Development of a New Class of Potent and Selective Non-Steroidal Inhibitors of 17β-Hydroxysteroid Dehydrogenase Type 1

Bis(hydroxyphenyl)substituted Azoles, Thiophenes, Benzenes and Aza-Benzenes

Dissertation

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Papers included in this thesis

The present thesis is divided into four publications which are referred to in the text by their Roman numerals:

I. **Design, Synthesis and Biological Evaluation of Bis(hydroxyphenyl)azoles as Potent and Selective Non-Steroidal Inhibitors of 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1) for the Treatment of Estrogen-Dependent Diseases**

Emmanuel Bey, Sandrine Marchais-Oberwinkler, Patricia Kruchten, Martin Frotscher, Ruth Werth, Alexander Oster, Oztekin Algül, Alexander Neugebauer and Rolf W. Hartmann

Bioorganic Medicinal Chemistry, *16*, 6423-6435 (**2008**).

II. **The Role of the Heterocycle in Bis(hydroxyphenyl)triazoles for Inhibition of 17β-Hydroxysteroid Dehydrogenase (17β-HSD) Type 1 and Type 2**

Yaseen A. Al-Soud, Emmanuel Bey, Alexander Oster, Sandrine Marchais-Oberwinkler, Ruth Werth, Patricia Kruchten, Martin Frotscher and Rolf W. Hartmann

Mo*lecular and Cellular Endocrinology,* (**2008**), doi:10.1016/j.mce.2008.09.012.

III. **Design, Synthesis, Biological Evaluation and Pharmacokinetics of Bis(hydroxyphenyl)substituted Azoles, Thiophenes, Benzenes and Aza-Benzenes as Potent and Selective Non-Steroidal Inhibitors of 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1)**

Emmanuel Bey, Sandrine Marchais-Oberwinkler, Ruth Werth, Matthias Negri, Yaseen A. Al-Soud, Patricia Kruchten, Alexander Oster, Martin Frotscher, Barbara Birk and Rolf W. Hartmann

Journal of Medicinal Chemistry, 51, 6725-6739 (**2008**).

IV. **New Insights into the SAR and Binding Modes of Bis(hydroxyphenyl)thiophenes and Benzenes: Influence of Additional Substituents on 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1) Inhibitory Activity and Selectivity**

Emmanuel Bey, Sandrine Marchais-Oberwinkler, Matthias Negri, Patricia Kruchten, Alexander Oster, Ruth Werth, Martin Frotscher, Barbara Birk and Rolf W. Hartmann

Journal of Medicinal Chemistry, (**2008**), manuscript submitted.

Contribution report

The author wishes to clarify his contributions to the papers **I**–**IV** in the thesis.

I. Significantly contributed to the synthetical concept. Synthesized and characterized most of the new compounds. Compounds **7** and **8** were prepared by Alexander Oster as a part of a PhD thesis. Compounds **10** and **15** were synthesized by Dr. Oztekin Algül. Significantly contributed to the interpretation of the results. Significantly contributed to writing of the manuscript.

II. Contributed to the inhibitor design concept. Significantly contributed to the interpretation of the results. Significantly contributed to writing of the manuscript.

III. Significantly contributed to the inhibitor design concept. Planned, synthesized and characterized all new compounds except for compounds **12** to **15** (synthesized by Dr. Yaseen A. Al-Soud) and compounds **16** and **24** (synthesized by Alexander Oster as a part of a PhD thesis). Significantly contributed to the interpretation of the results. Significantly contributed to writing of the manuscript.

IV. Significantly contributed to the inhibitor design concept. Planned, synthesized and characterized all new compounds except for compounds **35**, **39**, **41** and **42** synthesized by Alexander Oster as a part of a PhD thesis. Significantly contributed to the interpretation of the results. Significantly contributed to writing of the manuscript.

Abbreviations

17β-HSD1	17β-hydroxysteroid dehydrogenase type 1
17β-HSD2	17β-hydroxysteroid dehydrogenase type 2
AcTH	adrenocorticotropic hormone
AKR	aldo-keto reductase
Asn	asparagine
Asp	aspartic acid
AUC	area under the curve
CC	column chromatography
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
CNS	central nervous system
COF	cofactor binding site
DHEA	dehydroepiandrosterone
DME	di-methoxyethane
DMEM	Dulbecco's modified eagle medium
DMF	dimethylformamide
CD_3SOCD_3	deuterated dimethylsulfoxyde
E1	estrone
E2	17β-estradiol
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	ethylene diaminetetraacetate
ER	estrogen receptor
ESP	electrostatic potential
Eq	equivalent
Et	ethyl
FCS	fetal calf serum
Glu	glutamic acid
Gly	glycine
GnRH	gonatropin releasing hormone
His	histidine
HOBt	1-hydroxybenzotriazole
HSD	hydroxysteroid dehydrogenase
HYC	hybrid inhibitor O5'-[9-(3,17β-dihydroxy-1,3,5(10)-estratrien-16β-yl)-nonanoyl]adenosine
MEP	molecular electrostatic potential
Hz	hertz
Leu	leucine
LH	luteinizing hormone
Lys	lysine
Mm	micromolar
Me	methyl
MES	2-(morpholino)ethanesulfonic acid
Met	methionine
MHz	megahertz
mRNA	messenger ribonucleic acid
nM	nanomolar
NADP(H)	nicotinamide adenine dinucleotide phosphate
NAD(H)	nicotinamide adenine dinucleotide

P_{app}	apparent permeability coefficient
PDB-ID	protein data bank identification code
Ph	phenyl
PK	pharmacokinetic
ppm	parts per million
RBA	relative binding affinity
rt	room temperature
SAR	structure activity relationship
SDR	short dehydrogenase reductase
SEM	standard error of the mean
semi-QMAR	semi-quantitative MEP-activity relationship
SER	serine
SERM	selective estrogen receptor modulator
SRE	steroid response element
SUB	substrate binding site
THF	tetrahydrofurane
TE	Tris-EDTA
Thr	threonine
Tyr	tyrosine
Å	Ångström

Abstract

A new class of non-steroidal 17β-HSD1 inhibitors was designed and synthesized as potential therapeutics for the treatment of estrogen-dependent pathologies like breast cancer and endometriosis. Using the combination of a ligand- and a structure-based approach, a pharmacophore model was generated and a tricyclic core structure bearing two hydroxyphenyl moieties connected to an additional aromatic ring was derived. At a first stage, the scaffold of these steroidomimetics was optimized varying two different parameters: the OH-OH substitution pattern and the nature of the central aromatic system. At a second stage, supplementary substituents were added to the most active core to enhance activity and selectivity. Following this strategy, 120 new compounds were prepared, most of them being highly potent 17β-HSD1 inhibitors with IC_{50} values in the low nanomolar range. Besides an excellent selectivity over 17β-HSD2 and the estrogen receptors (ERs) α and β, the most promising compounds of this study showed also no estrogenicity, good cell permeability (T-47D), good metabolic stability in rat liver microsomes, high CaCo-2 permeability, moderate inhibition of CYP3A4 and CYP2D6 and excellent plasma-levels after peroral application to rats. In conclusion, the present thesis provides an extensive structure-activity study regarding 17β-HSD1 inhibition which can be used in the future development of new molecules to further investigate *in vivo* the concept 17β-HSD1 as a target for the treatment of estrogen-dependent diseases.

Zusammenfassung

Eine neue Klasse von nicht-steroidalen 17β-HSD1 Hemmstoffen wurde zunächst rational am PC entwickelt. Anschließend wurden die entsprechenden Verbindungen als potentielle Wirkstoffe zur Behandlung von estrogen-abhängigen Erkrankungen synthetisiert. Durch die Kombination eines Ligand- und Struktur-basierten Ansatzes wurde ein Pharmakophormodell erstellt und ein trizyklisches Grundgerüst abgeleitet. Die Leitstruktur besteht aus zwei Hydroxyphenylresten, die über einen zusätzlichen aromatischen Kern verknüpft sind. In einem ersten Schritt wurde die Struktur dieser Steroidomimetika durch die Variation zweier Parameter optimiert: das OH-OH Substitutionsmuster und die Art des zentralen Aromaten. Ausgehend von der aktivsten Verbindung wurden zusätzliche Substituenten eingefügt um Aktivität und Selektivität zu steigern. Im Verlauf dieses Projektes wurden 120 neue Moleküle hergestellt und deren biologische Aktivität untersucht. Die meisten der hier vorgestellten Substanzen sind hochpotente 17β-HSD1 Inhibitoren mit IC_{50}-Werten im nanomolaren Bereich. Außerdem verfügen diese Hemmstoffe über eine sehr gute Selektivität gegenüber 17β-HSD2 sowie den Estrogen-Rezeptoren α und β und zeigen selbst keine Estrogenizität. Weiterhin wurde eine hohe Zellgängigkeit (T-47D), eine gute metabolische Stabilität in Rattenlebermikrosomen, hohe CaCo-2 Permeabilität, nur eine moderate Hemmung der hepatischen CYP Enzyme 3A4 und 2D6 sowie hervorragende Plasmaspiegel nach peroraler Gabe an Ratten nachgewiesen. Zusammenfassend lässt sich feststellen, daß die vorliegende Arbeit einen tiefen Einblick in die Struktur-Wirkungsbeziehung eine neuer Klasse von 17β-HSD1 Hemmstoffe liefert, die eine Grundlage für die Entwicklung weiterer Inhibitoren zur *in-vivo* Validierung des Konzeptes 17β-HSD1 als Target für die Therapie estrogen-abhängiger Krankheiten darstellt.

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1. Introduction

1.1 Sex steroid hormones

1.1.1 General features

Natural sex steroids are produced by the gonads (ovaries or testes), by adrenal glands, or by conversion from other sex steroids in peripheral tissues such as liver or fatty tissues.[1, 2] These hormones are then released into the blood circulation and transported to the target organs. After passive diffusion of the cell membrane, they bind with a receptor protein, which then binds to the hormone response element on the nuclear DNA, activating or suppressing specific sequences in the regulatory regions of the gene responsive element that control cell growth and differentiation (Figure 1).

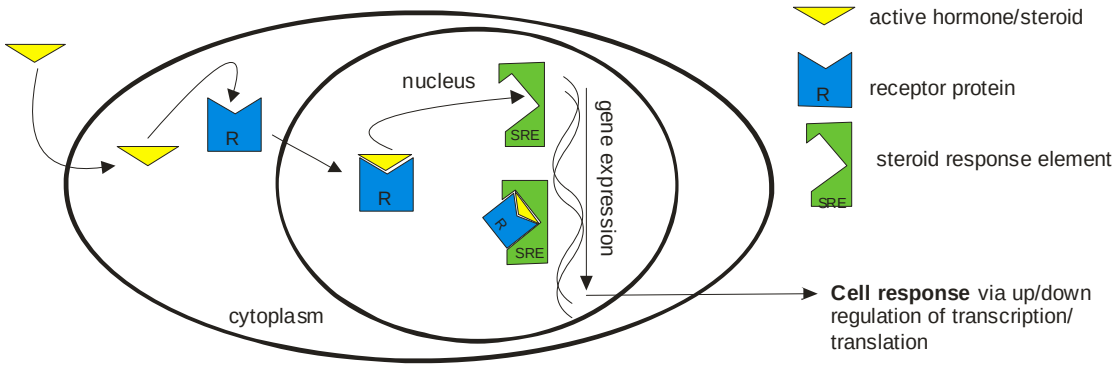


Figure 1: schematic representation of the hormone mechanism of action

Sex steroids play a crucial role in the induction of body changes known as primary and secondary sex characteristics. They are divided in two main classes: androgens and estrogens, of which the most important and active human derivatives are testosterone and estradiol (E2), respectively. In general, androgens are considered as "male sex hormones", since they have masculinizing effects, while estrogens are considered as "female sex hormones".

The strength of these hormones is modulated in the target cell with the aid of different enzymes. This implicate that besides to the classical way of action (endocrine), where the active hormones are secreted in specific glands and transported to distant cells to exert their action, it exists an another mechanism (intracrine),[3, 4] in which the active steroid is directly synthesized in the target cell without release into the pericellular compartment (Figure 2).

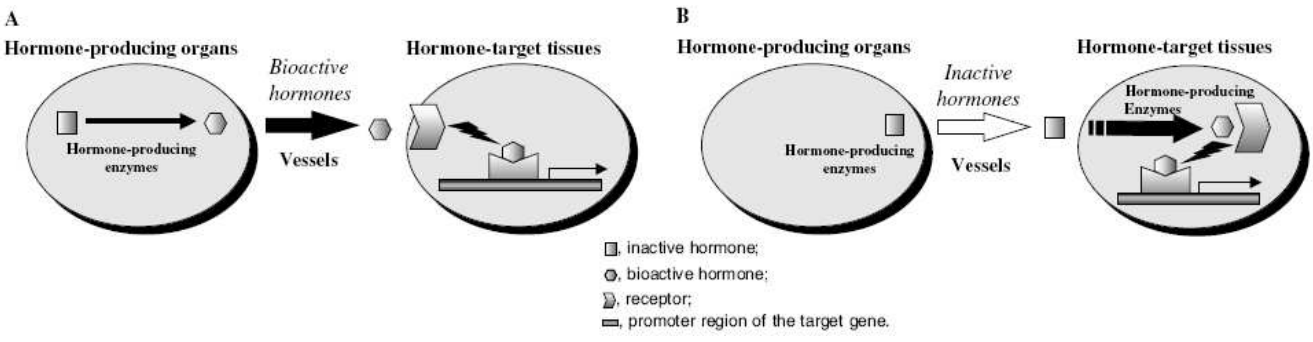


Figure 2: schematic representation of endocrine (A) and intracrine (B) mechanism (taken from[5])

1.1.2 Estrogens

The classical role of estrogens in female reproduction and development is well defined. In the ovary, E2 stimulates the proliferation of granulosa cells and the growth of follicles.[6, 7] In the uterus, E2 in combination with progesterone regulates the cyclic growth of the endometrium.[8] In addition, estrogens together with progesterone are also crucial for the maintenance of pregnancy and fetal development.[9, 10] Moreover, to their roles in reproduction and female development, estrogens affect a large number of other physiological systems: in the skeleton, for example, estrogens are important for maintaining bone mass in adult women's by suppressing bone remodelling and keeping a balance between the osteoblasts and the osteoclasts.[11] Moreover, a large number of studies have pointed out the influence of estrogens on the cardiovascular system,[12] central nervous system[13, 14] and immune system.[15]

Estrogens mainly exist in three molecular forms: estrone (E1), E2 and estriol (E3). Since the biological activity of E2 is much higher than that of the other types, E2 is physiologically the most important estrogen. The primary source of estrogen in premenopausal women is the ovary, but circulating E1 and androgens originating from the adrenal glands could also be converted into E2 in peripheral tissues[16] (Figure 3).

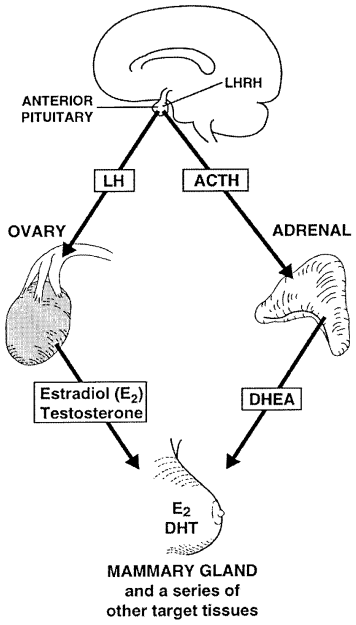


Figure 3: schematic representation of gonadal and adrenal sources of sex steroids in premenopausal women (taken from[17])

However, after menopause, estrogens are produced only through conversion of androgens by adrenal origins. It has been shown that the conversion of androgen to E1 occur principally in peripheral tissues, including skin, muscle, fat and bone.[18] This conversion is catalyzed by the aromatase enzyme. E1 is subsequently reduced to E2 by members of the 17β-hydroxysteroid dehydrogenase family (17β-HSD), which are also widely distributed in various peripheral tissues[19] (Figure 4).

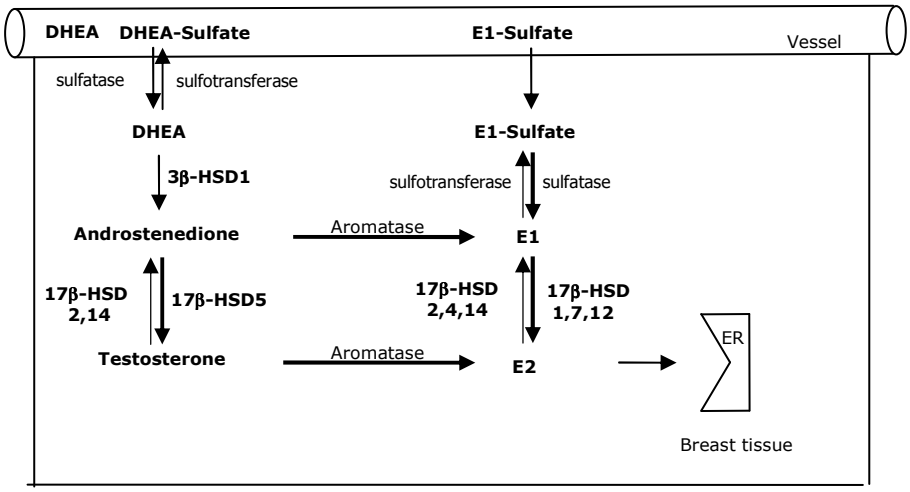


Figure 4: schematic representation of the local production of sex steroids in breast tissues

1.2 Hydroxysteroid dehydrogenases (HSDs)

The hydroxysteroid dehydrogenases (HSDs) belong to a group of pyridine nucleotide-dependent enzymes which catalyze stereoselective reactions at specific positions of the steroid backbone.[20] In fact, for each sex hormone an isoform pair is responsible for either inactivation or provision of an active ligand. This is achieved either by the oxidation of the alcohol group (oxidase activity) or by the reduction of the keto function (reductase activity, Figure 5) on the steroid skeleton. These reductase/oxidase activities allow, therefore, the HSDs to function as a molecular switch.

