration of the corresponding activity. Advantages of the resulting prolonged

pharmacodynamics include lower level and frequency of dosing, as well as suppressed extent of side effects. EMATE-based irreversible inhibitors, however, have the drawback that the STS inactivation process releases steroidal compounds bearing estrogenic hormonal effect [Reed et al. 2004]. In the case of a reversible inhibitor or drug, the mechanism will require sustained plasma concentrations to maintain efficacy at the target. A number of potential benefits lead to the use of irreversible compounds, including high potency, lower dose and extended duration of action [Baillie 2016].

1.3.8 Ligand-based approach of inhibitor development, prediction of inhibitory potential

Inhibitor design and prediction of inhibitor efficiency is a complex task concerning the enzymes of the estrogen biosynthesis. The three-dimensional crystal structures of human $P450_{17\alpha}$, aromatase, STS and 17β-HSD1 were explored, providing valuable information on molecular basis of substrate and inhibitor binding including key amino acid residues in the active sites and in the binding cavities involved. These structural data were essential to understand the catalytic mechanisms, however, they proved to be less useful in inhibitor design [Hong & Chen 2011; Hu & Hartmann 2014]. Majority of the inhibitors of enzymes of the estrogen biosynthesis have been developed by ligand-base approach.

Experimental testing and biochemical analysis of inhibition of novel compounds still remained a promising way of inhibitor development. Structure–inhibitory potential relationships provide further information for better understanding of binding and catalytic mechanisms of the enzymes.

2 Aims

The aim of this work was to investigate the inhibitory effect of novel steroid derivatives exerted on the key enzymes of estrogen biosynthesis. Newly synthesized compounds are the results of cooperating research groups. Test compounds possessed various structural modifications applied regularly in pharmacological research for the modulation of biological effect. Modifications included skeletal modifications such as isomerization and ring opening, as well as substitutions with heterocyclic and halogenated functional groups in rings A and D.

We aimed to develop *in vitro* radiosubstrate incubation methods to measure aromatase, STS and 17β-HSD1 activity and inhibition. (Measurement of the activity and inhibition of $C_{17,20}$-lyase activity of $P450_{17\alpha}$ enzyme was carried out using a method previously developed in our laboratory.)

Our goal was to collect data on inhibitory potentials of new test compounds (IC_{50}) and molecular structure–activity relationships. We also planned to investigate the enzyme specificity of the inhibitors and possible multiple inhibitory effects. We also aimed to investigate kinetic and mechanistic properties of the inhibitors. Final aim of this work was to provide further information to better understanding of ligand biding mechanisms of the enzymes. Our new data might be useful for the development of novel inhibitor compounds of estrogen biosynthesis and contribute to the exploration of new drug candidates.

During our research we investigated in detail the followings:

1. Inhibition of $C_{17,20}$-lyase activity with 16α-amino-pregnenolone, 17-heterocyclic and 17-azido compounds
2. Comparative investigations on cofactor dependent inhibition of 17β-HSD1 with A- and D-ring modified estrone derivatives
3. Inhibition of aromatase, STS and 17β-HSD1 with A-ring halogenated estrone analogs
4. Inhibition of aromatase, 17β-HSD1 and STS with steroidal ferrocenes

3 Experimental

3.1 General

Radioactive [1,2,6,7-^{3}H(N)]17OHProg, [1,2,6,7-^{3}H(N)]testosterone, [6,7-^{3}H(N)]estrone-3-sulfate ammonium salt and [6,7-^{3}H(N)]estrone was purchased from the American Radiolabeled Chemicals (St. Louis, MO, USA), respectively. Non-radioactive substrates, standards, reference inhibitors and cofactors were obtained from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland). Other chemicals and solvents with purity of analytical grade were purchased from Sigma (St. Louis, MO, USA). Kieselgel-G TLC layers (Si 254 F, 0.25 mm thick) were obtained from Merck (Darmstadt, Germany).

3.2 Preparation of enzyme sources

3.2.1 Rat testicular homogenate

Testicular tissue was obtained from male rats (12-week old, 200-250 g body mass) *via* surgeries under ether anaesthesia. Tissue specimens were washed with an isotonic solution of NaCl. 1.0 g pieces of the testicles of five animals were mixed and the tissue sample was homogenized with an Ultra-Turrax in 20.0 ml 0.1 M HEPES buffer (pH=7.3) containing 1 mM EDTA and 1 mM dithiothreitol. Homogenization was performed for 4×1 min with continuous cooling. The homogenate was centrifuged at 1500 *g* for 10 min at 4 °C. The supernatant was divided into portions for storage at -70 °C.

3.2.2 Human placental cytosol and microsomes

Human term placenta were collected immediately after delivery and stored frozen at -80 °C. Tissue specimens were washed with an isotonic solution of NaCl. 5 g pieces of the five placenta specimens were mixed and the tissue sample was homogenized with an Ultra-Turrax in 50 ml 0.1 M HEPES buffer (pH=7.3) containing 1 mM EDTA and 1 mM dithiothreitol. Homogenization was performed for 4×1 min with continuous cooling. Supernatant of subsequent cooled centrifugations of the homogenate at 1200 *g* for 7 min and 12000 *g* for 30 min was subjected to ultracentrifugation at 100000 *g* for 60 min at 4 °C. Obtained cytosol was drained and the microsomes sediment was resuspended in 12 ml HEPES buffer with ultrasonic homogenization. Cytosol and microsomes fraction was divided into portions for storage at -70 °C. Application of the human tissue was approved by the institutional Human Investigation Review Board.

3.3 Incubation procedures

Enzymatic incubations were carried out in the HEPES buffer medium at a final volume of 200 µl. The substrate was added to the incubate in 20 µl of a 25 v/v% propylene glycol (or acetonitrile for aromatase) in HEPES buffer solution, whereas test compounds were applied in 10 µl of dimethyl sulfoxide solution. In incubates of the inhibition studies substrate concentration was 1.0 µM. Incubations were performed at 37 °C with continuous shaking (200 rpm) and terminated by cooling and the addition of organic solvents of the subsequent extraction procedure. Radioactivity of the isolated product and remaining substrate was measured by means of liquid scintillation counting (Packard Tri-Carb 2200CA). Enzyme activity was calculated from the amount of the product formed with correction of the background and recovery.

3.3.1 $C_{17,20}$-lyase ($P450_{17\alpha}$) assay

$C_{17,20}$-lyase activity was measured with a method established previously by Szabó et al. [Szabó et al. 2015]. In the $P450_{17\alpha}$ assays the rat testicular homogenate was incubated using 17OHProg substrate with [^{3}H]17OHProg tracer and 0.1 mM NADPH cofactor excess. The incubation time was 20 min. The product androst-4-ene-3,17-dion was extracted with 100 µl ethyl acetate and isolated by a TLC method performed on silica gel plate and with dichloromethane/diisopropyl ether/ethyl acetate (75:15:10 v/v) eluent.

3.3.2 Aromatase assay

In the aromatase assay the microsomes suspension was incubated with appropriate Test substrate spiked with [^{3}H]testosterone tracer, and in the presence of 0.1 mM NADPH cofactor excess. Incubation time was 40 min. The incubation mixture was extracted with 700 µl toluene, then the toluene phase was drained and washed with equal volume of HEPES buffer. Aromatase products containing phenolic hydroxy group were extracted with 1.2 M sodium hydroxide solution from the toluene extract, and the alkaline phase was measured in the LSC [VII., IX.].

3.3.3 STS assay

STS assays were performed using microsomes suspension and estrone-3-sulfate substrate with [^{3}H]estrone-3-sulfate tracer with incubations lasting for 20 min. Reactions were terminated by the addition of equal volume of methanol, and the product estrone was extracted with 400 µl toluene [VII., IX.].

3.3.4 17β-HSD1 assay

In the 17β-HSD1 assay the cytosol was incubated using estrone substrate with [6,7-^{3}H]estrone tracer and 0.1 mM cofactor (NADH or NADPH) excess. The incubation time was 2.5 min. The product 17β-estradiol was extracted with 100 µl ethyl acetate and isolated by a TLC method performed on silica gel plate and with dichloromethane/diisopropyl ether/ethyl acetate (75:15:10 v/v) eluent. UV detection was used to trace the separated steroids and their spots were cut out for measurement of radioactivity [II., III.].

Table 1. Description of radiosubstrate enzyme incubation methods used for inhibition tests

Enzyme	**$C_{17,20}$-lyase**	**Aromatase**	**STS**	**17β-HSD1**
Enzyme source	Rat testicular homogenate	Human placental microsomes	Human placental microsomes	Human placental cytosol
Substrate	17OHProg	Testosterone	Estrone-3-sulfate	Estrone
Product	Androst-4-ene-3,17-dione	17β-Estradiol	Estrone	17β-Estradiol
Coenzyme	NADPH	NADPH	-	NADPH/NADH
Incubation time	20 min	40 min	20 min	2.5 min
Enzyme amount in mg tissue equivalent	3.2 mg	20 mg	0.2 mg	0.4 mg
Radioactivity of the tracer (dpm/incubate)	170000	250000	30000	250000

3.4 Inhibition studies

Relative conversions (RC-s) compared to non-inhibited controls (100%) were determined. The assays were performed in triplicates for the determination of the percentages of inhibited RC-s at a final inhibitor concentration of 10 µM or 50 µM. Mean of the inhibited RC-s and the standard deviations (SD-s) were calculated. IC_{50} values (the inhibitor concentration, which decreases enzyme activity to 50%) were determined for the most effective and other selected test compounds. In these cases, conversions were measured at 10–15 different concentrations in the appropriate interval between 0.001–50 µM. IC_{50} results were calculated using an unweighted iterative leastsquares logistic curve fitting by means of "absolute IC_{50} calculation" function of the GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, United States). IC_{50} of unlabeled substrates and reference compounds were measured as reference. Relative inhibitory potentials (RIP) of the test compounds were calculated using reference IC_{50} data

measured with the corresponding cofactor: RIP= IC_{50} of test compound / IC_{50} of unlabeled substrate.

3.4.1 Kinetic analyses

The Michaelis constant (K_M) of substrates was measured using increasing concentration of the unlabeled substrate. The K_M was calculated from Michaelis-Menten analysis (reaction velocity vs. substrate concentration) using the GraphPad Prism 4.0 software.
Inhibition constant (K_i) was determined *via* measurement of the enzymatic transformation using different fixed substrate concentrations and varied inhibitor concentrations. The Dixon's linear transformation (1/velocity *vs.* inhibitor concentration) was applied for evaluation and K_i was estimated from abscissa of intersection of the lines of different substrate concentrations. Mechanism of binding was identified according to the Dixon's graphs and the secondary plot of slopes of the Dixon's lines *vs.* 1/substrate concentration [Cortes et al. 2001; Segel 1993].

3.4.2 Reversibility studies

3.4.2.1 Charcoal adsorption method

Reversibility of potent $C_{17,20}$-lyase inhibitors was investigated *via* preincubation and charcoal adsorption method. Testicular homogenate in incubation mixture (200 µl) was preincubated with high concentration (10 µM and 50 µM) of the tested compounds, in the presence of NADPH cofactor for 10 and 40 minutes at 37 ºC. After the addition of 50 µl 3% dextran coated charcoal suspension in 0.6% final concentration, the preincubated samples were kept on ice for 15 minutes and centrifuged at 4 ºC. Supernatants were drained and used for inhibition tests.

3.4.2.2 Dilution method

Mechanistic studies for STS and 17β-HSD1 inhibitors were performed *via* preincubation and dilution method. The inhibitor compounds were preincubated with the microsomal fraction or placental cytosol in a volume of 4.0 µl at 37 °C for various time periods (2.5-20 min). Following this procedure samples were diluted with the incubation medium to 50-fold. Enzyme activity measurements were started immediately for the 17β-HSD1 and for STS after a 20 min secondary incubation time to allow dissociation. The enzymatic reactions were started by the addition of the substrate, and subsequent incubation procedures were then carried out as described. Conversions were compared to their respective controls, which were treated in a similar way but were made without inhibitors.

4 Results and discussion

During our research work we tested approximately 500 steroid compounds in more than 600 incubation tests. Only most interesting and valuable test compounds, inhibitory potentials and results are shown in the thesis. Data collections and entire analyzes can be found in our related publications [I.–IX.]. Certain compounds and inhibitory results remained unpublished until now.

4.1 Methodological results

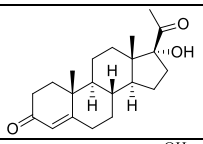
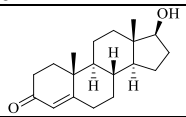
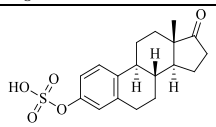
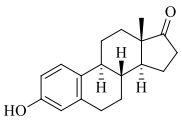
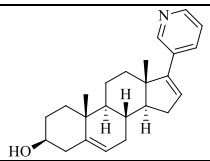
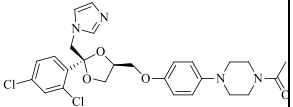
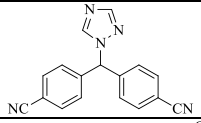
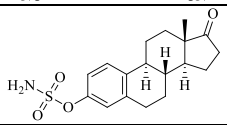
Human placenta tissue preparatums were appropriate enzyme sources for the enzymes investigated. We adapted and improved extraction techniques for enzyme products of aromatase and STS and developed a TLC separation substrate and product of 17β-HSD1 [Kellis & Vickery 1987; LaMorte et al. 1994; Leslie et al. 1994; Thompson & Siiteri 1974]. These isolation techniques provided high recovery, low blank and good reproducibility. Incubation protocols were suitable for rapid testing of numerous compounds [III.; VII; IX.].

With the selected incubation parameters the enzyme reactions satisfied conditions of initial velocity measurements. Conversion in the non-inhibited control incubates reached similar (10-13%) rate and the product formation was proportional to the enzyme concentration and the incubation time. Organic solvents present in the incubation medium did not reduce the enzyme activities substantially.

Parameters characterizing substrate affinity (K_M and IC_{50}) values were measured and potential of reference inhibitors were determined (Table 2.). In the case of aromatase with the Test IC_{50}= 0.52 μM and K_M= 0.60 μM, for STS with the estrone-3-sulfate IC_{50}= 5.2 μM and K_M= 8.5 μM and for 17β-HSD1 with estrone IC_{50}= 0.63 μM, K_M= 0.17 μM (in the presence of NADPH) and IC_{50}= 2.0 μM (in the presence of NADH) were measured using our methods. Similar data can be found in the literature when corresponding methodologies were applied for the aromatase [LaMorte et al. 1994], for the STS [Poirier & Boivin 1998], or for the 17β-HSD1 [III.] and K_M [Gangloff et al. 2001; Jin & Lin 1999]. IC_{50} values of the substrates and the test compounds were applied to calculate relative inhibitory potential (RIP = IC_{50} of test compound / IC_{50} of unlabeled substrate) and this parameter was used to assess the inhibitory effect and affinity of new test compounds towards the enzymes.

Reliability of our methods was inspected with reference enzyme parameters and inhibitor data published earlier in the literature. Reference inhibitor compounds were available for the aromatase and for the STS assays. In our methods the non-specific P_{450} inhibitor ketoconazole displayed IC_{50} value of 14.2 μM, which is close to the data published formerly by Ayub et al. (IC_{50}= 6 μM, with Test substrate) [Ayub & Level 1988]. We measured IC_{50} value of the specific aromatase inhibitor letrozole in the nanomolar range (0.0038 μM) as it is reported in numerous earlier studies [Caporuscio et al. 2011; Wang et al. 2013]. Inhibition parameters determined for the STS reference EMATE were found as IC_{50}= 0.0098 μM and K_i= 0.0044 μM which results are similar to data published in literature references [Numazawa et al. 2006; Purohit et al. 1998].

Table 2. Parameters of reference compounds for the investigated enzymes

Comp.	Structure	$C_{17,20}$-lyase	Aromatase	STS	17β-HSD1
		IC_{50} and/or K_M±SD (μM) or RC at 10 μM±SD (%)			
17OHProg		IC_{50}= 1.6±0.35 [Szabó et al. 2015]			
Test			IC_{50}= 0.52±0.14 K_M= 0.60±0.18		
Estrone-3-sulfate			87±4	IC_{50}= 5.2±1.2 K_M= 8.5±2.9	80±5
Estrone			78±7	63±2 IC_{50}= 24.1±10.2	IC_{50}= 0.63±0.11 (NADPH) K_M= 0.17±0.040 (NADPH) IC_{50}= 2.0±0.18 (NADH)
Abiraterone		IC_{50}= 0.0125±0.0015 [Szabó et al. 2015]			
Ketoconazole		IC_{50}= 0.32±0.02 [Szabó et al. 2015]	IC_{50}= 14.2±0.40		
Letrozole			IC_{50}= 0.0038±0.0010		
EMATE			95±8	IC_{50}= 0.0098±0.0038 K_i= 0.0044±0.0005	IC_{50}= 4.6±2.9

4.2 Investigations on inhibition of $C_{17,20}$-lyase activity of $P450_{17\alpha}$ with steroid derivatives substituted on ring D

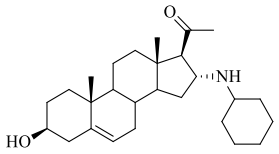
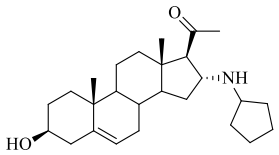
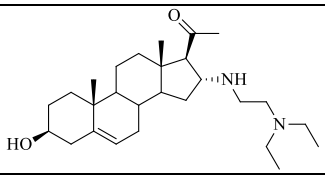
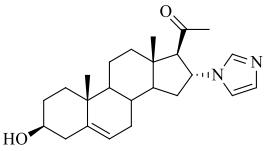
The $P450_{17\alpha}$ is a pivotal enzyme of the steroid biosynthesis. $C_{17,20}$-lyase process is the rate limiting step in the synthesis of the C_{19} preandrogens. Several 5-membered *N*-containing imidazole, pyrazole, triazole and tetrazole derivatives of androstane compounds were studied earlier as potential $C_{17,20}$-lyase inhibitors [Ling et al. 1997; Njar et al. 1998; Nnane et al. 1999; Moreira et al. 2008]. It was also observed with C-17β, and C-17β phenyl pyrazolinylandrostenes that the extended 17β heterocyclic side-chain with various substituted aromatic rings may have substantial inhibitory effect [Ondré et al. 2009]. Structural investigations propose the existence of a second hydrophobic binding pocket beside the heme structure, which could also support the inhibitor development strategy to use adjacent phenyl group on the C-17 substituent [DeVore & Scott 2012]. Example of galeterone also indicates that compounds with more spacious aromatic side chain may improve affinity and inhibitory effect towards the $P450_{17\alpha}$, and the extension of the side chain may be useful in modification of the activity profile of a pharmacon candidate [Handratta et al. 2005; Njar & Brodie 2015].

4.2.1 16α-Amino-pregnenolone compounds

We investigated the $C_{17,20}$-lyase inhibitory potential of 16α-amino-pregnenolone compounds synthesized at the Department of Organic Chemistry, Institute of Chemistry, University of Pannonia, Veszprém, Hungary [IV.].

The cyclohexylamine derivative **ML-147**, the cyclopentyl derivative **ML-208** and the *N,N*-diethyl-etilenediamine derivative **ML-214** exerted considerable inhibition, with IC_{50} values measured between 9-10 μM. The imidazole derivative (**ML-AD-46**) was found to be the most potent inhibitor, displaying an IC_{50} value of 1.8 μM (Table 3.). The other investigated derivatives exerted somewhat less effective inhibitions (IC_{50} values measured between 20-50 μM) or did not suppress RC to below 50% when the 50 μM test concentration was applied, resulting weak inhibitory effects with IC_{50} values exceeding 50 μM.

Table 3. Most relevant $C_{17,20}$-lyase inhibitory potentials of 16α-amino-pregnenolone compounds [IV.]

Comp.	Structure	IC_{50}±SD (μM)	RC at 10 μM±SD (%)	RC at 50 μM±SD (%)
ML-147		9.1±4.0	45±6	10±3
ML-208		9.5±1.3	47±1	42±8
ML-214		9.3±1.3	44±6	28±4
ML-AD-46		1.8±0.36	21±1	19±2

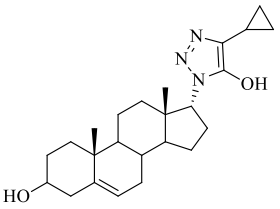
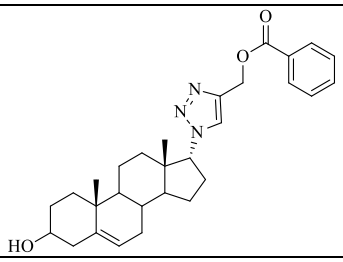
The imidazole derivative (**ML-AD-46**) had substantial inhibitory potential, which is close to that of reference ketoconazole. The presence of the *N*-containing unsaturated cyclic substituent on ring D may be the promoter of this increased effectivity. Interesting to note the considerable inhibitory potential of the **ML-208** and **ML-147**. These compounds bear saturated cyclopentyl and cyclohexyl cycles, respectively, which do not possess heteroatom or substituent to be able to donate lone electron pair. Their cyclopropyl counterpart, however, displayed weak inhibition.

4.2.2 17-Heterocyclic androstenes

We investigated $C_{17,20}$-lyase activity of $P450_{17\alpha}$ of new androst-5-en-3β-ol derivatives possessing diverse triazolyl substituents on 17α or 17β position. Compounds were synthesized at the Department of Organic Chemistry, University of Szeged [VI.].

In the series of the cycloalkyl substituted 17α-triazolyl compounds **GT-166** and **GT-70** were found to be the best inhibitors. Their IC_{50} values were 3.1 μM and 3.5 μM, respectively (Table 4.). Our results demonstrate substantial inhibitory effect of the carbomethoxy coupled phenyl substituted **GT-70** to the enzyme. IC_{50} value indicates affinity to the enzyme similar to that of the substrate. The corresponding 17β counterparts were found to be almost inactive in the $C_{17,20}$-lyase tests.

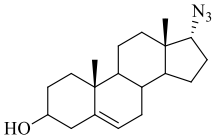
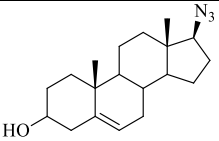
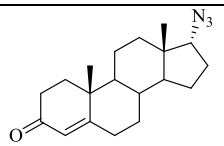
Table 4. Most relevant $C_{17,20}$-lyase inhibitory potentials of C-17α derivatives [VI.]

Comp.	Structure	IC_{50}±SD (μM)	RC at 10 μM±SD (%)	RC at 50 μM±SD (%)
GT-166		3.1±1.4	28±3	15±2
GT-70		3.5±0.8	32±5	6±1

4.2.3 17-Azido androstenes

We also investigated the precursor compounds of C-17-heterocyclic androstenes. Tested 17-azido-androstene compounds were able to inhibit the $C_{17,20}$-lyase activity and could bind to the $P450_{17\alpha}$ enzyme with considerable affinity (Table 5.).

Table 5. Most relevant $C_{17,20}$-lyase inhibitory potentials of C-17-azido compounds [VI.]

Comp.	Structure	IC_{50}±SD (μM)	RC at 10 μM±SD (%)	RC at 50 μM±SD (%)
SCH-1737		21.2±6.8	64±9	42±4
GT-101		0.60±0.18	15±2	17±3
GT-119		5.3±3.0	41±5	28±7

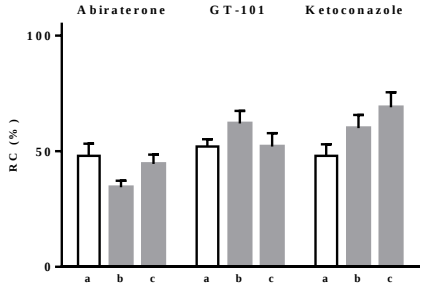
In the pair of the 5-en-3-hydroxy epimers, the 17β-azido **GT-101** was found to be more effective than the 17α-azido **SCH-1737**. In the case of the 17α-azido compound pair, the Δ^4-3-oxo derivative proved to be more effective compared to the Δ^5-3-hydroxy. This is against the general tendency that 17-heterocyclic inhibitors based on a

progesterone scaffold are normally weaker than the corresponding pregnenolone analogs [Iványi et al. 2012; Njar et al. 1998], but corresponds to other earlier examples found among 17β-oxazolidones and 17β-oxazolines [Ondré et al. 2008; Ondré et al. 2009]. The IC_{50} parameter of the most effective 17β-azido-androst-5-en-3β-ol **GT-101** indicates a 2.5-fold stronger binding compared to the substrate, and its inhibitory potential was found close to that of the nonsteroidal imidazole reference ketoconazole.

Potent inhibition of the 17β-azido **GT-101** is an interesting finding, therefore we further investigated inhibitory properties of this compound.

*4.2.3.1 Time dependence of inhibitory effect of **GT-101***

Possible time dependent change of inhibitory effect of **GT-101** was investigated in experiments in which the compound was applied in its IC_{50} concentration (0.60 μM) and preincubated with the testicular homogenate before the usual inhibition test. Difference was not found between the normal and the preincubated samples neither in case of the test compound **GT-101**, nor the references abiraterone and ketoconazole (Fig. 2). Presence of cofactor during the preincubation had also no influence on the measured inhibitory potentials. This result indicates that conversion which could modify inhibitory potential of 17β-azido-androst-5-en-3β-ol (**GT-101**) does not occur under the conditions of incubation procedures applied.



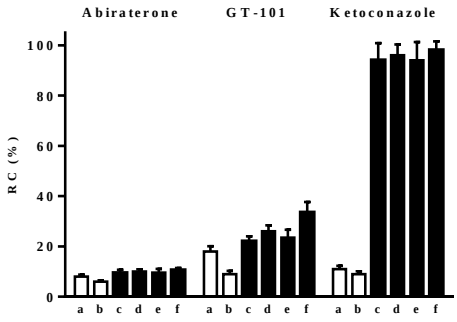
Experimental conditions:
a No preincubation
b Preincubation, 40 min
c Preincubation with NADPH, 40 min

Fig. 2 Inhibitory potential of the IC_{50} concentration of **GT-101** and reference compounds (abiraterone and ketoconazole) with or without 40 min preincubations with the rat testicular homogenate. Following the preincubation period the usual incubation procedure was applied. Mean±SD of three separate experiments

*4.2.3.2 Reversibility of inhibition of **GT-101***

Reversibility of the inhibition was studied *via* preincubation with high concentration of the test compounds followed by the removal of unbound and reversible bound inhibitor molecules by charcoal adsorption. Samples pretreated with the known reversible inhibitor compound ketoconazole reference displayed almost non-inhibited $C_{17,20}$-lyase activity [Lamber et al. 1986], whereas irreversible reference inhibitor compound abiraterone

[Jarman et al. 1998] caused blocked enzyme activity. Incubations pretreated with compound **GT-101** resulted in conversions close to those in the inhibited control inhibition tests with high inhibitor concentration (Fig. 3). These results indicate that **GT-101** molecules cannot be removed by charcoal treatment from the binding, and the binding to the enzyme has an irreversible mechanism.



Experimental conditions:
a No preincubation,10 μM
b No preincubation, 50 μM
c Preincubation, 10 μM, NADPH, 10 min, charcoal separation
d Preincubation, 50 μM, NADPH, 10 min, charcoal separation
e Preincubation, 10 μM, NADPH, 40 min, charcoal separation
f Preincubation, 50 μM, NADPH, 40 min, charcoal separation

Fig. 3 Investigation of reversibility of **GT-101** and reference compounds (abiraterone and ketoconazole). Inhibitor compounds were preincubated with rat testicular homogenate. Following a charcoal separation step, the usual incubation procedure was applied.

4.2.3.3 Aromatase inhibitory effect of azido compounds

Taking into account the aromatase inhibitory effect of 19-azido-4-androstene-3,17-dione [Wright et al. 1991] and certain $P450_{17\alpha}$ inhibitor abiraterone analogs [Brossard et al. 2013], we performed tests whether our 17-azido compounds are capable of inhibiting this enzyme. Incubations with high concentrations (10 μM and 50 μM) resulted in RC-s similar to the non-inhibited controls. These results indicate that 17-azido-androst-5-en-3β-ol (**GT-101**) does not inhibit aromatase activity substantially. Inhibitory effect towards the cytochrome P450 dependent steroid converting enzymes proved to be specific for the $P450_{17\alpha}$.

In the literature, a small number of steroid azides similar to those investigated by us can be found. The synthesis and antituberculosis activity of 17α-azido-5α-androstan-3β-ol was investigated by Merlani et al. [Merlani et al. 2008]. The neuroactive 17α- and 17β-azido-5β-androstan-3α-ol were studied by Vidrna et al. [Vidrna et al. 2011]. The 17-azidomethyl-androst-5,16-dien-3β-ol were synthesized by the group of Njar and Brodie during their $P450_{17\alpha}$ inhibitor development studies [Njar & Brodie 2015]. $P450_{17\alpha}$ or $C_{17,20}$-lyase inhibitory potential of these related compounds, nevertheless, were not reported in the corresponding publications.

Wright et al. investigated the inhibition of the 19-azido-4-androstene-3,17-dione against the aromatase, another cytochrome P450 dependent enzyme in the steroid

hormone metabolism [Wright et al. 1991]. They have found reversible inhibitor binding and hypothesized that the first nitrogen atom in the azide group (attached directly to the C-19 carbon) is most likely to be involved in coordination to the heme iron of aromatase.

Other, non-steroidal primary azides were found to be converted to the corresponding aldehydes *via* biocatalysis of myoglobin, another heme-dependent metalloprotein similar to cytochrome P450 [Giovanni et al. 2016]. The proposed binding and reaction mechanisms of the myoglobin catalysed azide reaction indicate also that the α-nitrogen is coordinated to the heme iron of the active center. Interesting to note, that in the case of abiraterone and several other C-17 heterocyclic $P450_{17\alpha}$ inhibitors the $\Delta^{16,17}$ double bond not only increases the inhibitory potency but also switches the binding mode from reversible to irreversible [Jarman et al. 1998]. The irreversible binding of 17β-azido-androst-5-en-3β-ol (**GT-101**), nevertheless, occurs in the absence of $\Delta^{16,17}$ double bond.

4.2.4 Conclusions

Results on $C_{17,20}$-lyase inhibitory effect of new 16α-amino-pregnenolone and 17-triazolyl androstene compounds revealed that compounds bearing 16α- or 17α- side chains may also have substantial affinity to this enzyme. Results verified that extension of these side chains may improve inhibitory potential. Our results revealed that stereoisomerism at the C-17 of the new androst-5-en-3β-ol derivatives influenced the $C_{17,20}$-lyase effect. Inhibitors were found only in the 17α-triazolyl series. Our data indicate that azido group on the steroidal C-17 is a suitable pharmacophore in the inhibition of the $C_{17,20}$-lyase activity. Potent inhibition of the azido compound is an interesting finding worth for further investigations. These discoveries indicate that similar derivatives may be capable of reasonable affinity to the enzyme and development of this group of compounds for studying the inhibition and for targeting new inhibitors of the $P450_{17\alpha}$ may be promising.

4.3 Comparative investigations on cofactor dependent inhibition of 17β-HSD1

17β-HSD1 catalyzes the final hormon activating step of the estrogen biosynthesis, the transformation of estrone to 17β-estradiol. The NADPH and the NADH are both able to promote the stereospecific reduction of the C-17 carbonyl of the substrate (Fig. 4) [Jin & Lin 1999; Mazza et al. 1998; Puranen et al. 1997].

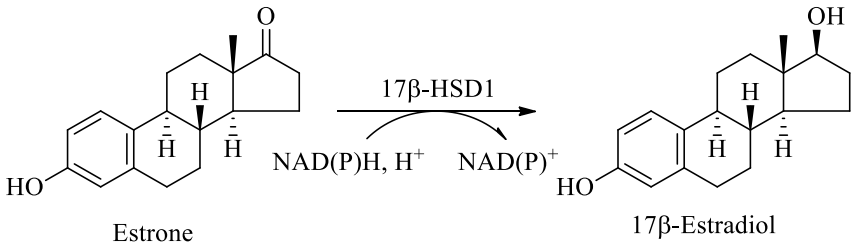


Fig. 4 Transformation of estrone to 17β-estradiol catalysed by 17β-HSD1

Both cofactor molecules, NADPH and NADH, bind to 17β-HSD1 in an extended conformation, with the nicotinamide moiety pointing towards the active site of the enzyme. Nicotinamide is relatively flexible in the complex and the major interactions between cofactor and enzyme occur at the adenine dinucleotide phosphate part [Huang et al. 2001]. Most of these interactions are common for both NADPH and NADH. A major difference between the two cofactors is, however, that the 2′-phosphate group of NADPH is stabilized mainly through hydrogen bonds with residues Ser11 and Arg37, whereas the free hydroxyl groups on adenosine ribose of non-phosphorylated cofactor NADH may form hydrogen bonds with Ser11, but not with Arg37. The presence or absence of 2′-phosphate in the cofactors causes differences in the structure of the holoenzymes [Breton et al. 1996; Mazza et al. 1998; Mazumdar et al. 2009].

When a cofactor binds to 17β-HSD1, structural changes are induced in the area of the substrate binding site as well. An otherwise disordered loop, which is composed of residues 189–200, may adopt a specific conformation to accommodate more space for the cofactor in the active center [Breton et al. 1996; Ghosh & Vihko 2001; Mazumdar et al. 2009]. The hydrophobic tunnel part of the substrate binding site (Leu149, Val255, Phe226, and Phe259) [Mazza et al. 1998; Mazumdar et al. 2009] and the catalytic triad (Ser142, Tyr155, and Lys159) may also change their conformation upon cofactor binding [Breton et al. 1996; Mazumdar et al. 2009]. These structural differences between the holo form and the apo form of the enzyme may modify interactions of ligands bound in the substrate binding site. Estrane-based inhibitors are assumed to occupy the substrate binding site of 17β-HSD1 [Messinger et al. 2009].

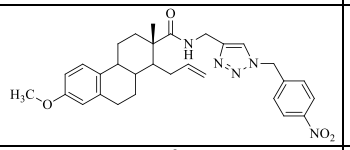
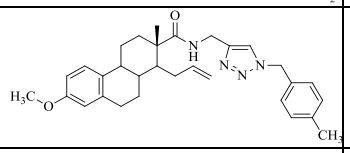
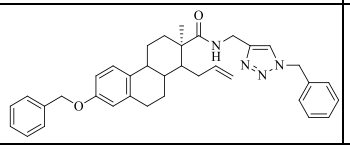
Considering the cofactor induced changes in the area of the substrate binding site and the differences in the binding mechanisms of the two cofactors, it seemed reasonable to investigate inhibitor candidates according to their potentials against NADPH- and NADH-complexed 17β-HSD1.

4.3.1 Triazole and tetrazole compounds

We investigated the 17β-HSD1 inhibitory potential of various D-seco-triazolyl compounds [II.], compounds bearing triazolyl group on ring A [Herman et al. 2015, unpublished results] and D-fused triazole and tetrazole derivatives [Jovanović-Šanta et al. 2018, unpublished results]. Compounds were synthesized at the Department of Organic Chemistry, University of Szeged and Department of Chemistry, Biochemistry and Environmental protection, University of Novi Sad.

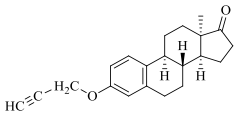
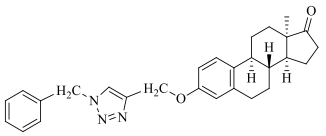
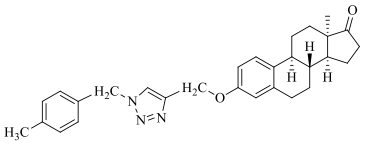
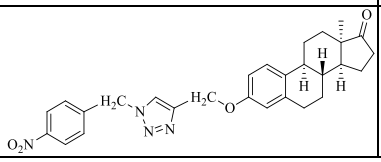
The examined seco-triazolyl compounds **BI-28**, **BI-42** and **BI-25** exerted considerable inhibitions in the presence of NADH cofactor (Table 6.). Inhibition potential of the *p*-nitrophenyl-derivative **BI-28** was similar to the unlabeled estrone, for which 2 μM reference IC_{50} was determined (Table 2., Methodological results). Compounds proved to be weak inhibitors when NADPH was used.

Table 6. Most relevant 17β-HSD1 inhibitory potentials of D-secoestrone compounds [II.]

Comp.	Structure	NADPH			NADH		
		IC_{50} ± SD (μM)	RC at 10μM ± SD (%)	RIP	IC_{50} ± SD (μM)	RC at 10μM ± SD (%)	RIP
BI-28		> 10	99±1	> 15	1.3±0.4	25±2	0.65
BI-42		> 10	109±2	> 15	4.5±0.8	42±11	2.25
BI-25		> 10	92±14	> 15	3.6±0.4	34±5	1.8

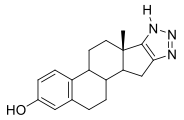
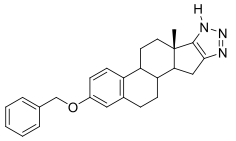
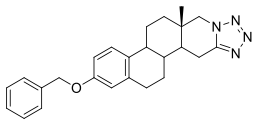
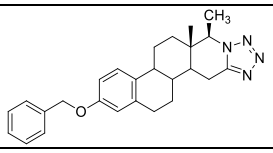
We screened more than twenty test compounds bearing triazolyl substituens on ring A, best examples are shown in Table 7. The nature of the cofactor used had no remarkable influence on the activities of the 17-keto-3-(*N*-benzyl-triazolylmethoxy) derivatives (**PZ-8, PZ-14** and **PZ-6**), they displayed moderate or weak inhibitory activities. There is an interesting difference between inhibitory potentials observed in the case of the precursor molecule, the propargyl ether derivative (**PZ-5**) of the 13α-estrone. The shift in the inhibitory potential in the presence of different cofactors was even more pronounced when the RIP data (1.8 and 14.3, respectively) were compared.

Table 7. Most relevant 17β-HSD1 inhibitory potentials of 3-triazolyl compounds [Herman et al. 2015, unpublished results]

Comp.	Structure	NADPH			NADH		
		IC_{50} ± SD (μM)	RC at 10μM ± SD (%)	RIP	IC_{50} ± SD (μM)	RC at 10μM ± SD (%)	RIP
PZ-5		9.0±2.4		14.3	3.6±0.6		1.8
PZ-8		7.8±1.4		13.4	9.7±2.8		4.85
PZ-14		> 10	66±2	> 15	> 10	50±4	> 5
PZ-6		> 10	67±5	> 15	> 10	56±10	> 5

We also tested D-fused triazole and tetrazole estrone analogs. Compounds containing 3-benzyloxy functions did not inhibit 17β-HSD1 substantially, whereas the 3-hydroxyl triazolyl estrone analog **MS/AN-3** exerted a potent inhibitory effect (Table 8). The inhibition in this case was found to be selective towards the NADH-complexed enzyme and the binding affinity was similar to that of the substrate estrone.

Table 8. Most relevant 17β-HSD1 inhibitory potentials of D-fused triazole and tetrazole derivatives [Jovanović-Šanta et al. 2018, unpublished results]

Comp.	Structure	NADPH			NADH		
		IC_{50} ± SD (μM)	RC at 10μM ± SD (%)	RIP	IC_{50} ± SD (μM)	RC at 10μM ± SD (%)	RIP
MS/AN-3		> 10	79±16	> 15	2.6±1.0		1.3
MS/AN-2		> 10	100±10	> 15	> 10	81±4	> 5
KPG/AO-1		> 10	94±9	> 15	> 10	89±6	> 5
KPG/AO-2		> 10	98±0.3	> 15	> 10	91±7	> 5

In the literature several triazolyl-derivatives of estrone [Allan et al. 2006; Messinger et al. 2009; Poirier 2011] and also numerous D-fused, differently substituted *N*-heterocyclic estratriene compounds have been reported as potent inhibitors of the 17β-HSD1 [Brozic et al. 2008, Day et al. 2008; Poirier 2010].

Effect of the 16,17-fused pyrazolyl compounds has been also investigated in detail [Allan et al. 2006; Fischer et al. 2005; Vicker et al. 2004; Vicker et al. 2006]. Molecular docking studies of a derivatized pyrazolyl estrone analog and a 17β-HSD1-NADPH complex indicated that nitrogens of the pyrazole ring could bind the Pro-S hydrogen atom on the nicotinamide ring of the cofactor [Vicker et al. 2006]. In another report, binding mechanism of the pyrazolyl estrone analog was interpreted as the pyrazole N-H donating a hydrogen bond to His210, with His213 adopting a tautomeric equilibrium in order to donate a hydrogen bond to the pyrazole nitrogen atom (numbered as His198 and His201 in references) [Meanwell 2011; Sweet et al. 1991]. It was also found that the binding

depends on contribution of groups on the A- and D-rings and compounds possessing phenolic hydroxyl function in the ring A show higher potential than the corresponding methyl ether derivatives [Sweet et al. 1991].

Our triazole and tetrazole test compounds (**MS/AN-2**, **MS/AN-3**, **KPG/AO-1**, **KPG/AO-2**) are close analogs of pyrazole fused inhibitors reported in previous references. Mechanisms proposed earlier for the binding of pyrazolyl estrone analog [Sweet et al. 1991; Vicker et al. 2006] can be regarded as plausible explanations for binding of our compounds. We may therefore assume that triazolyl nitrogens of our D-seco and D-fused estratrienes may form hydrogen bonds to the His210 and His213, and can also be able to establish connection to the Pro-S hydrogen atom on the nicotinamide ring of the NADH cofactor.

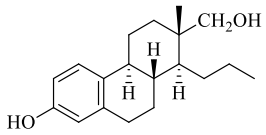
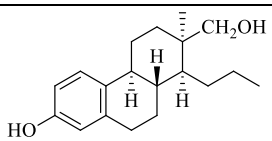
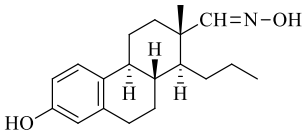
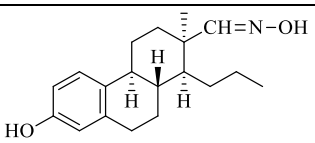
Cofactor dependent inhibitory effect of test compounds bearing a triazole functional group on ring D indicates that residues His210 and His213 and/or the nicotinoyl pyridine ring which might be involved in the binding are situated differently in the different holoenzymes and they are seized in proper positions for binding only in the NADH bound enzyme complex. These mechanisms are not applied to triazolyl side chains at the opposite site (ring A), the investigated estratriene molecules block binding and diminish affinity to the 17β-HSD1 complexed with either cofactor.

During our experimental work we identified estrone analogs which display 17β-HSD1 inhibitory effect with marked cofactor dependence. Results obtained with triazole and tetrazole compounds demonstrate that *in vitro* potentials obtained with the two cofactors may differ substantially compared to certain inhibitor compounds. In our experimental practice, these groups of compounds provided the first examples for cofactor dependent inhibition and these results focused our attention to this phenomenon.

4.3.2 D-secoalcohol and D-secooxime compounds

We investigated inhibitory potentials of 13α- and 13β-D-secoestrone derivatives (Table 9.) in the presence of NADPH and NADH [III.].

Table 9. Most relevant 17β-HSD1 inhibitory potentials of D-seco compounds [III.]

Comp.	Structure	NADPH		NADH	
		IC_{50} ± SD (μM)	RIP	IC_{50} ± SD (μM)	RIP
SZJ-36		3.4 ± 1.4	5.4	0.41 ± 0.24	0.21
JN-14		3.7 ± 1.3	5.8	1.7 ± 0.3	0.85
FG-7		0.070±0.027	0.11	0.077±0.036	0.039
FG-4		30±7	48	0.058±0.044	0.029

The two epimeric D-secoalcohols (**SZJ-36** and **JN-14**) displayed IC_{50} values in the low or submicromolar range. The 13β epimer of the D-secoalcohol (**SZJ-36**) exerted an 8–9-fold more effective inhibition with NADH according to the IC_{50} data, and a 25-fold stronger effect in the sense of the RIP values.

The epimeric 3-hydroxy-D-secooximes (**FG-7** and **FG-4**) displayed noteworthy inhibitory properties and C-13 chirality dependence. **FG-7** was found to be highly potent in the presence of either NADPH or NADH. The 13α counterpart (**FG-4**) was effective only when NADH was used as cofactor. The oxime epimer pairs of **FG-7** and **FG-4** displayed a large difference, demonstrating inhibition around 400-fold stronger of the 13β than that of the 13α epimer in the presence of NADPH, whereas they exerted similar effect with NADH. The 13α epimer of the D-secooxime (**FG-4**) displayed an outstanding cofactor dependence. This compound exerted only weak inhibition with NADPH, but it was highly effective in the presence of NADH. The difference between the IC_{50} values was more than 500-fold, whereas the RIP ratio exceeded 1200.

The complexities of the interaction mechanisms of 17β-HSD1 have the result that relatively small changes in the shape of the steroid substrate or inhibitor ligand, and/or in the protein conformation induced by the cofactor or by other modulators can significantly

affect the binding and catalytic arrangements, and consequently the binding affinity, the inhibitor potential and the selectivity. Natural estrone possesses a tetracyclic steroidal framework with *trans* junctions of rings B/C and C/D. The other characteristics of this classical steroid are the typical conformations of rings C (chair) and D (strongly restricted). The rigid structure of estrone contains two oxygen functionalities with well-defined distances, which are crucial in the binding of estrone or estradiol to its nuclear hormone receptors. In contrast with the natural 13β compound, the 13α epimer has a quasi-equatorial angular methyl group, a *cis* junction of rings C/D and a ring D that is directed to the β side [Schoenecker et al. 2000]. Ayan et al. reported the impact of inversion of the configuration at C-13 and/or C-17 of estradiols on their estrogenic activity [Ayan et al. 2011]. They concluded that 13 epimers have low relative binding affinity for estrogen receptor alpha and have no significant uterotropic activity. Accordingly, inversion at C-13 in the estrane skeleton could be a correct strategy in the design of estrone-based anticancer agents lacking estrogenic activity.

Since the two secooxime epimers **FG-7** and **FG-4** differ only in the configuration of carbon in position 13 and the oxime function, the orientation of this part of the molecule seems to be favorable in the NADH complex of the enzyme for both the 13α and the 13β epimer (**FG-4** and **FG-7**), but only for **FG-7** in the NADPH complex. Effective binding of the 13α counterpart (**FG-4**) is possibly prevented by the increased specificity of 13β compounds to the NADPH complex. The side chain at C-13 in **FG-4** may be directed into an unfavored position, which cannot be modified because of the limited flexibility of the oxime function caused by its double bond. The related alcohols **SZJ-36** and **JN-14** displayed similar inhibitory potencies, irrespectively of the orientation of the methyl group at C13, as the shorter and more flexible side chain may find its optimum position either in the NADH- or in the NADPH-complexed protein.

We assume that polar functionalities of short side chains of D-seco compounds studied might establish hydrophilic interactions or hydrogen bonds towards suitable amino acids of the enzyme present in close proximity of ring D region. Binding affinity of certain compounds in that series also displayed strong cofactor dependence and this phenomenon indicated that complexation with NADPH or NADH furnished different conformations to enzyme residues involved in the interactions.

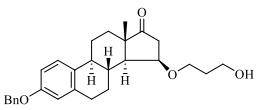
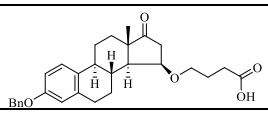
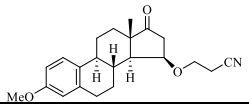
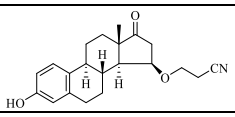
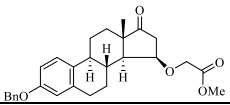
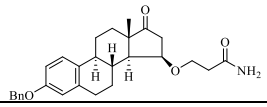
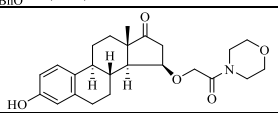
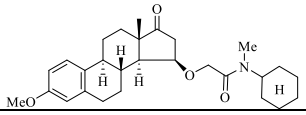
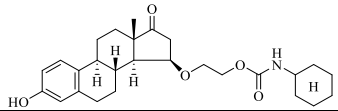
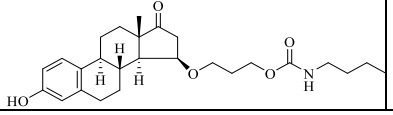
The very low *in vitro* IC_{50} of **FG-7** indicates that this compound is one of the most effective 17β-HSD1 inhibitors ever reported. The different *in vitro* inhibitory potentials observed for the C-13 epimer pairs, with the cofactor NADH instead of NADPH, are

interesting findings. Additional investigations with the aim of elucidating the binding mechanisms may provide new data clarifying the structure–function relationships of 17β-HSD1.

4.3.3 C-15 substituted estrone derivatives

We investigated a diverse group of substituted 15β-alkoxy estrone derivatives (Table 10.). Substantial inhibitory potentials and increased binding affinities were observed compared to the unsubstituted core molecules in the presence of both cofactors, and some of the compounds exerted different inhibitory potentials towards NADPH- or NADH-complexed 17β-HSD1 [VIII.].

Table 10. Most relevant 17β-HSD1 inhibitory potentials of C-15 substituted estrone derivatives [VIII.]

Comp.	Structure	NADPH			NADH		
		IC_{50} ± SD (μM)	RC at 10μM ± SD (%)	RIP	IC_{50} ± SD (μM)	RC at 10μM ± SD (%)	RIP
SCH-1478		0.78 ± 0.30		1.2	> 10	76 ± 7	> 5
SZE-5012		> 10	87 ± 4	> 15	3.5 ± 1.0		1.8
SZE-4013		0.56 ± 0.37		0.89	20 ± 9.0		10
SCH-1484		0.64 ± 0.18		1.01	3.2 ± 1.5		1.6
SZE-4033		1.5 ± 0.50		2.4	> 10	60 ± 3	> 5
SZE-4017		1.1 ± 0.42		1.7	12 ± 4		6.2
SCH-1054		> 10	67 ± 4	> 15	5.6 ± 2.2		2.8
SZE-5027		> 10	61 ± 2	> 15	7.0 ± 0.7		3.5
SCH-1544		0.42 ± 0.33		0.66	0.38 ± 0.22		0.19
SCH-1548		0.45 ± 0.23		0.71	1.4 ± 0.9		0.70

In our experiments, substantial inhibitory potentials and increased binding affinities were observed in presence of both cofactors. However, some of the compounds exerted different inhibitory potentials towards NADPH- or NADH-complexed 17β-HSD1. Substrate estrone displays different IC_{50} values depending on the cofactor applied (Table 2., Methodological results) and RIP parameters reflect more reliably the cofactor dependent differences in the inhibitor binding. Using the RIP values, derivative **SCH-1544** exerted similar effect in the presence of NADPH than estrone (RIP= 0.66 and 1.0, respectively), but this compound had a strong 5-fold increase in inhibitory potential, when NADH was applied (RIP= 0.19). Derivative **SZE-4013** also displayed a maintained effect with NADPH (RIP= 0.89), but showed diminished potential with NADH (RIP= 10). C-15 substituents increased the inhibitory potentials in the case of 3-benzyloxy compounds **SCH-1478**, **SZE-4033**, and **SZE-4017** when NADPH was applied (RIP= 1.2–2.4), but maintained effects were measured for these derivatives in the presence of NADH (RIP > 5.0). Compound **SZE-5012** had an opposite behavior, it showed improved inhibition with NADH (RIP= 1.8). On the other hand, **SCH-1484** and **SCH-1548** showed retained potentials with both cofactors compared to their unsubstituted core estrone with RIP parameters close to 1 (0.70–1.6). For some other compounds derivatized at C15, however, decreased inhibitory effects could be observed when either NADPH or NADH was applied. RIP parameters demonstrate 3–8 fold stronger binding with NADH than with NADPH for **SCH-1054, SZE-5027, SCH-1544** and **SZE-5012**. Binding of 3-benzyl-*O*-estrone compounds **SCH-1478**, **SZE-4033** or **SZE-4017** and 3-methyl-*O*-estrone derivative **SZE-4013** display similar (2–11 fold) cofactor preference in terms of RIP values, but these derivatives favor binding to the NADPH-bound enzyme.

In the course of binding, C-15 substituted compounds are assumed to be capable of establishing further contacts to the enzyme than the substrate molecule itself and in this way, they may modulate binding affinity and inhibitory potential [Marchais-Oberwinkler et al. 2011; Mazumdar et al. 2009; Poirier 2003; Poirier 2010; Srungboonmee et al. 2015]. Messinger et al. analysed the X-ray structure of the 17β-HSD1 [Breton et al. 1996] and identified a hole in the proximity of the enzymes' active site, which is composed of flexible amino acids Ser222, Leu219 and Met193 as well as Tyr218, Leu96, and Gly198 [Messinger et al. 2009]. The hole shows its opening towards the environment of C-15 of the steroidal backbone, and thought to be able to accommodate side chains with appropriate length, spacer unit, and capping group [Hirvelae et al. 2014; Messinger et al. patents]. The binding hole (Leu96, Met193, Gly198

Tyr218, Leu219 and Ser222) which is suitable to accommodate C-15 side chains [Messinger et al. 2009] shares Met193 and Gly198 with a loop element (amino acids no. 189–200) adopting a specific conformation upon cofactor binding [Breton et al. 1996; Mazumdar et al. 2009].

C-15 substituted compounds displayed considerable difference in binding affinities towards 17β-HSD1 complexed with NADPH or NADH. It is reasonable to assume that side chains of the potent compounds can be accommodated in the binding hole of 17β-HSD1 existing in proximity of the C-15 position of ring D of the steroidal ligands [Messinger et al. 2009]. This binding hole shares Met193 and Gly198 with a loop element which is known to adopt a specific conformation upon cofactor binding [Breton et al. 1996; Mazumdar et al. 2009]. We suppose that conformation of this loop may be different in NADPH- or NADH-complexed 17β-HSD1. Structural differences can be forwarded by the joint amino acids inducing different positioning and binding capabilities of the binding hole. Further structural investigations (e.g., molecular docking studies) may confirm mechanisms involved in binding of our inhibitor compounds and different binding affinities of the test compounds exerted towards the holoenzyme variants.

4.3.4 Conclusions

We identified several groups of compounds which may exert cofactor dependent inhibition towards the 17β-HSD1. Our results indicate that binding of the phosphorylated NADPH or the unphosphorylated NADH cofactor may exert different influence on certain areas and structural elements of the substrate binding site which are involved in inhibitor binding.

Enzyme residues able to form strong hydrophilic interactions or hydrogen bonds (e. g. His210 and His213), the loop element (amino acids 189-200) and the binding hole of C-15 substituents, as well as the cofactors' pyridine ring in the nicotinamide function may have different positions in the different holoenzymes. These structural differences involve different binding affinities of inhibitor molecules to NADH- or NADPH-complexed 17β-HSD1.

Early studies assigned NADH as a catalytic cofactor of 17β-HSD1 and numerous *in vitro* inhibition tests have been performed with this recognition [III.]. Later, however, it became accepted that NADPH might be the prevalent partner of 17β-HSD1 in its main *in vivo* function [III.; Huang et al. 2001; Jin & Lin 1999].

Our results indicate that the apparent *in vitro* potentials obtained with the two cofactors may differ substantially for several inhibitors belonging to diverse groups of compounds. Data on NADPH and NADH are not interchangeable and their direct comparison [Marchais-Oberwinkler et al. 2011] is not advised. The literature data must be reviewed with special attention to the cofactor supplementation, the screening systems [Kruchten et al. 2009] should be specified precisely, and NADPH should be preferred instead of NADH in cell-free *in vitro* inhibitor tests. The influence of cofactors might be an explanation for the altered, occasionally disappointingly decreased inhibition potentials obtained in cellular 17β-HSD1 inhibition assays performed following promising cell-free screening tests with NADH [Farhane et al. 2009]. Data measured in the presence of NADH must be evaluated with caution in inhibitor optimization and in lead selection. NADH results are less relevant to the potential *in vivo* effect, but could be valuable in facilitating the understanding of the mechanism of catalysis and the inhibition of 17β-HSD1.

3D modelling and molecular docking studies may give further explanations for the cofactor dependent behavior of 17β-HSD1 inhibitors. Comparative investigations on cofactor dependent inhibition offer a good experimental tool for mechanistic studies and in combination with theorethical methods may provide a better insight to the mechanisms of inhibitor binding of the 17β-HSD1.

4.4 Inhibition of aromatase, STS and 17β-HSD1 with halogenated estrone analogs

Earlier investigations indicate that steroidal compounds substituted with halogens or lipophilic groups in the position C-2 and/or C-4 may have high binding affinities towards aromatase, STS, and 17β-HSD1, and compounds in these series may also be able to inhibit estrogen biosynthesis substantially. Literature references describe potent aromatase inhibitors among the 2-halo substituted estrone derivatives, 2-bromo and 2-chloroestrone were found the most powerful members of the series investigated [Numazawa et al. 2005]. Earlier studies on STS revealed that certain halogens or other small electron withdrawing groups (cyano and nitro) in the position 2 and/or 4 of the steroidal ring A might enhance the inhibitory potential of either estrone- or EMATE-based compounds [Mostafa et al. 2015; Numazawa et al. 2006; Phan et al. 2011]. In the case of the 17β-HSD1, literature data indicates that estrone derivatives possessing chloro, cyano, ethyl or allyl substituents in C-2 position display similar affinity to that of their parent compound [Moeller et al. 2009].

Substituents in the ring A of estrone-based derivatives may suppress intrinsic estrogenicity of these compounds [Anstead et al. 1997; Kuruto-Niwa et al. 2007; Nakamura et al. 2006; Zhu et al. 2003]. Modifications of the D-ring region of estrone-based enzyme inhibitors, for instance absence of the 17β-hydroxyl or 17-oxo function [Anstead et al. 1997; Zhu et al. 2003], as well as inversion of the configuration at C-13 may result also a loss of hormonal activity [Ayan et al. 2011]. Estrone-based compounds possessing substituents in their ring A and bearing intact or modified ring D are favorable target molecules concerning development of inhibitors of the estrogen biosynthesis.

There is an increasing concern about the environmental fate and xenobiotic potential of estrone derivatives. Estrone, among other natural steroidal estrogens, is excreted into the environment in considerable amount by human beings and livestock. Estrone is also often the most abundant estrogen in the wastewater, and can be found in 0.1 – 0.2 nM concentrations both in influent and effluent the sewage treatment plants [Baronti et al. 2000; Khanal et al. 2006; Souissi et al. 2014]. Upon exposure to sunlight or UV irradiation used as disinfection tool in water treatment, 13α-estrone is produced photochemically from estrone [Nakamura et al. 2006; Souissi et al. 2014; Trudeau et al. 2011]. Estrone and 13α-estrone chlorinated by the reaction with disinfectant chlorine or hypochlorous acid and brominated derivatives are formed when bromide ions (e. g. from

sea water) are also present [Kuruto-Niwa et al. 2007; Nakamura et al. 2006; Zhu et al. 2003]. This way estrone and 13α-estrone, as well as their chlorinated and brominated derivatives are released into environmental waters and eventually drinking water reservoirs may be polluted [Souissi et al. 2014]. Aquatic and land ecosystems, as well as human populations could be compromised by the potential endocrine disrupting effects. We find therefore reasonable to evaluate influence of halogenated estrone derivatives on the estrogen biosynthesis in this context, too.

We investigated inhibitory potential of several estrone analogs, namely estrone, 13α-estrone and 17-deoxy-13α-estrone derivatives bearing diverse substituents in the C-2 and C-4 positions regarding their inhibitory potentials exerted against aromatase, STS and 17β-HSD1 [I.; VII.]. Compounds were synthesized at the Steroid Research Group, Department of Organic Chemistry, University of Szeged.

4.4.1 Inhibition of aromatase

Estrone derivatives halogenated in the ring A proved to be weak aromatase inhibitors with the exception of the 2-bromo **EM-1049I** and 2-chloro **BI-347 II 4/4** derivatives which exerted moderate inhibition with IC_{50}= 8.7 and 6.0 μM (Table 11.). In the series of 13α- and 13α-17-deoxy compounds were found weak inhibitors.

According to literature information, F, Cl and Br in the C-2 position of estrone offer compounds with high binding affinity to aromatase enzyme [Numazawa et al. 2005]. Exact correlations between inhibitory activity and size and/or electronegativity of substituents at C-2 could not be established. Halogenation at the C-4 position, except for fluorination, markedly decreased affinities [Osawa et al. 1997].

Our aromatase inhibition results obtained for the series of 2-halogenated estrone analogs reveal that the inhibitory potential is enhanced and the effect is increasing in the row of I, Br and Cl. (Worth to note that our results are in good agreement concerning IC_{50} values of 2-chloro- and 2-bromoestrone with those of Numazawa et al. [Numazawa et al. 2005].) Test compounds with modified ring D (13α- and 13α-17-deoxy-estrone) and their halogenated derivatives exerted very weak inhibitory effect.

4.4.2 Inhibition of STS

Estrone compounds halogenated in the ring A proved to be potent STS inhibitors with the exceptions of the weak inhibitor 2-iodo **EM-1048V** and 2,4-*bis*-iodo **BI-341 2/5** derivatives (Table 11.). Chloro and bromo substituted estrone derivatives displayed 8–

28-fold increased inhibitory potentials compared to the parent compound estrone (Table 2., estrone IC_{50}= 24.1 μM). The most potent inhibitor in this series, the 4-iodo derivative (**EM-1048III**) displayed outstanding IC_{50} results (0.23 μM) showing more than 100-fold improved inhibition than the estrone and 22-fold better binding affinity than the substrate estrone-3-sulfate (IC_{50}= 5.2 μM). Weak inhibitory effect of 13α-estrone and 17-deoxy-13α-estrone indicate their weak binding affinity to the STS. 4-Bromo, 4-iodo, and 2,4-*bis*-iodo compounds (**BI-116AF**, **BI-113FF** and **BI-76**) in the 13α-estrone series were found to be effective inhibitors. Halogenated 17-deoxy-13α-estrone derivatives displayed considerable inhibitory effect towards the STS enzyme with IC_{50} values similar to that of the substrate estrone-3-sulfate. The 2,4-*bis*-chloro derivative (**HK-27II**) was found to be somewhat more effective than other compounds in this series.

Table 11. Most relevant inhibitory potentials of halogenated estrone derivatives [I.; VII.]

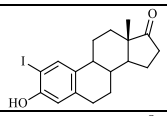
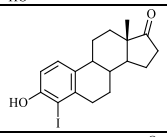
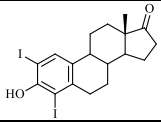
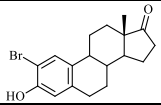
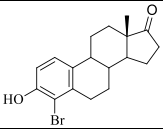
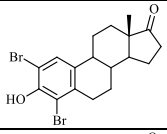
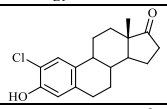
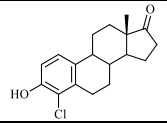
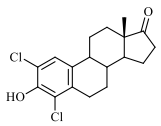
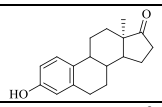
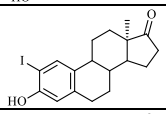
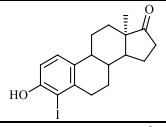
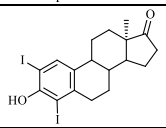
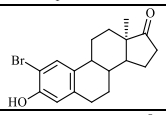
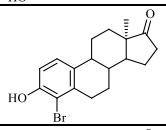
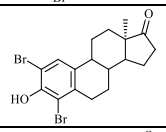
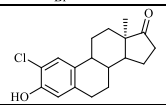
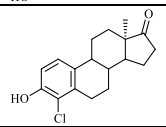
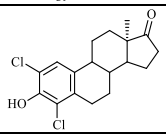
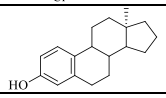
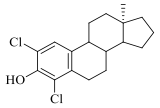
Comp.	Structure	Inhibition of enzyme activities RC±SD (%), IC_{50}±SD (μM) and K_i±SD (μM)		
		Aromatase	STS	17β-HSD1
EM-1048V		62±1	64±3	IC_{50}= 0.064±0.034
EM-1048III		88±2	IC_{50}= 0.23±0.09 K_i= 0.36±0.05	IC_{50}= 0.36±0.25
BI-341 2/5		86±6	80±13	55±7
EM-1049I		IC_{50}= 8.7±2.8	IC_{50}= 2.0±0.4	IC_{50}= 0.095±0.031
EM-1049II		91±6	IC_{50}= 0.89±0.3	IC_{50}= 0.30±0.20
BI-340 5/1		81±5	IC_{50}= 2.1±0.6	IC_{50}= 0.96±0.45
BI-347 II 4/4		IC_{50}= 6.0±1.2	IC_{50}= 2.4±0.4	IC_{50}= 0.18±0.02
BI-347 5/4		92±3	IC_{50}= 1.6±0.3	IC_{50}= 0.60±0.16
BI-347 D 5/1		82±4	IC_{50}= 3.0±0.9	IC_{50}= 0.59±0.16

Table 11. Continued

Comp.	Structure	Inhibition of enzyme activities RC±SD (%), IC_{50}±SD (μM) and K_i±SD (μM)		
		Aromatase	STS	17β-HSD1
PZ-1		85±13	96±1	IC_{50}= 1.2±0.2 K_i= 1.9±0.2
BI-113 AF		82±10	83±3	IC_{50}= 0.59±0.23
BI-113 FF		90±7	IC_{50}= 6.0±1.6	IC_{50}= 1.0±0.3 K_i= 2.2±0.3
BI-76		91±1	IC_{50}= 2.4±0.52	IC_{50}= 0.38±0.08 K_i= 0.94±0.15
BI-135		100±5	81±6	IC_{50}= 1.2±0.36
BI-116 AF		78±1	IC_{50}= 8.5±3.1	IC_{50}= 2.1±1.2
BI-116 FF		108±11	71±4	IC_{50}= 2.7±0.1
BI-323II		106±6	57±1	IC_{50}= 0.33±0.10
BI-123 7/3		98±3	80±7	IC_{50}= 2.6±1.0
BI-123 5/5		101±4	70±3	IC_{50}= 2.2±0.6
PZ-16		95±12	76±5	IC_{50}= 1.1±0.33 K_i= 2.0±0.4
HK-27 II		82±12	IC_{50}= 1.3±0.4 K_i= 1.9±0.2	53±2

Phan et al. investigated STS inhibitory potential of a series of 2- and 4-substituted estrone derivatives. They reported that the inhibition potential of 2-bromoestrone is modest, whereas its 4-bromo-counterpart displays considerable inhibition [Phan et al. 2011]. In our tests the base compound estrone displayed weak inhibition against STS. Estrone derivatives substituted with halogens in ring A, however, exerted substantial inhibition. Our results with 4-chloro, -bromo and -iodo (**BI-347 5/7**, **EM-1049II** and **EM-**

1048III) compounds reveal that C-4 substitution is more advantageous than the halogenation in position C-2. (Certain difference can be observed with the inhibitory potentials estimated by Phan et al. and measured in our experiments. This difference may be ascribed to different substrates used in the two methods. Phan et al. used an artificial substrate (4-methylumbelliferyl sulfate), whereas we applied the natural substrate estrone-3-sulfate. The different binding specificity of these substrates may result in different inhibition results.) It has to be emphasized that the 4-iodoestrone (**EM-1048III**) exerts an outstanding inhibition with submicromolar IC_{50} close to that of the reference EMATE (0.0098 μM).

Compounds in the D-ring modified series displayed decreased STS inhibition compared to their estrone based counterparts, however, 17-deoxy compounds exerted substantial inhibitory potentials in most of the cases. In the 13α-estrone series the *bis*-iodo derivative (**BI-76**) was the most potent inhibitor, whereas in the 17-deoxy series the *bis*-chloro compound (**HK-27II**) exerted the strongest inhibitory effect.

Our results demonstrate that smaller halogen substituents (Cl, Br) on the C-2 usually improve binding affinity of estrone analogs. Enhanced affinity to STS of C-2 halo-derivatives of EMATE and E_2MATE compared to the parent compounds was reported in the literature and it was explained by putative electrostatic or hydrogen bonding interactions to Lys134, Lys368, His136, His290, and His346 residues of the binding pocket in the proximity of the position 2 [Reed et al. 2004]. We may postulate that similar interactions might be involved in the improved binding of 2-chloro- and 2-bromoestrone (**BI-347 II 4/4** and **EM-1049I**) derivatives. Weak binding of their iodo counterpart (**EM-1048V**), however, may be explained with steric reasons.

As reported in the literature, the 3-hydroxyl function of the inhibitor may be involved in hydrogen bonding with certain amino acid residues, with His346 and/or formylGly75 hydrate among others. The electron-withdrawing properties of the introduced ring A substituents may greatly influence the polarisation and the acidity of the 3-hydroxyl group. This substituent effect depends on the position, number and nature of the introduced groups. Certain substituents at the *ortho* positions may additionally be involved in intramolecular hydrogen bonding with the 3-hydroxyl group, which may reduce the affinity of the inhibitor to the enzyme. Phan et al. have not found direct correspondence between the estimated pK_a values (taken from the corresponding *ortho*-substituted phenols) and the inhibitor potentials of their examined compounds [Phan et al. 2011].

Inhibitory data of estrone analogs possessing halogenated A ring offer the option for the investigation of the potential correlation between their predicted pK_a values and the measured inhibitory potentials. pK_a values were calculated using computer software available online [131]. Predicted pK_a values (data are not listed separately) suggest that our monosubstituted derivatives bear protonated 3-hydroxyl function (pK_a > 7.6), whereas *bis* compounds (pK_a= 6.8–7.4) are partially or completely deprotonated under the physiological conditions (pH= 7.3) applied in our experiments. The obtained inhibition potentials do not have apparent direct relationship in either estrone series with the number and electronegativity of the introduced halogens, neither reflect the predicted pK_a data. Differences observed between inhibitory potentials of the two regioisomers indicate that electronic properties of the introduced halogens are not the determining factors in binding interactions of the 3-hydroxyl group [Phan et al. 2011].

4.4.2.1 Mechanistic and kinetic investigations of STS inhibitors

Potent test compounds 4-iodoestrone and 2,4-dichloro-17-deoxy-13α-estrone (**EM-1048III** and **HK-27II**) were selected for mechanistic and kinetic investigations (Fig. 5). Reversibility tests show that they are bound to the enzyme during the preincubation period irreversibly. Time course experiments reveal that the binding is nearly completed within 2.5 min, in a relatively short period compared to the time of incubation.

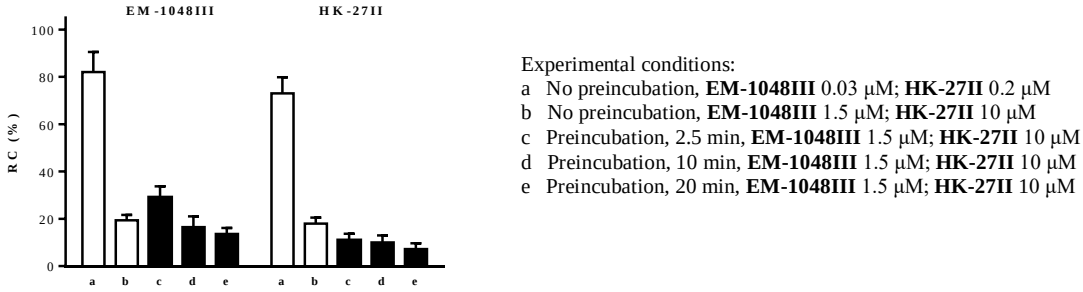


Fig. 5 Investigation of STS inhibition reversibility of selected compounds **EM-1048III** and **HK-27II**. Inhibitor compounds were preincubated with human placental microsomes for 2.5, 10 and 20 min. Following a 50-fold dilution step and 20 min secondary incubation to allow dissociation, the usual enzyme activity measurement was applied. Mean±SD of three separate experiments

In these compounds reactive functional group which could be able to form covalent bindings to the residues of the enzyme protein (as it is the case for EMATE) cannot be found. Inhibitors and the enzyme, therefore, are likely to establish non-covalent adducts, but the interactions can be so tight that the inhibitors are bound essentially irreversibly. The enzyme-inhibitor complex is formed rapidly (compared to the time course of the incubation), indicating that rearrangement or conformational change around

the inhibitor molecule which undergoes slower and often seen with other tight-binding inhibitors are not required in these cases [Szedlacsek & Duggleby 1995].

In kinetic investigations the Dixon's plot of inhibition of the selected compounds provided straight lines at different fixed substrate concentrations intersecting in the second quadrant characterizing the competitive inhibition mechanism (Fig. 6, part B). The replots of slopes vs. 1/substrate concentration resulted in a straight line through the origin which confirms the competitive binding manner [Cortés et al. 2001; Segel 1993].

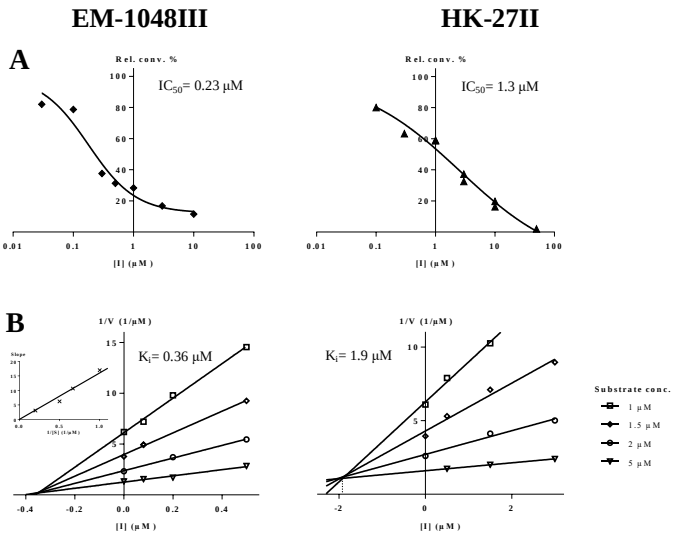


Fig. 6 Concentration-dependent STS inhibition (A) and Dixon's kinetic analysis (B) of selected compounds **EM-1048III** and **HK-27II**. Inset in B/**EM-1048III** shows the secondary plot of slopes of the Dixon's lines vs. 1/substrate concentration.