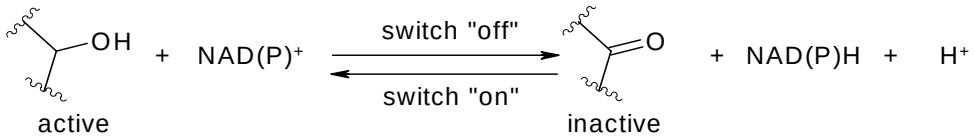


Figure 5: general reaction catalyzed by hydroxysteroid dehydrogenases

Aiming the structural characteristics, the HSD family is split into 2 superfamilies: the SDR (Short-Chain Dehydrogenase/Reductase) and the AKR (Aldo-Keto Reductase). The SDR family members often function as multimers and share a low sequence identity (less than 25%). Despite to this relatively low homology, SDR family members share identical protein folds.[21] A part of the protein fold includes an arrangement of α-helix and β-strands $(\beta\text{-}\alpha\text{-}\beta\text{-}\alpha\text{-}\beta)_2$ to produce the "Rossmann fold" motif for cofactor binding. They also contain a conserved catalytic motif of Tyr-X-X-X-Lys where Tyr is the catalytic general acid/base[22] of the reaction.

The AKRs are monomeric, soluble enzymes utilising NAD(P)(H) as cofactor. They share a high amino sequence identity (> 67%) compared to the SDR subfamily. Invariant to their active site is a catalytic tetrad consisting of Asp, Tyr, Lys and His motif. Site-directed mutagenesis supports the crucial role for the amino acids Tyr and Lys in the catalytic mechanism[23] in a manner similar to that described for the SDR family but the disposition of these residues on the enzyme structure is different compared to the SDRs.

1.3 17β-HSDs

1.3.1 General features

17β-Hydroxysteroid dehydrogenase (17β-HSD) are a group of enzymes responsible for the stereospecific oxido-reduction reaction of hydroxy or carbonyl groups at position 17 of the steroid backbone using NAD(P)H or $NAD(P)^+$ as cofactor.[23-25] They are expressed in the gonads as well as in peripheral tissues, where they serve as key enzymes in the regulation of potent androgens and estrogens.[17, 24] Until now, 15 mammalian 17β-HSDs have been described, 12 of them exist in humans.[19] They all belong the SDR superfamily except 17β-HSD type 5, which is an AKR.[19] The nomenclature of 17β-HSD enzymes follows the chronological order of description.[26] They have relatively low sequence identity (25-30 %) and differ in catalytic direction, substrate and cofactor specificity, subcellular localisation and tissue distribution.[27]

1.3.2 Importance of the 2'-phosphate group of the cofactor for the determination of the catalytic reaction

17β-HSDs drive unidirectional reactions in intact cells while in cell homogenates, it has been shown that these enzymes are able to catalyze either the oxidation or the reduction depending on which cofactor form (reducent or oxidant) is present.

In cells, NADPH is a major source of electrons, and reducing equivalents from NADPH are used in different metabolic reactions. In contrast NAD+ is a versatile electron acceptor, and most enzymatic oxidations deposit these electrons on NAD+. To maintain constant this cofactor abundance, the cell constantly recycles these compounds from other oxidation states. As shown in Figure 6, the cellular concentration of NADPH is higher (500 times) than the $NADP^+$ concentration.[28] In contrast, cells maintain the opposite concentration for the non phosphorylated NAD: NAD^+ is generally 700 times more abundant than NADH.[29, 30]

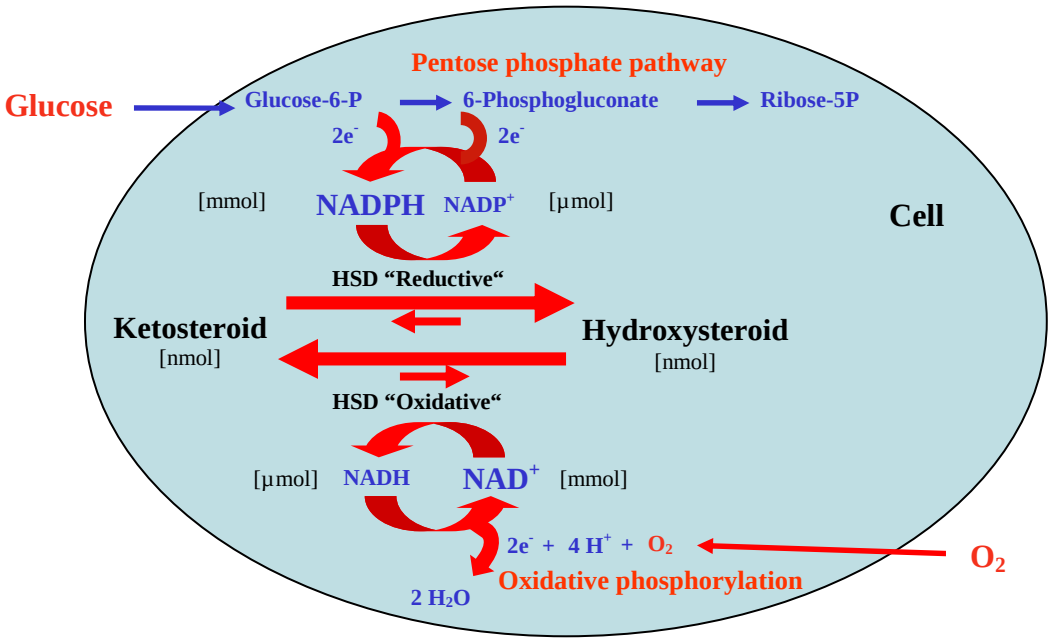


Figure 6: interplay between metabolism and HSD reactions

Besides these cofactor concentration differences observed in intact cells, kinetic studies showed that the 17β-HSDs exhibit huge K_M value differences between NADP(H) and NAD(H). This implicates that it exists in the cofactor binding sites of each 17β-HSD isoform amino acids which allow the enzyme to distinguish the non-phosphorylated from the phosphorylated cofactor form.[2, 31]

Crystal structure analysis of reductive HSD enzymes, such as 17β-HSD1, show that an arginine residue in the N-terminal region of the Rossmann fold is responsible for this discrimination.[32-35] The positively charged guanidinium group of the arginine forms a salt bridge with 2'-phosphate moiety of NADPH, which further enhance the affinity of these enzyme for NADPH (Figure 7).

In contrast, oxidative SDRs like 17β-HSD2, contain a negatively charged amino acid at the position corresponding to the stabilizing arginine of the reductive enzymes: the carboxylate groups of aspartate and glutamate induce repulsive interactions with the 2'-phosphate group of NADP+ but might be able to form hydrogen bonds with the 2'-OH function of NAD+ (Figure 7) enhancing therefore the affinity for the non-phosphorylated cofactor form.

Furthermore, mutagenesis experiments on these enzymes demonstrated that exchanging this arginine with an aspartate moiety in the case of a reductive HSD enzyme increases the affinity for NADH (vs. NADPH) while exchanging this aspartate with arginine moiety in the case of an oxidative HSD enzyme enhances the affinity for NADP+ (vs. NAD+).[36-38]

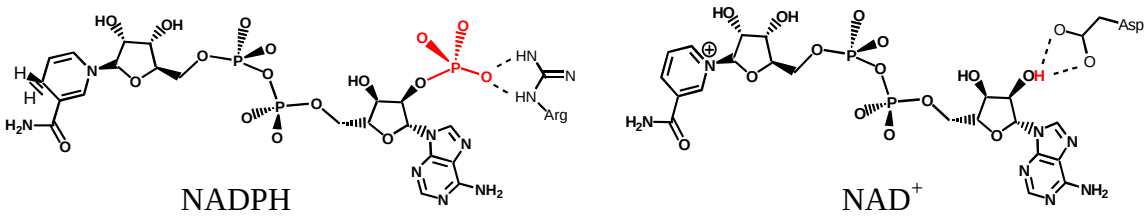


Figure 7: binding preferences of NADPH and NAD^+ for reductive/oxidative HSDs

1.3.3 Oxidizing / NAD^+ binding enzymes

As it is presented in chart 1, oxidizing 17β-HSDs are widespread distributed in the body and not especially only in steroidogenic tissues but also in case of 17β-HSD4 in the lung or in central nervous system for 17β-HSD10. As they catalyze a oxidation reaction, they lower the concentration of active sex hormone in the target tissues and therefore play a protective role.[19]

enzyme	localisation	substrates	physiological role
17β-HSD2	microsomes breast, endometrium, ovary, placenta	androgens estrogens	protective: decrease of active sex hormones in target tissues
17β-HSD4	peroxisomes breast, liver, lung	estrogens	decrease of E_2 level deficiency: Zellweger syndrome
17β-HSD8	microsomes liver, ovary	androgens estrogens	decrease of active hormone in target tissues, may play role in fatty acid metabolism
17β-HSD10	mitochondria central nervous system	androgens estrogens	pathogenesis of Alzheimer diseases

enzyme	localisation	substrates	physiological role
17β-HSD11	microsomes kidney, liver, lung	androgens	decrease of active androgens in target tissue, may play role in fatty acid metabolism
17β-HSD14	cytosol breast, kidney	androgens estrogens	decrease of active androgens in target tissue, may play role in fatty acid metabolism

Chart 1: characteristics of the oxidizing 17β-HSD isoforms

1.3.4 Reducing/NADPH binding enzymes

Until now, six reductive 17β-HSDs enzymes are described in the literature (Chart 2). They are widespread distributed and are responsible for a high level of active sex steroids in target tissues.

Aiming the estrogens, only three subtypes (1, 7 and 12) are involved in the biosynthesis of E2, the most important being 17β-HSD1. The primary physiological role of 17β-HSD7 and 17β-HSD12 is supposed to be in the cholesterol synthesis[39] and in the regulation of the lipid biosynthesis,[40] respectively. Recently, Day et al.[41] showed that 17β-HSD12, although highly expressed in breast cancer cell lines, is inefficient in E2 formation. These results strongly suggest that in case of breast cancer cells, only 17β-HSD1 is responsible for intracellular high E2 level and might therefore a good target for the treatment of hormone dependent breast cancers.

enzyme	localisation	substrates	physiological role
17β-HSD1	cytosol breast, endometrium, ovary, placenta	estrogens in a minor extent androgens	high concentration of active steroids (E_2 and T) in target tissues
17β-HSD3	microsomes testis	androgens	formation of T deficiency: pseudohermaphroditism
17β-HSD5	cytosol breast, liver, prostate	androgens	formation of active androgens responsible for the rising level of androgens at puberty in cases of pseudohermaphroditism
17β-HSD7	membrane breast, liver, placenta	estrogens	formation of active estrogens may play role in cholesterol synthesis, deficiency leads to similar malformation as CHILD syndrome
17β-HSD12	microsomes breast, kidney, liver, placenta	estrogens	formation of active estrogens, regulator of lipid biosynthesis
17β-HSD15	prostate	androgens	pathogenesis of prostate cancer

Chart 2: characteristics of the reducing human 17β-HSD enzymes

1.4 17β-HSD1

1.4.1 Biological characteristics

17β-HSD type 1 (EC 1.1.1.62) was first described in 1958 by Langer & Engel.[42] It is active as a soluble cytosolic homodimer,[43] both subunits have a molecular mass of 34.9 kDa and contain 327 amino acids.[44]

It catalyzes the final step of the E2 biosynthesis. The enzyme has also the ability to reduce some androgens,[45, 46] such as DHEA, into di-hydrotestosterone, but only in a minor extent (K_M(E1)= 0.03 μM vs. K_M(DHEA)= 33 μM).[47] Site directed mutagenesis experiments revealed that the androgen discrimination is due to the steric hindrance of the C19 methyl group of the steroidal β-face with Leu149.[47]

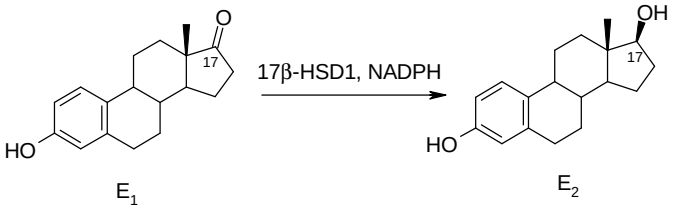


Figure 8: Conversion of E1 into E2 by 17β-HSD1

In vivo, 17β-HSD1 is only a unidirectional reductive enzyme, catalyzing the reduction of the weak active E1 into the most potent estrogen E2 (Figure 8), whereas the purified enzyme or cell homogenates can be driven to catalyze also the oxidative direction depending on the presence of the oxidative cofactor form.[28] The enzyme is widespread expressed, most abundantly in ovaries, placenta, breast tissues and endometrium but also in other peripheral tissues like adipose tissues and skin.

Using site-directed mutagenesis experiments, critical amino acid residues (Ser142, Tyr155, His221) for the enzyme activity and substrate binding were identified.[38, 48, 49]

1.4.2 Structural characteristics

The first X-ray structure of 17β-HSD1 as native form was published in 1995.[50] Since then, several crystal structures of binary or ternary complexes with estrogenic[34, 51, 52], androgenic[47, 53, 54] ligands or with steroid based inhibitors[35, 55] are available at the Protein Data Bank (PDB).[56]

The monomeric form of 17β-HSD1 consists of seven parallel β-strands, forming a β-sheet and 11 α-helices.[50, 51] The β-sheet is surrounded on both sides by three parallel α-helices bearing the classical Rossmann fold, which is associated with NAD(P)H binding (Figure 9).

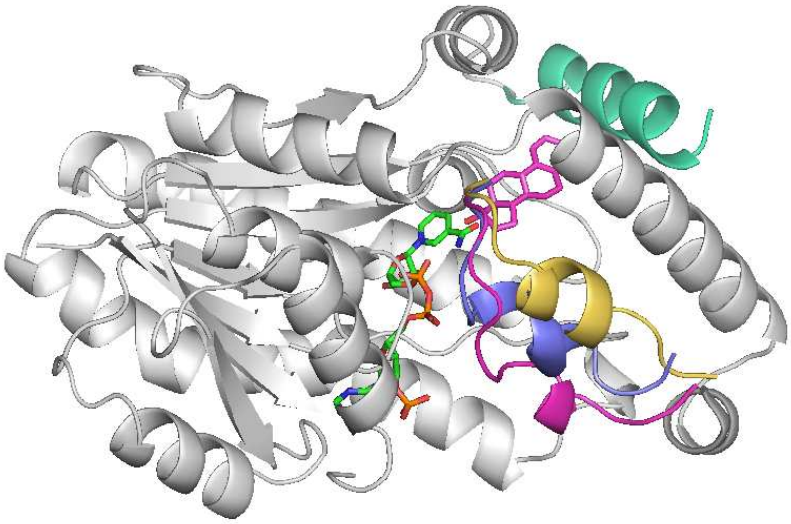


Figure 9: 3D structure of human 17β-HSD1 monomer crystallised with E2 (coloured in pink) and $NADP^+$ resolved to 2.2 Å (PDB-ID: 1FDT)

The analysis of the available crystal structures furnish useful information about the enzyme architecture: a substrate binding site, a cofactor binding pocket and an entry channel can be defined.

The 17β-HSD1 substrate binding cleft is a narrow hydrophobic tunnel showing a high degree of complementarity to the steroid. As a member of the SDR superfamily, 17β-HSD1 contains the highly conserved and catalytically crucial tetrad[57] Tyr-x-x-x-Lys sequence (Asn114, Ser 142, Tyr155 and Lys 159). E2, the product of the catalytic reaction, is stabilized into the active site via four hydrogen bond contacts[51] (OH at C3 position with His221 and Glu282, OH at C17 position with Ser142 and Tyr155), as well as via several hydrophobic interactions with the apolar amino acids of the binding cavity (Val143, Leu149, Pro187, Val225, Phe226, Phe259). Interestingly, two polar amino acids (Tyr218 and Ser222) are located in the same area without any interaction with the steroid (Figure 10).

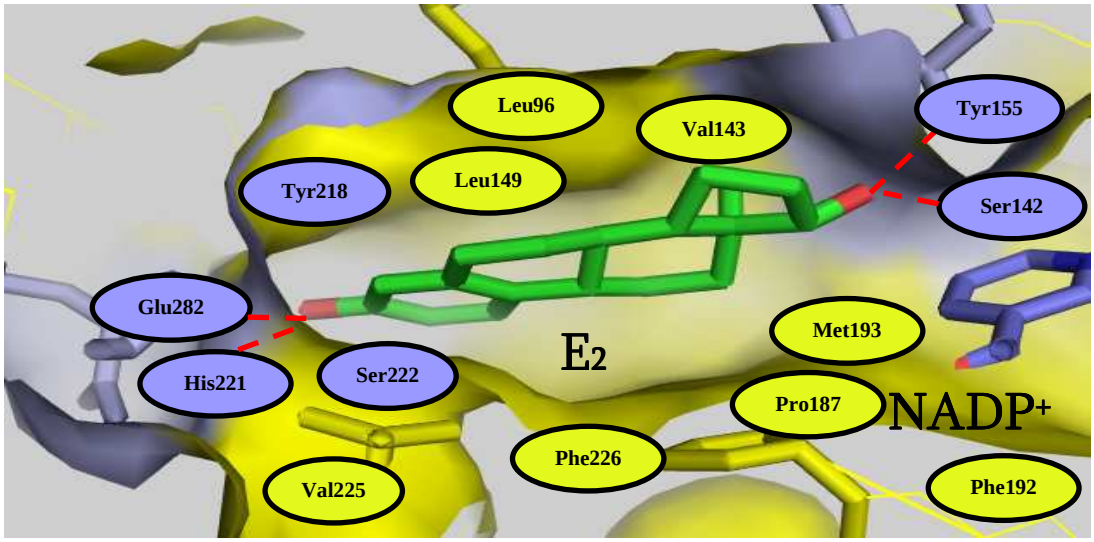


Figure 10: schematic representation of the active site of 17β-HSD1 containing E2 (PDB-code: 1A27). Blue labels denote polar amino acids and yellow labels stand for lipophilic amino acids. Hydrogen bonds are marked in red.