Phan and co-workers have found that in the case of 4-nitroestrone the binding is non-competitive, indicating that the compound is bound to the enzyme outside of its active site [Phan et al. 2011]. Our test results revealed that 4-iodoestrone and 2,4-*bis*-chloro-17-deoxy-13α-estrone (**EM-1048III** and **HK-27II**) bind to the enzyme in a similar manner, both displaying competitive mechanism which alludes to binding within the active site of the enzyme.

K_i parameters were determined from the intersections of the Dixon's plots, and these values were found to be 0.36 µM for **EM-1048III** and 1.9 µM for **HK-27II**. These measured K_i values reflected inhibitory potentials ranked according to the IC_{50} data.

4.4.3 Inhibition of 17β-HSD1

Estrone compounds halogenated in the ring A proved to be potent 17β-HSD1 inhibitors with the exception of the weak inhibitor 2,4-*bis*-iodo derivative (**BI-341 2/5**). 2-Chloro, -bromo and -iodo (**BI-347 II 4/4**, **EM-1049I** and **EM-1048III**) derivatives were the most

potent inhibitors in this series showing 3–10-fold improved affinity to the 17β-HSD1 than the parent compound and substrate estrone (Table 11.). In the 13α-estrone series, IC_{50} value of the base compound **PZ-1** was found to be 1.2 μM, indicating a considerable, but somewhat weaker affinity to the enzyme compared to that of the natural substrate estrone. 2-Chloro, 2-iodo and 2,4-*bis*-iodo (**BI-323II**, **BI-113AF** and **BI-76**) substitutions improved the inhibitory effect. Inhibitory potential of the parent compound was retained in the case of the 2-bromo and the 4-iodo derivatives (**BI-135** and **BI-113FF**), whereas 4-chloro, -bromo, and *bis*-chloro and -bromo derivatives (**BI-123 7/3**, **BI-116AF**, **BI-123 5/5** and **BI-116FF)** were somewhat weaker inhibitors. 17-deoxy-13α-estrone (**PZ-16**) displayed considerable inhibition against the 17β-HSD1 with IC_{50} value 1.1 μM. Investigated 2-halo derivatives exerted similar inhibition compared to the unsubstituted parent compound, whereas 4-substituted and *bis*-substituted 17-deoxy-13α-estrone compounds proved to be weaker inhibitors.

Moeller et al. investigated the 17β-HSD1 inhibitory potentials of 2-chloro and 2-bromoestrone [Moeller et al. 2009]. Under our experimental conditions inhibition results were obtained in similar IC_{50} interval, nevertheless, we found that 2-chloro and 2-bromo substitutions (**BI-347 II 4/4** and **EM-1049I**) enhance inhibition 3-fold and 7-fold, respectively. 2-Iodoestrone (**EM-1048V**) was not investigated before, and we found that it exerted an outstanding, 10-fold increased inhibitory effect compared to the parent compound. Binding ability of 17β-HSD1 towards D-ring modified estrones was demonstrated earlier with D-homo compounds [Moeller et al. 2009]. We found that 13α- and 17-deoxy-13α-estrone (**PZ-1** and **PZ-16**) were also able to bind 17β-HSD1 with binding affinity close to that of the substrate estrone. Similar binding affinity of 17-deoxy-13α-estrone (**PZ-16**) indicates that C-17 oxo function is not essential for the binding to the 17β-HSD1. These modifications of ring D do not change binding affinity of the basic compounds to the 17β-HSD1, however, modulate the inhibitory effect of the halogenated derivatives considerably. In the 13α-estrone series C-2 substitution seemed to be advantageous, nevertheless, the *bis*-iodo derivative is a potent compound in this group. Chloro-, bromo and iodo substituents in the position C-2 and/or C-4 of the 17-deoxy-13α-estrone compounds did not improve, but decreased inhibitory potentials. 2-Halo compounds remained more efficient than the C-4 regioisomers and the *bis* substituted analogs.

On the basis of the crystal structure of 17β-HSD1 in its complex with 17β-estradiol [Breton et al. 1996], molecular dynamic simulations and ligand–protein docking

studies showed that there is an unoccupied lipophilic tunnel to the exterior of the protein located near the C-2 atom of 17β-estradiol [Moeller et al. 2009]. These results may serve as the basis for the development of potent inhibitors, while the binding affinity of the potential inhibitor might be enhanced by introducing a lipophilic moiety to position 2.
Our results indicate that larger size and/or increasing lipophilicity of the halogens in this series are advantageous for the binding. It is reasonable to postulate that binding is promoted by accommodation of 2-chloro, -bromo and -iodo substituents (**BI-347 II 4/4**, **EM-1049I** and **EM-1048III**) in the lipophilic tunnel of the enzyme protein which is located in the proximity of the position 2 of the steroidal ligands and was previously identified in Moellers' publications [Moeller et al. 2009]. Among C-4 substituted derivatives the bromo and iodo compounds had enhanced binding compared to the parent compound, but it was less pronounced than for C-2 derivatives. Synergistic effect of the C-2 and C-4 halogens were not observed in the case of the *bis*-substituted compounds.

The 3-hydroxyl group of estrone and related inhibitors plays an important role in the binding to 17β-HSD1 forming a hydrogen bonding system with the His221 and Glu282 residues at the recognition end of the active site [Huang et al. 2001; Mazumdar et al. 2009]. Halogen substituents at C-2 and/or C-4 position modify binding abilities of the 3-hydroxyl substituent. The electron-withdrawing effect increases polarization of the O–H bond and may induce deprotonation and increase pK_a values of this group under physiological pH conditions. *Ortho* substituents might also be able to form intramolecular hydrogen bond with the 3-hydroxyl group, which is disadvantageous [Moeller et al. 2009]. Our inhibition results demonstrate that introduction of halogen atoms to C-2 and/or to C-4 position increases affinity to 17β-HSD1 of estrones and in certain 13α-estrone derivatives. However, there does not appear to be a direct relationship between the number and electronegativity of the halogens and the inhibitor potency.

4.4.3.1 Mechanistic and kinetic investigations of 17β-HSD1 inhibitors

Potent test compounds, 13α-estrone and its 4-iodo and 2,4-*bis*-iodo derivatives (**PZ-1**, **BI-113FF** and **BI-76**) were selected for mechanistic and kinetic investigations. Reversibility tests revealed that the inhibitors can be released from binding upon dilution that is they bind to the enzyme in a reversible manner (Fig. 7).

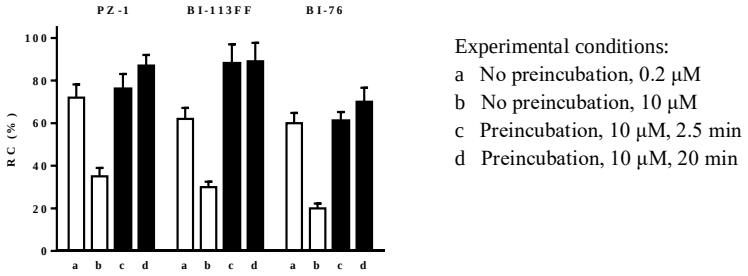


Fig. 7 Investigation of 17β-HSD1 inhibition reversibility of selected 13α-estrone compounds **PZ-1**, **BI-113FF**, **BI-76**. Inhibitor compounds were preincubated with human placental cytosol. Following a 50-fold dilution step, the usual enzyme activity measurement was applied. Mean±SD of three separate experiments

Kinetic experiments were performed for the selected inhibitors at different fixed substrate concentrations in the presence of cofactor excess. On the Dixon's plot, data for each substrate concentration fall on straight lines which intersect in the second quadrant, alluding to competitive inhibition mechanism (Fig. 8, part B) [Cortés et al. 2001; Segel 1993]. Inhibitors that bind to the steroid site of 17β-HSD1 can bind to both the free enzyme and the binary enzyme–cofactor complex by random kinetic mechanism. This mixed-type inhibition, nevertheless, is simplified if the enzyme is saturated with cofactor first and displays competitive patterns [Penning 2011]. Reversible and apparently competitive mechanism of the inhibition observed in our experiments shows that inhibitors 13α-estrone and its 4-iodo and 2,4-*bis*-iodo derivatives (**PZ-1**, **BI-113FF** and **BI-76**) are bound in the substrate-binding cavity of 17β-HSD1 with non-covalent interactions. K_i parameters were determined from the intersections of the Dixon's plots, and they were found to be comparable to those obtained by the IC_{50} values.

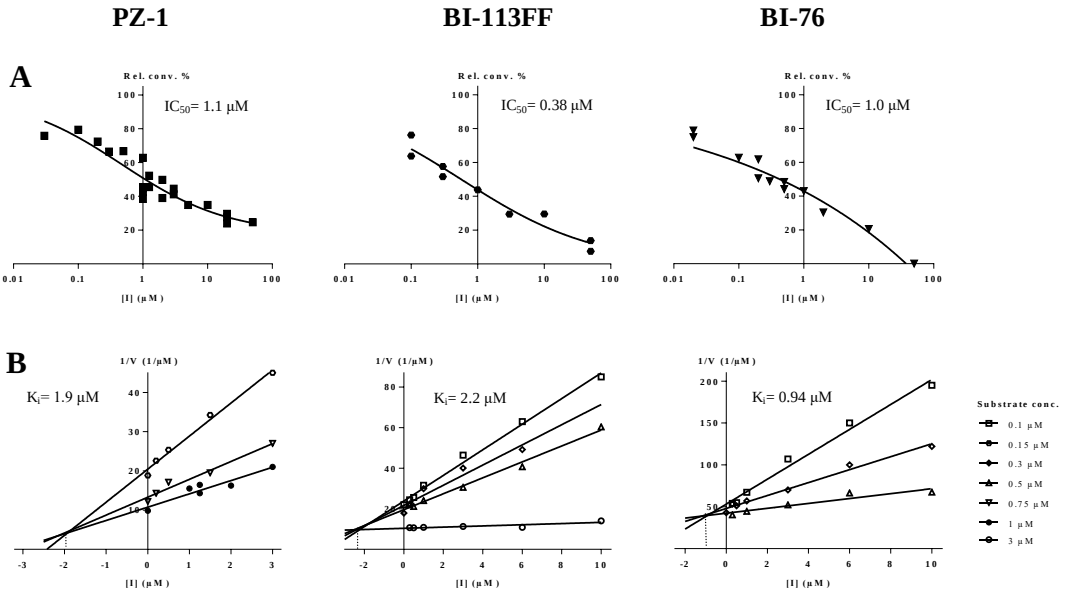


Fig. 8 Concentration-dependent 17β-HSD1 inhibition (A) and Dixon's kinetic analysis (B) of selected 13α-estrone compounds **PZ-1**, **BI-113FF** and **BI-76**.

4.4.4 Conclusions

We identified potent new inhibitors against the key enzymes of estrogen biosynthesis in the series of A-ring halogenated estrone derivatives. 2-Iodoestrone (**EM-1048V**) proved to be a specific and highly potent 17β-HSD1 inhibitor, whereas its 4-iodo counterpart (**EM-1048III**) has dual inhibition against STS and 17β-HSD1. Our results reveal that the 2-bromo derivative (**EM-1049I**) is a potent STS inhibitor and the 4-bromo derivative (**EM-1049II**) is effective against the 17β-HSD1. We confirmed the previously explored 17β-HSD1 inhibition of the 2-bromo (**EM-1049I**) and the STS inhibition of the 4-bromo derivative (**EM-1049II**), therefore, these compounds can be regarded as dual inhibitors.

We found that inverted configuration of C-13 and absence of the 17-oxo function decreases the inhibitory potentials in various degrees against aromatase, STS and 17β-HSD1 of most of the A-ring halogenated compounds, however, their substantial effect indicate that the 13α-estrone (**PZ-1**) may still serve as a promising scaffold of inhibitor development.

Our results obtained for aromatase, STS and 17β-HSD1 inhibitory effect of halogenated estrone analogs revealed that substituens in the position C-2 and/or C-4 and polarization of the phenolic OH in the position C-3 of ring A, as well as structural features of the ring D determine the binding affinity to the enzymes cooperatively. This complex binding mechanism seems to be relevant for the investigated enzymes owing rather different structures and operating distinct catalytic mechanisms. This complex binding

mechanism is also applicable for the inhibitor compounds possessing either substrate- or product-like structures.

Submicromolar IC_{50} parameters of the bromo and iodo substituted estrone analogs against STS and 17β-HSD1, together with their reduced estrogenicity known from literature indicate that these derivatives can be suitable bases for design of drug compounds suppressing estrogen biosynthesis.

Literature references demonstrate that some of the investigated compounds can be readily formed in considerable amount in the environment from excreted estrone. Our results reveal that particularly chloro and bromo derivatives of estrone may have xenobiotic effect on the estrogen biosynthesis of living organisms and the potential cumulative endocrine disrupting effect of the halogenated estrones can be a matter of concern.

4.5 Inhibition of aromatase, 17β-HSD1 and STS with steroidal ferrocenes

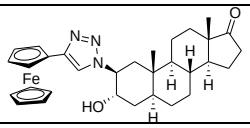
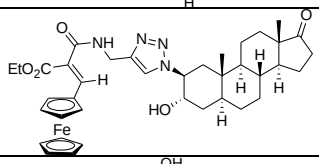
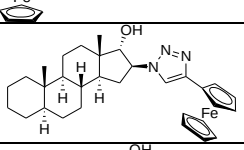
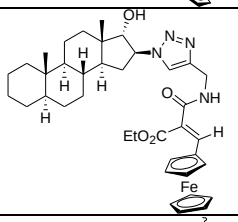
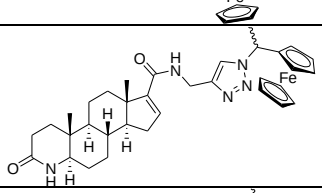
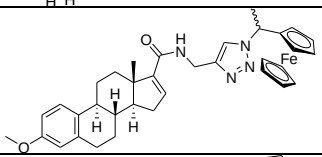
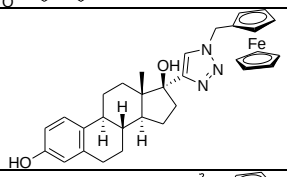
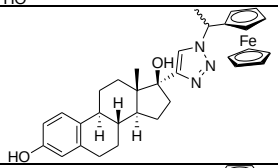
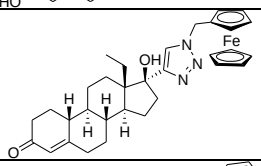
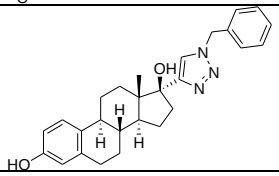
The ferrocene substituent features exceptional biomedical properties and its steroidal conjugates are believed to have a vast potential for medical application [Astruc 2017; Manosroi et al. 2010; Narvaez-Pita et al. 2017; Szánti-Pintér et al. 2015]. Steroidal ferrocenes were aimed mainly to be applied for vectorization of the DNA damaging effect of the ferrocene entity to produce cytotoxic effect in cancerous cells [Hillard et al. 2010]. C-2, C-16 and C-17 ferrocene derivatives of androgens, as well as C-3, C-7, C-16 and C-17 ferrocene derivatives of 17β-estradiol and estrone demonstrated considerable anti-proliferative effects (micromolar IC_{50} or GI_{50} values) against hormone independent cancerous cell lines [Hillard et al. 2010; Manosroi et al. 2010; Top et al. 2009; Vera et al. 2011; Vessières et al. 2006]. Concurrently, hormonal effects exerted on the specific sexual steroid receptors were also investigated. Low affinities to the androgen receptor was detected for the ferrocene derivatives of androgens [Astruc 2017; Vessières et al. 2006]. The C-7 and C-17 ferrocene derivatives of 17β-estradiol displayed estrogen receptor binding and estrogenic effect comparable to that of 17β-estradiol [Osella et al. 2001; Vessières et al. 1998] whereas the C-3 derivative has a predicted estrogen receptor antagonistic effect [Vera et al. 2011]. Potential inhibitory effect of the ferrocene steroid compounds exerted on enzymes of the steroid metabolism have not yet been investigated, to the best of our knowledge.

We investigated the potential inhibitory effect of diverse triazolyl-ferrocene steroids on key enzymes of the estrogen biosynthesis. Compounds were synthesized at the Department of Organic Chemistry, University of Pannonia, Institute of Chemistry, Veszprém, Hungary [IX.].

4.5.1 Aromatase inhibition

In our experiments, substances **KL-86**, **EV-74/6** and **EV-98/2** in the applied 10 μM test concentration suppressed aromatase activity to around 80% of the RC-s (Table 12.). These results mean IC_{50} values exceeding 10 μM, and indicate very weak inhibitory potentials. Other investigated compounds had even weaker effects, resulting in RC-s close to 90% or higher.

Table 12. Most relevant inhibitory potentials of steroidal ferrocenes [IX.]

Comp.	Structure	Inhibition of enzyme activities RC±SD (%), IC_{50}±SD (μM) and K_i±SD (μM)		
		Aromatase	STS	17β-HSD1
KL-105		100±11	41±5 IC_{50}= 4.6±1.5	81±8
KL-86		78±7	90±9	93±2
KL-99		93±6	85±6	94±7
KL-114		89±2	42±3 IC_{50}= 2.4±1.1	91±10
EV-74/6		82±7	87±4	65±13
EV-98/2		82±5	68±5	85±5
E-IL-249/5		89±8	8±2 IC_{50}= 0.084±0.043 K_i= 0.066±0.009	98±10
EV-95/3		117±15	1.0±0.5 IC_{50}= 0.035±0.006 K_i= 0.021±0.005	76±6
E-IL-250		93±5	77±7	89±9
Etöszba		90±5	17±5 IC_{50}= 1.0±0.60	100±7

Several diverse groups of compounds have been found to be potent aromatase inhibitors [Ayub & Level 1988; Chumsri et al. 2011; Numazawa et al. 2005; Yadav et al. 2015]. Among them potent inhibitory effect of 16β-imidazolyl androst-4-ene-3,17-dione derivatives has been reported, too [Bansal et al. 2012]. Aromatase inhibitors were also found among product-like compounds, and 2-chloro- and 6α-phenyl-estrones were the most powerful members of the series investigated [VI.; Numazawa et al. 2005]. Worth to mention that conjugated aromatic systems containing triazole cycle are common structural elements of non-steroidal aromatase inhibitors [Baston & Leroux 2007; Santen et al. 2009].

Various structural elements of referred aromatase inhibitor examples can be recognized in our test compounds. Similarities to known aromatase inhibitors made our compounds reasonable targets for aromatase inhibition tests. Experiments, however, revealed that none of the test compounds exerted substantial inhibitory effect against the human aromatase activity.

4.5.2 17β-HSD1 inhibition

Our test substances **EV-74/6** and **EV-95/3** inhibited 17β-HSD1 activity to RC-s 65% and 76%, respectively (Table 12.). These results showed weak inhibitions even in the relatively high 10 μM test concentration. Other test compounds displayed even weaker effects, RC-s were not decreased below 80% in the experiments where they were applied.

17β-HSD1 is able to bind compounds containing various steroidal cores, and suitable substituents, among them aromatic side chains in the position C-2, C-15, C-16 and C-17 may enhance the binding affinity of these compounds. Estrone and 17β-estradiol derivatives possessing aromatic substituents on C-2 or C-16 [Poirier 2011] and also those bearing triazolyl-aryl side chain on C-15 [Messinger et al. 2008] or on a D-seco structure [III.] were found to be efficient 17β-HSD1 inhibitors.

Several structural features of our steroidal ferrocenes resemble to known 17β-HSD1 inhibitors, nevertheless, our tested compounds did not exert considerable inhibition on the 17β-HSD1 activity under our experimental conditions.

4.5.3 STS inhibition

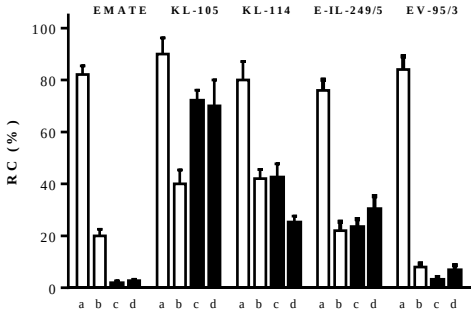
In our experiments, the 3α-hydroxy-5α-androstane-17-one compound derivatized with triazolyl-ferrocene in position 2β (**KL-105**) exerted potent inhibition, the IC_{50} result 4.6 μM (Table 12.) reveals a binding affinity similar to that of the substrate estrone-3-sulfate

(Table 2., IC_{50}= 5.2 μM). Insertion of an ethylacrylate group in the side chain of compound **KL-86** diminished the inhibitory effect almost completely. The 17α-hydroxy-5α-androstane compound with 16β-triazolyl-ferrocene (**KL-99**) had a weak effect, but its counterpart **KL-114** bearing the ethylacrylate elongated triazolyl-ferrocene side chain was a potent inhibitor. IC_{50} value of this compound (2.4 μM) indicates a two-fold affinity compared to the substrate. Substances **EV-74/6** and **EV-98/2** displayed RC results 87% and 68%, respectively. These values indicate that either isomers of the substituted 4-aza-3-oxo-androstene and the 3-methoxy-estratetraene compounds are very weak inhibitors.

The 17α-triazolyl-ferrocene derivative of 17β-estradiol (**E-IL-249/5**) exerted highly potent inhibition. IC_{50} value of this compound was 0.084 μM, indicating a more than 60-fold stronger binding compared to the substrate estrone-3-sulfate. Substance **EV-95/3** (consists of the methyl substituted isomer counterparts) displayed an even more potent inhibition, its apparent IC_{50} value of 0.035 μM is close to that of the reference EMATE (Table 2., IC_{50}= 0.0098 μM). A related ferrocenyl compound **E-IL-250** which possesses 4-ene-3-oxo structure in its ring A and an ethyl group on the C-13 had a very weak effect with RC result of 77%. The analog compound **Etöszba** bearing phenyl group attached to the triazole ring had also potent inhibitory effect showing an IC_{50} result of 1.0 μM.

1.4.3.1 Mechanistic and kinetic investigation of STS inhibitors

Potent test substances (**KL-105**, **KL-114**, **E-IL-249/5** and **EV-95/3**) and the reference competitive irreversible inhibitor EMATE were subjected to mechanistic and kinetic investigations. In reversibility tests performed with **KL-105**, the preincubated and diluted samples resulted in higher conversions (Fig. 9, c and d), similar to the results measured for lower concentration of the test compound (Fig. 9, a). This means that **KL-105** can be released from binding by dilution and this inhibitor binds to the enzyme in a reversible manner. In case of **KL-114**, **E-IL-249/5** and **EV-95/3**, results of the preincubated and diluted samples (Fig. 9, c and d) were similar to that obtained for incubations with higher concentration of the inhibitor (Fig. 9, b). This means that these inhibitors are not released from binding upon dilution and they are bound to the enzyme irreversibly during the preincubation period. Time course experiments reveal that the binding processes are completed within 2.5 min, in a relatively short period when compared to the time of incubation.



Experimental conditions:
a No preincubation, EMATE 0.001 μM; **KL-105**, **KL-114** 0.2 μM; **E-IL-249/5**, **EV-95/3** 0.01 μM
b No preincubation, EMATE 0.05 μM; **KL-105**, **KL-114** 10 μM; **E-IL-249/5**, **EV-95/3** 0.5 μM
c Preincubation, 2.5 min, EMATE 0.05 μM; **KL-105**, **KL-114** 10 μM; **E-IL-249/5**, **EV-95/3** 0.5 μM, secondary incubation 20 min
d Preincubation, 20 min, EMATE 0.05 μM; **KL-105**, **KL-114** 10 μM; **E-IL-249/5**, **EV-95/3** 0.5 μM, secondary incubation 20 min

Fig. 9 Investigation of STS inhibition reversibility of selected compounds. Inhibitor compounds were preincubated with human placental microsomes. Following a 50-fold dilution step, the usual enzyme activity measurement was applied. Mean±SD of three separate experiments

Kinetic investigations were performed with test substances **E-IL-249/5**, **EV-95/3** and reference EMATE. The Dixon's plot provided straight lines at different fixed substrate concentrations intersecting in the second quadrant characterizing the competitive inhibition mechanism (Fig. 10, part B). The replots of slopes vs. 1/substrate concentration resulted in a straight line through the origin (insets in part B of Fig. 10) which confirms the competitive binding manner that means that these inhibitors occupy the substrate binding place of the enzyme [Cortes et al. 2001; Segel 1993]. K_i parameters were found to be 0.066 μM for **E-IL-249/5**, 0.021 μM for **EV-95/3**, and 0.0044 μM for the reference EMATE (Fig. 10, part B). Inhibitory potentials on the basis of these K_i data were comparable to those obtained by the IC_{50} values. Determined K_i values are substantially lower than the K_M parameter of estrone-3-sulfate (8.5 μM), that reflects the significantly higher binding affinities of these inhibitors when compared to the substrate.

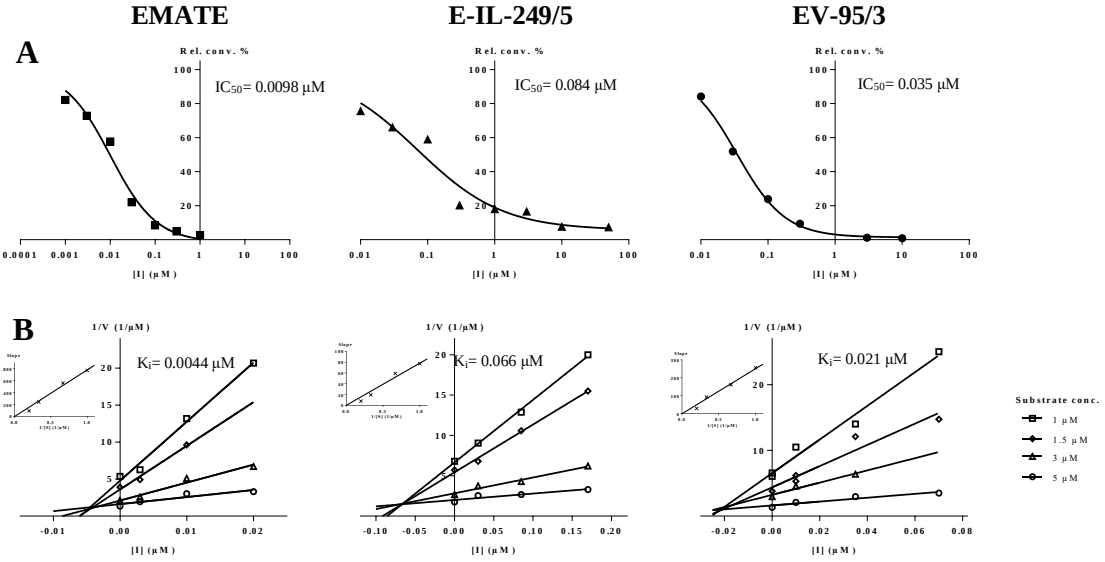


Fig. 10 Concentration dependent STS inhibition (A) and Dixon's kinetic analysis (B) of selected reference EMATE and compounds **E-IL-249/5** and **EV-95/3**. Insets in B show the secondary plot of slopes of the Dixon's lines vs. 1/substrate concentration.

Protein of the STS consists of a globular polar domain and a hydrophobic transmembrane stem domain, resembling to a mushroom in that overall tertiary shape of the enzyme. The substrate binding site situated in a cavity lies at the base of the globular domain, just at the top of the stem domain [Hernandez-Guzman et al. 2003]. STS hydrolyzes various physiological substrates to their unconjugated forms, including 3-sulfate ester of estrone, dehydroepiandrosterone, pregnenolone and cholesterol. Numerous other compounds proved to be capable for binding to the enzyme and demonstrated effective inhibition towards the sulfatase activity [Maltais & Poirier 2011; Nussbaumer & Billich 2004; Shah et al. 2016; Williams 2013]. A large group of these inhibitors are ester derivatives which apply the FGly75 inactivation mechanism by their acid residue. Most of these, either steroidal or non-steroidal compounds, are sulfamates, and the first and still the best known example of these irreversible inhibitors is EMATE [Reed et al. 2004].

Various estrane-, androstane- and pregnane-based compounds exert inhibitory effect towards the STS and their binding affinity to the enzyme can be largely modified by substituents on the steroid skeleton [Maltais & Poirier 2011; Nussbaumer & Billich 2004; Shah et al. 2016; Williams 2013]. Certain halogens or other small electron withdrawing groups (cyano and nitro) in the position 2 and/or 4 of the steroidal ring A may enhance the inhibitory potential [V.; Mostafa et al. 2015; Numazawa et al. 2006; Phan et al. 2011]. Reason of the enhanced binding is not known, nevertheless, existence of cavities able to accommodate these substituents could be postulated. Amino acids which might be involved in putative electrostatic or hydrogen-binding interactions in the proximity of the position 2 (Lys134, Lys368, His136, His290, and His346) and the position 4 (Lys368, Lys134 and Arg79) are also proposed [Mostafa et al. 2015; Phan et al. 2011; Reed et al. 2004].

Literature results have also revealed that appropriate side chains on the ring D might also improve binding affinity of the steroidal inhibitors [Shah et al. 2016]. 17α-benzyl or alkyl derivatives of 17β-estradiol display enhanced inhibitor potential [Fournier & Poirier 2011; Poirier & Boivin 1998]. Hydrophobic substituents on the aromatic cycle might further improve the inhibitory effect [Ciobanu et al. 1999] and relatively rigid 17α-moieties (cyclic substituent attached with only one rotatable bond, a methylene group) were more advantageous in the binding. 16α- And 16β-benzyl substituted compounds exert also augmented inhibition compared to their parent compound 17β-estradiol, although they are much less potent than their 17α counterparts. These findings indicated

non-specific hydrophobic interactions of the D-ring side chains with hydrophobic residues in a long, deep and narrow pocket in the D-ring area [Fournier & Poirier 2011]. Non-competitive or mixed type reversible inhibition of the 17α-benzyl-17β-estradiol suggested the existence of two proximate binding sites involved in the binding of this inhibitor [Ciobanu et al. 1999; Mostafa et al. 2015]. One of them allows the steroidal part to bind, whereas another one, an allosteric binding site, to establish non-specific hydrophobic interactions of the D-ring side chains. The latter is formed by hydrophobic residues in a long and narrow pocket located between the two hydrophobic α-helices and buried in the trans-membrane domain [Fournier & Poirier 2011; Mostafa & Taylor 2012]. Authors Fournier and Poirier postulated that inhibitory potency might be increased by maximizing interactions with both, the substrate and the allosteric binding sites [Fournier and Poirier 2011]. Considering enhanced inhibitor potency of further steroidal compounds bearing C-17 aryl group, a π-π type interaction to the Phe residues present at the entrance of α-helices of the binding site have also been hypothesized [Ciobanu et al. 2001; Shah et al. 2016]. Large affinity difference which was observed between 17β-arylsulfonamide and 17β-arylamide derivatives of estra-1,3,5(10)-trien-3-ol indicated that other, non-hydrophobic interactions of the linker which attach the aryl function might also play a key role in potent inhibition [Mostafa et al. 2015; Mostafa & Taylor, 2012].

STS inhibition data of the investigated steroidal ferrocene compounds reveal that the 17α-triazolyl-ferrocene derivative of 17β-estradiol (**E-IL-249/5**) is a highly potent inhibitor of this enzyme. The submicromolar IC_{50} and K_i parameters enroll this compound to the group of the most effective steroidal STS inhibitors known to date. Inhibitory potential of this compound is similar to that of the optimized 17α-benzyl-17β-estradiol derivatives (IC_{50}= 0.022–0.310 μM) reported by the Poirier's group, among which a bicyclic 4-benzyloxybenzyl derivative was the most effective inhibitor [Nussbaumer & Billich 2004; Poirier & Boivin 1998]. Our test compound **E-IL-249/5** also bears a bicyclic aromatic 17α substituent and both of its triazolyl and ferrocenyl moieties bear a pronounced pharmacophore character.

In order to investigate structural features contributing to the outstanding inhibitory potential of compound **E-IL-249/5** we may compare this finding with the results of the triazolyl-benzene derivative **Etöszba** and their parent compound 17β-estradiol. Both 17α substituted compounds have markedly enhanced effect when compared to 17β-estradiol (IC_{50}= 9.0 μM), and the presence of ferrocenyl moiety instead of a simple phenyl group results in a further 12-fold increase of the inhibition. Ferrocene and phenyl ring has been

used to replace each other mutually, and can be regarded as bioisosteric functional groups in pharmaca. Concerning inhibition towards STS, the ferrocenyl analog exerts a considerably higher potential, indicating a crucial and specific function of this moiety in the binding of the inhibitor to this enzyme.

Substance **EV-95/3** contains methylated derivatives of **E-IL-249/5**. Although substance **EV-95/3** is a mixture of epimers, it displays regular behavior in inhibition experiments. Measured apparent IC_{50} and K_i parameters can reliably reflect the inhibitory effect, and these values indicate further enhanced potential when compared to that of the non-methylated counterpart. Structural aspects cannot be identified precisely, nevertheless, results of substance **EV-95/3** emphasize that further optimization of the binding of 17α-triazolyl-ferrocene derivatives is possible even with minor modification(s) of the linker part between the triazolyl and ferrocenyl structures of the side chain.

In kinetic experiments, **E-IL-249/5** displayed a competitive behavior. It is most likely, that substrate binding cavity is occupied by the 17β-estradiol part of the molecule. Ring A accommodated in the active center of the enzyme and the ring D with the 17α-triazolyl-ferrocene side chain is directed towards the transmembrane domain of the STS. Literature references indicate that C-17 aryl substituents may interact with Phe and other hydrophobic amino acids of the α-helices in this domain [Ciobanu et al. 2001; Fournier and Poirier 1998; Shah et al. 2016]. We may also postulate that the 17α-triazolyl-ferrocene side chain of **E-IL-249/5** can have access to these residues, and the enhanced binding affinity of this compound can be explained by similar mechanisms, namely by π-π type interactions towards Phe residues present at the entrance of α-helices, as well as by non-specific hydrophobic interactions directed to suitable residues buried deeper in the tunnel of the α-helices. Competitive inhibition pattern also suggests that the 17α-triazolyl-ferrocene side chain is long and flexible enough to allow 17β-estradiol part to bind into the substrate binding cavity, which means that these types of derivatives may be able to maximize interactions with both binding sites.

16β-triazolyl-ferrocene-17α-hydroxy-5α-androstane (**KL-99**) has a weak inhibitory effect, whereas **KL-114** which bears an ethylacrylate triazolyl-ferrocene substituent on C-16 proved to be a potent inhibitor. It seems that an elongated ferrocenyl side chain is preferred for the binding of C-16 substituted derivatives. Ferrocenyl moiety attached to a longer and flexible linker may reach the binding pocket which otherwise is situated closer and positioned more appropriately for C-17 substituents, and this might be the reason for a better binding affinity of **KL-114** to the STS.

In the case of the tested two 2β substituted 3α-hydroxy-5α-androstane-17-one compounds, the shorter triazolyl-ferrocene substituent of **KL-105** proved to be advantageous concerning the affinity to the STS. It may be postulated that this triazolyl-ferrocene side chain is capable of interactions with residues of the binding cavity supposed to exist in the proximity of the ligands' position 2 [Mostafa et al. 2015; Reed et al. 2004].

Examples of the potent test compounds (**KL-105**, **KL-114** and **E-IL-249/5**) show that various steroidal backbones may provide suitable core for ferrocenyl derivative STS inhibitors. Comparison of the highly potent 17α-triazolyl-ferrocene derivative of 17β-estradiol (**E-IL-249/5**) and its 4-ene-3-oxo analog (**E-IL-250**), a weak inhibitor, reveal that in other cases the steroidal scaffold has a strong influence on the STS inhibitory potential.

Mechanistic experiments indicate a reversible binding mode for the C-2 substituted triazolyl-ferrocene derivative **KL-105** and an irreversible binding mechanism for the C-16 and C-17 derivatives **KL-114**, **E-IL-249/5** and substance **EV-95/3**. This binding difference approves that different interactions may be involved in the binding of the C-2 triazolyl-ferrocene derivative and the compounds substituted on their ring D. Our inhibitors do not possess arylsulfamate moiety, which is usually the active principle of the irreversible STS inhibitors, neither bear other reactive group capable to form covalent binding to certain enzyme residues. Steroidal ferrocenes and the enzyme, therefore, are likely to establish non-covalent adducts, but the interactions can be so tight in certain cases that the inhibitors will be bound essentially irreversibly.

4.5.4 Conclusions

We investigated *in vitro* inhibitory effects of diverse steroidal ferrocene substances exerted on key enzymes of the estrogen biosynthesis. Numerous structural elements of these derivatives resemble to known potent aromatase and 17β-HSD1 inhibitors, nevertheless, our test compounds were found weakly effective against these enzymes. We observed a potent inhibition against STS by three triazolyl-ferrocene derivatives which display stronger affinities to the enzyme than the substrate estrone-3-sulfate itself. Compounds with triazolyl-ferrocene substituent on C-2 bound in a reversible manner, whereas the C-16 and C-17 ferrocenyl derivatives are irreversible inhibitors. Related non-ferrocenyl compounds were found to exert lower potency, indicating that the ferrocene moiety may have a specific function in the enhanced affinity to the STS.

Based on literature references, interactions of the 16β- and 17α-triazolyl-ferrocenes with Phe residues and with other hydrophobic amino acids of the transmembrane helices can be proposed. Comparison of adequate counterparts revealed a profound influence of the steroidal backbone and of the linker part in the ferrocenyl side chains on the STS inhibition.

Our most potent STS inhibitor, the 17α-triazolyl-ferrocene derivative of 17β-estradiol (**E-IL-249/5**) presents submicromolar IC_{50} and K_i parameters, and therefore, this compound belongs to the group of the most effective STS inhibitors published so far. Our results indicate that optimized combination of structural elements (steroidal core, ferrocenyl side chain, position of the substitution) may further enhance STS inhibitory potential of the steroidal ferrocenes. STS inhibitory potential of the steroidal ferrocenes may lead to the development of novel compounds able to suppress *in situ* 17β-estradiol production in target tissues. Our finding is more valuable considering a presumed synergism between the hormone-independent cytotoxicity and the suppressed estrogen dependent cell proliferation can make this type of compounds particularly promising drug candidates for the pharmacological therapy of hormone dependent gynecological cancers.

5 Summary

The aim of this work was to investigate the inhibitory effect of novel steroid derivatives exerted on $C_{17,20}$-lyase, aromatase, STS and 17β-HSD1, the key enzymes of estrogen biosynthesis.

In the methodological developments we established *in vitro* radiosubstrate incubation methods to measure aromatase, STS and 17β-HSD1 activity and inhibition. We adapted and improved extraction techniques for enzyme products of aromatase and STS and developed a TLC separation for the substrate and product of 17β-HSD1. These isolation techniques provided high recovery, low blank and good reproducibility. Incubation protocols were suitable for rapid testing of numerous compounds. Parameters characterizing substrate affinity values (K_M and IC_{50}) were measured and relative potential of reference inhibitors were determined.

We investigated $C_{17,20}$-lyase inhibitory effect of novel 16α-amino-pregnenolone and 17-triazolyl-androst-5-en-3β-ol compounds. Several potent inhibitors were identified and results revealed that stereoisomerism of the substituent at the C-17 influenced the $C_{17,20}$-lyase inhibitory effect markedly. Results also verified that extension of side chains on position C-16 and C-17 may improve inhibitory potential. We found the parent compound 17β-azido-androst-5-en-3β-ol to be a potent $C_{17,20}$-lyase inhibitor, whereas aromatase (another cytochrome P450 dependent enzyme in the estrogen biosynthesis) was not inhibited by this compound. These new findings indicate that azido group on the steroidal C-17 is a suitable pharmacophore in the inhibition of the $P450_{17\alpha}$ worth for further investigations.

We investigated 17β-HSD1 inhibitory potential of several groups of diverse new steroidal compounds giving particular attention to the cofactor dependence of the presumed inhibitory effect. We identified numerous potent inhibitors and results revealed that the apparent *in vitro* potentials obtained with the cofactors NADPH or NADH may differ substantially. This finding indicates that binding of the phosphorylated or the unphosphorylated cofactors may exert different influence on structural elements of the substrate binding site which are involved in inhibitor binding. Results measured in the presence of NADH are less relevant to the potential *in vivo* effect, therefore these data must be evaluated with caution in inhibitor optimization and in lead selection. Comparative investigations on cofactor dependent inhibition offer a good experimental

tool for mechanistic studies and in combination with theorethical methods may provide a better insight to the mechanisms of inhibitor binding of the 17β-HSD1.

We investigated aromatase, STS and 17β-HSD1 inhibitory effect of A-ring halogenated estrone, 13α-estrone and 17-deoxy-13α-estrone derivatives. We identified numerous potent inhibitors exerting specific or dual inhibitory effect. 2-Iodoestrone was found to be a specific and highly potent 17β-HSD1 inhibitor, whereas its 4-iodo counterpart has dual inhibition against STS and 17β-HSD1. Our results reveal that 2- and 4-bromoestrone can be also regarded as dual inhibitors against STS and 17β-HSD1. The inverted 13α-configuration and absence of the 17-oxo function decreases the inhibitory potentials in various extents against aromatase, STS and 17β-HSD1 of most of the A-ring halogenated compounds, however, their effect still remained substantial. Our results obtained for aromatase, STS and 17β-HSD1 inhibitory effect of halogenated estrone analogs revealed that substituens in the position C-2 and/or C-4 and polarization of the phenolic OH in the position C-3 of ring A, as well as structural features of the ring D determine the binding affinity to the enzymes cooperatively. This complex binding mechanism seems to be relevant for the investigated enzymes owning rather different structures and operating distinct catalytic mechanisms. This complex binding mechanism is also applicable for the inhibitor compounds possessing either substrate- or product-like structures. Submicromolar IC_{50} parameters of these bromo and iodo substituted estrones together with their reduced estrogenicity known from literature indicate that these derivatives can be suitable bases for design of lead compounds in drug development. Our results reveal that cumulative xenobiotic effect of halogenated estrone derivatives exerted on the estrogen biosynthesis and the potential endocrine disrupting effect can be a matter of concern.

We investigated inhibitory effects of diverse steroidal ferrocene substances exerted on aromatase, STS and 17β-HSD1. Test compounds were found weakly effective against aromatase and 17β-HSD1, nevertheless, certain triazolyl-ferrocene derivatives exerted potent inhibitions against STS and displayed higher affinities to this enzyme than the substrate estrone-3-sulfate did. Kinetic and mechanistic investigations revealed that the compounds bearing triazolyl-ferrocene substituent in the C-2 position was bound in a reversible manner, whereas the C-16 and C-17 derivatives were irreversible inhibitors. Experiments demonstrated a key role of the ferrocenyl moiety in the enhanced binding affinity of the inhibitors. Our most potent STS inhibitors, the 17α-triazolyl-ferrocene derivatives of 17β-estradiol belong to the group of the most effective STS inhibitors

published so far. A presumed synergism between the suppressed estrogen dependent cell proliferation evolved by the STS inhibition and hormone-independent cytotoxicity known from the literature can make this type of compounds particularly promising drug candidates for the pharmacological therapy of hormone dependent gynecological cancers.

Investigations of this work identified numerous compounds exerting potent inhibitory effect against the key enzymes involved in the estrogen biosynthesis. Certain inhibitors displaying specific and dual inhibitory effect against $C_{17,20}$-lyase, aromatase, STS and 17β-HSD1, and could be suitable for either local or systemic estrogen deprivation in living organisms. Relations between molecular structure and inhibitory potential were observed. These new achievements may contribute to the development of new drug candidates to be applied for the suppression of estrogen biosynthesis.

6 References

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7 Appendix

I.

Bacsa I, Jójárt R, Schneider G, Wölfling J, Maróti P, **Herman BE**, Szécsi M, Mernyák E: Synthesis of A-ring halogenated 13α-estrone derivatives as potential 17β-HSD1 inhibitors, *Steroids*, **2015**, *104*, 230–236.

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ABSTRACT

13α-Estrone and its 3-methyl or benzyl ether were halogenated in ring A with *N*-bromo- or *N*-iodosuccinimide or 1,3-dibromo-5,5-dimethylhydantoin as electrophile triggers. The chemo- and regioselectivities of the reactions depended greatly on the nature of the substituent on C-3. Bromination of the ethers led to 2- and 4-regioisomers. Bis-halogenation occurred only in the case of the phenolic derivative. Iodination and bromination resulted in similar products, except that the 3-benzyl ether could not be iodinated under the applied conditions. The potential inhibitory action of the new halogenated 13α-estrones on human 17β-hydroxysteroid dehydrogenase 1 activity was investigated via *in vitro* radiosubstrate incubation. Some compounds proved to be effective inhibitors, with IC_{50} values in the submicromolar range.

1. Introduction

Estrogens play an important role in cell proliferation, and their overproduction stimulates the growth of hormone-sensitive cells, leading to hormone-dependent cancers such as breast, ovarian, uterine and endometrial [1,2]. One well-established route through which to prevent this action of estrogens is inhibition of the enzymes involved in the final steps of estrogen biosynthesis. Cytochrome P450 aromatase is responsible for the conversion of nonaromatic androgens to estrone (E1) or 17β-estradiol (E2). The biotransformation of E1 into E2 is catalyzed by 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1). Steroid sulfatase (STS) catalyzes the desulfation of inactive estrone sulfate to yield biologically active E1. The steroidal inhibitors of the above enzymes are mainly built around the estrane nucleus, and they therefore usually exhibit retained estrogenic activity. Substitution at position 2 or 4, D-ring expansion and/or epimerization of C-13 in E1 usually reduce the hormonal activity, but enhance the antitumor properties [3–6]. The presence of relatively small electron-withdrawing groups at position 4 in estrogens generally enhances the potency of estrogen-derived STS inhibitors [7]. Aromatase inhibitors are usually designed on the basis of the preferred substrate androstenedione, but product-like estrone derivatives have also been reported recently as effective competitive inhibitors [8]. The introduction of halogens onto C-2 in E1 proved to result in substantial inhibitory potencies, with IC_{50} values in the low micromolar range [9]. 2-Haloestrones are not only aromatase inhibitors, but can also suppress the conversion of E1 to the more potent E2. Möller et al. described that 2-haloestrones and -D-homoestrones are submicromolar inhibitors of human 17β-HSD1 [10]. Despite numerous efforts to design potent 17β-HSD1 inhibitors, none have yet reached the stage of clinical trials because of their retained estrogenic activity. The availability of a 17β-HSD1 inhibitor without hormonal behavior would be of particular interest.

We describe here the halogenation of ring A in the hormonally inactive 13α-estrone core with different protecting groups at position 3. Bromination or iodination was carried out and the regio- and chemoselectivities of the electrophilic additions were examined. We additionally investigated the potential *in vitro* inhibitory effects of the new halogenated 13α-estrones toward human placental 17β-HSD1.

2. Experimental

Melting points (mp) were determined with a Kofler hot-stage apparatus and are uncorrected. Elemental analyses were performed with a Perkin-Elmer CHN analyzer model 2400. Thin-layer chromatography: silica gel 60 F_{254}; layer thickness 0.2 mm

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(Merck); eluent: 80% diisopropylether/hexane; detection with I_2 or UV (365 nm) after spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid and heating at 100–120 °C for 10 min. Flash chromatography: silica gel 60, 40–63 μm (Merck). ^{1}H NMR spectra were recorded in DMSO-d_6, $CDCl_3$ or C_6D_6 solution with a Bruker DRX-500 instrument at 500 MHz, with Me_4Si as internal standard. ^{13}C NMR spectra were recorded with the same instrument at 125 MHz under the same conditions.

2.1. General procedure for the synthesis of 13α-bromoestrones (**2**–**4**, **9**, **10**, **14**, **15**)

13α-Estrone **1** (135 mg, 0.50 mmol) or its 3-ether **8** or **13** (142 mg or 180 mg, 0.50 mmol) was dissolved in dichloromethane (5 ml) or tetrahydrofuran (13 ml) or DMSO (5 ml), and *N*-bromosuccinimide (NBS; 0.50 mmol or 0.75 mmol) or 1,3-dibromo-5,5-dimethylhydantoin (DDH; 0.25 mmol) was added. The mixture was stirred at rt for 2–3 h, the solvent was then evaporated off, and the crude product **2**–**4** or **9**, **10** or **14**, **15** was subjected to flash chromatography.

2.1.1. 2-Bromo-3-hydroxy-13α-estra-1,3,5(10)-trien-17-one (**2**), 4-bromo-3-hydroxy-13α-estra-1,3,5(10)-trien-17-one (**3**) and 2,4-dibromo-3-hydroxy-13α-estra-1,3,5(10)-trien-17-one (**4**)

2.1.1.1. Reaction in DMSO. As described in Section 2.1, compound **1** (135 mg, 0.50 mmol) was dissolved in DMSO (5 ml), and NBS (89 mg, 0.50 mmol) was added. The mixture was stirred at rt for 3 h. The solvent was then evaporated off, and the crude product was purified by flash chromatography with EtOAc/hexane = 5/95 as eluent. The first-eluted **4** (22 mg, 10%) was obtained as a white solid. Mp 196–197 °C, R_f = 0.64. Anal Calcd. for $C_{18}H_{20}Br_2O_2$: C, 50.49; H, 4.71. Found C, 50.62, H, 4.86%. ^{1}H NMR (DMSO-d_6): δ_H 0.96 (s, 3H, 18-H_3), 2.56 and 2.78 (2 × m, 2 × 1H, 6-H_2), 7.41 (s, 1H, 1-H), 9.50 (s, 1H, OH). ^{13}C NMR (DMSO-d_6): δ_C 20.4, 24.3 (C-18), 27.5, 28.0, 31.2, 31.3, 32.8, 39.5, 40.7, 48.1, 49.2, 108.7 (C-2), 115.0 (C-4), 128.9 (C-1), 134.6 (C-10), 136.6 (C-5), 148.3 (C-3), 220.4 (C-17). Continued elution yielded first a mixture of **3** and **4** (25 mg (12%) and 48 mg (28%), the yields of the compounds in the mixture were determined by ^{1}H NMR spectroscopy), and then compound **3** (40 mg, 23%) as a white solid. Mp 214–216 °C, R_f = 0.55. Anal. Calcd. for $C_{18}H_{21}BrO_2$: C, 61.90; H, 6.06. Found C, 62.08, H, 5.92%. ^{1}H NMR (DMSO-d_6): δ_H 0.96 (s, 3H, 18-H_3), 2.57 and 2.83 (2 × m, 2 × 1H, 6-H_2), 6.74 (d, 1H, J = 8.5 Hz, 2-H), 7.10 (d, 1H, J = 8.5 Hz, 1-H), 9.83 (s, 1H, OH), ^{13}C NMR (DMSO-d_6): δ_C 20.4, 24.4 (C-18), 27.7, 28.1, 31.2, 31.5, 32.8, 39.8, 40.9, 48.2, 49.2, 112.0 (C-4), 113.2 (C-2), 125.4 (C-1), 132.1 (C-10), 136.7 (C-5), 151.7 (C-3), 220.5 (C-17). Finally, the eluted **2** was obtained as a white solid (42 mg, 24%). Mp 222–225 °C, R_f = 0.65. Anal. Calcd. for $C_{18}H_{21}BrO_2$: C, 61.90; H, 6.06. Found C, 61.75, H, 6.22%. ^{1}H NMR (DMSO-d_6): δ_H 0.96 (s, 3H, 18-H_3), 2.66 (m, 2H, 6-H_2), 6.61 (s, 1H, 4-H), 7.27 (s, 1H, 1-H), 9.81 (s, 1H, OH). ^{13}C NMR (DMSO-d_6): δ_C 20.3, 24.4 (C-18), 27.5, 27.9, 29.1, 31.4, 32.8, 39.8, 40.4, 48.3, 49.3, 106.5 (C-2), 115.8 (C-4), 129.8 (C-1), 132.0 (C-10), 137.2 (C-5), 151.4 (C-3), 220.4 (C-17).

2.1.1.2. Reaction in THF. As described in Section 2.1, compound **1** (135 mg, 0.50 mmol) was dissolved in tetrahydrofuran (15 ml) and NBS (114 mg, 0.75 mmol) was added. The mixture was stirred at rt for 3 h. The solvent was then evaporated off, and the crude product was purified by flash chromatography with EtOAc/hexane = 5/95 as eluent. Compound **4** (62 mg, 29%) was eluted first, and continued elution yielded a mixture of **3** and **4** (29 mg (17%) and 38 mg (18%)), and finally compound **3** (53 mg, 30%).

2.1.2. 2-Bromo-3-methoxy-13α-estra-1,3,5(10)-trien-17-one (**9**) and 4-bromo-3-methoxy-13α-estra-1,3,5(10)-trien-17-one (**10**)

As described in Section 2.1, compound **8** (142 mg, 0.50 mmol) was dissolved in dichloromethane (5 ml) and NBS (89 mg, 0.50 mmol) was added. The mixture was stirred at rt for 2 h, the solvent was then evaporated off, and the crude product was purified by flash chromatography with diisopropyl/hexane = 50/50 as eluent. The first-eluted **10** was obtained as a white solid (77 mg, 43%). Mp 193–196 °C, R_f = 0.70. Anal. Calcd. for $C_{19}H_{23}BrO_2$: C, 62.82; H, 6.38. Found C, 62.97, H, 6.25%. ^{1}H NMR ($CDCl_3$): δ_H 1.06 (s, 3H, 18-H_3), 2.69 and 3.00 (2 × m, 2 × 1H, 6-H_2), 3.86 (s, 3H, OCH_3), 6.73 (d, 1H, J = 8.6 Hz, 2-H), 7.21 (d, 1H, J = 8.6 Hz, 1-H). ^{13}C NMR ($CDCl_3$): δ_C 21.1, 25.0 (C-18), 28.4 (2C), 31.6, 32.0, 33.4, 40.7, 41.7, 49.1, 50.0, 56.3 (OCH_3), 109.2 (C-2), 114.4 (C-4), 125.4 (C-1), 134.1 (C-10), 137.8 (C-5), 153.9 (C-3), 221.4 (C-17). Continued elution yielded a mixture of **9** and **10** (67 mg, 37%), and finally **9** as a white solid (24 mg, 13%). Mp 192–194 °C, R_f = 0.53. Anal. Calcd. for $C_{19}H_{23}BrO_2$: C, 62.82; H, 6.38. Found C, 63.05, H, 6.42%. ^{1}H NMR ($CDCl_3$): δ_H 1.05 (s, 3H, 18-H_3), 2.78 (m, 2H, 6-H_2), 3.83 (s, 3H, OCH_3), 6.56 (s, 1H, 4-H), 7.40 (s, 1H, 1-H). ^{13}C NMR ($CDCl_3$): δ_C 20.9, 25.0 (C-18), 28.1, 28.2, 30.1, 31.9, 33.3, 41.2 (2C), 49.1, 50.0, 56.1 (OCH_3), 108.8 (C-2), 112.1 (C-4), 130.7 (C-1), 133.5 (C-10), 137.2 (C-5), 153.6 (C-3), 221.2 (C-17).

2.1.3. 3-Benzyloxy-2-bromo-13α-estra-1,3,5(10)-trien-17-one (**14**) and 3-benzyloxy-4-bromo-13α-estra-1,3,5(10)-trien-17-one (**15**)

2.1.3.1. Reaction with NBS. As described in Section 2.1, compound **13** (180 mg, 0.50 mmol) was dissolved in tetrahydrofuran (15 ml) and NBS (114 mg, 0.75 mmol) was added. The mixture was stirred at rt for 3 h, the solvent was then evaporated off, and the crude product was purified by flash chromatography with EtOAc/hexane = 5/95 as eluent. Compound **14** was obtained as a white solid (200 mg, 91%). Mp 154–155 °C, R_f = 0.60. Anal. Calcd. for $C_{25}H_{27}BrO_2$: C, 68.34; H, 6.19. Found C, 68.47, H, 6.32%. ^{1}H NMR ($CDCl_3$): δ_H ppm 1.05 (s, 3H, 18-H_3), 2.76 (m, 2H, 6-H_2), 5.09 (s, 2H, OCH_2), 6.64 (s, 1H, 4-H), 7.31 (t, 1H, J = 7.3 Hz, 4'-H), 7.37 (t, 2H, J = 7.3 Hz, 3'-H and 5'-H), 7.43 (s, 1H, 1-H), 7.47 (d, 2H, J = 7.3 Hz, 2'-H and 6'-H). ^{13}C NMR ($CDCl_3$): δ_C ppm 21.0, 25.1 (C-18), 28.1, 28.2, 30.1, 32.0, 33.4, 41.2, 41.3, 49.2, 50.1, 70.8 (OCH_2), 109.7 (C-2), 114.0 (C-4), 127.0 (2C: C-2', C-6'), 127.8 (C-4'), 128.5 (2C: C-3', C-5'), 130.8 (C-1), 133.9 (C-10), 136.7 (C-1'), 137.1 (C-5), 152.8 (C-3), 221.2 (C-17).

2.1.3.2. Reaction with DDH. As described in Section 2.1, compound **13** (180 mg, 0.50 mmol) was dissolved in dichloromethane (5 ml) and 1,3-dibromo-5,5-dimethylhydantoin (71 mg, 0.25 mmol) was added. The mixture was stirred at rt for 2 h, the solvent was then evaporated off, and the crude product was purified by flash chromatography with diisopropylether/hexane = 30/70 as eluent. The first-eluted **15** was obtained as a white solid (78 mg, 36%). Mp 192–194 °C, R_f = 0.72. Anal. Calcd. for $C_{25}H_{27}BrO_2$: C, 68.34; H, 6.19. Found C, 68.52, H, 6.15%. ^{1}H NMR ($CDCl_3$): δ_H 1.06 (s, 3H, 18-H_3), 2.69 and 3.02 (2 × m, 2 × 1H, 6-H_2), 5.13 (s, 2H, OCH_2), 6.76 (d, 1H, J = 8.4 Hz, 2-H), 7.17 (d, 1H, J = 8.4 Hz, 1-H), 7.32 (t, 1H, J = 7.3 Hz, 4'-H), 7.38 (t, 2H, J = 7.3 Hz, 3'-H and 5'-H), 7.47 (d, 2H, J = 7.3 Hz, 2'-H and 6'-H). ^{13}C NMR ($CDCl_3$): δ_C 21.0, 25.0 (C-18), 28.4, 28.5, 31.6, 32.0, 33.4, 40.6, 41.7, 49.1, 50.0, 70.9 (OCH_2), 111.1 (C-2), 115.3 (C-4), 125.3 (C-1), 126.9 (2C: C-2', C-6'), 127.7 (C-4'), 128.5 (2C: C-3', C-5'), 134.4 (C-10), 136.8 (C-1'), 137.9 (C-5), 153.0 (C-3), 221.3 (C-17). Continued elution yielded a mixture of **14** and **15** (76 mg, 35%), and finally **14** (46 mg, 21%).

*2.2. General procedure for the synthesis of 13α-iodoestrones (**5**–**7** and **11**–**12**)*

13α-estrone **1** (135 mg, 0.50 mmol) or its 3-ether **8** or **13** (142 mg or 180 mg, 0.50 mmol) was dissolved in trifluoroacetic acid (5 ml) and *N*-iodosuccinimide (NIS; 0.50 mmol or 0.75 mmol) was added. The mixture was stirred at room temperature for 2 h, and then poured onto 100 ml water and extracted with dichloromethane. The organic phase was separated, neutralized with ammonia solution and washed with a saturated solution of sodium thiosulfate and water. The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated. The crude product **5**–**7** or **11**–**12** was subjected to flash chromatography.

*2.2.1. 2-Iodo-3-hydroxy-13α-estra-1,3,5(10)-trien-17-one (**5**) and 4-iodo-3-hydroxy-13α-estra-1,3,5(10)-trien-17-one (**6**)*

As described in Section 2.2, compound **1** (135 mg, 0.50 mmol) was dissolved in trifluoroacetic acid (5 ml) and NIS (114 mg, 0.50 mmol) was added. The crude product was purified by flash chromatography with diisopropyl ether/hexane = 50/50 as eluent. The first-eluted **6** was obtained as a white solid (36 mg, 18%). Mp 209–211 °C, R_f = 0.52. Anal. Calcd. for $C_{18}H_{21}IO_2$: C, 54.56; H, 5.34. Found C, 54.69, H, 5.22%. ^{1}H NMR ($CDCl_3$): δ_H 1.06 (s, 3H, 18-H_3), 2.64 and 2.86 (2 × m, 2 × 1H, 6-H_2), 5.39 (s, 1H, OH), 6.82 (d, 1H, *J* = 8.5 Hz, 2-H), 7.18 (d, 1H, *J* = 8.5 Hz, 1-H). ^{13}C NMR ($CDCl_3$): δ_C 21.0, 24.9 (C-18), 28.5, 29.0, 31.9, 33.4, 37.4, 39.3, 40.8, 41.7, 48.9, 94.6 (C-4), 112.1 (C-2), 127.3 (C-1), 133.7 (C-10), 136.4 (C-5), 152.7 (C-3), 221.5 (C-17). Continued elution yielded a mixture of **5** and **6** (40 mg (20%) and 54 mg (27%)), and finally **5** as a white solid (46 mg, 23%). Mp. 205–208 °C, R_f = 0.36 (80% diisopropyl ether/hexane). Anal. Calcd. for $C_{18}H_{21}IO_2$: C, 54.56; H, 5.34. Found C, 54.72, H, 5.18%. ^{1}H NMR ($CDCl_3$): δ_H 1.05 (s, 3H, 18-H_3), 2.77 (m, 2H, 6-H_2), 5.09 (s, 1H, OH), 6.70 (s, 1H, 4-H), 7.50 (s, 1H, 1-H). ^{13}C NMR ($CDCl_3$): δ_C 21.0, 25.0 (C-18), 28.1, 28.2, 29.8, 31.9, 33.4, 39.2, 41.1, 41.2, 49.2, 81.6 (C-2), 114.6 (C-4), 133.3 (C-10), 135.6 (C-1), 137.9 (C-5), 154.3 (C-3), 221.5 (C-17).

*2.2.2. 2,4-Diiodo-3-hydroxy-13α-estra-1,3,5(10)-trien-17-one (**7**)*

As described in Section 2.2, compound **1** (135 mg, 0.50 mmol) was dissolved in trifluoroacetic acid (5 ml) and NIS (225 mg, 1.00 mmol) was added. The crude product was purified by flash chromatography with diisopropyl ether/hexane = 50/50 as eluent. The first-eluted **7** was obtained as a white solid (98 mg, 37.5%), mp. 198–201 °C, R_f = 0.66. Anal. Calcd. for $C_{18}H_{20}I_2O_2$: C, 41.40; H, 3.86. Found C, 41.62; H, 3.95%. ^{1}H NMR (DMSO-d_6): δ_H 0.96 (s, 3H, 18-H_3), 2.53 and 2.68 (2 × m, 2 × 1H, 6-H_2), 7.60 (s, 1H, 1-H), 9.21 (s, 1H, OH). ^{13}C NMR (DMSO-d_6): δ_C 20.4, 24.3 (C-18), 28.2, 28.3, 31.4, 32.8, 37.4, 39.5, 40.6, 48.1, 49.2, 83.5 (C-2), 95.9 (C-4), 135.8 (C-10), 136.0 (C-1), 140.6 (C-5), 152.9 (C-3), 220.6 (C-17). Continued elution yielded a mixture of **5** + **6** and **7** (93 mg (47%) and 25 mg (9.6%)).

*2.2.3. 2-Iodo-3-methoxy-13α-estra-1,3,5(10)-trien-17-one (**11**) and 4-iodo-3 methoxy-13α-estra-1,3,5-(10)-trien-17-one (**12**)*

As described in Section 2.2, compound **8** (135 mg, 0.50 mmol) was dissolved in trifluoroacetic acid (5 ml) and NIS (114 mg, 0.50 mmol) was added. The crude product was purified by flash chromatography with diisopropyl ether/hexane = 50/50 as eluent. The first-eluted **12** was obtained as a white solid (41 mg, 20%). Mp 151–152 °C, R_f = 0.68. Anal. Calcd. for $C_{19}H_{23}IO_2$: C, 55.62; H, 5.65. Found C, 55.78; H, 5.46%. ^{1}H NMR ($CDCl_3$): δ_H 1.06 (s, 3H, 18-H_3), 2.67 and 2.94 (2 × m, 2 × 1H, 6-H_2), 3.86 (s, 3H, OCH_3), 6.65 (d, 1H, *J* = 8.6 Hz, 2-H), 7.24 (d, 1H, *J* = 8.6 Hz, 1-H), ^{13}C NMR ($CDCl_3$): δ_C 21.1, 25.0 (C-18), 28.5, 29.2, 32.0, 33.4, 37.6, 40.9, 41.8, 49.1, 50.0, 56.5 (OCH_3), 94.6 (C-4), 108.4 (C-2), 126.7 (C-1), 134.2 (C-10), 140.9 (C-5), 156.1 (C-3), 221.4 (C-17). Continued elution yielded a mixture of **11** and **12** (71 mg, 35%), and finally **11** as a white solid (68 mg, 33%). Mp 184–185 °C, R_f = 0.53. Anal. Calcd. for $C_{19}H_{23}IO_2$: C, 55.62; H, 5.65. Found C, 55.81; H, 5.53%. ^{1}H NMR ($CDCl_3$): δ_H 1.05 (s, 3H, 18-H_3), 2.80 (m, 2H, 6-H_2), 3.82 (s, 3H, OCH_3), 6.51 (s, 1H, 4-H), 7.63 (s, 1H, 1-H), ^{13}C NMR ($CDCl_3$): δ_C 21.0, 25.1 (C-18), 28.1, 28.3, 30.3, 32.0, 33.4, 41.2, 41.3, 49.2, 50.1, 56.3 (OCH_3), 82.8 (C-2), 111.1 (C-4), 134.3 (C-10), 137.0 (C-1), 138.4 (C-5), 156.3 (C-3), 221.3 (C-17).

2.3. Determination of the activity of 17β-HSD1 and its inhibition in the human placenta cytosol

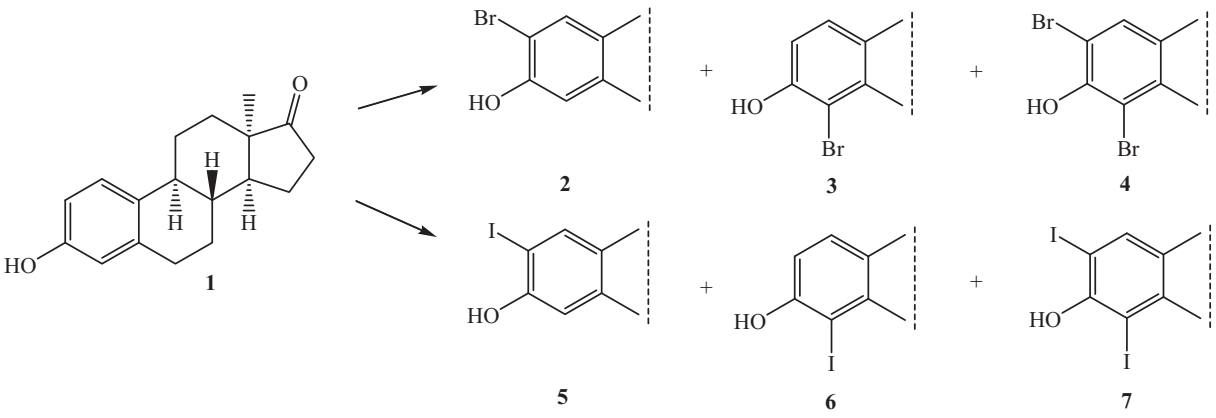
[6,7-^{3}H(N)]Estrone, S.A. = 50 Ci/mmol, was purchased from the American Radiolabeled Chemicals (St. Louis, MO, USA). Nonradioactive E1 and E2 standards, NADPH cofactor, other chemicals and solvents of analytical grade purity were purchased from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland). Kieselgel-G TLC layers (Si 254 F, 0.25 mm thick) were obtained from Merck (Darmstadt, Germany). Human term placenta specimens were collected and used with the ethical approval of the Institutional Human Investigation Review Board.

Inhibitory effects exerted on the activity of 17β-HSD1 by the newly synthesized compounds were investigated via the conversion of E1 to E2 *in vitro*. Human placental cytosol served as the source of the isozyme [11–14]. Human term placenta specimens were combined and homogenized with an Ultra-Turrax in 0.1 M HEPES buffer (pH = 7.3) containing 1 mM EDTA and 1 mM dithiotreitol, and the cytosol was obtained by fractionated centrifugation. The substrate tritium-labeled E1 (1 μM, 250,000 dpm) was added to the incubate in 10 μl of 25 v/v% propylene glycol in HEPES buffer solution, whereas the test compounds were applied in 10 μl of dimethyl sulfoxide solution. (These organic solvent contents in the 200 μl final volume of the HEPES buffer incubation medium did not reduce the enzyme activity substantially.) Cofactor NADPH was used in excess concentration (100 μM). The enzymatic reaction was started by the addition of the cytosol aliquots. Incubation was carried out at 37 °C for 2.5 min and was then stopped by the addition of ethyl acetate and freezing. After extraction, unlabeled carriers of E1 and the product E2 were added to the samples. The two steroids were separated by TLC with the solvent system dichloromethane/diisopropyl ether/ethyl acetate (70:15:15 v/v) and UV spots were used to trace the separated steroids. Spots were cut out and the radioactivity of the E2 formed and of the E1 remaining was measured by means of liquid scintillation counting. Test compounds were applied at 10 μM and their inhibitory effects were expressed by relative conversions, in comparison with the transformation measured in the noninhibited control incubation. Two measurements were performed for each test compound and the mean was calculated. IC_{50} values (the inhibitor concentration which decreases the enzyme activity to 50%) were determined for the most effective test compounds. In these cases, conversions were measured at different concentrations in the interval 0.001–50 μM. IC_{50} results were calculated by using unweighted iterative least-squares logistic curve-fitting by means of the "absolute IC_{50} calculation" function of GraphPad Prism 4.0. The IC_{50} of unlabeled E1 was measured as reference.

3. Results and discussion

3.1. Chemistry

The first electrophilic bromination of E1 to furnish 4-bromo derivatives was described by Slaunwhite et al. who used bromine in aqueous acetic acid and powdered iron as catalyst [15]. Replacement of the aqueous acetic acid by glacial acetic acid led

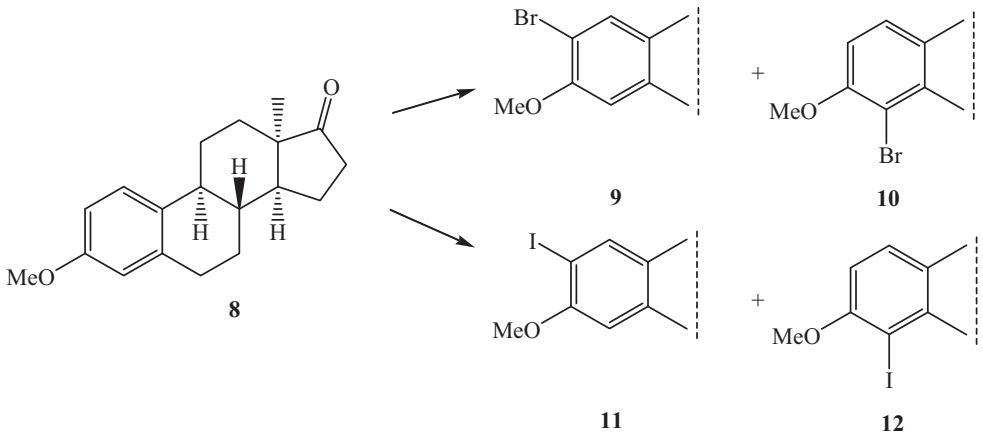


Scheme 1. Bromination and iodination of 13α-estrone (**1**).

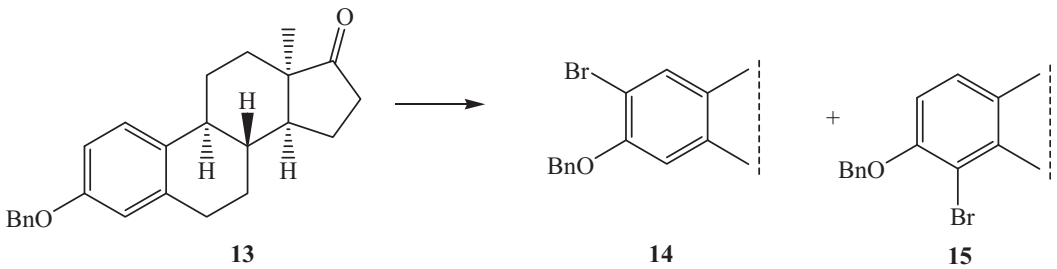
Table 1
Synthesis of halogenated 13α-estrone derivatives (**2**–**7**, **9**–**12**, **14**, **15**).

Entry	Starting compound	Electrophilic reagent	Product	Yield (%)
1	**1**	NBS (THF)	**3** + **4**	47 + 47
2	**1**	NBS (DMSO)	**2** + **3** + **4**	24 + 35 + 38
3	**1**	NIS	**5** + **6**	43 + 45
4	**1**	NIS (2 equiv.)	**5** + **6** + **7**	10 + 38 + 47
5	**8**	NBS	**9** + **10**	23 + 70
6	**8**	NIS	**11** + **12**	47 + 41
7	**13**	NBS	**14**	91
8	**13**	DDH	**14** + **15**	31 + 61

to the 2-bromo compound with high regioselectivity. Chen et al. performed the bromination with NBS in chloroform, and obtained the two monosubstituted bromo regioisomers in 1:1 ratio [16]. Iodination of aromatic compounds is usually carried out with iodine and an activating agent, needed because of the moderate reactivity of iodine [17]. The recently developed direct iodinations are based on iodonium-donating agents, but require high reaction temperatures and long reaction times [18–20]. Page et al. described a direct arene thalliation with thallic trifluoroacetate and subsequent substitution of the estrogen-thallium(III) bis(trifluoroacetate) intermediate with copper(I) halides [21]. This reaction provided 2-halogenated derivatives exclusively, but its major drawback was the toxicity of the reagent. Another powerful aromatic iodination method applies NIS with a catalytic amount of trifluoroacetic acid, which leads in situ to iodine trifluoroacetate, which acts as a very reactive electrophile [22]. We decided to carry out the halogenation of 13α-estrone (**1**) or its 3-ethers (**8**, **13**) with NBS or DDH or NIS as electrophile triggers (Scheme 1, Table 1). Our aim was to obtain more regioisomers with a view to diversifying the drug structure. Halogenations were carried out in different



Scheme 2. Bromination and iodination of 13α-estrone 3-methyl ether (**2**).