Additionally, a flexible loop delimited in its extend by Pro187 and Pro200 can be visualized (Figure 11). This loop can adopt various conformations or is not resolved at all depending on which X-ray structure is considered. Since the loop borders both substrate and cofactor binding sites, its conformational variation influences strongly the volume and shape of each binding cavity and its location might be of relevance for the design of inhibitors.

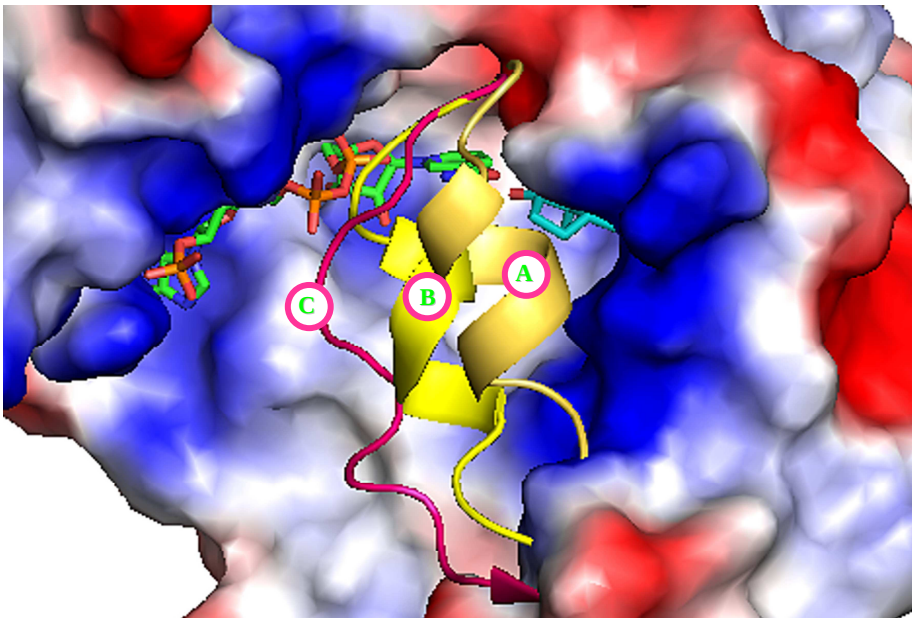


Figure 11: representation of the border loop conformations
(A: PDB: 1IOL, B: PDB: 1FDT_B, C: PDB: 1I5M)

In 1FDT two distinct conformations of the loop are present referred as 1FDT_A and 1FDT_B, respectively. The main difference between the two loops concerns the four amino acids Phe192, Met193, Glu194 and Lys195. In 1FDT_A Phe192 and Met193 are turned towards the outer part of the enzyme, while Glu194 and Lys195 are oriented towards the substrate and cofactor, extending the area of the substrate binding site into the cofactor binding area. On the other hand, in 1FDT_B these two couples of residues show a reversed orientation limiting significantly the length (and so the volume) of the substrate binding pocket.

1.4.3 Postulated mechanism of the hydride transfer

In the postulated mechanism, the *pro*-S hydride of the cofactor nicotineamide moiety is transferred to the α-face of the steroid at the C17 carbon, whereas the keto oxygen at C17 forms a strong hydrogen bond with the OH group of Tyr155. The proton transfer between C17 oxygen and Tyr155 is facilitated by the close neighbourhood of the protonated side chain of Lys159, the 2'-OH of the ribose and a water molecule bound to the backbone carbonyl group of Asn114 which are responsible for the decrease of the apparent pKa of the tyrosine residue allowing the proton transfer (Figure 12).[50, 51]

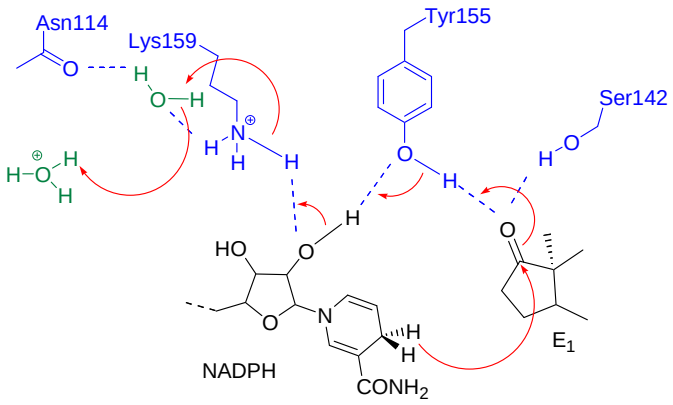


Figure 12: scheme of the postulated mechanism of reduction of E1 into E2. Hydrogen bonds are represented in dashed lines.

1.5 Estrogen-dependent diseases

1.5.1 Breast cancer

Most breast cancers are hormone-dependent, i.e. tumor cells express estrogen receptors (ER), and it is well known that estrogens, especially E2, have a pivotal role in the development and progression of these diseases.[58]

Breast carcinomas can be divided in two categories: estrogen receptor positive (ER+) and estrogen receptor negative (ER-) tumors. Around 50% of breast cancers in premenopausal women and 75% of breast cancers in postmenopausal women are ER+,[59] i.e. the progression of the tumor is dependent on the physiological concentration of estrogens present in the diseased tissue.

In addition to surgery, chemotherapy and radiotherapy, hormone-dependent breast cancers can be treated via different endocrine therapies:[60-62] SERMs (Selective Estrogen Receptor Modulators), like tamoxifene and raloxifene constrain the estrogen action at the receptor level, aromatase inhibitors (e.g. fadrozole, letrozole) suppress the estrogen formation by inhibiting the last step of E1 biosynthesis and GnRH analogues completely block the ovarian steroid formation. (Figure 13).

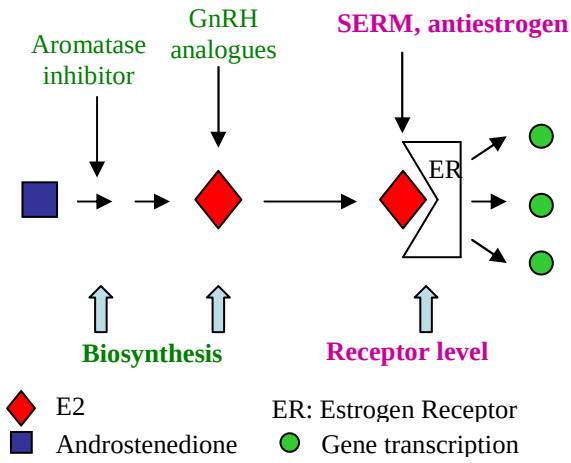


Figure 13: schematic representation of the existing endocrine therapies

These strategies are already used in therapy but they show some limitations. Many breast tumors fail to respond to anti-estrogen therapy or progress after a period of time. SERMs are also known to induce carcinoma in other tissues like endometrium, where tamoxifene acts as an estrogen agonist. Aromatase inhibitors block the formation of estrogens but do not prevent the formation of the non-aromatic estrogen 5-androstene-3β-diol from DHEA which also stimulates the proliferation of cancer cells after binding to the ER. Aromatase inhibitors are restricted to postmenopausal women because in premenopausal women they induce a strong ovarian stimulation by hypothalamic/pituitary feedback. GnRH analogues do not affect adrenal formation of androgens which are converted into estrogens by peripheral aromatase.

1.5.2 Endometriosis

Endometriosis is one of the most common causes of pelvic pain and infertility in women. In this condition endometrial tissue grows abnormally outside the uterus, often in locations such as the ovaries, fallopian tubes and abdominal cavity. It causes adhesions and scarring, pain, heavy bleeding and can damage the reproductive organs leading to infertility. The specific causes of endometriosis are still not clear. The most widely accepted theory is that the disorder originates from retrograde menstruation of endometrial tissue through the fallopian tubes into the peritoneal cavity.[63, 64]

Currently available medical therapies are designed to suppress the estrogen biosynthesis. Oral contraceptive, androgenic agents and GnRH-analogues are used to inhibit the menstruation, a source of much of the pain associated with endometriosis and to restrain the growth of endometriotic tissue.[61] Analgesics are also applied in combination to the classical hormonal therapy in order to relieve the pain triggered by endometriosis. However, the available therapies focused on the symptoms of the disease but do not provide a cure. Due to the alteration of the hormone balance, it leads also to several side effects such as weight gain and acne.[65]

1.6 Is 17β-HSD1 a good target for the treatment of EDD ?

As it has been described in chapter 1.5.1, estrogens have a crucial role in supporting the growth of hormone-dependent breast cancer in women. Although both 17β-HSD1 and 17β-HSD2 are present in healthy women, several studies have indicated that the ratio of 17β-HSD1 to 17β-HSD2 is increased in the tumors of patients with ER+ breast cancer.[66] This results in increased levels of E2 which drive the proliferation of the tumor tissue via activation of the ERs. Additionally, several studies indicate that patients with tumors showing high 17β-HSD1 expression have significantly shortened disease free and overall survival.[67, 68]

Recently, the *in vivo* efficacy of 17β-HSD1 inhibitors to reduce E1 induced tumour growth on two different mouse models[41, 69, 70] has been demonstrated suggesting that the 17β-HSD1 inhibition is a suitable concept for the treatment of estrogen dependent breast cancers which could be applied in both pre- and postmenopausal womens.

In endometriotic tissues, a change in the expression of the steroidogenic enzymes is responsible for a high concentration of E2, which stimulates proliferation of the tissues.[71] A recent study, indicate that there is down regulation of 17β-HSD2 mRNA expression in endometriotic lesions, while both aromatase and 17β-HSD1 activity are up regulated, in comparison to normal tissues.[72, 73] Thus, therapies aiming the inhibition of 17β-HSD1, the enzyme which catalyze the last step of the E2 biosynthesis, would give an innovative approach to the treatment of this disorder.

In conclusion, development of potent, selective and *in vivo* active inhibitors of 17β-HSD1 should be attractive for the treatment of estrogen dependent diseases and should be useful tools to get further insight into the intracrine regulation of estrogen-dependent tissues.

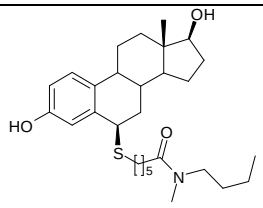
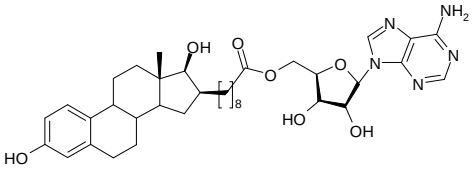
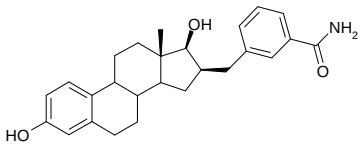
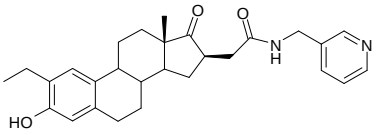
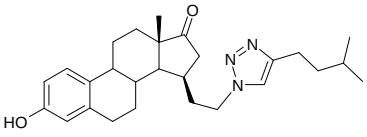
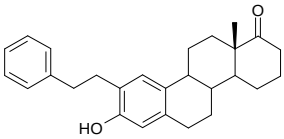
1.7 State of the art on 17β-HSD1 inhibition

During the last decade, the number of patents[74-79] and publications (for reviews see[80, 81]) dealing with 17β-HSD1 inhibitors increased constantly. Most of the known inhibitors are based on steroidal structures, while only a few numbers of non-steroidal scaffolds are described.

An inhibitor of 17β-HSD1 should be selective over 17β-HSD2 and, most importantly, should not trigger unwanted estrogenic effects. Antiestrogenic effects or inhibitory activity against aromatase or E1-sulfatase can be considered as an extra benefit. Furthermore, a good 17β-HSD1 inhibitor should exhibit drug-like properties in order to have sufficient absorption and permeation in biological systems (Lipinski rule of five).[82]

Aiming at steroid based 17β-HSD1 inhibitors large libraries based on E1 and E2 cores with substitution at the C2, C6, C7, C15, C16 positions were synthesized. Table 3 lists representative 17β-HSD1 inhibitors exhibiting high inhibitory activity toward the enzyme. From the biological results, it becomes apparent that the C15 and C16 positions and especially the corresponding β-isomers are suitable for introduction of side chains (Table 3). In order to reduce the estrogenic activity of these compounds, small or flexible hydrophobic groups were introduced in C2 position. Docking studies showed that these types of inhibitors establish interactions with both binding sites of the enzyme: the steroid part being anchored in the substrate binding cleft while the side chain is able to interact with a part of the cofactor.[83]

Table 3: selection of described highly active steroidal 17β-HSD1 inhibitors

structure	IC_{50}(17β-HSD1)
	170 nM
	52 nM
	44 nM
	27 nM
	92% at 1μM
	15 nM

Concerning the non-steroidal cores, less work has been published. As compared with inhibitors based on steroid structures non-steroidal compounds could have advantages such as synthetic accessibility, drug-likeness, selectivity and non-estrogenicity.

Phytoestrogens like coumestrol (**1**) and gossypol (**2**) derivatives are the first fairly active (IC_{50} values in the micromolar range) non-steroidal 17β-HSD1 inhibitors which have been described.[84, 85] Their major drawback is that they suffer from a lack of selectivity, as they are also inhibitors of numerous other enzymes.[85, 86]

Based on the knowledge they had gained in the steroid based inhibitors,[83, 87-89] Sterix Limited synthesized a library of biphenyl ethanone derivatives[90] as steroidomimetic of E1. The most active inhibitor (**3**) shows a moderate inhibition of 17β-HSD1 (IC_{50}= 1.7 μM) and exhibits low selectivity over 17β-HSD2.

More recently, thienopyrimidinones[91, 92] have been developed as inhibitors of 17β-HSD1 by Solvay Pharmaceuticals. The most potent compound of this series (**4**) appears to have an excellent inhibitory activity (IC_{50}= 5 nM) in the cell free assay but its potency is significantly reduced (25 % inhibition at 1 μM)[91] in the cell assay indicating poor bioavailability of these compounds.

In the current year, 1-substituted hydroxyphenyl naphthols[93-95] were found by our group to be highly selective and potent inhibitors of the enzyme. In addition to a high activity (**5**, IC_{50}= 20 nM), good selectivity over 17β-HSD2 and the ERs, the compound shows excellent pharmacokinetic properties in the rat (Figure 14).

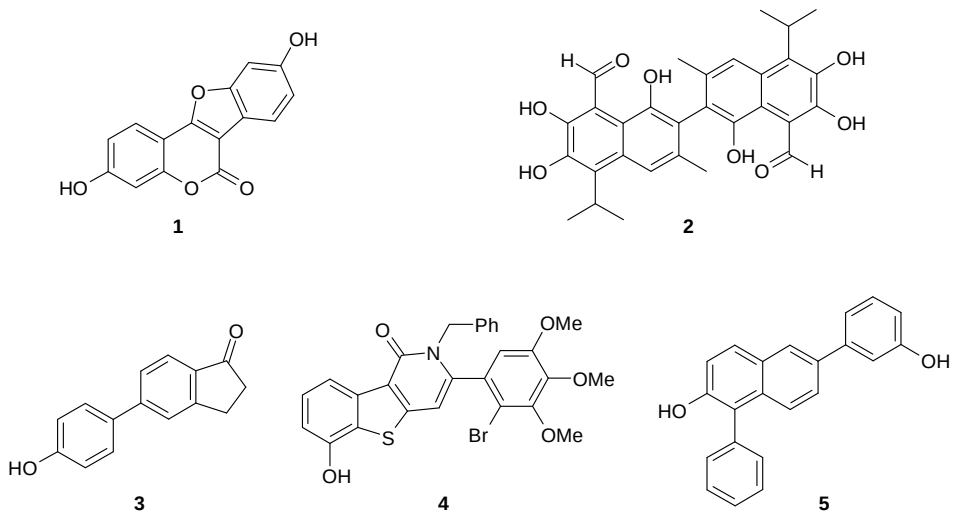


Figure 14: described non-steroidal 17β-HSD1 inhibitors

2. Outline of this thesis

2.1 Scientific goal

Estrogen target cells – i.e. cells expressing ERα and/or β - express to varying degrees 17β-HSD1 and 17β-HSD2. The former one activates E1, the most prevalent estrogen in circulation, upon entering the cell into E2, which stimulates the cell proliferation. 17β-HSD2 inactivates intracellular E2 by oxidizing it to E1 and thus protects the cell from too strong stimulation. As 17β-HSD1 is very often strongly overexpressed in breast cancer or endometriosis, this enzyme is discussed to be a promising target for the treatment of these diseases.

This therapeutical concept might offer a softer approach compared to the existing therapies: fewer side effects can be expected as only the last step of the E2 biosynthesis will be inhibited. Recently the *in vivo* efficacy of 17β-HSD1 inhibitors to reduce E1 stimulated tumour growth on two different mouse models has been demonstrated,[41, 69, 70] highlighting the fact that the inhibition of 17β-HSD1 is a suitable concept for the treatment of estrogen-dependent pathologies.

Accordingly, the aim of the present thesis was to develop highly potent and selective inhibitors of 17β-HSD1 which are applicable *in vivo* in order to provide scientific tools for the elucidation of intracellular estrogen regulation and lead compounds for a novel strategy to treat hormone-dependent diseases.

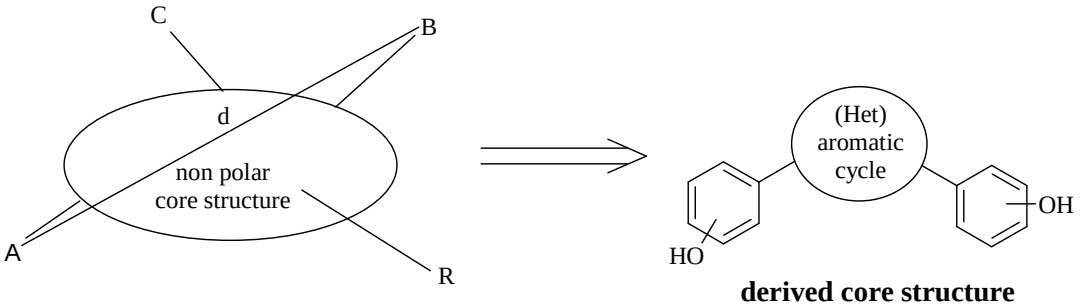
To achieve this goal, novel 17β-HSD1 inhibitors have been designed using a combination of a structure and a ligand based approach. Regarding the therapeutical concept it is important that inhibitors of 17β-HSD1 are selective toward: 1. 17β-HSD2, which inactivates E2 to E1, thus acting contra-productive to the type 1 enzyme; 2. the estrogen receptors α and β to avoid intrinsic estrogenic effects.

2.2 Working strategy

In the design process, it was decided that steroidal structures should be avoided to limit the side effects due to interaction with steroid hormone receptors. However, the compounds should be capable of mimicking E1.

Based on the X-ray structure analysis of the enzyme active site (PDB-ID: 1FDT), our group established a pharmacophore model for non-steroidal inhibitors of 17β-HSD1. It contains two polar points A and B, which could be hydrogen bond donor or acceptor, mimicking the steroid O-3 and O-17 positions, respectively. A distance of about 11 Å, in the same range as observed for the steroid, should be optimum. Another polar group, C, equidistant to A and B could also be considered for interaction as two polar amino acids Ser222 and Tyr218 were identified in the X-ray structure. These three points A, B and C should be connected to a non polar core. Upon identification of the most appropriate core structure, a substituent R could be introduced on the hydrophobic scaffold to increase activity and selectivity.

From the previously established pharmacophore model, a tricyclic core structure was derived. The compounds consist of two hydroxyphenyl or two methoxyphenyl moieties, which could imitate the A-ring (interaction with His221 and Glu282) and the D-ring (Ser142 and Tyr155) of the steroid. To keep a planar shape, similar to E1, the two hydroxyphenyl moieties should be linked via an aromatic ring. Furthermore, different position isomers of the hydroxy groups at the phenyl ring (*para-/meta-, meta-/para- and para-/para-*) should be investigated in order to find out the one which fits best to 17β-HSD1.



A: H-bond donor/acceptor mimicking the 3-O position of the steroid
B: H-bond acceptor mimicking the 17-O position of the steroid
C: polar group => H-bond with Tyr218/Ser222
R: additional substituent
d: distance about 11 Å, like in E1

(Het) aromatic cycle:
5-membered and 6-membered aromatic rings which can contain N, O, S, Se, C, NH

Figure 15: proposed pharmacophore model and derived core structure

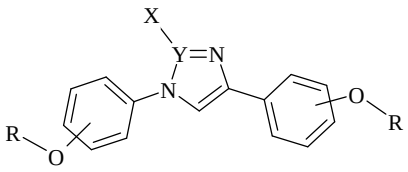
In the first project of the present work (Paper I), different azoles containing nitrogen and/or oxygen were considered as central aromatic rings (the nitrogen and/or the oxygen might be able to establish additional interactions with the amino acids Tyr218 and/or Ser222). Position, number and nature of the heteroatoms in the heterocycle as well as the location of the OH groups on the hydroxyphenyl moieties were varied to determine which "molecular geometry" (combination of heteroatoms and most suitable OH-OH substitution pattern) is in correlation with a high 17β-HSD1 inhibitory activity (Figure 16). The results of these investigations are presented in chapter **3.I**.

To obtain further insight into the impact of an additional nitrogen atom, and on the distribution of the nitrogen atoms in the central ring on inhibitory activity, a small study was performed with bis(hydroxyphenyl) triazoles (Paper II, chapter **3.II**, Figure 16). Furthermore, it was investigated, whether an additional substituent on the heterocycle might be able to increase the potency of the inhibitors.

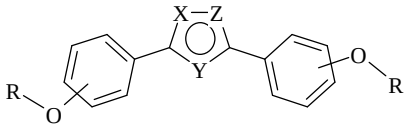
In the third part of this thesis (Paper III, chapter **3.III**), the significance of the OH groups was further evaluated on other five-membered heterocycles, especially sulfur containing ones. Moreover it was evaluated, whether aromatic six-membered rings were also appropriate to connect the two hydroxyphenyl moieties (Figure 16).

From the previous subproject, 2,5-disubstituted thiophenes, thiazoles and 1,4-disubstituted benzenes proved to be highly potent inhibitors of 17β-HSD1 (IC_{50} in the nanomolar range) and therefore especially suitable hits for the introduction of further substituents.

Aiming to increase activity and selectivity of this class of compounds, a series of molecules bearing various substituents either on the central aromatic ring or on the hydroxyphenyl moieties have been synthesized and their biological properties evaluated (Paper IV, chapter **3.IV**, Figure 16).

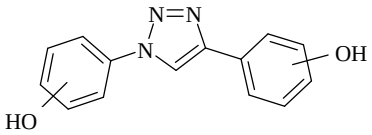


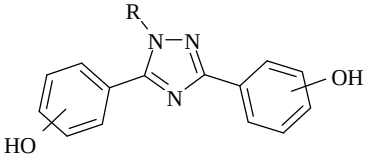
X= SH, H
Y= C, N
R= H, CH_3



X= CH, N, O
Y= C, N, O
Z= CH, NH
R= H, CH_3

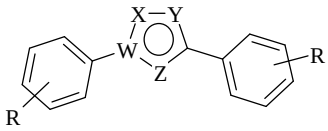
project I



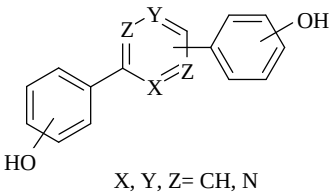


R= Me, Ph

project II

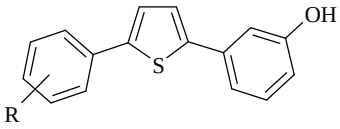


W, X, Y, Z= C, CCH_3, N, S, Se
R= H, OH

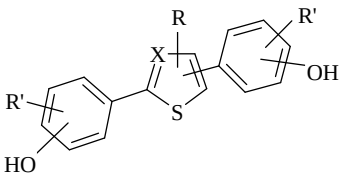


X, Y, Z= CH, N

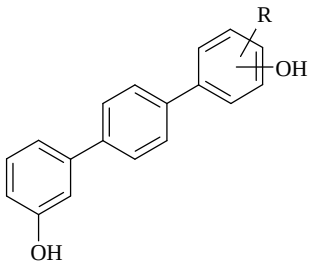
project III



R= NH_2, SH, CN, F
$NHSO_2CH_3$, $NHSO_2PhCH_3$



X= CH, N
R= H, Me, Ph, Ph(OH)
R'= H, Me, Ph, OH, F, CF_3,
acrylamide, propanamide
acrylester



R= F, Me

project IV

Figure 16: structure overview of the molecules described in this thesis

3. Results

3.I Design, Synthesis and Biological Evaluation of Bis(hydroxyphenyl) azoles as Potent and Selective Non-Steroidal Inhibitors of 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1) for the Treatment of Estrogen-Dependent Diseases

Emmanuel Bey, Sandrine Marchais-Oberwinkler, Patricia Kruchten, Martin Frotscher, Ruth Werth, Alexander Oster, Oztekin Algül, Alexander Neugebauer and Rolf W. Hartmann

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Bioorganic and Medicinal Chemistry, *16*, 6423-6435 (**2008**)

Paper I

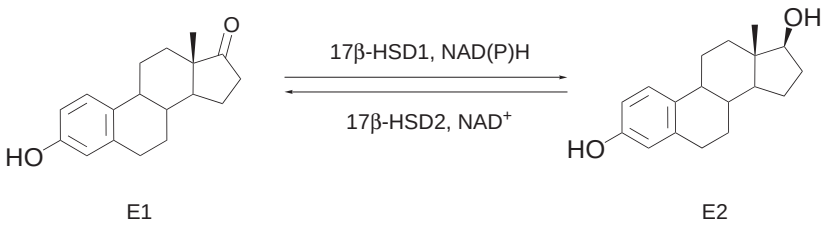
abstract: The 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) catalyses the reduction of the weakly active estrone (E1) into the most potent estrogen, 17β-estradiol (E2). E2 stimulates the growth of hormone-dependent diseases via activation of the estrogen receptors (ERs). 17β-HSD1 is often over-expressed in breast cancer cells. Thus, it is an attractive target for the treatment of mammary tumors. The combination of a ligand- and a structure-based drug design approach led to the identification of bis(hydroxyphenyl) azoles as potential inhibitors of 17β-HSD1. Different azoles and hydroxy substitution patterns were investigated. The compounds were evaluated for activity and selectivity with regard to 17β-HSD2, ERα and ERβ. The most potent compound is 3-[5-(4-hydroxyphenyl)-1,3-oxazol-2-yl]phenol (**18**, IC_{50}= 0.31 μM), showing very good selectivity, high cell permeability and medium CaCo-2 permeability.

Introduction

The naturally occurring steroidal hormones, estrone (E1) and 17β-estradiol (E2) are responsible for the development and differentiation of estrogen-sensitive tissues. It is well known, however, that E2, the most active estrogen, also plays a pivotal role in the growth of estrogen-dependent breast cancer[1] and is involved in the pathophysiology of endometriosis.[2]
Until now three endocrine therapies have been established for the treatment of breast cancer.[3,4] SERMs (Selective Estrogen Receptor Modulators) and pure antiestrogens[5] like fulvestrant block the estrogen action at the receptor level while aromatase inhibitors and GnRH-analogues restrain the formation of estrogens. This strong reduction of systemic estrogen action is a rather radical approach. A softer therapy could be the inhibition of the enzyme catalysing the last step of the E2 biosynthesis: 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1).

17β-HSD1 is a member of the 17β-hydroxysteroid dehydrogenase family which is responsible for the intracellular regulation of steroidal sex hormones activities.[6,7] Until now, fourteen members of this enzyme class are known.[8] In humans, eleven of them regulate the concentration of active androgens and estrogens in a tissue-specific manner.[8,9]
17β-HSD1 (EC1.1.1.62) is a cytosolic enzyme, which converts the weakly active estrone (E1) into the highly potent E2 using NAD(P)H as cofactor (Chart 1). The enzyme is expressed in different organs like ovaries, placenta, breast, endometrium[10] and often overexpressed in many breast cancer tissues.[11-14] Inhibition of 17β-HSD1 should be a good strategy to selectively reduce the E2 level in diseased tissues and might therefore be a new therapeutic approach with probably less side effects for the treatment of estrogen dependent diseases.
As a biological counterpart the membrane-bound 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) catalyses the NAD^+-dependent oxidation of E2 to E1 (Chart 1). This enzyme should not be affected by potential inhibitors of 17β-HSD1, as it might protect the cell from excessively high concentrations of active estrogen.[15] Nor should 17β-HSD1 inhibitors show affinity to the estrogen receptors α and β (ERα and ERβ) to avoid intrinsic estrogenic effects.

Chart 1: interconversion of estrone (E1) to 17β-estradiol (E2)



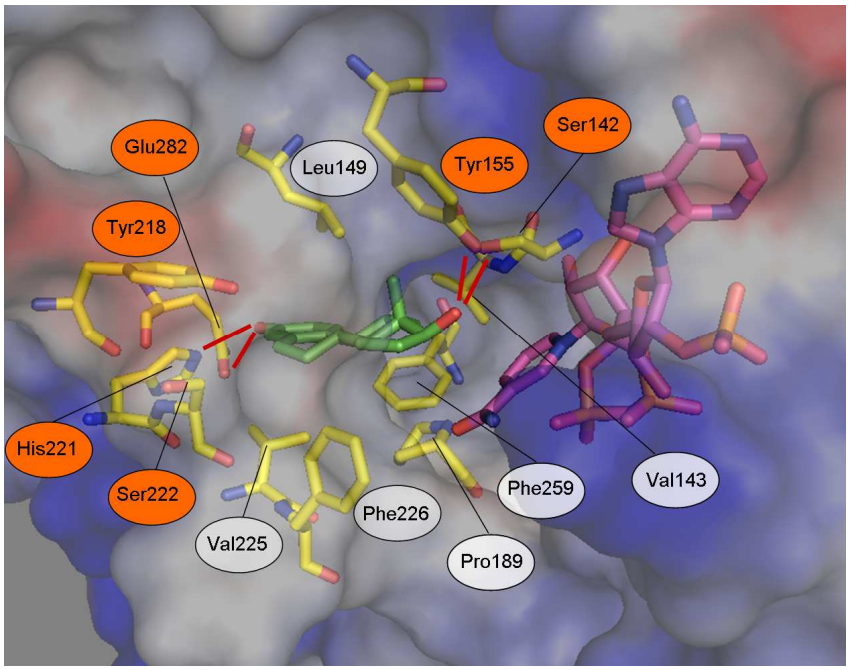
Over the last decade, several groups have reported on 17β-HSD1 inhibitors, most of them showing steroidal structures.[6, 16-30] Until now, only three classes of non-steroidal compounds have been described.[31-34]
In the following we will report on the design, synthesis and biological evaluation of potent and selective non-steroidal inhibitors of 17β-HSD1 that are appropriate for drug development.

Design of the inhibitors

Characterisation of the binding site

Sixteen crystal structures[35-43] with different steroidal ligands are available from the Protein Data Bank.[44] The three dimensional architecture of the enzyme was investigated using the ternary complex of 17β-HSD1 with E2 and $NADP^+$ (PDB-ID: 1FDT).[35] A substrate binding site, a cofactor binding pocket and an entry channel can be defined. The former is a narrow hydrophobic tunnel showing a high degree of complementarity to the steroid. Two polar regions can be identified at each extremity of the substrate binding site corresponding to the binding positions of the 3- and 17-hydroxy group of E2. Both of them establish four hydrogen bonds with His221/Glu282 and Ser142/Tyr155, respectively. This area also contains a mainly apolar region, corresponding to the B/C ring of the steroid (Leu149, Val225, Phe226, Phe259). Interestingly, two polar amino acids (Tyr218, Ser222) are located in the same domain (Figure 1).

Figure 1: schematic presentation of the active site of 17β-HSD1 containing E2 (PDB-code: 1FDT). Orange labels denote polar amino acids and white labels stand for lipophilic amino acids. Hydrogen bonds are marked in red and cofactor in purple.



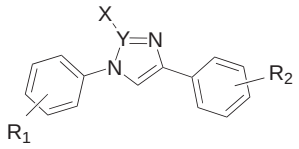
The catalytic centre of the enzyme is formed by a tetrad of amino acids[45] (Asn114, Ser142, Tyr155 and Lys159) which stabilises the steroid and the nicotinamide moiety during hydride transfer. The active site is limited by a flexible loop (amino acids 188-201) which is not well resolved in all of the X-ray structures.

Design of steroidomimetics

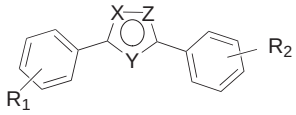
In the design process, the substrate binding site was defined as a binding region for the potential inhibitors as a lack of selectivity is expected for the compounds targeting only the cofactor binding pocket: this area consists of Rossmann fold motifs which are highly conserved in the majority of dehydrogenases. We focused on non-steroidal structures as it is known that steroidal compounds often show side effects which are caused by agonistic or antagonistic effects at steroid receptors. As the compounds have to mimic the steroidal substrate, they should contain two hydroxyphenyl (or two methoxyphenyl) moieties to imitate the A-ring (interaction with His221/Glu282) and the D-ring (interaction with Ser142/Tyr155) of the steroid. Additionally, the O-O distance should be in the same range as observed for the steroid (*d*= 11 Å). Different substitution patterns concerning the position of the phenolic OH groups were therefore investigated to find out the one which fits best into the active site (Chart 2). To mimic the flat shape of the substrate, the two substituted benzenes should be linked by an additional aromatic ring. The five-membered heterocycles imidazole, triazole, pyrazole, isoxazole and oxazole seemed to be especially suitable as their heteroatoms might be able to establish additional interactions with the amino acids Tyr218 and/or Ser222, which are located close to the C6 position of the steroidal B-ring. Position, number and nature of the heteroatoms in the five-membered ring were varied in order to identify the most appropriate heterocycle. 1,2- and 1,3-bis(hydroxyphenyl) and tris(hydroxyphenyl) azoles have been described by Fink et al.[46] as novel ER ligands. The fact that the compounds bearing two hydroxyphenyl moieties did not show any binding affinity to the ERs[46] is supportive of our design concept. In the following we describe the synthesis of compounds **1** to **19** (Chart 2) and their biological

evaluation using human 17β-HSD1, 17β-HSD2, ERα and ERβ as well as T-47D and CaCo2-cell lines.