Scheme 3. Bromination of 13α-estrone 3-benzyl ether (**3**).

solvents. Bromination of compound **1** with 1 equivalent of reagent in tetrahydrofuran led to a 1:1 mixture of **3** and **4**; the 2-regioisomer (**2**) was not detected (Table 1, entry 1). Compound **2** could be isolated only when the solvent was changed to dimethyl sulfoxide (Table 1, entry 2). Iodination of **1** with 1 equivalent of NIS in trifluoroacetic acid yielded the two regioisomers (**5** and **6**) in a ratio of 1:1 (Table 1, entry 3). Iodination with 2 equivalents reagent led to a mixture of **5**, **6** and **7** (Table 1, entry 4).

Bromination of the methyl ether **8** with 1 equivalent of NBS in dichloromethane led to a mixture of the two regioisomers **9** and **10** (Table 1, entry 5, Scheme 2). The ratio of the products differed from that obtained for the phenolic starting compound; here, the 4-regioisomer (**10**) was the main product. Iodination of compound **8** led to a mixture of the two regioisomers (Table 1, entry 6). Iodination proceeded with the same regioselectivity. Bis-halogenations did not occur in the 3-methyl ether series, which may be ascribed to the difference in the electronic properties of the substituent at position 3.

The 3-benzyl ether (**13**) was first brominated with NBS, which furnished solely the 2-regioisomer (Table 1, entry 7, Scheme 3). With DDH as electrophile trigger, a 1:2 mixture of **14** and **15** was formed (Table 1, entry 8). Surprisingly, the main product was the 4-regioisomer (**15**). The 3-benzyl ether (**13**) could not be iodinated through the use of NIS in trifluoroacetic acid. The bulk of the benzyl function may explain the moderate reactivity of compound **13** in the halogenation reactions.

The structures of the new compounds (**2**–**7**, **9**–**12**, **14**, **15**) were confirmed by ^{1}H and ^{13}C NMR measurements. In the ^{1}H NMR spectra, the number and the multiplicity of the signals in the aromatic region clearly indicated the substitution pattern of ring A. In the ^{13}C NMR spectra, the signals of halogenated C-2 and C-4 appeared at lower chemical shifts than in the spectra of the nonhalogenated derivatives. These downfield shifts were pronounced in the case of the iodo derivatives (**5**–**7**, **11**, **12**).

3.2. Inhibition of 17β-HSD1

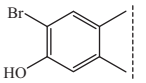
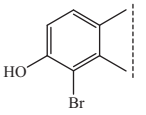
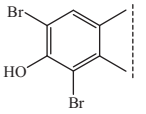
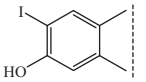
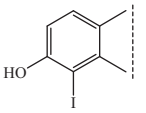
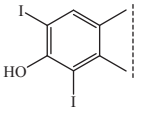
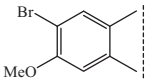
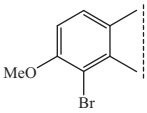
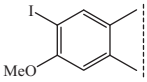
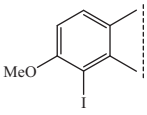
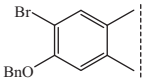
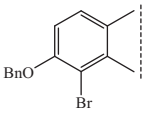
The crystal structure of 17β-HSD1 in its complex with E2 has been reported [23]. On the basis of this structure, Möller et al. performed molecular dynamic simulations and ligand–protein docking studies, focusing on position 2 of estrane core, which is located in a lipophilic environment consisting of the side-chains of Val143, Met147, Phe259, Leu262 and Met279 [10]. There is a lipophilic tunnel to the exterior of the protein, which may bind a lipophilic residue at position 2 of the potential inhibitor. This encouraged Möller et al. to introduce halogen substituents onto C-2. 2-Chloro- and 2-bromoestrone proved to be potent inhibitors, with IC_{50} values in the submicromolar range. Additional substitution on C-16, leading to 2-chloro-16β-fluoroestrone, significantly improved the inhibitory potential, resulting in an IC_{50} of 35 nM. The potential role of the fluorine introduced near the 17-keto function was to protect it from metabolic degradation.

The above literature results led us to perform aromatic halogenations on ring A in 13α-estrone (**1**) or its methyl (**8**) or benzyl ether (**13**) in order to obtain potential 17β-HSD1 inhibitors on a hormonally inactive 13-epimeric estrone core. We were interested in the effects of different substituents at position 3 on the inhibitory potential. We recently published a series of D-secoestrone 3-methyl and 3-benzyl ethers, where some compounds efficiently inhibited 17β-HSD1, even though they did not possess a 3-OH function [14]. We performed halogenations at positions 2 and/ or 4 to diversify the structure and determined the ability of differently halogenated 13α-estrones to suppress the E1–E2 conversion.

In the 3-OH series, all the synthesized halogenated derivatives (**2**–**7**) proved to be effective inhibitors, but their IC_{50}-s depended on the number, the nature and the position of the halogens introduced (Table 2). The iodo derivatives (**5**–**7**) displayed a more substantial inhibitory potential than that of their bromo counterparts (**2**–**4**). The 2,4-bis-iodo compound (**7**) inhibited the E1–E2 conversion most effectively, with a submicromolar IC_{50} of 0.38 μM. The test compounds gave rise to concentration-dependent decreases in the enzyme activity (examples of experimental data are presented in Fig. 1).

In the 3-OMe series, exclusively the 4-substituted derivatives (**10**, **12**) inhibited the enzyme efficiently, irrespectively of the

Table 2
Inhibition of human placental 17β-HSD1. Relative conversions measured in the presence of the compounds tested at 10 μM. Mean of two measurements; control incubation with no inhibition is taken as 100%. SD: standard deviation.

Comp.	Structure	Relative conversion ± SD (%) or IC_{50} ± SD (μM)
3-OH derivatives		
2		IC_{50} = 1.2 ± 0.36
3		IC_{50} = 2.1 ± 1.2
4		IC_{50} = 2.7 ± 0.3
5		IC_{50} = 0.59 ± 0.23
6		IC_{50} = 1.0 ± 0.3
7		IC_{50} = 0.38 ± 0.08
3-OMe derivatives		
9		79 ± 2
10		IC_{50} = 0.37 ± 0.08
11		92 ± 2
12		IC_{50} = 0.56 ± 0.17
3-OBn derivatives		
14		64 ± 10
15		98 ± 1
Reference		
E1		IC_{50} = 0.63 ± 0.11

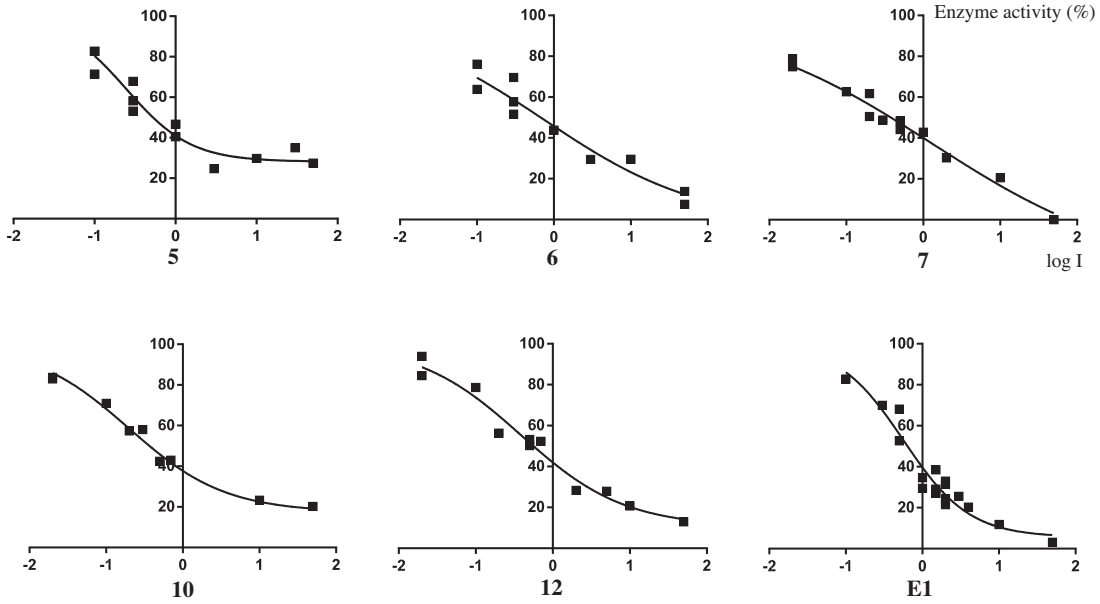


Fig. 1. Concentration-dependent inhibitory effects of the test compounds (**5**–**7**, **10**, **12**) on the *in vitro* activity of 17β-HSD1. I: inhibitor concentration in μM.

nature of the halogen. The IC_{50}-s of these regioisomers (**10**, **12**) are commensurate with that obtained for the best 3-OH inhibitor (**7**). The 3-OBn derivatives (**14**, **15**) did not decrease the relative conversion to below 50% at a test concentration of 10 μM.

When all the results on the halogenated 13α-estrone derivatives are taken into consideration, it can be stated that the 17β-HSD1 inhibitory potential of the compounds depends markedly on the nature and the size of the protecting group on the phenolic OH function. The presence of H or Me is advantageous, but the more bulky Bn results in a loss of inhibitory potential. Consequently, in the case of the 3-OH derivatives it was the nature of the halogen, whereas in the 3-OMe series it was the position of the introduced bromine or iodine substituent that was the determining factor.

4. Conclusions

13α-Estrones with substantial 17β-HSD1 inhibitory potential are promising scaffolds for the design of estrone-based anticancer agents lacking hormonal activity. To the best of our knowledge, such an effective 13-epimeric E1 derivative as 17β-HSD1 inhibitor has not been published previously. Since the presented halogen derivatives were designed on the basis of the literature analogy of similarly halogenated 13β-estrones as nanomolar inhibitors of 17β-HSD1, further investigations with 13α-estrones may well be interesting and may lead to selective, nonestrogenic inhibitors appropriate for clinical trials.

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II.

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RESERACH ARTICLE

Synthesis and *in vitro* pharmacological evaluation of *N*-[(1-benzyl-1,2,3-triazol-4-yl)methyl]-carboxamides on D-secoestrone scaffolds

Johanna Szabó[1], Ildikó Bacsa[1], János Wölfling[1], Gyula Schneider[1], István Zupkó[2], Mónika Varga[3], Bianka E. Herman[4], László Kalmár[5], Mihály Szécsi[4], and Erzsébet Mernyák[1]

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Abstract

An efficient synthesis of several *N*-[(1-benzyl-1,2,3-triazol-4-yl)methyl]carboxamides in the 13β- and 13α-D-secoestrone series is reported. Novel triazoles were synthesized via the Cu(I)-catalyzed azide–alkyne cycloaddition of steroidal alkynyl carboxamides and *p*-substituted benzyl azides. Each of the products was evaluated *in vitro* by means of MTT assays for antiproliferative activity against a panel of human adherent cancer cell lines (HeLa, MCF-7, A431 and A2780). Some of them exhibited activities similar to those of the reference agent cisplatin. On change of the substitution pattern of the benzyl group of the azide, great differences in the cell growth-inhibitory properties were observed. The *p*-alkylbenzyl-substituted triazoles selectively exerted high cytostatic action against A2780 cells, with IC_{50} values of 1 μM. We investigated the potential inhibitory action exerted on the human 17β-HSD1 activity of the new secosteroids. Three triazoles effectively suppressed the estrone to 17β-estradiol conversion with IC_{50} values in low micromolar range.

Keywords

Antiproliferative effect, azide–alkyne cycloaddition, A2780, 17β-HSD1 inhibition, steroid

History

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Introduction

The development of anticancer drugs based on naturally occurring biologically active substances is one of the most important objectives of modern medicinal chemistry[1–3]. Structural modifications of steroidal hormones may lead to compounds with diverse biological activities[4–6]. The challenge of such drug design is the discovery of lead compounds with selective biological potency: when the new action is not followed by the original hormonal activity. The literature reveals that the homologization or opening of ring D or inversion of the configuration at C-13 of the estrane skeleton may lead to the complete loss of estrogenic activity[7–9]. We recently reported that 3-benzyloxy-D-secoestrone alcohols and oximes display high *in vitro* antiproliferative potential against a number of cancer cell lines, with IC_{50} values in the low micromolar or submicromolar range[10,11]. The most potent compounds were found to induce apoptosis via the enhancement of tubulin polymerization or to cause disturbance in a cell cycle. Furthermore, 16-oximes of 13α-estrone proved to inhibit the proliferation of particular cancer cell lines effectively, with high selectivity for cancer cell lines[12]. As D-seco- and 13α-estrones have been reported to possess no estrogenic activity, but to exert growth-inhibitory action, we chose these compounds as candidates for the design of potentially cytostatic derivatives with selective biological activity. To diversify the structure, starting compounds bearing different protecting groups at C-3 were used. D-Secoestrone carboxylic acids are readily available in both the 13α- and the 13β-estrone series[10,13–15]. We therefore set out to build additional structural elements, such as a triazole moiety, onto the carboxylic acid scaffolds, in order to obtain compounds with enhanced antiproliferative properties[10]. The triazole structure is a good mimic of peptide bonding with improved metabolic and proteolytic stability[16]; moreover, there is literature evidence of steroidal triazoles with moderate or enhanced antiproliferative behaviour[17–21]. Introduction of a terminal alkyne function onto the carboxyl group was planned by using propargylamine and peptide coupling reagents, followed by the formation of triazoles from the alkynes by using differently substituted benzyl azides as dipoles. The resulting *N*-[(1-benzyl-1,2,3-triazol-4-yl)methyl]carboxamide structural moiety is completely or partially present in a number of literature drug candidate examples: in small molecules and in triterpenoids with diverse biological activities, such as antitumour or antiviral[22–24]. Certain carboxamido- or triazolyl-derivatives of estrone (C15 or C16 substituted) are able to inhibit the 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) isozyme[5,25,26] and to block the estrone to 17β-estradiol conversion. This inhibition suppresses both the gonadal and the peripheral 17β-estradiol synthesis and the hormone ablation might be applied for the treatment of estrogen-dependent diseases such as endometriosis, as well as breast and ovarian cancers.

In view of these promising literature evidences, we planned to determine the *in vitro* antiproliferative activities of the newly synthesized secoestrones by means of MTT assays against a panel of human adherent cancer cell lines (HeLa, MCF-7,

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A431 and A2780). We also planned to study a potential inhibition of the human 17β-HSD1 enzyme.

Experimental

Chemistry

Melting points (mp) were determined with a Kofler hot-stage apparatus and are uncorrected. Elemental analyses were performed with a Perkin-Elmer CHN analyzer model 2400. Thin-layer chromatography: silica gel 60 F_{254}; layer thickness 0.2 mm (Merck, Darmstadt, Germany); detection with iodine or UV (365 nm) after spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid and heating at 100–120 °C for 10 min. Flash chromatography: silica gel 60, 40–63 μm (Merck, Darmstadt, Germany). ^{1}H NMR spectra were recorded in $CDCl_3$ solution (if not otherwise stated) with a Bruker DRX-500 instrument at 500 MHz, with Me_4Si as internal standard. ^{13}C NMR spectra were recorded with the same instrument at 125 MHz under the same conditions. Full scan mass spectra of the compounds were acquired in the range 50 to 800 *m/z* with an Agilent 500MS Ion trap mass spectrometer equipped with an electrospray ionization source.

*General procedure for the preparation of N-(prop-2-yn-1-yl) carboxamides **5–8***

Compounds **1** or **3** (314 mg, 1.00 mmol) or **2** or **4**[15] (390 mg, 1.00 mmol) were dissolved in *N*,*N*-dimethylformamide (DMF, 5 ml), and 1-hydroxybenzotriazole (HOBt, 148.6 mg, 1.1 mmol), *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 417.2 mg, 1.1 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 0.17 ml, 1.00 mmol) were added. After 15 minutes of stirring, propargylamine (0.06 ml, 1.00 mmol) in DMF (1 ml) was added. The mixture was stirred at room temperature for 2 h, and then diluted with water and extracted with CH_2Cl_2. The combined organic phases were washed with water until neutral and dried over Na_2SO_4. The crude products **5–8** were subjected to flash chromatography with CH_2Cl_2 as eluent.

*3-Methyloxy-14β-(prop-2-en-yl)-*N*-(prop-2-yn-1-yl)-des-D-estra-1,3,5(10)-trien-13α-carboxamide **5***. Compound **1** (314 mg, 1.00 mmol). Yield: 274 mg (78%), oil, $R_f = 0.40$ (dichloromethane). ^{1}H NMR (500 MHz, $CDCl_3$): δ [ppm] = 1.18(s, 3H, 18-H_3); 2.24(s, 1H, C≡CH); 2.83(m, 2H, 6-H_2); 3.78(s, 3H, OCH_3); 4.06(m, 2H, HN–CH_2); 4.97(m, 2H, 16 a-H_2); 5.85(m, 1H, 16-H); 5.91(m, 1H, NH); 6.63(s, 1H, $J = 2.2$ Hz, 4-H); 6.71(dd, 1H, $J = 8.6$ Hz, $J = 2.2$ Hz, 2-H); 7.18(d, 1H, $J = 8.6$ Hz, 1-H). ^{13}C NMR δ [ppm] = 15.2(C-18); 25.9; 27.2; 29.5; 30.2; 35.3; 37.9; 40.9; 42.9; 45.7; 47.3, 55.2(OCH_3); 71.6(C≡CH); 79.8(C≡CH); 111.7(C-2); 113.5(C-4); 115.0(C-16a); 126.3(C-1); 132.2(C-10); 137.8(C-5); 138.9(C-16); 157.5(C-3); 177.9(C-17). MS *m/z* (%): 352 (MH^+, 100), 236 (38). Anal. Calcd. for $C_{23}H_{29}NO_2$: C, 78.59; H, 8.32. Found: C, 78.67;H, 8.45%.

Analytical data on the described propargyl derivatives **5–8** are presented in supporting information (SI).

*General procedure for the synthesis of triazoles **10–13***

To a stirred solution of the appropriate terminal alkyne (**5–8**, 1.00 mmol) in toluene (5 ml), benzyl azide (**9**, 1.00 mmol), triphenylphosphine (Ph_3P) (52 mg, 0.2 mmol), copper(I) iodide (CuI) (19 mg, 0.1 mmol) and DIPEA (0.52 ml, 3.00 mmol) were added. The reaction mixture was refluxed for 2 h, the solvent was evaporated off, and the residue was subjected to flash chromatography over silica gel with 50% ethyl acetate/50% CH_2Cl_2 as eluent.

*3-Methyloxy-*N*-[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]-14β-(prop-2-en-yl)-des-D-estra-1,3,5(10)-trien-13α-carboxamide **10a***. Compound **5** (351 mg, 1.00 mmol). Yield: 464 mg (96%), mp 61–63 °C, $R_f = 0.44$ (50% ethyl acetate/50% dichloromethane). ^{1}H NMR (500 MHz, DMSO-d_6): δ [ppm] = 1.05(s, 3H, 18-H_3); 2.74(m, 2H, 6-H_2); 3.68(s, 3H, OCH_3); 4.29(m, 2H, HN–CH_2); 4.81(m, 2H, 16a-H_2); 5.55(s, 2H, N-CH_2); 5.71(m, 1H, 16-H); 6.59(d, 1H, $J = 2.2$ Hz, 4-H); 6.67(dd, 1H, $J = 8.6$ Hz, $J = 2.2$ Hz, 2-H); 7.16(d, 1H, $J = 8.6$ Hz, 1-H); 7.29–7.36 overlapping multiplets: 2″-H, 3″-H, 4″-H, 5″-H, 6″-H; 7.84(m, 1H, C = CH); 8.12(m, 1H, NH). ^{13}C NMR δ [ppm] = 15.2(C-18); 25.8; 26.9; 29.6; 34.6; 34.7; 36.9; 40.8; 42.5; 45.0; 46.7; 52.6; 54.8(OCH_3); 111.6(C-2); 113.0(C-4); 114.3(C-16a); 122.8(C = CH); 126.3(C-1); 127.8(2C: C-2″, C-6″); 128.0(C-4″); 128.7(2C, C-3″, C-5″); 131.8(C-10); 136.1(C-1″); 137.3(C-5); 139.5(C-16); 145.8(C = CH); 157.0(C-3); 177.4(C-17). MS *m/z* (%): 485 (MH^+, 100), 486 (36). Anal. Calcd. for $C_{30}H_{36}N_4O_2$: C, 74.35; H, 7.49. Found: C, 74.51; H, 7.63%.

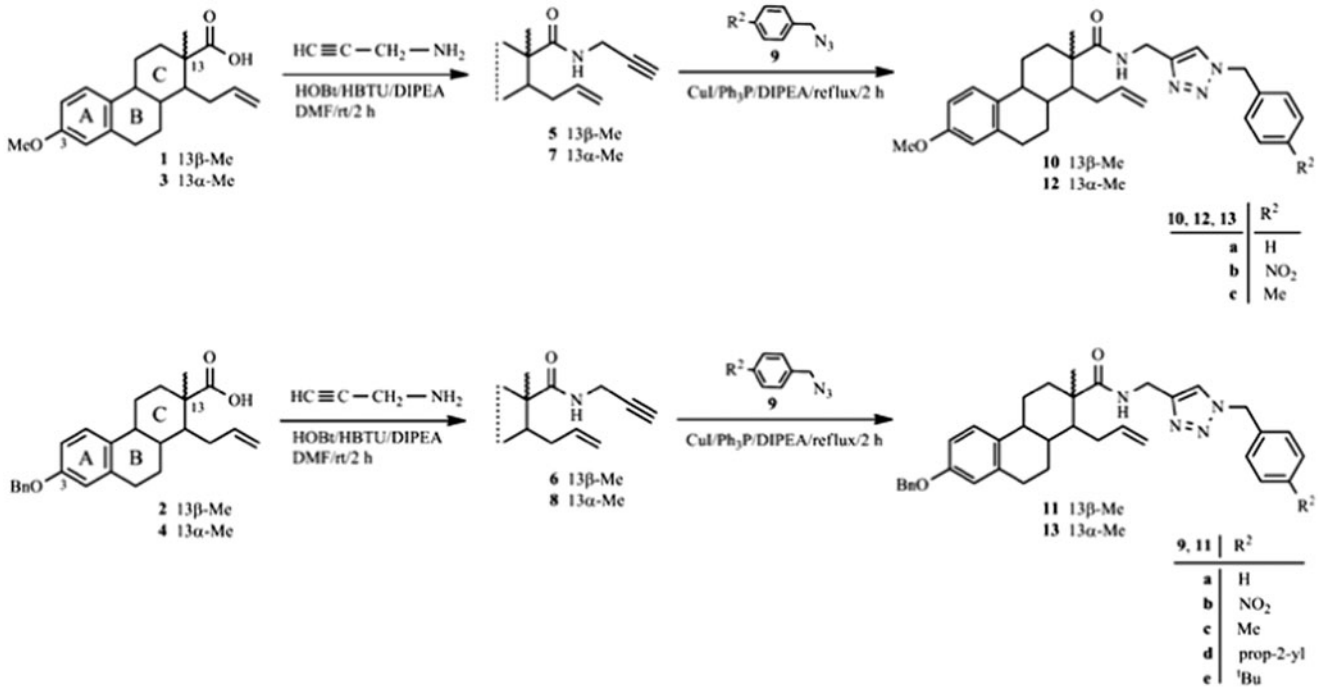
Analytical data on the described triazolyl compounds **10–13** are presented in supporting information (SI).

MTT assay for determination of antiproliferative activity

The growth-inhibitory effects of the test compounds were determined on HeLa, MCF-7, A2780 and A431 cells isolated from cervical, breast, ovarian and skin cancer, respectively[27]. These cell lines were obtained from the European Collection of Cell Cultures, Salisbury, UK. The cells were cultivated in minimal essential medium (Sigma-Aldrich, Budapest, Hungary) supplemented with 10% fetal bovine serum, 1% non-essential amino acids and an antibiotic-antimycotic mixture. All media and supplements were obtained from Life Technologies (Paisley, Scotland, UK). All cell types were seeded into 96-well plates at a density of 5000 cells/well and incubated with two concentrations (10 and 30 μM) of the compounds at 37 °C under cell culturing conditions. After a 72-h incubation, cells were treated with 5.0 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution for 4 h. The precipitated formazan crystals were dissolved in dimethyl sulfoxide and the absorbance was read at 545 nm with a microplate reader. Wells with untreated cells were utilized as controls. For the selected molecules the assay was repeated with a set of dilutions and sigmoidal concentration–response curves were fitted to the measured data, and IC_{50} values were calculated by means of GraphPad Prism 4 (GraphPad Software, San Diego, CA). Cisplatin was used as a positive control in same concentrations as the test compounds.

Determination of 17β-HSD1 activity and its inhibition in the human placenta cytosol

Radioactive [6,7-^{3}H(N)]estrone, S. A. = 50 Ci/mmol was purchased from the American Radiolabeled Chemicals (St. Louis, MO). Non-radioactive estrone and 17β-estradiol standards, NADH coenzyme, other chemicals and solvents with purity of analytical grade were purchased from Sigma (St. Louis, MO) and Fluka (Buchs, Switzerland). Kieselgel-G TLC layers (Si 254 F, 0.25 mm thick) were obtained from Merck (Darmstadt, Germany). Human term placenta specimens were collected and used under ethical approval of the institutional Human Investigation Review Board. Inhibitory effects exerted on the 17β-HSD1 activity by the newly synthesized compounds **5–8**, and **10–13** were investigated via conversion of estrone to 17β-estradiol *in vitro*. Our previously published radioincubation methods for 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (Δ^5-3β-HSD)[28,29] and the



Scheme 1. Synthesis of *N*-(prop-2-yn-1-yl)carboxamides **5–8** and the related triazoles **10–13**.

17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2)[30] were modified and adapted to the 17β-HSD1 measurements. Human placental cytosol served as feasible source of the isozyme[31–33]. Human term placenta specimens were combined and homogenized with an Ultra-Turrax in 0.1 M HEPES buffer (pH = 7.3) containing 1 mM EDTA and 1 mM dithiotreitol and the cytosol were obtained with fractionated centrifugation. The substrate was added to the incubate in 10 µl of a 25 v/v% propylene glycol in HEPES buffer solution, whereas test compounds were applied in 10 µl of dimethyl sulfoxide solution. (These organic solvent contents of 200 µl final volume of the HEPES buffer incubation medium did not reduce the enzyme activity substantially.) The incubates contained estrone substrate in 1µM final concentration with 250 000 dpm activity of tritium labelled tracer, and the coenzyme NADH was used in an excess 100 µM concentration. Enzymatic reaction was started by the addition of the cytosol aliquots. Incubation was carried out at 37 °C for 2.5 min and then was stopped by the addition of ethyl acetate and freezing. After extraction, unlabelled carriers of estrone and the product 17β-estradiol were added to the samples. The two steroids were separated by TLC with the solvent system dichloromethane/diisopropyl ether/ethyl acetate (70:15:15 v/v) and UV spots were used to trace the separated steroids. Spots were cut out and the radioactivity of the 17β-estradiol formed and of the estrone remaining was measured by means of liquid scintillation counting. Control incubates without test substances were prepared in every series. In these non-inhibited incubations 7–9% of estrone was transformed, therefore the substrate concentration could be accepted constant during the enzyme reaction. Test compounds were applied at 10 µM and their inhibitory effects were expressed by relative conversions, in comparison to the transformation measured in non-inhibited control incubation. IC_{50} values were determined for the most effective test compounds. In these cases, conversions were measured at six-eight different concentrations between 0.1–10 µM. IC_{50} results were calculated using an unweighted iterative leastsquares method for four-parameter logistic curve fitting. IC_{50} of unlabelled estrone was measured as reference.

Results and discussion

Chemistry

Two epimeric pairs of steroidal carboxylic acids **1–4** were chosen as starting materials for the introduction of a terminal alkyne function. The starting δ-alkenyl carboxylic acids **1–4** were obtained from the appropriate δ-alkenyl aldehydes via Jones oxidation, as described earlier[15]. The coupling reactions were carried out by using propargylamine and peptide coupling reagents: HOBt and HBTU (Scheme 1) and proceeded in a chemoselective manner, yielding the desired carboxamides **5–8** in high yields, without the formation of side-products. The resulting *N*-(prop-2-yn-1-yl)carboxamides **5–8** were transformed into triazoles **10–13** with benzyl azides (**9**) under ''click'' reaction conditions (using CuI as catalyst, triphenylphosphane as stabilizing ligand, DIPEA as base and toluene as solvent). Benzyl azides **9a–e** were prepared from benzyl bromides as described in the literature[34–37]. The structures of the triazoles were diversified by using benzyl azides containing different *p*-substituents, with the aim of determining the dependence of the biological activity on the structure of the triazoles. All the cycloadditions proceeded with full conversion of the starting compounds, no side-products were formed and the nature of the *p*-substituent of the benzyl ring did not influence the efficiency or the rate of the reactions. The structures of the newly synthesized compounds **5–8** and **10–13** were established from ^1H and ^{13}C NMR measurements.

Determination of antiproliferative properties of the newly synthesized compounds

Compounds **5–8** and **10–13** were evaluated *in vitro* by means of MTT assays for antiproliferative activity against HeLa (cervical carcinoma), A2780 (ovarian carcinoma), A431 (skin epidermoid carcinoma) and MCF-7 (breast adenocarcinoma) cells. Compounds **5–8** did not display substantial antiproliferative effects against the tested tumour cell lines (Table 1). The inhibitions of proliferation were generally under 50% at both applied concentrations, the 3-benzyl ether derivatives **6** and **8**

Table 1. Antiproliferative properties of the synthetized compounds **5–8** and **10–13**.

	Inhibition of proliferation (%) ± SEM							
	HeLa		A431		A2780		MCF-7	
	10 μM	30 μM	10 μM	30 μM	10 μM	30 μM	10 μM	30 μM
5	15.5 ± 2.4	40.7 ± 1.6	12.3 ± 1.8	19.4 ± 0.9	16.0 ± 2.5	41.2 ± 1.9	12.7 ± 2.7	18.0 ± 2.7
6	26.7 ± 2.5	28.7 ± 32	19.6 ± 1.0	67.7 ± 1.5	42.4 ± 1.7	49.5 ± 0.3	21.7 ± 0.7	40.3 ± 1.4
7	<10*	15.4 ± 1.9	<10	10.3 ± 3.4	<10	12.9 ± 3.0	<10	<10
8	42.6 ± 1.8	61.5 ± 1.4	14.4 ± 1.9	28.4 ± 1.2	25.3 ± 1.9	48.7 ± 1.4	11.0 ± 1.3	33.3 ± 0.5
10a	36.1 ± 1.3	48.2 ± 1.9	15.1 ± 1.6	45.4 ± 1.3	29.9 ± 0.8	52.1 ± 1.0	24.8 ± 2.1	48.2 ± 1.1
10b	26.8 ± 2.8	36.9 ± 1.1	18.3 ± 2.2	34.6 ± 1.1	<10	41.9 ± 1.6	10.8 ± 2.3	35.0 ± 0.8
10c	23.7 ± 1.3	32.1 ± 1.9	<10	57.9 ± 0.6	29.1 ± 1.5	92.7 ± 0.5	11.3 ± 1.6	90.4 ± 0.6
11a†	27.9 ± 1.5	31.3 ± 1.4	13.9 ± 1.2	24.4 ± 1.8	79.8 ± 0.5	88.6 ± 0.1	44.6 ± 0.4	72.3 ± 0.4
11b	32.1 ± 1.9	29.6 ± 2.1	14.9 ± 0.4	13.0 ± 0.7	75.9 ± 0.4	78.3 ± 0.1	52.5 ± 0.6	56.4 ± 0.2
11c‡	19.3 ± 1.1	26.5 ± 1.1	15.5 ± 1.6	17.4 ± 1.0	80.6 ± 0.4	81.3 ± 0.5	51.7 ± 1.9	63.7 ± 0.8
11d	52.0 ± 1.2	61.0 ± 0.7	15.2 ± 2.1	<10	72.3 ± 0.4	95.1 ± 0.2	53.9 ± 1.5	72.3 ± 0.3
11e	52.0 ± 0.7	54.1 ± 1.2	11.9 ± 3.2	13.3 ± 2.3	80.4 ± 0.3	94.3 ± 0.3	63.2 ± 1.6	72.6 ± 0.5
12a	37.7 ± 2.1	51.7 ± 2.2	22.3 ± 2.3	32.5 ± 2.1	25.9 ± 1.7	33.5 ± 1.5	17.0 ± 1.8	34.8 ± 2.2
12b	24.5 ± 5.6	34.3 ± 3.3	25.3 ± 2.0	29.3 ± 1.2	35.8 ± 1.9	60.1 ± 1.2	20.1 ± 3.2	68.1 ± 0.4
12c	37.2 ± 1.5	38.0 ± 2.0	12.3 ± 2.2	55.7 ± 1.1	23.6 ± 2.1	64.9 ± 0.3	25.0 ± 0.5	90.0 ± 0.6
13a	35.7 ± 0.9	44.9 ± 2.2	<10	45.1 ± 5.4	<10	47.7 ± 3.3	<10	47.9 ± 1.5
13b	38.6 ± 1.6	48.9 ± 1.8	<10	20.6 ± 0.9	<10	36.6 ± 1.5	13.3 ± 0.9	30.1 ± 1.4
13c	43.0 ± 1.6	58.5 ± 1.3	<10	<10	22.2 ± 0.3	35.9 ± 4.4	23.5 ± 2.0	41.8 ± 0.7
CP‖	42.6 ± 2.3	99.9 ± 0.3	88.6 ± 0.5	90.2 ± 1.8	83.6 ± 1.2	95.0 ± 0.3	53.0 ± 2.3	86.9 ± 1.2

*Inhibition values <10% are not detailed.
†Experimentally determined IC_{50}§ value on A2780: 1.1 μM.
‡Experimentally determined IC_{50} value on A2780: 0.8 μM.
§Mean values from two independent determinations with five parallel wells; standard deviation <15%, reference agent: cisplatin (IC_{50} value on A2780: 0.8 μM).
‖Cisplatin (reference compound).

Table 2. *In vitro* inhibition of human placenta cytosol 17β-HSD1 activity by test compounds **5–8** and **10–13**.

Compound	Relative conversion (%) ± S.D. ($n = 3$)	IC_{50} (μM) ± S.D.
5	68 ± 13	
6	73 ± 23	
7	75 ± 12	
8	78 ± 10	
10a	53 ± 16	
10b	25 ± 2	1.3 ± 0.4
10c	42 ± 11	4.5 ± 0.8
11a	60 ± 9	
11b	60 ± 9	
11c	72 ± 12	
11d	86 ± 12	
11e	64 ± 8	
12a	87 ± 10	
12b	89 ± 9	
12c	80 ± 15	
13a	34 ± 5	3.6 ± 0.4
13b	71 ± 17	
13c	78 ± 6	
Estrone (reference)		1.8 ± 0.2

Relative conversions (control incubation with no inhibition is 100%) measured in the presence of 10 μM concentration of the compound tested. S.D.: standard deviation.

proving to be slightly more potent than their 3-methyl ether counterparts **5**, **7**. Compound **6** at 30 μM exerted a close to 70% growth-inhibitory effect against A431 cells, but was inactive on HeLa, the proliferation of which was effectively inhibited by the epimeric counterpart (**8**). The inhibitory potencies of the triazoles **10–13**, however, were greatly affected by the structural differences between the heterocyclic products. As concerns the 3-methyl ether derivatives **10** and **12**, the determining moiety is the *p*-substituent on the *N*-benzyl ring. The *p*-methyl compound in both the 13α- and 13β-series **12c** and **10c** displayed the highest activity, 90% inhibition of the MCF-7 and A2780 cell lines being attained at 30 μM. The difference in growth-inhibitory behaviour depended on the orientation of the angular methyl group and was most marked in the case of the 3-benzyloxytriazoles **11** and **13**. The results of the MTT assays of the 13α derivatives **13** revealed their substantially lower activities than those of the 13β compounds **11**, and only **13c** at 30 μM exhibited >50% inhibition on HeLa cells. In contrast, the three 13β-triazoles **11a–c** selectively displayed high activity against A2780 cells. In view of the latter promising results, two additional *p*-alkyl-*N*-benzyl-substituted derivatives **11d**,**e** were synthesized in order to examine whether the alkyl group facilitates the biological action. The MTT results revealed improved growth-inhibitory effects for **11d** and **11e** (95% inhibition at 30 μM on A2780 cells).

Inhibition of 17β-HSD1

Argenta et al. assayed high activity of 17β-HSD1 in a series of ovarian epithelial carcinomas[38] and they concluded that 17β-HSD1-specific inhibitors may have role in the therapy of ovarian cancer. Reed et al. reported that the 17β-HSD1 mRNA is expressed in MCF-7 and T47D breast cancer cell lines, and there is high association between mRNA expression and enzyme activity[39]. The literature reveals that selective inhibition of 17β-HSD1 reduces estrogen responsive cell growth of the mentioned cell lines[40]. Since our present results of the MTT assays demonstrated remarkable activities for the potent compounds on ovarian cancer (A2780) and breast cancer (MCF-7) cell lines, herein we investigated potential *in vitro* inhibitory effect of the new secoestrone derivatives **5–8** and **10–13** towards the human placental 17β-HSD1. Compounds **10b**,**c** and **13a** exerted considerable inhibitions, IC_{50} values were found 1.3, 4.5 and 3.6 μM, respectively (Table 2). Inhibition potential of the *p*-nitrophenyl-derivative **10b** is similar to the unlabelled estrone, for which

1.8 μM reference IC_{50} was determined. Other compounds displayed weak inhibitions, relative conversions were decreased to 53–89% at the 10 μM test concentration.

Conclusion

Novel antiproliferative triazolyl D-secoestrone derivatives were synthesized. Introduction of the *N*-(prop-2-yn-1-yl)carboxamide structural element led to compounds **5–8** with moderate cytostatic behaviour, but through the ''clicking'' of benzyl azides to the terminal alkyne function, the antiproliferative action was intensified. Among the 3-methoxytriazoles **10** and **12**, irrespectively of the orientation of the angular methyl group, the substitution pattern on the *N*-benzyl ring substantially influenced the antiproliferative behaviour: the presence of the *p*-alkyl group appeared to be advantageous. In contrast, in the 3-benzyl ether series **11** and **13**, the configuration of C-13 was the determining moiety: the 13β derivatives **11** exhibited much higher potency than that of their epimer counterparts **13**, and displayed selective growth-inhibitory action against A2780 cells. It can be concluded that the combination of the latter structural components in the D-secoestrone series, i.e. the 3-benzyl protecting group, the triazolyl function and the 13β-methyl group, enhances the cytostatic potential. 17β-HSD1 inhibition tests performed i*n vitro* with human placenta cytosol revealed that **10b**,**c** and **13a** effectively suppress estrone–estradiol conversion catalyzed by this isozyme. 17β-HSD1 inhibitory potential of D-secoestrone derivatives is a novel finding.

Although there is no correlation between the effects of the compounds on enzyme activity and on cell proliferation, secoestrones with high 17β-HSD1 inhibitory potential are promising scaffolds for the design of estrone-based antitumor agents with no estrogenic activity. To the best of our knowledge, no 17β-HSD1 inhibitor has entered the clinical trial step, because of the retained estrogenicity of the candidate compounds. Further investigations with secoestrones can be interesting and may provide valuable inhibitor structure-activity data.

Declaration of interest

The authors report no declarations of interest. The authors are grateful for financial support from the Hungarian Scientific Research Fund (OTKA K101659 and K109293).

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Supplementary material available online

III.

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RESEARCH ARTICLE

Comparative investigation of the *in vitro* inhibitory potencies of 13-epimeric estrones and D-secoestrones towards 17β-hydroxysteroid dehydrogenase type 1

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Abstract

The inhibitory effects of 13-epimeric estrones, D-secooxime and D-secoalcohol estrone compounds on human placental 17β-hydroxysteroid dehydrogenase type 1 isozyme (17β-HSD1) were investigated. The transformation of estrone to 17β-estradiol was studied by an *in vitro* radiosubstrate incubation method. 13α-Estrone inhibited the enzyme activity effectively with an IC_{50} value of 1.2 μM, which indicates that enzyme affinity is similar to that of the natural estrone substrate. The 13β derivatives and the compounds bearing a 3-hydroxy group generally exerted stronger inhibition than the 13α and 3-ether counterparts. The 3-hydroxy-13β-D-secoalcohol and the 3-hydroxy-13α-D-secooxime displayed an outstanding cofactor dependence, i.e. more efficient inhibition in the presence of NADH than NADPH. The 3-hydroxy-13β-D-secooxime has an IC_{50} value of 0.070 μM and is one of the most effective 17β-HSD1 inhibitors reported to date in the literature.

Keywords

13α-estrone, 17β-HSD1 inhibition, D-secoestrone, NADH, NADPH

History

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Introduction

The human 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1, EC 1.1.1.62) protein is comprised of 328 amino acids and exists as a cytosolic functional homodimer with a subunit molecular mass of 34 950 Da[1,2]. Amino acid sequence alignments and homology studies have revealed that it belongs to the short-chain dehydrogenase/reductase (SDR) superfamily. 17β-HSD1 is a pluripotent enzyme in terms of substrate, cofactor, and the oxidative and reductive direction of the 17β-hydroxy-17-oxo interconversion. This isozyme is capable of the 3β-hydroxy reduction of substrates bound in reverse mode[3].

Under *in vivo* conditions, as in living cells, however, the isoenzyme functions unidirectionally[4–6] and predominantly catalyze the NADPH-promoted stereospecific reduction of estrone (**1a**) to 17β-estradiol (E2) (Scheme 1), the final hormone-activating process in estrogen biosynthesis[5,7]. The highest expression and activity of the isozyme may be observed in the female steroidogenic reproductive tissues, such as the ovaries and the placenta[8]. This isozyme makes a major contribution to the general gonadal supply and to the circulating level of E2 in the blood. 17β-HSD1 is also expressed and active in peripheral tissues, where it regulates the intracellular accumulation of E2 and consequently the intracrine estrogen effect[3,9]. 17β-HSD1 has been reported to be responsible for the intracellular overproduction of E2 in various neoplasms. The pathophysiological accumulation of E2 then contributes to the development and progression of estrogen-dependent forms of endometriosis, breast cancer and ovarian cancer. The inhibition of 17β-HSD1 with suitable pharmacons may suppress both the systemic and the local or *in situ* synthesis of E2. The evoked pre-receptorial anti-hormonal effect offers a suitable option for the therapy of estrogen-dependent diseases. 17β-HSD1 inhibitors may serve as interesting drug targets of anti-estrogen therapy[2,10].

Numerous earlier studies have demonstrated that various estrone and 17β-estradiol derivatives inhibit 17β-HSD1 activity effectively[2,9,11,12]. Inhibitor design based on the estrane core is nonetheless limited, because they must be devoid of estrogenic activity[13–17].

Certain structural modifications of the estrane skeleton, such as the opening of ring D or inversion of the configuration at C-13, may lead to the complete loss of hormonal activity[18–20]. We recently described the synthesis and *in vitro* investigation of the 17β-HSD1-inhibitory activities of C-13 epimeric 17-(triazolylmethyl)carboxamido D-secoestrone derivatives bearing ether protecting groups on C-3[21]. The nature of the functional groups on C-3 and/or C-17 and the orientation of the angular methyl group influence the enzyme inhibitory potential substantially. Certain 3-methoxy-13β-D-secoestrones and one 3-benzyloxy-13α derivative displayed low micromolar 17β-HSD1 inhibitory

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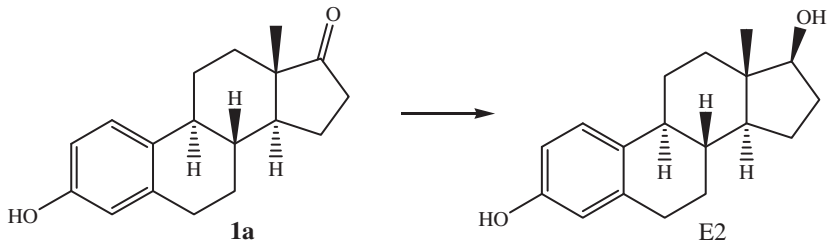
Scheme 1. Stereospecific reduction of estrone (**1a**) to 17β-estradiol (E2) by 17β-HSD1.



potentials. This biological activity of D-secoestrones was a novel finding and alludes to the design of hormonally inactive 17β-HSD1 inhibitors on these scaffolds in both the 13α- and 13β-estrone series.

We recently described the halogenation of ring A of 13α-estrone with different protecting groups at position 3[22]. The halogen derivatives were designed on the basis of the literature analogy of similarly halogenated 13β-estrones as nanomolar inhibitors of 17β-HSD1. The inhibitory potential of the 13α-compounds depended markedly on the nature and the size of the protecting group on the phenolic OH function. The presence of H or methyl was advantageous relative to the more bulky benzyl group. Effective 13α-estrones such as these 17β-HSD1 inhibitors had not been published previously.

We now report an investigation of the inhibitory potentials of C-13 epimeric 3-OH and 3-ether estrone derivatives bearing an intact or seco ring D towards human placental 17β-HSD1. *In vitro* inhibition tests were performed with both the cofactors NADPH and NADH that are regularly applied in these assays.

Materials and methods

Chemistry

Compounds **1a** and **1c** were purchased from Sigma (St. Louis, MO) and **1b** from Steraloids (Newport, RI). 13α-Derivatives (**2b** and **2c**) were obtained by the epimerization of **1b** or **1c** using the literature methods[18],S3. 13α-Estrone (**1a**) was obtained by debenzylation of **1b**[S3]. The experimental details for the chemical synthesis and data on the compounds (**3–6**) are presented in the Supplemental Information.

Determination of 17β-HSD1 activity and its inhibition in the human placenta cytosol

Radioactive [6,7-3H(N)]estrone, S.A. = 50 Ci/mmol, was purchased from American Radiolabeled Chemicals (St. Louis, MO). Non-radioactive estrone (**1a**) and E2 standards, NADH and NADPH cofactors, other chemicals and solvents of analytical grade purity were purchased from Sigma (St. Louis, MO) or Fluka (Buchs, Switzerland). Kieselgel-G TLC layers (Si 254 F, 0.25 mm thick) were from Merck (Darmstadt, Germany). Human term placenta specimens were collected and used with the ethical approval of the Institutional Human Investigation Review Board.

The inhibitory effects of the newly synthesized compounds on the 17β-HSD1 activity were investigated via the conversion of **1a** to E2 *in vitro*. Human placental cytosol served as a source for the isozyme[21,23]. Human term placenta specimens were combined and homogenized with an Ultra-Turrax in 0.1 M HEPES buffer (pH = 7.3) containing 1 mM EDTA and 1 mM dithiotreitol and the cytosol was obtained by fractionated centrifugation. Substrate **1a** (1 μM) with its tritiated tracer (250 000 dpm) was added to the incubator in 10 μL of 25 v/v% propylene glycol in HEPES buffer solution, whereas the test compounds were applied in 10 μL of dimethyl sulfoxide solution. (These organic solvent contents in the 200 μL final volume of the HEPES buffer incubation medium did not reduce the enzyme activity substantially.) The cofactor, either NADH or NADPH, was used in an excess concentration of 100 μM. The enzymatic reaction was started by the addition of the cytosol aliquots. Incubation was carried out at 37 °C for 2.5 min and was then stopped by the addition of ethyl acetate and freezing. After extraction with ethyl acetate, unlabelled carriers of **1a** and the product E2 were added to the samples. The two steroids were separated by TLC with the solvent system dichloromethane/diisopropyl ether/ethyl acetate (70:15:15 v/v) and UV spots were used to trace the separated steroids. Spots were cut out and the radioactivity of the E2 formed and the **1a** remaining was measured by means of liquid scintillation counting. 17β-HSD1 activity was calculated from the radioactivity of the E2 with correction for the recovery.

The assays were performed in triplicate for determination of the percentages of relative inhibited conversions at a final inhibitor concentration of 10 μM, and the standard deviations (SDs) were also calculated. IC_{50} values (the inhibitor concentration that decreases the enzyme activity to 50%) were determined for the most effective and other selected test compounds. In these cases, conversions were measured at 10–15 different concentrations in the appropriate interval 0.001–50 μM. IC_{50} results were calculated by using unweighted iterative least squares logistic curve fitting by means of the ''absolute IC_{50} calculation'' function of the GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). The IC_{50} of unlabelled estrone (**1a**) was measured as reference. The relative inhibitory potentials (RIPs) of the test compounds were calculated by using reference IC_{50} data measured with the corresponding cofactor: RIP = IC_{50} of test compound/IC_{50} of unlabelled estrone (**1a**).

With the selected incubation parameters, the enzyme reaction satisfied the conditions of the initial velocity measurements. The conversions in the non-inhibited control incubates reached similar rates (10–13%) with both cofactors, and the product formation was proportional to the enzyme concentration and the incubation duration. The 1 μM substrate was a saturation concentration in the presence of NADPH, whereas it was on the declining proportional phase with NADH (data not shown).

Results and discussion

17β-HSD1 inhibition

The steroid ligand binding site of 17β-HSD1[24,25] has been described as a hydrophobic tunnel with polar residues at each end. The surface of the tunnel is complementary to the C_{18} steroidal scaffold and ensures selectivity towards estrogenic substrates[26]. At the C-terminal recognition end, hydrophilic amino acids form hydrogen-bonds to the 3-hydroxy group of the substrate[27,28]. These interactions fix the substrate and its C-17 oxo into an appropriate orientation for the catalytic transformation[7,24,29], but they have been found not to be essential for the binding, and they may even establish a catalytically unfavourable position for non-cognate substrates[30]. Residues of the N-terminal catalytic end form triangular hydrogen-bond contacts with the C-17 carbonyl

oxygen and facilitate a charge-equalizing proton transfer following the hydride donation from the nicotinamide moiety of the cofactor[6,24,31].

NADPH and NADH bind in the same extended conformation to 17β-HSD1, pointing towards the active site with their nicotinamide ring[27,30], and both cofactors are able to promote the stereospecific reduction of the C-17 carbonyl of the substrate. Despite these resemblances, NADPH and NADH are not interchangeable as cofactors of 17β-HSD1. Their different interactions and the different ground and transition state structures[32] suggest that different modes of binding exist for the phosphorylated and the unphosphorylated cofactors. Binding differences induce different conformational changes in the cofactor binding cleft, which extends towards the catalytic cleft of the active centre in its close proximity[33].

Estrane-based inhibitors are assumed to occupy the substrate-binding site of 17β-HSD1, and are able to form other contacts to the enzyme than substrate molecules[2,9,12]. These interactions may improve the binding affinity and modulate the inhibitory potential[34]. The complexities of the interaction mechanisms of 17β-HSD1 have the result that relatively small changes in the shape of the steroid substrate or inhibitor ligand, and/or in the protein conformation induced by the cofactor or by other modulators can significantly affect the binding and catalytic arrangements, and consequently the binding affinity, the inhibitor potential and the selectivity.

Natural estrone possesses a tetracyclic steroidal framework with *trans* junctions of rings B/C and C/D. The other characteristics of this classical steroid are the typical conformations of rings C (chair) and D (strongly restricted). The rigid structure of estrone contains two oxygen functionalities with well-defined distances, which are crucial in the binding of estrone or estradiol to its nuclear hormone receptors. In contrast with the natural 13β compound, the 13 epimer has a quasi-equatorial angular methyl group, a *cis* junction of rings C/D and a ring D that is directed to the β side[18]. Poirier et al. reported the impact of inversion of the configuration at C-13 and/or C-17 of estradiols on their estrogenic activity[20]. They concluded that 13 epimers have low relative binding affinity for estrogen receptor alpha and have no significant uterotropic activity. Accordingly, inversion at C-13 in the estrane skeleton could be a correct strategy in the design of estrone-based anticancer agents lacking estrogenic activity.

In this work, we determined the *in vitro* inhibitory potencies on human placental 17β-HSD1 of the 3-hydroxy and the 3-ether derivatives of 13α- and 13β-estrones (**1**, **2**) and D-secoestrones (**3**–**6**) (Figure 1) in the presence of NADPH or NADH.

The reference IC_{50} data determined for unlabelled estrone (**1a**) were found to be 2.0 μM in the presence of NADH and 0.63 μM when NADPH was applied as cofactor (Table 1). These IC_{50} results are similar to those of the earlier published results by other authors (for placental 17β-HSD1)[5,23,35–37].

13α-Estrone (**2a**) proved here to be a potent inhibitor, displaying low micromolar IC_{50} values similar to those of the unlabelled reference estrone (**1a**). The 13α epimer (**2a**) of the natural estrogenic prehormone **1a** has long been known[38], but its inhibitory properties against the 17β-HSD1 activity have not been reported so far. Recently, 17β-HSD1 inhibition of 16-substituted derivatives of the 13α-estradiol has been investigated[39].

As concerns the inhibitory activities of the test compounds bearing an intact ring D (**1a–c** and **2a–c**), the nature of the substituent on C-3 was the determining factor. Similar to the earlier established relationships[22], the presence of the phenolic OH or the small methyl ether function was more advantageous than the bulky apolar benzyl group. The 13α methyl ether **2c** displayed a lower range activity than that of its 13β counterpart (**1c**). Our results tend to confirm the earlier observations[22] that the hydrogen-bonds of a phenolic OH function in this position might be beneficial, but not absolutely necessary for efficient inhibition.

As concerns the secoestrones, the two epimeric D-secoalcohols (**3a** and **4a**) display IC_{50} values in the low or submicromolar range. Of the 3-methyl ethers of the secoalcohol (**3c** and **4c**), only the 13β counterpart (**3c**) was proved effective, but with higher IC_{50} value than that of its 3-OH derivative (**3a**). The epimeric 3-hydroxy-D-secooximes (**5a** and **6a**) displayed noteworthy inhibitory properties and C-13 chirality dependence. **5a** was found to be highly potent in the presence of either NADPH or NADH, with IC_{50} values of 0.070 μM and 0.077 μM, respectively. The 13α counterpart (**6a**) was effective only when NADH was used as a cofactor ($IC_{50} = 0.058$ μM). The oxime epimer pairs of **5a** and **6a** displayed a large difference, demonstrating inhibition around 400-fold stronger of the 13β than that of the 13α epimer in the presence of NADPH, whereas they exerted similar effect with NADH.

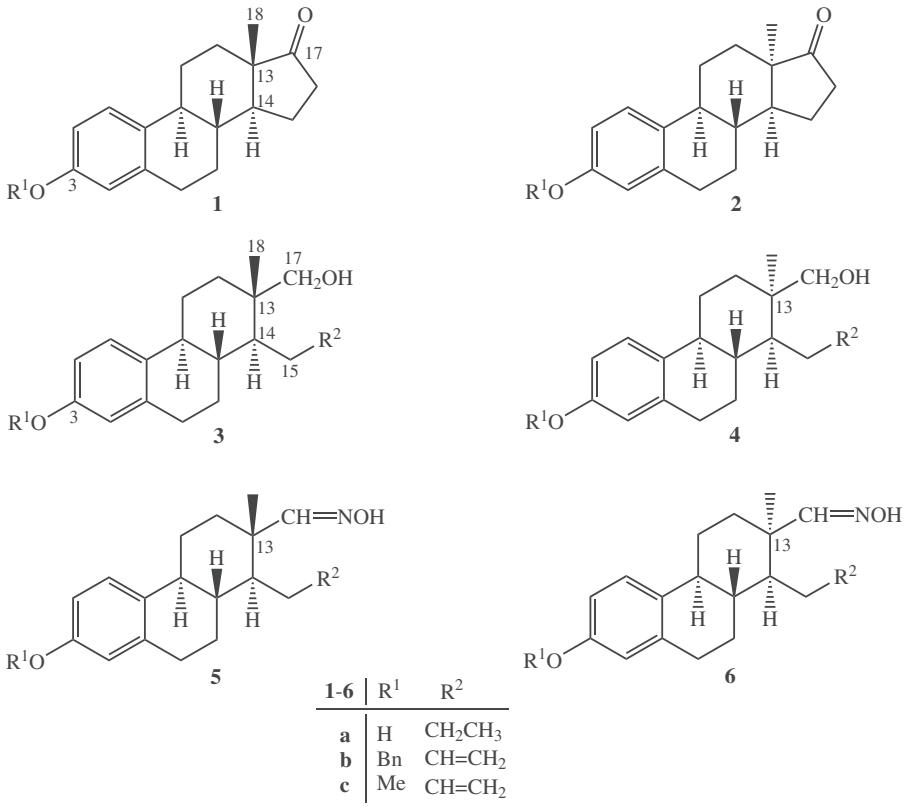
The inhibitory data of the D-seco compounds reveal that the nature of the 3 substituent has a crucial influence on the activities. Of the epimeric oxime ethers (**5b**, **c** and **6b**, **c**), only one 13β epimer (**5c**), bearing a small methyl group on the phenolic OH function, exerted substantial inhibitory effect, which was more pronounced than that observed for the 13β secoalcohol 3-methyl ether (**3c**).

Molecular mechanic and semi-empirical energy minimizations of the most potent 3-OH derivatives (**2a**–**6a**) were performed to demonstrate their structural features and differences (Figure 2). Figure 2 reveals that the epimerization of C-13 modifies the ring D region considerably. Functional groups in this region (carbonyl, hydroxymethyl or oxime) display alterations in position, direction and distance from those of 3-OH in the epimer pairs. Despite these structural differences, 13α-estrone (**2a**) binds to 17β-HSD1 with similar affinity as for the cognate substrate. As concerns the D-secoestrones (**3a**–**6a**), the 13β compounds (**3a** and **5a**) possess an axial angular methyl group and an equatorial functional group on C-13. In contrast with the 13β derivatives, the angular methyl group of 13α-D-secoestrones (**4a** and **6a**) has an equatorial orientation, and the oxime or primary alcoholic function is axial[40]. The oxime function has a double bond with *E* or *Z* orientation, but the primary alcoholic group can rotate freely. The difference in the inhibitory activities of the oximes and alcohols may therefore reflect the differences in the nature and the position of the C-17 functional groups. It may be postulated that in **5a** or **6a**, this oxime side-chain may take up an appropriate position to form strong hydrophilic interactions or hydrogen-bonds to certain amino acids of the enzyme, and these interactions may cause the high affinity and outstanding inhibitory potentials observed for the oximes. The noteworthy effectiveness of the oximes may be ascribed to the capability of the oxime function to form strong interactions with certain amino acid residues of the target proteins. Further investigations might identify the amino acid residues which are involved in these interactions.

Cofactor dependence

The 17β-HSD1 inhibition results of the test compounds demonstrate the influence of the cofactor partner. 13β-Estrone 3-methyl ether (**1c**) exerted a five-fold stronger inhibition in the presence of the phosphorylated cofactor. 13α-Estrone (**2a**) and its methyl ether (**2c**) displayed similar IC_{50} values with the two cofactors, but the RIP data demonstrated a 2–3-fold higher potential with NADH in comparison with the reference **1a**. The cofactor dependence was more pronounced among the D-seco compounds. The IC_{50} values were found to be 2–3-fold lower, indicating a 3–7-fold higher inhibition effect in terms of the RIP measured with NADH. The difference was further enhanced for the 13β epimer

Figure 1. Structural formulae of the test compounds (**1**–**6**).



1-6	R^1	R^2
a	H	CH_2CH_3
b	Bn	$CH{=}CH_2$
c	Me	$CH{=}CH_2$

of the D-secoalcohol (**3a**), which exerted an 8–9-fold more effective inhibition with NADH according to the IC_{50} data, and a 25-fold stronger effect in the sense of the RIP values.

The 13α epimer of the D-secooxime (**6a**) displayed an outstanding cofactor dependence. This compound exerted only weak inhibition with NADPH, but it was highly effective in the presence of NADH. The difference between the IC_{50} values was more than 500-fold, whereas the RIP ratio exceeded 1200.

Since the two secooxime epimers, **5a** and **6a**, differ only in the position of the angular methyl and the oxime function, the orientation of this part of the molecule seems to be favourable in the NADH complex of the enzyme for both the 13α and the 13β epimers (**6a** and **5a**), but only for **5a** in the NADPH complex. Effective binding of the 13α counterpart (**6a**) is possibly prevented by the increased specificity towards 13β compounds of the NADPH complex. The side chain at C-13 in **6a** may be directed into an unfavoured position, which cannot be modified because of the limited flexibility of the oxime function caused by its double bond. The related alcohols **3a** and **4a** display similar inhibitory potencies, irrespective of the orientation of the angular methyl group, as the shorter and more rotatable side chain may find its optimum position either in the NADH or in the NADPH complexed protein.

Other SDR enzymes feature NADH for the catalytic process and early studies annotated this cofactor to the reductive direction of 17β-HSD1[30,41,42]. Numerous *in vitro* inhibition tests have been performed with supplementation of the unphosphorylated cofactor too. Higher affinity for 17β-HSD1 of NADPH over NADH[5,29,43,44] and considerations of the abundance and metabolic roles of nicotinamide cofactors[4,45,46] have made it evident that NADPH might be the prevalent partner of 17β-HSD1 in its *in vivo* function, in the **1a**–E2 conversion[4,46].

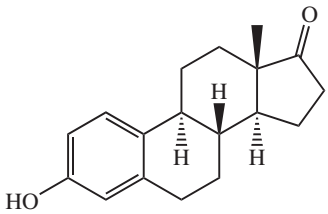
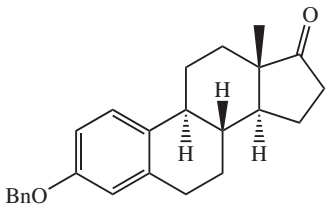
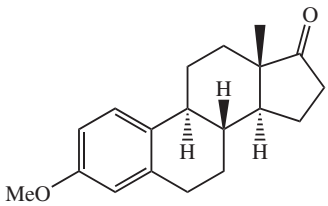
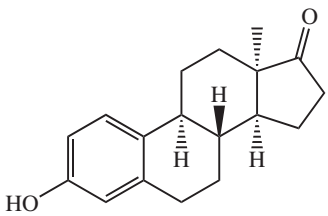
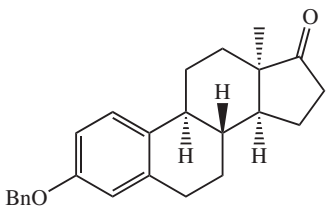
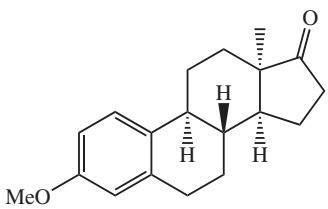
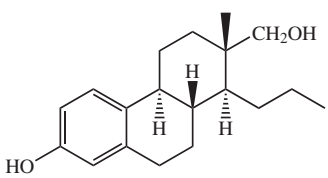
Only a few data are to be found in the literature as concerns the direct comparison of inhibitory potencies with NADPH versus NADH. The hybrid inhibitor EM-1745, in which an unphosphorylated cofactor-mimicking moiety was coupled to the estradiol core, and which was therefore planned to act on both the active centre and the cofactor binding site of the enzyme, proved to be a weaker inhibitor of 17β-HSD1 when NADPH was used as cofactor rather than NADH[47]. This difference, however, was explained specifically that the adenosine moiety of EM-1745 does not bind the cofactor-binding site of 17β-HSD1 as strongly as the phosphorylated adenosine moiety of NADPH, and thus the bisubstrate inhibitor EM-1745 (without a phosphate group) cannot compete efficient enough against the cofactor NADPH. Our D-seco compounds **3a** and **6a** do not possess a cofactor-mimicking moiety, but they display large differences in inhibitory potential measured in the presence of NADPH or NADH.

These inhibition results indicate that the apparent *in vitro* potentials obtained with the two cofactors may differ substantially for certain compounds. Data on NADPH and NADH are not interchangeable and their direct comparison (e.g. in one table[48]) is not advised. The literature data must be reviewed with special attention to the cofactor supplementation, the screening systems[49] should be specified precisely, and NADPH should be preferred instead of NADH in cell-free *in vitro* inhibitor tests. The influence of cofactors might be an explanation for the altered, occasionally disappointingly decreased inhibition potentials obtained in cellular 17β-HSD1 inhibition assays performed following promising cell-free screening tests with NADH[50]. Data measured in the presence of NADH must be evaluated with caution in inhibitor optimization and in lead selection. NADH results are less relevant to the potential *in vivo* effect, but could be valuable in facilitating the understanding of the mechanism of catalysis and the inhibition of 17β-HSD1.

17β-HSD1 inhibition and antiproliferative effects

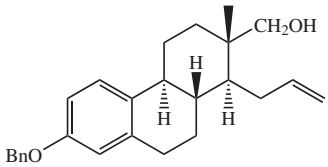
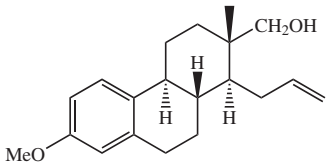
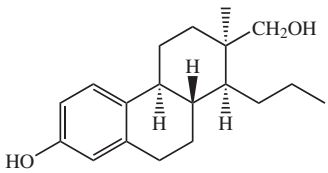
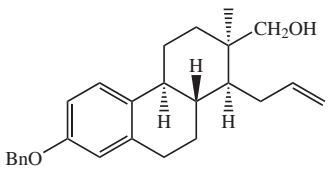
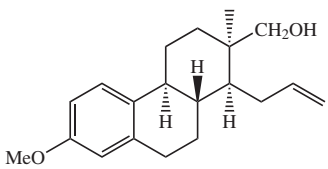
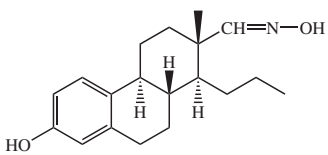
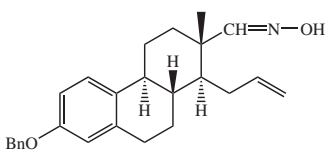
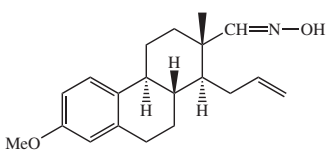
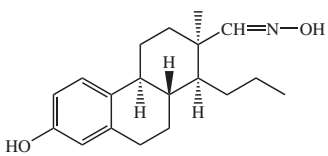
The synthesis and *in vitro* investigation of the antiproliferative potentials of the D-secoestrone derivatives tested here for their

Table 1. Inhibition results on 17β-HSD1.

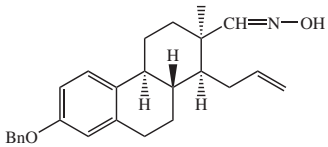
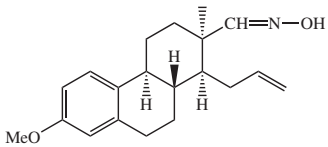
Compd.	Structure	IC_{50}±SD (μM) and RIP or relative conversion at 10 μM ± SD (%) NADPH	NADH
1a		$IC_{50} = 0.63 \pm 0.11$ RIP = 1.0	$IC_{50} = 2.0 \pm 0.18$ RIP = 1.0
1b		52 ± 2	52 ± 5
1c		$IC_{50} = 0.77 \pm 0.29$ RIP = 1.2	$IC_{50} = 4.2 \pm 1.6$ RIP = 2.1
2a		$IC_{50} = 1.2 \pm 0.2$ RIP = 1.7	$IC_{50} = 1.1 \pm 0.3$ RIP = 0.59
2b		55 ± 3	65 ± 10
2c		$IC_{50} = 5.5 \pm 1.5$ RIP = 8.8	$IC_{50} = 7.5 \pm 2.5$ RIP = 3.7
3a		$IC_{50} = 3.4 \pm 1.4$ RIP = 5.4	$IC_{50} = 0.41 \pm 0.24$ RIP = 0.21
3b		76 ± 7	70 ± 4

(*continued*)

Table 1. Continued

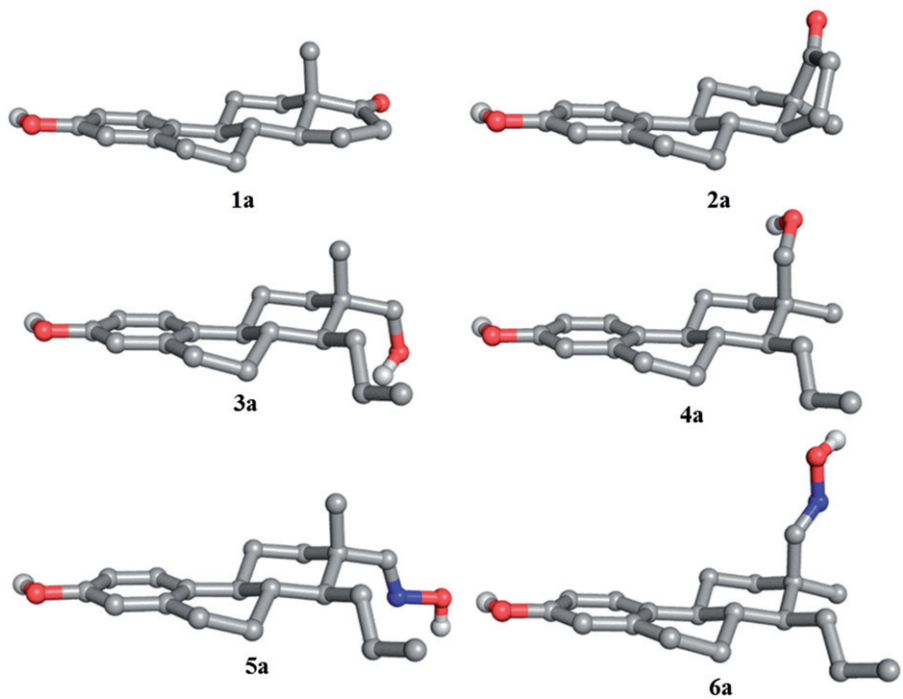
Compd.	Structure	IC_{50}±SD (μM) and RIP or relative conversion at 10 μM ± SD (%) NADPH	NADH
			
3c		$IC_{50} = 9.0 \pm 1.7$ RIP = 14.3	$IC_{50} = 5.5 \pm 1.9$ RIP = 2.7
4a		$IC_{50} = 3.7 \pm 1.3$ RIP = 5.8	$IC_{50} = 1.7 \pm 0.3$ RIP = 0.85
4b		84 ± 6	83 ± 7
4c		66 ± 10	67 ± 6
5a		$IC_{50} = 0.070 \pm 0.027$ RIP = 0.11	$IC_{50} = 0.077 \pm 0.036$ RIP = 0.039
5b		76 ± 10	82 ± 0.4
5c		$IC_{50} = 3.1 \pm 1.7$ RIP = 4.9	$IC_{50} = 1.9 \pm 0.8$ RIP = 0.93
6a		$IC_{50} = 30 \pm 7$ RIP = 48	$IC_{50} = 0.058 \pm 0.044$ RIP = 0.029

(*continued*)

Compd.	Structure	IC_{50}±SD (μM) and RIP or relative conversion at 10 μM ± SD (%) NADPH	NADH
6b		90 ± 17	77 ± 5
6c		IC_{50} = 21 ± 7 RIP = 34	IC_{50} = 24 ± 10 RIP = 12

Relative conversions (control incubation with no inhibition is 100%) measured in the presence of 10 μM of the compound tested. IC_{50}: The inhibitor concentration that decreases the enzyme activity to 50%. RIP: relative inhibition potency compared to reference E1. SD: standard deviation (for relative conversion $n = 3$).

Figure 2. Molecular structures of compounds **1a**–**6a**.



17β-HSD1 inhibitory properties have recently been reported[51,52]. Our potential anticancer agents were designed on the hormonally inactive D-seco- and/or 13α-estrone core. 3-Benzyloxy-D-secoestrone alcohol (**3b**), the first D-secoestrone in the literature, displays substantial *in vitro* antiproliferative effects against a number of human reproductive cancer cell lines with good tumour selectivity[51]. The debenzylated secoalcohol (**3a**) containing a 3-phenolic group did not inhibit tumour cell growth markedly. These results led to further D-secoestrone derivatives as potential antitumour agents. 17-Oxime derivatives of the potent secoalcohol (**3b**) were synthesized and diversified at several sites of the molecule: 3-ethers (**5b**,**c** and **6b**,**c**) or 3-hydroxy derivatives (**5a** and **6a**) were investigated in both the 13β- and the 13α-estrone series[52]. None of the 13α epimers (**6**) or the 3-hydroxy derivatives (**5a**) exerted substantial antiproliferative activities, but the 13β-D-secoestrone-3-ethers (**5b**,**c**) proved to be effective against various cell lines (HeLa, A2780, A431 and MCF-7) with IC_{50} values in the low μM range. Tests were performed on cell lines with diverse steroidogenic and steroid responsive properties, and the results suggested that the cytotoxic effect is most probably independent of the estrogen hormonal mechanisms, and the 17β-HSD1 inhibition among them.

The literature reveals that it is possible to combine direct cytostatic activity with 17β-HSD1-inhibitory potential, resulting in dual action against estrogen-dependent tumours. It is therefore reasonable to evaluate our present results in this sense too (Figure 3). Depending on the nature of the substituent at C-3, 13β-methyl-D-secoestrone oxime (**5**) is able to exert different

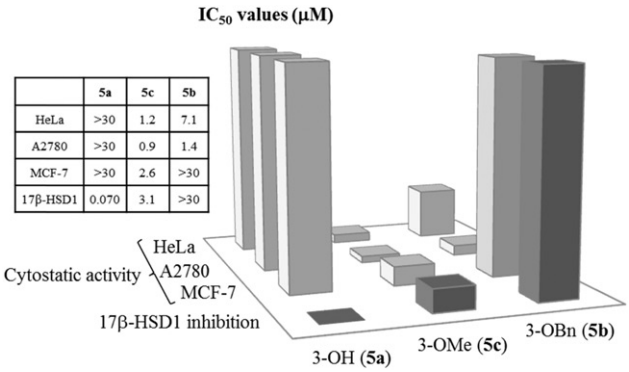


Figure 3. 17β-HSD1 inhibition (in the presence of NADPH) and cytostatic potentials[51,52] of the tested D-secooximes (IC_{50} values on a relative scale).

important biological activities: bearing an unsubstituted 3-OH (**5a**), which belongs to the highly potent 17β-HSD1 inhibitors with unmarked antiproliferative action on the examined cell lines. Compound **5b**, possessing a bulky apolar benzyl protecting group, substantially inhibits the growth of certain cell lines, but does not influence the estrone–estradiol conversion catalysed by 17β-HSD1. The methyl ether (**5c**) behaves dually by inhibiting both the cell growth and 17β-HSD1 as enzyme. The same tendency appears in the results of the antiproliferative and 17β-HSD1 inhibitory measurements as concerns 13β-methyl-D-secoestrone alcohol (**3**), but it can be stated that the biological activities of the secooximes are more pronounced than those of their alcoholic counterparts. Compound **5a** or **5b**, however, is a selective 17β-HSD1 inhibitor or an antiproliferative agent respectively, in this comparison.