Chart 2: title compounds



1-8



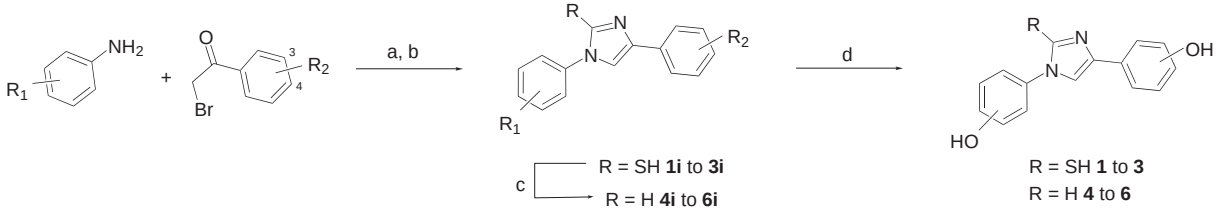
9-19

compound	X	Y	Z	R_1	R_2
1	SH	C		4-OH	3-OH
2	SH	C		4-OH	4-OH
3	SH	C		3-OH	4-OH
4	H	C		4-OH	3-OH
5	H	C		4-OH	4-OH
6	H	C		3-OH	4-OH
7		N		4-OH	3-OH
8		N		3-OH	4-OH
9	N	NH	CH	4-OH	3-OH
10	N	NH	CH	4-OH	4-OH
11	N	NH	CH	3-OH	4-OH
12	NH	CH	N	4-OH	3-OH
13	NH	CH	N	4-OH	4-OH
14	O	CH	N	4-OH	3-OH
15	O	CH	N	4-OH	4-OH
16	O	CH	N	3-OH	4-OH
17	N	O	CH	4-OH	4-OH
18	N	O	CH	3-OH	4-OH
19	O	N	CH	3-OH	4-OH

Chemistry

The synthesis of the title compounds is depicted in Schemes 1 to 4. 1,4-Bis(hydroxyphenyl) imidazoles were synthesised according to the route presented in Scheme 1. Intermediates **1i** to **3i** were prepared by nucleophilic substitution followed by cyclisation with potassium thiocyanate as described by Prakash et al.[47] The sulfur removal (**4i** to **6i**) was performed under strong acidic conditions according to Dodson and Ross.[48] Compounds **1i** to **6i** were submitted to ether cleavage with boron trifluoride dimethyl sulfide complex[46] as reagent.

Scheme 1: synthesis of compounds **1** to **6**[a]

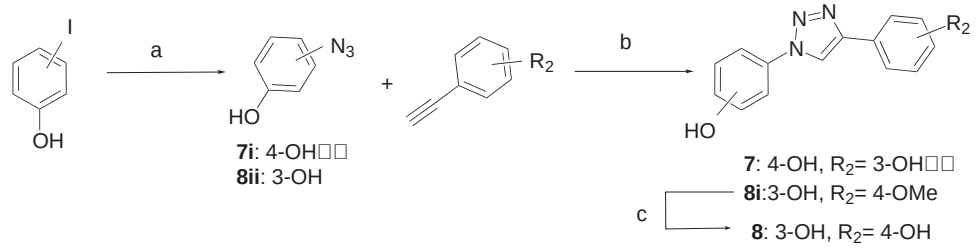


compound	R_1	R_2
1i	4-OMe	3-OMe
2i	4-OMe	4-OMe
3i	3-OMe	4-OMe
4i	4-OMe	3-OMe
5i	4-OMe	4-OMe
6i	3-OMe	4-OMe

[a]**Reagents and conditions:** a.: NEt_3, DMF, rt, 7 h; b.: KSCN, cat. HCl, MeOH, reflux, 18 h; c.: HNO_3, $NaNO_2$, glacial acetic acid, 0° C, 20 min; d.: $BF_3 \cdot SMe_2$, CH_2Cl_2, rt, 20 h.

Synthesis of the 1,2,3-triazoles **7** and **8** was performed according to the synthetic pathway shown in Scheme 2. Aromatic nucleophilic substitution of iodophenol derivatives by sodium azide led to intermediates **7i** and **8ii**. The resulting azides were converted to compounds **7** and **8i** in the presence of the corresponding phenyl acetylene derivative using the method of Sharpless.[49] The methoxy group of **8i** was cleaved with boron trifluoride dimethyl sulfide complex.

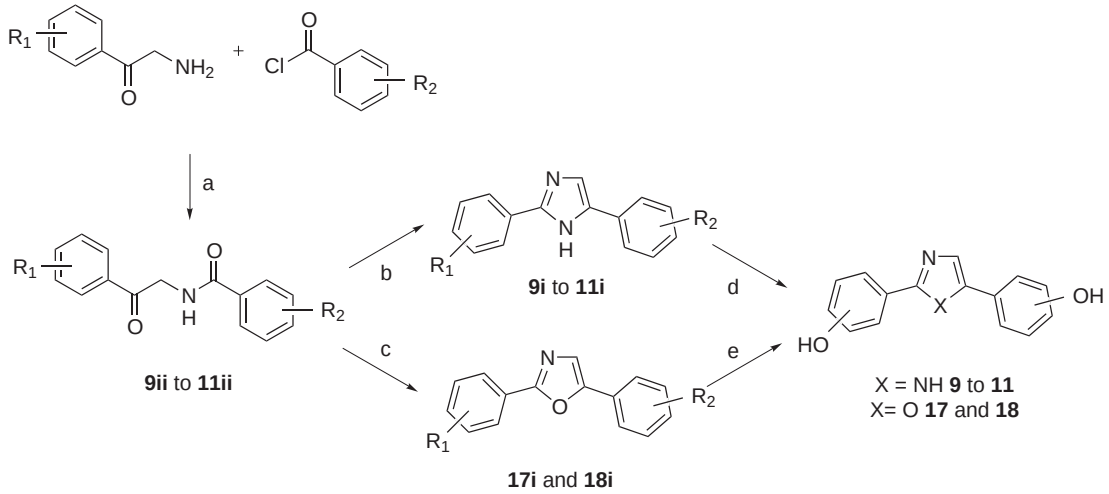
Scheme 2: synthesis of compounds **7** and **8**[a]



[a]**Reagents and conditions:** a.: NaN_3, CuI, L-Proline, NaOH, DMSO, 60 °C, 10 h; b.: Na-ascorbate, $CuSO_4$, H_2O/*t*-BuOH (1:1) , 60 °C, 24 h; c.: $BF_3 \cdot SMe_2$, CH_2Cl_2, rt, 20 h.

The synthesis of the 2,5-bis(hydroxyphenyl) imidazoles and 2,5-bis(hydroxyphenyl) oxazoles is presented in Scheme 3. The commercially available amine derivatives were *N*-acylated with two different acid chlorides to build the key intermediates **9ii** to **11ii**. The latter were cyclised to the 2,4-disubstituted imidazoles (**9i** to **11i**) according to Suzuki et al.[50] and to the 2,5-disubstituted oxazoles (**17i** and **18i**) following Nicolaou et al.[51] Ether cleavage was successful with boron trifluoride dimethyl sulfide complex for the imidazole compounds **9** to **11**, pyridinium hydrochloride for **17** and boron tribromide for **18**.

Scheme 3: synthesis of compounds **9** to **11**, **17** and **18**[a]

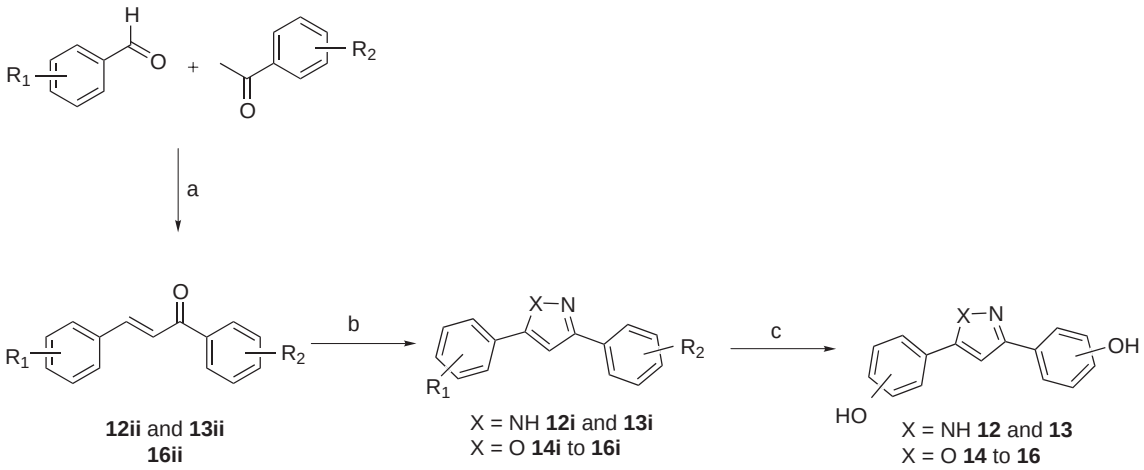


compound	R_1	R_2	compound	R_1	R_2
9ii	4-OMe	3-OMe	**9i**	4-OMe	3-OMe
10ii	4-OMe	4-OMe	**10i**	4-OMe	4-OMe
11ii	3-OMe	4-OMe	**11i**	3-OMe	4-OMe
			17i	4-OMe	4-OMe
			18i	3-OMe	4-OMe

[a]**Reagents and conditions:** a.: NEt_3, rt, 30 min.; b.: NH_4OAc, AcOH, reflux, 2 h; c.: $POCl_3$, pyridine, reflux, 8 h; d.: $BF_3 \cdot SMe_2$, CH_2Cl_2, rt, 20 h; e.: for **17i**: Pyridinium hydrochloride, 220 °C, 18h, for **18i**: BBr_3, CH_2Cl_2, -78 °C to rt, 20 h.

The pyrazoles **12** and **13**, the isoxazoles **14** and **16** were synthesised according to the route shown in Scheme 4. Key intermediates **12ii**, **13ii** and **16ii** were prepared via Claisen condensation of the commercially available methoxylated acetophenone derivatives with the appropriate benzaldehydes under strongly basic conditions. The cyclisation step for the pyrazoles[52] **12i** and **13i** was carried out with hydrazine monohydrate (Method A). For compounds **14i** to **16i,** the α,β-unsaturated ketones **12ii**, **13ii** and **16ii** were first activated with bromine and cyclised to isoxazole with hydroxylamine hydrochloride (Method B). Ether cleavage was performed with boron tribromide to yield **12** to **16**.

Scheme 4: synthesis of compounds **12** to **16**[a]



compound	R_1	R_2	method
12i	4-OMe	3-OMe	A
13i	4-OMe	4-OMe	A
14i	4-OMe	3-OMe	B
15i	4-OMe	4-OMe	B
16i	3-OMe	4-OMe	B

[a]**Reagents and conditions**: a.: EtOH, Na, rt, 30 min; b.: Method A: hydrazine monohydrate, AcOH, EtOH, reflux, 24 h; Method B: bromine, diethylether, 0 °C, 1 h, hydroxylamine hydrochloride, abs. EtOH, reflux 24 h; c: BBr_3, CH_2Cl_2, -78 °C to rt, 20 h.

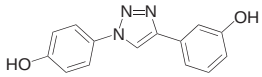
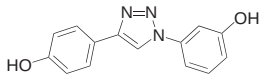
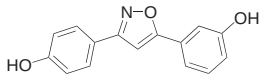
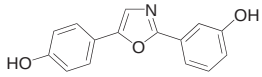
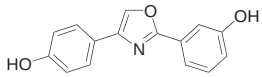
Compound **19** was prepared in a one pot synthesis following the procedure of Lee et al.[53] Briefly, 4-methoxyacetophenone, [hydroxy(2,4-dinitrobenzensulfonyloxy)iodo]benzene and 3-methoxybenzamide were heated under reflux in acetonitrile for 10 h to yield the intermediate **19i**. The ether groups of **19i** were cleaved with boron tribromide.

Biological results

Inhibition of human 17β-HSD1

Placental enzyme was isolated following a described procedure.[54] Tritiated E1 was incubated with 17β-HSD1, cofactor and inhibitor. The amount of formed E2 was quantified by HPLC. All methoxy compounds and *para-para* di-hydroxylated derivatives are inactive (data not shown). In contrast, some of the unsymmetrically substituted compounds were active (Table 1). Interestingly the *para-meta* di-substituted isoxazole **14** is inactive while its *meta-para* analogue **16** (IC_{50}= 1.61 μM) shows inhibitory activity. This exemplifies the importance of the positions of the OH groups. The triazole **7**, isoxazole **16** and 2,4-disubstituted oxazole **19** are only weak inhibitors of 17β-HSD1 while derivatives **8** and **18** show IC_{50} values in the nanomolar range. The 2,5-disubstituted oxazole **18** was the most potent compound identified with an IC_{50} value of 0.31 μM.

Table 1: inhibition of human 17β-HSD1 and 17β-HSD2 by selected inhibitors

compound	structure	IC_{50} (μM)[a]		selectivity factor[d]
		17β-HSD1[b]	17β-HSD2[c]	
7		1.32	8.12	6
8		0.84	7.28	9
16		1.61	0.27	0.1
18		0.31	17.5	56
19		1.85	0.25	0.1

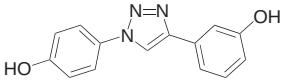
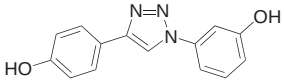
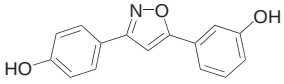
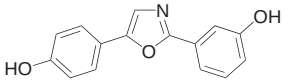
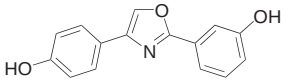
[a]Mean value of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate E1, 500 nM, cofactor NADH, 500 μM; [c]Human placenta, microsomal fraction, substrate E2, 500 nM, cofactor NAD^+, 1500 μM; [d]IC_{50} HSD2/IC_{50} HSD1.

It is striking that only compounds containing a heterocycle which can function as a hydrogen bond acceptor (nitrogen and/or oxygen) are active. The position of the heteroatoms in the heterocyclic skeleton is also a decisive criterion for inhibitory activity. The 2,5-disubstituted oxazole (**18**, IC_{50}= 0.31 μM) showed a stronger inhibition of the enzyme as its 2,4-disubstituted oxazole (**19**, IC_{50}= 1.85 μM) and 3,5-disubstituted isoxazole (**16**, IC_{50}= 1.61 μM) analogues.

Selectivity

Since 17β-HSD2 catalyses the inactivation of E2 to E1, inhibitory activity toward this enzyme must be avoided. The 17β-HSD2 inhibition was determined using an assay similar to the 17β-HSD1 test. Placental microsomes were incubated with tritiated E2 in the presence of NAD^+ and inhibitor. Quantification of labelled product (E1) was performed by HPLC and following radio detection. IC_{50} values and selectivity factors (IC_{50} HSD2/ IC_{50} HSD1) are presented in Table 1.
Compound **18** is only a weak inhibitor of the type 2 enzyme (IC_{50}= 17.5 μM) with a selectivity factor of 56, while the isoxazole **16** (IC_{50}= 0.27 μM) and the oxazole **19** (IC_{50}= 0.25 μM) isomers show stronger inhibition for the type 2 versus type 1 enzyme (selectivity factors: 0.1).
Furthermore, as 17β-HSD1 inhibitors should have no or low affinity to ERα and ERβ, binding affinity was measured for selected compounds. Using recombinant human protein, a competition assay applying tritium labelled E2 (RBA= 100 %) and hydroxyapatite was performed. The triazoles **7** and **8**, isoxazole **16** and oxazole **19** show very low binding affinity to both ERs, whereas for the most interesting compound, the 2,5-disubstituted oxazole **18,** a very low affinity to ERα (0.01< RBA(%)< 0.1) and a marginally higher (RBA= 0.5 %, Table 2) affinity to ERβ is observed.

Table 2: binding affinities for the estrogen receptors α and β by selected compounds

compound	structure	RBA[a] (%)	
		ERα	ERβ
7		<0.01	<0.01
8		0.01< RBA< 0.1	0.01< RBA< 0.1
16		0.01< RBA< 0.1	<0.01
18		0.01< RBA< 0.1	0.5
19		0.01< RBA< 0.1	0.01< RBA< 0.1

[a]RBA: relative binding affinity, E2: 100 %, mean value of three determinations, standard deviation less than 10 %.

Further biological evaluation of compound 18 using T-47D and CaCo-2 cell lines.

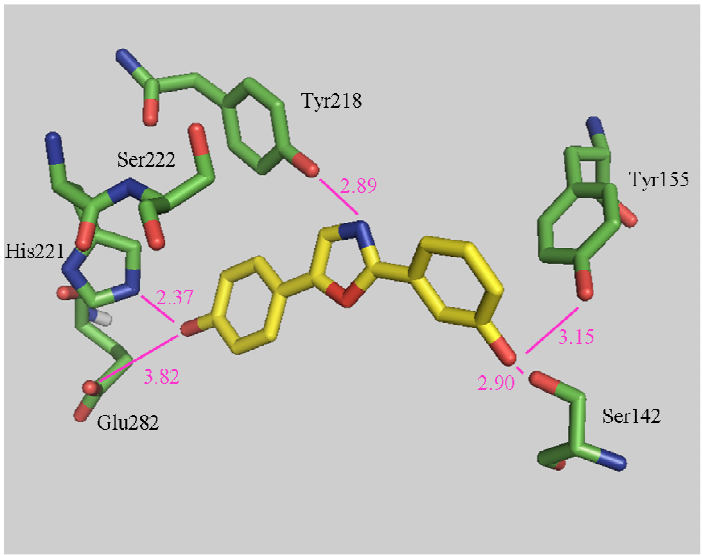
The most promising inhibitor of this series, compound **18** was evaluated for estrogenic effects on the ER-positive, mammary tumor T-47D cell line. No agonistic, i.e. stimulatory effect was observed after application of compound **18** even at a concentration 1000 fold higher compared to E2.

Compound **18** was additionally evaluated using the same cell line which expresses both 17β-HSD1 and 17β-HSD2. The oxazole **18** inhibits the formation of E2 after incubation with labelled E1 exhibiting an IC_{50} value of 0.38 µM. As this value is very similar to the one observed in the cell free assay (0.31 µM, see Table 1) it can be concluded that the compound is capable of unfolding its activity in intact cells.
Further investigations were performed using CaCo-2 cells. These cells exhibit morphological and physiological properties of the human small intestine[55] and are generally accepted to be an appropriate model for the prediction of peroral absorption. Depending on the P_{app} data obtained, compounds are usually classified as low (P_{app} (10^{-6} cm/sec) < 1), medium (1 < P_{app} < 10) or highly permeable (P_{app} > 10). Compound **18**, showing a P_{app} value of $7.9 \cdot 10^{-6}$ cm/sec, is a medium cell permeator like for example acetyl salicylic acid.

Molecular Modeling

In order to get a better insight into the molecular interactions between the most potent steroidomimetic **18** and 17β-HSD1, the compound was docked into the active site of the protein (PDB: 1FDT, E2 removed) using the docking software Gold 3.0 (rigid protein, flexible ligand).
Two different binding modes can be expected because of the pseudo-symmetry of our steroidomimetic: each hydroxyphenyl group could mimic the A-ring of the steroid. The energetically most favourable pose is depicted in Figure 2. The *para* hydroxyphenyl substituent and the heterocycle are in the same plane while the *meta* hydroxyphenyl moiety is rotated 32° out of this plane. This conformation allows the inhibitor to establish hydrogen bond interactions with His221/Glu282 (*para* hydroxyphenyl moiety) and Ser142/Tyr155 (*meta* hydroxyphenyl substituent). Interestingly, the $N_{oxazole}$-O_{Tyr218} distance is 2.89 Å making an additional hydrogen bond interaction very likely. On the other hand an interaction with the O_{Ser222} cannot be observed as in the rigid protein structure the CH_2OH moiety is turned away from the heterocycle. However, it cannot be excluded that there is a conformational change in that functional group after binding of this ligand. Additionally, it is likely that hydrophobic interactions (Van der Waals and π–π stacking) are also involved in the binding of **18** into the active site.

Figure 2: 17β-HSD1 binding pocket (green amino acids) with docked compound **18** (yellow). Hydrogen bonding interactions are marked by violet lines. All distances are expressed in Å. For clarity, only selected amino acids are represented.



Discussion and Conclusion

Looking at the SARs of the synthesised compounds, it becomes apparent that the positions of the OH groups at the hydroxyphenyl moieties are crucial for the activities of the corresponding derivatives: all *para-para* substituted compounds are inactive, while several of the *meta-para* /*para-meta* isomers are active. This suggests that 17β-HSD1 is not as flexible to adjust its geometry to the two OH groups as it is reported for the ERs[56] and is in agreement with the non-flexibility already observed for 17β-HSD1 inhibitors in the class of 6-(hydroxyphenyl)naphthalenes.[34] The fact that not all *meta-para* /*para-meta* bis-hydroxyphenyl derivatives (with O-O distances comparable to E2) are active, indicates that the nature of the heterocycle is also playing an important role for the inhibitory potencies of the compounds.
Obviously hydrogen bond donors are unfavourable for activity (e.g. imidazoles **9** to **11**, pyrazoles **12** and **13**) while several compounds with only hydrogen bond acceptor groups show reasonable activities (e.g. triazoles **7** and **8**, isoxazole **16**, oxazoles **18** and **19**).
It is interesting to have a closer look at the inactive isoxazole **14** (*para-meta*) and its highly active isomer, the oxazole **18** (*para-meta*). Provided that both compounds interact with the active site in the same manner, e.g. the *para*-hydroxyphenyl moiety establishes hydrogen bond contacts with His221/Glu282, the structure of the inhibitors only differ by the position of the oxygen on the heterocycle. It has been described[57] that only the nitrogen of isoxazole and oxazole (and not the oxygen) is able to establish hydrogen bond interaction. The position of the oxygen on the azole structure seems to be determinant for the ability of the compound for forming hydrogen bonds involving the nitrogen: in case of the isoxazole, the electronic density on the nitrogen is reduced compared to the oxazole. This is obviously due to the electronegative effect of the oxygen. It can therefore be assumed that only in case of compound **18** hydrogen bond interactions with Tyr218 and Ser222 (Figure 2) are possible making the latter compound a potent inhibitor. These explanations are supported by the fact that the oxazole **19** shows a weak inhibitory activity.
The insertion of a third nitrogen (triazoles **7** and **8**) in the heterocycle increases the inhibitory potency of the correspondingly substituted imidazoles **4, 6** and imidazoles-2-thiol **1, 3** which are inactive. Obviously, this nitrogen plays the same role for the interaction with the amino acid residues in the binding site as observed for compound **18**.

The most interesting compound of the present study is **18** showing a high 17β-HSD1 inhibition and a good selectivity toward 17β-HSD2. In addition, compound **18** exhibits very low affinity to ERα and ERβ. It is striking that the high binding affinity of similar ER ligands like the tris(hydroxyphenyl) pyrazoles described by Katzenellenbogen's group[46, 58-60] depends on the presence of three hydroxyphenyl moieties. No polar amino acids in the B/C ring region of E2 can be identified in the X-ray structures of the ERs as groups to establish interactions with the heterocycle. Obviously, the pyrazole is playing a passive role in this class of ER ligands. In case of our bis(hydroxyphenyl) substituted azoles the combination of at least two parameters is implicated in the 17β-HSD1 inhibitory potency: an optimal OH substitution pattern and an appropriate heterocycle.
Activation of the ERs would be detrimental for the treatment of estrogen-dependent diseases. Therefore agonism must be avoided. Antagonistic activity would be less critical. The diseased cells certainly could benefit from compounds with dual activity. However, antiestrogens would also exert systemic effects in other healthy steroidogenic tissues leading to unwanted effects. Consequently, we focussed on the discovery of compounds without affinity to the ERs.
The fact that the 2,5-disubstituted oxazole **18** inhibits the cellular formation of E2 with an IC_{50} value in the nanomolar range, shows that this compound is able to enter the cell and to be active at the target enzyme. The fact that the inhibitor shows a good permeation is supported by the CaCo-2 data. These results are indicative of a sufficient intestinal absorption.

Compound **18** might therefore be a good lead compound for the development of a clinically applicable therapeutic for the treatment of estrogen-dependent diseases.

Experimental section

Chemical Methods

Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Lancaster, Roth, Merck or Fluka and were used without purification.
Column chromatography (CC) was performed on silica gel (70-200 µm) coated with silica, preparative thin layer chromatography (TLC) on 1 mm SIL G-100 UV_{254} glass plates (Macherey-Nagel) and reaction progress was monitored by TLC on Alugram SIL G UV_{254} (Macherey-Nagel).
Melting points were measured on a Mettler FP1 melting point apparatus and are uncorrected. IR spectra were recorded on a Bruker Vector 33 spectrometer (neat sample).
^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker AM500 spectrometer (500 MHz) at 300 K. Chemical shifts are reported in δ (parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard ($CDCl_3$: δ = 7.24 ppm (^{1}H-NMR) and δ = 77 ppm (^{13}C-NMR), CD_3OD: δ = 3.35 ppm (^{1}H-NMR) and δ = 49.3 ppm (^{13}C-NMR), CD_3COCD_3: δ = 2.05 ppm (^{1}H-NMR) and δ = 29.9 ppm (^{13}C-NMR), CD_3SOCD_3 δ = 2.50 ppm (^{1}H-NMR) and δ = 39.5 ppm (^{13}C-NMR). Signals are described as s, d, t, dd, m, dt, q for singlet, doublet, triplet, doublet of doublets, multiplet, doublet of triplets and quadruplet respectively. All coupling constants (*J*) are given in hertz (Hz).
Mass spectra (ESI and APCI) were recorded on a TSQ Quantum (Thermo Finnigan) instrument. Elemental analyses were performed at the Department of Instrumental Analysis and Bioanalysis, Saarland University.

The following compounds were prepared according to previously described procedures: 1,4-bis-(4-methoxyphenyl)-1*H*-imidazol-2-thiol (**2i**),[47] 4-(3-methoxyphenyl)-1-(4-methoxyphenyl)-1*H*-imidazole (**4i**),[61] 1,4-bis-(4-methoxyphenyl)-1*H*-imidazole (**5i**),[62] 4-azidophenol (**7i**),[63] 3-[1-(4-hydroxyphenyl)-1*H*-1,2,3-triazol-4-yl]phenol (**7**),[63] 3-azidophenol (**8ii**),[63] 3-[4-(4-hydroxyphenyl)-1*H*-1,2,3-triazol-1-yl]phenol (**8**),[63] 3-methoxy-*N*-[2-(4-methoxyphenyl)-2-oxo-ethyl]-benzamide (**9ii**),[64] 4-methoxy-*N*-[2-(4-methoxyphenyl)-2-oxo-ethyl]benzamide (**10ii**),[65] 2,5-bis-(4-methoxyphenyl)-1*H*-imidazole (**10i**),[66] 3-methoxy-*N*-[2-(4-methoxyphenyl)-2-oxo-ethyl]-benzamide (**11ii**),[67] (2*E*)-1-(3-methoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**12ii**),[68] (2*E*)-1,3-bis(4-methoxyphenyl)prop-2-en-1-one (**13ii**),[68] 3,5-bis-(4-methoxyphenyl)-1*H*-pyrazole (**13i**),[69] 4,4'-(1*H*-pyrazol-3,5-diyl)diphenol (**13**),[46] 3-(3-methoxyphenyl)-5-(4-methoxyphenyl)isoxazole (**14i**),[70] 3-[5-(4-hydroxyphenyl)isoxazol-3-yl]phenol (**14**),[71] 3,5-bis-(4-methoxyphenyl)isoxazole (**15i**),[71] 4,4'-(isoxazol-3,5-diyl)diphenol (**15**),[71] (2*E*)-3-(3-methoxyphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (**16ii**),[72] 3-[3-(4-hydroxyphenyl)isoxazol-5-yl]phenol (**16**),[71] 2,5-bis-(4-methoxyphenyl)-oxazole (**17i**).[64]

General synthesis procedure for compounds 1i to 3i. A mixture of methoxyaniline (1 eq), methoxyphenacyl bromide (1 eq) and triethylamine (1 eq) were stirred at rt in 2 mL DMF for 7 h. The crude material was poured into ice water. The resulting precipitate was filtered, dried overnight in a desiccator. To a stirred solution of the precipitate (1 eq) in 20 mL methanol, potassium thiocyanate (1 eq) and 60 µL concentrated chlorhydric acid were added. The resulting mixture was refluxed for 18 h. After cooling to rt, the precipitate was filtered off, dried overnight in a desiccator. The crude product was purified by CC.

4-(3-Methoxyphenyl)-1-(4-methoxyphenyl)-1*H*-imidazol-2-thiol (1i). The title compound was prepared by reaction of *para*-methoxyaniline (751 mg, 6.11 mmol), 3-methoxyphenacyl bromide (1.38 g, 6.11 mmol), triethylamine (0.89 mL, 6.11 mmol) and potassium thiocyanate (593 mg, 6.11 mmol) according to the procedure reported above. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 28 % (500 mg); yellow powder; mp: 248 °C; ^{1}H NMR ($CDCl_3$): 7.36 (d, *J* = 9.40 Hz, 2H), 7.30-7.26 (m, 2H), 7.10 (d, *J* = 7.80 Hz, 2H), 6.84 (m, 1H), 6.81 (d, *J* = 8.80 Hz, 2H), 3.82 (s, 3H, OMe), 3.72 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$): 175.45, 160.05, 159.65, 129.70, 127.55, 117.45, 114.15 (2C), 113.95, 110.00, 55.45, 55.40. IR: 1626, 1514, 1222, 1037, 824 cm^{-1}; MS (APCI): 313 $(M+H)^{+}$.

1-(3-Methoxyphenyl)-4-(4-methoxyphenyl)-1*H*-imidazol-2-thiol (3i). The title compound was prepared by reaction of *meta*-methoxyaniline (356 mg, 2.90 mmol), 4-methoxyphenacyl bromide (658 mg, 2.90 mmol) and triethylamine (0.37 mL, 2.90 mmol), potassium thiocyanate (282 mg, 2.90 mmol) according to the procedure reported above. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 16 % (140 mg); white powder; mp: 250 °C; ^{1}H NMR ($CDCl_3$): 7.51 (d, *J* = 8.50 Hz, 2H), 7.38 (t, *J* = 7.80 Hz, 1H), 7.27 (s, 1H), 7.18 (d, *J* = 7.80 Hz, 1H), 7.02 (m, 1H), 6.97 (dd, *J* = 2.50 Hz and *J* = 8.50 Hz, 1H), 6.89 (d, *J* = 8.50 Hz, 2H), 3.84 (s, 3H, OMe), 3.79 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$): 188.00, 160.00, 129.95, 126.50, 118.00, 114.65 (2C), 111.80, 55.60, 55.35; IR: 3055, 1601, 1455, 1181, 825 cm^{-1}; MS (ESI): 313 $(M+H)^+$.

4-(4-Methoxyphenyl)-1-(3-methoxyphenyl)-1*H*-imidazole (6i). The title compound was prepared by reaction of sodium nitrite (11 mg, 0.16 mmol, 1 eq), nitric acid (152 µL, 87 µmol, 0.5 eq) and 1-(3-methoxyphenyl)-4-(4-methoxyphenyl)-1*H*-imidazol-2-thiol (**3i**) (150 mg, 0.48 mmol, 3 eq) mixed in glacial acetic acid (15 mL) at 0 °C and stirred for 20 min. The reaction was quenched with ammonium hydroxide (20 mL) and the resulting precipitate was filtered, dried overnight in a desiccator. The crude product was purified by CC (ethyl acetate/methanol 98:2); yield: 48 % (22 mg); yellow powder; mp: 246 °C; ^{1}H NMR ($CDCl_3$): 8.90 (s, 1H) 7.60 (s, 1H), 7.53 (s, 1H), 7.48 (m, 3H), 7.32 (t, *J* = 7.80 Hz, 1H), 7.02 (d, *J* = 8.50 Hz, 2H), 6.99 (dd, *J* = 1.80 Hz and *J* = 8.20 Hz, 1H), 3.95 (s, 3H, OMe), 3.85 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$): 161.15, 160.55, 136.15, 133.10, 130.20, 127.20, 123.85, 117.90, 117.15, 115.85, 110.50, 56.25, 55.60; IR: 3200, 2966, 1520, 1255, 855 cm^{-1}; MS (ESI): 281 $(M+H)^+$.

3-[4-(4-Methoxyphenyl)-1*H*-1,2,3-triazol-1-yl]phenol (8i). 3-Azidophenol (**8ii**) (500 mg, 3.70 mmol, 1 eq), 1-ethynyl-4-methoxybenzene (489 mg, 3.70 mmol, 1 eq) were stirred in a mixture of water/*t*-butanol (1:1, 20 mL) for 2 min. Copper(II)sulfate (9.24 mg, 0.04 mmol, 0.01 eq) was suspended in a freshly prepared solution of sodium ascorbate (1 M, 73.7 mg, 0.37 mmol, 0.1 eq) and added dropwise to the latter mixture. The reaction mixture was stirred at 60 °C for 24 h. After cooling to rt, the mixture was washed with water and the aqueous layer washed with ethyl acetate. The combined organic layers were dried over sodium sulfate and solvent was removed under reduced pressure. The crude product was purified by CC (ethyl acetate/methanol 9:1); yield: 37 % (350 mg); yellow powder; ^{1}H NMR (CD_3COCD_3): 8.80 (s, 1H) 7.91 (d, *J* = 8.80 Hz, 2H), 7.46 (s, 1H), 7.41 (d, *J* = 7.90 Hz, 2H), 7.04 (d, *J* = 8.80 Hz, 2H), 6.96 (m, 1H), 3.85 (s, 3H, OMe); ^{13}C NMR (CD_3COCD_3): 170.90, 159.45, 131.60, 127.80 (2C), 118.50, 116.30, 115.10 (2C), 111.80, 108.10, 55.65; IR: 3148, 2928, 1614, 1499, 1257, 833 cm^{-1}.

General synthesis procedure for compounds 9i to 11i. Benzamides **9ii** to **11ii** (1 eq) were refluxed in acetic acid (10 mL) with ammonium acetate (8 eq) for 2 h. Solvent was evaporated under reduced pressure and the crude product was suspended in a mixture of ethanol/water/dichloromethane (1:1:1). The dichloromethane layer was separated, washed with brine, dried over magnesium sulfate, evaporated under reduced pressure and purified by CC.

2-(4-Methoxyphenyl)-5-(3-methoxyphenyl)-1*H*-imidazole (9i). The title compound was prepared by reaction of 3-methoxy-*N*-[2-(4-methoxyphenyl)-2-oxo-ethyl]-benzamide (**9ii**) (917 mg, 3.20 mmol) and ammonium acetate (1.90 g, 25.6 mmol) according to the procedure reported above. The product was purified by CC (hexane/ethyl acetate 5:5); yield: 25 % (224 mg); white powder; mp: 198 °C; ^{1}H NMR (CD_3SOCD_3): 8.07 (d, *J* = 2.50 Hz, 1H), 7.98 (d, *J* = 8.50 Hz, 2H), 7.78 (d, *J* = 8.50 Hz, 1H), 7.57 (s, 1H), 7.37 (s, 1H), 7.12-7.08 (m, 2H), 6.75 (s, 1H), 3.83 (s, 3H, OMe), 3.82 (s, 3H, OMe); ^{13}C NMR (CD_3SOCD_3): 162.10, 160.85, 151.40, 137.50, 128.65 (2C), 127.25, 125.40, 123.35, 120.75, 115.75 (2C), 113.70, 56.80, 56.50; IR: 3070, 2950, 1578, 1242, 742 cm^{-1}; MS (ESI): 281 $(M+H)^+$.

2-(3-Methoxyphenyl)-5-(4-methoxyphenyl)-1*H*-imidazole (11i). The title compound was prepared by reaction of 3-methoxy-*N*-[2-(4-methoxyphenyl)-2-oxo-ethyl]-benzamide (**11ii**) (917 mg, 3.20 mmol) and ammonium acetate (1.90 g, 25.6 mmol) according to the procedure reported above. The product was purified by CC (hexane/ethyl acetate 5:5); yield: 6 % (54 mg); white powder; mp: 202 °C; ^{1}H NMR ($CDCl_3$): 8.04 (s, 1H), 7.85 (d, *J* = 8.20 Hz, 2H), 7.28-7.24 (m, 3H), 6.88 (d, *J* = 8.20 Hz, 2H), 6.78 (dq, *J* = 1.50 Hz and *J* = 7.60 Hz, 1H), 3.79 (s, 3H, OMe), 3.77 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$): 164.00,

132.35 (2C), 130.15, 120.60, 119.85, 113.75, 112.65, 55.60, 55.50; IR: 3077, 2965, 1678, 1468, 1240, 1031, 742 cm^{-1}; MS (ESI): 281 $(M+H)^{\cdot+}$.