The 3-methyl ether of D-secoestrone oxime (**5c**) may be considered as a compound with a dual mode of action, as it displays a noteworthy direct antiproliferative effect against a number of human reproductive cancer cell lines (independently of their 17β-HSD1 or ER status), and exerts substantial inhibitory potential against 17β-HSD1. Since 17β-HSD1 inhibition is a promising approach for the treatment of estrogen-dependent tumours, it decreases the level of estradiol in the tumour cells, a compound with a dual mode of action may be superior to simple 17β-HSD1 inhibitors.

Conclusions

17β-HSD1 has been studied for more than half a century, but none of its inhibitor candidates have yet reached clinical trials for the treatment of estrogen-dependent diseases. Since breast cancer is the most common cancer among women in the Western world, further intensive research efforts are demanded. In order to develop potent and selective 17β-HSD1 inhibitors, a profound understanding of the enzymatic mechanisms and the structure–function relationships is essential.

The present study has revealed that 13α-estrone (**2a**) and some D-secoestrone derivatives (**3a**–**6a**, **3c** and **5c**), might be promising inhibitors. The very low *in vitro* IC_{50} of **5a** indicates that this compound is one of the most effective 17β-HSD1 inhibitors ever reported. Its 3-methyl ether (**5c**) may be regarded as the first published D-secoestrone that exerts dual independent pre-receptorial antihormonal and antiproliferative effects. Further derivatization of the promising 13α-estrone and D-secoestrone oxime scaffold may lead to drug candidates that possess a beneficial combination of direct cytostatic and endocrine disruptor behaviour. The different *in vitro* inhibitory potentials observed for the C-13 epimer pairs, with the cofactor NADH instead of NADPH, are interesting findings. Additional investigations with the aim of elucidating the binding mechanisms may provide new data clarifying the structure–function relationships of 17β-HSD1.

Declaration of interest

The authors report no declarations of interest. The authors are grateful for the financial support from the Hungarian Scientific Research Fund [OTKA K113150].

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Supplementary materials online only

IV.

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Synthesis of 16α-amino-pregnenolone derivatives via ionic liquid-catalyzed aza-Michael addition and their evaluation as $C_{17,20}$-lyase inhibitors

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ABSTRACT

Aza-Michael addition of 16-dehydropregnenolone was studied in the presence of a basic ionic liquid, [DBU][OAc] as catalyst and solvent. The reaction was carried out using different primary and secondary amines as *N*-nucleophiles. The products were obtained in moderate to good yields and were characterized by ^{1}H and ^{13}C NMR, MS and IR. The ionic liquid was found to be an efficient and recyclable catalyst that was reused five times. The products were investigated for the inhibition of *in vitro* $C_{17,20}$-lyase activity and displayed moderate inhibitory effect.

1. Introduction

Ionic liquids (ILs) are environmentally friendly alternatives of organic solvents due to their low melting point, good chemical stability and high solubility in polar organic and inorganic compounds. Basic ILs can play the dual role of reaction medium and catalyst, replacing traditional base catalysts such as KOH or NaOH [1]. Aza-Michael reactions are usually carried out using transition metal salts and complexes or Brønsted acids as catalysts [2]. These methods have many drawbacks due to the high price and toxicity of catalysts, therefore the attention was focused on mild, environmentally benign processes. In addition, earlier studies prove that amines exhibit higher nucleophilicity in ionic liquids than in organic solvents [3]. Accordingly, 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU) derived ionic liquids were found to be efficient catalysts in aza-Michael addition of aliphatic [4] and aromatic amines [5] to produce α,β-unsaturated ketones under solvent-free conditions. In case of aliphatic amines the ionic liquid was reused without significant loss of activity [4]. Imidazolium and DABCO based ionic liquids were also found to promote aza-Michael addition of different *N*-nucleophiles to α,β-unsaturated compounds [6,7].

In order to improve the methodology of the synthesis of steroidal derivatives, our goal was the investigation of aza-Michael addition of different *N*-nucleophiles to 16-dehydropregnenolone in the presence of [DBU][OAc] as catalyst and reaction medium.

Michael addition of electron deficient steroidal alkenes, *e.g.* 16-dehydropregnenolone provides an efficient route for the introduction of heteroatoms into the side chain of steroids and often leads to compounds with pharmacological effect. Michael adducts of 16-dehydropregnenolone with different alcohols showed anti-oxidant and anti-dyslipidemic activity [8]. 16α-Heteroaryl-pregnenolone derivatives were found to be effective *in vitro* against cervical HeLa, prostate DU 205 and breast cancer MCF-7 cell lines [9]. Others are potential DPP-4 inhibitors, which can be used for the treatment of diabetes mellitus type 2 [10]. The synthesis of these compounds were often effected by hetero-Michael addition using $BF_3{\cdot}Et_2O$ catalyst or microwave irradiation.

Gould and co-workers described the synthesis of 16α-amino-substituted pregnanes using KOH as catalyst [11]. Kumar and co-workers carried out aza-Michael addition of 16-DHP mainly with aliphatic primary amines, in this case the amine served both as reactant and solvent [10]. At the same time, this method cannot be used for the addition of solid amines. During the present work, we compared this strategy with the use of a basic ionic liquid as solvent and catalyst.

The enzyme 17α-hydroxylase-$C_{17,20}$-lyase ($P450_{17\alpha}$) is a key regulatory enzyme in androgen biosynthesis. Inhibitors of $P450_{17\alpha}$ have potential application for the treatment of androgen-dependent diseases, the steroid type compounds are similar in structure to the natural

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substrates of this enzyme. Highly selective inhibitors, for example abiraterone [12] and galeterone [13] contain *N*-heterocyclic ring at C-17. Earlier studies support that the coordination of the lone pair of nitrogen in the heterocyclic ring with the heme iron of $P450_{17\alpha}$ results in tight binding. Several steroid inhibitors were developed in the last years with different modifications mainly at the C-17 side chain [14]. On the other hand, incorporation of substrates with α orientation at C-16 may also interact with the enzyme's active site [15], so we decided to explore the $C_{17,20}$-lyase inhibitory effect of some of the 16α-amino-pregnenolone derivatives obtained via the ionic liquid catalyzed aza-Michael addition.

2. Experimental

2.1. General methods

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 500 spectrometer at 500.15 MHz and 125.78 MHz, or on a Bruker Avance 400 spectrometer at 400.13 MHz and 100.62 MHz, respectively. Chemical shifts (δ) are reported in ppm relative to $CHCl_3$ (7.26 and 77.00 ppm for ^{1}H and ^{13}C, respectively). HRMS spectra were recorded using a Q-TOF Premier mass spectrometer (Waters Corporation, Milford, MA, USA), which was operated in positive electrospray ionization mode. GC–MS of **3l** was recorded on a Shimadzu GCMS-QP2010 SE instrument. Elemental analysis of **3l** was measured on a 1108 Carlo Erba apparatus. IR spectra were made using a Thermo Nicolet Avatar 330 FT-IR instrument. Samples were prepared as KBr pellets. The ionic liquids [DBU][OAc] and [DBU][Lac] were prepared by a described method [4].

2.2. General procedure for the aza-Michael addition of N-nucleophiles to 16-dehydropregnenolone

16-Dehydropregnenolone **1** (0.2 mmol, 62.8 mg), solid amine (2 mmol) and [DBU][OAc] or [DBU][Lac] (300 mg) were placed under argon atmosphere in a Schlenk tube equipped with a magnetic stirrer, a septum inlet and a balloon on the top. Liquid amines (as indicated in Table 1) were added through the septum inlet. The reaction mixture was heated at 65 °C for 8 or 15 h. The product was extracted with an organic solvent (**3a-g** and **3j-n**: diethyl ether (5 × 3 ml), **3h**, **3i**, **3o** and **3p**: toluene (5 × 3 ml)) and the solvent was removed in vacuo. During the synthesis of **3h**, **3o** and **3p** in [DBU][OAc], the reaction mixture was dissolved in dichloromethane and the organic phase was washed with water to remove the ionic liquid, then the organic solvent was evaporated in vacuo. The crude product was purified by column chromatography (silica, eluent: toluene/MeOH (4:1, v/v) (**3a**, **3b**, **3f**, **3g, 3h, 3i**), toluene/MeOH (2:1, v/v) (**3c**, **3j**), toluene/MeOH (3:2, v/v) (**3d**), chloroform/MeOH (1:1, v/v) (**3k**), chloroform/MeOH (9:1, v/v) (**3l**), MeOH/chloroform (6:1, v/v) (**3m**, **3o**), chloroform/MeOH (9:2, v/v) (**3n**); toluene/MeOH/EtOAc (3:1:3, v/v) (**3p**)) Table 2.

Compound **3i** is a known compound, its analytical data corresponded well to the literature [10].

2.2.1. 16α-[N,N-(1′,5′-(3′-Oxapentanediyl))]-amino-3β-hydroxypregn-5-ene-20-one (3a)

^{1}H NMR (*δ*, $CDCl_3$, 500.15 MHz): 5.32–5.39 (m, 1H, 6-H); 3.63–3.75 (m, 4H, $O(CH_2)_2$); 3.49–3.63 (m, 2H, 16-H, 3-H); 2.67 (d, J = 8.1 Hz, 1H, 17-H); 1.03–2.54 (m, 22H, ring protons, $N(CH_2)_2$, OH); 2.20 (s, 3H, $COCH_3$); 1.01 (s, 3H, 19-H_3); 0.67 (s, 3H, 18-H_3). ^{13}C NMR (*δ*, $CDCl_3$, 125.78 MHz): 208.1; 140.8; 121.1; 71.6; 66.9 (2C); 65.6; 64.9; 55.1 (2C); 51.4; 49.9; 44.7; 42.2; 38.9; 37.2; 36.5; 31.8; 31.7; 31.6 (2C); 29.1; 20.8; 19.4; 14.5. HRMS: calculated for $C_{25}H_{40}NO_3$ $[M+H]^+$ 402.3008, found 402.3000. IR (KBr, $\nu(cm^{-1})$): 3387, 2941, 2853, 1700, 1115, 1069, 735. R_f(toluene/MeOH (4:1, v/v)): 0.34.

Table 1
Product yields in the aza-Michael reaction of **1** in ionic liquids.[a]

Entry	Nucleophile	Reaction time (h)	Product	Yield (%)[b]
1[c]	Morpholine (**2a**)	8	**3a**	69
2[d]	Morpholine (**2a**)	8	**3a**	89
3[e]	Morpholine (**2a**)	8	**3a**	91
4	Morpholine (**2a**)	8	**3a**	91
5	Morpholine (**2a**)	4	**3a**	53
6[f]	Morpholine (**2a**)	8	**3a**	Traces
7	Piperidine (**2b**)	8	**3b**	60
8[g]	Piperidine (**2b**)	15	**3b**	86
9	Piperidine (**2b**)	15	**3b**	93
10	*N*-methyl-piperazine (**2c**)	8	**3c**	90
11	Dibutylamine (**2d**)	15	**3d**	23
12	Diisopropylamine (**2e**)	8	–	–
13	Aniline (**2f**)	8	**3f**	46
14	Aniline (**2f**)	15	**3f**	55
15[f]	Aniline (**2f**)	8	**3f**	Traces
16	4-methylaniline (**2g**)	8	**3g**	42
17	4-methylaniline (**2g**)	15	**3g**	52
18	4-methoxyaniline (**2h**)	15	**3h**	77[h]
19[i]	4-methoxyaniline (**2h**)	15	**3h**	64
20[i]	Benzylamine (**2i**)	8	**3i**	82
21	Cyclohexylamine (**2j**)	8	**3j**	74
22	Cyclopentylamine (**2k**)	15	**3k**	85
23	Cyclopropylamine (**2l**)	8	**3l**	68
24	*N,N*-Diethyl-ethylenediamine (**2m**)	8	**3m**	48
25	4-(2-Aminoethyl)morpholine (**2n**)	8	**3n**	76
26	1-(3-Aminopropyl)imidazole (**2o**)	8	**3o**	70[h]
27[i]	1-(3-Aminopropyl)imidazole (**2o**)	8	**3o**	65
28	Imidazole (**2p**)	8	**3p**	72[h]
29[i]	Imidazole (**2p**)	8	**3p**	70

[a] Reaction conditions: 0.2 mmol of substrate **1** in 300 mg [DBU][OAc], **1**/nucleophile (**2a–2j**) = 1/10, 65 °C.
[b] (mmol isolated product (**3a–3j**))/(mmol substrate **1**) × 100.
[c] Ratio of **1**/**2a** = 1/1.
[d] Ratio of **1**/**2a** = ½.
[e] Ratio of **1**/**2a** = 1/5.
[f] In the absence of ionic liquid.
[g] Ratio of **1**/**2b** = ½.
[h] Isolated via dilution with CH_2Cl_2 and removal of the ionic liquid with water.
[i] Using [DBU][Lac] as solvent and catalyst.

Table 2
Inhibition of rat testicular $C_{17,20}$-lyase activity by aza-Michael adducts.

Compound	Relative conversion ± S.D.[a] (%)	IC_{50} ± S.D. (μM)
3a	36 ± 3	32 ± 8
3b	56 ± 6	> 50
3c	48 ± 3	46 ± 9
3f	59 ± 5	> 50
3g	63 ± 6	> 50
3h	72 ± 6	> 50
3i	38 ± 4	22 ± 9
3j	10 ± 3	9.1 ± 4
3k	42 ± 8	9.5 ± 1.3
3l	62 ± 11	> 50
3m	28 ± 4	9.3 ± 1.3
3n	41 ± 5	21 ± 8
3o	62 ± 12	> 50
3p	19 ± 2	1.8 ± 0.36
Abiraterone (reference)		0.0125 ± 0.0015
Ketoconazole (reference)		0.32 ± 0.02

[a] Measured in the presence of compound tested at 50 μM; control incubation with no inhibition is taken as 100%, S.D.: standard deviation of the mean, n = 2.

*2.2.2. 16α-[N,N-(1′,5′-Pentanediyl)]-amino-3β-hydroxypregn-5-ene-20-one **(3b)***

^{1}H NMR (δ, $CDCl_3$, 500.15 MHz): 5.33–5.38 (m, 1H, 6-H); 3.48–3.58 (m, 2H, 16-H, 3-H); 2.69 (d, J = 8.2 Hz, 1H, 17-H); 1.02–2.45 (m, 28H, ring protons, $N(CH_2)_3$, OH); 2.20 (s, 3H, $COCH_3$); 1.01 (s, 3H, 19-H_3); 0.67 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 208.5; 140.8; 121.3; 71.6; 65.4 (2C); 55.0 (2C); 52.2; 49.9; 44.7; 42.3; 38.9; 37.2; 36.5; 31.9; 31.7; 31.6; 31.5; 30.1; 26.1 (2C); 24.5; 20.8; 19.4; 14.6. HRMS: calculated for $C_{26}H_{42}NO_2$ $[M+H]^+$ 400.3216, found 402.3210. IR (KBr, ν(cm^{-1})): 3390, 2932, 2852, 1702, 1062, 801. R_f(toluene/MeOH (4:1, v/v)): 0.33.

*2.2.3. 16α-[N,N-(1′,5′-(3′-Azamethyl)-pentanediyl)]-amino-3β-hydroxypregn-5-ene-20-one **(3c)***

^{1}H NMR (δ, $CDCl_3$, 500.15 MHz): 5.33–5.38 (m, 1H, 6-H); 3.65–3.72 (m, 1H, 16-H); 3.50–3.58 (m, 1H, 3-H); 2.70 (d, J = 8.1 Hz, 1H, 17-H); 1.03–2.67 (m, 26H, ring protons, $N(CH_2)_2$, OH); 2.28 (s, 3H, N-CH_3); 2.20 (s, 3H, $COCH_3$); 1.02 (s, 3H, 19-H_3); 0.68 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 208.3; 140.8; 121.2; 71.6; 64.7; 64.4; 55.2 (2C); 54.9 (2C); 50.1; 49.8; 45.7; 44.8; 42.2; 38.8; 37.2; 36.5; 31.8; 31.7; 31.6 (2C); 29.3; 20.8; 19.4; 14.5. HRMS: calculated for $C_{26}H_{43}N_2O_2$ $[M+H]^+$ 415.3325, found 415.3324. IR (KBr, ν(cm^{-1})): 3413, 2947, 2808, 1686, 1054, 792. R_f(toluene/MeOH (2:1, v/v)): 0.33.

*2.2.4. 16α-(N,N-Dibutylamino)-3β-hydroxypregn-5-ene-20-one **(3d)***

^{1}H NMR (δ, $CDCl_3$, 400.13 MHz): 5.29–5.35 (m, 1H, 6-H); 3.85–3.97 (m, 1H, 16-H); 3.44–3.55 (m, 1H, 3-H); 2.51–2.64 (m, 1H, 17-H); 0.99–2.37 (m, 30H, ring protons, $N((CH_2)_3CH_3)_2$, OH); 2.13 (s, 3H, $COCH_3$); 0.98 (s, 3H, 19-H_3); 0.87 (t, J = 7.2 Hz, 6H, $N((CH_2)_3CH_3)_2$); 0.64 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 208.4; 140.7; 121.3; 71.6; 70.9; 57.2; 54.5; 49.7; 49.5; 45.3; 44.9; 44.6; 42.2; 38.7; 37.2; 37.1; 36.5; 32.2; 31.8; 31.6 (2C); 31.5; 30.0; 28.5; 27.0; 23.4; 20.8; 19.4; 14.3. HRMS: calculated for $C_{29}H_{50}NO_2$ $[M+H]^+$ 444.3842, found 444.3829. IR (KBr, ν(cm^{-1})): 3439, 2930, 2851, 1638, 1414, 805. R_f(toluene/MeOH (3:2, v/v)): 0.49.

*2.2.5. 16α-(N-Phenylamino)-3β-hydroxypregn-5-ene-20-one **(3f)***

^{1}H NMR (δ, $CDCl_3$, 400.13 MHz): 7.10–7.17 (m, 2H, 3′,5′-H); 6.65–6.71 (m, 1H, 4′-H); 6.53–6.60 (m, 2H, 2′,6′-H); 5.29–5.33 (m, 1H, 6-H); 4.38–4.49 (m, 1H, 16-H); 3.45–3.57 (m, 1H, 3-H); 2.42 (d, J = 7.5 Hz, 1H, 17-H); 1.01–2.33 (m, 19H, ring protons, NH, OH); 2.14 (s, 3H, $COCH_3$); 1.00 (s, 3H, 19-H_3); 0.72 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 207.7; 147.5; 140.8; 129.2 (2C); 121.2; 117.7; 113.9 (2C); 72.8; 71.6; 55.1; 53.3; 50.0; 44.6; 42.2; 39.0; 37.2; 36.5; 33.9; 31.9; 31.7; 31.6; 31.5; 20.8; 19.4; 14.3. HRMS: calculated for $C_{27}H_{38}NO_2$ $[M+H]^+$ 408.2903, found 408.2903. IR (KBr, ν(cm^{-1})): 3522, 3342, 2913, 2844, 1703, 1602, 1042, 751. R_f(toluene/MeOH (4:1, v/v)): 0.38.

*2.2.6. 16α-[N-(4-Methylphenylamino)]-3β-hydroxypregn-5-ene-20-one **(3g)***

^{1}H NMR (δ, $CDCl_3$, 400.13 MHz): 6.95 (d, J = 8.1 Hz, 2H, 3′,5′-H); 6.50 (d, J = 8.4 Hz, 2H, 2′,6′-H); 5.30 (d, J = 5.1 Hz, 1H, 6-H); 4.35–4.44 (m, 1H, 16-H); 3.43–3.56 (m, 1H, 3-H); 2.40 (d, J = 7.5 Hz, 1H, 17-H); 1.01–2.34 (m, 19H, ring protons, NH, OH); 2.20 (s, 3H, 4′-H_3); 2.13 (s, 3H, $COCH_3$); 0.99 (s, 3H, 19-H_3); 0.71 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 207.8; 145.2; 140.8; 129.7 (2C); 127.0; 121.2; 114.2 (2C); 72.7; 71.6; 55.1; 53.6; 50.0; 44.5; 42.2; 39.0; 37.2; 36.5; 33.8; 31.9; 31.7; 31.6; 31.5; 20.8; 20.4; 19.4; 14.3. HRMS: calculated for $C_{28}H_{40}NO_2$ $[M+H]^+$ 422.3059, found 422.3055. IR (KBr, ν(cm^{-1})): 3393, 3233, 2927, 2859, 1683, 1519, 1052, 811. R_f(toluene/MeOH (4:1, v/v)): 0.38.

*2.2.7. 16α-[N-(4-Methoxyphenylamino]-3β-hydroxypregn-5-ene-20-one **(3h)***

^{1}H NMR (δ, $CDCl_3$, 500.15 MHz): 6.78 (d, J = 8.9 Hz, 2H, 3′,5′-H); 6.59 (d, J = 8.9 Hz, 2H, 2′,6′-H); 5.33–5.37 (m, 1H, 6-H); 4.37–4.45 (m, 1H, 16-H); 3.76 (s, 3H, OCH_3); 3.50–3.60 (m, 1H, 3-H); 2.45 (d, J = 7.5 Hz, 1H, 17-H); 1.05–2.40 (m, 19H, ring protons, NH, OH); 2.16 (s, 3H, $COCH_3$); 1.03 (s, 3H, 19-H_3); 0.75 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 207.9; 152.5; 141.7; 140.8; 121.2; 115.5 (2C); 114.9 (2C); 72.7; 71.6; 55.8; 55.1; 54.3; 50.0; 44.5; 42.2; 39.0; 37.2; 36.5; 33.7; 32.0; 31.7; 31.6; 31.5; 20.8; 19.4; 14.3. HRMS: calculated for $C_{28}H_{40}NO_3$ $[M+H]^+$ 438.3008, found 438.3014. IR (KBr, ν(cm^{-1})): 3479, 3324, 2927, 2848, 1692, 1515, 1248, 1038, 820. R_f(toluene/MeOH (4:1, v/v)): 0.36.

*2.2.8. 16α-(N-Cyclohexylamino)-3β-hydroxypregn-5-ene-20-one **(3j)***

^{1}H NMR (δ, $CDCl_3$, 500.15 MHz): 5.33–5.39 (m, 1H, 6-H); 3.89–4.01 (m, 1H, 16-H); 3.49–3.61 (m, 1H, 3-H); 2.48 (d, J = 7.2 Hz, 1H, 17-H); 1.03–2.41 (m, 30H, ring protons, cHex, NH, OH); 2.19 (s, 3H, $COCH_3$); 1.02 (s, 3H, 19-H_3); 0.67 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 208.3; 140.7; 121.3; 72.2; 71.6; 55.6; 54.6; 54.2; 49.9; 44.6; 42.2; 38.8; 37.2; 36.5; 33.5; 33.2; 33.1; 31.8; 31.6 (2C); 31.5; 26.0; 25.2; 25.1; 20.8; 19.4; 14.3. HRMS: calculated for $C_{27}H_{44}NO_2$ $[M+H]^+$ 414.3372, found 414.3369. IR (KBr, ν(cm^{-1})): 3392, 3289, 2928, 2852, 1695, 1063, 735. R_f(toluene/MeOH (2:1, v/v)): 0.46.

*2.2.9. 16α-(N-Cyclopentylamino)-3β-hidroxy-pregn-5-ene-20-one **(3k)***

^{1}H NMR (δ, $CDCl_3$, 500.15 MHz): 5.32–5.38 (m, 1H, 6-H); 3.95–4.02 (m, 1H, 16-H); 3.50–3.59 (m, 1H, 3-H); 3.07–3.14 (m, 1H, NH-C*H*); 2.83 (d, J = 6.0 Hz, 1H, 17-H); 1.02–2.35 (m, 27H, ring protons, Cyp, NH, OH); 2.19 (s, 3H, $COCH_3$); 1.02 (s, 3H, 19-H_3); 0.65 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 207.3; 140.7; 121.2; 71.6; 69.8; 58.1; 55.7; 54.4; 49.8; 44.7; 42.2; 38.6; 37.2; 36.5; 31.6 (2C); 31.5 (2C); 31.4; 31.3; 31.1; 24.1; 24.0; 20.8; 19.4; 14.2. HRMS: calculated for $C_{26}H_{42}NO_2$ $[M+H]^+$ 400.3216, found 400.3205. IR (KBr, ν(cm^{-1})): 3583, 3403, 2961, 2905, 1695, 1533, 1058, 657. R_f(chloroform /MeOH (1:1, v/v)): 0.38.

*2.2.10. 16α-(N-Cyclopropylamino)-3β-hidroxy-pregn-5-ene-20-one **(3l)***

^{1}H-NMR (δ, $CDCl_3$, 400.13 MHz): 5.28–5.34 (m, 1H, 6-H); 3.84–3.95 (m, 1H, 16-H); 3.44–3.57 (m, 1H, 3-H); 2.42 (d, J = 7.6 Hz, 1H, 17-H); 0.97–2.34 (m, 20H, ring protons, NH-CH, OH); 2.15 (s, 3H, $COCH_3$); 0.97 (s, 3H, 19-H_3); 0.64 (s, 3H, 18-H_3); 0.24–0.44 (m, 4H, NH-CH(CH_2)$_2$). ^{13}C-NMR (δ, $CDCl_3$, 100.62 MHz): 208.0; 140.3; 120.8; 71.5; 71.1; 57.7; 54.3; 49.4; 44.5; 41.7; 38.4; 36.7; 36.0; 32.7; 31.5; 31.1 (3C); 29.4; 20.3; 18.9; 14.0; 6.4; 5.4.

MS (*m/z*/rel. int.): 371 $(M)^+$/40; 356/21; 342/20; 328/36; 281/21; 207/21; 139/100; 124/24; 105/22; 96/38; 58/28; 43/49. Analysis calculated for $C_{24}H_{37}NO_2$ (371.56): C, 77.58; H, 10.04; N, 3.77; Found: C, 77.69; H, 10.12; N, 3.66. IR (KBr, ν(cm^{-1})): 3401, 3305, 3081, 2933, 2850, 1702, 1355, 1059, 754. R_f(chloroform/MeOH (9:1, v/v)): 0.39.

*2.2.11. 16α-[N-(2-(N,N-Diethylamino)-ethyl)amino]-3β-hidroxy-pregn-5-ene-20-one **(3m)***

^{1}H NMR (δ, $CDCl_3$, 400.13 MHz): 5.29–5.33 (m, 1H, 6-H); 3.66–3.73 (m, 1H, 16-H); 3.44–3.55 (m, 1H, 3-H); 2.37–2.57 (m, 9H, 17-H, $CH_2CH_2N(CH_2)_2$); 0.96–2.31 (m, 19H, ring protons, NH, OH); 2.14 (s, 3H, $COCH_3$); 0.97 (s, 3H, 19-H_3); 0.63 (s, 3H, 18-H_3); 0.96 (t, J = 7.1 Hz, 6H, $N(CH_2CH_3)_2$). ^{13}C NMR (δ, $CDCl_3$, 100.62 MHz): 208.2; 140.3; 120.8; 71.7; 71.1; 57.3; 54.1; 52.0; 49.4; 46.3 (2C); 45.8; 44.5; 41.8; 38.4; 36.7; 36.0; 32.6; 31.5; 31.1 (2C); 31.0; 20.4; 18.9; 14.0; 11.1 (2C). HRMS: calculated for $C_{27}H_{47}N_2O_2$ $[M+H]^+$ 431.3638, found 431.3638. IR (KBr, ν(cm^{-1})): 3391, 3293, 2933, 2849, 1702, 1065. R_f(MeOH/chloroform (6:1, v/v)): 0.27.

*2.2.12. 16α-[N-(2-(4-Morpholino)-ethyl)amino]-3β-hidroxy-pregn-5-ene-20-one (**3n**)*

^{1}H NMR (δ, $CDCl_3$, 500.15 MHz): 5.33–5.39 (m, 1H, 6-H); 3.67–3.78 (m, 5H, 16-H, $O(CH_2)_2$); 3.49–3.58 (m, 1H, 3-H); 2.63–2.74 (m, 1H, 17-H); 1.01–2.63 (m, 27H, ring protons, $(CH_2)_2N(CH_2)_2$, NH, OH); 2.19 (s, 3H, $COCH_3$); 1.01 (s, 3H, 19-H_3); 0.68 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 208.4; 140.8; 121.2; 71.7; 71.5; 67.0 (2C); 57.7; 54.6; 53.5 (2C); 53.3; 49.8; 44.9; 44.7; 42.3; 38.8; 37.2; 36.5; 32.4; 31.7; 31.6 (2C); 31.5; 20.8; 19.4; 14.3. HRMS: calculated for $C_{27}H_{45}N_2O_3$ $[M+H]^+$ 445.3430, found 445.3416. IR (KBr, ν(cm^{-1})): 3392, 3301, 2934, 2853, 1701, 1117. R_f(chloroform/MeOH (9:2, v/v)): 0.34.

*2.2.13. 16α-[N-(3-(1H-Imidazol-1-yl)-propyl)amino]-3β-hidroxy-pregn-5-ene-20-one (**3o**)*

^{1}H NMR (δ, $CDCl_3$, 400.13 MHz): 7.46 (s, 1H, 2′-H); 7.02 (s, 1H, 4′-H); 6.88 (s, 1H, 5′-H); 5.29–5.33 (m, 1H, 6-H); 3.89–4.04 (m, 2H, N-CH_2); 3.65–3.74 (m, 1H, 16-H); 3.44–3.56 (m, 1H, 3-H); 0.97–2.57 (m, 24H, ring protons, 17-H, $NH(CH_2)_2$, OH); 2.11 (s, 3H, $COCH_3$); 0.97 (s, 3H, 19-H_3); 0.62 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 100.62 MHz): 208.0; 140.4; 136.7; 128.8; 120.6; 118.4; 71.7; 71.0; 56.7; 54.3; 49.4; 44.3; 44.2 (2C); 41.7; 38.4; 36.7; 36.0; 32.1; 31.3; 31.1 (3C); 30.5; 20.3; 18.9; 13.8. HRMS: calculated for $C_{27}H_{42}N_3O_2$ $[M+H]^+$ 440.3277, found 440.3273. IR (KBr, ν-(cm^{-1})): 3420, 2925, 2848, 1708, 1507, 727. R_f(MeOH/chloroform (6:1, v/v)): 0.38.

*2.2.14. 16α-(1H-Imidazol-1-yl)-3β-hidroxy-pregn-5-ene-20-one (**3p**)*

^{1}H NMR (δ, $CDCl_3$, 400.13 MHz): 7.49 (s, 1H, 2′-H); 7.01 (s, 1H, 4′-H); 6.88 (s, 1H, 5′-H); 5.31–5.36 (m, 1H, 6-H); 5.20–5.27 (m, 1H, 16-H); 3.47–3.58 (m, 1H, 3-H); 2.75 (d, J = 8.0 Hz, 1H, 17-H); 1.01–2.39 (m, 18H, ring protons, OH); 2.07 (s, 3H, $COCH_3$); 1.01 (s, 3H, 19-H_3); 0.71 (s, 3H, 18-H_3). ^{13}C NMR (δ, $CDCl_3$, 125.78 MHz): 205.8; 141.0; 136.3; 129.9; 120.7; 116.7; 73.1; 71.5; 56.5; 55.6; 49.8; 45.2; 42.2; 38.7; 37.2; 36.5; 33.5; 31.7; 31.6; 31.5 (2C); 20.7; 19.4; 13.9. HRMS: calculated for $C_{24}H_{35}N_2O_2$ $[M+H]^+$ 383.2699, found 383.2697. IR (KBr, ν(cm^{-1})): 3403, 2929, 2848, 1704, 1503, 809. R_f(toluene/MeOH/EtOAc (3:1:3, v/v)): 0.35.

2.3. X-ray structural analysis

Suitable crystals of **3f** were grown from dichloromethane. Data were collected on a Gemini diffractometer (Oxford Diffraction Ltd) equipped with a Ruby CCD detector using Enhance Mo X-ray Source. Structure has been refined on F^2 using the SHELXL-2014 [16] suite of programs and data analysis was performed with PLATON [17]. A multi-scan procedure was applied to correct for absorption effects. Hydrogen atom positions were calculated and refined isotropically using a riding model. CCDC-1520874 entry contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre *via* www.ccdc.cam.ac.uk/data_request/cif.

*2.3.1. Crystal data for 16α-(N-phenyl-amino)-3β-hydroxypregn-5-ene-20-one (**3f**)*

Orthorhombic, $P2_12_12_1$, a = 6.2182(4), b = 17.0180(11), c = 24.4629(15) Å, V = 2588.7(3) Å^3, Z = 4, ρ_{calc} = 1.164 g cm^{-3}, F_{000} = 972, λMo Kα = 0.71072 Å, θ_{max} = 25.02°, 12385 total measured reflections, 4248 independent reflections (R_{int} = 0.033), 3486 observed reflections (I > 2 σ(I)), μ = 0.170 mm^{-1}, 298 parameters, R_1 (observed data) = 0.0742, R_1 (all data) = 0.0876, S = GooF = 1.045, Δ/s.u. = 0.000, residual ρ_{max} = 0.65 e Å^{-3}, ρ_{min} = –0.27 e Å^{-3}.

2.4. Determination of the inhibitory effect on rat testicular $C_{17,20}$-lyase

An *in vitro* radiosubstrate incubation method was used for the measurement of rat testicular $C_{17,20}$-lyase activity and inhibition [18,19]. In brief, tissue of testes dissected from adult Wistar rats was homogenized with an Ultra-Turrax in 0.1 M HEPES buffer (pH = 7.3) containing 1 mM EDTA and 1 mM dithiotreitol. Aliquots of this homogenate were incubated in 200 μL final volume at 37 °C for 20 min in the presence of 0.1 mM NADPH. 1 μM [^{3}H] 17-hydroxyprogesterone was added to the incubate in 20 μL of a 25 v/v% propylene glycol solution. Test compounds were introduced in 10 μL of DMSO. (These organic solvent contents did not reduce the enzyme activity substantially.) Following incubation, the androst-4-ene-3,17-dione formed and the 17-hydroxyprogesterone remaining were isolated through extraction and TLC. $C_{17,20}$-lyase activity was calculated from the radioactivity of the androst-4-ene-3,17-dione obtained. At least two experiments were performed with 50 μM concentration of each test compound. IC_{50} values were determined for the more active inhibitors. In this case, the conversion was measured at 8–10 different concentrations of the test compound. IC_{50} results were calculated using an unweighted iterative least squares method for logistic curve fitting. Reference IC_{50} parameters for the abiraterone and for the non-steroidal ketoconazole were also determined and that values were found 0.0125 μM and 0.32 μM, respectively.

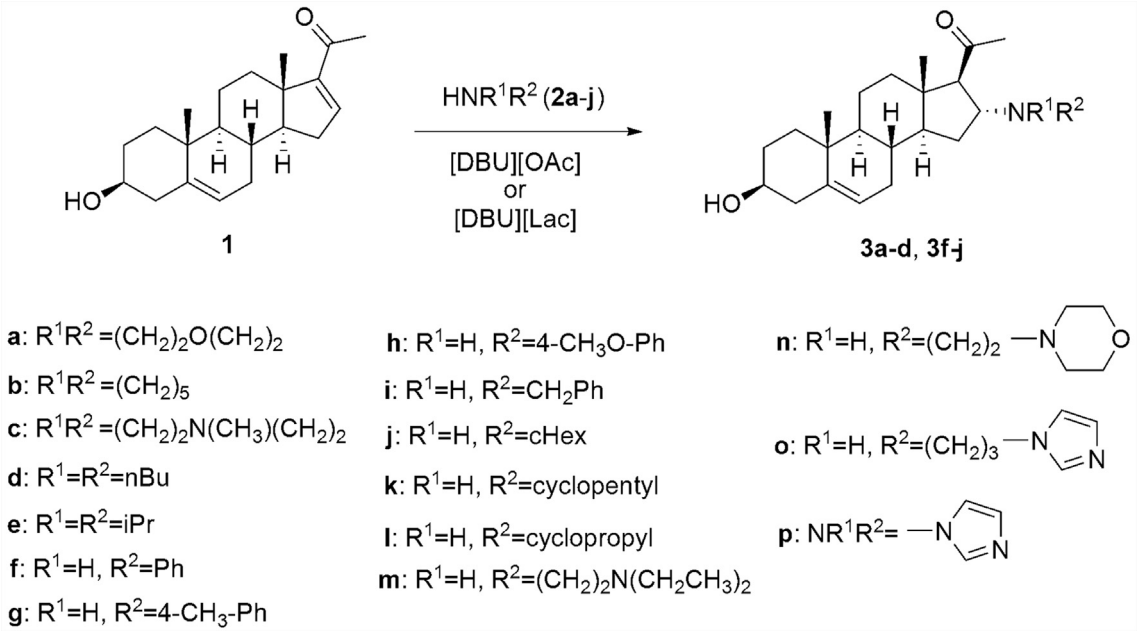
3. Results and discussion

3.1. Aza-Michael addition of different N-nucleophiles to 16-dehydropregnenolone

Aza-Michael addition of 16-dehydropregnenolone (**1**, Scheme 1) was carried out in the presence of [DBU][OAc] as catalyst and solvent. First, an equimolar amount of morpholine (**2a**) was used as nucleophile. In this case, the product (**3a**) could be isolated in 69% yield after 8 h at 65 °C (Table 1). An increase in the amount of morpholine led to compound **3a** in 89–91% yields (entries 2–4). Considerably lower conversion was observed in shorter reaction time even in the presence of a high excess of the nucleophile (entry 5).

Aza-Michael addition of different cyclic secondary amines led to the desired products in excellent yields (Table 1, entries 7–10), although a longer reaction time had to be used to achieve good conversion in case of piperidine (**2b**) (entries 8–9). Acyclic secondary amines (**2d**, **2e**) showed poor reactivity: the use of *N*,*N*-dibutylamine (**2d**) led to 23% of compound **3d** after 15 h (entry 11) and no reaction occurred in the presence of *N*,*N*-diisopropyl-amine (**2e**) (entry 12). This shows the decisive effect of the steric bulk of the amine on the outcome of the reaction. As a comparison, in the presence of piperidine with similar basicity, excellent yields were achieved under similar conditions (entries 7, 9). At the same time, the effect of basicity can be demonstrated by the results obtained using the less basic primary aromatic amines. The adducts could be obtained only in moderate to good yields even in longer reactions (entries 13, 14, 16–18). The use of primary aliphatic amines, such as cyclohexylamine (**2j**, entry 21), cyclopentylamine (**2k**, entry 22), cyclopropylamine (**2l**, entry 23), *N*,*N*-diethyl-ethylenediamine (**2m**, entry 24), 4-(2-aminoethyl)morpholine (**2n**, entry 25) also led to the products in acceptable yields.

Because of the low solubility of products **3h, 3o** and **3p** in diethyl ether, they could not be extracted from the ionic liquid using this solvent. The reaction mixtures were dissolved in dichloromethane and the organic phases were washed with water to remove [DBU][OAc]. The solvent was evaporated in vacuo and the crude products were purified by column chromatography leading to the products in 77% (**3h**, entry 18), 70% (**3o**, entry 26) and 72% (**3p**, entry 28) yield. To facilitate the separation of the products from the ionic liquid catalyst, [DBU][Lac] was chosen as catalyst and solvent in these reactions (entries 19, 27 and 29). Contrary to [DBU][OAc], this ionic liquid is insoluble in toluene, so products **3h, 3o** and **3p** could easily be extracted with this solvent. It should be mentioned however that [DBU][OAc] was found to be a more efficient catalyst. The aza-Michael addition with benzylamine was carried out in [DBU][Lac]



Scheme 1. Aza-Michael addition of different *N*-nucleophiles to 16-dehydropregnenolone (**1**) in the presence of basic ionic liquids.

too (entry 20), because of similar solubility problems of steroid **3i** in diethyl ether.

The efficiency of the ionic liquid-catalyzed reaction was compared to the use of the solvent-free conditions applied by Kumar et al. for the aza-Michael addition of primary amines [10]. Therefore the solvent-free reaction was studied using two different amines, morpholine (entry 6) and aniline (entry 15), but the formation of only traces of products was observed in both cases. As a comparison, in the presence of [DBU][OAc] the appropriate products, **3a** (entry 4) and **3f**, (entry 13) were isolated in 91 and 46% yield, respectively. This clearly shows the catalytic activity of [DBU][OAc] and the necessity of using a catalyst in the reactions of less basic amines.

The recyclability of the ionic liquid catalyst was also studied. Upon completion of the reaction, the product was extracted with diethyl ether, and the ionic liquid was dried to remove the solvent under vacuum. The recovered ionic liquid was used in the next run. The ionic liquid was reused four times efficiently (Fig. 1). A decrease of activity was observed only during the fifth use of [DBU][OAc], this could be explained by a small loss of ionic liquid upon reuse.

The products were characterized using ^{1}H and ^{13}C NMR spectroscopy and high resolution mass spectrometry. The stereochemistry of the products was confirmed by COSY and NOESY experiments showing

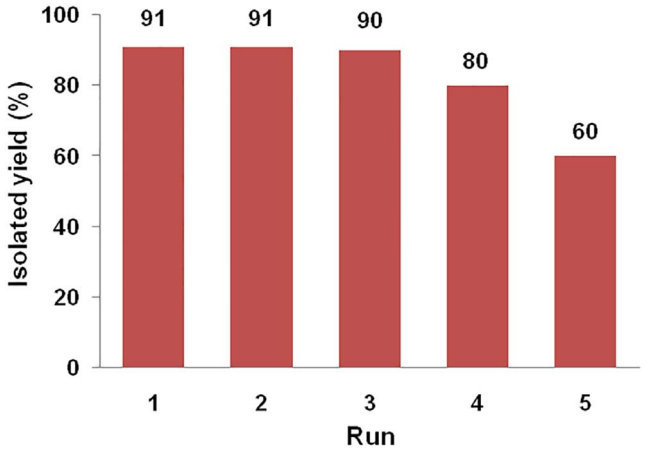


Fig. 1. Reuse of [DBU][OAc] in aza-Michael addition of **1** and morpholine (**2a**).

the selective formation of 17β-acetyl-16α-amino derivatives. In case of compound **3f**, crystals, suitable for X-ray measurements could be grown in dichloromethane. The compound crystallized as a solvate. The dichloromethane solvent molecule is highly disordered in the structure. However, the X-ray data supported the proposed structure of the adducts. In particular, the crystal structure of **3f** confirms the α-disposition of the C-16 substituent (Fig. 2).

3.2. $C_{17,20}$-lyase inhibition

The aza-Michael adducts were studied against the *in vitro* $C_{17,20}$-lyase activity of the rat testicular $P450_{17\alpha}$. The imidazole derivative (**3p**) was found to be the most potent inhibitor, displaying an IC_{50} value of 1.8 μM. The cyclohexylamine (**3j**), the cyclopentylamine (**3k**) and the *N,N*-diethyl-ethylenediamine (**3m**) compounds showed moderate inhibitory effect, their IC_{50} values were 9.1 μM, 9.5 μM and 9.3 μM, respectively. The morpholine (**3a**), *N*-methyl-piperazine (**3c**), benzylamine (**3i**) and 4-(2-aminoethyl)morpholine (**3n**) derivatives found to be somewhat less effective inhibitions. Derivatives possessing piperidine (**3b**), aniline (**3f**), 4-methylaniline (**3g**), 4-methoxyaniline (**3h**), cyclopropylamine (**3l**) or 1-(3-aminopropyl)imidazole (**3o**) groups in the C-16 side chains proved to be weak inhibitors under our experimental conditions. These compounds did not suppress relative conversion to below 50% when the 50 μM test concentration was applied, hence their IC_{50} values exceed 50 μM.

Investigated compounds have moderate inhibitory potential in comparison to the reference inhibitors abiraterone and ketoconazole which are applied in the medical practice. Interesting to note that some of the more potent inhibitors, **3j** and **3k** bear a saturated ring with no heteroatom or substituent to be able to donate lone electron pair. This finding indicates that similar derivatives may show reasonable affinity to the enzyme and development of this group of compounds for studying the inhibition and for targeting new inhibitors of the $P450_{17\alpha}$ may be promising.

4. Conclusions

A series of 16-dehydropregnenolone derivatives were synthesized *via* an ionic liquid promoted aza-Michael addition of different *N*-nucleophiles. A DBU based ionic liquid was found to be efficient and reusable catalyst of the conjugate addition. The Michael adducts were

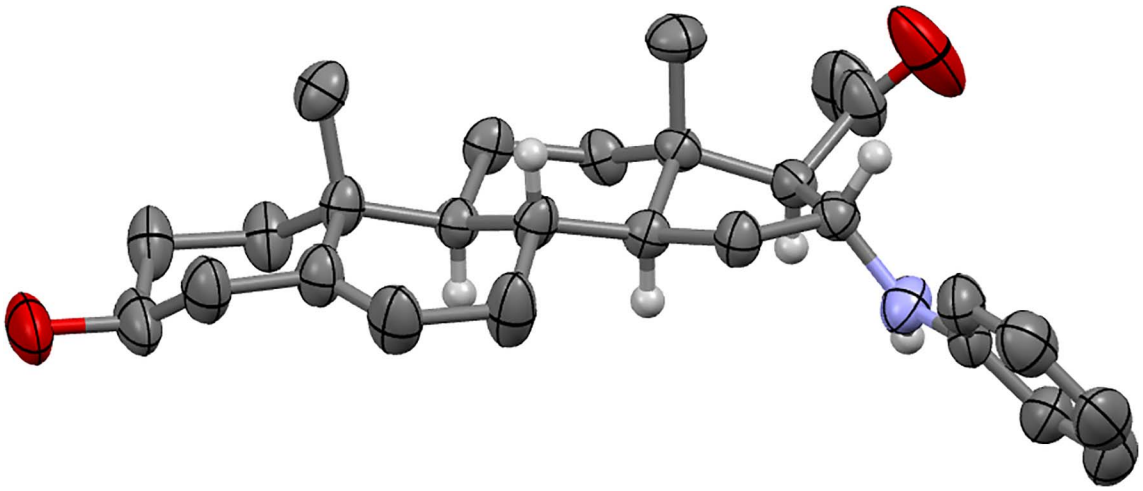


Fig. 2. Solid state structure (ORTEP diagram drawn at 50% probability) of **3f.**

screened for their inhibitory effect of $P450_{17\alpha}$ *in vitro* and **3p** was the most active in this study. The X-ray structure of **3f** confirmed the α-disposition of the C-16 substituent.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2017.05.006.

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BEILSTEIN JOURNAL OF ORGANIC CHEMISTRY

Synthesis of novel 13α-estrone derivatives by Sonogashira coupling as potential 17β-HSD1 inhibitors

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Abstract

Novel 13α-estrone derivatives were synthesized by Sonogashira coupling. Transformations of 2- or 4-iodo regioisomers of 13α-estrone and its 3-methyl ether were carried out under different conditions in a microwave reactor. The 2-iodo isomers were reacted with *para*-substituted phenylacetylenes using $Pd(PPh_3)_4$ as catalyst and CuI as a cocatalyst. Coupling reactions of 4-iodo derivatives could be achieved by changing the catalyst to $Pd(PPh_3)_2Cl_2$. The product phenethynyl derivatives were partially or fully saturated. Compounds bearing a phenolic OH group furnished benzofurans under the conditions used for the partial saturation. The inhibitory effects of the compounds on human placental 17β-hydroxysteroid dehydrogenase type 1 isozyme (17β-HSD1) were investigated by an in vitro radiosubstrate incubation method. Certain 3-hydroxy-2-phenethynyl or -phenethyl derivatives proved to be potent 17β-HSD1 inhibitors, displaying submicromolar IC_{50} values.

Introduction

Synthetic modifications of the naturally occurring female prehormone estrone may lead to compounds with diverse biological activities, for example with antitumor effect [1]. One of the main requirements of estrone anticancer derivatives is the lack of their hormonal activity. Several core-modified estrones have recently been produced and diversified in order to get selectively acting compounds [2-4]. One opportunity for that is the inversion of the configuration at C-13, which is accompanied by drastic conformational change for the overall molecule resulting from the *cis* junction of rings C and D [2]. The influence of inversion of the configuration at C-13 in 3,17-estradiols on their in vivo and in vitro estrogenic activity was shown by Poirier et al. [5]. They demonstrated that 13 epimers exhibit no substantial binding affinity for the estrogen receptor alpha and no uterotropic activity. Accordingly, the 13α-estrane core may serve as fundamental moiety for the design of hormonally inactive estrone derivatives bearing promising biological activities. We recently published the syntheses and the in vitro biological

evaluations of several 13α-estrone derivatives [6-9]. Certain compounds proved to be biologically active, bearing substantial antiproliferative or enzyme inhibitory potential [7,8]. Most literature data are mainly about 13α-estrones substituted in ring D, but compounds modified in ring A are rarely described [10,11]. More recently we have disclosed ring A halogenations in this series [12]. Electrophilic brominations or iodinations were carried out, furnishing 2-, 4- or 2,4-bis-halogenated compounds. All the halogenated 3-hydroxy and the 4-substituted regioisomers of 3-methyl ethers displayed substantial inhibitory activity against the 17β-hydroxysteroid dehydrogenase type 1 enzyme (17β-HSD1). Certain derivatives displayed a similar or more pronounced effect than those of their parent compounds 13α-estrone or 13α-estrone 3-methyl ether [13]. The 17β-HSD1 enzyme is responsible for the stereospecific reduction of prehormone estrone into the main estrogenic hormone 17β-estradiol [14,15]. 17β-Estradiol may enhance the proliferation of certain cancer cells [16]. The inhibition of 17β-HSD1 provokes an antitumor effect in hormone dependent cancers, hence 17β-HSD1 inhibitors could have good prospects as anti-estrogen therapeutics [17,18]. The recently synthesized halogenated 13α-estrones, in addition to their pharmacological importance, may serve as appropriate starting compounds for Pd-catalyzed C–C coupling reactions. Some Sonogashira couplings on estrane, but not on the 13α-estrane core have been performed at C-2, -3, -11, -16 and -17. To the best of our knowledge, 4-coupled regioisomers have not been synthesized to date [19]. Couplings of steroidal alkynes with small molecular halides are already described, and reactions of steroidal halides or triflates with small molecular alkynes also exist [20]. Certain phenethynyl estrone derivatives described in the literature possess substantial biological activities. Möller et al. performed the couplings of 2-iodoestrone-3-acetate with phenylacetylene using $Pd(OAc)_2$ and CuI as catalysts [21]. They did not investigate the influence of the nature of the substituent on the phenyl ring of the acetylene on the course of the reactions. They carried out the full saturation of the C≡C bond of the 2-phenethynyl estrone with palladium on charcoal, furnishing the 2-phenethyl-substituted derivative. However, they did not study the partial saturation of the estrone alkyne moiety. The 2-phenethyl and 2-phenethynyl derivatives proved to be potent 17β-HSD1 inhibitors with the fully-saturated compound being slightly more potent.

The aim of the present study was to develop facile and effective Sonogashira coupling methods for the preparation of 2- or 4-phenethynyl derivatives in the 13α-estrone series. 2- or 4-iodo-13α-estrone and their 3-methyl ethers were chosen as starting compounds. The partial or full saturation of the C≡C bond of certain 2- or 4-regioisomeric phenethynyl compounds was also planned. We intended to investigate the potential inhibitory effects of the novel 13α-estrones toward human placental 17β-HSD1 activity in vitro.

Results and Discussion

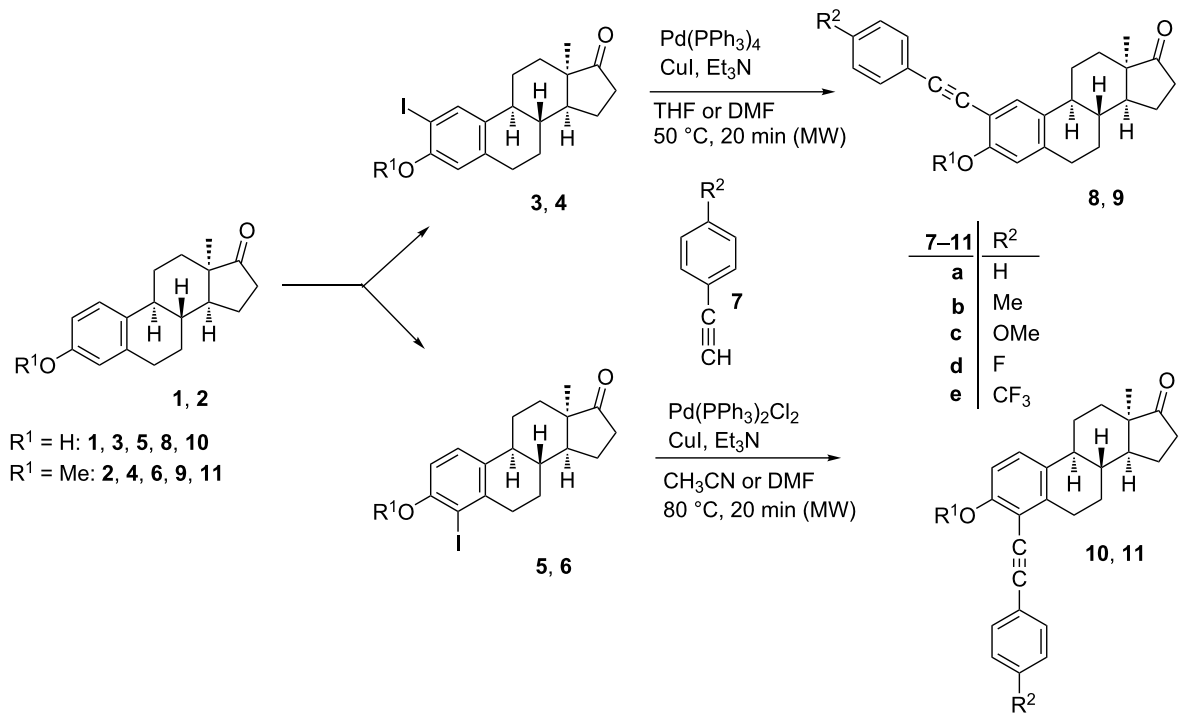
Synthetic work

Sonogashira coupling

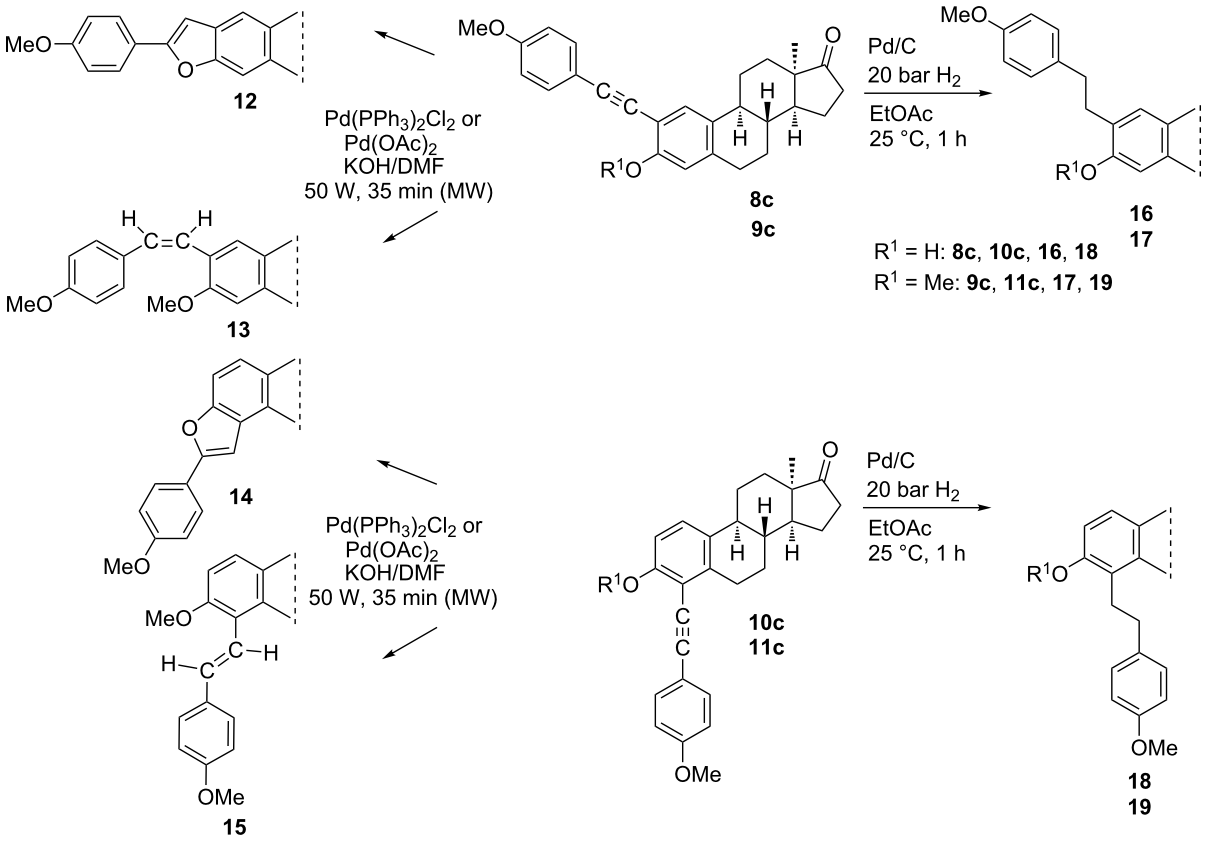
Iodo compounds **3**–**6** synthesized recently have been chosen as starting materials for the Sonogashira couplings, since the reactivity of the aryl iodides is higher than that of their bromo counterparts (Scheme 1) [22]. The optimizations of the coupling reactions were carried out using phenylacetylene (**7a**) as a model reagent. The optimal reaction conditions were found to differ depending on the position of the iodo substituent on the sterane skeleton (Scheme 1). Couplings at C-2 could efficiently be achieved using 0.1 equiv of $Pd(PPh_3)_4$ and CuI in tetrahydrofuran (THF) or dimethylformamide (DMF) as solvent in the presence of Et_3N as a base at 50 °C for 20 min in a microwave reactor. 4-Phenylalkynyl regioisomers (**10a**, **11a**) were obtained in high yields using 0.05 equiv of $Pd(PPh_3)_2Cl_2$ and CuI in CH_3CN or DMF, in the presence of Et_3N as a base at 80 °C for 20 min in a microwave reactor. After establishing the most favorable reaction conditions, the Sonogashira reactions (of both regioisomers) were carried out with several *para*-substituted phenylacetylenes (**7b**–**e**). All the couplings resulted in the desired products (**8**–**11**) in high yields. The newly synthesized 4-phenethynyl derivatives are the first 4-substituted Sonogashira coupled estrones in the literature. The structures of the new compounds were confirmed by ^1H, ^{13}C and two-dimensional NMR measurements (see Supporting Information File 1).

Full and partial saturation of the alkyne moiety

We have chosen four 4”-methoxy-substituted phenylalkynyl compounds (**8c**–**11c**) for partial or full saturation of the C≡C bond in both the 3-OH and the 3-OMe series (Scheme 2). The *trans* counterpart of the resulting diphenylethenyl moiety is related to the fully-methoxylated derivative of resveratrol (3,5,4’-trihydroxystilbene), a compound exhibiting diverse biological activities [23,24]. The chemo- and stereoselective semihydrogenation of internal alkynes may be achieved by two main catalytic methods: with molecular hydrogen using Lindlar’s catalyst [25,26] or by transfer hydrogenation with hydrogen donors [27,28]. Additionally, alkynes undergo reduction with diimide to produce *cis*-alkenes [29]. Li et al. carried out the semihydrogenation of different arylacetylenes using $Pd(OAc)_2$ or $Pd(PPh_3)_2Cl_2$ as the catalyst and DMF/KOH as a hydrogen source, under conventional heating [30]. The first catalyst afforded *cis*-alkenes in high yields with excellent chemo- and stereoselectivity. The latter catalyst displayed lower catalytic activity and stereoselectivity. The stereoselectivity of the semihydrogenation process may play a crucial role concerning the biological activity of the resulting alkenes, since geometrical



Scheme 1: Syntheses of 2- or 4-phenethynyl-13α-estrones (**8**–**11**) by Sonogashira coupling.



Scheme 2: Partial or full hydrogenation of compounds **8c**–**11c**.

isomers may possess different biological functions [31]. Here we performed the partial saturation of compounds **8c**–**11c** by the modified procedure of Li et al. using $Pd(OAc)_2$ or $Pd(PPh_3)_2Cl_2$ as a catalyst, and DMF/KOH as a hydrogen source, in a microwave reactor. The *cis*-alkene **13** and the *trans*-alkene **15** were formed chemo- and stereoselectively under the applied conditions. The different stereochemical outcome of the hydrogenations of the two regioisomers presumably arose from the steric hindrance caused by the vicinity of ring B in the case of compound **15**.

The *cis* or *trans* orientation of the resulting geometric isomers was deduced from the vicinal coupling constants according to the literature data, because *cis* and *trans* couplings across a double bond are very reliable indicators of stereochemistry [32,33]. In the case of the 2-regioisomer **13**, the signals of the vicinal olefinic protons appear as a singlet with double intensity, similar to those of 2,4'-dimethoxystilbene [32,33]. In the ^{1}H NMR spectrum of the 4-substituted counterpart **15**, the olefinic protons are shown as doublets with a large coupling constant of 12.2 Hz, which refers to their *trans* arrangement. Under the conditions used for the partial saturation, the ethynyl derivatives bearing a phenolic OH group (**8c**, **10c**) furnished benzo[*b*]furans **12** and **14**. There are literature reports about similar transition-metal-catalyzed cyclizations of *o*-alkynylphenols to construct benzofurans [34,35]. These heterocycles are important structural units in a variety of biologically active natural or synthetic compounds [36,37]. Full hydrogenation of the 2- or 4-phenethynyl intermediates (**8c**–**11c**) with palladium-on-charcoal furnished the 2- or 4-phenethyl-substituted derivatives (**16**–**19**).

In vitro 17β-HSD1 enzyme inhibition test

With the new compounds in hand (**8**–**19**, Table 1), we also determined their in vitro inhibitory potencies on human placental 17β-HSD1. In the 3-OH series, all the 2-phenylalkynyl regioisomers **8a**–**e** proved to be effective inhibitors with IC_{50} values depending on the nature of the 4"-functional group. The most potent compound was unsubstituted **8a** with an IC_{50} of 0.15 μM. The 4-substituted regioisomers **10a**–**e** inhibited the enzyme scarcely, suppressing the conversion by less than 15%. The phenylalkynyl derivatives in the 3-OMe series **9a**–**e** and **11a**–**e** exerted weak inhibitions. Phenylalkenyl compounds **13** and **15** and benzofuran compounds **12** and **14** displayed weaker inhibitory activity than their alkynyl counterparts **8c** and **10c**. The full saturation (leading to compounds **16**–**19**) did not influence the inhibitory potential markedly. The weak inhibitory activities of **9c**, **10c** or **11c** were not improved in compounds **17**, **18** or **19**, whereas the good inhibitory effect of the 2-regioisomer **8c** was retained in compound **16**.

Table 1: 17β-HSD1 inhibition data of Sonogashira coupled compounds and their precursors (**1**–**6**) [12,13] indicated with an asterisk (*).

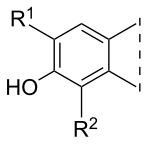
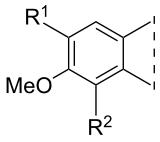
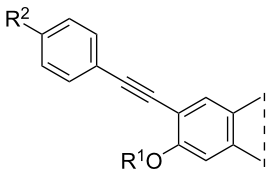
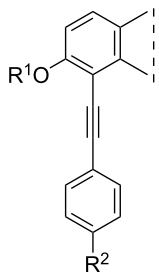
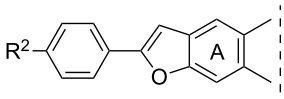
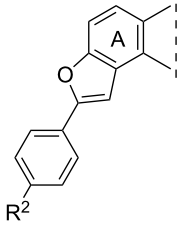
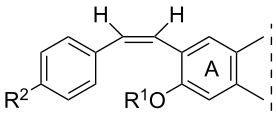
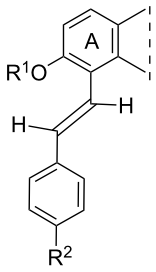
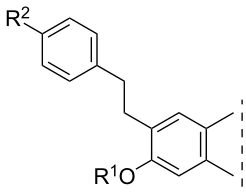
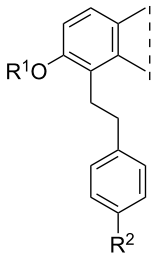
Structure	Compound	R^1	R^2	Relative conversion[a] ± SD (%) or IC_{50} ± SD (μM)
	**1**	H	H	IC_{50} = 1.2*
	3	I	H	IC_{50} = 0.59*
	5	H	I	IC_{50} = 1.0*
	**2**	H	H	IC_{50} = 5.5*
	4	I	H	>10*
	6	H	I	IC_{50} = 0.56*
	**8a**	H	H	IC_{50} = 0.15 ± 0.02
	8b		Me	IC_{50} = 1.40 ± 0.78
	8c		OMe	IC_{50} = 0.23 ± 0.03
	8d		F	IC_{50} = 0.30 ± 0.08
	8e		CF_3	IC_{50} = 0.93 ± 0.13
	9a		H	88 ± 12
	9b		Me	84 ± 5
	9c	Me	OMe	85 ± 1
	9d		F	94 ± 5
	9e		CF_3	76 ± 1

Table 1: 17β-HSD1 inhibition data of Sonogashira coupled compounds and their precursors (**1**–**6**) [12,13] indicated with an asterisk (*). (continued)

	**10a**	H	H	88 ± 12
	10b		Me	84 ± 5
	10c		OMe	85 ± 1
	10d		F	94 ± 5
	10e		CF_3	76 ± 1
	11a	Me	H	92 ± 15
	11b		Me	89 ± 0.4
	11c		OMe	91 ± 2
	11d		F	96 ± 7
	11e		CF_3	85 ± 1
	**12**	–	OMe	92 ± 2
	**14**			102 ± 6
	**13**	Me	OMe	70 ± 6
	**15**			80 ± 12
	**16**	H	OMe	IC_{50} = 0.47 ± 0.04
	17	Me		63 ± 8
	**18**	H	OMe	98 ± 3
	19	Me		94 ± 1

[a]At 10 μM, non-inhibited control 100%. Reference for precursors (**1**–**6**) [12,13].

When all the inhibition data of the novel compounds and their precursors from Table 1 are taken into consideration, some valuable structure–activity relationships appear. 13α-Estrone (**1**) displays 17β-HSD1 inhibitory potential similar to that of the natural substrate estrone. Iodination at C-2 of **1** improves the inhibitory potential, resulting in a submicromolar IC_{50} for compound **3**. Phenylalkynylation of the 2-iodo compound **3** retains or further improves the inhibition, depending on the nature of the substituent at C-4". Concerning the 4-regioisomers, iodination leads to an efficiency similar to that of compound **1**, whereas the inhibition is lost following C–C coupling. 13α-Estrone 3-methyl ether **2** possesses a weaker inhibitory effect than the 3-hydroxy compound **1**. Iodination or phenylalkynylation at C-2 diminishes inhibition of **2**. Introducing iodine onto C-4 of compound **2** leads to a 10-fold decrease in its IC_{50} value. 4-Phenylalkynyl derivatives **10** and **11**, nevertheless, exert weak inhibitions on the estrone to 17β-estradiol conversion

The results reveal a great influence of the 2,4-regioisomerism on the inhibition potential of the iodinated 3-methyl ethers **4** and **6**, the phenylalkynyl **8** and **10** and the phenylalkyl **16** and **18** 3-hydroxy compounds.

Conclusion

In conclusion, we described here an efficient synthetic microwave procedure for the synthesis of novel phenylalkynyl derivatives of 13α-estrone (**1**) and its 3-methyl ether **2**. The steroidal alkynes were chemo- and stereoselectively hydrogenated by transfer hydrogenation in a microwave reactor, furnishing alkenes or benzofurans depending on the nature of the substituent at C-3. Full hydrogenations of certain phenethynyl derivatives were also achieved. The newly-synthesized potent 17β-HSD1 inhibitors may serve as suitable tools for ligand-based enzyme studies. Further derivatizations of our compounds may provide promising candidates for drug development in order to get nanomolar inhibitors.

Supporting Information

Supporting Information File 1

Experimental procedures for compounds **8**–**19** and their ^{1}H, ^{13}C NMR, MS, elemental analysis data.
[http://www.beilstein-journals.org/bjoc/content/private/download/AXE7TONJJKIGDWQUZXHNHYVKZ4.pdf]

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License and Terms

VI.

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Synthesis of novel 17-triazolyl-androst-5-en-3-ol epimers via Cu(I)-catalyzed azide-alkyne cycloaddition and their inhibitory effect on 17α-hydroxylase/$C_{17,20}$-lyase

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ABSTRACT

The regioselective Cu(I)-catalyzed 1,3-dipolar cycloaddition of 17α- and 17β-azidoandrost-5-en-3β-ol epimers (**3b** and **5b**) with different terminal alkynes afforded novel 1,4-substituted triazolyl derivatives (**8a–k** and **9a–k**). For the preparation of 5′-iodo-1′,2′,3′-triazoles (**8m–n** and **9m–n**), an improved method was developed, directly from steroidal azides and terminal alkynes, in reaction mediated by CuI and ICl as iodinating agents. Acetolysis and subsequent hydrolysis of **8n** and **9n** yielded 5′-hydroxy-1′,2′,3′-triazoles **8o** and **9o**. The inhibitory effect of **8a–o**, **9a–o**, **3**, and **5** on rat testicular $C_{17,20}$-lyase was investigated by means of an *in vitro* radioincubation technique. The results revealed that the C-17 epimers of steroidal triazoles influence the $C_{17,20}$-lyase effect. Inhibitors were found only in the 17α-triazolyl series (**8a–o**), whereas in the C-17 azide pair the 17β compound (**5b**) was more potent.

1. Introduction

Prostate cancer is an age-related disease and a major cause of cancer-related mortality worldwide. It is androgen dependent in the majority of cases and can be treated with androgen ablation. Steroidal precursors are transformed to preandrogens by the 17α-hydroxylase-$C_{17,20}$-lyase enzyme ($P450_{17\alpha}$), which is highly active in both testes and adrenals [1]. Inhibitors of this enzyme can block androgen synthesis in its early steps, and may be suitable for pharmacological treatment of androgen dependent disorders, among them the prostate cancer [2–4]. In the past decades a large number of compounds were developed as $P450_{17\alpha}$ inhibitors [5,6]. The most promising compounds have been designed by the ligand-base approach. These are steroidal derivatives possessing heterocycle on the C-17 position. Heterocycles containing sp^2 hybrid N were demonstrated to be the most effective coordinating groups to the heme iron of the enzyme active center. The $\Delta^{16,17}$ double bond can significantly increase inhibitory potency [7]. Abiraterone [17-(3-pyridyl)androsta-5,16-dien-3β-ol] and Galeterone [17-(1H-benzimidazole-1-yl)androsta-5,16-dien-3β-ol] have become the most successful drug candidates of this group of compounds [8,9].

We previously described the synthesis of series of C-17β heterocyclic steroids, and investigated their inhibitory activity against $C_{17,20}$-lyase *in vitro* [10–15]. As a continuation of our research program we set out to prepare 1,4-substituted-1,2,3-triazolyl derivatives in the androstane series, in which the heterocycle is attached to the steroid C-17 position.

The Huisgen 1,3-dipolar cycloaddition of organic azides and terminal alkynes has been of considerable interest in recent years following the independent introduction of Cu(I) catalysis in 2002 by the research groups of Sharpless [16] and Meldal [17]. The catalyst dramatically improves both the rate and the regioselectivity of the reaction leading exclusively to 1,4-disubstituted 1,2,3-triazoles.

The synthetic routes involve the introduction of an azide group into the C-17 position of the sterane framework and the subsequent ring closure of the steroidal azid with a terminal acetylene in the presence of a Cu(I) source. The copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) is convenient for the regioselective construction of 1,4-disubstituted triazoles in which the heterocycle is attached directly to the steroid nucleus through a nitrogen atom.

The position and steric orientation of the azide group are determined by the synthetic method applied. The C-17 azide group is generally in the α position, because the nucleophilic exchange reaction

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of 17β-tosylate or mesylate by sodium azide takes place with Walden inversion [18]. Here we decided to synthesize not only the 17α-azides, but a novel series of 17β-azide epimers, as starting materials for CuAAC in order to obtain novel 1,4-disubstituted triazolyl androstane derivatives with varied terminal alkynes as reagents.

We set out to obtain answers to the following questions: (1) how the CuAAC process influenced by the steric structure of the steroidal azides, and (2) how the inhibitory activity against $C_{17,20}$-lyase in the C-17 epimer series differ.

2. Experimental

2.1. General

Melting points (mp) were determined on a Kofler block and are uncorrected. Specific rotations were measured in $CHCl_3$ (*c* 1) at 20 °C with a POLAMAT-A (Zeiss-Jena) polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. Elementary analysis data were determined with a Perkin-Elmer CHN analyzer model 2400. The reactions were monitored by TLC on Kieselgel-G (Merck Si 254F) layers (0.25 mm thick); solvent systems (ss): (A) isopropyl ether, (B) acetone/toluene/hexane (30:35:35, v/v). The spots were detected by spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid. The R_f values were determined for the spots observed by illumination at 254 and 365 nm. Flash chromatography: silica gel 60, 40–63 μm. All solvents were distilled prior to use. NMR spectra were recorded on a Bruker DRX 500 instrument at 500 (^{1}H NMR) or 125 MHz (^{13}C NMR). Chemical shifts are reported in ppm (δ scale), and coupling constants (*J*) in Hertz. For the determination of multiplicities, the *J*-MOD pulse sequence was used.