5-(4-Methoxyphenyl)-2-(3-methoxyphenyl)-oxazole (18i). The title compound was prepared by reaction of 3-methoxy-*N*-[2-(4-methoxyphenyl)-2-oxo-ethyl]-benzamide (**9ii**) (347 mg, 1.16 mmol, 1 eq) and phosphorous oxychloride (12 mL, 0.89 mmol, 0.8 eq) in pyridine (20 mL) and refluxed for 8 h. After cooling to rt, ethyl acetate (40 mL) was added and the crude material was poured into a saturated sodium hydrogencarbonate solution. The aqueous layer was extracted two times with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, evaporated under reduced pressure and purified by CC (hexane/ethyl acetate 5:5); yield: 36 % (117 mg); yellow oil; ^{1}H NMR (CD_3COCD_3): 7.79 (d, *J* = 8.80 Hz, 2H), 7.70 (dt, *J* = 1.00 Hz and *J* = 8.80 Hz, 1H), 7.64 (q, *J* = 1.00 Hz, 1H), 7.53 (s, 1H, H-oxazole), 7.44 (t, *J* = 7.90 Hz, 1H), 7.08 (m, 3H), 3.90 (s, 3H, OMe), 3.86 (s, 3H, OMe); ^{13}C NMR (CD_3COCD_3): 161.05, 160.95, 152.40, 130.95, 129.85, 126.65, 123.15, 121.65, 119.15, 116.95, 115.40, 111.85, 55.75, 55.70; IR: 2937, 1612, 1253, 1010, 872 cm^{-1}.

General synthesis procedure for compounds 12i and 13i (method A). A solution of hydrazine monohydrate (4 eq) in glacial acetic acid (4 eq) was added dropwise to the propenone intermediate **12ii** and **13ii** (1 eq). The reaction mixture was heated at reflux for 24 h. After cooling to rt, the precipitate was filtered off. A mixture of water/ethyl acetate (1:1) was added to the filtrate. The combined organic layers were washed with brine, dried over magnesium sulfate, evaporated under reduced pressure and purified by CC.

3-(4-Methoxyphenyl)-5-(3-methoxyphenyl)-pyrazole (**12i**). The title compound was prepared by reaction of (2*E*)-1-(3-methoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**12ii**) (250 mg, 0.93 mmol) and hydrazine monohydrate (212 µL, 3.72.mmol) according to described method A. The product was purified by CC (hexane/ethyl acetate 5:5) followed by preparative TLC (dichloromethane/methanol 99:1); yield: 25 % (60 mg); yellow oil; ^{1}H NMR ($CDCl_3$): 7.55 (d, *J* = 8.80 Hz, 2H), 7.21-7.19 (m, 2H), 7.18 (t, *J* = 7.80 Hz, 1H), 6.78-6.75 (m, 3H), 6.62 (s, 1H), 3.74 (s, 3H, OMe), 3.62 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$): 159.85, 159.55, 129.70, 126.85, 126.85, 118.10, 114.15 (2C), 114.10, 110.50, 99.35, 55.20, 55.05; IR: 2933, 2837, 1601, 1439, 1250, 1033, 834 cm^{-1}.

General synthesis procedure for compounds 14i to 16i (method B): Propenone intermediate (**12ii**, **13ii** and **16ii,** 1 eq) was stirred at 0 °C in 5 mL dry diethylether and bromine (1 eq) was added dropwise. After 1 h at 0 °C, the reaction was warmed up to rt, precipitate was filtered and washed with diethylether. The resulting di-brominated propenone was obtained in quantitative yield and used without further purification. The latter (1 eq) was refluxed in 10 mL absolute ethanol with 1 eq hydroxylamine hydrochloride and 1 eq potassium hydroxide for 24 h. After cooling to rt, the mixture was poured into a cold water solution. The resulting precipitate was filtered, washed with cold water, dried overnight in a desiccator and purified by CC.

5-(3-Methoxyphenyl)-3-(4-methoxyphenyl)-isoxazole (**16i**). The title compound was prepared by reaction of (2*E*)-3-(3-methoxyphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one (**16ii**) (275 mg, 1.03 mmol), bromine (52 µL, 1.03 mmol), hydroxylamine hydrochloride (71.6 mg, 1.03.mmol) and potassium hydroxide (57.8 mg, 1.03 mmol) according to method B. The product was purified by CC (hexane/ethyl acetate 9:1) followed by preparative TLC (dichloromethane/methanol 1%); yield: 45 % (130 mg); yellow powder; mp: 161 °C; ^{1}H NMR ($CDCl_3$): 7.20 (m, 2H), 6.75 (dd, *J* = 2.00 Hz and *J* = 7.50 Hz, 1H), 6.80 (d, *J* = 7.50 Hz, 1H), 6.76 (s, 1H), 6.74 (s, 1H), 6.70 (m, 1H), 6.45 (d, *J* = 7.50 Hz, 2H), 3.72 (s, 3H, OMe), 3.56 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$): 171.65, 164.15, 162.40, 161.25, 131.80, 131.20, 128.70 (2C), 121.60, 120.60, 117.35, 115.70, 113.00, 97.50; IR: 2925, 2853, 1602, 1248, 746 cm^{-1}.

4-(4-Methoxyphenyl)-2-(3-methoxyphenyl)oxazole (**19i**). 4-Methoxy-acetophenone (500 mg, 3.33 mmol, 1 eq) was refluxed for 2 h together with [hydroxy(2,4-dinitrobenzensulfonyloxy)-iodo]benzene (1.88 g, 3.99 mmol, 1.2 eq) in acetonitrile (20 mL). After cooling to rt, 3-methoxybenzamide (1.52 g, 9.99 mmol, 3 eq) was added and the reaction mixture was refluxed for 10 h. Acetonitrile was evaporated under reduced pressure. The crude product was suspended in dichloromethane. The

resulting organic layer was washed with a saturated sodium bicarbonate solution and dried over magnesium sulfate. Solvent was removed under reduced pressure and the product was purified by CC (hexane/ethyl acetate 7:3); yield 50 % (465 mg); white powder; mp: 165 °C; ^{1}H NMR (CD_3COCD_3): 8.23 (s, 1H, H-oxazole), 7.90 (d, *J* = 9.20 Hz, 2H), 7.32 (m, 2H), 7.20 (t, *J* = 7.50 Hz, 1H), 6.93 (d, *J* = 9.20 Hz, 2H), 6.76 (m, 1H), 3.73 (s, 3H, OMe), 3.70 (s, 3H, OMe); IR: 3015, 2925, 1625, 789 cm^{-1}.

Ether cleavage - general procedure for compounds 1 to 6 and 9 to 11: To a solution of bis-methoxyphenyl derivative (1 eq) in dry dichloromethane was added dropwise boron trifluoride dimethyl sulfide complex (75 eq). The reaction mixture was stirred at rt for 20 h. To quench the reaction water was added and the aqueous layer was washed with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, evaporated under reduced pressure and purified by preparative TLC.

3-[1-(4-Hydroxyphenyl)-2-sulfanyl-1*H*-imidazol-4-yl]phenol (**1**). The title compound was prepared by reaction of 4-(3-methoxyphenyl)-1-(4-methoxyphenyl)-1*H*-imidazol-2-thiol (**1i**) (100 mg, 0.32 mmol) according to the procedure reported above. The product was purified by preparative TLC (ethyl acetate); yield: 61 % (55 mg); orange powder; mp: 265 °C; ^{1}H NMR (CD_3SOCD_3): 12.76 (s, 1H, SH), 7.64 (s, 1H), 7.39 (d, *J* = 8.50 Hz, 2H), 7.19-7.15 (m, 2H), 7.09 (s, 1H), 6.84 (d, *J* = 8.50 Hz, 2H), 6.71-6.69 (m, 1H); ^{13}C NMR (CD_3SOCD_3): 162.30, 157.60, 156.85, 129.90, 129.20, 128.95, 127.15, 116.15, 115.10 (2C), 114.85, 111.10; IR: 3214, 1604, 1514, 1395, 1101, 833, 750 cm^{-1}; MS (APCI): 284 $(M)^+$; Anal. calcd $C_{15}H_{12}N_2O_2S$ C. 63.36; H, 4.25; N, 9.85. Found C, 63.20; H, 3.99; N, 9.80.

4,4'-(2-Sulfanyl-1*H*-imidazol-1,4-diyl)diphenol (**2**). The title compound was prepared by reaction of 1,4-bis-(4-methoxyphenyl)-1*H*-imidazol-2-thiol (**2i**) (100 mg, 0.32 mmol) according to the procedure reported above. The product was purified by preparative TLC (ethyl acetate); yield: 36 % (35 mg); white powder; mp: 268 °C; ^{1}H NMR (CD_3OD): 7.49 (d, *J* = 8.80 Hz, 2H) 7.42 (d, *J* = 8.80 Hz, 2H), 7.34 (s, 1H), 6.92 (d, *J* = 8.80 Hz, 2H), 6.87 (d, *J* = 8.80 Hz, 2H); ^{13}C NMR (CD_3OD): 162.00, 159.05, 158.80, 131.20, 131.10, 131.00, 128.55, 127.30, 120.55, 116.90, 116.50, 115.95; IR: 3135, 2469, 2072, 1511, 1116, 973, 836 cm^{-1}; MS (APCI): 284 $(M)^+$, 285 $(M+H)^+$; Anal. calcd $C_{15}H_{12}N_2O_2S$. C, 63.36; H, 4.25; N, 9.85. Found C, 63.40; H, 4.24; N, 9.95.

3-[4-(4-Hydroxyphenyl)-2-sulfanyl-1*H*-imidazol-1-yl]phenol (**3**). The title compound was prepared by reaction of 1-(3-methoxyphenyl)-4-(4-methoxyphenyl)-1*H*-imidazol-2-thiol (**3i**) (100 mg, 0.32 mmol) according to the procedure reported above. The product was purified by preparative TLC (ethyl acetate); yield: 37 % (40 mg); yellow powder; ^{1}H NMR (CD_3SOCD_3): 12.75 (s, 1H, SH), 7.62 (s, 1H), 7.40 (d, *J* = 8.50 Hz, 2H), 7.18-7.13 (m, 2H), 7.07 (s, 1H), 6.83 (d, *J* = 8.50 Hz, 2H), 6.69-6.66 (m, 1H); ^{13}C NMR (CD_3SOCD_3): 162.35, 157.65, 156.95, 129.85, 129.00, 128.90, 127.20, 116.20 (2C), 115.05, 114.90, 111.25; IR: 3213, 1600, 1514, 1392, 1100, 845, 750 cm^{-1}; MS (APCI): 284 $(M)^+$; Anal. calcd $C_{15}H_{12}N_2O_2S$. C, 63.36; H, 4.25; N, 9.85. Found C, 63.12; H, 4.22; N, 9.84.

3-[1-(4-Hydroxyphenyl)-1*H*-imidazol-4-yl]phenol (**4**). The title compound was prepared by reaction of 4-(3-methoxyphenyl)-1-(4-methoxyphenyl)-1*H*-imidazole (**4i**) (100 mg, 0.36 mmol) according to the procedure reported above. The product was purified by preparative TLC (ethyl acetate); yield: 28 % (25 mg); yellow oil; ^{1}H NMR (CD_3COCD_3): 9.32 (d, *J* = 1.20 Hz, 1H), 8.33 (d, *J* = 1.20 Hz, 1H), 7.70 (dd, *J* = 2.20 Hz and *J* = 8.80 Hz, 2H), 7.36-7.33 (m, 2H), 7.29 (t, *J* = 1.90 Hz, 1H), 7.06 (dd, *J* = 2.20 Hz and *J* = 8.80 Hz, 2H), 6.99 (m, 1H); ^{13}C NMR (CD_3COCD_3): 159.70, 158.95, 134.95, 131.60, 129.10, 128.10, 124.95, 118.25, 117.95, 117.80, 117.35, 113.35; IR: 3563, 1684, 1629, 1048, 836 cm^{-1}; MS (ESI): 253 $(M+H)^+$; Anal. calcd $C_{15}H_{12}N_2O_2$. C, 71.42; H, 4.79; N, 11.10. Found C, 71.11; H, 4.62; N, 11.01.

4,4'-bis-(1*H*-Imidazol-1,4diyl)-diphenol (**5**). The title compound was prepared by reaction of 1,4-bis-(4-methoxyphenyl)-1*H*-imidazole (**5i**) (100 mg, 0.36 mmol) according to the procedure reported above. The product was purified by preparative TLC (ethyl acetate), yield: 26 % (24 mg); yellow powder; ^{1}H NMR (CD_3COCD_3): 9.43 (d, *J* = 1.50 Hz, 1H), 8.32 (d, *J* = 1.50 Hz, 1H), 7.74 (dd, *J* = 2.20 Hz and *J* = 8.80 Hz, 2H), 7.71 (dd, *J* = 2.20 Hz and *J* = 8.80 Hz, 2H), 7.10 (dd, *J* = 2.20 Hz and *J* = 8.80 Hz, 2H), 7.08 (dd, *J* = 2.20 Hz and *J* = 8.80 Hz, 2H); ^{13}C NMR (CD_3COCD_3): 160.40, 128.70 (2C), 125.30,

117.70 (2C), 117.45 (2C), 117.25; IR: 3563, 3155, 1684, 1048, 931, 836 cm^{-1}; MS (ESI): 253 $(M+H)^+$; Anal. calcd $C_{15}H_{12}N_2O_2$. C, 71.42; H, 4.79; N, 11.10. Found C, 71.43; H, 4.85; N, 11.11.

3-[4-(4-Hydroxyphenyl)-1*H*-imidazol-4-yl]phenol (**6**). The title compound was prepared by reaction of 4-(4-methoxyphenyl)-1-(3-methoxyphenyl)-1*H*-imidazole (**6i**) (100 mg, 0.36 mmol) according to the procedure reported above. The product was purified by preparative TLC (ethyl acetate); yield: 26 % (24 mg); yellow oil; ^{1}H NMR (CD_3COCD_3): 9.50 (d, *J* = 1.50 Hz, 1H), 8.40 (d, *J* = 1.50 Hz, 1H), 7.77 (m, 2H), 7.50 (t, *J* = 8.20 Hz, 1H), 7.36-7.34 (m, 2H), 7.16 (dd, *J* = 2.20 Hz and *J* = 8.80 Hz, 1H), 7.04 (dt, *J* = 2.20 Hz and *J* = 8.20 Hz, 2H); ^{13}C NMR (CD_3COCD_3): 137.45, 132.50, 128.80 (2C), 118.35, 117.50, 114.35, 110.70; IR: 3542, 3160, 2955, 1699, 1630, 1062, 841 cm^{-1}; MS (ESI): 253 $(M+H)^+$; Anal. calcd $C_{15}H_{12}N_2O_2$. C, 71.42; H, 4.79; N, 11.10. Found C, 71.59; H, 4.92; N, 10.97.

3-[2-(4-Hydroxyphenyl)-1*H*-imidazol-5-yl]phenol (**9**). The title compound was prepared by reaction of 2-(4-methoxyphenyl)-5-(3-methoxyphenyl)-1*H*-imidazole (**9i**) (40 mg, 0.14 mmol) according to the procedure reported above. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 45 % (15 mg); yellow powder; ^{1}H NMR (CD_3COCD_3): 8.58 (s, 1H), 7.42 (t, *J* = 7.80 Hz, 2H), 7.40 (m, 1H), 7.33 (m, 1H), 7.27 (t, *J* = 7.80 Hz, 2H), 7.05 (dd, *J* = 0.90 Hz and *J* = 1.50 Hz, 1H), 6.90 (dd, *J* = 0.90 Hz and *J* = 1.50 Hz, 1H), 6.47 (s, 1H, N-H); ^{13}C NMR (CD_3COCD_3): 168.95, 168.90, 158.25, 137.90, 136.95, 130.10 (2C), 119.40, 119.10, 118.70, 115.25; IR: 3450, 2950, 1604, 1580, 785 cm^{-1}. MS (ESI): 253 $(M+H)^+$; Anal. calcd $C_{15}H_{12}N_2O_2$. C, 71.42; H, 4.79; N, 11.10. Found C, 71.23; H, 4.64; N, 10.98.

4,4'-(1*H*-Imidazol-2,5-diyl)diphenol (**10**). The title compound was prepared by reaction of 2,5-bis-(4-methoxyphenyl)-1*H*-imidazole (**10i**) (82 mg, 0.29 mmol) according to the procedure reported above. The product was purified by preparative TLC (dichloromethane/methanol 99:1); yield: 17 % (12 mg); yellow powder; ^{1}H NMR (CD_3OD): 7.83 (d, *J* = 8.70 Hz, 2H), 7.62 (d, *J* = 8.70 Hz, 2H), 7.60 (s, 1H), 7.03 (d, *J* = 8.70 Hz, 2H), 6.92 (d, *J* = 8.70 Hz, 2H); ^{13}C NMR (CD_3OD): 131.30, 129.15, 120.15 (2C), 119.80 (2C), 115.55 (2C), 114.75 (2C), 114.30; IR: 2590, 1645, 1488, 1114, 841 cm^{-1}; MS (ESI): 253 $(M+H)^+$; Anal. calcd $C_{15}H_{12}N_2O_2$. C, 71.42; H, 4.79; N, 11.10. Found C, 71.30; H, 4.52; N, 11.00.

3-[5-(4-Hydroxyphenyl)-1*H*-imidazol-2-yl]phenol (**11**). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-5-(4-methoxyphenyl)-1*H*-imidazole (**11i**) (40 mg, 0.14 mmol) according to the procedure reported above. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 45 % (15 mg); yellow powder; ^{1}H NMR (CD_3COCD_3): 8.56 (s, 1H), 7.40 (t, *J* = 7.80 Hz, 2H), 7.39 (m, 1H), 7.37 (m, 1H), 7.25 (t, *J* = 7.80 Hz, 2H), 6.99 (dd, *J* = 0.90 Hz and *J* = 1.50 Hz, 1H), 6.97 (dd, *J* = 0.90 Hz and *J* = 1.50 Hz, 1H), 6.47 (s, 1H, NH); ^{13}C NMR (CD_3COCD_3): 168.95, 168.90, 158.25, 137.95, 136.90, 130.15 (2C), 119.30 (2C), 118.95, 115.40; IR: 3350, 3045, 2922, 1664, 1582, 760 cm^{-1}; MS (ESI): 253 $(M+H)^+$; Anal. calcd $C_{15}H_{12}N_2O_2$. C, 71.42; H, 4.79; N, 11.10. Found C, 71.42; H, 4.89; N, 11.08.