2.2. 3β-Acetoxy-androst-5-ene-17β-p-toluenesulfonate (2)

3β-Acetoxy-androst-5-en-17β-ol (**1**, 16.6 g, 50 mmol) was dissolved in pyridine (100 m), and a solution of *p*-toluenesulfonyl chloride (14.25 g, 75 mmol) in anhydrous pyridine (50 ml) was added during cooling with ice. The reaction mixture was allowed to stand for 24 h and then poured onto a mixture of ice (500 g) and concentrated H_2SO_4 (50 ml). The crystalline precipitate separating out was filtered off, washed thoroughly with water, and recrystallized from a mixture of acetone and water to give pure **2** (21.55 g, 88%). Mp: 165–166 °C, R_f = 0.80 (ss A); $[\alpha]_D^{20}$ = −70 (*c* 1 in $CHCl_3$) (Ref. [19] 162–164 °C, $[\alpha]_D^{20}$ = −72, *c* 1 in ethanol). (Found C, 68.95; H, 7.94. $C_{28}H_{38}O_5S$ requires C, 69.10; H, 7.87%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.80 and 0.99 (s, 3H, 18-H_3, and s, 3H, 19-H_3), 2.01 (s, 3H, Ac-CH_3), 2.44 (s, 3H, Ts-CH_3), 4.24 (t, 1H, *J* = 8.0 Hz, 17-H), 4.57 (m, 1H, 3-H), 5.33 (d, 1H, *J* = 4.5 Hz, 6-H), 7.32 (d, 2H, *J* = 8.0 Hz, 3′- and 5′ -H), 7.77 (d, 2H, *J* = 8.0 Hz, 2′- and 6′-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 11.6 (C-18), 19.3 (C-19), 20.3, 21.4, 21.6, 23.3, 27.6, 31.2, 31.6, 35.9, 36.6(C-3), 36.9, 38.0, 42.8 (C-13), 49.8, 50.2, 73.7 (Ac-CH_3), 89.9 (C-17), 121.9 (C-6), 127.8 (C-2′ and C-6′), 129.6 (C-3′ and C-5′), 134.2 (C-4′), 139.8, 144.3 (C-1′), 170.4 (C═O).

2.3. 3β-Acetoxy-androst-5-en-17α-azide (3a)

Compound **2** (9.73 g, 20 mmol) was dissolved in *N,N*-dimethylformamide (150 ml), and NaN_3 (5.2 g, 80 mmol) was added. The mixture was stirred for 48 h at 100 °C, then poured into water (600 ml). The precipitate separating out was filtered off and washed with water. The residue obtained was dissolved in CH_2Cl_2 and chromatographed on silica gel with CH_2Cl_2/hexane (1:1, v/v) to give **3a** (5.6 g, 78%) as a white solid. Mp: 153–155 °C, R_f = 0.85 (ss A); $[\alpha]_D^{20}$ = −113 (*c* 1, in $CHCl_3$). (Found: C, 70.76; H, 8.62; N, 11.93. $C_{21}H_{31}N_3O_2$ requires C, 70.55; H, 8.74; N, 11.75%.) ^{1}H NMR (δ, ppm, $CDCl_3$): 0.75 and 1.01 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 2.02 (s, 3H, Ac-H_3), 3.52 (d, 1H, *J* = 6.5 Hz, 17-H), 4.60 (m, 1H, 3-H), 5.37 (d, 1H *J* = 4.5 Hz, 6-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.4 (Ac-CH_3, 19.3 (C-18), 20.5, 21.4 (C-19), 24.7, 27.7, 28.6, 32.0, 32.1 (C-8), 32.4, 36.6, 37.0, 38.0, 45.7 (C-13), 49.6 (C-9), 49.8 (C-14), 71.4 (C-17), 73.8 (C-3), 122.3 (C-6), 139.6 (C-5), 170.5 (C═O).

2.4. 17α-Azido-androst-5-en-3β-ol (3b)

Compound **3a** (3.57 g, 10 mmol) was dissolved in methanol (100 ml) containing $NaOCH_3$ (108 mg, 2 mmol), and the solution was allowed to stand for 24 h. It was then diluted with water and the white precipitate separating out was separated by filtration and recrystallized from a mixture of acetone/water to give **3b** (3.15 g, 92%). Mp: 132–134 °C, R_f = 0.65 (ss B); $[\alpha]_D^{20}$ = −118 (*c* 1, in $CHCl_3$). (Found: C, 72.47; H, 9.05; N, 13.51. $C_{19}H_{29}N_3O$ requires C, 72.34; H, 9.27; N, 13.32%.) ^{1}H NMR (δ, ppm, $CDCl_3$): 0.77 and 1.01 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.51 (m, 2H, 3-H and –OH), 5.35 (t, 1H, *J* = 2.5 Hz, 6-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.4 (C-18), 19.4 (C-19), 20.7, 24.8, 28.7, 31.6, 31.7, 32.1, 32.3, 32.6, 36.6, 37.3, 42.3, 45.8, 49.9, 50.0, 71.6 (C-17), 71.7 (C-3), 121.4 (C-6), 140.8 (C-5).

2.5. 3β-Acetoxy-androst-5-en-17α-iodide (4)

Compound **1** (5 g, 15 mmol), Ph_3P (15.78 g, 60.15 mmol), and imidazol (4.10 g, 60.15 mmol) were dissolved in toluene (250 ml) and I_2 (15.26 g, 60.15 mmol) was added in two portions. The reaction mixture was stirred at 80 °C for 2 h and allowed to cool to room temperature. A saturated aqueous Na_2SO_3 solution (150 ml) was then added and the resulting mixture was stirred until all solids dissolved. EtOAC (100 ml) was added, and the organic phase was washed with saturated aqueous $NaHCO_3$ (2 × 100 ml) and brine (100 ml), dried over Na_2SO_4 and evaporated *in vacuo*. The residue was subjected to column chromatography on silica gel in CH_2Cl_2/hexane (1:3, v/v) to yield **4** (5.27 g, 79%) as a white solid. Mp: 162–164 °C, (Ref. [7] 164–166 °C), R_f = 0.80 (ss A); $[\alpha]_D^{20}$ = –138 (*c* 1 in $CHCl_3$). (Found: C, 56.93; H, 7.27. $C_{21}H_{31}IO_2$ requires C, 57.02; H, 7.06%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.84 and 1.02 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 2.30 (s, 3H, Ac-CH_3), 2.79 (m, 1H, 6-H), 4.36 (d, 1H, *J* = 6.5 Hz, 1 = H), 4.60 (m, 1H, 3-H), 5.39 (s, 1H, 6-H). ^{13}C NMR (δ, ppm, $CDCl_3$):15.5 (C-18), 19.4 (C-19), 21.4 (Ac-CH_3), 21.8, 25.2, 27.7, 31.9, 32.5, 36.6, 36.8, 37.0, 38.1, 40.6, 45.1, 47.7, 49.4 (C-2), 73.8 (C-3), 122.3 (C-6), 139.6 (C-5), 170.4 (Ac-C═O).

2.6. 3β-Acetoxy-androst-5-en-17β-azide (5a) and 3β-acetoxy-androst-5,16-diene (6)

Compound **4** (4.42 g, 10 mmol) was dissolved in *N,N*-dimethylformamide (150 ml), and NaN_3 (5 g, 75 mmol) was added. The mixture was stirred for 32 h at 60 °C, then poured onto ice (500 g). The resulting emulsion was extracted with CH_2Cl_2 (3 × 150 ml). The CH_2Cl_2 phase was washed with water, dried over Na_2SO_4, evaporated *in vacuo* and subjected to chromatographic separation on silica gel in CH_2Cl_2/hexane (1:3 v/v) to yield **6** (1.32 g, 42%) as a slowly-crystallizing colourless oil. Mp: 45–46 °C, R_f = 0.85 (ss A); $[\alpha]_D^{20}$ = – 92 (*c* 1 in $CHCl_3$). (Found: C, 80.37; H, 9.46. $C_{21}H_{30}O_2$ requires: C, 80.21; H, 9.62%). ^{1}H NMR (δ ppm, $CDCl_3$): 0.78 and 1.06 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 2.03 (s, 3H, Ac-CH_3), 2.33 (m, 2H, 15-H_2), 4.61 (m, 1H, 3-H), 5.39 (d, 1H, *J* = 5.0 Hz, 6-H), 5.70 (m, 1H, 16-H), 5.84 (dd, 1H, *J* = 5.0 Hz, *J* = 1.0 Hz, 17-H). ^{13}C NMR (δ, ppm $CDCl_3$): 16.8 (Ac-CH_3), 19.2 (C-18), 21.4 (C-19), 27.7, 30.4 (C-8), 31.7, 32.1, 35.8, 36.9, 26.9, 38.1, 45.3 (C-13), 50.8 (C-9), 56.1 (C-14), 73.9 (C-3), 122.5 (C-6), 129.3 (C-16), 139.9 (C-5), 143.8 (C-17), 170.5 (C═O). Continued elution resulted in **5a** (1.64 g, 46%) as a white solid. Mp: 143–145 °C, R_f = 0.75 (ss A); $[\alpha]_D^{20}$ = −68 (*c* 1 in $CHCl_3$). (Found: C, 70.76; H, 8.55; N, 11.94. $C_{21}H_{31}N_3O_2$ requires C, 70.55; H, 8.74; N, 11.75%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.76 and 1.02 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.32 (t, 1H, *J* = 9.0 Hz, 17-H), 4.59 (m, 1H, 3-H), 5.36 (d, 1H,

J = 4.5 Hz, 6-H).

^{13}C NMR (δ, ppm, $CDCl_3$): 12.1 (Ac-CH_3), 19.3 (C-18), 20.5, 21.4 (C-19), 23.6, 26.9, 27.7, 31.5, 31.9 (C-8), 36.6, 37.0, 38.0, 44.2 (C-13), 49.9 (C-9), 52.3 (C-14), 71.2 (C-17), 73.8 (C-3), 122.1 (C-6), 139.7 (C-5), 170.5 (C═O).

2.7. 17β-Azido-androst-5-en-3β-ol (5b)

Compound **5a** (7.15 g, 20 mmol) was dissolved in methanol (200 ml) containing $NaOCH_3$ (270 mg, 5 mmol), and the solution was allowed to stand for 24 h. It was then diluted with water, the white precipitate separating out was filtered off, recrystallized from a mixture of acetone/hexane to give **5a** (5.45 g, 86%) as a white solid. Mp: 78–80 °C, R_f = 0.60 (ss B); $[\alpha]_D^{20}$ = −67 (*c* 1 in $CHCl_3$). (Found: C, 72.55; H, 9.42; N, 13.65. $C_{19}H_{29}N_3O$ requires C, 72.34; H, 9.27; N, 13.32%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.76 and 1.00 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.31 (t, 1H, J = 8.5 Hz, 17-H), 3.50 (m, 1H, 3-H) 5.34 (s, 1H, 6-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 12.1 (C-18), 19.4 (C-19), 20.6, 23.6, 26.9, 31.5, 31.6, 32.0, 37.1, 37.2, 42.1, 42.2, 44.2, 50.0, 52.4, 71.2 (C-17), 71.6 (C-3), 121.1 (C-6), 140.9 (C-5).

2.8. General procedure for the synthesis of triazoles (8a–k and 9a–k)

17α-Azido-androst-5-en-3β-ol (**3b**) (315.45 mg, 1 mmol) or 17β-azido-androst-5-en (**5b**) (315.45 mg, 1 mmol) was dissolved in CH_2Cl_2 (20 ml), then CuI (19 mg, 0.10 mmol), Et_3N (0.2 ml, 2 mmol), and the appropriate terminal alkyne (**7a–k**, 2 mmol) were added. The mixture was stirred under reflux for 24 h, then diluted with water (30 ml) and extracted with CH_2Cl_2 (2 × 30 ml). The combined organic phases were dried over Na_2SO_4 and evaporated *in vacuo*. The crude product was purified by flash chromatography using ethyl acetate/CH_2Cl_2 in different concentrations.

2.8.1. 17α-(4′-Phenyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (8a)

Compound **3b** (315.45 mg, 1 mmol) and phenylacetylene (**7a**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (1:99 v/v) to yield pure **8a** (234 mg, 56%) as a white solid. Mp: 234–236 °C; R_f = 0.45 (ss B); $[\alpha]_D^{20}$ = −73 (*c* 1 in $CHCl_3$). (Found: C, 77.52; H, 8.26; N, 9.87. $C_{27}H_{35}N_3O$ requires C, 77.66; H, 8.45; N, 10.06%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.97 and 0.98 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.47 (m, 1H, 3-H), 4.63 (d, 1H, J = 7.5 Hz, 17-H), 5.33 (s, 1H, 6-H), 7.32 (t, 1H, J = 7.4 Hz, 4″-H), 7.41 (t, 1H, J = 7.4 Hz, 3″- and 5″-H), 7.68 (s, 1H, 5′-H), 7.83 (d, 2H, J = 7.4 Hz, 2″-H and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.4 (C-18), 19.3 (C-19), 20.4, 25.3, 28.6, 31.5, 31.9, 32.2, 32.4, 36.4, 37.1, 42.1, 46.1, 49.4, 50.2, 70.4 (C-17), 71.5 (C-3), 119.8 (C-5′), 121.1 (C-6), 125.6 and 128.8 (4C, C-2″, C-3″, C-5″ and C-6″), 128.0 (C-4″), 130.6 (C-1″), 140.7 (C-5), 146.8 (C-4′).

2.8.2. 17α-[4′-(4″-Tolyl)-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (8b)

Compound **3b** (315.45 mg, 1 mmol) and 4-tolylacetylene (**7b**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (1:99 v/v) to yield pure **8b** (193 mg, 45%) as a colorless crystalline solid. Mp: 258–261 °C; R_f = 0.45 (ss B); $[\alpha]_D^{20}$ = −75 (*c* 1 in $CHCl_3$). (Found: C, 78.05; H, 8.42; N, 9.87. $C_{28}H_{37}N_3O$ requires C, 77.92; H, 8.64; N, 9.74%). ^{1}H NMR (δ, pm, $CDCl_3$): 0.99 and 1.03 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 2.40 (s, 3H, 4″−CH_3), 3.49 (m, 1H, 3-H), 4.74 (d, 1H, J = 6.5 Hz, 17-H), 5.35 (s, 1H, 6-H), 7.27 and 7.84 (d, 2H, J = 7.0 Hz, 3″- and 5″-H, and d, 2H, J = 7.0 Hz, 2″-H and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.4 (C-18), 19.3 (C-19), 20.5, 21.3 (4″−CH_3), 25.6, 29.1, 31.5, 31.8, 32.1, 32.5, 36.4, 37.1, 42.1, 46.8, 49,2, 49.7, 69.8 (C-17), 71.5 (C-3), 78.9 (C-4′), 121.5 (C-6), 127.5 and 129.1 (4C, C-2″, C-3″, C-5″ and C-6″), 127.5 (C-1″), 138.3 (C-4″), 140.5 (C-5), 187.7 (C-5″).

2.8.3. 17α-[4′-(4″-Methoxyphenyl)-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (8c)

Compound **3b** (315.45 mg, 1 mmol) and 4-methoxyphenyl acetylene (**7b**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield pure **8c** (170 mg, 38%) as a white solid. Mp: 246–248 °C ; R_f = 0.50 (ss B); $[\alpha]_D^{20}$ = –74 (*c* 1 in $CHCl_3$). (Found: C, 75.34; H, 8.17; N, 9.55. $C_{28}H_{37}N_3O_2$ requires C, 75.13; H, 8.33; N, 9.39%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.97 and 0.99 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.47 (m, 1H, 3-H), 3.83 (s, 3H, 4″−OCH_3), 4.61 (d, 1H, J = 8.5 Hz, 17-H), 5.34 (d, 1H, J = 2.3 Hz, 6-H), 6.95 (d, 2H, 8.2 Hz, 3″-5″−H_2), 7.58 (s, 1H, 5′-H), 7.76 (d, 2H, J = 8.2 Hz, 2″-H and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.5 (C-18), 19.5 (C-19), 20.5, 25.4, 28.8, 31.7, 32.1, 32.3 (4′-OCH_3), 32.6, 36.6, 37.3, 42.3, 46.3, 49.6, 50.3, 55.5, 70.4 (C-17), 114.3 (C-2″ and -6″), 119.0 (C-5′), 121.3 (C-6), 123.6 (C-1″), 127.05 (C-3″ and -5″), 140.8 (C-5), 146.9 (C-4″), 159.6 (C-4′).

2.8.4. 17α-(4′-Cyclopropyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (8d)

Compound **3b** (315.45 mg, 1 mmol) and cyclopropylacetylene (**7d**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield pure **8d** (360 mg, 96%). Mp: 197–200 °C; R_f = 0.55 (ss B); $[\alpha]_D^{20}$ = −70 (*c* 1 in $CHCl_3$). (Found: C, 75.81; H, 9.08; N, 10.94. $C_{24}H_{35}N_3O$ requires C, 75.55; H, 9.25; N, 11.01%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.98 and 0.99 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.49 (m, 1H, 3-H), 4.57 (t, 1H, J = 8.1, 2.5 Hz, 17-H), 5.33 (d, 1H, J = 2.3 Hz, 6-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 7.3, 7.4, 7.8, 18.5 (C-18), 19.5 (C-19), 20.6, 22.0, 25.7, 29.1, 29.8, 31.7, 32.0, 32.2, 32.6, 36.6, 37.3, 42.4, 46.9, 49.3, 49.8, 69.7 (C-17), 71.7 (C-3), 121.7 (C-6), 140.6 (C-5).

2.8.5. 17α-(4′-Cyclopentyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (8e)

Compound **3b** (315.45 mg, 1 mmol) and cyclopentylacetylene (**7e**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield pure **8e** (300 mg, 45%). Mp: 108–110 °C; R_f = 0.53 (ss B); $[\alpha]_D^{20}$ = –68 (*c* 1 in $CHCl_3$). (Found: C, 76.38, H, 9.47; N, 10.03. $C_{26}H_{39}N_3O$ requires C, 76.24; H, 9.60; N, 10.26%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.93 and 0.98 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.15 (t, 1H, J = 8.2 Hz, 1′-H), 3.48 (m, 1H, 3-H), 4.52 (t, 1H, J = 8.6 Hz, 17-H), 5.34–5.33 (m, 1H, 6-H),7.13 (s, 1H, 5′-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.5 (C-18), 19.5 (C-19), 20.6, 22.8, 25.3, 25.5, 28.8, 31.7, 32.1, 32.4, 32.5, 33.4, 33.4, 36.6, 36.9, 37.3, 42.3, 46.2, 49.6, 50.3, 70.1 (C-17), 71.7 (C-3), 120.0 (C-5′), 121.3 (C-6), 140.9 (C-5), 151.9 (C-4′).

2.8.6. 17α-(4′-Cyclohexyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (8f)

Compound **3b** (315.45 mg, 1 mmol) and cyclohexylacetylene (**7f**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield pure **8f** (240 mg, 71%) as white crystalline product. Mp: 127–129 °C; R_f = 0.50 (ss B); $[\alpha]_D^{20}$ = –65 (*c* 1 in $CHCl_3$). (Found C, 76.73; H, 9.55; N, 10.06. $C_{27}H_{41}N_3O$ requires C, 76.55; H, 9.76; N, 9.92%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.92 and 0.98 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 2.73 (m, 1H, 1″-H), 3.48 (m, 1H, 3-H), 4.52 (d, 1H, J = 8.5 Hz, 17-H), 5.33 (t, 1H, J = 2.3 Hz, 5-H), 7.12 (s, 1H, 5′-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.5 (C-18), 19.5 (C-19), 20.5, 25.5, 26.2, 26.2, 26.3, 28.7, 31.7, 32.1, 32.3, 32.5, 33.2, 33.2, 35.4, 36.6, 37.3, 42.3, 46.2, 49.6, 50.3, 70.1 (C-17), 71.7 (C-3), 119.6 (C-5′), 121.3 (C-6), 140.9 (C-5), 152.9 (C-4′).

*2.8.7. 17α-[4′-(2″-Pyridyl)-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (**8g**)*

Compound **3b** (315.45 mg, 1 mmol) and 2-ethynylpyridine (**7g**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (10:90 v/v) to yield pure **8 g** (270 mg, 64%) as a white solid. Mp: 211–213 °C; R_f = 0.30 (ss B); $[\alpha]_D^{20}$ = –88 (*c* 1 in $CHCl_3$). (Found C, 74.48; H, 8.35; N, 13.12. $C_{26}H_{34}N_4O$ requires C, 74.61; H, 8.19; N, 13.39%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.97 (s, 6H, 18–19-H_6), 3.46 (m, 1H, 3-H), 4.71–4.70 (m, 1H, 17-H), 5.32–5-31 (m, 1H, 6-H), 7.22–7.20 (m, 1H, 5″-H), 7.76 (m, 1H, 4″-H), 8.09 (s, 1H, 5′-H), 8.19 (d, 1H, *J* = 7.9 Hz, 3″-H), 8.56–8.55 (m, 1H, 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 19.0 (C-19), 20.0, 24.9, 28.4, 31.1, 31.49, 31.8, 32.1, 36.1, 36.7, 41.8, 45.7, 49.0, 49.9, 70.0 (C-17), 71.1 (C-3), 120.0, 120.8 (C-6), 121.3 (C-1′), 122.4, 136.6, 140.3 (C-5), 147.1 (C-1″), 148.9 (C-3″), 150.1 (C-4′).

*2.8.8. 17α-(4′-Phenoxymethyl-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (**8h**)*

Compound **3b** (315.45 mg, 1 mmol) and phenyl propargyl ether (**7h**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (15:85 v/v) to yield pure **8 h** (332 mg, 76%) as white crystals. Mp: 173–175 °C; R_f = 0.50 (ss B); $[\alpha]_D^{20}$ = –54 (*c* 1 in $CHCl_3$). (Found C, 75.05; H, 8.51; N, 9.952. $N_{28}H_{37}N_3O_2$ requires C, 75.13; H, 8.33; N, 9.39%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.91 and 0.95 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.46 (m, 1H, 3-H), 4.55 (d, 1H, 17-H), 5.18 (s, 2H, 4′-CH_2-O-), 5.31 (t, 1H, 6-H), 6.93 (m, 3H, 2″-H, 4″-H and 6″-H), 7.24 (t, 2H, 3″-, 5″-H), 7.50 (s, 1H, 5′-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.3 (C-18), 19.3 (C-19), 20.4, 25.3, 28.6, 31.5, 32.2, 32.4, 36.4, 37.1, 42.2, 46.1, 49.3, 50.0, 62.2, 70.3 (C-17), 71.5 (C-3), 114.9 (C-2″ and -6″), 121.2 (C-6 and C-4″), 122.8 (C-5′), 129.4 (C-3″ and -5″), 140.7 (C-5), 143.4 (C-4′), 158.1 (C-1″).

*2.8.9. 17α-(4′-Benzoyloxymethyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (**8i**)*

Compound **3b** (315.45 mg, 1 mmol) and propargyl benzoate (**7i**, 0.20 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (10:90 v/v) to yield pure **8i** (385 mg, 81%) as a white solid. Mp: 222–223 °C; R_f = 0.45 (ss B); $[\alpha]_D^{20}$ = −56 (*c* 1 in $CHCl_3$). (Found C, 73.48; H, 7.66; N, 8.68. $C_{29}H_{37}N_3O_3$ requires C, 73.23; H, 7.84; N, 8.83%). ^{1}H NMR (δ, ppm, DMSO): 0.90 and 0.92 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.21 (m, 1H, 3-H), 4.61 (d, 1H, *J* = 4.5 Hz, −OH), 4.62 (d, 1H, *J* = 7.5 Hz, 17-H), 5.26 (s, 1H, 6-H), 5.39 (s, 2H, −CH_2−O−), 7.51 (t, 2H, *J* = 7.5 Hz, 3″- and 5″ -H), 7.66 (t, 1H, *J* = 7.5 Hz, 4″-H), 7.95 (d, 2H, *J* = 7.5 Hz, 2″-H and 6″-H), 8.24 (s, 1H, 5′-H). ^{13}C NMR (δ, ppm, DMSO): 17.7 (C-18), 19.1 (C-19), 19.9, 24.9, 28.0, 31.3, 31.5, 31.6, 31.9, 36.0, 36.8, 42.1, 45.4, 49.3, 49.6, 58.0 (4′-CH2-), 69.0 (C-17), 69.9 (C-3), 120.2 (C-6), 125.2 (C-5′), 128.7 (C-2″ and -6″), 129.1 (C-3″ and -5″), 129.3 (C-1″), 133.4 (C-4″), 141.1 (C-4′), 141.1 (C-5), 165.4 (C═O).

*2.8.10. 17α-[4′-(4″-Toluoyloxymethyl)-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (**8j**)*

Compound **3b** (315.45 mg, 1 mmol) and propargyl 4-methylbenzoate (**7j**, 0.20 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (10:90 v/v) to yield pure **8j** (430 mg, 88%) as a white crystalline solid. Mp: 250–252 °C; R_f = 0.48 (ss B); $[\alpha]_D^{20}$ = –55 (*c* 1 in $CHCl_3$). (Found C, 73.72; H, 7.95; N, 8.44. $C_{30}H_{39}N_3O_3$ requires C, 73.59; H, 8.03; N, 8.58%). ^{1}H NMR (δ, ppm, DMSO): 0.90 and 0.92 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 2.37 (s, 3H, 4″−H_3), 4.60 (d, 1H, *J* = 4.5 Hz, 3-H), 4.69 (d, 1H, *J* = 8.0 Hz, 17-H), 5.27 (d, 1H, *J* = 3.0 Hz, 6-H), 5.36 (s, 2H, 4′-H_2), 7.32 (d, 2H, *J* = 8.0 Hz, 3″- and 5″-H), 7.84 (d, 2H, *J* = 8.0 Hz, 2″- and 6″-H), 8.22 (s, 1H, 5′-H). ^{13}C NMR (δ, ppm, DMSO): 17.7 (C-18), 19.1 (C-19), 19.9, 24.9, 28.0, 31.3, 31.5, 31.6, 31.9, 36.0, 36.8, 42.1, 45.4, 49.3, 49.6, 58.0, 69.0, (C-17), 69.9 (C-3), 120.1 (C-6), 125.2 (C-5′), 128.7 and 129.1 (C-4, C-2″, C-3″, C-5″ and C-6″), 133.5 (C-1″), 141.0 (C-5), 141.1 (C-4′), 165.4 (C═O).

*2.8.11. 17α-[4′-(4″-Nitrobenzoyloxymethyl)-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (**8k**)*

Compound **3b** (315.45 mg, 1 mmol) and propargyl 4-nitrobenzoate (**7k**, 411 mg) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (10:90 v/v) to yield pure **8 k** (360 mg, 62%) as pale yellow crystals. Mp: 201–202 °C; R_f = 0.45 (ss B); $[\alpha]_D^{20}$ = –63 (*c* 1 in $CHCl_3$). (Found C, 67.02; H, 6.75; N, 10.85. $C_{29}H_{36}N_4O_5$ requires C, 66.90; H, 6.97; N, 10.76%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.95 and 0.97 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.47 (m, 1H, 3-H), 4.59 (d, 1H, *J* = 7.5 Hz, 6-H), 5.49 (s, 2H, 4′-H_2), 7.61 (s, 1H, 5′-H), 8.20 (d, 2H, *J* = 9.0 Hz, 3″- and 5″-H), 8.26 (d, 2H, *J* = 9.0 Hz, 2″- and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.4 (C-18), 19.3 (C-19), 20.3, 25.3, 28.7, 31.5, 31.9, 32.1 (C-8), 32.4, 36.4, 37.1, 42.1, 46.1 (C-13), 49.4 (C-9), 50.1 (C-14), 58.9 (4′-CH_2-), 70.4, (C-17), 71.5 (C-3), 121.1 (C-6), 123.5 (C-2″ and C-6″), 124.4 (C-5′), 130.9 (C-3″ and C-5″), 135.1 (C-1′), 140.7 (C-5), 141.2 (C-4′), 150.6 (C-4″), 164.6 (C═O).

*2.8.12. 17α-(4′-Hydroxymethyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (**8l**)*

Compound **8k** (520 mg, 1 mmol) was dissolved in methanol (10 ml) containing $NaOCH_3$ (27 mg, 0.5 mmol), and the solution was allowed to stand for 24 h. It was then diluted with water, and the white precipitate separating out was filtered off, dried and recrystallized from a mixture of ethyl acetate/hexane to afford **8l** (350 mg, 94%) as a white solid. Mp: 217–219 °C; R_f = 0.20 (ss B); $[\alpha]_D^{20}$ = – 38 (*c* 1 in ethanol). (Found C, 71.25; H, 9.03; N, 11.16. $C_{22}H_{33}N_3O_2$ requires C, 71.12; H, 8.95; N, 11.31%). ^{1}H NMR (δ, ppm, DMSO): 0.89 and 0.93 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 4.49 (d, 2H, *J* = 5.0 Hz, 4′-CH_2-), 5.14 (d, 1H, *J* = 5.0 Hz, 17-H), 5.27 (s, 1H, 6-H), 7.92 (s, 1H, 5′-H). ^{13}C NMR (δ, ppm, DMSO): 17.8 (C-18), 19.1 (C-19), 19.9, 24.9, 28.0, 31.3, 31.5, 31.6 (C-8), 31.9, 36.0, 36.8, 42.1, 45.3, 49.3 (C-9), 49.6 (C-14), 55.0 (4′-CH_2-), 68.8 (C-17), 69.9 (C-3), 120.2 (C-6), 123.0 (C-5′), 141.1 (C-4′), 147.1 (C-5).

*2.9. General procedure for the synthesis of 5′-iodotriazoles (**8m-k** and **9m–k**)*

Compound **3b** or **5b** (315.45 mg, 1 mmol) was dissolved in CH_2Cl_2 (20 ml), then Et_3N (0.2 ml, 2 mmol), substituted acetylene derivative (**7m–k**, 2 mmol), ICl (1.5 mmol) and, finally, CuI (190 mg, 10 mol) were added. The heterogeneous reaction mixture was stirred under N_2 for 24 h, then diluted with 1% $N_2S_2O_3$ solution (30 ml), and extracted with CH_2Cl_2 (2 × 30 ml). The combined organic phases were dried over Na_2SO_4 and evaporated *in vacuo*. The crude product was purified by flash chromatography using ethyl acetate/CH_2Cl_2 (5:95 v/v) to obtain the steroidal 5′-iodotriazoles (**8m–n** and **9m–n**).

*2.9.1. 17α-[4′-(4″-Nitrobenzoyloxymethyl)-5′-iodo-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (**8m**)*

Compound **3b** (315.45 mg, 1 mmol) and propargyl 4-nitrobenzoate (**7k**, 204 mg) were used for the synthesis as described in Section 2.9. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield a pale yellow crystalline material (320 mg, 49%). Mp: 278–281 °C; R_f = 0.60 (ss B); $[\alpha]_D^{20}$ = +81 (*c* 1 in $CHCl_3$). (Found C, 54.02; H, 5.37; N, 8.55. $C_{29}H_{35}IN_4O_5$ requires C, 53.87; H, 5.46; N, 8.67%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.99 and 1.02 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.50 (m, 1H, 3-H), 4.64 (dd, 1H, *J* = 9.0 Hz, *J* = 4.5 Hz, 17-H), 5.35 (d, 1H, *J* = 6.5 Hz, 6-H), 5.47 (d, 2H, *J* = 5.5 Hz, 4′-H_2), 8.22 (d, 2H, *J* = 11.0 Hz, 3″-H and 5″-H), 8.27 (d, 2H, *J* = 11.0 Hz, 2″-H and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.4 (C-18), 19.4 (C-19), 20.5, 25.6, 29.1, 31.5, 31.8, 32.1, 32.5, 36.4, 37.1,

42.2 (C-13), 46.9, 49.2 (C-9), 49.7 (C-14), 59.1 (4′-CH_2-), 70.3 (C-17), 71.6 (C-3), 121.4 (C-5′), 121.5 (C-6), 123.5 (C-2″ and C-6″), 11.0 (C-3″ and C-5″), 135.1 (C-1′), 140.5 (C-5), 145.1 (C-4′), 150.6 (C-4″), 164.3 (C═O).

2.9.2. 17α-(4′-Cyclopropyl-5′-iodo-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (8n)

Compound **3b** (315.45 mg, 1 mmol) and cyclopropylacetylene (**7d**, 0.22 ml) were used for the synthesis as described in Section 2.9. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield **8n** (285 mg, 57%) as white crystals. Mp: 176–179 °C; R_f = 0.55 (ss B); $[\alpha]_D^{20}$ = +43 (*c* 1 in $CHCl_3$). (Found C, 56.63; H, 6.89; N, 8.42. $C_{24}H_{34}IN_3O$ requires C, 56.80; H, 6.78; N, 8.28%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.92 and 0.98 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.50 (m, 1H, 3-H), 4.51 (d, 1H, *J* = 5.5 Hz, 17-H), 5.33 (d, 1H, *J* = 4.5 Hz, 6-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 7.2 and 7.7 (C-2″ and C-3″), 18.4 (C-18), 19.3 (C-19), 20.4, 25.3, 28.6, 31.5, 31.9, 32.1, 32.3, 36,5, 37.1, 42.2, 46.0 (C-13), 49.4 (C-14), 50.1, 70.0 (C-17), 71.5 (C-3), 121.5 (C-6), 140.4 (C-5), 149.2 (C-4′), 180.5 (C-5′).

2.10. General procedure for the synthesis of 5′-hydroxytriazoles (8o, 9o)

Compound **8 m** or **9 m** (127 mg, 0.25 mmol) was dissolved in Ac_2O (3 ml) containing KOAc (196 mg, 2 mmol), and the mixture was treated at reflux temperature for 4 h. The progress of the reaction was monitored by TLC. After completion of the transformation, the reaction mixture was diluted with water, then extracted with CH_2Cl_2 (2 × 30 ml). The CH_2Cl_2 solution was washed with $NaHCO_3$ solution and then with water, dried and evaporated *in vacuo*. The residual yellow oil was dissolved in methanol (15 ml) containing KOH (56 mg, 1 mmol) and the solution was treated at reflux temperature for 2 h. The precipitate separating out was filtered off and recrystallized from a mixture of acetone/water.

2.10.1. 17α-(4′-Cyclopropyl-5′-hydroxy-1′H-1′,2′,3′-triazol-1′-yl) androst-5-en-3β-ol (8o) Compound 8n (127 mg, 0.25 mmol) were used for the synthesis described in Section 2.10

8o (68 mg, 69%). Mp: 193–195 °C; R_f = 0.15 (ss B); $[\alpha]_D^{20}$ = –40 (*c* 1 in ethanol). (Found C, 72.68; H, 8.45; N, 10.72. $C_{24}H_{35}N_3O_2$ requires C, 72.51; H, 8.87; N, 10.57%). ^{1}H NMR (δ, ppm, DMSO): 0.87 and 0.92 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 2.33 (m, 1H, 3-H), 4.04 (d, 1H, *J* = 8.5 Hz, 17-H), 4.28 (d, 1H, *J* = 8.0 Hz, 6-H), 5.27 (s, 1H, 5′-OH). ^{13}C NMR (δ, ppm, DMSO): 5.0 (C-18), 6.2, 6.6, 10.0 (C-19), 17.9, 19.1 (C-8), 19.9, 25.1, 27.1, 31.3, 31.6 (C-9), 36.0, 36.8, 42.1 (C-13), 45.3 (C-5′), 49.3 (C-14), 49.4 (C-1″), 50.8, 61.6, 63.6 (C-17), 69.9 (C-3), 120.2 (C-6), 141.1 (C-4′), 172.5 (C-5).

2.10.2. 17β-(4′-Phenyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (9a)

Compound **5b** (315.45 mg, 1 mmol) and phenylacetylene (**7a**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (1:99 v/v) to yield pure **9a** (283 mg, 68%) as a white crystalline product. Mp: 280–283 °C; R_f = 0.40 (ss B); $[\alpha]_D^{20}$ = –40 (*c* 1 in $CHCl_3$). (Found: C, 77.48; H, 8.31; N, 10.27. $C_{27}H_{35}N_3O$ requires C, 77.66; H, 8.45; N, 10.6%). ^{1}H NMR (δ, ppm, DMSO): 0.95 and 1.24 (s, 3H, 18-H_3 and 19-H_3), 4.52 (t, 1H, *J* = 9.5 Hz, 17-H), 4.61 (d, 1H, *J* = 4.0 Hz, 3-H), 5.31 (dd, 1H, *J* = 4.0 Hz, *J* = 1.5 Hz, 6-H), 7.32 (t, 1H, *J* = 7.5 Hz, 4″-H), 7.44 (t, 2H, *J* = 7.5 Hz, 3″-H and 5″-H). ^{13}C NMR (δ, ppm, DMSO): 12.5 (C-18), 19.6 (C-19), 21.0, 23.8, 26.1, 31.4, 31.7, 32.4, 36.9, 37.2, 37.6, 42.1 (C-13), 44.7, 50.4, (C-9), 53.5 (C-14), 71.2 (C-17), 71.4 (C-3), 120.0 (C-6), 121.2 (C-5′), 126.0 (C-2″ and C-6″), 128.6, 129.2 (C-3″ and C-5″), 130.6, 141.4, (C-5), 147.4 (C-4′).

2.10.3. 17β-[4′(4″-Tolyl)-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (9b)

Compound **5b** (315.45 mg, 1 mmol) and 4-tolylacetylene (**7b**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (99:1 v/v) to yield pure **9b** (210 mg, 77%) as a white crystalline material. Mp: 293–295 °C; R_f = 0.45 (ss B); $[\alpha]_D^{20}$ = –42 (*c* 1 in $CHCl_3$). (Found C, 77.78; H, 8.83; N, 9.54. $C_{28}H_{37}N_3O$ requires C, 77.92; H, 8.64, N, 9.79%). ^{1}H NMR (δ, ppm, DMSO): 0.51 and 0.94 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 4.49 (s, 1H, 17-H), 4.53 (d, 1H, *J* = 2.0 Hz, 3-H), 5.30 (d, 2H, *J* = 2.0 Hz, 6-H_2), 7.24 (d, 2H, *J* = 1.5 Hz, 3″- H and 5″- H), 7.75 (d, 2H, *J* = 1.5 Hz, 2″- H and 6″- H), 8.59 (s, 1H, 5′- H).

2.10.4. 17β-[4′(4″-Methoxyphenyl)-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (9c)

Compound **5b** (315.45 mg, 1 mmol) and 4-methoxyphenyl acetylene (**7c**, 0.22 ml)) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield pure **9c** (143 mg, 67%) as a white solid. Mp: 260–262 °C; R_f = 0.40 (ss B); $[\alpha]_D^{20}$ = –30 (*c* 1 in $CHCl_3$). (Found C, 75.27; H, 8.22; N, 9.46. $C_{28}H_{37}N_3O_2$ requires C, 75.13; H, 8.33; N, 9.39%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.62 and 1.01 (s, 3H, 18-H_3 and s, 3H, 19-H_3) 3.53 (m, 1H, 3-H), 3.84 (—OCH_3), 4.43 (t, 1H, *J* = 9.5 Hz, 17-H), 5.38 (s, 1H, 6-H), 6.95 (d, 2H, *J* = 8.5 Hz, 2″- and 6″-H), 7.67 (s, 1H, 5′-H), 7.76 (d, 2H, *J* = 8.5 Hz, 3″-H and 5″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 12.0 (C-18), 19.4 (C-19), 20.7, 23.5, 25.9, 31.5, 31.6, 32.0, 36.9, 37.2, 42.2, 44.3, 50.1, 53.2, 55.3, 63.3, 65.2, 70.2, 71.6 (C-3), 114.2 (2C, C-2″ and C-6″), 118.3 (C-5′) 121.0 (C-6), 126.9 (2C, C-3″ and C-5″), 140.9 (C-5), 149.0, 159.6.

2.10.5. 17β-(4′-Cyclopropyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (9d)

Compound **5b** (315.45 mg, 1 mmol) and cyclopropylacetylene (**7d**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (1:99 v/v) to yield pure **9d** (170 mg, 45%). Mp: 242–244° C; R_f = 0.48 (ss B); $[\alpha]_D^{20}$ = −36 (*c* 1 in $CHCl_3$). (Found C, 75.62; H, 9.03; N, 11.25. $C_{24}H_{35}N_3O$ requires C, 75.55; H, 9.25; N, 11.01%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.55 and 1.00 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.52 (m, 1H, 3-H), 4.32 (t, 1H, *J* = 9.5 Hz, 17-H), 5.35 (d, 1H, *J* = 5.0 Hz, 6-H), 7.23 (s, 1H, 5′-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 6.6 (C-18), 7.8 (C-2″ and C-3″), 11.4 (C-19), 19.4 (C-8), 20.6, 23.4, 25.8, 29.7, 31.5, 31.6, 32.0 (C-9), 36.3, 36.9, 37.2, 42.2, 44.2 (C-13), 50.0 (C-14), 53.1 (C-1″), 70.5 (C-17), 71.6 (C-3), 119.3 (C-6), 121.0 (C-4′), 141.0 (C-5), 149.2 (C-5′).

2.10.6. 17β-(4′-Cyclopentyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (9e)

Compound **5b** (315.45 mg, 1 mmol) and cyclopentylacetylene (**7e**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield **9e** (290 mg, 71%) as a white crystalline product. Mp: 275–277 °C; R_f = 0.45 (ss B); $[\alpha]_D^{20}$ = −48 (*c* 1 in $CHCl_3$). (Found C, 76.38; H, 9.83; N, 10.12. $C_{26}H_{39}N_3O$ requires C, 76.24; H, 9.60; N, 10.26%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.53 and 0.98 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.50 (m, 1H, 3-H), 4.33 (t, 1H, *J* = 9.5 Hz, 17-H), 5.34 (s, 1H, 6-H), 7.21 (d, 2H, *J* = 8.0 Hz, 2″- and 6″-H), 7.67 (s, 1H, 5′-H), 7.92 (d, 2H, *J* = 8.0 Hz, 3″- and 5″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 11.9 (C-18), 19.3 (C-19), 20.6, 23.4, 25.1, 25.8, 31.4 (C-13), 32.0, 33.1, 33.2, 36.5, 36.7, 36.8, 37.2, 42.0, 44.1, 50.0, 53.1, 70.4 (C-17), 71.4 (C-3), 119.1 (C-5′), 121.0 (C-6), 141.0 (C-5), 151.8 (C-4′).

2.10.7. 17β-(4′-Cyclohexyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (9f)

Compound **5b** (315 mg, 1 mmol) and cyclohexyacetylene (**7f**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield **9f** (250 mg, 59%) as a white solid. Mp:

296–297 °C; R_f = 0.40 (ss B); $[\alpha]_D^{20}$ = −49 (*c* 1 in $CHCl_3$). (Found C, 76.73; H, 9.55; N, 9.76. $C_{27}H_{41}N_3O$ requires C, 76.55; H, 9.76; N, 9.92%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.55 (s, 3H, 18-H_3), 1.01 (s, 3H, 19-H_3), 1.84 (m, 8H, 2″-, 3″-, 5″- and 6″-H_2), 3.40 (m, 1H, 3-H), 4.45 (t, 1H, *J* = 9.5 Hz, 17-H), 5.34 (dd, 1H, *J* = 3.5 Hz, *J* = 2.5 Hz, 6-H), 7.71 (s, 1H, 4′-H).

2.10.8. 17β-[4′(2″-Pyridyl)-1′H-1′,2′,3′-triazol-1′-yl)]androst-5-en-3β-ol (9g)

Compound **5b** (315 mg, 1 mmol) and 2-ethynylpiridine (**7g**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (10:90 v/v) to yield **9g** (330 mg, 79%) as a white crystalline material. Mp: 254–256 °C; R_f = 0.35 (ss B); $[\alpha]_D^{20}$ = –34 (*c* 1 in $CHCl_3$). (Found C, 74.38; H, 8.25; N, 13.55. $C_{26}H_{34}N_4O$ requires C, 74.61; H, 8.19; N, 13.39%). ^{1}H NMR (δ, ppm, $CDCl_3$): 1.00 and 1.24 (s, 3H, 18-H_3 and s, 3H 19-H_3), 3.53 (m, 1H, 3-H), 4.46 (t, 1H, *J* = 9.5 Hz, 17-H), 5.36 (s, 1H, 6-H), 7.21 (t, 1H, *J* = 6.1 Hz, 5″-H), 7.77 (m, 1H, 4″-H), 8.18 (s, 1H, 5′-H), 8.20 (s, 1H, 3″-H), 8.57 (d, 1H *J* = 4.1 Hz, 5″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 12.0 (C-18), 19.4 (C-19), 20.6, 23.5, 25.9, 29.7, 31.5, 31.6, 32.0, 36.9, 37.2, 42.2, 44.3 (C-13), 50.0, 53.2, 70.7 (C-17), 71.6 (C-3), 120.2, 121.0 (C-6), 121.4 (C-5′), 122.7, 131.9, 141.0 (C-5), 147.4 (C-1″), 149.3 (C-3″), 150.5 (C-4′).

2.10.9. 17β-(4′-Phenoxymethyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (9h)

Compound **5b** (315 mg, 1 mmol) and phenyl propargyl ether (**7h**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield **9h** (356 mg, 79%) as white crystals. Mp: 198–201 °C; R_f = 0.48 (ss B); $[\alpha]_D^{20}$ = – 46 (*c* 1 in $CHCl_3$). (Found C, 75.36; H, 8.22; N, 9.51. $C_{28}H_{37}N_3O_2$ requires C, 75.13; H, 8.33; N, 9.39%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.56 and 1.00 (s, 1H, 18-H_3 and s, 1H, 19-H_3, and q, *J* = 11.0 Hz, 1H), 3.52 (m, 1H, 3-H), 4.39 (t, 1H, *J* = 9.5 Hz, 17-H), 5.21 (s, 1H, 4′-CH_2-), 3.53 (d, 1H, *J* = 2.5 Hz, 6-H), 5.45 (s, 1H, 4′-CH_2-) 6.97 (overlapping multiplets, 3H, 3″-,4″- and 5″-H), 7.29 (t 2H, *J* = 8.3 Hz, 2″-H and 6″-H), 7.60 (s, 1H, 5′-H). ^{13}C (δ, ppm, $CDCl_3$): 12.0 (C-18), 19.4 (C-19), 20.6, 23.5, 25.9, 31.4, 31.5, 32.0, 36.5, 36.8, 37.2, 42.2, 44.2, 50.0, 53.1, 62.2 (4′-CH_2-), 70.6, 71.6 (C-3), 114.8 (2C, C-2″ and C-6″) 121.0 (C-6), 121.2, 122.1, 1129.5 (2C, C-3″ and C-5″), 141.0 (C-5), 143.4, 158.2.

2.10.10. 17β-(4′-Benzoyloxymethyl-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (9i)

Compound **5b** (315 mg, 1 mmol) and propargyl benzoate (**7i**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (10:90 v/v) to yield **9i** (230 mg, 48%) as a white solid. Mp 203–206 °C; R_f = 0.40 (ss B); $[\alpha]_D^{20}$ = –50 (*c* 1 in $CHCl_3$). (Found C, 73.42; H, 8.02; N, 8.67. $C_{29}H_{37}N_3O_3$ requires C, 73.23; H, 7.84; N, 8.83%). ^{1}H NMR (δ, ppm, $CDCl_3$) 0.55 and 0.99 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.52 (m, 1H, 3-H), 4.40 (t, 1H, *J* = 9.5 Hz, 17-H), 5.35 (s, 1H, 6-H), 5.47 (s, 2H, −CH_2-O-), 7.42 (t, 2H, *J* = 7.5 Hz, 3″-H and 5″-H), 7.55 (t, 1H, *J* = 7.5 Hz, 4″-H), 7.69 (s, 1H, 5′-H), 8.03 (d, 2H, *J* = 7.5 Hz, 2″-H and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$) 12.0 (C-18), 19.4 (C-19), 20.6, 23.4, 25.9, 29.7, 31.4, 31.5, 32.0, 36.5, 36.8, 37.2, 42.1, 44.2, 50.0, 53.1, 58.2, 70.6 (C-17), 71.5 (C-3), 121.0 (C-6), 123.5 (C-5′), 128.3 and 129.7 (4C, C-2″, C-3″, C-5″ and C-6″), 133.1 (C-4″), 140.9, 142.0, 166.5 (C═O).

2.10.11. 17β-[4′-(4″-Toluoyloxymethyl)-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (9j)

Compound **5b** (315 mg, 1 mmol) and propargyl 4-methylbenzoate (**7j**, 0.22 ml) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (10:90 v/v) to yield **9j** (250 mg, 51%) as a white solid. Mp: 212–215 °C; R_f = 0.45 (ss B); $[\alpha]_D^{20}$ = – 48 (*c* 1 in $CHCl_3$). (Found C, 73.70; H, 9.87, N, 8.75. $C_{30}H_{39}N_3O_3$ requires C, 73.59, H, 8.03; N, 8.58%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.55 and 0.99 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 2.39 (s, 3H, 4″−H_3), 2.51 (m, 1H), 3.53 (m, 1H, 3-H), 4.39 (t, 1H, *J* = 9.5 Hz, 17-H), 5.35 (d, 1H, *J* = 2.5 Hz, 6-H), 5.45 (s, 2H, 4′-CH_2-), 7.21 and 7.92 (d, 2H, *J* = 8.0 Hz, 3″-H and 5″-H and d, 2H, *J* = 8.0 Hz, 2″- and 6″-H), 7.67 (s, 1H, 5′-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 12.0 (C-18), 19.4 (C-19), 20.6, 21.6 (4″−CH_3), 23.4, 25.9, 31.4, 31.5, 32.0, 36.5, 36.8, 37.2, 42.2, 44.2, 50.0, 53.1, 58.0 (4′-CH_2-), 70.6 (C-17), 71.6 (C-3), 121.0 (C-6), 123.4 (C-5′), 127.1 (C-1″), 129.0 and 129.7 (4C, C-2″, C-3″, C-5″ and C-6″), 140.1 (C-5′), 141.2 (C-4′), 143.8 (C-1″), 166.5 (C═O).

2.10.12. 17β-[4′-(4″-Nitrobenzoyloxymethyl)-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (9k)

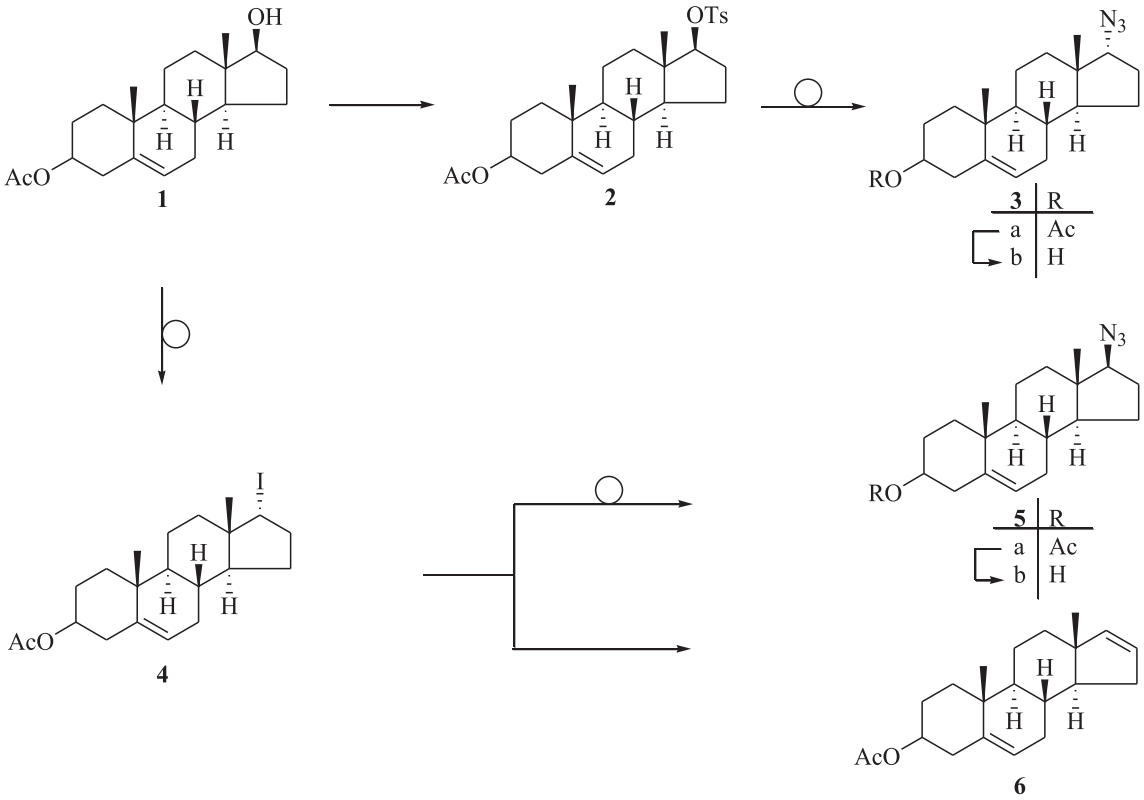
Compound **5b** (315 mg, 1 mmol) and propargyl 4-nitrobenzoate (**7k**, 411 mg) were used for the synthesis as described in Section 2.8. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (10:90 v/v) to yield **9k** (375 mg, 72%) as a pale yellow solid. Mp: 198–199 °C; R_f = 0.42 (ss B); $[\alpha]_D^{20}$ = −43 (*c* 1 in $CHCl_3$). (Found C, 66.82; H, 7.12; N, 10.64. $C_{29}H_{36}N_4O_5$ requires C, 66.90; H, 6.97; N, 10.76%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.56 and 0.99 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.52 (m, 1H, 3-H), 4.41 (t, 1H, *J* = 9.5 Hz, 17-H), 5.35 (d, 1H, *J* = 5.0 Hz, 6-H), 5.51 (s, 2H, 4′-H_2), 7.71 (s, 1H, 5′-H), 8.20 (d, 2H, *J* = 9.0 Hz, 3″-H and 5″-H), 8.26 (d, 2H, *J* = 9.0 Hz, 2″-H and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 12.0 (C-18), 19.4 (C-18), 20.6, 23.4, 25.9, 29.6, 31.4, 31.5, 32.0 (C-8), 36.8, 37.2, 42.2, 44.3 (C-13), 50.0 (C-9), 53.1 (C-14), 58.8 (4′-CH_2-), 70.7 (C-17), 71.5 (C-3), 120.9 (C-6), 123.5 (C-2″ and C-6″), 123.7 (C-5′), 130.9 (C-3″ and C-5″), 135.2 (C-1″), 141.0 (C-5), 141.2 (C-4′), 150.6 (C-4″), 164.6 (C═O).

2.10.13. 17β-(4′-Hydroxymethyl-1′H-1′,2′,3′ -triazol-1′-yl)androst-5-en-3β-ol (9l)

Compound **9 k** (520 mg, 1 mmol) was dissolved in methanol (10 ml) containing $NaOCH_3$ (27 mg, 0.5 mmol), and the solution was allowed to stand for 24 h. It was then diluted with water, and the precipitate separating out was filtered off, and recrystallized from a mixture of ethyl acetate/hexane to afford **9l** (320 mg, 86%) as a white crystalline product. Mp: 244–245 °C; R_f = 0.18 (ss B); $[\alpha]_D^{20}$ = −18 (*c* 1 in ethanol). (Found C, 70.98; H, 9.11; N, 11.46. $C_{22}H_{33}N_3O_2$ requires C, 71.12; H, 8.95; N, 11.31%). ^{1}H NMR (δ, pp, DMSO): 0.47 and 0.94 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 4.51 (s, 2H, 4′-CH_2-), 4.61 (s, 1H, 3-H), 5.14 (s, 1H, 17-H), 5.29 (s, 1H, 6H), 7.98 (s, 1H, 5′-H). ^{13}C NMR (δ, ppm, DMSO): 11.7 (C-18), 19.1 (C-19), 20.1, 23.0, 25.1, 30.9, 31.3, 31.5 (C-8), 36.1, 36.2, 36.9, 42.1, 43.5, 49.6 (C-9), 52.2 (C-14), 55.0 (4′-CH_2-), 69.3 (C-17), 69.9 (C-3), 120.1 (C-6), 122.1 (C-5′), 141.3 (C-4′), 147.1 (C-5).

2.10.14. 17β-[4′-(4″-Nitrobenzoyloxymethyl)-5′-iodo-1′H-1′,2′,3′-triazol-1′-yl]androst-5-en-3β-ol (9m)

Compound **5b** (315.45 mg, 1 mmol) and propargyl 4-nitrobenzoate (**7k**, 204 mg) were used for the synthesis as described in Section 2.9. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield **9m** (375 mg, 72%) as a white solid. Mp: 198–200 °C; R_f = 0.55 (ss B); $[\alpha]_D^{20}$ = +89 (*c* 1 in $CHCl_3$). (Found C, 53.62; H, 5.68; N, 8.72. $C_{29}H_{35}IN_4O_5$ requires C, 53.87; H, 5.46; N, 8.67%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.77 and 1.01 (s, 3H, 18-H_3 and 19-H_3), 3.53 (m, 1H, 3-H), 4.49 (t, 1H, *J* = 8.5 Hz, 17-H), 5.38 (d, 1H, *J* = 5.0 Hz, 6-H), 5.49 (dd, 1H, *J* = 20.0 Hz, *J* = 13.0 Hz, 4′-CH_2-), 8.24 (t, 2H, *J* = 7.0 Hz, 3″-H and 5″-H), 8.28 (t, 2H, *J* = 7.0 Hz, 2″- and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 14.1 (C-18), 19.4 (C-19), 20.8, 22.7, 23.6, 27.1, 29.4 (C-8), 31.5, 31.6, 31.9 , 32.0, 36.6 (C-9), 37.2, 38.2, 42.2, 45.6, (C-13), 50.0 (C-14), 53.7 (C-17), 59.1 (4′-CH_2-), 71.6 (C-3), 121.1 (C-6), 123.5 (C-2″ and C-6″), 131.0 (C-3″ and C-5″), 135.1 (C-1′), 140.9 (C-5), 164.4 (C═O).



Scheme 1. Reagents and conditions: (i) TsCl, pyridine, rt, 24 h; (ii) NaN_3, DMF, 100 °C, 24 h; (iii) Ph_3P, imidazole, I_2, toluene, 80 °C, 2 h; (iv) NaN_3, DMF, 60 °C, 24 h.

*2.10.15. 17β-(4′-Cyclopropyl-5′-iodo-1′H-1′,2′,3′-triazol-1′-yl)androst-5-en-3β-ol (**9n**)*

Compound **5** (315.45 mg, 1 mmol) and cyclopropylacetylene (**7d**, 0.22 ml) were used for the synthesis as described in Section 2.9. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (5:95 v/v) to yield **9n** (260 mg, 53%) as a white crystalline material. Mp: 210–213 °C; R_f = 0.52 (ss B); $[\alpha]_D^{20}$ = +63 (*c* 1 in $CHCl_3$). (Found C, 56.63; H, 6.91; N, 8.42. $C_{24}H_{34}IN_3O$ requires C, 6.80; H, 6.75, N, 8.28%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.70 and 0.98 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 3.51 (m, 1H, 3-H), 4.40 (t, 1H, *J* = 9.5 Hz, 17-H), 5.34 (d, 1H, *J* = 4.5 Hz, 6-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 7.24, 7.26, 7.63 7.63, 12.7 (C-18), 19.4 (C-19), 20.8, 23.5, 26.9, 29.7, 31.5, 32.0 (C-9), 37.2, 38.2, 42.2, 45.4 (C-13), 50.0 (C-14), 53.7 (C-1″), 70.0 (C-17), 71.6 (C-3), 114.0 (C-5′), 121.1 (C-6), 140.9 (C-4′), 151.7 (C-5).

*2.10.16. 17β-(4′-Cyclopropyl-5′-hydroxy-1′H-1′2′,3′-triazol-1′-yl) androst-5-en-3β-ol (**9o**)*

Compound **9n** (127 mg, 0.25 mmol) were used for the synthesis described in Section 2.10. **9r** (76 mg, 76%). Mp: 178–180 °C; R_f = 0.12 (ss B); $[\alpha]_D^{20}$ = −31 (*c* 1 in ethanol). (Found C, 72.67; H, 8.55, N, 10.68. $C_{24}H_{35}N_3O_2$ requires C, 72.51; H, 8.87; N, 10.57%).^{1}H NMR (δ, ppm, DMSO): 0.56 and 0.97 (s, 3H, 18-H_3 and s, 3H, 19-H_3), 1.98 (s, 4H, 2″-H and 3″-H), 2.62 (dd, 1H, *J* = 21.5 Hz, *J* = 10.5 Hz, 3-H), 4.18 (t, 1H, *J* = 9.5 Hz, 17-H), 4.45 (m, 1H, 6-H), 5.37 (s, 1H, 5′-OH). ^{13}C NMR (δ, ppm, DMSO): 5.0 (C-18), 6.1 and 6.5 (C-2″ and C-3″), 12.2 (C-19), 18.9 (C-8), 20.1, 23.0, 24.3, 27.2, 30.9, 31.4 (C-9), 31.6, 36.4, 36.7, 37.6, 43.8, 49.3 (C-14), 52.4 (C-1″), 61.5 (C-5′), 64.7 (C-17), 73.0 (C-3), 121.8 (C-6), 139.5 (C-4′), 169.6 (C-5).

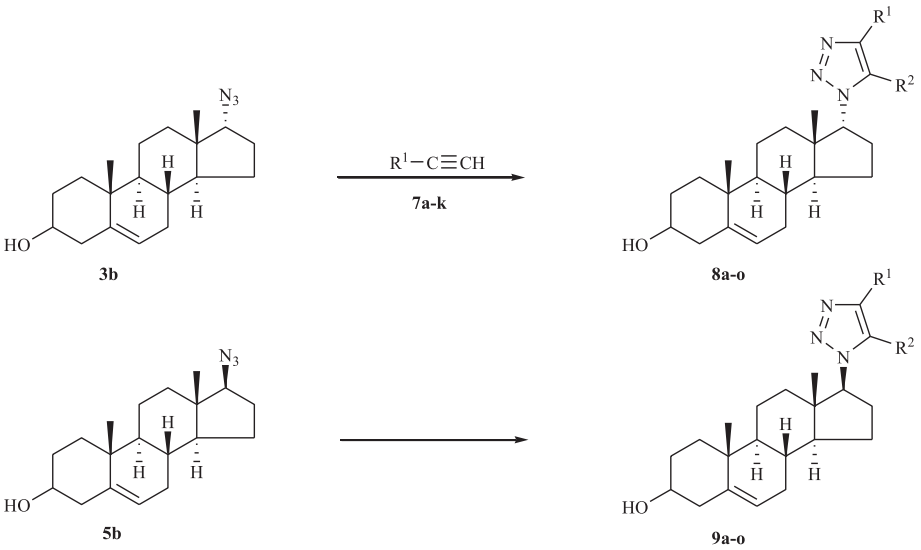
2.11. Determination of the $C_{17,20}$-lyase inhibition

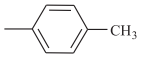
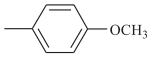
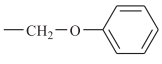
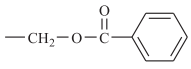
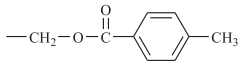
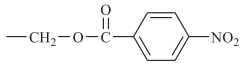
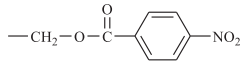
The inhibitory effect on the rat testicular $C_{17,20}$-lyase enzyme exerted by the new compounds were determined via an *in vitro* radiosubstrate incubation method [20]. In brief, adult Wistar rat testicular tissue was homogenized with an Ultra-Turrax in 0.1 M HEPES buffer (pH = 7.3) containing 1 mM EDTA and 1 mM dithiotreitol. Aliquots of this homogenate were incubated in 200 µl final volume at 37 °C for 20 min in the presence of 0.1 mM NADPH. 1 µM [^{3}H]-17-hydroxyprogesterone was added to the incubate in 20 µl of a 25% (v/v) propylene glycol solution. Test compounds were applied at 50 µM and introduced in 10 µl of DMSO. (Such minor quantities of organic solvents used here did not reduce the enzyme activity substantially). Control incubates without test substances, and incubates with the reference compound ketoconazole and abirateron were also prepared in every series. Following

incubation, the androst-4-ene-3,17-dione formed and the 17-hydroxyprogesterone remaining were isolated through extraction and TLC. $C_{17,20}$-lyase activity was calculated from the radioactivity of the androst-4-ene-3,17-dione obtained. Two experiments were performed with each test compound and the standard deviations of the mean enzyme activity results (S.D.) were within ± 15%. IC_{50} values were determined for the more active inhibitors. In this case, the conversion was measured at 5 or 6 different concentrations of the test compound. IC_{50} results were calculated using an un-weighted iterative least squares method for four-parameter logistic curve fitting. IC_{50} of unlabelled 17-hydroyprogesterone substrate, as well as abiraterone, and another clinically relevant, nonsteroidal imidazole $P450_{17\alpha}$ inhibitor, ketoconazole were measured as reference. Reference IC_{50} values were determined for unlabelled 17-hydroxyprogesterone substrate (1.6 µM) as well as for abiraterone (0.0125 µM) and ketoconazole (0.32 µM).

2.11.1. Time dependence of inhibitory effect

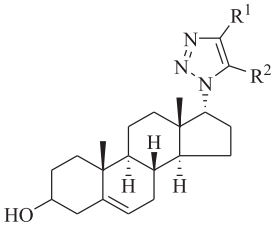
Time-dependent change in the inhibitory effect was investigated by preincubation with the testicular homogenate. Testicular homogenate in incubation mixture was preincubated with IC_{50} concentration in the presence or the absence of NADPH cofactor for 40 min at 37 °C, then the inhibition test was started by the addition of the substrate.



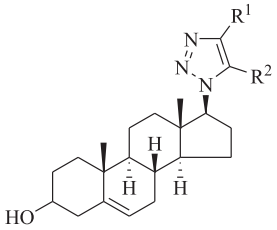
No. 8, 9	R^1	R^2
a		H
b		H
c		H
d		H
e		H
f		H
g		H
h		H
i		H
j		H
k		H
l		H
m		I
n		I
o		OH

Scheme 2. Reagents and conditions: Ph_3P or Et_3N, CuI, CH_2Cl_2, rt, 24 h.

Table 1
In vitro inhibition of rat testicular $C_{17,20}$-lyase by test compounds **8a–o** and **9a–o**.



8a-o



9a–o

No. **8, 9**	IC_{50}	Relative conversion % 50 μM	Relative conversion % 10 μM	IC_{50}	Relative conversion % 50 μM	Relative conversion % 10 μM
a		88 ± 4	92 ± 3		95 ± 5	95 ± 7
b		89 ± 6	89 ± 1		107 ± 10	100 ± 8
c		81 ± 3	97 ± 5		87 ± 4	92 ± 5
d	21.6 ± 7.5	40 ± 7	83 ± 5		76 ± 7	74 ± 3
e		70 ± 8	82 ± 3		91 ± 8	90 ± 10
f		89 ± 11	85 ± 8		80 ± 6	92 ± 2
g		58 ± 3	77 ± 7		74 ± 7	90 ± 5
h		61 ± 7	62 ± 4		86 ± 8	86 ± 5
i	3.5 ± 0.8	5 ± 1	32 ± 5		79 ± 5	82 ± 6
j		71 ± 7	86 ± 12		94 ± 11	90 ± 4
k	9.2 ± 3.4	5 ± 2	31 ± 4		88 ± 8	100 ± 10
l	33 ± 23	49 ± 2	63 ± 3		74 ± 7	90 ± 5
m		72 ± 8	84 ± 7		75 ± 3	85 ± 7
n		98 ± 10	86 ± 7		72 ± 4	93 ± 9
o	3.1 ± 1.4	15 ± 2	26 ± 5		94 ± 7	98 ± 5

2.11.2. Investigation of binding mechanism

Reversibility of test compounds was investigated via preincubation and charcoal adsorption method. Testicular homogenate in incubation mixture was preincubated with high concentration (10 μM and 50 μM) of the tested compounds, in the presence of NADPH cofactor for 10 and 40 min at 37 °C. After the addition of charcoal suspension in 0.6% final concentration, the preincubated samples were kept on ice for 15 min and centrifuged at 4 °C. Supernatants were drained and used for inhibition tests.

3. Results and discussion

3.1. Synthetic studies

To prepare novel steroid triazoles via 1,3-dipolar cycloaddition, 17α- and 17β-azidoandrost-5-en-3β-ol (**3b** and **5b**) were selected as starting compounds. The synthetic strategy for the preparation of the starting azides is illustrated in Scheme 1 and the synthesis of steroidal 1,2,3-triazoles by CuAAC is outlined in Scheme 1 and 2.

3β-Acetoxy-androst-5-en-17β-ol (**1**) was esterified with *p*-toluenesulfonyl chloride to give tosylate **2**, which then underwent facile S_N2 substitution with NaN_3 in *N,N*-dimethylformamide to furnish the corresponding 17α-azido compound **3a**. Deacetylation of **3a** by the Zemplén method produced 17α-azido-androst-5-en-3β-ol **3b**. Iodination of **1** by the Appel reaction proceeded with Walden inversion to yield 3-acetoxy-androst-5-en-17α-iodide **4** [7]. Further nucleophilic exchange reaction with NaN_3 in *N,N*-dimethylformamide furnished 17β-azido compound **5a** in moderate yield. The exchange reaction was accompanied by elimination to give 3-acetoxy-androst-5,16-diene (**6**) too.

The 17-azido derivatives (**3b** or **5b**) were subjected to azide–alkyne CuAAC reactions with substituted phenylacetylenes (**7a–c**), small cycloalkyl acetylenes (**7d–f**), 2-piridylacetylene (**7g**), phenyl propargyl ether (**7h**), and propargyl phenyl esters (**7i-k**). The CuAAC of **3b** or **5b** with substituted acetylenes (**7a–k**) were carried out in refluxing CH_2Cl_2 with CuI as catalyst in the presence of Et_3N (0.2 equivalent) to obtain the required 1,4-disubstituted triazoles (**8a–k** and **9a–k**) in moderate yields after 24 h.

Alkaline hydrolysis of 4′-benzoyloxymethyl triazoles **8i–k** and **9i–k** yielded polar 4′-hydroxymethyltriazolyl derivatives **8l** and **9l**.

We found earlier that the CuAAC reaction in the presence of Et_3N is accompanied by building of 5′-iodo-1,4-triazoles in small quantities [21]. When 1.5 equivalent of ICl was used as electrophile in the presence of 10 equivalents of CuI and Et_3N, the quantity of 5′-iodo-triazoles increased to 49–72%. With this method we prepared 4′-*p*-nitrobenzoyloxymethyl-5′-iodo- and 4′-cyclopropyl-5′-iodo triazoles **8m–n** and **9m–n**. Acetolysis of **8n** and **9n** in acetic anhydride in the presence of KOAc followed by alkaline hydrolysis yielded 4′-cyclopropyl-5′ hydroxy triazolyl derivatives **8o** and **9o**.

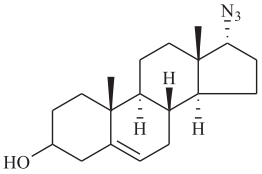
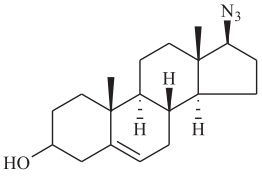
3.2. $C_{17,20}$-lyase inhibition studies

The $P450_{17\alpha}$ catalyzed biotransformation of C_{21} steroids consists of hydroxylation and a subsequent side-chain cleavage at the C-17 carbon. This second $C_{17,20}$-lyase process is the rate-limiting step in the synthesis of the C_{19} preandrogens. Inhibition of the $C_{17,20}$-lyase activity is, therefore, the primary evaluation criteria of compounds with presumed androgen-withdrawing effect achieved by blockade of the $P450_{17\alpha}$ [1,5,22]. In this study we investigated inhibition against $C_{17,20}$-lyase activity of $P450_{17\alpha}$ exerted by the new androst-5-en-3β-ol derivatives possessing diverse C-substituted triazolyl substituents or the azide group in 17α or 17β position.

3.2.1. Inhibition effect of the17-heterocyclic compounds

Several related 5-membered *N*-containing imidazole, pyrazole, triazole, and tetrazole isomers of androstane compounds were studied as potential $C_{17,20}$-lyase inhibitors [5,6,23–25]. We have previously observed with C-17β and C-17β phenyl-substituted pyrazolinylandrostenes that the extended 17β heterocyclic side-chain with various substituted aromatic rings may have substantial inhibitory effect [11]. Structural investigations proposed the existence of a second hydrophobic binding pocket beside the heme structure, which also supports the inhibitor development strategy involving an adjacent phenyl group on the C-17 substituent [26]. As a further example, related studies with galeterone also indicate that compounds with more spacious aromatic side chains may improve affinity and inhibitory effect towards $P450_{17\alpha}$,

Table 2
In vitro inhibition of rat testicular $C_{17,20}$-lyase by test compounds **3b** and **5b**.

No.		IC_{50}	Relative conversion % 50 μM	Relative conversion % 10 μM
3b		21.1 ± 6.8	42 ± 4	64 ± 9
5b		0.60 ± 0.18	17 ± 3	15 ± 2

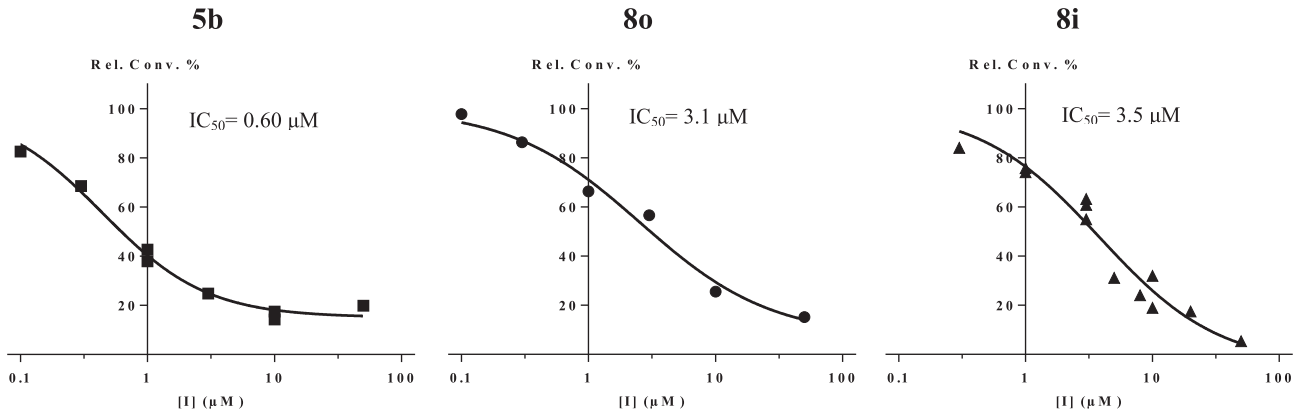
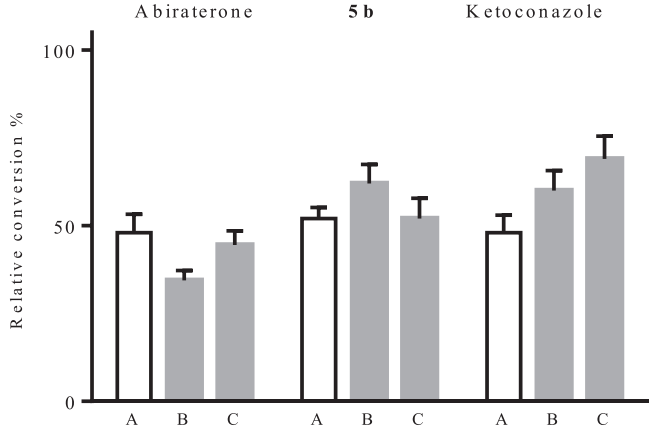


Fig. 1. Concentration-dependent inhibitory effects of test compounds **5b**, **8o** and **8i** on the *in vitro* activity of $C_{17,20}$-lyase. [I]: inhibitor concentration in μM.



Mean±SD of three separate experiments

Experimental conditions:

A No preincubation

B Preincubation, 40 min

C Preincubation with NADPH, 40 min

Fig. 2. Inhibitory potential of the IC_{50} concentration of **5b** and reference compounds (abiraterone and ketoconazole) with or without 40 min pre-incubations with the rat testicular homogenate. Following the preincubation period, the usual incubation procedure was applied.

and the extension of the side chain may be useful in modification of the activity profile of a pharmacon candidate [27,28].

In this report we investigated inhibition against $C_{17,20}$-lyase activity of $P450_{17\alpha}$ exerted by the new androst-5-en-3β-ol derivatives (**8** and **9**) possessing diverse C-substituted triazolyl substituents in the 17α and 17β position (Tables 1 and 2).

Compounds bearing 17β-triazolyl substituents on the androst-5-en-3β-hydroxy skeleton (**9**) exerted weak inhibition with the relative conversions more than 70% even in the relatively high 50 μM test concentration (Table 1). In the 17α-triazolyl series two cyclopropyl derivatives (**8d**, **8o**) and three phenyl ester derivatives (**8i**, **8k**, **8l**) exerted more pronounced inhibition (Table 1). Compounds **8o** and **8i** were found to be the best inhibitors among them with IC_{50} values 3.1 μM and 3.5 μM, respectively (Table 1, Fig. 1). Compounds **8g** in the 17α-triazolyl series and **9g** in the 17β series containing heteroaryl substituents at the C-4′ carbon were also weak inhibitors. In the series of the cycloalkyl-substituted 17α-triazolyl compounds (**8d–f**) a decreasing inhibition could be observed with increasing carbon numbers in the cycle. An additional iodo substituent on the triazole ring (**8-m**,**n**, **9 m**,**n**) was disadvantageous for inhibition, whereas a hydroxyl group at the same position (**8o**) improved the effect.

These results demonstrate substantial inhibitory effect of the C-4′-hydroxymethyl benzoate **8i** and *p*-nitrobenzoate **8k** to the enzyme. Their IC_{50} values indicate affinities to the enzyme similar to that of the substrate. The presence as well as the flexible and appropriate positioning of phenyl group seems to be essential for the good binding such as the precursor alcohol **8l**. The C-4′ coupled phenyl group (**8a**), and the $C^{4'}$-phenoxymethyl ether (**8h**) derivatives were weak inhibitors. The *p*-nitro function on the phenyl group (**8k**) decreased the inhibition in a lesser, whereas the *p*-methyl group (**8j**) decreased in a higher extent.

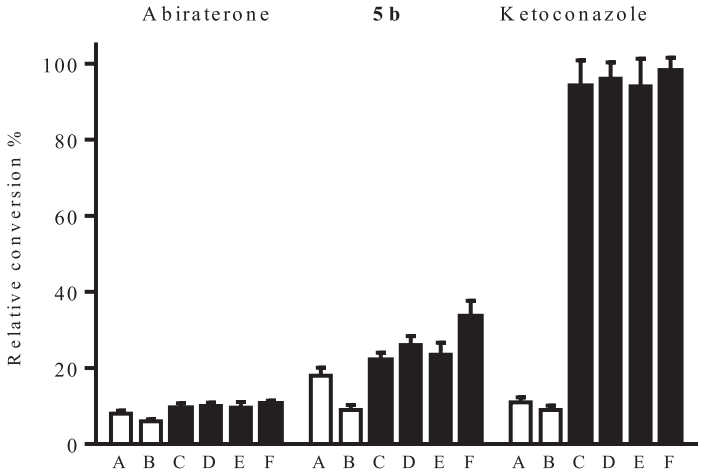


Fig. 3. Investigation of inhibition reversibility of **5b** and reference compounds (abiraterone and ketoconazole). Compounds were preincubated with rat testicular homogenate. Following a charcoal separation step, the usual incubation procedure was applied.

Our results reveal that stereoisomerism at the C-17 of the new androst-5-en-3β-ol derivatives influences the $C_{17,20}$-lyase effect. Inhibitors were found only in the 17α-triazolyl series, whereas the 17β counterparts (**9**) were found to be almost inactive in the $C_{17,20}$-lyase test.

3.2.2. Inhibition effect of the 17-azido compounds

Only a few steroid azides similar to those investigated here can be found in the literature. The synthesis and antituberculosis activity of 17α-azido-5α-androstan-3β-ol was investigated by Merlani et al. [29]. The neuroactive 17α- and 17β-azido-5β-androstan-3α-ols were studied by Vidnra et al. [30]. 17-Azidomethyl-androst-5,16-dien-3β-ol was synthesized by the group of Njar and Brodie during their $P450_{17\alpha}$ inhibitor development studies [31]. $P450_{17\alpha}$ or $C_{17,20}$-lyase inhibitory potential of these related compounds, however, was not reported in either of the corresponding publications.

Wright et al. investigated the inhibition of 19-azido-4-androstene-3,17-dione against the aromatase, another cytochrome P450 dependent enzyme in the steroid hormone metabolism [32]. They have found reversible inhibitor binding and hypothesized that the first nitrogen atom in the azide group (attached directly to the C-19 carbon) is most likely to be involved in coordination to the heme iron of aromatase.

Other, non-steroidal primary azides were found to be converted to the corresponding aldehydes *via* biocatalysis of myoglobin, another heme-dependent metalloprotein similar to cytochrome P450 [33]. The proposed binding and reaction mechanisms of the myoglobin-catalyzed azide reaction also indicate that the first-nitrogen is coordinated to the heme iron of the active center.

In this report we investigated $C_{17,20}$-lyase inhibition of the two androst-5-en-3β-ol compounds possessing azide group in the 17α or 17β position. Our results revealed that the 17-azido androstene compounds (**3b**, **5b**) tested here were able to inhibit the $C_{17,20}$-lyase activity. Stereoisomerism at the C-17 atom influenced the effect in the pair of the 3β-hydroxy-5-androstene compounds (**3b**, **5b**). 17β-Azido derivative **5b** was found to be more effective (IC_{50} = 0.60 μM) than 17α-azide **3b** (IC_{50} = 21 μM) (Table 2, Fig. 1). The IC_{50} parameter of **5b** indicated a 2.5-fold stronger binding compared to the substrate, and its inhibitory potential was found to be close to that of the nonsteroidal imidazole reference ketoconazole.

Potent inhibition of the 17β-azido **5b** is an interesting finding; therefore, we further investigated inhibitory properties of this compound.

*3.2.2.1. Time dependence of inhibitory effect of **5b**.* The possible time-dependent change of inhibitory effect of **5b** was investigated in experiments in which the compound was applied in its IC_{50} concentration (0.60 μM) and preincubated with the testicular homogenate before the usual inhibition test. No difference was found between the normal and the preincubated samples either in case of test compound **5b** or the reference abiraterone and ketoconazole (Fig. 2). The presence of cofactor during the preincubation had no influence either on the measured inhibitory potentials. This observation indicates that conversion of the tested compound, which could modify the inhibitory potential of 17β-azido-androst-5-en-3β-ol **5b**, does not occur under the conditions of incubation procedures applied.

*3.2.2.2. Reversibility of inhibition of **5b**.* Reversibility of the inhibition

was studied *via* preincubation with high concentration of the test compounds followed by the removal of unbound and reversibly bound inhibitor molecules by charcoal adsorption. Samples pretreated with the known reversible inhibitor compound ketoconazole as reference [34] displayed almost non-inhibited $C_{17,20}$-lyase activity [34], whereas irreversible reference inhibitor compound abiraterone [7] blocked enzyme activity (Fig. 3). Incubations pretreated with compound **5b** resulted in conversions close to those in the inhibited control inhibition tests with high inhibitor concentration. These results indicate that **5b** molecules cannot be removed by charcoal treatment from the binding, that is binding to the enzyme takes place *via* an irreversible mechanism. Note that in the case of abiraterone and several other C-17 heterocyclic $P450_{17\alpha}$ inhibitors, the $\Delta^{16,17}$ double bond not only increases the inhibitory potency but also switches the binding mode from reversible to irreversible [7]. Nevertheless, the irreversible binding of 17β-azido-androst-5-en-3β-ol (**5b**) occurs in the absence of $\Delta^{16,17}$ double bond.

*3.2.2.3. Aromatase inhibitory effect of **5b**.* Taking into account of the aromatase inhibitory effect of 19-azido-4-androstene-3,17-dione [32] and certain $P450_{17\alpha}$ inhibitor abiraterone analogs [35], we performed tests whether **5b** is capable of inhibiting this enzyme. The *in vitro* radiosubstrate incubations were based on similar incubation methodology as described here for the $C_{17,20}$-lyase. Incubations with high concentrations (10 μM and 50 μM) resulted in relative conversions similar to the non-inhibited controls (results are not shown). These results indicate that 17β-azido-androst-5-en-3β-ol **5b** does not inhibit aromatase activity substantially.

4. Summary

$P450_{17\alpha}$ is a pivotal enzyme of steroid biosynthesis. Inhibition of its $C_{17,20}$-lyase activity leads to suppressed androgen production, which can be applied for antiandrogen therapies. Results on $C_{17,20}$-lyase inhibitory effect of our new aryl-substituted 17-triazolyl androstenes revealed that compounds bearing a 17α-heterocyclic side chain may also have substantial affinity to this enzyme. Our results verified that extension of the C-17 side chain may improve inhibitory potential. In addition, an azido group on the steroidal C-17 atom is a suitable pharmacophore in the inhibition of the $C_{17,20}$-lyase activity. The potent inhibition of the azido compound is an interesting finding worth further investigations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.steroids.2018.03.006.

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VII.

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RESEARCH PAPER

Synthesis and structure–activity relationships of 2- and/or 4-halogenated 13β- and 13α-estrone derivatives as enzyme inhibitors of estrogen biosynthesis

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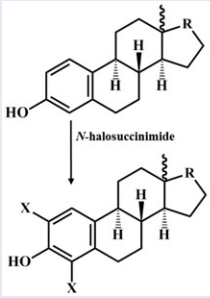
ABSTRACT

Ring A halogenated 13α-, 13β-, and 17-deoxy-13α-estrone derivatives were synthesised with N-halosuccinimides as electrophile triggers. Substitutions occurred at positions C-2 and/or C-4. The potential inhibitory action of the halogenated estrones on human aromatase, steroid sulfatase, or 17β-hydroxysteroid dehydrogenase 1 activity was investigated via *in vitro* radiosubstrate incubation. Potent submicromolar or low micromolar inhibitors were identified with occasional dual or multiple inhibitory properties. Valuable structure–activity relationships were established from the comparison of the inhibitory data obtained. Kinetic experiments performed with selected compounds revealed competitive reversible inhibition mechanisms against 17β-hydroxysteroid dehydrogenase 1 and competitive irreversible manner in the inhibition of the steroid sulfatase enzyme.



	R	X		Substantial inhibitory potential against		
		2	4	Aromatase	Steroid sulfatase	17β-HSD1
13β-Me	C=O	I	H			+
		H	I		+	+
		Br	H	+	+	+
		H	Br		+	+
		Cl	H	+	+	+
13α-Me	C=O	I	H			+
		I	I		+	+
		Cl	H			+
	CH_2	Cl	Cl		+	

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Introduction

Estrogens play an important role in cell proliferation and their overproduction stimulates the growth of hormone-sensitive cells, leading to hormone-dependent diseases, such as breast and endometrial cancer[1]. Inhibition of enzymes involved in the final steps of estrogen biosynthesis is a powerful route to prevent the proliferative action of estrogens. Cytochrome P450 aromatase is responsible for the conversion of nonaromatic androgens **1** and **2** to estrone (E1, **4**, Scheme 1) or 17β-estradiol (E2, **5**), respectively. Estrogens are originated not only from nonaromatic steroids, but also from estrone-3-sulfate (E1S) **3**, which exists as a large circulatory reservoir. E1S is transported into cells by organic anion transporters (OATPs) and several other members of the SoLute Carrier (SLC) protein family[2]. After entering the cells, E1 is released from the sulfate ester by steroid sulfatase (STS). The next, hormone-activating process is the formation of active hormone E2 from E1, which is mainly catalyzed by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1). Activities of STS and 17β-HSD1 are higher in breast cancer tissue compared to other tissues, that is this may be the main route of local estrogen production in the tumors[3,4].

Inhibition of the above-mentioned enzymes may be achieved by inhibitors designed on the estrane core. A-ring halogenation of E1 results in 2- and 4-regioisomeric (**6**, **7**) and/or 2,4-*bis*-substituted compounds (**8**, Figure 1)[5–7]. The substitution pattern of the aromatic ring and the nature of the introduced halogen greatly influence the inhibitory properties of estrone derivatives halogenated at the A-ring. Compounds obtained by introduction of the same substituent to different positions of the aromatic ring may

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Supplemental data for this article can be accessed here.

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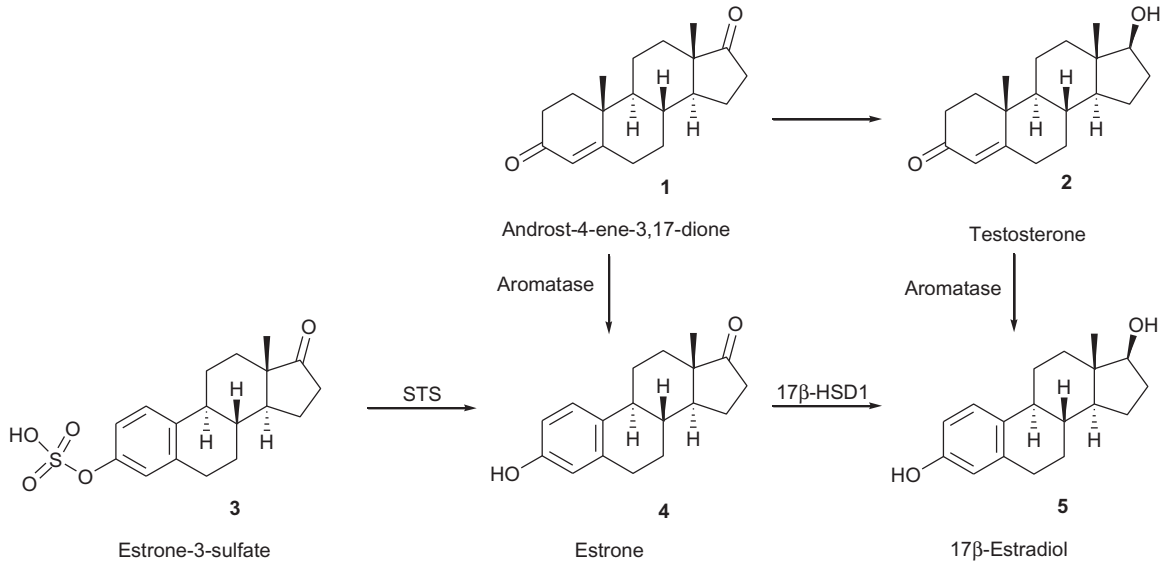


Scheme 1. The role of aromatase, STS and 17β-HSD1 enzymes in the estrogen biosynthesis.

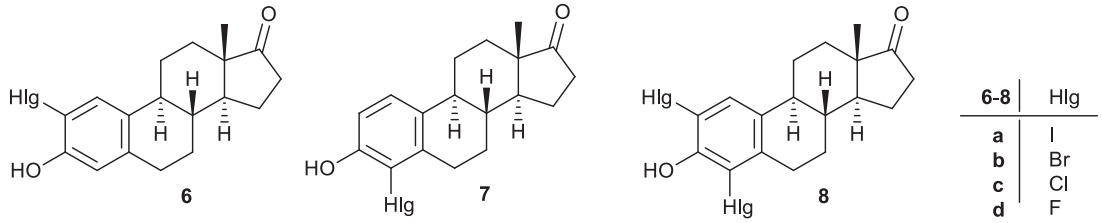


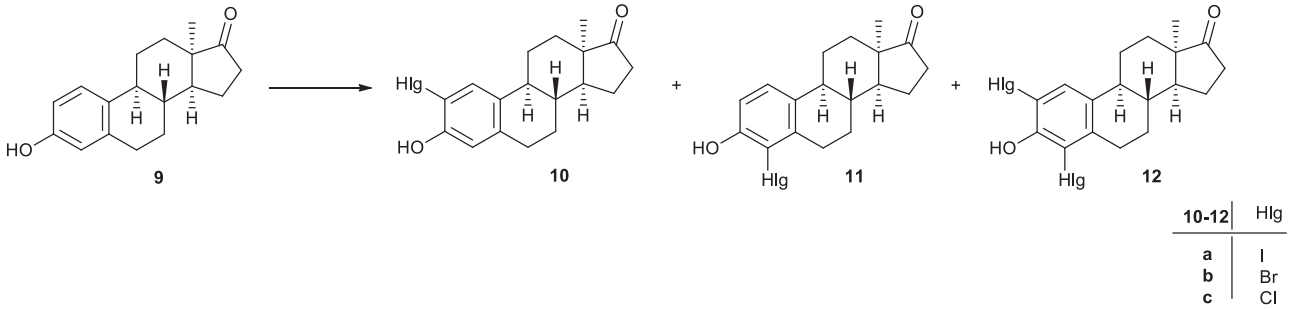
Figure 1. Ring A halogenated derivatives **6–8** of estrone.

possess high binding affinities to different enzymes. 2-Bromo- (**6b**) or 2-chloroestrone (**6c**) are potent aromatase inhibitors with low micromolar IC_{50} values[5]. Their 4-substituted counterparts (**7b**,**c**) display only moderate aromatase inhibitory potential. Möller *et al*. described that 2-haloestrones (**6b**,**c**) as 17β-HSD1 inhibitors can suppress the E1–E2 conversion with IC_{50} values in the submicromolar range[6]. 4-Halogenated counterparts (**7**) have not been tested against 17β-HSD1 by this research group. However, the 4-haloestrones (**7b**,**d**) are known to be efficient STS inhibitors[7]. Thus, introduction of Br or F onto C-4 of E1 led to a significant increase in STS inhibitory potential. None of the mentioned references discusses the affinity of 2-iodo- and 4-iodoestrones (**6a**, **7a**) or 2,4-*bis*-compounds (**8**) for these three enzymes.

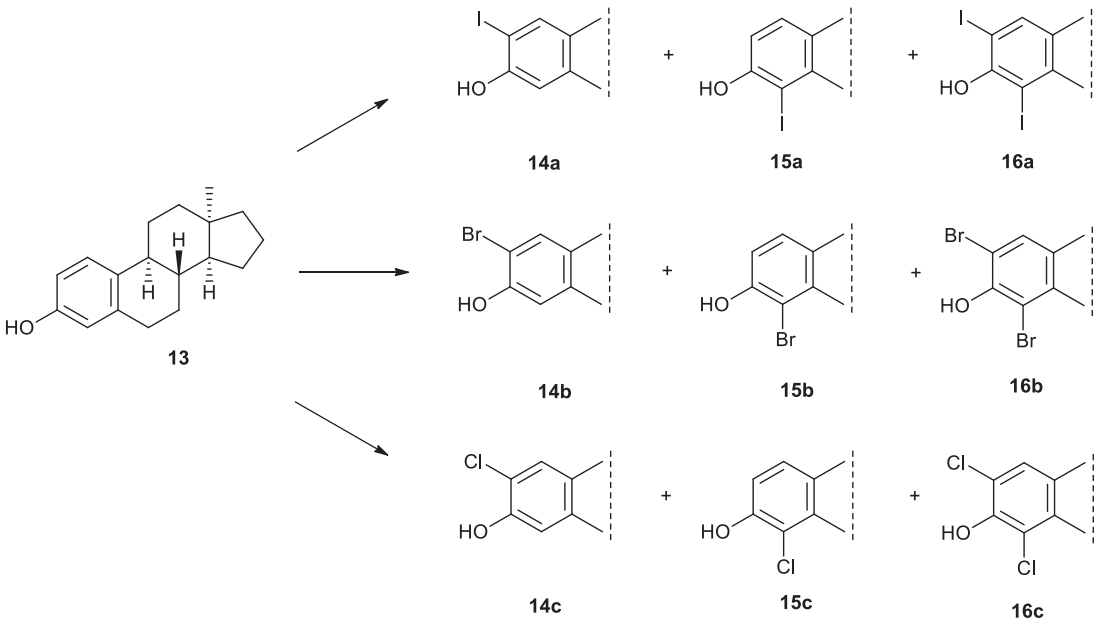
Concerning the results obtained so far for certain A-ring halogenated estrones it seems that substitution of E1 at C-2 may enhance aromatase and 17β-HSD1 inhibitory potential, but 4-halogenation may lead to efficient STS inhibitors.

The use of the estrone-based inhibitors of the mentioned steroidogenic enzymes in the therapy is limited because of their retained estrogenic activity. The availability of inhibitors acting selectively without hormonal behavior would be of particular interest. Literature data reveal that estrogenic effect of estrone is a 5–35% extent of that of 17β-estradiol[8–10]. Chloro substitution at position C-4 of estrone or 17β-estradiol retains the estrogenicity, however, 4-bromo and 4-iodo, as well as 2-chloro, -bromo, and -iodo compounds exert suppressed effect compared to that of the parent compounds. Data vary according to the methods applied, but it can be stated that estrogenic effect decreases with the increasing size of the introduced halogen. C-2 substituted estrone or 17β-estradiol compounds are usually less estrogenic than their C-4 counterparts. The 2,4-disubstituted analogs, nevertheless, exert negligible estrogenic potential[8,10,11]. Certain other chemical modifications of the estrane skeleton, such as the inversion of the configuration at C-13 or the opening of ring D, may result in the complete loss of hormonal activity[12–15]. We have promising preliminary results concerning the design, synthesis and biochemical evaluation of 17β-HSD1 inhibitors based on hormonally inactive 13α-estrane core[16]. 13α-Estrone (**9**, Scheme 2) itself was proved to be a potent inhibitor with an IC_{50} comparable to that of the natural substrate E1. Additionally, the previously synthesised 13α-estrone derivatives (**10a**,**b**–**12a**,**b**) brominated or iodinated in the A-ring exerted low micromolar or submicromolar inhibitory potential[17]. Chlorinations in the 13α-estrone series leading to **10c**–**12c** have not been performed up to now. Concerning recent promising results, that 13α-estrone derivatives might possess valuable 17β-HSD1 inhibitory potential, it seemed rational to evaluate these compounds as potential aromatase or STS inhibitors, too.

Based on the above-mentioned literature results here we aimed to perform aromatic halogenations on 13α-estrone (**9**) and its 17-deoxy counterpart (**13**, Scheme 2, 3). Chlorination of 13α-estrone (**9**) and introduction of chlorine, bromine or iodine onto positions 2 and/or 4 of 17-deoxy-13α-estrone (**13**) were planned. Synthesis of the halogenated 13β-estrone derivatives (**6a**,**b**,**c**–**8a**,**b**,**c**) was also designed with the aim of preparing the 13β-counterparts for comparative investigations (Figure 1). We additionally intended to investigate the potential *in vitro* inhibitory effects of the basic and the halogenated compounds (**6a**,**b**,**c**–**8a**,**b**,**c**; **9**–**16**) towards aromatase, STS and 17β-HSD1 enzymes. Comparison of the inhibitory results and evaluation of structure–activity relationships were also planned. Representatives from the most effective test compounds were selected for mechanistic and kinetic investigations.



Scheme 2. Ring A substitutions in the 13α-estrone series.



Scheme 3. Introduction of different halogens onto the aromatic ring of 17-deoxy-13α-estrone

Materials and methods

Chemical syntheses and characterisation data of the reported compounds, as well as experimental conditions of enzymatic assays performed are described in the Supporting Information.

Results and discussion

Chemistry

We recently described the halogenations of the aromatic ring of **9** with different groups at position 3 (OH, OMe, OBn)[17]. Bromination or iodination was carried out with *N*-halosuccinimides as electrophile triggers in different solvents. In the 3-OH series both 2- and 4-substituted regioisomers were formed and *bis*-substitutions also occurred. The mixtures of the desired products (**10a**,**b**–**12a**,**b**) could efficiently be separated by flash chromatography.

As a completion of our previous results, here we performed the chlorination of the aromatic ring of **9** in order to get the appropriate 2-, 4-, or 2,4-*bis*-chloro derivatives (**10c**–**12c**) for further structure–activity examinations. Reactions with *N*-chlorosuccinimide led to a mixture of products (**10c**–**12c**, Scheme 2, Table 1, Entries 1, 2), which were separated by flash chromatography.

Expecting to acquire valuable structure–activity relationship data, it seemed reasonable to synthesise the appropriate halogenated 13α compounds in the 17-deoxy series, too. The halogenations of 17-deoxy-13α-estrone **13** were carried out using *N*-halosuccinimides in different solvents (Scheme 3, Table 1). Iodination of **13** with 1.5 equivalent of NIS in trifluoroacetic acid yielded the mixture of the two regioisomers (**14a** and **15a**) and the *bis*-compound (**16a**) in a ratio of 2:1:1 (Table 1, Entry 3). Bromination of **13** with 1.1 equivalent of reagent in dichloromethane led to a 4:3:1 mixture of **14b**, **15b** and **16b** (Table 1, Entry 4). Chlorination of **13** with 1.5 equivalent of NCS yielded the 2:1 mixture of the two regioisomers **14c** and **15c** (Table 1, Entry 5). The *bis*-chloro compound (**16c**) could be synthesised by using 3.0 equivalent of NCS (Table 1, Entry 6).

Table 1. Synthesis of halogenated compounds (**6–8, 10c–12c, 14–16**)

Entry	Starting compound	Electrophilic reagent (solvent)	Product	Yield (%)
1	**9**	NCS (1.0 equiv., TFA)	**10c** + **11c**	16 + 63
2	**9**	NCS (2.0 equiv., TFA)	**11c** + **12c**	57 + 19
3	**13**	NIS (1.5 equiv., TFA)	**14a** + **15a** + **16a**	48 + 24 + 24
4	**13**	NBS (1.1 equiv., CH_2Cl_2)	**14b** + **15b** + **16b**	44 + 33 + 11
5	**13**	NCS (1.5 equiv., CH_3CN)	**14c** + **15c**	48 + 24
6	**13**	NCS (3.0 equiv., CH_3CN)	**15c** + **16c**	26 + 53
7	**4**	NIS (1.0 equiv., TFA)	**6a** + **7a**	58 + 30
8	**4**	NIS (2.0 equiv., TFA)	**7a** + **8a**	59 + 30
9	**4**	NBS (1.0 equiv., CH_2Cl_2)	**6b** + **7b**	40 + 41
10	**4**	NBS (2.0 equiv., CH_2Cl_2)	**7b** + **8b**	12 + 72
11	**4**	NCS (1.1 equiv., MeCN/TFA)	**6c** + **7c**	30 + 45
12	**4**	NCS (2.2 equiv., CH_2Cl_2)	**7c** + **8c**	41 + 40

In order to have the appropriate 13β-estrone derivatives (**6a**,**b**,**c**–**8a**,**b**,**c**) for the comparative enzyme inhibition studies, halogenations were carried out in this series, too. Our primary goal was to get the two regioisomers and the *bis*-compounds for the *in vitro* tests; therefore the regioselectivity was inessential. Different conditions were needed for the convenient synthesis of mono- and disubstituted 13β compounds. Monosubstitutions occurred using nearly 1 equiv. of *N*-halosuccinimide (Table 1, Entries 7, 9, 11), but the excess (nearly 2 equiv.) of the halogenating agent led to disubstitution (Table 1, Entries 8, 10, 12), too. The aimed halogenations at C-2 and/or C-4 were achieved and the flash chromatographic separations of the reaction mixtures furnished the desired compounds (**6**–**8**).

Enzyme inhibition studies

Aromatase, STS and 17β-HSD1 belong to different enzyme families with distinct catalytic mechanisms[18–20]. Specific inhibitors of these enzymes may be developed because of the differences in their active sites[4]. Dual or multiple inhibition might also be of value since inhibition of only one of these three enzymes is not adequate in treatment of hormone-dependent breast cancer. Since aromatase is needed for the synthesis of E1, hormone-dependent breast cancer may be more effectively treated by dual inhibition of aromatase and STS. Among the inhibitors of the mentioned three enzymes, aromatase inhibitors are clinically the most effective for hormone-dependent breast cancer.

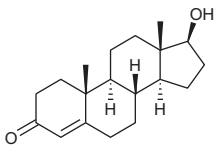
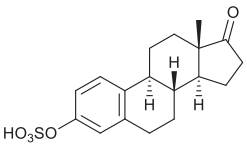
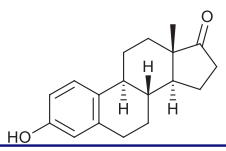
Beside their efficiency, the estrogen deprivation is accompanied with resistance and side effects. It would be crucial to design new drug candidates without such side effects. Innovative treatment strategies combining inhibitors of STS or 17β-HSD1 with aromatase inhibitor could lower the dose of the latter and extend the disease progression. The tumor-selective lowering of E2 levels could be achieved by the use of inhibitors of these two enzymes together with the aromatase inhibitor.

Literature data reveal that three-dimensional structures of these enzymes have not met expectations in drug design, but they are useful in understanding the catalytic mechanisms and inhibitor binding[4]. Aside from the differences in the active sites and catalytic mechanisms, these three enzymes may be inhibited by similar, estrone-based inhibitors. Slight differences in the structures of the potential inhibitors, involving regioisomerism, may influence not only the extent but also the nature of inhibition. This seems to be true for the ring A halogenated derivatives in the 13β-estrone series[5–7]. Literature data suggest that substitutions at C-2 are advantageous concerning 17β-HSD1 and aromatase, but 4-regioisomers are better STS inhibitors. Nevertheless, there is no thorough comparative investigation in the literature concerning the aromatase, STS and 17β-HSD1 inhibitory activities of 2-, 4-, and 2,4-*bis*-chloro, -bromo, and -iodo estrones. The literature data are incomplete in this sense. The involvement of 17-keto- and 17-deoxy-13α-estrone compounds in the structure–activity determinations seems also reasonable. To the best of our knowledge, there has not been found any exact correlation between the structural characteristics of the investigated estrone derivative (regarding the conformation and the presence of the 17-keto function) and good aromatase, STS, or 17β-HSD1 inhibitory potential.

As a part of our efforts to get valuable pieces of information, which could help the development of more potent single or multiple inhibitors of estrogen biosynthesis, we describe here the evaluation of halogenated 17-keto-13β-, 17-keto-13α-, and 17-deoxy-13α-estrone derivatives (**6a**,**b**,**c**–**8a**,**b**,**c**, **9**–**16**) as 17β-HSD1, aromatase, and/or STS inhibitors.

Enzyme inhibition studies were performed by *in vitro* radiosubstrate incubations using human term placenta cytosol and microsomas as enzyme sources. Aromatase inhibition was measured on testosterone (**2**) to E2 (**5**) conversion, STS inhibition was investigated via hydrolytic release of E1 (**4**) from E1S (**3**), whereas the influence on 17β-HSD1 was tested by the transformation of (**4**) to E2 (**5**). Relative conversions compared to non-inhibited controls (100%) were measured in the presence of 10 μM concentration of the test compound. For more efficient compounds, IC_{50} values were determined and inhibitory potentials were assessed also in comparison to IC_{50} data of the corresponding substrate. Reference IC_{50} parameters measured for the substrates and the basic compound E1 (**4**) are listed in Table 2. Mechanistic and kinetic

Table 2. Reference IC50 parameters of the substrate compounds. Relative conversions (Rel. conv., control incubation with no inhibition is 100%) measured in the presence of 10 μM concentration of the compound tested. Mean ± SD, n = 3.

Comp.	Structure	Arom IC_{50} ± SD (μM)	STS IC_{50} ± SD (μM)	17β-HSD1 IC_{50} ± SD (μM)
Testosterone (**2**)		0.52 ± 0.14		
E1S (**3**)			5.2 ± 1.2	
E1 (**4**)		>10 Rel. conv. 78 ± 7%	24 ± 10	0.63 ± 0.11

IC_{50}: the inhibitor concentration decreasing the enzyme activity to 50%. K_i: inhibitor constant determined from Dixon plot; SD: standard deviation.

investigations were performed and inhibitory constants (K_i) were determined for selected compounds.

Aromatase

According to literature information, introduction of F, Cl, Br, Me, and formyl groups to C-2 of E1 affords compounds with high binding affinity to aromatase enzyme[5]. 2-Bromoestrone (**6b**) proved to be an efficient inhibitor with an IC_{50} value of 2.4 μM, whereas 2-chloroestrone (**6c**, $K_i = 0.13$ μM) seemed to be more potent displaying about a 20-fold enhancement in its affinity compared to E1 ($K_i = 2.50$ μM). Concerning **6c** as a powerful competitive inhibitor, no evidence of enzymatic generation of a reactive substance was observed. Exact correlations between inhibitory activity and size and/or electronegativity of substituents at C-2 could not be established. Inhibitory activities of estrone analogs were found to be higher than those of the corresponding estradiol derivatives. Consequently, a 17-carbonyl function plays a crucial role in the binding of estrogens to the active site of aromatase enzyme, as observed in the cases of the androgen derivatives[21–24]. Halogenation at the C-4 position, except for fluorination, markedly decreased affinities. Osawa *et al*. suggested that substrates bind to the active site of aromatase through two conformations [β-side up (normal) and α-side up (upside down)], or have the opportunity and space to rotate around the binding site[25]. In the β-side up binding mode, C-2 is located close to the heme. This binding allows the catalysis of 2-hydroxylation. The estradiol molecule may rotate by 180° through the O(3)–O(17) axis, resulting in the α-side up binding mode, which allows 4-hydroxylation but to a lesser extent. The higher inhibitory potential of C-2-substituted E1 analogs compared with those of their C-4 substituted counterparts may be related to the high aromatase C-2 hydroxylation activities. These literature results indicate that concerning estrone-based aromatase inhibitors, the nature of the C-17 substituent, the substitution pattern of the aromatic ring, and the conformation of the compound greatly influence their inhibitory behavior.

In this contribution we also report *in vitro* aromatase inhibition tests of the synthesised 13β- and 13α-estrone derivatives. Certain 2-halogenated 13β-estrone derivatives (**6b** and **6c**) displayed low micromolar inhibition (Table 3). 2-Chloroestrone (**6c**) was found to be the most effective with its IC_{50} value of 6.0 μM. 2-Bromoestrone (**6b**) was slightly less potent ($IC_{50} = 8.7$ μM). These results are in a good agreement with those of Numazawa *et al*.[5]. 2-Iodoestrone (**6a**) displayed weaker inhibition with a relative conversion of 66% at a 10 μM test concentration ($IC_{50} > 10$ μM). Nevertheless, both derivatives have enhanced efficiency compared to their parent compound E1. The results obtained for the 2-halogenated 13β-estrone derivatives reveal that the inhibitory potential decreases with the increasing size of the halogen substituent.

Other test compounds including 13α-estrone (**9**), its 17-deoxy counterpart (**13**), and their halogenated derivatives (**10**–**12**, **14**–**16**) exerted very weak inhibitory effect: their relative conversion data are higher than 80% at a 10 μM test concentration. The empirical rules previously established in the 13β-series have not been observable in the 13α-estrone series, while the affinity for aromatase enzyme of the two basic 13α-estrone derivatives (**9** and **13**) could not be improved by attaching halogens onto ring A. This might be explained by the lack of ability of 13α-estrones for binding to the active site, because of their core-modified structure.

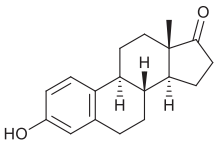
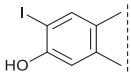
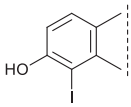
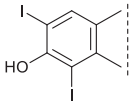
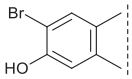
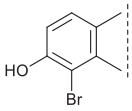
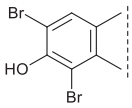
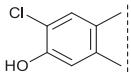
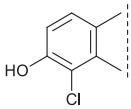
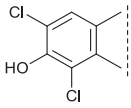
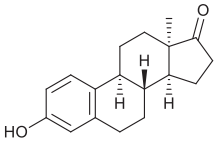
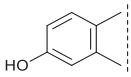
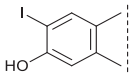
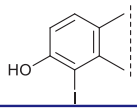
STS

Numerous STS inhibitors have already been described in the literature[7]. Estrone aryl sulfamates are known as irreversible, suicide inhibitors. EMATE is a highly potent STS inhibitor, but because of its estrogenic activity it is not an adequate antitumor drug candidate. As literature data show the 17-deoxy analog of EMATE (NOMATE) displays similar STS inhibitory potential as its 17-keto counterpart[26,27]. This suggests that the presence of the 17-keto function is not essential for the effective inhibition of 3-sulfamates. E1 displays weak binding to STS, but its certain counterparts substituted in ring A exert substantial inhibition. This proves that appropriately substituted 3-OH E1 derivatives may also be good inhibitor candidates. It was established that substitution at C-4 of E1 with relatively small electron withdrawing-groups, such as F, Br, CN, formyl, or NO_2, lead to improvement in inhibitory potency, which may be attributed to H-bonding and/or steric or other interactions. It is known that 4-formylestrone is a time- and concentration-dependent irreversible inhibitor of STS[7], and it inactivates the enzyme by reacting with active site residues. Phan et al. proposed that the 4-formyl function is involved in Schiff base formation with amino groups in appropriate side-chains including Lys-368, Lys-134, and Arg-79[7]. As reported in the literature, the 3-OH function of the inhibitor may be involved in H-bonding with certain amino acid residues, with His346 and/or formylGly75 hydrate among others. Concerning hydrogen-bonding abilities, these side-chains are bifunctional. His346 may accept proton through its π-cloud and nitrogen, but may donate its NH proton. FormylGly75 hydrate may establish hydrogen bonds with its carbonyl group as a proton acceptor, whereas its OH group may behave as a proton donor. On the part of the steroid, both the OH function and its phenolate may form H-bonds or specific interactions. The electron-withdrawing properties of the introduced ring A substituents may greatly influence the polarisation and the acidity of the 3-OH group. This substituent effect depends on the position, number and nature of the introduced groups. Certain substituents at the *ortho* positions may additionally be involved in intramolecular H-bonding with the 3-OH group, which may reduce the affinity of the inhibitor to the enzyme. Phan et al. have not found direct correspondence between the estimated pK_a values (taken from the corresponding *o*-substituted phenols) and the inhibitor potentials of their examined compounds[7].

Taking into account the above-mentioned literature results, it can be stated that not only the presence of a 3-*O*-sulfamoyl group but also the introduction of a relatively small electron-withdrawing group to carbon 4 of E1 may be a general possibility to enhance the potency of estrone-based STS inhibitors.

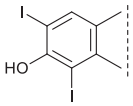
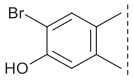
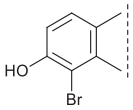
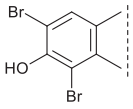
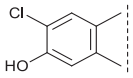
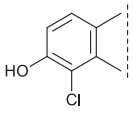
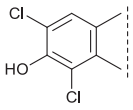
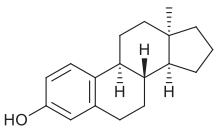
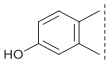
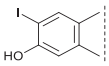
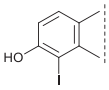
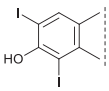
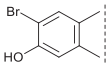
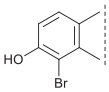
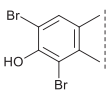
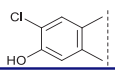
Here we start with the evaluation of the 2- and/or 4-chlorinated, brominated or iodinated 13β-estrone derivatives (**6a**,**b**,**c**–**8a**,**b**,**c**). The 4-iodo compound (**7a**) exerted outstanding submicromolar inhibition (Table 3). Its 0.23 μM IC_{50} value indicates a 22-fold higher affinity compared to the E1S substrate and an affinity increased by 100-fold compared to E1. **6a** its 2-sbstituted counterpart exerted 100-fold weaker inhibition according to its IC_{50} value. 4-Bromo derivative **7b** displayed submicromolar inhibitory potential with an IC_{50} value nearly twice as high as that of its 2-counterpart (**6b**). Phan et al. recently reported that the inhibition potential of **6c** is modest, whereas its 4-counterpart **7b** displays considerable inhibition[7]. Even so we found that both isomers are potent inhibitors. This difference may be ascribed to different substrates used in the two methods. Phan et al. used an artificial substrate (4-methylumbelliferyl sulfate), whereas we applied the natural substrate estrone-3-sulfate (**3**). The different binding specificity of these substrates may result in different

Table 3. *In vitro* inhibition of enzyme activities by the test compounds.

		Arom		STS		17β-HSD1	
Comp.	Structure	Rel.conv. ± SD (%)	IC_{50} ± SD (μM)	Rel.conv. ± SD (%)	IC_{50} ± SD (μM)	Rel.conv. ± SD (%)	IC_{50} ± SD (μM)
							
6a		62 ± 1		64 ± 3			0.064 ± 0.034
7a		88 ± 2			0.23 ± 0.09 K_i = 0.36 ± 0.05 μM		0.36 ± 0.25
8a		86 ± 6		80 ± 13		55 ± 7	
6b			8.7 ± 2.8		2.0 ± 0.4		0.095 ± 0.031
7b		91 ± 6			0.89 ± 0.3		0.30 ± 0.20
8b		81 ± 5			2.1 ± 0.6		0.96 ± 0.45
6c			6.0 ± 1.2		2.4 ± 0.4		0.18 ± 0.02
7c		92 ± 3			1.6 ± 0.3		0.60 ± 0.16
8c		82 ± 4			3.0 ± 0.9		0.59 ± 0.16
							
9		85 ± 13		96 ± 1			1.2 ± 0.2 [12] K_i = 1.9 ± 0.2 μM 0.59 ± 0.23 [13]
10a		82 ± 10		83 ± 3			
11a		90 ± 7			6.0 ± 1.6		1.0 ± 0.3 [13] K_i = 2.2 ± 0.3 μM

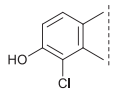
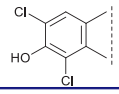
(*continued*)

Table 3. Continued.

		Arom		STS		17β-HSD1	
Comp.	Structure	Rel.conv. ± SD (%)	IC_{50} ± SD (μM)	Rel.conv. ± SD (%)	IC_{50} ± SD (μM)	Rel.conv. ± SD (%)	IC_{50} ± SD (μM)
12a		91 ± 1			2.4 ± 0.5		0.38 ± 0.08 [13] K_i= 0.94 ± 0.15 μM
10b		100 ± 5		81 ± 6			1.2 ± 0.3 [13]
11b		78 ± 1			8.5 ± 3.1		2.1 ± 1.2 [13]
12b		108 ± 11		71 ± 4			2.7 ± 0.1 [13]
10c		106 ± 6		57 ± 1			0.33 ± 0.10
11c		98 ± 3		80 ± 7			2.6 ± 1.0
12c		101 ± 4		70 ± 3			2.2 ± 0.6
							
13		95 ± 12		76 ± 5			1.1 ± 0.33 K_i = 2.0 ± 0.4μM
14a		89 ± 9			3.9 ± 1.6		2.9 ± 1.6
15a		92 ± 7			2.7 ± 1.3	57 ± 9	
16a		94 ± 2		59 ± 13		61 ± 7	
14b		97 ± 5			4.1 ± 1.3		1.3 ± 0.8
15b		90 ± 8			3.7 ± 1.2	49 ± 12	11 ± 4
16b		82 ± 6			7.5 ± 2.0		4.1 ± 2.5
14c		88 ± 10			7.0 ± 1.9		2.6 ± 1.3

(*continued*)

Table 3. Continued.

Comp.	Structure	Arom		STS		17β-HSD1	
		Rel.conv. ± SD (%)	IC_{50} ± SD (μM)	Rel.conv. ± SD (%)	IC_{50} ± SD (μM)	Rel.conv. ± SD (%)	IC_{50} ± SD (μM)
15c		89 ± 1			6.3 ± 1.8		4.5 ± 2.0
16c		82 ± 12			1.3 ± 0.4 K_i = 1.9 ± 0.2 μM	53 ± 2	

inhibition results. Nevertheless, our results are in good agreement with those obtained recently by Phan et al. concerning the effect of the regioisomerism on the inhibitory potential[7]. The two chlorinated regioisomers (**6c** and **7c**) displayed commensurate inhibitory properties in the low micromolar range. Concerning all data obtained for the monosubstituted compounds (**6**, **7**), it can be stated that introduction of a large halogen substituent onto C-4 is particularly advantageous. The 2,4-*bis*-iodo compound (**8a**) had no marked influence on the conversion of E1S–E1. The *bis*-bromo compound (**8b**) displayed low micromolar inhibition, commensurable with that of its *bis*-chloro counterpart (**8c**).

With all compounds and their inhibitory data in hand we were additionally interested in the investigation of the potential correlation between their predicted pK_a values and the measured inhibitory potentials. pKa values were calculated using computer software available online[28]. Data are not listed separately. Predicted pK_a values suggest that our monosubstituted derivatives bear protonated 3-OH function ($pK_a > 7.6$), whereas *bis* compounds ($pK_a = 6.8–7.4$) are partially or completely deprotonated under the physiological conditions (pH =7.3) applied in our experiments. The obtained inhibition potentials do not have apparent direct relationship with the number and electronegativity of the introduced halogens, neither reflect the predicted pK_a data. Differences observed between inhibitory potentials of the two regioisomers indicate that electronic properties of the introduced halogens are not the determining factors in binding interactions of the 3-OH group[7].

In the 13α-estrone series, the inhibitory potential of the compounds tested greatly depended on both the nature and the position of the halogen introduced. Introduction of iodine onto ring A was advantageous over substitution with smaller halogens (Br or Cl). 4-Bromo and 4-iodo compounds (**11a** and **11b**) were effective inhibitors with IC_{50} values of 8.5 and 6.0 μM. These regioisomers displayed better inhibitory potentials than their two counterparts (**10a** and **10b**). In the iodinated series, the best inhibitor was *bis*-iodo derivative **12a** with its low micromolar IC_{50} value. The chlorinated compounds (**10c**–**12c**) displayed weak inhibition with relative conversions over 50%.

The data obtained for the halogenated 17-deoxy-13α-estrone derivatives (**14**–**16**) reveal that all monohalogenated compounds (**14**, **15**), independently from the regioisomerism, are potent STS inhibitors in the low micromolar range. In general, these compounds displayed higher inhibitory potential than those of their 17-keto counterparts (**10**, **11**). We found that the presence of the 17-keto function on the halogenated 13α-estrane core is not essential for STS inhibitory activity. Additionally, empirical rules established previously do not predominate in this series. Furthermore, the IC_{50} values of the two regioisomers are comparable and the nature of introduced halogen is not crucial. Concerning the *bis*-substituted compounds (**16a**–**16c**), their

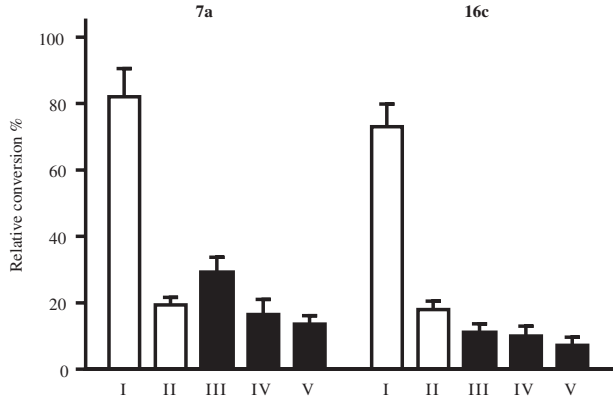


Figure 2. Investigation of STS inhibition reversibility of selected 13β-estrone compounds **7a**, **16c**. Inhibitor compounds were preincubated with human placental microsomes for 10 and 20 min. Following a 50-fold dilution step and 20 min secondary incubation to allow dissociation, the usual enzyme activity measurement was applied. Mean ± SD of three separate experiments. Experimental conditions: I No preincubation, **7a** 0.03 μM; **16c** 0.2 μM, II No preincubation, **7a** 1.5 μM; **16c** 10 μM, III Preincubation, 2.5 min, **7a** 1.5 μM; **16c** 10 μM, IV Preincubation, 10 min, **7a** 1.5 μM; **16c** 10 μM, V Preincubation, 20 min, **7a** 1.5 μM; 16c 10 μM.

inhibitory potential greatly depended on the nature of the halogen introduced. Surprisingly, the most potent **16c** compound in the 17-deoxy series, displaying an IC_{50} value of 1.3 μM, bears the smallest halogens. Compound **16c** exerts inhibition comparable to that of 4-bromoestrone **7b** (IC_{50} = 1.3 and 0.89 μM, respectively), but lower to that of 4-iodoestrone **7a** (IC_{50} = 0.23 μM).

The most potent representative test compounds **7a** and **16c** were selected for mechanistic and kinetic investigations. In the reversibility tests, placental microsomas were preincubated with high concentration of the inhibitor and enzyme activities were measured after dilution of the samples. Relative conversions in these preincubated samples (Figure 2, III and V) show suppressed enzyme activities, similar to those obtained for incubations with high inhibitor concentrations (Figure 2, II). Results indicate that **7a** and **16c** molecules do not dissociate upon dilution and they are bound to the enzyme in an irreversible manner. The time dependence of the reversibility test reveals that irreversible binding is nearly completed within a short 2.5 min time for both compounds (Figure 2, III).

Kinetic analyses of selected compounds **7a** and **16c** were performed by measurement of enzyme activities using different fixed substrate concentrations and varied inhibitor concentrations. Lines of the Dixon's plots intersect in the second quadrant revealing competitive inhibition mechanisms (Figure 3, B). To confirm the competitive nature of the inhibition of **7a**, replot of slopes vs. 1/substrate concentration was constructed (inset B/**7a** in Figure 3). The resulting straight line passing through the origin supports the kinetics obtained from the Dixon's plot[29,30]. Phan and coworkers

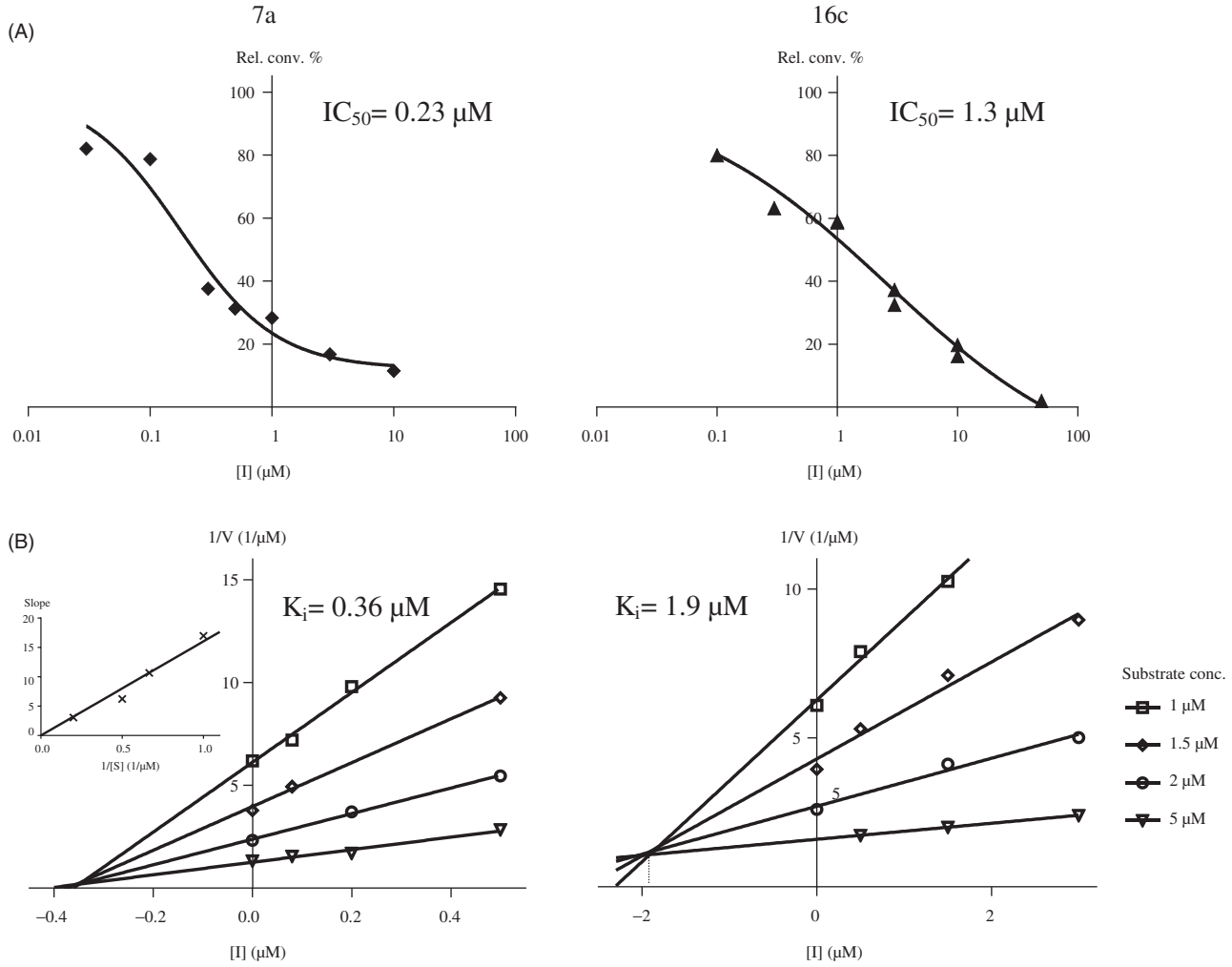


Figure 3. Concentration-dependent STS inhibition (A) and Dixon's kinetic analysis (B) of selected 13β-estrone compounds **7a**, **16c**. Inset in B/7a shows the secondary plot of slopes of the Dixon's lines vs. 1/substrate concentration.

investigated 4-nitroestrone as a representative estrone derivatives substituted at position C-4 with various electron-withdrawing functions. They have found the binding to be noncompetitive indicating that 4-nitroestrone is bound to the enzyme outside of its active site[7]. Our test compounds, 4-iodoestrone **7a** and 2,4-*bis*-chloro-17-deoxy-13α-estrone **16c** selected for the mechanistic investigations displayed a competitive mechanism, which alludes to binding within the active site of the enzyme.

K_i parameters were determined from the intersections of the Dixon's plots, and these values were found to be 0.36 µM for **7a** and 1.9 µM for the **16c**. These measured K_i values reflected inhibitory potentials ranked according to the IC_{50} data.

17β-hsd1

The crystal structure of 17β-HSD1 in its complex with E2 has been recently reported[31]. On the basis of this structure, molecular dynamic simulations and ligand–protein docking studies showed that there is an unoccupied lipophilic tunnel to the exterior of the protein located near the C-2 atom of E2[6]. These results may serve as the basis for the development of potent inhibitors, while the binding affinity of the potential inhibitor might be enhanced by introducing a lipophilic moiety to position 2. Möller *et al.*[6] have found that 2-bromo and 2-chloro derivatives of E1 exerted potent inhibition and displayed similar binding affinity to that of the parent compound. We previously extended their research to the 13α-estrone series by performing brominations or iodinations on its aromatic ring[17]. All synthesised 3-OH derivatives (**10a**,**b**–**12a**,**b**) displayed outstanding low or submicromolar 17β-HSD1 inhibitory activities, but their IC_{50} values depended on the number, the nature and the position of the A-ring substituents (Table 3). The iodo derivatives (**10a**–**12a**) proved to be more potent than their bromo counterparts (**10b**–**12b**). The finding, that the 2,4-*bis*-iodo compound (**12a**) displays a submicromolar IC_{50} of 0.38 µM, was a novel result[17].

Concerning that the iodo derivatives in the 13α-estrone series displayed high inhibitory potential, the iodo counterparts in the 13β-estrone series (**6a**–**8a**) have also been tested. Both iodo regioisomers (**6a**, **7a**) exerted outstanding inhibition, but the 2-iodo compound (**6a**) proved to be more potent with an IC_{50} value of 0.064 µM (Table 3). 2-Bromo- and 2-chloro-13β-estrone (**6b** and **6c**) exerted somewhat weaker potentials as their iodo counterpart (**6a**), nevertheless, we observed their improved binding affinity compared to the parent compound E1.

Efficiency of the 4-substituted counterparts (**7a**, **7b**) was found to be slightly weaker. These results are in a good agreement with empirical rules established previously concerning the two substitution to be up against the four substitution. New inhibition results of the iodo compounds (**6a**–**7a**) are particularly remarkable, and the inhibition potential of the 2-iodo regioisomer is outstanding.

Additionally, all chlorinated derivatives (**6c**–**8c**) displayed outstanding commensurate submicromolar inhibition.

The three newly synthesised chloro 13α-estrone compounds (**10c**–**12c**) proved to be potent inhibitors. The 2-regioisomer (**10c**) seemed to be the most prominent with its submicromolar IC_{50} value of 0.33 μM. This value is commensurate with that of compound **6c**. This result indicates a twofold better binding than that of E1, and an affinity increased by fourfold in comparison to parent compound 13α-estrone **9**. The 4-chloro and the 2,4-*bis*-chloro derivatives (**11c** and **12c**) exerted somewhat weaker inhibitions (IC_{50} values were found to be 2.6 and 2.2 μM, respectively).

Comparing recent results obtained for bromo and iodo compounds in both the 13α- and 13β-series with those of the chloro-13α derivatives, it can be stated that there is less difference in the inhibitory potential of the two regioisomers in the 13α-series than in the natural estrone series except for the chloro-13α compounds. The inhibitory potential of 13α-estrone (**9**) increased four-fold by adding chlorine, a relatively small electron-withdrawing group, to C-2.

We were interested in the comparison of the inhibitory results concerning the 17-keto and the 17-deoxy-13α derivatives. The basic deoxy compound **13** displayed a surprising but outstanding inhibitory potential comparable with that of reference E1, the natural substrate of the enzyme. We expected that this promising result might be improved by the introduction of halogens to the aromatic ring of **13**. Nevertheless, the 1.1 μM IC_{50} value of **13** could not be lowered significantly. All halogenated derivatives (**14**–**16**) displayed comparable or higher values than that obtained for compound **13**. In this series the 2-halogenated compounds (**14a**–**c**) were found to be the best inhibitors (IC_{50} values 2.6, 1.3, and 2.9 μM, respectively). The 4-chloro (**15c**) and the 2,4-*bis*-bromo derivative (**16b**) exerted modest inhibition (IC_{50}=4–5 μM), whereas other 17-deoxy-13α derivatives displayed weaker inhibition: their relative conversions exceeded 50% indicating $IC_{50} > 10$ μM.

It was established that the advantage of the 2-substitution over the 4-halogenation shows up in the 17-deoxy series, too. However, none of the new 17-deoxy inhibitor candidates displayed submicromolar IC_{50} values.

Potent 13α-estrone derivatives, basic compound **9**, its 4-iodo (**11a**) and 2,4-*bis*-iodo derivative **12a**, were selected for mechanistic and kinetic studies. To provide some information about the mechanism of action, we performed reversibility tests by preincubation of inhibitors with human placental cytosol. Figure 4 shows relative conversions obtained for the selected inhibitors according to preincubation conditions I–IV. The results indicate that the relative conversions in preincubated and diluted samples (Figure 4, III and IV), were similar to that obtained for incubation with the lower concentration of the inhibitor (Figure 4, I). This means that inhibitors **9**, **11a** and **12a** can be released from binding by dilution that is they bind to the enzyme in a reversible manner.

In order to characterise the inhibition type and determine the K_i, inhibition experiments were performed for the selected inhibitors **9**, **11a** and **12a** at different fixed substrate concentrations in the presence of cofactor excess. On the Dixon's plot, data for each substrate concentration fall on straight lines which intersect in the second quadrant, alluding to competitive inhibition mechanism (Figure 5, B)[29,30]. Inhibitors that bind to the steroid site of 17β-HSD1 can bind to both the free enzyme and the binary enzyme–-cofactor complex by random kinetic mechanism. This mixed-type inhibition, nevertheless, is simplified if the enzyme is saturated with cofactor first and displays competitive patterns[32]. Reversible and apparently competitive mechanism of the inhibition observed in our experiments shows that inhibitors **9**, **11a**, and **12a** are

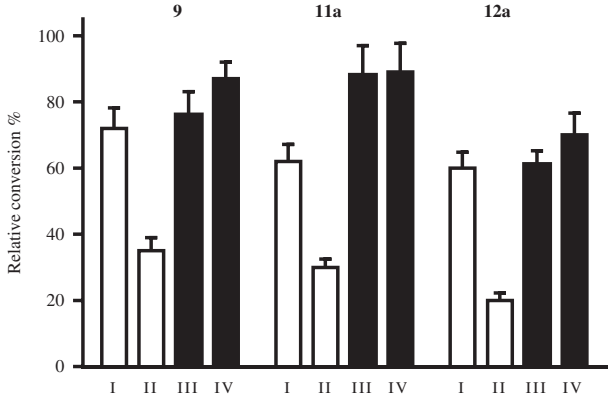


Figure 4. Investigation of 17β-HSD1 inhibition reversibility of selected 13α-estrone compounds **9**, **11a**, **12a**. Inhibitor compounds were preincubated with human placental cytosol. Following a 50-fold dilution step, the usual enzyme activity measurement was applied. Mean ± SD of three separate experiments. Experimental conditions: I No preincubation, 0.2 μM, II No preincubation, 10 μM, III Preincubation, 10 μM, 2.5 min, IV Preincubation, 10 μM, 20 min.

bound in the substrate-binding cavity of 17β-HSD1 with non-covalent interactions.

K_i parameters were determined from the intersections, and they were found to be 1.9, 2.2, and 0.94 μM for compounds **9**, **11a**, and **12a**, respectively. Inhibitory potentials on the basis of these K_i data were comparable to those obtained by the IC_{50} values.

The 3-OH group of E1 and related inhibitors plays an important role in the binding to 17β-HSD1 forming a hydrogen-bonding system with the His221 and Glu282 residues at the recognition end of the active site[33,34]. However, literature data show high affinity of certain 3-OMe derivatives indicating that ligands may establish effective binding even in the absence of proton on the C-3 substituent[16,17,35–37]. Halogen substituents at C-2 and/or C-4 position modify binding abilities of the 3-OH substituent. The electron-withdrawing effect increases polarisation of the O–H bond and may induce deprotonation of this group under physiological pH conditions. *ortho* Substituents might also be able to form intramolecular hydrogen bond with the 3-OH group, which is disadvantageous[6].

Our inhibition results demonstrate that introduction of halogen atoms to C-2 and/or to C-4 position increases affinity to 17β-HSD1. However, there does not appear to be a direct relationship between the number and electronegativity of the halogens and the inhibitor potency.

Multiple or specific inhibition

Certain dual 17β-HSD1 and STS inhibitors were identified in both the 13β- and 13α-estrone series. Two 4-halo-17-keto-13β compounds (**7a** and **7b**) elicited submicromolar inhibitory effect towards both enzymes. Certain additional 17-keto compounds (**6b**, **6c**, **7c**, **8c**, **12a**) possess dual inhibitory properties with IC_{50} values in submicromolar or low micromolar range. In the 17-deoxy-13α-estrone series, all two-halogenated compounds (**14**), the 2,4-*bis*-bromo- (**16b**) and 4-chloro derivative (**15c**) exerted potent low micromolar dual action. Two compounds, namely 2-bromo- and 2-chloro-13β-estrones **6b** and **6c** exerted considerable inhibitions towards the three investigated enzymes.

It is interesting to note that inhibitory potentials of iodo derivatives in the 13β-estrone series display outstanding variations. 2-Iodo compound **6a** is a highly specific 17β-HSD1 inhibitor, but **7a** its 4-counterpart has dual STS and 17β-HSD1 inhibitory

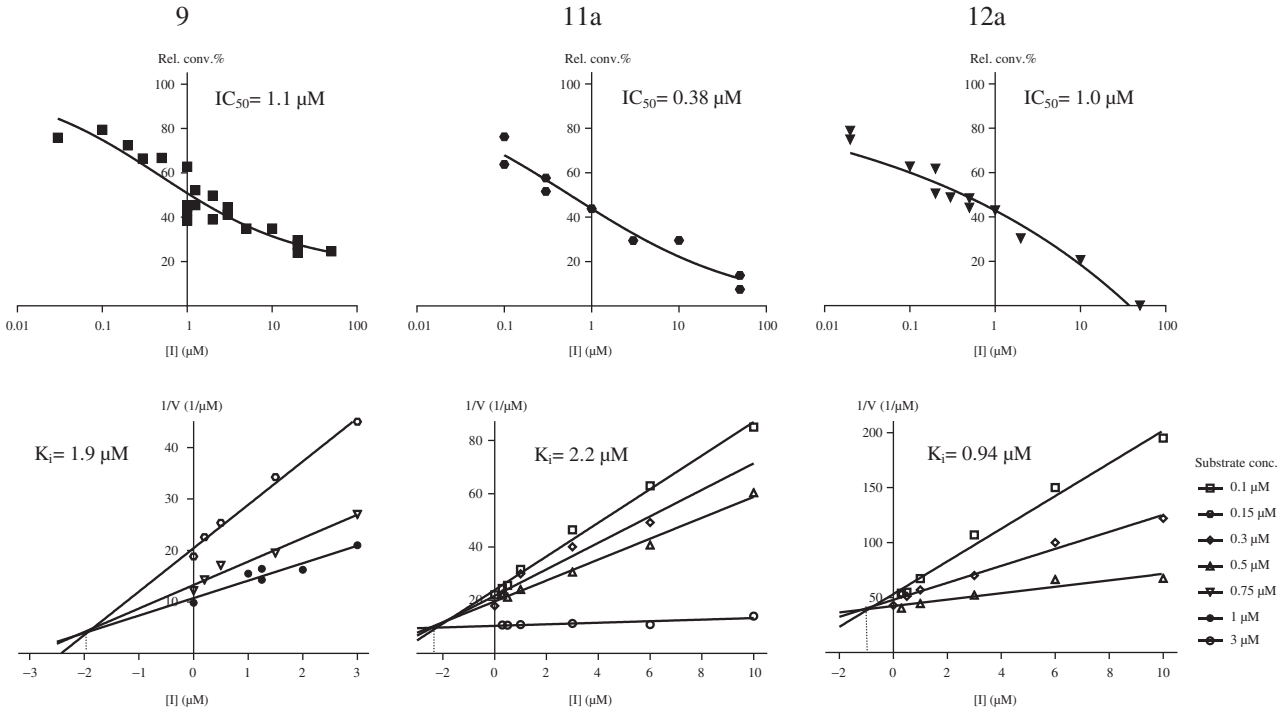


Figure 5. Concentration-dependent 17β-HSD1 inhibition (A) and Dixon's kinetic analysis (B) of selected 13α-estrone compounds **9, 11a, 12a**.

potential. The disubstituted derivative (**8a**) exerts weak effects towards the enzymes investigated.

Our results confirm that structurally different enzymes with distinct catalytic mechanisms might be inhibited by the same inhibitor compounds. Newly detected multiple 17β-HSD1, STS and/or aromatase inhibitors might be superior to compounds affecting the action of only a single enzyme. These multiple inhibitors may serve as good candidates for efficient suppression of local estrogen production in breast cancer tissues.

Conclusions

Extensive research has been carried out in recent decades concerning enzyme inhibitors able to block estrogen biosynthesis. An armament of aromatase inhibitors is available by now in the medical practice; nevertheless, research work is continued to eliminate side effects and resistance developed by these medications[38]. Numerous compounds have also been evaluated as potential STS or 17β-HSD1 inhibitors, but these efforts have not been crowned with success, as none of the drug candidates has been clinically introduced for the treatment of estrogen-dependent diseases[3,32,39]. In order to develop potent new inhibitors of estrogen biosynthesis, a profound understanding of the enzymatic mechanisms and the structure–function relationships is essential. Our results obtained for aromatase, STS and 17β-HSD1 inhibition of 13α- and 13β-estrone compounds bearing halo substituents on their ring A make valuable contribution to this aim.

Disclosure statement

No potential conflict of interest was reported by the authors.

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VIII.

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Synthesis of substituted 15β-alkoxy estrone derivatives and their cofactor-dependent inhibitory effect on 17β-HSD1

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Abstract

17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) is a key enzyme in the biosynthesis of 17β-estradiol. Novel estrone-based compounds bearing various 15β-oxa-linked substituents and hydroxy, methoxy, benzyloxy, and sulfamate groups in position C3 as potential 17β-HSD1 inhibitors have been synthesized. In addition, *in vitro*inhibitory potentials measured in the presence of excess amount of NADPH or NADH were investigated. We observed substantial inhibitory potentials for several derivatives (IC_{50} < 1 μM) and increased binding affinities compared to unsubstituted core molecules. Binding and inhibition were found to be cofactor-dependent for some of the compounds and we propose structural explanations for this phenomenon. Our results may contribute to the development of new 17β-HSD1 inhibitors, potential drug candidates for antiestrogen therapy of hormone-dependent gynecological cancers.

Keywords: Michael addition; substituted 15β-alkoxy-estrone derivatives; 17β-HSD1; estrogen biosynthesis; NADPH and NADH

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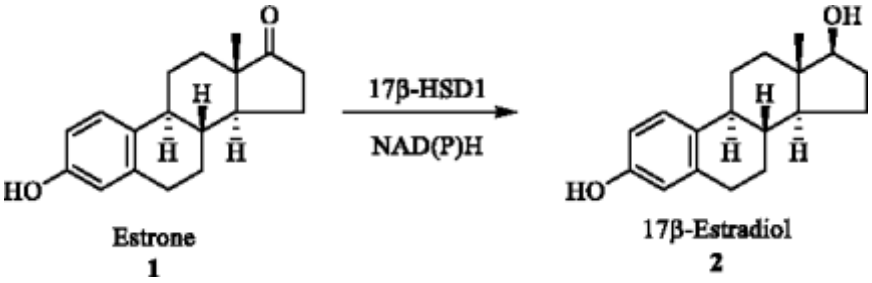
Introduction

The 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1, EC 1.1.1.62) catalyzes the conversion of estrone (**1**) to highly active estrogen 17β-estradiol (**2**) (Scheme 1). Human 17β-HSD1 is expressed in tissues of female reproductive organs (such as placenta, ovarian follicles, mammary gland and uterus)[1]. The expression of 17β-HSD1 was shown to be elevated and to have prognostic significance in gynecological malignancies, e.g. in hormone-dependent breast cancer[2–4]. The enzyme is involved in progression of these diseases due to the increase of local 17β-estradiol (**2**) levels. Inhibition of the enzyme is able to control estrogen actions at the pre-receptor level; therefore, a suppression of the

17β-HSD1 activity has a significant therapeutic potential[5]. Numerous 17β-HSD1 inhibitors based on either steroidal or non-steroidal structures have been developed, but none of them have been introduced to the medical practice, so far[6–11].

Scheme 1. Transformation of estrone to 17β-estradiol catalyzed by 17β-HSD1.



Estrone
1

17β-Estradiol
2

Extensive earlier studies indicated that attachment of C15 substituents on the estrone scaffold might be a successful way for the synthesis of inhibitors against 17β-HSD1[12,13]. In addition to improving binding affinity to 17β-HSD1, appropriate side chains may confer selectivity towards 17β-HSD type 2[7,10,14]. 17β-HSD type 2 catalyzes inactivation of 17β-estradiol (**2**) to estrone (**1**) and is considered to be an important enzyme for the control of proliferation of breast cancer cells[15]. C15 substituents may also suppress inherent estrogenicity of the estrone core[9,10,12,16]. These features of C15 derivatives make this substitution strategy particularly attractive for the development of estrone-based 17β-HSD1 inhibitors.

It was shown that neither the presence of the phenolic hydroxyl group nor the hydrogen bonding of the C3 function is essential to the effective 17β-HSD1 binding of estrone derivatives[17,18]. This tolerance provides further options for the modulation of enzyme inhibition and other biological effects of the candidate compounds. Accordingly, several 3-methoxy analogues were tested as 17β-HSD1 inhibitors[12,13] presumably that they exert reduced estrogenicity compared to estrone possessing phenolic hydroxy group[18]. Estrone 3-sulfamate analogues in this series tend to show moderate 17β-HSD1 inhibition[13]. However, the sulfamate moiety may lead to an inhibitory effect against steroid sulfatase (STS), another enzyme playing a central role in 17β-estradiol biosynthesis. Such a dual inhibitory effect was recently proposed to be beneficial as it should result in a stronger suppression of estrogen biosynthesis compared to selective inhibition of 17β-HSD1[19]. Steroidal sulfamates may be delivered to the tumour by the carbonic anhydrase II, and evolve targeted actions[20].

In this paper, we report the synthesis and chemical characterization of new substituted 15β-alkoxy estrone derivatives. We also aimed to investigation 17β-HSD1 inhibitory potentials of these compounds, including comparison of their inhibitor potentials measured in the presence of NADPH or NADH.

Experimental

General

Melting points (mp) were determined on a Kofler block and are uncorrected. Specific rotations were measured in $CHCl_3$, or MeOH (*c* 1) at 25 C with a POLAMAT-A (Zeiss-Jena) polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. Elementary analysis data were determined with a PerkinElmer CHN analyzer model 2400. Reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick); solvent systems (ss): (A) (ethyl acetate/CH_2Cl_2 (1:1 v/v), (B) acetone/toluene/hexane (30:35:35 v/v), (C) ethyl acetate/CH_2Cl_2 (5:95 v/v), (D) ethyl acetate. The spots were detected by spraying with 5% phosphomolybdic acid in 50% aqueous H_3PO_4. The R_f values were determined for the spots observed by

illumination at 254 and 365 nm. Flash chromatography: silica gel 60, 40–63 μm. All solvents were distilled immediately prior to use. NMR spectra were recorded on a Bruker DRX 500 instrument at 500 (^{1}H NMR) or 125 MHz (^{13}C NMR). Chemical shifts are reported in ppm (δ scale), and coupling constants (*J*) in Hz. For the determination of multiplicities, the *J*-MOD pulse sequence was used.

Materials for enzyme experiments

Radiolabelled steroids [6,7-^{3}H(N)]estrone (S. A. = 52 Ci/mmol), was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). Non-radioactive estrone, 3-methyl-*O*-estrone, 3-benzyl-*O*-estrone and estrone-3-sulfamate (EMATE) standards and other chemicals and solvents ofanalytical grade purity were purchased from Sigma (St. Louis, MO, USA), from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany).

Human placenta microsomal fraction and cytosol were produced and applied with the permission of the Human Investigation Review Board of the University of Szeged.

General procedure for the synthesis of 3-methoxy-, and 3-benzyloxy-15β-alkoxyestra-1,3,5(10)-trien-17-ones (8–12)

A solution of **6**[23] or **7**[12] (2 mmol) in CH_2Cl_2 (10 ml) and ethane-1,2-diol, propane-1,3-diol or butane-1,4-diol (15 ml), containing 5% aqueous sodium hydroxide (1 ml) was stirred at room temperature for 6 h. The reaction mixture, after the addition of CH_2Cl_2 (50 ml), was diluted with water (100 ml). The organic phase was separated, washed with water, dried over Na_2SO_4, and evaporated *in vacuo.* The residual product was purified by flash chromatography using ethyl acetate/CH_2Cl_2 in different proportions.

3-Methoxy-15β-(2'-hydroxy)ethoxy-estra-1,3,5(10)-trien-17-one (8)

Compound **6** (565 mg, 2 mmol) and ethane-1,2-diol (15 ml) were used for the synthesis as described in general procedure. The crude product was chromatographed on silica gel with dichloromethane/hexane (1:1 v/v) to yield pure **8** (580 mg, 84%). Mp: 139–140 °C; R_f = 0.55 (ss B); $[\alpha]_D^{20}$ + 54 (*c* 1 in $CHCl_3$). Found: C, 73.45; H, 7.98. $C_{21}H_{28}O_4$ (344.45) requires: C,73.23; H, 8.19%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.16 (s, 3H, 18-H_3), 2.92 (m, 2H, 6-H_2), 3.42 and 3.64 (2xm, 2x1H, linker H_2), 3.72 (m, 2H, linker OCH_2), 3.77 (s, 3H, 3-OCH_3), 4.22 (t, 1 H, *J* = 5.3 Hz, 15-H), 6.64 (d, 1H, *J* = 2.2 Hz, 4-H), 6.71 (dd, 1H, *J* = 8.6 Hz, *J* = 2.2 Hz, 2-H), 7.19 (d, 1H, *J* = 8.6 Hz, 1-H).^{13}C NMR (δ, ppm, $CDCl_3$):17.5 (C-18), 25.6, 26.2, 29.4, 32.5, 34.8, 43.1, 44.0, 47.2 (C-13), 54.4, 55.1 (3-OCH_3), 61.9 (CH_2-OH), 70.7 (linker CH_2), 75.1 (C-15), 111.4 (C-2), 113.8 (C-4), 126.0 (C-1), 132.0 (C-10), 137.6 (C-5), 157.5 (C-3), 219.4 (C-17).

3-Methoxy-15β-(3'-hydroxy)propoxy-estra-1,3,5(10)-trien-17-one (9)

Compound **6** (565 mg, 2 mmol) and propane-1,3-diol (15 ml) were used for the synthesis as described in general procedure. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (1:99 v/v) to yield pure **9** (575 mg, 80%). Mp: 83–84 °C; R_f = 0.50 (ss B); $[\alpha]_D^{20}$ + 48 (*c* 1 in $CHCl_3$). Found: C, 73.92; H, 8.26%. $C_{22}H_{30}O_4$ requires: C, 73.71; H, 8.44%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.15 (s, 3H, 18-H_3), 2.94 (m, 2H, 6-H_2), 3.42 (m, 1H) and 3.74 (m, 3H): 2xlinker H_2, 3.77 (s, 3H, 3-OCH_3), 4.17 (t, 1 H, *J* = 5.3 Hz, 15-H), 6.64 (d, 1H, *J* = 2.2 Hz, 4-H), 6.71 (dd, 1H, *J* = 8.6 Hz, *J* = 2.2 Hz, 2-H), 7.19 (d, 1H, *J* = 8.6 Hz, 1-H).^{13}C NMR (δ, ppm, $CDCl_3$):17.4 (C-18), 25.7, 26.2, 29.4, 32.3, 32.6, 34.9, 42.8, 44.1, 47.2 (C-13), 54.2, 55.1 (3-OCH_3), 61.5 (CH_2-OH), 68.3 (linker CH_2), 75.1 (C-15), 111.4 (C-2), 113.8 (C-4), 126.1 (C-1), 131.9 (C-10), 137.7 (C-5), 157.5 (C-3), 219.5 (C-17).

3-Benzyloxy-15β-(2'-hydroxy)ethoxy-estra-1,3,5(10)-trien-17-one (10)

Compound **7** (717 mg, 2 mmol) and ethane-1,2-diol (15 ml) were used for the synthesis as described in general procedure. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (30:70 v/v) to yield pure **10** (690 mg, 82%). Mp: 102–104 °C; R_f = 0.45 (ss B); $[\alpha]_D^{20}$ + 73 (*c* 1 in $CHCl_3$). Found: C, 76.95; 7.84. $C_{27}H_{32}O_4$ (420.54) requires: C, 77.11; H, 7.67%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.17 (s, 3H, 18-H_3), 2.91 (m, 2H, 6-H_2), 3.42 and 3.65 (2xm, 2x1H, linker H_2), 3.73 (m, 2H, linker OCH_2), 4.23 (t, 1 H, *J* = 5.7 Hz, 15-H), 5.04 (s, 2H, Bn-H_2), 6.74 (d, 1H, *J* = 2.2 Hz, 4-H), 6.79 (dd, 1H, *J* = 8.6 Hz, *J* = 2.2 Hz, 2-H), 7.19 (d, 1H, *J* = 8.6 Hz, 1-H), 7.32 (t, 1H, *J* = 7.3 Hz, 4-H of Bn), 7.39 (t, 2H, *J* = 7.3 Hz, 3-H and 5-H of Bn), 7.45 (d, 2H, *J* = 7.3 Hz, 2-H and 6-H of Bn).^{13}C NMR (δ, ppm, $CDCl_3$): 17.6 (C-18), 25.7, 26.2, 29.5, 32.7, 34.9, 43.1, 44.2, 47.2 (C-13), 54.5, 62.0 (CH_2-OH), 70.0 (linker CH_2), 70.8 (Bn-CH_2), 75.2 (C-15), 112.4 (C-2), 114.9 (C-4), 126.1 (C-1), 127.4 (2 C: C-2 and C-6 of Bn), 127.8 (C-4 of Bn), 128.5 (2 C: C-3 and C-5 of Bn), 132.4 (C-10), 137.2 (C-1 of Bn), 137.7 (C-5), 156.9 (C-3), 219.1 (C-17).

3-Benzyloxy-15β-(3'-hydroxy)propoxy-estra-1,3,5(10)-trien-17-one (11)

Compound **7** (717 mg, 2 mmol) and propane-1,3-diol (15 ml) were used for the synthesis as described in general procedure. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (30:70 v/v) to yield pure **11** (742 mg, 78%). Mp: 144–146 °C; R_f = 0.35 (ss B); $[\alpha]_D^{25}$ + 88 (*c* 1 in $CHCl_3$). Found: C, 77.54; H, 8.02. $C_{28}H_{34}O_4$ (434.57) requires: C, 77.39; H, 7.89%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.15 (s, 3H, 18-H_3), 2.94 (m, 2H, 6-H_2), 3.36 (t, 2H, *J* = 6.0 Hz, linker H_2), 3.72 (m, 2H, linker

H_2), 4.17 (t, 1 H, *J* = 6.5 Hz, 15-H), 5.03 (s, 2H, H_2 of Bn), 6.73 (d, 1H, *J* = 3.0 Hz, 4-H), 6.78 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.18 (d, 1H, *J* = 10.5 Hz, 1-H), 7.37 (m, 5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 26.2, 26.6, 30.0, 32.8, 33.2, 35.4, 43.3 (linker CH_2), 44.7, 47.7 (C-13), 54.8, 62.1 (CH_2-OH), 68.9 (OCH_2), 70.4 (Bn-CH_2), 75.7 (C-15), 112.8 (C-2), 115.4 (C-4), 126.6 (C-1), 127.9 (C-2 and -6 of Bn), 128.3 (C-4 of Bn), 129.0 (C-3 and C-5 of Bn), 132.8 (C-10), 138.0 (C-1 of Bn), 138.3 (C-5), 157.4 (C-3), 219.7 (C-17)

3-Benzyloxy-15β-(4'-hydroxy)butoxy-estra-1,3,5(10)-trien-17-one (12)

Compound **7** (717 mg, 2 mmol) and butane-1,4-diol (15 ml) were used for the synthesis as described in general procedure. The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (1:1 v/v) to yield pure **12** (720 mg, 80%). Mp: 131–130 °C; R_f = 0.30 (ss B); $[\alpha]_D^{25}$ + 58 (*c* 1 in $CHCl_3$). Found: C, 77.48; H, 9.15. $C_{29}H_{36}O_4$ (448.59) requires: C, 77.64; H, 8.09%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.16 (s, 3H, 18-H_3), 3.34 (m, 2H, linker H_2, 3.64 (m, 2H, O-CH_2), 4.14 (t, 1 H, *J* = 6.5 Hz, 15-H), 5.03 (s, 2H, H_2 of Bn), 6.73 (d, 1H, *J* = 3.0 Hz, 4-H), 6.78 (dd, 1H, *J* = 10.5 Hz, 2-H), *J* = 3.0 Hz, 2H), 7.18 (d, 1H, *J* = 10.5 Hz, 1-H), 7.36 (m, 5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 26.2, 26.8, 27.0, 30.0, 33.2, 35.3, 41.9, 43.7 (linker CH_2), 44.7, 47.7 (C-13), 55.0, 63.0 (CH_2OH), 69.9 (OCH_2), 70.5 (CH_2 of Bn), 75.2 (C-15), 112.8 (C-2), 115.4 (C-4), 126.6 (C-1), 126.6 (C-2 and C-6 of Bn), 127.8 (C-4 of Bn), 128.3 (C-3 and C-5 of Bn), 132.9 (C-10), 138.0 (C-1 of Bn), 138.3 (C-5), 157.3 (C-3), 220.2 (C-17).

3-Methoxy-15β-(carboxyl)methoxy-estra-1,3,5(10)-trien-17-one (13)

Compound **8** was dissolved in acetone (15 ml). Jones reagent (1 ml) was added while cooling with ice. The mixture was diluted with ice-water, and the precipitate was filtered off, washed with water and dried. The crude product was dissolved in CH_2Cl_2 and was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (25:75 v/v), yielding pure **13** (285 mg, 39%). Mp: 142–144 °C; R_f = 0.32 (ss B); $[\alpha]_D^{25}$ + 42 (*c* 1 $CHCl_3$). Found C, 70.54; H, 7.43. $C_{21}H_{26}O_5$ (358.43) requires: C, 70.73; H, 7.31%. ^{1}H NMR (δ, ppm, DMSO-d_6): 1.08 (s, 3H, 18-H_3), 2.82 (m, 2H, 6-H_2), 3.69 (s, 3H, 3-OCH_3), 4.05 (m, 2H, O-CH_2), 4.29 (t, 1 H, *J* = 5.5 Hz, 15-H), 6.63 (s, 1H, 4-H), 6.68 (dd, 1H, *J* = 8.5 Hz, *J* = 2.0 Hz, 2-H), 7.17 (d, 1H, *J* = 8.5 Hz, 1-H), 12.60 (brs, 1H, OH). ^{13}C NMR (δ, ppm, DMSO-d_6): 17.2 (C-18), 25.3, 25.4, 29.1, 32.4, 34.7, 42.5 (C-13), 43.7, 46.5, 53.2, 54.8 (3-OCH_3), 65.8 (O-CH_2), 74.9 (C-15), 111.4 (C-2), 113.5 (C-4), 126.0 (C-1), 131.8 (C-10), 137.4 (C-5), 157.1 (C-3), 171.9 (COOH), 218.8 (C-17).

3-Methoxy-15β-(2'-carboxyl)ethoxy-estra-1,3,5(10)-trien-17-one (14)

Compound **9** was dissolved in acetone (15 ml). Jones reagent (1 ml) was added during cooling with ice. The mixture was diluted with ice-water, and extracted with CH_2Cl_2. The organic phase was evaporated to dryness and subjected to chromatographic separation on silica gel in ethyl acetate/CH_2Cl_2 (1:1 v/v), yielding pure **14** (346 mg, 46%). Mp: 150–152 °C; R_f = 0.30 (ss B); $[\alpha]_D^{25}$ + 46 (*c* 1 in $CHCl_3$). Found: C, 71.15; H, 7.32. $C_{22}H_{28}O_5$ (372.46) requires: C, 70.94; H, 7.58%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.14 (s, 3H, 18-H_3), 2.91 (m, 2H, 6-H_2), 3.59 (m, 1H, 14-H), 3.80 (s, 4H, 2x linker H_2), 4.21 (t, 1 H, *J* = 6.5 Hz, 15-H), 6.66 (s, 1H, 4-H), 6.73 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.21 (d, 1H, *J* = 10.5 Hz, 1-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 26.2, 26.6, 29.9, 33.1, 35.2, 35.5, 43.3, 44.6 (linker CH_2), 47.7 (C-13), 54.9, 55.6 (3-OCH_3), 65.0 (linker CH_2), 75.4 (C-15), 111.7 (C-2), 114.5 (C-4), 126.6 (C-1), 132.6 (C-10), 138.3 (C-5), 158.0 (C-3), 177.2 (COOH), 220.1 (C-17).

3-Benzyloxy-15β-(2'-carboxyl)ethoxy-estra-1,3,5(10)-trien-17-one (15)

Compound **11** (435 mg, 1 mmol) was dissolved in acetone (10 ml) and Jones reagent (1 ml) was added while cooling with ice. The mixture was diluted with ice-water, the precipitate separating out was filtered, dried and recrystallized from CH_2Cl_2/hexane, yielding **15** (342 mg, 76%). Mp: 184–186 °C; R_f = 0.30 (ss B); $[\alpha]_D^{20}$ + 98 (*c* 1 in $CHCl_3$). (Found: C, 74.86; H, 7.35. $C_{28}H_{32}O_5$ (448.55) requires: C, 74.79; H, 7.19%). ^{1}H NMR (δ, ppm, $CDCl_3$): 1.15 (s, 3H, 18-H_3), 2.93 (m, 2H, 6-H_2), 3.60 (m, 1H, O-CH_2), 3.81 (m, 1H, O-CH_2), 4.21 (t, 1H, *J* = 6.5 Hz, 15-H), 5.06 (s, 2H, Bn-H_2), 6.75 (s, 1H, 4-H), 6.80 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.21 (d, 1H, *J* = 10.5 Hz, 1-H), 7.40 (m, -5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.8 (C-18), 26.2, 26.6, 29.9, 33.2, 35.2, 35.5, 43.3 (linker CH_2), 44.6, 47.7 (C-13), 54.9, 65.0 (O-CH_2), 70.4 (Bn-CH_2), 75.4 (C-15), 112.6 (C-2), 115.5 (C-4), 126.6 (C-1), 127.9 (C-2 and C-6 of Bn), 128.3 (C-4 of Bn), 129.0 (C-3 and C-5 of Bn), 132.9 (C-10), 138.0 (C-1 of Bn), 138.4 (C-5), 157.3 (C-3), 177.3 (COOH), 220.1 (C-17).

3-Hydroxy-15β-(2'-carboxyl)ethoxy-estra-1,3,5(10)-trien-17-one (16)

Compound **15** (448 mg, 1 mmol) was dissolved in ethyl acetate (30 ml) and the solution containing Pd/C (300 mg, 10%) was hydrogenated under 5 bar for 6 h at room temperature. The solution was filtered off, the residue was crystallized from methanol to yield **16** (320 mg, 89%). Mp: 200–202 °C; R_f = 0.2 (ss B); $[\alpha]_D^{25}$ + 29 (*c* 1 in $CHCl_3$). Found C, 70.18; H, 7.45. $C_{21}H_{26}O_5$ requires: C, 70.37; H, 7.31%. ^{1}H NMR (δ, ppm, DMSO): 0.78 (s, 3H, 18-H_3), 2.52 (m, 2H, 6-H_2), 3.18 (m, 6H, 2x linker H_2), 3.90 (t, 1 H, *J* = 6.5 Hz, 15-H), 6.24 (d, 1H, *J* = 2.5 Hz, 4-H), 6.29 (dd, 1H, *J* = 10.5 Hz, *J* = 2.5 Hz, 2-H), 6.81 (d, 1H, *J* = 10.5 Hz, 1-H). ^{13}C NMR (δ, ppm, DMSO-d_6): 17.6 (C-18), 25.8, 26.0, 29.3, 32.7, 35.1, 35.4, 43.1 (linker CH_2), 43.9, 46.9 (C-13), 53.6, 65.1 (linker CH_2), 74.6 (C-15), 113.1 (C-2), 115.4 (C-4), 126.2 (C-1), 130.6 (C-10), 137.6 (C-5), 155.4 (C-3), 173.1 (COOH), 219.3 (C-17).

3-Benzyloxy-15β-(3'-carboxyl)propoxy-estra-1,3,5(10)-trien-17-one (17)

Compound **12** (448 mg, 1 mmol) was dissolved in acetone (10 ml) and Jones reagent (1 ml) was added while cooling with ice. The mixture was diluted with ice-water, the precipitate separating out was filtered, dried, and crystallized from acetone/hexane to yield **17** (390 mg, 84%). Mp: 138–140 °C; R_f = 0.25 (ss B); $[\alpha]_D^{25}$ + 57 (*c* 1 in $CHCl_3$). Found: C, 75.22; H, 7.67. $C_{29}H_{34}O_5$ (462.58) requires: C, 70.30; H, 7.41%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.14 (s, 3H, 18-H_3), 3.29 (m, 1H, O-CH_2), 3.56 (m, 1H, O-CH_2), 4.12 (t, 1 H, *J* = 6.5 Hz, 15-H), 5.02 (s, 2H, Bn-H_2), 6.73 (d, 1H, *J* = 3.0 Hz, 4-H), 6.77 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.18 (d, 1H, *J* = 10.5 Hz, 1-H), 7.36 (m, 5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 25.5, 26.2, 26.8, 29.9, 31.3, 33.1, 35.3, 43.5 (linker CH_2), 44.7, 47.7 (C-13), 55.0, 68.6 (O-CH_2), 70.4 (Bn-CH_2), 75.3 (C-15), 112.8 (C-2), 115.4 (C-4), 126.6 (C-1), 127.8 (C-2 and C-6 of Bn), 128.3 (C-4 of Bn), 129.0 (C-3 and C-5 of Bn), 132.9 (C-10), 137.7 (C-1 of Bn), 138.3 (C-5), 157.3 (C-3), 179.5 (COOH), 220.2 (C-17).

3-Methoxy-15β-(2'-cyano)ethoxy-estra-1,3,5(10)-trien-17-one (18)

Compound **6** (282 mg, 1 mmol) was dissolved in CH_2Cl_2 (10 ml) and 3-hydroxypropionylnitrile (10 ml), containing 5% aqueous NaOH (1 ml), was stirred at room temperature for 8 h. CH_2Cl_2 (50 ml) was added to the reaction mixture and then it was diluted with water (100 ml). The organic phase was separated, washed with water, dried over Na_2SO_4, and evaporated *in vacuo.* The residual product was purified by flash chromatography using CH_2Cl_2 to yield **18** (305 mg, 86%). Mp: 197–200 °C; R_f = 0.50 (ss B); $[\alpha]_D^{25}$ + 63 (*c* 1 in $CHCl_3$). Found C, 74.92; H, 7.55. $C_{22}H_{27}NO_3$ (353.45) requires C, 74.76; H, 7.70%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.17 (s, 3H, 18-H_3), 2.60 (s 2H, linker H_2), 3.00 (m, 2H, 6-H_2), 3.65 (s, 3H, 3OCH_3), 3.77 (s, 2'H, linker H_2), 16-H_2), 4.23 (t, 1 H, *J* = 6.4 Hz, 15-H), 6.65 (s, 1H, 4-H), 6.71 (d, 1H, *J* = 10.5 Hz, 2-H), 7.19 (d, 1H, *J* = 10.5 Hz, 1-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.2 (C-18), 20.0, 26.4, 27.1, 30.1, 33.4, 35.4, 43.5, 44.9 (linker CH_2), 47.9 (C-13), 55.1 (3-OCH_3), 55.9, 65.0 (O-CH_2), 72.2 (C-15), 112.2 (C-2), 114.6 (C-4), 118.3 (CN), 126.8 (C-1), 132.7 (C-10), 138.5 (C-5), 158.3 (C-3), 219.2 (C-17).

3-Benzyloxy-15β-(2'-cyano)ethoxy-estra-1,3,5(10)-trien-17-one (19)

Compound **7** (358 mg, 1 mmol) dissolved in CH_2Cl_2 (10 ml) and 3-hydroxypropionytrile (10 ml), containing 5% aqueous NaOH (1 ml), was stirred at room temperature for 8 h. After adding CH_2Cl_2 (50 ml) to the reaction mixture, the organic phase was separated, washed with water, dried over Na_2SO_4, and evaporated *in vacuo*. The residual product was purified by flash chromatography using ethyl acetate/CH_2Cl_2 (2.5/97.5 v/v) to yield **19** (346 mg, 80%). Mp: 183–185 °C; R_f = 0.45 (ss B); $[\alpha]_D^{25}$ + 54 (*c* 1 in $CHCl_3$). Found C, 78.52; H, 7.42. $C_{28}H_{31}NO_3$ (429.55) requires: C, 78.29; H, 7.27%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.20 (s, 3H, 18-H_3), 3.54 (m, 1H, O-CH_2), 3.78 (m, 1H, O-CH_2), 4.27 (t, 1 H, *J* = 6.5 Hz, 15-H), 5.07 (s, 2H, Bn-H_2), 6.78 (s, 1H, 4-H), 6.81 (d, 1H, *J* = 11.0 Hz, 2-H), 7.22 (d, 1H, *J* = 11.0 Hz, 1-H), 7.41 (m, 5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 19.8, 26.2, 26.8, 29.9, 33.2, 35.2, 43.3 (CN), 44.7, 47.7 (C-13), 54.9, 64.8 (O-CH_2), 70.4 (Bn-CH_2), 76.0 (C-15), 112.8 (C-2), 115.4 (C-4), 118.1 (CN), 126.6 (C-1), 127.9 (C-2 and C-6-of Bn), 128.3 (C-4-of Bn), 129.0 (C-3 and C-5 of Bn), 132.7 (C-10), 137.7 (C-1 of Bn), 138.4 (C-5), 157.4 (C-3), 219.1 (C-17).

3-Hydroxy-15β-(2'-cyano)ethoxy-estra-1,3,5(10)-trien-17-one (20)

Compound **19** (430 mg, 1 mmol) was dissolved in ethyl acetate (30 ml) and the solution containing Pd/C (300 mg, 10%) was hydrogenated under 5 bar pressure for 6 h, at room temperature. The reaction mixture was filtered off, evaporated *in vacuo* and crystallized from CH_2Cl_2/hexane to yield **20** (286 mg, 84%). Mp: 221–223 °C; R_f = 0.25 (ss A); $[\alpha]_D^{25}$ + 37 (*c* 1 in MeOH). Found C, 74.62; H, 7.35. $C_{21}H_{25}O_3N$ (339.43) requires: C, 74.31; H, 7.42%. ^{1}H NMR (δ, ppm, DMSO-d_6): 1.07 (s, 3H, 18-H_3), 2.76 (m, 2H, 6-H_2), 3.33 (s, 3H, CN-H_2), 15-H), 3.70 (m, 1H, O-CH_2), 4.04 (m, 1H, O-CH_2), 4.21 (t, 1 H, *J* = 6.5 Hz, 15-H), 6.47 (d, 1H, *J* = 3.0 Hz, 4-H), 6.53 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.05 (d, 1H, *J* = 10.5 Hz, 1-H), 9.00 (brs, 1H, 3-OH). ^{13}C NMR (δ, ppm, DMSO-d_6): 17.2 (C-18), 18.5, 25.4, 25.7, 28.9, 32.3, 34.7, 42.7 (linker CH_2), 43.5, 46.5 (C-13), 53.1, 63.7 (O-CH_2), 74.5 (C-15), 112.7 (C-2), 115.0 (C-4), 119.2 (CN), 125.9 (C-1), 130.1 (C-10), 137.2 (C-5), 155.0 (C-3), 218.6 (C-17).

3-Sulfamoyloxy-15β-(2'-cyano)ethoxy-estra-1,3,5(10)-trien-17-one (21)

Compound **20** (340 mg, 1 mmol) was dissolved in dimethylformamide (20 ml), and 575 mg (5 mmol) sulfamoylchloride was added dropwise during cooling with ice. The reaction mixture was allowed to stand 6 h and then poured onto ice (300 g). The precipitate was filtered off and dried. The product was crystallized from ethyl acetate to yield **21** (360 mg, 86%). Mp: 78–80 °C; R_f = 0.30 (ss A); $[\alpha]_D^{25}$ + 52 (*c* 1 in $CHCl_3$). Found: C, 60 55; H, 6.42. $C_{21}H_{26}N_2O_5S$ (418.51) requires: C, 60.27; H, 6.26%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.10 (s, 3H, 18-H_3), 3.45 (m, 1H, O-CH_2), 3.70 (m, 1H, O-CH_2), 4.19 (t, 1 H, *J* = 7.0 Hz, 15-H), 5.25 (s, 2H, NH_2), 6.99 (d, 1H, *J* = 3.0 Hz, 4-H), 7.02 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.23 (t, 1 H, *J* = 10.5 Hz, 1-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.9 (C-18), 19.8, 26.0, 26.4, 29.6, 33.0, 34.7, 43.2 (linker CH_2), 44.7, 47.6 (C-13), 54.8, 64.7 (OCH_2), 75.9 (C-15), 118.3 (CN), 119.5 (C-2), 122.5 (C-4), 127.0 (C-1), 139.3 (C-10), 139.4 (C-5), 148.5 (C-3), 219.3 (C-17).

3-Benzyloxy-15β-(2'-methoxy-2'-oxoethoxy)-estra-1,3,5(10)-trien-17-one (22)

Compound **7** (717 mg, 2 mmol) in CH_2Cl_2 (10 ml) and ethane-1,2 diol (15 ml), containing 5% aqueous sodium hydroxide (1 ml) was stirred at room temperature for 6 h. The reaction mixture was diluted with water (100 ml). The organic phase

was separated, washed with water, dried and evaporated *in vacuo*. The crude 3-benzyloxy-15β-(2'-hydroxy)ethoxy-estra-1,3,5(10)-trien-17-one was dissolved in acetone (15 ml) and Jones reagent (1 ml) was added cooling with ice. The mixture was diluted with ice-water, the precipitate was filtered off, and dried. The crude 3-benzyloxy-15β-(carboxyl)methoxy-estra-1,3,5(10)-trien-17-one was dissolved in tetrahydrofurane (10 ml) and diethyl ether containing 1% diazomethane (50 ml) was added during cooling with ice. After standing 6 h, the solution was evaporated and the residue was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (2.5/97.5 v/v) to yield **22** (265 mg, 29%). Mp: 101–103 °C; R_f = 0.55 (ss C); $[\alpha]_D^{25}$ + 71 (*c* 1 in $CHCl_3$). Found: C, 75.12; H, 7.35. $C_{28}H_{32}O_5$ (448.55) requires: C, 74.97; H, 7.19%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.21 (s, 3H, 18-H_3), 2.93 (m, 2H, 6-H_2), 3.76 (s, 3H, $COOCH_3$), 4.10 (m, 2H, O-CH_2), 4.36 (t, 1 H, *J* = 6.5 Hz, 15-H), 5.05 (s, 2H, Bn-H_2), 6.99 (s, 1H, 4-H), 6.76 (d, 1H, *J* = 3.5 Hz, 2-H), 7.21 (d, 1H, *J* = 10.5 Hz, 1-H), 7.38 (m, 5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 16.9 (C-18), 25.2, 25.6, 29.0, 32.3, 34.3, 42.4, 43.9, 46.8 (C-13), 51.2, 54.1 (OCH_3), 66.0 (O-CH_2), 69.5 (Bn-CH_2), 75.2 (C-15), 111.8 (C-2), 114.5 (C-4), 125.6 (C-1), 126.9 (C-2 and C-6 of Bn), 127.3 (C-4-of Bn), 128.0 (C-3 and C-5 of Bn), 131.9 (C-10), 136.9 (C-1'), 137.5 (C-5), 156.4 (C-3), 170.2 (C = O), 218.4 (C-17).

3-Methoxy-15β-(3'-methoxy-3'-oxopropoxy)-estra-1,3,5(10)-trien-17-one (23)

Compound **14** (373 mg, 1 mmol) was dissolved in tetrahydrofuran (10 ml) and diethylether containing 1% diazomethane (50 ml) was added during cooling with ice. After standing 6 h, the solution was evaporated and the residue was crystallized from MeOH, to yield **23** (370 mg, 95%). Mp: 95–97 °C; R_f = 0.58 (ss C); $[\alpha]_D^{25}$ + 64 (*c* 1 in $CHCl_3$). Found: C, 71.62; H, 8.04; $C_{23}H_{30}O_5$ (386.48) requires: C, 71.48; H, 7.82%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.13 (s, 3H, 18-H_3), 2.40 (m, 2H, linker H_2), 2.90 (m, 2H, 6-H_2), 3.59 (m, 2H, linker H_2) 3.69 (s, 3H, 3-OCH_3), 3.80 (s, 3H, $COOCH_3$), 4.20 (t, 1 H, *J* = 6.5 Hz, 15-H), 6.68 (s, 1H, 4-H), 6.74 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.21 (d, 1H, *J* = 10.5 Hz, 1-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.8 (C-18), 26.2, 26.6, 30.0, 33.2, 35.2, 35.6, 43.3, 44.6 (linker CH_2), 47.7 (C-13), 52.1, 54.9, 55.6 (3-OCH_3), 65.3 (O-CH_2), 75.3 (C-15), 111.9 (C-2), 114.3 (C-4), 126.6 (C-1), 132.6 (C-10), 138.3 (C-5), 158.1 (C-3), 172.4 (C = O), 219.9 (C-17).

3-Hydroxy-15β-(3'-methoxy-3'-oxopropoxy)-estra-1,3,5(10)-trien-17-one (24)

Compound **15** (448 mg, 1 mmol) was dissolved in tetrahydrofuran (10 ml) and diethyl ether containing 1% diazomethane (50 ml) was added during cooling with ice. After standing 6 h, the solution was evaporated and the residue was crystallized from MeOH, to yield crude 3-benzyloxy-15β-(3'-methoxy-3'-oxopropoxy)-estra-1,3,5(10)-trien-17-one. This compound was dissolved in ethyl acetate (30 ml) and the solution containing Pd/C (300 mg, 10%) was hydrogenated under 5 bar pressure for 6 h, at room temperature. The reaction mixture was filtered off, evaporated *in vacuo* and the residue was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (10:90 v/v) to yield **24** (196 mg, 52%). Mp: 140–142 °C; R_f = 0.25 (ss A); $[\alpha]_D^{20}$ + 51 (*c* 1 in $CHCl_3$). Found: C, 71.08; H, 7.76. $C_{22}H_{28}O_5$ (372.45) requires: C, 70.94; H, 7.58%. ^{1}H NMR (δ, ppm, DMSO-d_6): 0.99 (s, 3H, 18-H_3), 2.75 (m, 2H, 6-H_2), 3.34 (s, 4H, 2 x linker H_2), 3.58 (s, 3H, COO-H_3), 4.13 (t, 1 H, *J* = 6.5 Hz, 15-H), 6.48 (s, 1H, 4-H), 6.52 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.04 (d, 1H, *J* = 10.5 Hz, 1-H), 8.99 (brs, 1H, 3-OH). ^{13}C NMR (δ, ppm, DMSO-d_6): 18.0 (C-18), 26.3, 26.5, 29.9, 33.2, 35.6, 35.6, 43.4 (linker CH_2), 44.4, 47.4 (C-13), 52.1, 54.0, 65.3 (O-CH_2), 75.2 (C-15), 113.6 (C-2), 115.9 (C-4), 126.8 (C-1), 131.0 (C-10), 138.0 (C-5), 155.9 (C-3), 172.6 (C = O), 219.7 (C-17).

General procedure for the synthesis of 3-hydroxy-, and 3-benzyloxy-15β-(carboxamido)alkoxy-estra-1,3,5(10)-trienes with ammonium hydroxide, morpholine or *N*-cyclohexyl,*N*-methylamine (25–33)

To the solution of the corresponding 3-hydroxy-, or 3-benzyloxy-15β-(carboxyl)alkoxy-estra-1,3,5(10)-triene (1 mmol) in CH_2Cl_2 (20 ml) 0.2 ml (2 mmol) oxalyl chloride was added dropwise while cooling in ice under continuous stirring. The solution was allowed to stand at room temperature for 2 h. After evaporation *in vacuo* the residue was dissolved in CH_2Cl_2 (20 ml) and 4 mmol of the corresponding amine component was added while cooling in ice under continuous stirring. After 1 h, water (100 ml) was added and the mixture was extracted with CH_2Cl_2 (2x 50 ml). The organic phase was washed with water, dried and evaporated. The residual material was chromatographed on a silica gel column with ethyl acetate/CH_2Cl_2 in different concentrations.

3-Benzyloxy-15β-(3'-amino-3-oxopropoxy)-estra-1,3,5,(10)-trien-17-one (25)

Compound **15** (448 mg, 1 mol) was used for synthesis as described in general procedure. The amine component was ammonium hydroxide solution (20 ml). The crude product was chromatographed on silica gel with ethyl acetate/CH_2Cl_2 (1:1 v/v) to yield **22** (238 mg, 53%). Mp: 183–185 °C; R_f = 0.55 (ss C); $[\alpha]_D^{25}$ + 61 (*c* 1 in $CHCl_3$). Found: C, 75.36; H, 7.26. $C_{28}H_{33}NO_4$ (447.57) requires: C, 75.14; H, 7.43%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.26 (s, 3H, 18-H_3), 2.30 (m, 2H, linker H_2), 2.96 (m, 2H, 6-H_2),3.60 (m, 2H, linker H_2), 4.43 (t, 1 H, *J* = 6.0 Hz, 15-H), 5.07 (s, 2H, Bn-H_2), 6.78 (s, 1H, 4-H), 6.83 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.24 (d, 1H, *J* = 10.5 Hz, 1-H), 7.38 (m, 5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 15.5 (C-18), 22.0, 23.9, 24.2, 27.8, 30.9, 33.2, 33.8, 40.9, 41.9 (linker CH_2), 45.9 (C-13), 50.1, 51.8, 64.7 (linker CH_2), 73.9 (C-15), 112.2 (C-2), 114.6 (C-2), 125.8 (C-1), 123.0 (C-10), 126.8 (C-2 and C-6 of Bn), 127.1 (C-4 of Bn), 129.0 (C-3 and C-5 of Bn), 137.4 (C-5), 155.8 (C-3), 171.8 (C = O), 220.1 (C-17).

3-Hydroxy-15β-(3'-amino-3'-oxopropoxy)-estra-1,3,5(10)-trien-17-one (26)

Compound **25** (448 mg, 1 mmol) was dissolved in ethyl acetate (30 ml) and the solution containing Pd/C (300 mg, 10%) was hydrogenated under 5 bar pressure for 6 h, at room temperature. The reaction mixture was filtered off and evaporated *in vacuo.* The residue was chromatographed on silica gel column with ethyl acetate/CH_2Cl_2(1:1 v/v) to yield **26** (308 mg, 86%). Mp: 124–126 °C; R_f = 0.20 (ss A); $[\alpha]_D^{25}$ + 54 (*c* 1 in MeOH). Found: C, 70.38; H, 7.85. $C_{21}H_{27}NO_4$ (357.44) requires: C, 70.56; H, 7.61%. ^{1}H NMR (δ, ppm, DMSO-d_6): 1.03 (s, 3H, 18-H_3), 2.77 (m, 2H, 6-H_2), 3.33 (s, 2H, linker H_2), 3.47 (m, 1H, O-CH_2), 3.68 (m, 1H, O-CH_2), 4.11 (t, 1 H, *J* = 6.5 Hz, 15-H), 6.47 (d, 1H, *J* = 3.5 Hz, 4-H), 6.52 (dd, 1H, *J* = 10.5 Hz, *J* = 3.5 Hz, 2-H), 6.78 (brs, 1H, NH_2), 7.04 (d, 1H, *J* = 10.5 Hz, 1-H), 7.22 (brs, 1H, NH_2), 7.90 (brs, 1H, 3-OH). ^{13}C NMR (δ, ppm, DMSO-d_6): 16.5 (C-18), 24.7, 24.9, 28.3, 31.7, 34.0, 35.3, 42.0, 42.8 (linker CH_2), 45.8 (C-13), 52.5, 64.4 (linker CH_2), 73.5 (C-15), 112.0 (C-2), 114.2 (C-2), 125.1 (C-1), 129.5 (C-10), 136.5 (C-5), 154.3 (C-3), 171.6 (C = O), 218.3 (C-17).

3-Benzyloxy-15β-(2'-morpholino-2'-oxoethoxy-estra-1,3,5(10)-trien-17-one (27)

3-Benzyloxy-15β-(carboxyl)methoxy-estra-1,3,5(10)-triene (434 mg, 1 mmol) was used for the synthesis as described in general procedure. The crude steroidal carbonyl chloride was dissolved in CH_2Cl_2 (20 ml), and morpholine (0.35 ml, 4 mmol) was added dropwise during cooling with ice under continuous stirring. After 1 h, water (100 ml) was added, and extracted with CH_2Cl_2 (2 x 50 ml). The organic solution was washed with water, dried, and evaporated. The residual material was chromatographed on silica gel column with ethyl acetate/CH_2Cl_2 (25:75 v/v) to yield **27** (432 mg, 85%). Mp: 121–123 °C; R_f = 0.45 (ss D); $[\alpha]_D^{25}$ + 37 (*c* 1 in MeOH). Found: C, 74.14; H, 7.53. $C_{31}H_{37}NO_5$ (503.63) requires: C, 73.93; H, 7.41%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.16 (s, 3H, 18-H_3), 3.58 (m, 8H, 4x morpholine H_2), 4.08 (d, 1H, *J* = 13.0 Hz, O-CH_2), 4.20 (d, 1H, *J* = 13.0 Hz, O-CH_2), 4.35 (t, 1 H, *J* = 5.5 Hz, 15-H), 5.04 (s, 2H, Bn-H_2), 6.75 (s, 1H, 4-H), 6.80 (dd, 1H, *J* = 8.5 Hz, J = 2.5 Hz, 2-H), 7.20 (d, 1H, *J* = 8.5 Hz, 1-H), 7.32 (t, 1H, *J* = 7.5 Hz, Bn 4-H), 7.38 (t, 2H, *J* = 7.5 Hz, Bn 3- and 5-H), 7.43 (d, 2H, *J* = 7.5 Hz, Bn-2- and 6-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.5 (C-18), 25.6, 26.3, 29.5, 32.7, 34.8, 42.1, 43.0, 44.3, 45.9 (C-13), 47.2, 54.4, 66.7 (morpholine CH_2), 66.7 (morpholine CH_2), 69.1 (O-CH_2), 69.9 (Bn-CH_2), 76.0 (C-15), 112.4 (C-2), 114.8 (C-4), 126.2 (C-1), 127.4 (C-2 and C-6 of Bn), 127.8 (C-4 of Bn), 128.5 (C-3 and C-5 of Bn), 132.1 (C-10), 137.1 (C-1 of Bn), 137.5 (C-5), 156.9 (C-3), 167.5 (C = O), 218.7 (C-17).

3-Hydroxy-15β-(carboxmorpholydo)methoxy-2'-morpholino-2'oxoethoxy)-estra-1,3,5(10)-trien-17-one (28)

Compound **27** (503 mg, 1 mmol) was dissolved in ethyl acetate (30 ml) and the solution containing Pd/C (448 mg, 10%) was hydrogenated under 5 bar pressure for 6 h at room temperature. The reaction mixture was filtered off, evaporated *in vacuo*, and the residue was crystallized from MeOH to yield **28** (350 mg, 69%). Mp: 215–220 °C; R_f = 0.40 (ss D); $[\alpha]_D^{25}$ + 54 (*c* 1 in MeOH). Found: C, 73.07; H, 7.92. $C_{25}H_{33}NO_4$ (411.53) requires: C, 72.96; H, 8.08%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.14 (s, 3H, 18-H_3), 3.51 (s, 2H, linker H_2), 3.58 (m, 2H, 2x H_2 of morpholine), 3.60 (m 2H, 2x H_2 of morpholine), 4.14 (t, 1 H, *J* = 6.5 Hz, 15-H), 6.60 (d, 1H *J* = 2.5 Hz, 4-H), 6.65 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.12 (d, 1H, *J* = 10.5 Hz, 1-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 26.0, 26.2, 26.7, 29.9, 30.1, 33.1, 35.4, 42.5, 43.7, 44.5, 46.4, 47.7 (C-13), 54.9, 66.6 (linker CH_2), 67.3 (linker CH_2), 75.2 (C-15), 113.4 (C-2), 115.8 (C-4), 126.7 (C-1), 132.1 (C-10), 138.2 (C-5), 154.2 (C-1), 172.0 (linker C = O), 220.5 (C-17).

3-Benzyloxy-15β-(3'-morpholino-3'oxopropoxy)-estra-1,3,5(10)-trien-17-one (29)

Compound **15** (448 mg, 1 mmol) was used for synthesis as described in general procedure. The crude steroidal carbonyl chloride was dissolved in CH_2Cl_2 (20 ml), and morpholine (0.35 ml, 4 mmol) was added dropwise during cooling with ice under continuous stirring. After 1 h, water (100 ml) was added, and extracted with CH_2Cl_2 (2x 50 ml). The organic solution was washed with water, dried, and evaporated. The residual material was chromatographed on silica gel column with ethyl acetate/CH_2Cl_2(2.5:97.5 v/v) to yield **29** (372 mg, 72%). Mp: 128–130 °C; R_f = 0.42 (ss D); $[\alpha]_D^{25}$ + 37 (*c* 1 in $CHCl_3$). Found: C, 74.46; H, 7.72. $C_{32}H_{39}NO_5$ (517.66) requires: C, 74.24; H, 7.59%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.12 (s, 3H, 18-H_3), 2.89 (m, 2H, 6-H_2), 3.51(m, 2H, linker H_2), 4.20 (t, 1H, *J* = 6.5 Hz, 15-H), 5.04 (s, 2H, Bn-H_2), 6.74 (d, 1H, *J* = 3.5 Hz, 4-H), 6.79 (dd, 1H, *J* = 10.5 Hz, *J* = 3.5 Hz, 2-H), 7.19 (d, 1H, *J* = 10.5 Hz, 1-H), 7.31 (t, 1 H, *J* = 8.5 Hz, Bn 4-H), 7.38 (t, 2H, *J* = 8.5 Hz, Bn 3- and 5-H), 7.43 (d, 2H, *J* = 8.5 Hz, Bn 2- and 6-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.9 (C-18), 26.2, 26.6, 30.0, 33.1, 33.7, 35.4, 42.4 (morpholine CH_2), 43.5 (linker CH_2), 44.6, 46.5 (morpholine CH_2), 47.7 (C-13), 54.9, 66.2 (O-CH_2), 67.1 (morpholine CH_2), 67.3 (morpholine CH_2), 70.4 (Bn-CH_2), 75.5 (C-15), 112.8 (C-2), 115.4 (C-4), 126.6 (C-1), 127.8 (C-2 and C-6 of Bn), 128.3 (C-4 of Bn), 129.0 (C-3 and C-5 of Bn), 132.9, 137.9 (C-1'), 138.2 (C-5), 157.3 (C-3), 169.9 (C = O), 219.9 (C-17).

3-Sulfamoyloxy-15β-(3'-morpholino-3'-oxopropoxy)-estra-1,3,5(10)-trien-17-one (30)

Compound **29** (517 mg, 1 mmol) was dissolved in ethyl acetate (30 ml) and the solution containing Pd/C (300 mg, 10%) was hydrogenated under 5 bar pressure for 6 h, at room temperature. The reaction mixture was filtered off and evaporated *in vacuo.* The residue was dissolved in dimethylformamide (20 ml), and 575 mg (5 mmol) sulfamoyl chloride was added dropwise during cooling with ice. The reaction mixture was allowed to stand 6 h and then poured onto ice (300 g). The precipitate separated out was filtered off, and subjected to chromatographic separation on silica gel column with ethyl acetate/CH_2Cl_2 (1:1 v/v) to yield **30** (240 mg, 45%). Mp: 104–108 °C; R_f = 0.35 (ss D); $[\alpha]_D^{25}$ + 17 (*c* 1 in $CHCl_3$). Found: C, 59.43; H, 6.54. $C_{25}H_{34}N_2O_7S$ (506.61) requires: C, 59.27; H, 6.76%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.12 (s, 3H, 18-H_3), 2.74 (m, 2H, linker CH_2), 4.46 (m, 4H, 2x H_2 of morpholine), 3.56 (m, 2H, O-CH_2), 3.61 (m, 4H, 2x H_2 of morpholine), 3.82 (t, 1 H, *J* = 6.5 Hz, 15-H), 5.42 (s, 2H, NH_2), 6.78 (d, 1H, *J* = 3.5 Hz, 4-H), 7.20 (dd, 1H, *J* = 10.0 Hz, *J* = 3.5 Hz, 2-H), 7.30 (t, 1 H, *J* = 10.0 Hz, 1-

H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.8 (C-18), 26.1, 26.7, 30.5, 33.4, 33.9, 35.5, 42.2 (morpholine CH_2), 43.4 (linker CH_2), 45.1, 46.7 (morpholine CH_2), 47.5 (C-13), 55.8, 64.3 (O-CH_2), 66.7 (morpholine CH_2), 67.5 (morpholine CH_2), 75.7 (C-15), 112.5 (C-2), 115.1 (C-4), 126.8 (C-1), 133.0, 140.5 (C-5), 159.1 (C-3), 170.9 (C = O), 220.2 (C-17).

3-Benzyloxy-15β-(4'-morpholino-4'oxobutoxy)-estra-1,3,5(10)-trien-17-one (31)

Compound **17** (462 mg, 1 mmol) was used for the synthesis as described in general procedure. The crude steroidal carbonyl chloride was dissolved in CH_2Cl_2 (20 ml), and morpholine (0.35 ml, 4 mmol) was added dropwise during cooling with ice under continuous stirring. After 1 h, water (100 ml) was added, and extracted with CH_2Cl_2(2 x 50 ml). The organic solution was washed with water, dried and evaporated *in vacuo.* The residual material was chromatographed on silica gel column with ethyl acetate/CH_2Cl_2 (30:70 v/v) to yield **31** (810 mg, 76%). Mp: 135–138 °C; R_f = 0.42 (ss D); $[\alpha]_D^{25}$ + 20 (*c* 1 in $CHCl_3$). Found: C, 74.87; H, 7.43. $C_{33}H_{41}NO_5$ (531.68) requires: C, 74.55; H, 7.77%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.14 (s, 3H, 18-H_3), 1.91 (m, 4H, 2 x morpholine H_2), 2.37 (m, 4H, 2 x morpholine H_2), 3.36 (t, 2H, *J* = 6.5 Hz, O-CH_2), 4.15 (t, 1 H, *J* = 6.5 Hz, 15-H), 5.04 (s, 2H, Bn-H_2), 6.73 (d, 1H, *J* = 3.5 Hz, 4-H), 6.79 (dd, 1H, *J* = 10.5 Hz, *J* = 3.5 Hz, 2-H), 7.20 (d, 1H, *J* = 10.5 Hz, 1-H), 7.36 (m, 5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 26.0, 26.2, 26.7, 30.0, 33.1, 34.7, 35.4, 42.3 (morpholine CH_2), 43.7 (linker CH_2), 44.6, 46.3 (morpholine CH_2), 47.7 (C-13), 54.9, 67.0 (morpholine CH_2), 67.3 (morpholine CH_2), 69.0 (O-CH_2), 70.4 (Bn-CH_2), 75.1 (C-15), 112.8 (C-2), 115.4 (C-4), 126.6 (C-1), 127.8 (C-2 and C-6 of Bn), 128.3 (C-4'), 129.0 (C-3 and C-5 of Bn), 132.9 (C-10), 137.9 (C-1 of Bn), 138.1 (C-5), 157.4 (C-3), 171.6 (C = O), 220.0 (C-17).

3-Hydroxy-15β-(4'-morpholino-4'-oxobutoxy)-estra-1,3,5(10)-trien-17-one (32)

Compound **31** (531 mg, 1 mmol) was dissolved in ethyl acetate (30 ml) and the solution containing Pd/C (300 mg, 10%) was hydrogenated under 5 bar pressure for 6 h, at room temperature. The reaction mixture was filtered off, evaporated *in vacuo* and crystallized from MeOH to yield **32** (390 mg, 88%). Mp: 80–84 °C; R_f = 0.35 (ss D); $[\alpha]_D^{25}$ + 46 (*c* 1 in $CHCl_3$). Found: C, 70.58; H, 8.12. $C_{26}H_{35}NO_5$ (441.57) requires: C, 70.72; H, 7.99%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.14 (s, 3H, 18-H_3), 3.33 (t, 2H, *J* = 6.0 Hz, linker H_2), 3.48 (m, 4H, 2x morpholine H_2), 361 (m, 4H, 2x morpholine H_2), 4.14 (t, 1 H, *J* = 6.5 Hz, 15-H), 6.60 (d, 1H *J* = 2.5 Hz, 4-H), 6.65 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.12 (d, 1H, *J* = 10.5 Hz, 1-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 26.0, 26.2, 26.7, 29.9, 30.1, 33.1, 35.4, 42.5, 43.7, 44.5, 46.4, 47.7 (C-13), 54.9, 66.6 (linker CH_2), 67.3 (linker CH_2), 69.0 (linker CH_2), 75.2 (C-15), 113.4 (C-2), 115.8 (C-4), 126.7 (C-1), 132.1 (C-10), 138.2 (C-5), 154.2 (C-1), 172.0 (linker C = O), 220.5 (C-17).

3-Methoxy-15β-(2'*N*-cyclohexyl,*N*-methyl-2'oxoethoxy)-estra-1,3,5(10)-trien-17-one (33)

Starting from 3-methoxy-15β-(carboxyl)methoxy-estra-1,3,5(10)-trien-17-one (**13**) 358 mg (1 mmol) was used for synthesis as described in general procedure. The crude steroidal carbonyl chloride was dissolved in CH_2Cl_2 (20 ml), and *N*-cyclohexyl, *N*-methylamine (4.5 ml, 4 mmol) was added dropwise during cooling with ice under continuous stirring. After 1 h, water (100 ml) was added, and extracted with CH_2Cl_2 (2 x 50 ml). The organic solution was washed with water, dried, and evaporated. The residual material was chromatographed on silica gel column with ethyl acetate/CH_2Cl_2 (25:75 v/v) to yield **33** (385 mg, 84%). Mp: 53–58 °C (foam); R_f = 0.50 (ss B); $[\alpha]_D^{25}$ + 61 (*c* 1 in $CHCl_3$). Found: C, 73.92; H, 8.82. $C_{28}H_{39}NO_4$ (453.61) requires: C, 74.14; H, 8.67%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.18 (s, 3H, 18-H_3), 3.30 (s, 3H, N-CH_3), 3.77 (s, 3H, 3-OCH_3), 4.12 (m, 2H, O-CH_2), 4.39 (m, 1H, 15-H), 6.65 (s, 1H, 4-H), 6.71 (d, 1H, *J* = 11.0 Hz, 2-H), 7.19 (d, 1H, *J* = 11.0 Hz, 1-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.9 (C-18), 25.8, 26.0, 26.2, 27.6, 29.3, 29.9, 30.0, 31.3, 33.2, 35.4, 43.5, 43.8 (C-13), 44.8, 47.7, 53.0, 55.0, 55.6, 69.9 (O-CH_2), 75.7 (C-15), 112.0 (C-2), 114.3 (C-4), 126.6 (C-1), 132.5 (C-10), 138.1 (C-5), 158.1 (C-3), 166.3 (C = O), 219.5 (C-17).

General procedure for the synthesis of 3-benzyloxy-15β-(carbamoyloxy)alkoxy-estra-1,3,5(10)-trien-17-one (34–40)

To the solution of 3-benzyloxy-15β-(2'-hydroxy)ethoxy-estra-1,3,5(10)-trien-17-one (**10**) or 3-benzyloxy-15β-(3'-hydroxy)propoxy-estra-1,3,5(10)-trien-17-one (**11**) mmol) in CH_2Cl_2 (30 ml) containing 0.2 ml triethylamine, 4 mmol of the corresponding alkyl or aryl isocyanate was added under continuous stirring. After the addition of the reagent the reaction mixture was heated at reflux for 1 h, poured into water (100 ml), and then extracted with CH_2Cl_2 (2x 30 ml). The organic phase was washed with $NaHCO_3$ solution, water, dried and evaporated *in vacuo*. The residual material was chromatographed on a silica gel column with ethyl acetate/CH_2Cl_2 in different concentrations.

3-Benzyloxy-15β-(2'-cyclohexylcarbamoyloxy)ethoxy-estra-1,3,5(10)-trien-17-one (34)

Compound **10** (420 mg, 1 mmol) was used for the synthesis as described in general procedure. The reagent was cyclohexyl isocyanate (4 mmol). The crude product was chromatographed with ethyl acetate/CH_2Cl_2 (2.5:97.5 v/v) to yield **34** (430 mg, 78%). Mp: 96–98 °C; R_f = 0.55 (ss B); $[\alpha]_D^{25}$ + 39 (*c* 1 in $CHCl_3$). Found: C, 74.65; H, 8.14. $C_{34}H_{43}NO_5$ (545.71) requires: C, 74.83; H, 7.94%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.16 (s, 3H, 18-H_3), 3.65 (m, 2H, 16-H_2), 3.68 (m, 1H, OCH_2), 4.19 (m, 3H, OCH_2), 4.55 (d, 1H, *J* = 7.5 Hz, 15-H), 5.03 (s, 2H, Bn-H_2), 6.73 (d, 1H, *J* = 3.0 Hz, 4-H), 6.78 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.19 (d, 1H, *J* = 10.5 Hz, 1-H), 7.37 (m, 5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.9 (C-18), 24.8, 25.2, 25.9, 26.2, 30.0, 33.2, 33.8, 35.2, 43.7 (O-CH_2), 44.6, 47.8 (C-13), 50.2, 53.8, 55.0, 64.0, 68.3 (O-CH_2), 70.4, 70.5 (Bn-CH_2), 75.4 (C-15), 112.7 (C-2),

115.4 (C-4), 126.6 (C-1), 127.8 (C-2and C-6 of Bn), 127.8 (C-4 of Bn), 129.0 (C-3 and C-5 of Bn), 133.0 (C-10), 137.8 (C-1'), 138.3 (C-5), 157.3 (C-3), 220.0 (C-17).

3-Hydroxy-15β-(2'-cyclohexylcarbamoyloxy)ethoxy-estra -1,3,5(10)-trien-17-one (35)

Compound **34** (545 mg, 1 mmol) was dissolved in ethyl acetate (30 ml) and the solution containing Pd/C (300 mg, 10%) was hydrogenated under 5 bar for 6 h, at room temperature. The reaction mixture was filtered off and evaporated *in vacuo.* The residual material was chromatographed on silica gel column with ethyl acetate/CH_2Cl_2(25:75 v/v) to yield **35** (390 mg, 85%). Mp: 95–96 °C; R_f = 0.40 (ss D); $[\alpha]_D^{25}$ + 51 (*c* 1 in $CHCl_3$). Found: C, 71.37; H, 8.02. $C_{27}H_{37}NO_5$ (455.60) requires: C, 71.18; H, 8.19%.1H NMR (δ, ppm, $CDCl_3$): 1.12 (s, 3H, 18-H_3), 1. 22 (m, 4H, 2x H_2 of cyclohexyl), 1. 56 (m, 4H, 2x H_2 of cyclohexyl), 3.62 (m, 2H, 16-H_2), 3.66 (m, 2H, O-CH_2), 4.28 (m, 2H, O-CH_2), 4.55 (d, 1H, *J* = 7.5 Hz, 15-H), 6.61 (d, 1H, *J* = 3.0 Hz, 4-H), 6.71 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.10 (d, 1H, *J* = 10.5 Hz, 1-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.9 (C-18), 24.6, 25.4, 25.9, 25.9, 26.1, 30.1, 33.3, 33.7, 35.4, 44.1 (O-CH_2), 44.8, 48.9 (C-13), 50.4, 54.0, 55.0, 64.2, 68.4 (O-CH_2), 70.5, 75.6 (C-15), 112.7 (C-2), 115.5 (C-4), 126.8 (C-1), 133.3 (C-10), 138.6 (C-5), 157.5 (C-3), 220.0 (C-17).

3-Benzyloxy-15β-(2'-t-butylcarbamoyloxy)ethoxy-estra-1,3,5(10)-trien-17-one (36)

Compound **10** (420 mg, 1 mmol) was used for the synthesis as described in general procedure. The reagent was *t*-butyl isocyanate (4 mmol). The crude product was chromatographed with ethyl acetate/CH_2Cl_2 (2.5:97.5 v/v) to yield **36** (265 mg, 51%). Mp: 206–207 °C; R_f = 0.45 (ss B); $[\alpha]_D^{25}$ +8 (*c* 1 in $CHCl_3$). Found: C, 73.82; H, 8.15. $C_{32}H_{41}NO_5$ (519.67) requires: C, 73.95; H, 7.95%.1H NMR (δ, ppm, $CDCl_3$): %) ^{1}H NMR (δ, ppm, $CDCl_3$): 1.15 (s, 3H, 18-H_3), 1.42 (s, 9H, *t*-Bu), 3.62 (m, 2H, O-CH_2), 3.81 (m, 2H, O-CH_2), 4.21 (t, 1 H, *J* = 7.0 Hz, 15-H), 5.12 (s, 2H, Bn-H_2), 6.62 (d, 1H, *J* = 3.5 Hz, 4-H), 6.80 (dd, 1H, *J* = 11.0 Hz, *J* = 3.5 Hz, 2-H), 7.19 (d, 1H, *J* = 11.0 Hz, 1-H), 7.40 (m, 5 H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 13 C NMR (δ, ppm, $CDCl_3$): 18.2 (C-18), 26.0, 26.5, 29.3 (3 x CH_3 of *t*-Bu), 29.6, 33.0, 35.1, 41.9, 43.9 (O-CH_2), 44.7, 48.0 (C-13), 55.0, 56.7 (quaterner C of *t*-Bu), 64.9 (O-CH_2), 67.1 (O-CH_2), 70.3 (Bn-CH_2), 75.3 (C-15), 112.8 (C-2), 115.2 (C-4), 124.4 (C-1), 126.8 (C-2 and C-6 of 3-Bn), 128.1 (C-4 of 3-Bn), 129.5 (C-3 and C-5 of 3-Bn), 133.1 (C-10), 138.0 (C-1 of 3-Bn), 133.5 (C-5), 158.1 (C-3), 220.0 (C-17).

3-Benzyloxy-15β-(2'-phenylcarbamoyloxy)ethoxy-estra-1,3,5(10)-trien-17-one (37)

Compound **10** (420 mg, 1 mmol) was used for the synthesis as described in general procedure. The reagent was phenyl isocyanate (4 mmol). The crude product was chromatographed with ethyl acetate/CH_2Cl_2 (25:75 v/v) to yield **37** (310 mg, 57%). Mp: 73–76 °C (foam); R_f = 0.65 (ss B); $[\alpha]_D^{25}$ + 35 (*c* 1 in $CHCl_3$). Found: C, 75.83; H, 7.12. $C_{34}H_{37}NO_5$ (539.66) requires: C, 75.67; H, 6.91% ^{1}H NMR (δ, ppm, $CDCl_3$): 1.16 (s, 3H, 18-H_3), 3.60 (m, 1H, O-CH_2), 3.73 (m, 1H, O-CH_2), 4.21 (t, 1 H, *J* = 6.5 Hz, 15-H), 4.30 (m, 2H, O-CH_2), 5.02 (s, 2H, Bn-H_2), 6.64 (s, 1H, NH), 6.67 (d, 1H, *J* = 3.0 Hz, 4-H), 6.77 (dd, 1H, *J* = 10.5 Hz, *J* = 3.0 Hz, 2-H), 7.06 (t, 1H, *J* = 9.0 Hz, 4H of N-Ph), 7.17 (d, 1H, *J* = 10.5 Hz, 1-H), 7.34 (m, 9 H, 5x CH of Bn and 4x CH of N-Ph). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 26.2, 26.7, 29.9, 33.2, 35.2, 41.8, 43.7 (O-CH_2), 44.6, 47.8 (C-13), 55.0, 64.7 (O-CH_2), 68.1 (O-CH_2), 70.4 (Bn-CH_2), 75.5 (C-15), 112.8 (C-2), 115.4 (C-4), 121.1 (C-4" of N-Ph), 124.1 (C-1), 126.6 (C-2' and C-6'), 127.9 (C-4'), 128.3 (C-2 and C-6 of 3Bn), 129.0 (C-3 and C-5 of 3Bn), 129.5 (C-3 and C-5 of N-Ph), 132.9 (C-10), 137.7 (C-1 of 3-Bn), 132.8 (C-5), 154.8 (C-1), 157.3 (C-3), 220.0 (C-17).

3-Benzyloxy-15β-(2'-4''-chlorophenylcarbamoyloxy)ethoxy-estra-1,3,5(10)-trien-17-one (38)

Compound **10** (420 mg, 1 mmol) was used for the synthesis as described in general procedure. The reagent was 4-chlorophenyl isocyanate (4 mmol). The crude product was chromatographed with ethyl acetate/CH_2Cl_2 (25:75 v/v) to yield **38** (380 mg, 66%). Mp: 103–106 °C; R_f = 0.62 (ss B); $[\alpha]_d$25 + 32 (*c* 1 in $CHCl_3$) . Found: C, 71.38; H, 6.55. $C_{34}H_{35}ClNO_5$ (574.11) requires: C, 71.13; H, 6.32%. ^{1}H NMR (δ, ppm, $CDCl_3$): 1.18 (s, 3H, 18-H_3), 3.60 (m, 1H, O-CH_2), 3.76 (m, 1H, O-CH_2), 4.23 (t, 1 H, *J* = 7.0 Hz, 15-H), 4.33 (m, 2H, O-CH_2), 5.06 (s, 2H, Bn-H_2), 6.70 (s, 2H, 4-H, NH), 6.81 (dd, 1H, *J* = 11.0 Hz, *J* = 3.0 Hz, 2-H), 7.21 (d, 1H, *J* = 11.0 Hz, 1-H), 7.37 (m, 9H, 5x CH of 3-Bn and 6'-H, 4x CH of N-Ph). ^{13}C NMR (δ, ppm, $CDCl_3$): 18.0 (C-18), 26.2, 26.7, 27.5, 29.9, 33.2, 35.2, 42.2, 43.7 (O-CH_2), 44.6, 47.8 (C-13), 55.0, 62.4, 64.8, 68.1 (O-CH_2), 70.4 (Bn-CH_2), 75.6 (C-15), 112.8 (C-2), 115.3 (C-4), 118.5 (C-4 of 3-Bn), 126.6 (C-1), 127.9 (C-2 and C-6 of 3-Bn), 128.3 (C-2 and C-6 of N-Ph), 129.0 (C-3 and C-5 of 3-Bn), 129.3 (C-3 and C-5 of N-Ph),129.5 (C-4 of N-Ph), 157.3 (C-3), 160.1 (C = O), 219.9 (C-17).

3-Benzyloxy-15β-(3'-butylcarbamoyloxy)propoxy-estra-1,3,5(10)-trien-17-one (39)

Compound **11** (434 mg, 1 mmol) was used for the synthesis as described in general procedure. The reagent was *n*-butyl isocyanate (4 mmol). The crude product was chromatographed with ethyl acetate/CH_2Cl_2 (2.5:97.5 v/v) to yield **39** (460 mg, 86%). Mp: 101–103 °C; R_f = 0.40 (ss B); $[\alpha]_D^{25}$ + 58 (*c* 1 in $CHCl_3$). Found: C, 74. 43; H, 8.39. $C_{33}H_{43}NO_5$ (533.70) requires: C, 74.27; H, 8.12%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.88 (t, 3H, *J* = 6.5 Hz, $(CH_2)_3$-H_3), 11.1 (s, 3H, 18-H_3, 2.88 (m, 2H, 6-H_2), 3.12 (d, 1H, *J* = 8.0 Hz, NH-CH_2), 3.29 (m, 1H, O-CH_2), 3.55 (m, 1H, O-CH_2), 4.09 (m, 3H, O-CH2, 15-H), 4.57 (brs, 1H, NH), 4.99 (s, 2H, Bn-H_2), 6.70 (d, 1H, *J* = 3.0 Hz, 4-H), 6.74 (dd, 1H, *J* = 11.0 Hz, *J* = 3.0 Hz, 2-H), 7.15 (d, 1H, *J* = 11.0 Hz, 1-H), 7.33 (m, 5H, 5x CH of Bn). ^{13}C NMR (δ, ppm, $CDCl_3$): 13.6 $(CH_2)_3$-CH_3), 17.4 (C-18), 19.7, 25.6, 26.2, 29.4, 29.6, 31.9, 32.6, 34.7, 40.6, 43.0, 44.1, 47.1 (C-13), 54.4, 61.7 (O-CH_2), 65.7 (O-CH_2), 69.8 (Bn-CH_2), 74.6 (C-15), 112.2 (C-2), 114.8 (C-4), 126.0 (C-1), 127.3 (C-2

and C-6 of Bn), 127.7 (C-4 of Bn), 128.4 (C-3 and C-5 of Bn), 132.4 (C-10), 137.2 (C-1 of Bn), 137.7 (C-5), 156.4 (C = O), 156.8 (C-3), 219.5 (C-17).

3-Hydroxy-15β-(3'-n-butylcarbamoyloxy)propoxy-estra-1,3,5(10)-trien-17-one (40)

Compound **39** (533 mg, 1 mmol) was dissolved in ethyl acetate (30 ml) and the solution containing Pd/C (300 mg, 10%) was hydrogenated under 5 bar for 6 h, at room temperature. The reaction mixture was filtered off and evaporated *in vacuo.* The residual material was chromatographed on silica gel column with ethyl acetate/CH_2Cl_2(25:75 v/v) to yield **40** (290 mg, 65%). Mp: 65–70 °C (foam); R_f = 0.42 (ss D); $[\alpha]_D^{25}$ + 63 (*c* 1 in $CHCl_3$). Found: C, 70.27; H, 8.63. $C_{26}H_{37}NO_5$ (443.58) requires: C, 70.40; H, 8.41%. ^{1}H NMR (δ, ppm, $CDCl_3$): 0.91 (s, 3H, *J* = 6.5 Hz, NH-$(CH_2)_2$-H_3), 1.15 (s, 3H, 18-H_3), 3.13 (m, 2H, 6-H_2), 3.20 (t, 2H, *J* = 6.5 Hz, N-$H_{2)}$, 3.33 (t 2H, *J* = 6.0 Hz, linker H_2) 4.75 (s, 1H, 15-H), 6.17 (s, 1H, 4-H), 6.64 (dd, 1H, *J* = 10.5 Hz, *J* = 3.5 Hz, 2-H), 7.11 (d, 1H, *J* = 10.5 Hz, 1-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 11.6 (NH-$(CH_2)_2$-CH_3), 13.9, 18.0 (C-18), 23.6, 26.2, 26.7, 29.8, 30.1, 33.1, 35.3, 43.2 (C-13), 43.6 (linker CH_2), 44.6, 47.8 (linker CH_2), 55.0, 62.5 (linker CH_2), 66.2 (linker CH_2), 75.2 (C-15), 113.3 (C-2), 115.8 (C-4), 126.6 (C-1), 132.1 (C-10), 138.4 (C-5), 154.5 (C-3), 220.8 (C-17).

Measurement of inhibition of 17β-HSD1

Our previously published methods were used for the measurement of 17β-HSD1 inhibition[21,22]. In brief, human placental cytosol was incubated as enzyme source with 1 μM [^{3}H]-labelled estrone substrate at 37 °C. The cofactor, either NADH or NADPH, was used in an excess concentration of 100 μM. The buffer medium consisted of 0.1 M HEPES (pH = 7.3), 1 mM EDTA, and 1 mM dithiotreitol. The substrate was added to the incubate in 10 μl of a 25 v/v% propylene glycol in HEPES buffer solution, whereas test compounds were applied in 10 μl of dimethyl sulfoxide solution.

After an incubation time of 2.5 min, the enzymatic reaction was stopped and the product 17β-estradiol was isolated by TLC. Radioactivity of the 17β-estradiol (**2**) formed was measured by means of liquid scintillation counting. Test compounds were usually applied in 10 μM concentration, whereas concentrations of 0.1–50 μM were used during determination of IC_{50} values. The inhibitor effect was assessed with relative conversion results calculated in comparison to non-inhibited controls (100%). IC_{50}results were calculated by using unweighted iterative least squares logistic curve fitting by means of the "absolute IC_{50} calculation" function of the GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). The IC_{50} of unlabelled estrone (**1**) was measured as reference. The relative inhibitory potential (RIP) values of the test compounds were calculated by using reference IC_{50} data measured with the corresponding cofactor: RIP = IC_{50} of test compound/IC_{50} of unlabelled estrone (**1**).

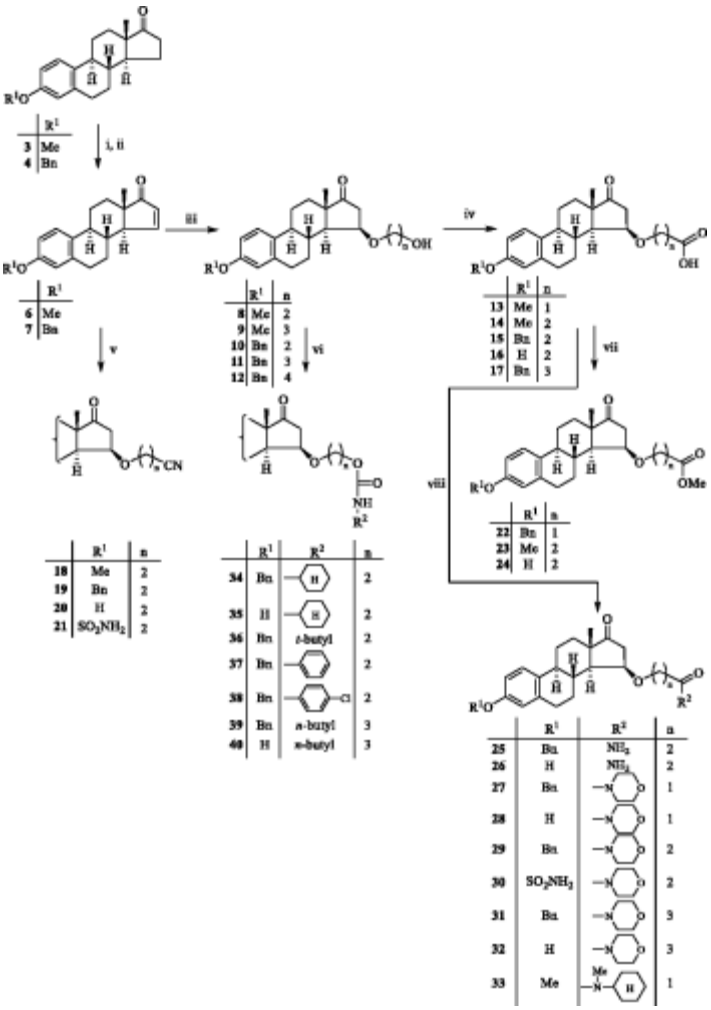
Results and discussion

Synthetic studies

To prepare novel substituted 15β-alkoxy steroids, 3-methoxy-estra-1,3,5(10),15-tetraen-17-one (**6**), and 3-benzyloxy-estra-1,3,5(10),15-tetraen-17-one (**7**) were chosen as starting compounds[12,23]. The synthetic strategy for the preparation of the different type of compounds is illustrated in Scheme 2.

Scheme 2. Reagents and conditions: (i) and (ii)[12,23]; (iii) CH_2Cl_2, hydroxyl alcohol, NaOH; (iv) acetone, Jones reagent; (v) CH_2Cl_2, hydroxyl alkylnitrile, NaOH; (vi) CH_2Cl_2, TEA, hydroxyl alkyl-,

or arylisocyanate; (vii) THF, diethylether, CH_2N_2; (viii) CH_2Cl_2, oxalyl chloride, amines.



Treatment of Δ^{15}-17-one compound **6** with aqueous potassium hydroxyde in methanol afforded 3,15β-dimethoxy-estra-1,3,5(10)-trien-17-one via 1,4 addition in practically quantitative yield. On the basis of an earlier observation from W. S. Johnson and W. F. Johns[23], we extended this 1,4-addition process to 1,2-ethanediol, 1,3-propanediol and 1,4-butanediol to receive from compound **6** the corresponding 3-methoxy-15β-(2'-hydroxy)ethoxy-, and 3-methoxy-15β-(3'-hydroxy)propoxy-estra-1,3,5(10)-triene-17-ones (**8** and **9)**, from compound **7** the 3-benzyloxy-15β-(2'-hydroxy)ethoxy-, 3-benzyloxy-15β-(3'-hydroxy)propoxy-, and 3-benzyloxy-15β-(4'-hydroxy)butoxy-estra-1,3,5(10)-trien-17-ones (**10–12**). The addition of different nucleophiles is highly stereospecific, giving 15β substituted estranes in all cases[12,23]. Jones oxidation of these compounds (**8–12**) furnished the corresponding 3-methoxy-, and 3-benzyloxy-15β-(carboxyl)-alkoxy-estra-1,3,5(10)-trien-17-one derivatives (**13–17**). The 1,4-addition process of compounds **6** and **7** with 3-hydroxypropionitrile afforded the corresponding 3-methoxy-, and 3-benzyloxy-15β-(2'-cyano)ethoxy-estra-1,3,5(10)-trien-17-ones (**18**, **19**). Cleavage of the 3-benzyloxy group of **19** yielded **20**, which reacted with sulfamoyl chloride to yield **21**. Esterification of 15β-(carboxyl)alkoxy derivatives by diazomethane yielded the corresponding methyl esters **22–24**. The 15β-(carboxyl)alkoxy compounds were reacted with oxalyl chloride to give carboxylic acid chloride which, upon reaction with ammonium hydroxide, morpholine or *N*-cyclohexyl, *N*-methylamine yielded the corresponding carboxamides **25–33**. The 3-benzyloxy-15β-(2'-hydroxy)ethoxy- and 3-benzyloxy-15β-(3'-

hydroxy)propoxy-estra-1,3,5(10)-triene-17-ones (**10** and **11**) reacted with different alkyl- and aryl isocyanates to furnish alkyl- and aryl urethane derivatives **34–40**.

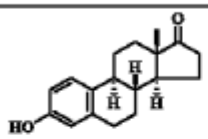
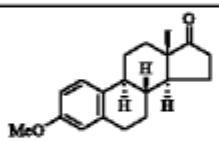
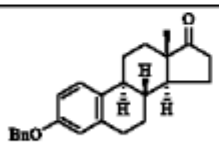
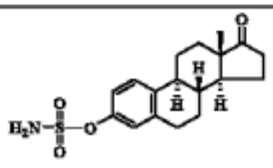
Inhibitory potentials of the C15 estrone derivatives towards 17β-HSD1

Ligand-based approach and the role of reference compounds

Experimental testing and biochemical analysis of the inhibition of novel compounds provide a feasible way for the development of inhibitors against 17β-HSD1. This ligand-based approach may also give valuable information on the molecular basis of substrate binding and catalytic mechanisms[24].

In our present experiments, thirty substituted 15β-alkoxy estrone derivatives possessing hydroxy, methoxy, benzyloxy and sulfamate groups in position C3 were investigated. *In vitro* conversion of estrone (**1**) to 17β-estradiol (**2**) was measured and cofactors, either NADPH or NADH were present in excess amounts in the incubations. Inhibitory effects were evaluated first by relative conversion measured at 10 µM test concentration. For more potent inhibitors IC_{50} values were determined. Inhibitory potentials and binding affinities were evaluated in comparison to those of natural substrate estrone (**1**) and the parent unsubstituted core molecules of the inhibitors, including 3-methoxy- and 3-benzyloxy-estrone (**3** and **4**) and estrone-3-sulfamate (**5)** (EMATE). Comparative evaluation of inhibitory potentials measured with different cofactors was performed on the basis of RIP parameters which were calculated in comparison to substrate estrone (Table 1).

Table 1. Reference parameters of *in vitro* 17β-HSD1 inhibition of the unsubstituted compounds.

Nr.	Structure	17β-HSD1 inhibition in the presence of NADPH			17β-HSD1 inhibition in the presence of NADH		
		IC_{50} ± SD (µM)	Rel. conv. (%) at 10µM ± SD	RIP	IC_{50} ± SD (µM)	Rel. conv. (%) at 10µM ± SD	RIP
1		0.63 ± 0.11		1.0	2.0 ± 0.18		1.0
3		0.77 ± 0.29		1.2	4.2 ± 1.6		2.1
4		>10	52 ± 2	> 15	>10	52 ± 5	> 5
5		0.98 ± 0.71		1.6	2.6 ± 1.6		1.3

IC_{50}: concentration which decreases the enzyme activity to 50%.

Relative conversions (Rel. conv., control incubation with no inhibition is 100%) measured in the presence of 10 µM concentration of the compound tested. Mean ± SD, *n* = 3.

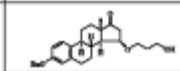
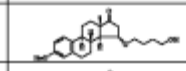
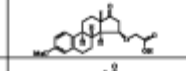
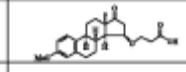
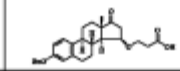
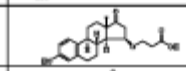
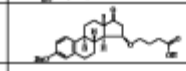
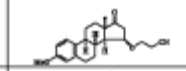
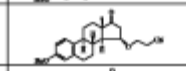
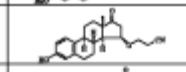
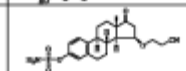
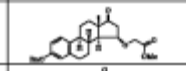
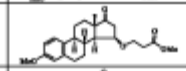
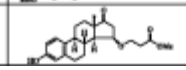
RIP: relative inhibitory potential compared to estrone; SD: standard deviation.

17β-HSD1 inhibition results of the test compounds

Results obtained with NADPH

In the NADPH supplemented incubations compounds **18**, **20**, and **40** were found to be the most potent inhibitors (Table 2). Their IC_{50} values are below 1 µM (0.42–0.64 µM), indicating inhibitory effects similar to those of the unsubstituted parent compounds estrone (**1**) and 3-methyl-*O*-estrone (**3**) (0.63 and 0.77 µM, respectively). Compounds **11**, **21**, **22**, and **25** proved to be effective inhibitors displaying IC_{50} close to 1 µM (0.78–1.5 µM). Three compounds of this group (**11**, **22**, **25**) are derivatives of 3-benzyl-*O*-estrone, and they exert substantially stronger inhibition in comparison to the unsubstituted core itself. The IC_{50} value was found to be 2.7 µM for another 3-benzyloxy compound (**9**), and that shows a somewhat weaker effect, but still an improved inhibition when compared to the parent molecule (**4**). IC_{50} values of **23**, **24**, and **26** were found also in the micromolar range (2.2–5.1 µM). These results, however, indicate decreased potentials compared to the unsubstituted estrone (**1**) and 3-methyl-*O*-estrone (**3**) cores. Relative conversions measured for the 10 µM test concentration of the other compounds in the presence of NADPH were higher than 50%. These results mean IC_{50} values higher than 10 µM and reveal a weak inhibitory effect against the 17β-HSD1.

Table 2. *In vitro* 17β-HSD1 inhibition of the C15 derivatized test compounds.

Nr.	Structure	17β-HSD1 inhibition in the presence of NADPH			17β-HSD1 inhibition in the presence of NADH		
		IC_{50} ± SD (μM)	Rel. conv. (%) at 10μM ± SD	RIP	IC_{50} ± SD (μM)	Rel. conv. (%) at 10μM ± SD	RIP
11		0.78 ± 0.30		1.2	> 10	76 ± 7	> 5.0
12		2.7 ± 1.6		4.3	> 10	77 ± 5	> 5.0
13		> 10	90 ± 9	> 15	> 10	78 ± 7	> 5.0
14		> 10	88 ± 9	> 15	> 10	76 ± 3	> 5.0
15		> 10	78 ± 6	> 15	10 ± 3		5.1
16		> 10	76 ± 15	> 15	9.7 ± 4.5		4.9
17		> 10	87 ± 4	> 15	3.5 ± 1.0		1.8
18		0.56 ± 0.37		0.89	20 ± 9.0		10
19		> 10	65 ± 4	> 15	> 10	83 ± 1	> 5.0
20		0.64 ± 0.18		1.01	3.2 ± 1.5		1.6
21		1.1 ± 0.14		1.7	4.0±1.5		2.0
22		1.5 ± 0.50		2.4	> 10	60 ± 3	> 5.0
23		5.1 ± 2.3		8.1	> 10	78 ± 2	> 5.0
24		2.2 ± 0.73		3.5	7.0 ± 4.2		3.5

(*Continued*)

Results obtained with NADH

In experiments performed with NADH in excess, an outstanding inhibitory potential was measured for 3-hydroxy compound (**35**) (IC_{50} = 0.38 μM). Two other 3-hydroxy compounds (**20** and **40**) were found to be somewhat less potent, and their IC_{50} values (3.2 and 1.4 μM, respectively) reflect similar effects as the unsubstituted estrone. The 3-benzyloxy compound (**17**) and the 3-sulfamate compound (**21**) also displayed medium strengths with IC_{50} values 3.5 μM and 4.0 μM, respectively. The result of **17**indicates an improved inhibition compared to the basic molecule 3-benzyl-*O*-estrone (**4**). Compounds **24**, **28**, and **33** displayed moderate inhibitions with IC_{50} values between 5.6 and 7.0 μM. Other compounds exerted weak inhibition when NADH was applied as cofactor. In these cases, relative conversions were not suppressed below 50% at the 10 μM test concentration (IC_{50}>10 μM).

Results with NADPH versus NADH

Results measured with NADPH or NADH showed different tendencies for some of the test compounds when the inhibitory effects of the C15 derivatives were compared to those of their unsubstituted core compounds. Using the RIP values, derivative **35** exerted similar effect in the presence of NADPH than estrone (**1**) (RIP = 0.66 and 1.0, respectively), but this compound had a

strong 5-fold increase in inhibitory potential, when NADH was applied (RIP = 0.19). Derivative **18** also displayed a maintained effect with NADPH (RIP = 0.89), but showed diminished potential with NADH (RIP = 10) in comparison to its unsubstituted core 3-methyl-*O*-estrone (**3**). The latter displayed RIP values 1.2 with NADPH and 2.1 with NADH). C15 substituents increased the inhibitory potentials in the case of 3-benzyloxy compounds **11**, **22**, and **25** when NADPH was applied (RIP = 1.2–2.4), but maintained effects were measured for these derivatives in the presence of NADH (RIP > 5.0). Compound **17** had an opposite behavior, that is, it showed improved inhibition with NADH (RIP = 1.8) compared to the unsubstituted core 3-benzyl-*O*-estrone (**4**) ((RIP (NADPH) > 15 and RIP (NADH) > 5)). On the other hand, **20** and **40** showed retained potentials with both cofactors compared to their unsubstituted core estrone (**1**) with RIP parameters close to 1 (0.70–1.6). For some other compounds derivatized at C15, however, decreased inhibitory effects could be observed when either NADPH or NADH was applied.

Biomedical evaluation of the inhibitory potentials obtained with NADPH

Literature background

Estrane-based inhibitors, as C15-substituted derivatives are assumed to occupy the substrate binding site of 17β-HSD1. Side chains are capable of establishing further contacts to the enzyme than the substrate molecule itself and, in this way, they may modulate binding affinity and inhibitory potential this way[5,7,9,25,26]. Messinger et al. analysed the X-ray structure of the 17β-HSD1[27] and identified a hole in the proximity of the enzyme's active site, which is composed of flexible amino acids Ser222, Leu219 and Met193 as well as Tyr218, Leu96, and Gly198[12]. The hole shows its opening towards the environment of C15 of the steroidal backbone, and thought to be able to accommodate side chains with appropriate length, spacer unit, and capping group. This finding inspired the Messinger group to synthesize numerous C15-substituted estrone derivatives as presumed 17β-HSD1 inhibitors and they have identified several compounds with high potential, displaying IC_{50} values in the low nanomolar range[12,13].

Further studies also established that 17β-HSD1 accomplished complex processes in ligand binding sites[11,28]. That is, the enzyme protein could change its conformation depending on the inhibitor molecule offered[12]. These mechanisms indicate that a very small change in inhibitor structure can make large differences in the course of binding to the enzyme[12]. Inhibitor studies, therefore, may give ambiguous picture with regard to the binding properties of the compounds and complete structure–activity relations could be revealed sometimes scarcely[9,11,12].

17β-HSD1 can use either NADPH or NADH as hydride donor for the estrone (**1**) to 17β-estradiol (**2**) transformation. NADPH, *in vivo,* seems to be the prevailing cofactor in the process[29,30]. It is, therefore, reasonable to make biomedical evaluation of inhibitor candidates according to their potentials exerted in NADPH supplemented medium[22].

Discussion of test compounds.

Among the test compounds, we investigated four 15β-(2'-cyano)ethoxy derivatives. Three of them, the 3-methoxy-, 3-hydroxy-, and 3-sulfamate compounds (**18, 20**, and **21**, respectively) proved to be potent inhibitors. These results indicate that 15β-(2'-cyano)ethoxy substituent can be a beneficial

side chain concerning 17β-HSD1 inhibitory effect of estrone derivatives possessing different functionalities in their C3 position.

Four 3-benzyloxyestrone derivatives exerted substantial inhibitory effects against 17β-HSD1. Two compounds with 15β-(3'-hydroxy)propoxy-, and 15β-(4'-hydroxy)butoxy side chain (**11** and **12** respectively), as well as the 15β-(1'-methoxycarbonyl)methoxy derivative **22** and 15β-(2'-carbonylamido)ethoxy derivative **25** were found to be potent inhibitors. Potentials observed for these compounds are interesting, since very few effective 3-benzyl-*O*-estrone derivatives have been published in the literature[9,13,21].

Some of the compounds tested display structural similarities to C15 derivatized estrone-based compounds published earlier as 17β-HSD1 inhibitor candidates[12,13] and interesting conclusions can be arrived at by comparison our inhibitors with their counterparts. In previous studies 15β-propanolyl and 15β-pentanolyl substituents were found to be beneficial substituents on estrone concerning 17β-HSD1 inhibition[12,13]. In our case, the corresponding 15β-(3'-hydroxy)propoxy- and 15β-(4'hydroxy)butoxy-substituted 3-benzyl-*O*-estrone **11** and **12** also displayed considerable inhibitions. We found that the 3-methyl-*O*-estrone compound bearing a C15 substituent with a cyclohexyl capping group **33** was a poor inhibitor. In Messingers' experiments, however, its non-oxa analogue exerted potent inhibition[12,13]. A related cyclohexyl derivative of estrone **35**, on the other hand, showed also a strong inhibitory effect in our tests. The Messinger group found efficient 17β-HSD1 inhibitors of estrone and 3-methyl-*O*-estrone compounds that bore 15β-substituents containing a morpholino capping group and long chain with 3–5 methylene units[12,13]. In comparison, 15β-oxy-coupled morpholino compounds in our tests displayed only weak inhibitions. The comparison of oxa-coupled derivatives with earlier non-oxa analogues indicates the importance of the C15 linker unit in the 17β-HSD1 inhibitory effect of estrone derivatives. Benchmarking of recent results against earlier data is not easy, since different studies may be performed with different methodologies and reference inhibition parameters may be missing in previous studies. Our best inhibitors, nevertheless, may be estimated to be equipotent with some of related earlier C15 derivatives of estrone compounds and non-oxa analogues studied by Messinger et al[12,13].

Our investigations reveal that C15 substituents of the tested estrogen derivatives have decisive influence in the binding to 17β-HSD1. These results further indicate that remote fragments on position C3 also determine affinity of the investigated inhibitor molecules. C15 substituents of the test compounds show high variety in their chemical nature. Compounds found to be potent inhibitors also possess diverse side chains and it seems difficult to identify chain length, capping groups or spacer units which can be definitely beneficial in 17β-HSD1 binding. C15 substituents of the compounds can be, however, regarded as side chains both long and flexible. Considering these common features, we may assume that binding may be promoted by accommodation of these side chains in the binding hole of 17β-HSD1 that exists in proximity of C15 positions of ring D as described by Messinger et al[12].

Our experiments identified several potent 17β-HSD1 inhibitors among 15β-derivatized estrone-based compounds tested. The results demonstrate definitive influence of C15 substituents as well as crucial role of different functionalities in position C3. Structural diversity of the test compounds makes difficult to give complete structure–activity relationship conclusions, nevertheless, several interesting observations can be established from the inhibition data set.

Cofactor dependence of the 17β-HSD1 inhibition, a comparative evaluation

Literature background

Both cofactor molecules, NADPH and NADH, bind to 17β-HSD1 in an extended conformation, with the nicotinamide moiety pointing towards the active site of the enzyme. Nicotinamide is relatively flexible in the complex and themajor interactions between cofactor and enzyme occur at the adenine dinucleotide phosphate part[30]. Most of these interactions are common for both NADPH and NADH[25,27,31]. A major difference between the two cofactors is, however, that the 2'-phosphate group of NADPH is stabilized mainly through hydrogen bonds with residues Ser11 and Arg37[27,31], whereas the free hydroxyl groups on adenosine ribose of non-phosphorylated cofactor NADH may form hydrogen bonds with Ser11, but not with Arg37[31]. When a cofactor binds to 17β-HSD1, structural changes are induced in the area of the substrate binding site as well. An otherwise disordered loop, which are composed of residues 189–200, may adopt a specific conformation to accommodate more space for the cofactor in the active centre[25,27]. Structural differences between the holo form and the apo form of the enzyme may modify interactions of ligands bound in the substrate binding site. For instance, side chain of a potent inhibitor 3-[3',17'*β*-dihydroxyestra-1',3',5'(10')-trien-16'*β*-methyl]-3-benzamide occupies a different position in the ternary inhibitor complex, which is different from that in the binary complex[25]. Differences in binding of substrate or inhibitor ligands may also occur when different cofactors are complexed. The NADPH-bound holoenzyme exerts higher affinity to the substrate estrone than the NADH-bound complex[29,30]. In an earlier study we observed highly different inhibitory potential and binding affinity of various D-secoestrones depending on which cofactor (either NADPH or NADH) was applied in the *in vitro* experiments[22].

Discussion of test compounds, NADPH versus NADH

In our experiments, substantial inhibitory potentials and increased binding affinities were observed in presence of both cofactors. However, some of the compounds exerted different inhibitory potentials towards 17β-HSD1 complexed to NADPH- or NADH-complexed 17β-HSD1. Substrate estrone displays different IC_{50} values depending on the cofactor applied and RIP parameters reflect more reliably the cofactor-dependent differences in the inhibitor binding.

RIP parameters demonstrate 3–8 fold stronger binding with NADH than with NADPH for estrone derivatized with a 15β-(1'-morpholinocarbonyl)methoxy- side chain (**28**), for 3-methoxy-estrone and estrone derivatized with 15β-(1'-*N*-methyl,cyclohexylamino carbonyl)methoxy- and 15β-(2'-cyclohexylcarbamoyloxy)ethoxy chains (**33**and **35**), as well for 3-benzyl-*O*-estrone derivative possessing a 15β-(3'-carboxylic)propoxy substituent (**17**). Binding of 3-benzyl-*O*-estrone compounds substituted with a 15β-(3'-hydroxy)propoxy, 15β-(1'-methoxycarbonyl)methoxy, or a 15β-(2'-aminocarbonyl)ethoxy side chain (**11**, **22**, and **25)** and 3-methyl-*O*-estrone bearing a 15β-(2'-cyano)methoxy side chain (**18**) display similar (2–11 fold) cofactor preference in terms of RIP values, but these derivatives favour binding to the NADPH bound enzyme.

Cofactor-dependent affinities of some of the investigated compounds indicate that binding capabilities of the binding hole may be different depending on the nature of cofactor the enzyme complexed with. The binding hole suitable to accommodate C15 side chains is known to be formed by amino acids Leu96, Met193, Gly198, Tyr218, Leu219, and Ser222[12]. Residues Met193 and Gly198 are also constituents of the disordered loop (amino acids 189–200) which adopt a specific

conformation following cofactor binding[25,27]. The presence or absence of 2'-phosphate in the cofactors causes differences in the structure of the holoenzymes. Furthermore, the area of the substrate binding site may also be affected in a different way in these complexes[25,27,31]. We may also assume that binding of NADPH or NADH modifies the conformation of the loop of residues 189–200 differently. Joint residues Met193 and Gly198 of the two structural elements transmit this difference from the loop to the binding hole, and these processes induce different positioning and binding capabilities of the binding hole in the holoenzyme variants. Hosting certain C15 side chains, therefore, might be, therefore, favoured or unfavoured in the binding hole altered differently upon binding of phosphorylated or unphosphorylated cofactors.

In our earlier investigation, potent 17β-HSD1 inhibitory effects of ring D modified seco-oxime and seco-alcohol estrones were identified[22]. In that case we assumed that polar functionalities of short side chains of compounds studied might establish hydrophilic interactions or hydrogen bonds towards suitable amino acids of the enzyme present in close proximity of ring D region. Binding affinity of certain compounds in that series also displayed strong cofactor dependence and this phenomenon indicated that complexation with NADPH or NADH furnished different conformations to enzyme residues involved in the interactions.

In our present study we identified another group of compounds, C15 derivatized estrones, which may exert cofactor-dependent inhibition towards the 17β-HSD1. Structural features and binding mechanisms of side chains in the region of ring D differ to a great extent for those compounds investigated in our earlier report and for those presented in this study. Cofactor dependence of inhibitor binding, however, could be observed in both series. These results suggest that binding of the phosphorylated or the unphosphorylated cofactor may exert different influence on more areas and structural elements of the substrate binding site.

Early studies assigned NADH as a catalytic cofactor of 17β-HSD1 and numerous *in vitro* inhibition tests have been performed with this recognition. (See corresponding references in our earlier work[22].) Later, however, it became accepted that NADPH might be the prevalent partner of 17β-HSD1 in its main *in vivo* function in the catalysis of the estrone–17β-estradiol conversion[22,29,30]. Our present results obtained with C15 estrone derivatives support that *in vitro* potentials obtained with the two cofactors may differ substantially for certain inhibitor compounds. Data measured in the presence of NADPH are more relevant in inhibitor optimization and in lead selection, but NADH results could be valuable in understanding of the mechanism the inhibition of 17β-HSD1.

Conclusions

17β-HSD1 inhibitory potential of 15β-oxa-coupled estrone derivatives possessing hydroxy, methoxy, benzyloxy and sulfamate functionalities in position C3 has been investigated. Thirty inhibitor candidates were tested via *in vitro* radioincubations. We found several potent 17β-HSD1 inhibitors and the results demonstrated that potent inhibitory effect could be achieved with both various C15 substituents, and different C3 functional groups. We identified four 3- benzyloxyestrone derivatives (**11**, **22**, **25**), which exerted substantial inhibitory effect. We also found that 15β-(2'-cyano)ethoxy was a beneficial substituent of compounds bearing different functionalities in position C3 (**18**, **19**, **20**, and **21**). A comparison with earlier non-oxa analogues indicates that beyond the effect of capping groups and spacer units, there might be a strong influence of the presence or absence of oxygen in the 15β linker on the inhibitory potential.

Some of the compounds displayed considerable difference in binding affinities towards 17β-HSD1 complexed with NADPH or NADH. It is reasonable to assume that side chains of the potent compounds can be accommodated in the binding hole of 17β-HSD1 existing in proximity of the C15 position of ring D of the steroidal ligands[12]. This binding hole shares Met193 and Gly198 with a loop element which is known to adopt a specific conformation upon cofactor binding[25,27]. We suppose that conformation of this loop may be different in NADPH- or NADH-complexed 17β-HSD1. Structural differences can be forwarded by the joint amino acids inducing different positioning and binding capabilities of the binding hole. Further structural investigations (e.g., molecular docking studies) may confirm mechanisms involved in binding of our inhibitor compounds and different binding affinities of the test compounds exerted towards the holoenzyme variants.

Our investigations provide valuable data on binding processes of the enzyme and may contribute to the development of new 17β-HSD1 inhibitors, as novel drug molecules acting on enzyme level.

Disclosure statement

The authors declare no conflict of interest.

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IX.

Herman BE, Gardi J, Julesz J, Tömböly Cs, Szánti-Pintér E, Fehér K, Skoda-Földes R, Szécsi M: Steroidal ferrocenes as potential enzyme inhibitors of the estrogen biosynthesis, *Biol Fut*, **2019** (submitted)

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Steroidal ferrocenes as potential enzyme inhibitors of the estrogen biosynthesis

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Summary

The potential inhibitory effect of diverse triazolyl-ferrocene steroids on key enzymes of the estrogen biosynthesis was investigated. Inhibition of human aromatase, steroid sulfatase (STS) and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) activities were investigated with *in vitro* radiosubstrate incubations. Some of the test compounds were found to be potent inhibitors of the STS. A compound bearing ferrocenyl side chain on the C-2 displayed a reversible inhibition, whereas C-16 and C-17 derivatives displayed competitive irreversible binding mechanism towards the enzyme. 17α-triazolyl-ferrocene derivatives of 17β-estradiol exerted outstanding inhibitory effect and experiments demonstrated a key role of the ferrocenyl moiety in the enhanced binding affinity. Submicromolar IC_{50} and K_i parameters enroll these compounds to the group of the most effective STS inhibitors published so far. STS inhibitory potential of the steroidal ferrocenes may lead to the development of novel compounds able to suppress *in situ* biosynthesis of 17β-estradiol in target tissues.

Introduction (611 words)

The ferrocene substituent features exceptional biomedical properties and its steroidal conjugates are believed to have a vast potential for medical application (Astruc, 2017, Manosroi et al. 2010; Author et al. 2015; Narvaez-Pita et al. 2017). Steroidal ferrocenes were aimed mainly to be applied for vectorization of the DNA damaging effect of the ferrocene entity to produce cytotoxic effect in cancerous cells (Hillard et al. 2010). C-2, C-16 and C-17 ferrocene derivatives of androgens, as well as C-3, C-7, C-16 and C-17 ferrocene derivatives of 17β-estradiol and estrone demonstrated considerable anti-proliferative effects (micromolar IC_{50} or GI_{50} values) against hormone independent cancerous cell lines (Hillard et al. 2010; Manosroi et al. 2010; Top et al. 2009; Vera et al. 2011; Vessières et al. 2006). Concurrently, hormonal effects exerted on the specific sexual steroid receptors were also investigated. Low affinities to the androgen receptor was detected for the ferrocene derivatives of androgens (Astruc, 2017; Vessières et al. 2006). The C-7 and C-17 ferrocene derivatives of 17β-estradiol displayed estrogen receptor binding and estrogenic effect comparable to that of 17β-estradiol (Osella et al. 2001; Vessières et al. 1998) whereas the C-3 derivative has a predicted estrogen receptor antagonistic

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effect (Vera et al. 2011). Potential inhibitory effect of the ferrocene steroid compounds exerted on enzymes of the steroid metabolism have not yet been investigated, to the best of our knowledge.
Estrogens are biosynthesized from their steroidal precursors via aromatization of their ring A. The process is catalyzed by the cytochrom P450 dependent aromatase (Fig. 1). This enzyme converts mainly androst-4-ene-3,17-dione and a minor amount of testosterone into estrone and 17β-estradiol, respectively. The estrogen reservoir estrone-3-sulfate is formed from estrone, which can be then regenerated by a steroid sulfatase (STS)-catalyzed hydrolysis. Estrone is converted to the active hormone 17β-estradiol by the stereospecific reduction of the C-17 oxo function accomplished by the 17β-hydroxysteroid dehydrogenases type 1 (17β-HSD1) (Hong & Chen, 2011). Estrogens have a major role in the development and progression of gynecological pathologies, with hormone dependent forms of breast cancer among them. Breast cancer tissues have been shown to express enzymes of the estrogen producing machinery in an exaggerated manner (Africander & Storbeck, 2018). Increased expression resulted in elevated local 17β-estradiol concentration, which is believed to promote the pathophysiological cell proliferation. Estrogen ablation achieved by blocked biosynthesis can be successfully applied in the pharmacotherapy of the hormone dependent tumors, therefore aromatase, STS and 17β-HSD1 are interesting topics of anticancer drug development (Hong & Chen, 2011; Purohit & Foster, 2012; Thomas & Potter, 2015). The three-dimensional crystal structures of human aromatase, STS and 17β-HSD1 have been explored and binding cavities and key amino acid residues involved in the binding have been identified (Ghosh et al. 2009; Hernandez-Guzman et al. 2003; Ghosh et al. 1995). These investigations provided valuable information on the molecular basis of substrate binding and catalytic mechanisms, however, they proved to be less useful in the inhibitor design (Hong & Chen 2011). Majority of the inhibitors of enzymes of the estrogen biosynthesis have been developed by ligand-based approach. Experimental testing and biochemical analysis of inhibition of novel compounds are, therefore, still a feasible way of inhibitor development.
In this study, we aimed to investigate the inhibitory effects of diverse triazolyl-ferrocene steroids exerted on the activity of key enzymes of the estrogen biosynthesis. We investigated a newly synthesized ferrocenyl steroid, other derivatives were prepared previously and the details were reported in former publications (Author et al. 2012; Author et al. 2011; Author et al. 2012b). A related phenyl-triazolyl compound (Author et al. 2018b) and cited literature therein) and two non-ferrocenyl steroid precursors (Author et al. 2011) were also included in the present experiments for investigation of structure-activity aspects.

Materials and Methods (793 words)

Test substances

1 – 2β-(4-Ferrocenyl-1,2,3-triazol-1-yl)-3α-hydroxy-5α-androstane-17-one (Author et al. 2012).
2 – 2β-(4-((E)-Ethyl 3-ferrocenylacrylate-2-methylcarbamoyl))-1,2,3-triazol-1-yl)-3α-hydroxy-5α-androstan-17-one (Author et al. 2012).
3 – 6β-(4-((E)-Ethyl 3-ferrocenylacrylate-2-methylcarbamoyl)-1,2,3-triazol-1-yl)-3α,5α-dihydroxy-androstan-17-one (Author et al. 2012).
4 – 16β-(4-Ferrocenyl-1,2,3-triazol-1-yl)-17α-hydroxy-5α-androstane (Author et al. 2012).
5 – 16β-(4-((E)-Ethyl 3-ferrocenylacrylate-2-methylcarbamoyl)-1,2,3-triazol-1-yl)-17α-hydroxy-5α-androstane (Author et al. 2012).
6 – 17-(*N*-(1-(1-ferrocenyl-ethyl)-1,2,3-triazol-4-yl)-methyl-carbamoyl)-4-aza-5α-androst-16-en-3-one, 1/1 mixture of two epimers (Author et al. 2011)
7 – 17-(*N*-(prop-2-ynyl)-carbamoyl)-4-aza-5α-androst-16-en-3-one (Author et al. 2011)
8 – 17-(*N*-(1-(1-ferrocenyl-ethyl)-1,2,3-triazol-4-yl)-methyl-carbamoyl)-3-methoxy-estra-1,3,5(10),16-tetraene, 1/1 mixture of two epimers (Author et al. 2011)
9 – 17-(*N*-(prop-2-ynyl)-carbamoyl)-3-methoxy-estra-1,3,5(10),16-tetraene (Szánti-Pintér et al. 2011)
10 – 17α-(1-Ferrocenylmethyl-1*H*-1,2,3-triazol-4-yl)estradiol (Author et al. 2018b).
11 – 17α-(1-(1-Ferrocenyl-ethyl)-1,2,3-triazol-4-yl)-3,17β-dihydroxy-estra-1,3,5(10)-triene, 1/1 mixture of two epimers (Author et al. 2012b)
12 – 17α-(1-Ferrocenylmethyl-1*H*-1,2,3-triazol-4-yl)-17β-hydroxy-18a-homo-19-nor-androst-4-ene-3-one. Compound **12** was synthesized via reaction of levonorgestrel and azidomethylferrocene and was purified by column chromatography. Synthesis method in detail and characterization data are described in the Supplementary Material.
13 – 17α-(1-Benzyl-1*H*-1,2,3-triazol-4-yl)estradiol (Author et al. 2018b).

Enzyme experiments

Activity and inhibition of the aromatase, 17β-HSD1 and STS enzymes were assessed with radioincubation methods based on techniques described in our previous publications (Author et al. 2018a; Author et al. 2016a). Here we provide short descriptions of methods applied in the enzyme experiments. (Chemicals and materials are listed in the Supplementary Material.)

Incubation procedures and measurement of inhibitory effect

Enzymatic incubations were carried out in 0.1 M HEPES buffer medium at a final volume of 200 µl. The substrate was added to incubate in 20 µl of 25 v/v% propylene glycol in HEPES buffer solution, whereas test compounds were applied in 10 µl of dimethyl sulfoxide solution. Incubations were performed at 37 °C and terminated by cooling and by the addition of organic solvents of the subsequent extraction procedure. The radioactivity of the isolated enzyme products was measured by means of liquid scintillation counting. Control samples with no inhibitor and blank samples were incubated simultaneously.
In incubates of the inhibition studies substrate concentration was 1.0 µM, and test compounds were applied at 10 µM. Relative conversions compared to non-inhibited controls (100%) were determined. The assays were performed in triplicate, the mean value and the standard deviation (SD) were calculated.
IC_{50} values (the inhibitor concentration that decreases the enzyme activity to 50%) were determined for the most effective test compounds and for certain reference compounds. In these cases, conversions were measured at 10–15 different concentrations in the appropriate interval between 0.001–50 µM. IC_{50} results were calculated by using unweighted iterative least squares logistic curve fitting by means of the "absolute IC_{50} calculation" function of the GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA).

Aromatase assay

In the aromatase inhibition assays the human placental microsoma suspension was incubated with testosterone substrate spiked with [1,2,6,7-^{3}H]testosterone tracer, in the presence of 0.1 mM NADPH cofactor excess. Incubation time was 40 min. The incubation mixture was extracted with 700 µl toluene, then the toluene phase was drained and washed with equal volume of HEPES buffer. Aromatase products containing phenolic hydroxy group were extracted with 1.2 M sodium hydroxide solution from the toluene extract.

17β-HSD1 assay

In the 17β-HSD1 inhibition assays human placental cytosol was incubated using estrone substrate containing [6,7-^{3}H]estrone tracer, in the presence of 0.1 mM NADPH cofactor excess. The incubation time was 2.5 min. The product 17β-estradiol was extracted with 100 µl ethyl acetate and isolated by a TLC method performed on silica gel plate and with dichloromethane/diisopropyl ether/ethyl acetate (75:15:10 v/v) eluent.

STS assay

STS assays were performed using human placental microsomas and estrone-3-sulfate substrate with [6,7-^{3}H]estrone-3-sulfate tracer. Incubations lasted for 20 min. Reactions were terminated by the addition of equal volume of methanol, and the product 17β-estradiol was extracted with 400 µl toluene.

Reversibility studies and kinetic analyses of STS inhibitors

In the reversibility studies inhibitor compounds were preincubated with the human microsomal placenta fraction in a volume of 4.0 µl at 37 °C for various time periods (2.5–40 min). Following this procedure, samples were diluted with the incubation medium to 50-fold. Enzyme activity measurements were performed after 20 min secondary incubation time to allow dissociation. The enzymatic reactions were started by the addition of the substrate (1.0 µM final concentration of estrone-3-sulfate with tracer), and subsequent incubation procedures were then carried out as described. Conversions were compared to their respective controls, which were treated in a similar way but without inhibitors.
Kinetic analyses were performed via measurement of the enzymatic transformation using different fixed substrate concentrations (1–5 µM) and varied inhibitor concentrations (0–0.17 µM). The K_M parameter of the STS was calculated from Michaelis-Menten analysis (non-inhibited reaction velocity *vs*. substrate concentration) using the GraphPad Prism 4.0 software. The Dixon's linear transformation (1/velocity *vs*. inhibitor concentration) was applied for evaluation and the inhibition constant (K_i) was determined from abscissa of intersection of the lines of different substrate concentrations. Mechanism of binding was identified

according to the Dixon's graphs and the secondary plot of slopes of the Dixon's lines *vs.* 1/substrate concentration.

Results (906 words)

Enzyme inhibition experiments of the steroidal ferrocenes were performed by *in vitro* radiosubstrate incubations using human term placental microsoma suspension and cytosol as enzyme sources; (Author et al. 2018a; Author et al. 2016a; and corresponding references therein). Aromatase inhibition was tested by the transformation of testosterone to 17β-estradiol, STS inhibition was investigated via hydrolytic release of estrone from estrone-3-sulfate, whereas the influence on 17β-HSD1 was measured on estrone to 17β-estradiol conversion. Relative conversions compared to non-inhibited controls (100%) were measured in the presence of 10 μM concentration of the test compounds; for more efficient compounds, IC_{50} values were determined. Inhibitory potentials and binding affinities of the test compounds were assessed also in comparison with IC_{50} data of the corresponding substrate. IC_{50} parameters of 17β-estradiol and estrone-3-sulfamate (EMATE) as references were also determined in our STS assay (results are listed in Table 1). Mechanistic and kinetic investigations were performed and inhibitory constants (K_i) determined for selected compounds.

Aromatase inhibition results of the test compounds

In our experiments, substances **2**, **6** and **8** in the applied 10 μM test concentration suppress aromatase activity to around 80% of the relative conversions (Table 2). These results mean IC_{50} values exceeding 10 μM, and indicate very weak inhibitory potentials. Other investigated compounds have even weaker effects, resulting in relative conversions close to 90% or higher.

17β-HSD1 inhibition results of the test compounds

Our test substances **6** and **11** inhibited 17β-HSD1 activity to relative conversions 65% and 76%, respectively (Table 2). These results show weak inhibitions even in the relatively high 10 μM test concentration. Other test compounds displayed even weaker effects, relative conversions were not decreased below 80% in the experiments where they were applied.

STS inhibition results of the test compounds

In our experiments, the 3α-hydroxy-5α-androstane-17-one compound derivatized with triazolyl-ferrocene in position 2β (**1**) exerts potent inhibition, the IC_{50} result 4.6 μM (Table 2) reveals a binding affinity similar to that of the substrate estrone-3-sulfate (IC_{50} result 5.2 μM). Insertion of an ethylacrylate group in the side chain of the compound **2** diminishes the inhibitory effect almost completely. Another 3α-hydroxy-5α-androstane-17-one derivative bearing a similar elongated ferrocenyl side chain in the position 6β and additionally a 5α-hydroxy group (**3**) is found to be also ineffective against STS. The 17α-hydroxy-5α-androstane compound with 16β-triazolyl-ferrocene (**4**) has a weak effect, but its counterpart **5** bearing the ethylacrylate elongated triazolyl-ferrocene side chain is a potent inhibitor. IC_{50} value of this compound (2.4 μM) indicates a two-fold affinity compared to the substrate estrone-3-sulfate. Substances **6** and **8** display relative conversion results 87% and 68%, respectively. These values indicate that either isomers of the substituted 4-aza-3-oxo-androstene and the 3-methoxy-estratetraene compounds are very weak inhibitors. These triazolyl-ferrocene derivatized substances, nevertheless, exert somewhat more potent inhibition than their carbamoyl-propene precursor compounds 7 and 9 (relative conversions are 93% and 75%, respectively).
The 17α-triazolyl-ferrocene derivative of 17β-estradiol (**10**) exerts highly potent inhibition. IC_{50} value of this compound is 0.084 μM, indicating a more than 60-fold stronger binding compared to the substrate estrone-3-sulfate (Table 2, and Fig. 2, part A). Substance **11** (consists of the methyl substituted isomer counterparts) displays an even more potent inhibition, its apparent IC_{50} value of 0.035 μM is close to that of the reference EMATE (0.0098 μM). A related ferrocenyl compound **12** which possesses 4-ene-3-oxo structure in its ring A and an ethyl group on the C-13 has a very weak effect with a relative conversion result of 77%. The analogue compound **13** bearing phenyl group attached to the triazole ring has also potent inhibitory effect showing an IC_{50} result of 1.0 μM.
Potent test substances (**1**, **5**, **10** and **11**) and the reference competitive irreversible inhibitor EMATE were subjected to mechanistic and kinetic investigations. In reversibility tests performed with **1**, the preincubated and diluted samples resulted in higher conversions (Fig. 3, III and IV), similar to the results measured for lower concentration of the test compound (Fig. 3, I). This means that **1** can be released from binding by dilution and this inhibitor binds to the enzyme in a reversible manner. In case of **5**, **10** and **11**, results of the preincubated and diluted samples (Fig. 3, III and IV) were similar to that obtained for incubations with higher

concentration of the inhibitor (Fig. 3, II). This means that these inhibitors are not released from binding upon dilution and they are bound to the enzyme irreversibly during the preincubation period. Time course experiments reveal that the binding processes are completed within 2.5 min, in a relatively short period when compared to the time of incubation.
Kinetic investigations were performed with test substances **10**, **11** and reference EMATE. The Dixon's plot provided straight lines at different fixed substrate concentrations intersecting in the second quadrant characterizing the competitive inhibition mechanism (Fig. 2, part B). The replots of slopes vs. 1/substrate concentration resulted in a straight line through the origin (insets in part B of Fig. 2) which confirms the competitive binding manner73, that means that these inhibitors occupy the substrate binding place of the enzyme. K_i parameters were found to be 0.066 μM for **10**, 0.021 μM for **11**, and 0.0044 μM for the reference EMATE (Fig. 2 part B). Inhibitory potentials on the basis of these K_i data were comparable to those obtained by the IC_{50} values. Determined K_i values are substantially lower than the K_M parameter of estrone-3-sulfate (8.5 μM), that reflects the significantly higher binding affinities of these inhibitors when compared to the substrate.

Discussion (2417 words)

Inhibition of aromatase

Structural studies of aromatase have revealed that structure of substrate binding site provides specificity towards androgens (Ghosh et al. 2009). Numerous derivatives of the preferred substrate androst-4-ene-3,17-dione have been found potent inhibitors, and the 4-hydroxy- and the 6-methylidene-1,4-diene analogues of the androst-4-ene-3,17-dione (formestane and exemestane, respectively) have been applied in the medical practice (Yadav et al. 2015). Structure-activity investigations have found that planarity of the ring A (4-ene or 1,4-diene structure), preferred anellation of the ring A and ring B junction (5α-epimers), and presence of either apolar or polar substituents on the C-4, C-6 and C-7 carbons enhance the binding affinity of the inhibitors (Cepa et al., 2005). It has been also revealed that androst-4-ene-3,17-dione derivatives were able to bind the enzyme in the absence of 3-oxo function. Potent inhibitory effect of 16β-imidazolyl androst-4-ene-3,17-dione derivatives has been reported, too (Bansal et al. 2012). Aromatase inhibitors were also found among product-like compounds, and 2-chloro- and 6α-phenyl-estrones were the most powerful members of the series investigated (Author et al. 2018a; Numazawa et al. 2005). Worth to mention that conjugated aromatic systems containing triazole cycle are common structural elements of non-steroidal aromatase inhibitors (Baston & Leroux, 2007, Santen et al. 2009;). Corresponding further literature references can be found in the comprehensive review articles on aromatase inhibitors published by Baston and Leroux (2007), Santen et al. (2009), and Yadav et al. (2015).
Various structural elements of referred aromatase inhibitor examples can be recognized in our test compounds. Certain test compounds bear substrate-like 4-en-3-oxo structure or possess ring A with similar planarity to the 4-aza-3-oxo moiety, whereas others possess product-like estrane core. Some investigated compounds have side chain in position C-2, C-6 and C-16, and the ferrocenyl substituents bear a triazolyl group. Similarities to known aromatase inhibitors made our compounds reasonable targets for aromatase inhibition tests. Experiments, however, revealed that none of the test compounds exerted substantial inhibitory effect against the human aromatase activity.

Inhibition of 17β-HSD1

The substrate binding site of 17β-HSD1 has been identified as a hydrophobic tunnel with polar residues at each end (Hernandez-Guzman et al. 2003). Further extensive inhibitor research works demonstrated that the binding cavity is complementary to the C18 steroidal scaffold of the main natural substrate estrone. 17β-HSD1 is multispecific in terms of binding other, noncognate steroidal substrates, e. g. C19 androstenes or androstanes, even in alternative binding orientations. Adjacent cavities and subpockets can be found around the substrate binding site which are able to accommodate various side chains on the steroidal C-2 position or on the ring D (positions C-15, C-16 and C-17). These interactions improve binding affinity and enhance inhibitory potential considerably. Estrone and 17β-estradiol derivatives possessing aromatic substituents on C-2 or C-16 (Poirier, 2011) and also those bearing triazolyl-aryl side chain on C-15 (Messinger et al. 2008) or on a D-seco structure (Author et al. 2016b) were found to be efficient 17β-HSD1 inhibitors. Readers may find reference details of these literature data in our previous publications (Author et al. 2018a; Author et al. 2016) and in review articles on 17β-HSD1 inhibitors from Brožič et al. (2008) and Poirier (2011).

17β-HSD1 is able to bind compounds containing various steroidal cores, and suitable substituents, among them aromatic side chains in the position C-2, C-15, C-16 and C-17 may enhance the binding affinity of these compounds. Several structural features of our steroidal ferrocenes resemble to known 17β-HSD1 inhibitors, nevertheless, our tested compounds did not exert considerable inhibition on the 17β-HSD1 activity under our experimental conditions.

Inhibition of STS

Protein of the STS consists of a globular polar domain and a hydrophobic transmembrane stem domain, resembling to a mushroom in that overall tertiary shape of the enzyme. The substrate binding site situated in a cavity lies at the base of the globular domain, just at the top of the stem domain (Hernandez-Guzman et al. 2003). STS hydrolyzes various physiological substrates to their unconjugated forms, including 3-sulfate ester of estrone, dehydroepiandrosterone, pregnenolone and cholesterol. Numerous other compounds proved to be capable for binding to the enzyme and demonstrated effective inhibition towards the sulfatase activity (Maltais & Poirier, 2011; Nussbaumer & Billich, 2004; Shah et al. 2016; Williams, 2013). A large group of these inhibitors are ester derivatives which apply the FGly75 inactivation mechanism by their acid residue. Most of these, either steroidal or non-steroidal compounds, are sulfamates, and the first and still the best known example of these irreversible inhibitors is EMATE (Reed et al. 2004).
Various estrane-, androstane- and pregnane-based compounds exert inhibitory effect towards the STS and their binding affinity to the enzyme can be largely modified by substituents on the steroid skeleton (Maltais & Poirier, 2011; Nussbaumer & Billich, 2004; Shah et al. 2016; Williams, 2013). Certain halogens or other small electron withdrawing groups (cyano and nitro) in the position 2 and/or 4 of the steroidal ring A may enhance the inhibitory potential (Author et al. 2018a; Mostafa et al. 2015; Numazawa et al. 2006; Phan et al. 2011). Reason of the enhanced binding is not known, nevertheless, existence of cavities able to accommodate these substituents could be postulated. Amino acids which might be involved in putative electrostatic or hydrogen-binding interactions in the proximity of the position 2 (Lys134, Lys368, His136, His290, and His346) and the position 4 (Lys368, Lys134 and Arg79) are also proposed (Mostafa et al. 2015; Phan et al. 2011; Reed et al. 2004).
Literature results have also revealed that appropriate side chains on the ring D might also improve binding affinity of the steroidal inhibitors (Shah et al. 2016). 17α-benzyl or alkyl derivatives of 17β-estradiol display enhanced inhibitor potential (Poirier & Boivin, 1998; Fournier & Poirier, 2011). Hydrophobic substituents on the aromatic cycle might further improve the inhibitory effect (Ciobanu et al. 1999) and relatively rigid 17α-moieties (cyclic substituent attached with only one rotatable bond, a methylene group) were more advantageous in the binding. 16α-benzyl and 16β-benzyl substituted compounds exert also augmented inhibition compared to their parent compound 17β-estradiol, although they are much less potent than their 17α counterparts. These findings indicated non-specific hydrophobic interactions of the D-ring side chains with hydrophobic residues in a long, deep and narrow pocket in the D-ring area (Fournier & Poirier, 2011). Non-competitive or mixed type reversible inhibition of the 17α-benzyl-17β-estradiol suggested the existence of two proximate binding sites involved in the binding of this inhibitor (Ciobanu et al. 1999; Mostafa et al. 2015). One of them allows the steroidal part to bind, whereas another one, an allosteric binding site, to establish non-specific hydrophobic interactions of the D-ring side chains. The latter is formed by hydrophobic residues in a long and narrow pocket located between the two hydrophobic α-helices and buried in the trans-membrane domain (Fournier & Poirier, 2011; Mostafa & Taylor, 2012). Authors Fournier and Poirier postulated that inhibitory potency might be increased by maximizing interactions with both, the substrate and the allosteric binding sites (Fournier and Poirier, 2011). Considering enhanced inhibitor potency of further steroidal compounds bearing C-17 aryl group, a π-π type interaction to the Phe residues present at the entrance of α-helices of the binding site have also been hypothesized (Ciobanu et al. 2001; Shah et al. 2016). Large affinity difference which was observed between 17β-arylsulfonamide and 17β-arylamide derivatives of estra-1,3,5(10)-trien-3-ol indicated that other, non-hydrophobic interactions of the linker which attach the aryl function might also play a key role in potent inhibition (Mostafa et al. 2015; Mostafa & Taylor, 2012).
STS inhibition data of the investigated steroidal ferrocene compounds reveal that the 17α-triazolyl-ferrocene derivative of 17β-estradiol (**10**) is a highly potent inhibitor of this enzyme. The submicromolar IC_{50} and K_i parameters enroll this compound to the group of the most effective steroidal STS inhibitors known to date56–59. Inhibitory potential of this compound is similar to that of the optimized 17α-benzyl-17β-estradiol derivatives (IC_{50}= 0.022–0.310 μM) reported by the Poirier's group, among which a bicyclic 4-benzyloxybenzyl derivative was the most effective inhibitor (Nussbaumer & Billich, 2004; Poirier & Boivin, 1998). Our test compound **10** also bears a bicyclic aromatic 17α substituent and both of its triazolyl and ferrocenyl moieties bear a pronounced pharmacophore character.
In order to investigate structural features contributing to the outstanding inhibitory potential of compound **10** we may compare this finding with the results of the triazolyl-benzene derivative **13** and their parent compound

17β-estradiol. Both 17α substituted compounds have markedly enhanced effect when compared to 17β-estradiol (IC_{50}= 9.0 μM), and the presence of ferrocenyl moiety instead of phenyl group results in a further 12-fold increase of the inhibition. Ferrocene and phenyl ring has been used to replace each other mutually, and can be regarded as bioisosteric functional groups in pharmaca. Concerning inhibition towards STS, the ferrocenyl analogue exerts a considerably higher potential, indicating a crucial and specific function of this moiety in the binding of the inhibitor to this enzyme.

Substance **11** contains methylated derivatives of **10**. Although substance **11** is a mixture of epimers, it displays regular behavior in inhibition experiments (Fig. 2). Measured apparent IC_{50} and K_i parameters can reliably reflect the inhibitory effect, and these values indicate further enhanced potential when compared to that of the non-methylated counterpart. Structural aspects cannot be identified precisely, nevertheless, results of substance **11** emphasize that further optimization of the binding of 17α-triazolyl-ferrocene derivatives is possible even with minor modification(s) of the linker part between the triazolyl and ferrocenyl structures of the side chain.

In kinetic experiments, **10** displayed a competitive behavior. It is most likely, that substrate binding cavity is occupied by the 17β-estradiol part of the molecule. Ring A accommodated in the active center of the enzyme and the ring D with the 17α-triazolyl-ferrocene side chain is directed towards the transmembrane domain of the STS. Literature references indicate that C-17 aryl substituents may interact with Phe and other hydrophobic amino acids of the α-helices in this domain (Fournier and Poirier, 1998; Ciobanu et al. 2001; Shah et al. 2016). We may also postulate that the 17α-triazolyl-ferrocene side chain of **10** can have access to these residues, and the enhanced binding affinity of this compound can be explained by similar mechanisms, namely by π-π type interactions towards Phe residues present at the entrance of α-helices, as well as by non-specific hydrophobic interactions directed to suitable residues buried deeper in the tunnel of the α-helices. Competitive inhibition pattern also suggests that the 17α-triazolyl-ferrocene side chain is long and flexible enough to allow 17β-estradiol part to bind into the substrate binding cavity, which means that these types of derivatives may be able to maximize interactions with both binding sites.

16β-triazolyl-ferrocene-17α-hydroxy-5α-androstane (**4**) has a weak inhibitory effect, whereas 5 which bears an ethylacrylate triazolyl-ferrocene substituent on C-16 proved to be a potent inhibitor. It seems that an elongated ferrocenyl side chain is preferred for the binding of C-16 substituted derivatives. Ferrocenyl moiety attached to a longer and flexible linker may reach the binding pocket which otherwise is situated closer and positioned more appropriately for C-17 substituents69, and this might be the reason for a better binding affinity of 5 to the STS.

In the case of the tested two 2β substituted 3α-hydroxy-5α-androstane-17-one compounds, the shorter triazolyl-ferrocene substituent of **1** proved to be advantageous concerning the affinity to the STS. It may be postulated that this triazolyl-ferrocene side chain is capable of interactions with residues of the binding cavity supposed to exist in the proximity of the ligands' position 2 (Reed et al. 2004; Mostafa et al. 2015).

Examples of the potent test compounds (**1**, **5** and **10**) show that various steroidal backbones may provide suitable core for ferrocenyl derivative STS inhibitors. Comparison of the highly potent 17α-triazolyl-ferrocene derivative of 17β-estradiol (**10**) and its 4-ene-3-oxo analogue (**12**), a weak inhibitor, reveal that in other cases the steroidal scaffold has a strong influence on the STS inhibitory potential.

Mechanistic experiments indicate a reversible binding mode for the C-2 substituted triazolyl-ferrocene derivative **1** and an irreversible binding mechanism for the C-16 and C-17 derivatives **5**, **10** and substance **11**. This binding difference approves that different interactions may be involved in the binding of the C-2 triazolyl-ferrocene derivative and the compounds substituted on their ring D. Our inhibitors do not possess arylsulfamate moiety, which is usually the active principle of the irreversible STS inhibitors, neither bear other reactive group capable to form covalent binding to certain enzyme residues. Steroidal ferrocenes and the enzyme, therefore, are likely to establish non-covalent adducts, but the interactions can be so tight in certain cases that the inhibitors will be bound essentially irreversibly.

Biomedical relevance

In cancerous breast tissues, 17β-estradiol can be formed *in situ* from estrone, which is generated also locally either via the aromatase route (aromatization of adrenal androst-4-ene-3,17-dione) or the sulfatase route (desulfation of estrone-3-sulfate). It is considered, that sulfatase pathway predominates, and around 10-fold greater amount of estrone may be produced via the sulfatase route rather than via the aromatase pathway (Hong & Chen, 2011; Numazawa et al. 2006). Inhibition of STS is, therefore, a particularly attractive manner of antiestrogenic pharmacotherapy of the estrogen dependent breast cancer (Rizner, 2016; Potter, 2018). Numerous STS inhibitors were developed in the last decades, but none of them have been introduced into the medical practice till now (Potter, 2018; Rizner, 2016; Thomas & Potter, 2015). There still remains a

considerable demand for a new class of STS inhibitor compounds. Our results on STS inhibitory potential of certain ferrocenyl steroids seem therefore to be particularly promising.
When an irreversible inhibitor is used as medicinal agent, effect can be maintained after its clearance from the system, and a de novo biosynthesis of the enzyme protein is required for the restoration of the original enzyme activity. Advantages of the resulting prolonged pharmacodynamics include lower level and frequency of dosing, as well as suppressed extent of side effects. EMATE-based irreversible inhibitors, however, have the drawback that the STS inactivation process releases steroidal compounds bearing estrogenic hormonal effect (Reed et al. 2004). Binding of steroidal ferrocenes does not transform the inhibitor molecule, and the irreversible inhibition without generating hormonally active agents makes steroidal ferrocenes superior to EMATE derivatives in a presumed pharmacological application.
Various steroidal ferrocene compounds bear cytotoxic effects which evolve hormone independent antiproliferative effects in cancerous cells (Hillard et al. 2010; Manosroi et al. 2010; Top et al. 2009; Vera et al. 2011; Vessières et al. 2006). Recently tested derivatives and their analogues may also bear such a cytotoxic potential. A hypothesized synergism between the hormone-independent cytotoxicity and the suppressed estrogen dependent cell proliferation evolved by the inhibited STS activity might be particularly advantageous in the treatment of breast cancer.

Conclusion for Future Biology (289 words)

We investigated *in vitro* inhibitory effects of ten diverse steroidal ferrocene substances exerted on key enzymes of the estrogen biosynthesis. Numerous structural elements of these derivatives resemble to that of known potent aromatase and 17β-HSD1 inhibitors, nevertheless, our test compounds were found weakly effective against these enzymes. We observed a potent inhibition against STS by three triazolyl-ferrocene derivatives which display stronger affinities to the enzyme than the substrate estrone-3-sulfate itself. Compound with triazolyl-ferrocene substituent on C-2 is bound in a reversible manner, whereas the C-16 and C-17 derivatives are irreversible inhibitors. Related non-ferrocenyl compounds are found to exert lower potency, indicating that the ferrocene moiety may have a specific function in the enhanced affinity to the STS. Based on literature references, interactions of the 16β- and 17α-triazolyl-ferrocenes with Phe residues and with other hydrophobic amino acids of the transmembrane helices can be proposed. Comparison of adequate counterparts revealed a profound influence of the steroidal backbone and of the linker part in the ferrocenyl side chains on the STS inhibition. Our most potent STS inhibitor, the 17α-triazolyl-ferrocene derivative of 17β-estradiol (**10**) presents submicromolar IC_{50} and K_i parameters, and therefore, this compound belongs to the group of the most effective STS inhibitors published so far. Our results indicate that optimized combination of structural elements (steroidal core, ferrocenyl side chain, position of the substitution) may further enhance STS inhibitory potential of the steroidal ferrocenes. STS inhibitory potential of the steroidal ferrocenes may lead to the development of novel compounds able to suppress in situ 17β-estradiol production in target tissues. A presumed synergism between the hormone-independent cytotoxicity and the suppressed estrogen dependent cell proliferation can make this type of compounds particularly promising drug candidates for the pharmacological therapy of hormone dependent breast cancer.

Ethical Statement
Human placenta specimens were applied with the permission of the Human Investigation Review Board of the University of Szeged no. 3262.

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Data Accessibility
The data supporting Materials and Methods section of this article have been uploaded as part of the Supplementary Material.

Competing Interests
The authors declare no competing interests.

Authors' Contributions
Conceptualisation and design: B. E. H. and M. Sz.; acquisition and analysis of data: B. E. H., J. G., Cs. T., E. Sz-P., K. F., R. S-F., and M. Sz.; interpretation of the results: B. E. H. and M. Sz.; drafting and revising the article: B. E. H., J. J., E. Sz-P., K. F.; R. S-F., and M. Sz.

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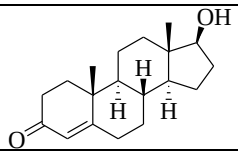
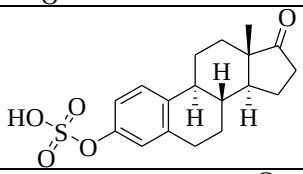
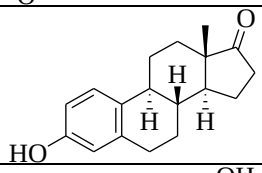
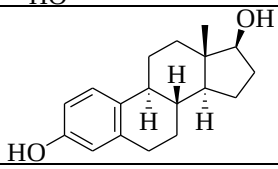
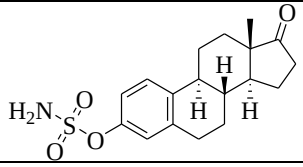
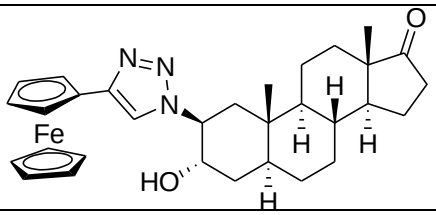
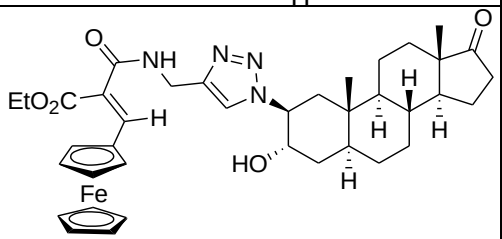
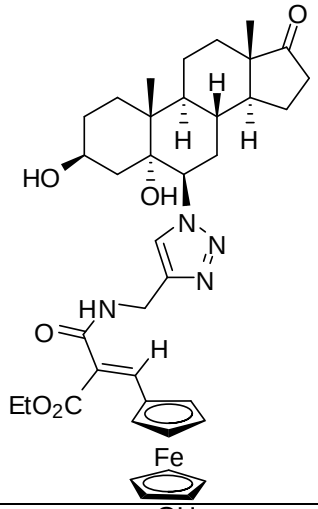
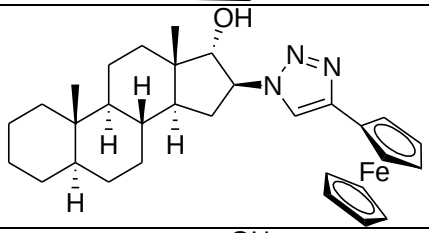
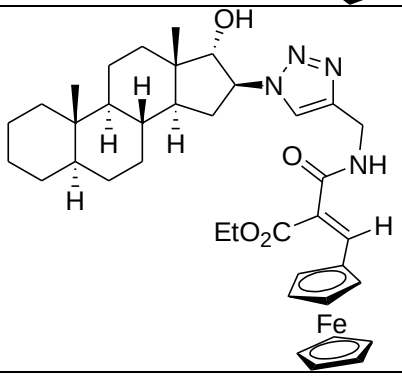
Tables

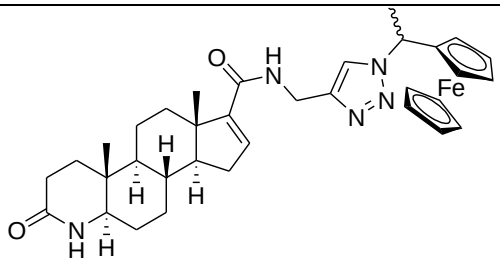
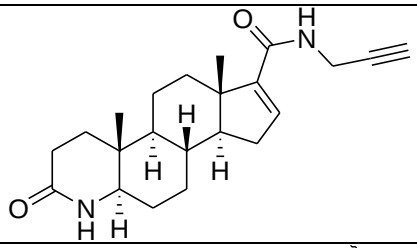
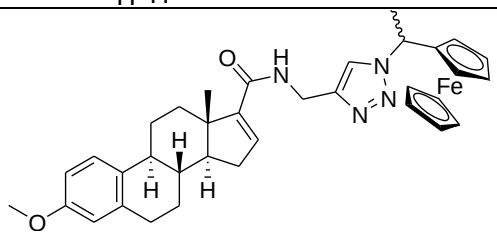
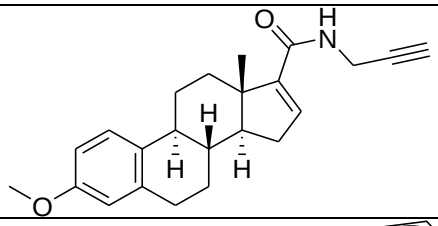
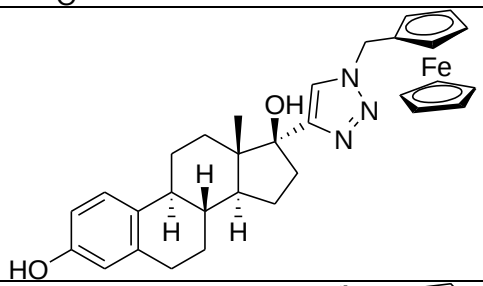
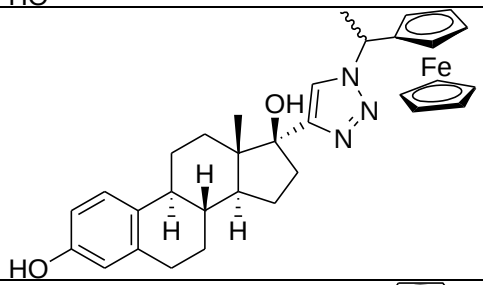
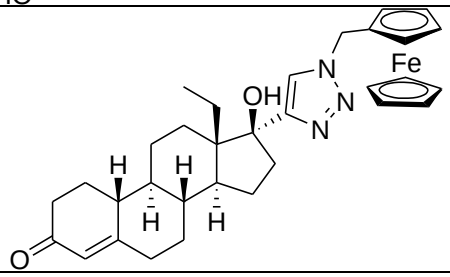
Table 1. Inhibition and kinetic parameters of the enzyme activities determined for the substrate and reference compounds.

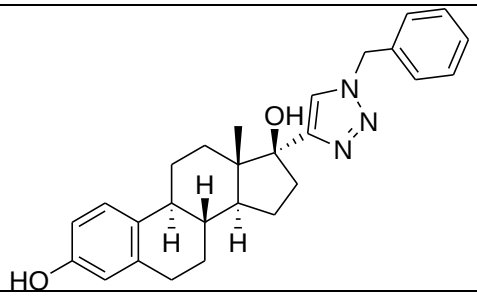
Compound	Structure	Inhibition and kinetic parameters of enzyme activities IC_{50}±SD (μM), K_i±SD (μM) or K_M±SD (μM)		
		Aromatase	Steroid sulfatase	17β-HSD1
Testosterone		IC_{50}= 0.52±0.14		
Estrone-3-sulfate			IC_{50}= 5.2±1.2 K_M= 8.5±2.9	
Estrone				IC_{50}= 0.63±0.11
17β-Estradiol			IC_{50}= 9.0±4.0	
EMATE			IC_{50}= 0.0098±0.0038 K_i= 0.0044±0.0005	

IC_{50}: concentration which decreases the enzyme activity to 50%.
K_M: The Michaelis constant (K_M) of substrates was measured using increasing concentration of the unlabelled substrate.
K_i: Inhibitor constant determined from Dixon plot.
SD: standard deviation.

Table 2. *In vitro* inhibition of enzyme activities by the test compounds.
Relative conversions (Rel. conv., control incubation with no inhibition is 100%) measured in the presence of 10 μM concentration of the compound tested. Mean±SD, n=3.

No.	Structure	Inhibition of enzyme activities Rel.conv.±SD (%), IC_{50}±SD (μM) and K_i±SD (μM)		
		Aromatase	Steroid sulfatase	17β-HSD1
1		100±11	41±5 IC_{50}= 4.6±1.5	81±8
2		78±7	90±9	93±2
3		101±10	97±10	89±4
4		93±6	85±6	94±7
5		89±2	42±3 IC_{50}= 2.4±1.1	91±10

6		82±7	87±4	65±13
7		83±4	93±6	101±8
8		82±5	68±5	85±5
9		94±3	75±8	91±6
10		89±8	8±2 IC_{50}= 0.084±0.043 K_i= 0.066±0.009	98±10
11		117±15	1.0±0.5 IC_{50}= 0.035±0.006 K_i= 0.021±0.005	76±6
12		93±5	77±7	89±9

13		90±5	17±5 IC_{50}= 1.0±0.60	100±7

IC_{50}: the inhibitor concentration which decreases the enzyme activity to 50%.
K_i: Inhibitor constant determined from Dixon plot.
SD: standard deviation.

Figures

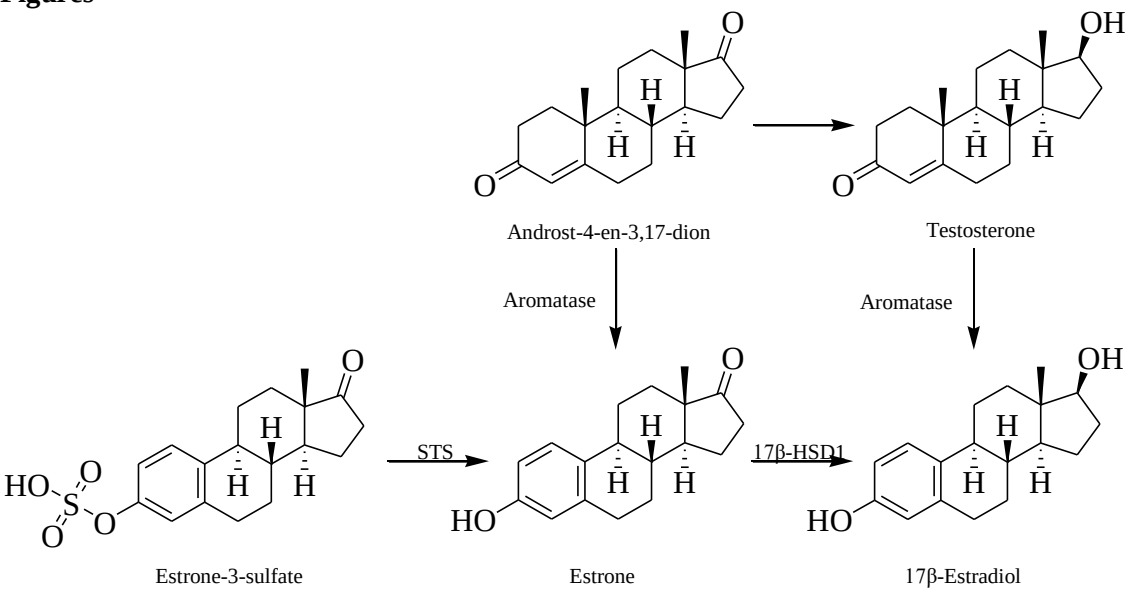


Fig. 1. Key enzymes of the estrogen biosynthesis

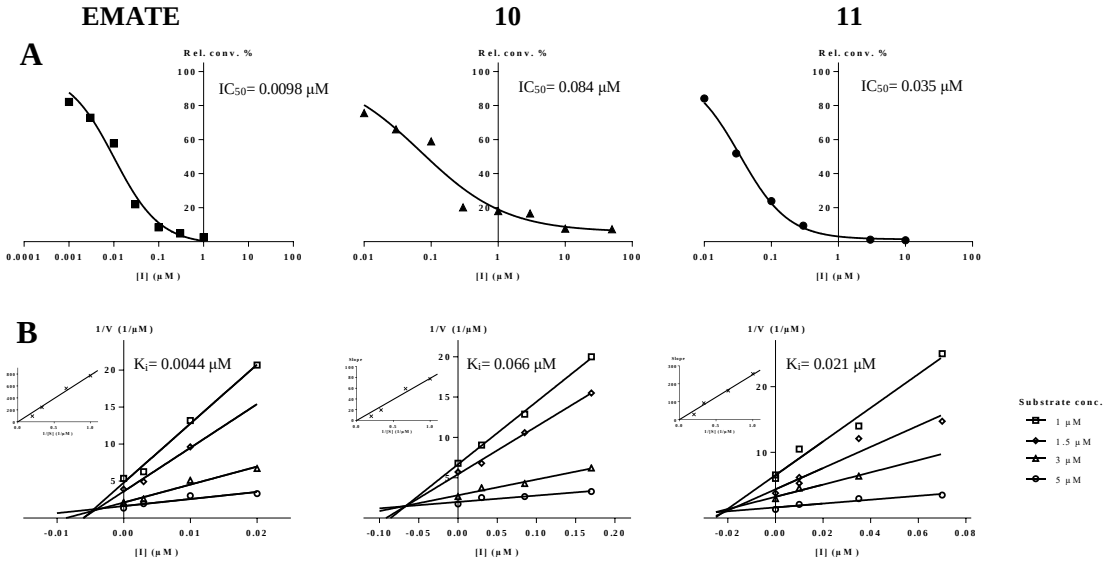


Fig. 2. Concentration dependent STS inhibition (A) and Dixon's kinetic analysis (B) of selected reference EMATE and compounds **10** and **11**. Insets in B show the secondary plot of slopes of the Dixon's lines vs. 1/substrate concentration.

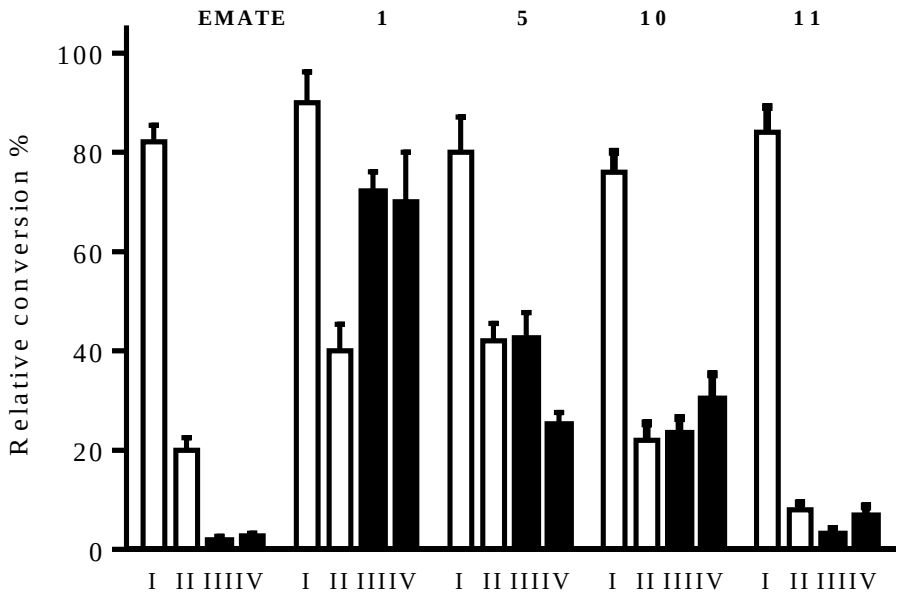


Fig. 3. Investigation of STS inhibition reversibility of selected compounds. Inhibitor compounds were preincubated with human placental microsomes. Following a 50-fold dilution step, the usual enzyme activity measurement was applied.

Mean±SD of three separate experiments
Experimental conditions:

I No preincubation, EMATE 0.001 μM; **1**, **5** 0.2 μM; **10**, **11** 0.01 μM
II No preincubation, EMATE 0.05 μM; **1**, **5** 10 μM; **10**, **11** 0.5 μM
III Preincubation, 2.5 min, EMATE 0.05 μM; **1**, **5** 10 μM; **10**, **11** 0.5 μM, secondary incubation 20 min
IV Preincubation, 20 min, EMATE 0.05 μM; **1**, **5** 10 μM; **10**, **11** 0.5 μM, secondary incubation 20 min

Figure and table captions

Figure 1. Key enzymes of the estrogen biosynthesis

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Table 1. Inhibition and kinetic parameters of the enzyme activities determined for the substrate and reference compounds.

Table 2. *In vitro* inhibition of enzyme activities by the test compounds. Relative conversions (Rel. conv., control incubation with no inhibition is 100%) measured in the presence of 10 μM concentration of the compound tested. Mean±SD, n=3.