Ether cleavage – general synthesis for compounds 12, 18 and 19: To a solution of bis(methoxyphenyl) derivative (1 eq) in dry dichloromethane at -78°C (dry ice /acetone bath), boron tribromide (1 M in dichloromethane, 6 eq) were added dropwise and the reaction mixture was stirred for 20 h. To quench the reaction, water was added and the aqueous layer was washed with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, evaporated under reduced pressure and purified by preparative TLC.

3-[3-(4-Hydroxyphenyl)-1*H*-pyrazol-5-yl]phenol (**12**). The title compound was prepared by reaction of 3-(4-methoxyphenyl)-5-(3-methoxyphenyl)-pyrazole (**12i**) (82 mg, 0.29 mmol) according to the procedure reported above. The product was purified by preparative TLC (dichloromethane/methanol 92:8); yield: 55 % (40 mg); orange powder; mp: 262 °C; ^{1}H NMR (CD_3OD): 7.65 (d, *J* = 8.50 Hz, 2H), 7.22 (m, 1H), 6.83 (d, *J* = 8.50 Hz, 2H), 6.81 (s, 1H), 6.72-6.74 (m, 3H); ^{13}C NMR (CD_3OD): 160.50, 131.85, 122.10, 122.10, 117.05, 116.80, 116.00, 113.30 (2C), 102.15; IR: 3500, 2935, 1620, 790 cm^{-1}; MS (ESI): 253 $(M+H)^+$; Anal. calcd $C_{15}H_{12}N_2O_2$. C, 71.42; H, 4.79; N, 11.10. Found C, 71.38; H, 4.71; N, 11.25.

3-[5-(4-Hydroxyphenyl)-1,3-oxazol-2-yl]phenol (**18**). The title compound was prepared by reaction of 5-(4-methoxyphenyl)-2-(3-methoxyphenyl)-oxazole (**18i**) (100 mg, 0.35 mmol) according to the procedure reported above. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 65 % (59 mg); yellow powder; ^{1}H NMR (CD_3COCD_3): 8.80 (s, 1H, OH), 8.75 (s, 1H, OH), 7.69 (d, *J* = 8.20 Hz, 2H), 7.60 (m, 2H), 7.46 (s, 1H, H-oxazole), 7.35 (t, *J* = 8.20 Hz, 1H), 6.98-6.95 (m, 3H); ^{13}C NMR (CD_3COCD_3): 160.90, 158.95, 158.75, 152.55, 130.95, 129.80, 126.80, 122.50, 120.65, 118.25 (2C), 118.15, 116.85, 115.40, 113.55; IR: 3480, 1602, 1510, 852 cm^{-1}; MS (ESI): 254 $(M+H)^+$; Anal. calcd $C_{15}H_{11}NO_3$. C, 71.14; H, 4.38; N, 5.53. Found C, 70.92; H, 4.35; N, 5.60.

3-[4-(4-Hydroxyphenyl)-1,3-oxazol-2-yl]phenol (**19**). The title compound was prepared by reaction of 4-(4-methoxyphenyl)-2-(3-methoxyphenyl)oxazole (**19i**) (100 mg, 0.36 mmol) according to the procedure reported above. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 82 % (74 mg); yellow powder; ^{1}H NMR (CD_3COCD_3): 8.27 (s, 1H, H-oxazole), 7.93 (d, *J* = 8.50 Hz, 2H), 7.37 (s, 1H), 7.33 (d, *J* = 7.60 Hz, 1H), 7.20 (t, *J* = 7.60 Hz, 1H), 6.97 (d, *J* = 8.50 Hz, 2H), 6.79 (m, 1H); ^{13}C NMR (CD_3COCD_3): 161.80, 159.70, 157.75, 141.55, 133.70, 132.90, 129.70, 128.05, 119.25, 116.70, 115.75, 114.90, 112.35; IR: 3300, 1595, 1259, 804 cm^{-1}; MS (ESI): 252 $(M-H)^-$; Anal. calcd $C_{15}H_{11}NO_3$. C, 71.14; H, 4.38; N, 5.53. Found C, 71.00; H, 4.48; N, 5.58.

4,4'-(1,3-Oxazol-2,5-diyl)diphenol (**17**). The title compound was prepared by reaction of 2,5-bis-(4-methoxyphenyl)-oxazole (**17i**) (260 mg, 0.90 mmol, 1 eq) and pyridinium hydrochloride (2.90 g, 25.7 mmoles, 37 eq) heated to 220 °C for 18 h. After cooling to rt, water (10 mL) and ethyl acetate (20 mL) were added. The aqueous layer was washed with ethyl acetate. The combined organic layers were dried over sodium sulfate, filtered, evaporated under reduced pressure and purified by preparative TLC (hexane/ethyl acetate 5/5); yield: 82 % (186 mg), yellow powder; mp: 163 °C; ^{1}H NMR (CD_3OD): 7.89 (d, *J* = 7.80 Hz, 2H), 7.60 (d, *J* = 8.80 Hz, 2H), 7.32 (s, 1H), 6.91-6.86 (m, 4H); ^{13}C NMR (CD_3OD): 162.35, 161.30, 159.35, 152.78, 132.80, 129.00 (2C), 126.80 (2C), 125.80 (2C), 116.90 (2C); IR: 3387, 1611, 1506, 1170, 834 cm^{-1}; MS (ESI): 254 $(M+H)^+$; Anal. calcd $C_{15}H_{11}NO_3$. C, 71.14; H, 4.38; N, 5.53. Found C, 71.02; H, 4.18; N, 5.63.

Biological Methods

[2, 4, 6, 7-^{3}H]-E2 and [2, 4, 6, 7-^{3}H]-E1 were bought from Perkin Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt.
17β-HSD1 and 17β-HSD2 were obtained from human placenta according to previously described procedures.[40, 73] Fresh human placenta was homogenised and the enzymes were separated by centrifugation. For the purification of 17β-HSD1, the cytosolic fraction was precipitated with ammonium sulfate. 17β-HSD2 was obtained from the microsomal fraction.

1. Inhibition of 17β-HSD1

Inhibitory activities were evaluated by an established method with minor modifications.[54, 74, 75] Briefly, the enzyme preparation was incubated with NADH [500µM] in the presence of potential inhibitors at 37 °C in a phosphate buffer (50 mM) supplemented with 20 % of glycerol and EDTA 1mM**.** Inhibitor stock solutions were prepared in DMSO. The final concentration of DMSO was adjusted to 1 % in all samples. The enzymatic reaction was started by addition of a mixture of unlabelled- and [2, 4, 6, 7-^{3}H]-E1 (final concentration: 500 nM, 0.15 µCi). After 10 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with diethylether. After evaporation, the steroids were dissolved in acetonitrile. E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 reverse phase chromatography column (Nucleodur C18 Gravity, 3 µm, Macherey-Nagel, Düren) connected to a HPLC-system (Agilent 1100 Series, Agilent Technologies, Waldbronn). Detection and quantification of the steroids were performed using a radioflow detector (Berthold Technologies, Bad Wildbad). The conversion rate was calculated after analysis of the resulting chromatograms according to following equation: $\%conversion = \frac{AUC(E2)}{AUC(E2)+AUC(E1)} \times 100$. Each value was calculated from at least three independent experiments.

2. Inhibition of 17β-HSD2

The 17β-HSD2 inhibition assay was performed similarly to the 17β-HSD1 procedure. The microsomal fraction was incubated with NAD^+ [1500 µM], test compound and a mixture of unlabelled- and [2, 4, 6, 7-^{3}H]-E2 (final concentration: 500 nM, 0.11 µCi) for 20 min at 37°C. Further treatment of the samples and HPLC separation was carried out as mentioned above.
The conversion rate was calculated after analysis of the resulting chromatograms according to following equation: $\%conversion = \frac{AUC(E1)}{AUC(E1) + AUC(E2)} \times 100$.

3. ER affinity

The binding affinity of select compounds to the ERα and ERβ was determined according to Zimmermann et al.[76] Briefly, 0.25 pmol of ERα or ERβ, respectively, were incubated with [2, 4, 6, 7-^{3}H]-E2 (10 nM) and test compound for 1 h at room temperature. The potential inhibitors were dissolved in DMSO (5 % final concentration). Non-specific-binding was performed with diethylstilbestrol (10 µM). After incubation, ligand-receptor complexes were selectively bound to hydroxyapatite (5 g/ 60 mL TE-buffer). The complex formed was separated, washed and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (Rack Beta Primo 1209, Wallac, Turku). For determination of the relative binding affinity (RBA), inhibitor and E2 concentrations required to displace 50 % of the receptor bound labelled E2 were determined. RBA values were calculated according to the following equation: $RBA[\%] = \frac{IC_{50}(E2)}{IC_{50}(compound)} \times 100$. The RBA value for E2 was arbitrarily set at 100 %.

4. T-47D cell assays

4.1 Evaluation of the estrogenic activity

Phenol red-free medium was supplemented with sodium bicarbonate (2 g/L), streptomycin (100 µg/mL), insuline zinc salt (10 µg/mL), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/mL) and DCC-FCS 5% (vol/vol). RPMI 1640 (without phenol red) was used for the experiments. Cells (7500 cells/96-wellplate) were grown for 48 h in phenol red-free medium. Compound **18** was added at a final concentration of 100 nM. Inhibitors and E2 were diluted in ethanol (final ethanol concentration was adjusted to 1%). As a positive control E2 was added at a final concentration of 0.1 nM. Ethanol was used as negative control. Medium was changed every two to three days and supplemented with the respective additive. After eight days of incubation, the cell viability was evaluated measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT). The cleavage of MTT to a blue formazan by mitochondrial succinat-dehydrogenase was quantified spectrophotometrically at 590 nm as described by Denizot and Lang[77] with minor modifications. The control proliferation was arbitrarily set at 1 and the stimulation induced by the inhibitor was calculated according to following equation: $\%stimulation = \frac{[proliferation(compound-induced)-1]}{[proliferation(E2-induced)-1]} \times 100\%$. Each value is calculated as a mean value of at least three independent experiments.

4.2 Evaluation of the 17β-HSD1 activity

A stock culture of T-47D cells was grown in RPMI 1640 medium supplemented with 10 % FCS, L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL), insulin-zinc-salt (10 µg/mL) and sodium pyruvate (1 mM) at 37 °C under 5 % CO_2 humidified atmosphere.

The cells were seeded into a 24-well plate at $1x10^6$ cells/well in DMEM medium with FCS, L-glutamine and the antibiotics added in the same concentrations as mentioned above. After 24 h the medium was changed for fresh serum free DMEM and a solution of test compound in DMSO was added. Final concentration of DMSO was adjusted to 1 % in all samples. After a pre-incubation of 30 min at 37°C with 5 % CO_2, the incubation was started by addition of a mixture of unlabelled- and [2, 4, 6, 7-^{3}H]- E1 (final concentration : 50 nM, 0.15 µCi). After 2.5 h incubation, the enzymatic reaction was stopped by removing of the supernatant medium. The steroids were extracted into diethylether. Further treatment of the samples was carried out as mentioned for the 17β-HSD1 assay.

5. CaCo-2 assay

CaCo-2 cell culture and transport experiments were performed according to Yee[78] with small modifications. Cell culture time was reduced from 21 to 10 days by increasing seeding density from $6.3 \cdot 10^4$ to $1.65 \cdot 10^5$ cells per well. Four reference compounds (atenolol, testosterone, ketoprofen, erythromycin) were used in each assay for validation. The compounds were applied to the cells as a mixture (cassette dosing) to increase the throughput. The initial concentration of the compounds in the donor compartment was 50 µM (0.2 M MES, pH: 6.5, containing either 1 % ethanol or DMSO). Samples were taken from the acceptor side after 0 min, 60, 120 and 180 min and from the donor side after 0 and 180 min. Each experiment was run in triplicate. The integrity of the monolayers was checked by measuring the transepithelial electrical resistance (TEER) before the transport experiments and by measuring lucifer yellow permeability after each assay. All samples of the CaCo-2 transport experiments were analysed by LC/MS/MS after dilution with buffer of the opposite transwell chamber (1:1, containing 2 % acetic acid). The apparent permeability coefficients (P_{app}) were calculated using equation $P_{app} = \frac{dQ}{dtAc_0}$, where $\frac{dQ}{dt}$ is the appearance rate of mass in the acceptor compartment, A the surface area of the transwell membrane, and c_0 the initial concentration in the donor compartment.

Molecular Modeling

The X-ray structure of 17β-HSD1 (PDB-code: 1FDT) was obtained from the Protein Data Bank.[44] Water molecules, E2 and sulfate ions were removed from the PDB file and hydrogen atoms and neutral end groups were added. Close contacts were fixed (Arg37) and correct atom types were set.
Docking of inhibitors into the substrate binding site was performed by the automated docking program GOLD 3.0.[79]

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References

1. Travis, R. C.; Key, T. J. *Breast Cancer Res.* **2003**, *5*, 239.
2. Dizerega, G. S.; Barber, D. L.; Hodgen, G. D. *Fertil. Steril.* **1980**, *33*, 649.
3. Miller, W. R.; Bartlett, J. M.; Canney, P.; Verrill, M. *Breast Cancer Res. Treat.* **2007**, *103*, 149.
4. Bush, N. J. *Semin. Oncol. Nurs.* **2007**, *23*, 46.
5. Adamo, V.; Iorfida, M.; Montalto, E.; Festa, V.; Garipoli, C.; Scimone, A.; Zanghi, M.; Caristi, N. *Ann. Oncol.* **2007**, 18 Suppl 6, vi53.
6. Penning, T. M. *Endocr. Relat. Cancer* **1996**, *3*, 41.
7. Luu-The, V. *J. Steroid Biochem. Mol. Biol.* **2001**, *76*, 143.
8. Lukacik, P.; Kavanagh, K. L.; Oppermann, U. *Mol. Cell. Endocrinol.* **2006**, *248*, 61.
9. Labrie, F. *Mol. Cell. Endocrinol.* **1991**, *78*, C113.

10. Vihko, P.; Isomaa, V.; Ghosh, D. *Mol. Cell. Endocrinol.* **2001**, *171*, 71.
11. Gunnarsson, C.; Hellqvist, E.; Stål, O. *Br. J. Cancer*. **2005**, *92*, 547.
12. Gunnarsson, C.; Olsson, B. M.; Stål, O. *Cancer Res.* **2001**, *61*, 8448.
13. Miyoshi, Y.; Ando, A.; Shiba, E.; Taguchi, T.; Tamaki, Y.; Noguchi, S. *Int. J. Cancer* **2001**, *94*, 685.
14. Suzuki, T.; Moriya, T.; Ariga, N.; Kaneko, C.; Kanazawa, M.; Sasano, H. *Br. J. Cancer* **2000**, *82*, 518.
15. Vihko, P.; Harkonen, P.; Soronen, P.; Torn, S.; Herrala, A.; Kurkela, R.; Pulkka, A.; Oduwole, O.; Isomaa, V. *Mol. Cell. Endocrinol.* **2004**, *215*, 83.
16. Cadot, C.; Laplante, Y.; Kamal, F.; Luu-The, V.; Poirier, D. *Bioorg. Med. Chem*. **2007**, *15*, 714.
17. Poirier, D. *Curr. Med. Chem.* **2003**, *10*, 453.
18. Vihko, P.; Isomaa, V. WO2004080271, **2004**.
19. Hirvelä, L. WO2005032527, **2005**.
20. Messinger, J.; Thole, H.; Husen, B.; Van Steen, B. J.; Schneider, G.; Hulshof, J.; Koskimies, P. WO2005047303, **2005**.
21. Hillisch, A.; Regenhardt, W.; Gege, C.; Peters, O.; Bothe, U.; Adamski, J.; Möller, G.; Rosinus, A.; Elger, W.; Schneider, B. WO2006003013A2, **2006**.
22. Vicker, N.; Lawrence, H. R.; Allan, G. M.; Bubert, C.; Smith, A.; Tutill, H. J.; Purohit, A.; Day, J. M.; Mahon, M. F.; Reed, M. J.; Potter, B. V. *ChemMedChem* **2006**, *1*, 464.
23. Poirier, D.; Boivin, R. P.; Tremblay, M. R.; Berube, M.; Qiu, W.; Lin, S.-X. *J. Med. Chem.* **2005**, *48*, 8134.
24. Tremblay, M. R.; Boivin, R. P.; Luu-The, V.; Poirier, D. *J. Enzyme Inhib. Med. Chem.* **2005**, *20*, 153.
25. Lawrence, H. R.; Vicker, N.; Allan, G. M.; Smith, A.; Mahon, M. F.; Tutill, H. J.; Purohit, A.; Reed, M. J.; Potter, B. V. *J. Med. Chem*. **2005**, *48*, 2759.
26. Fischer, D. S.; Allan, G. M.; Bubert, C.; Vicker, N.; Smith, A.; Tutill, H. J.; Purohit, A.; Wood, L.; Packham, G.; Mahon, M. F.; Reed, M. J.; Potter, B. V. *J. Med. Chem.* **2005**, *48*, 5749.
27. Allan, G. M.; Bubert, C.; Vicker, N.; Smith, A.; Tutill, H. J.; Purohit, A.; Reed, M. J.; Potter, B. V. *Mol. Cell. Endocrinol*. **2006**, *248*, 204.
28. Allan, G. M.; Lawrence, H. R.; Cornet, J.; Bubert, C.; Fischer, D. S.; Vicker, N.; Smith, A.; Tutill, H. J.; Purohit, A.; Day, J. M.; Mahon, M. F.; Reed, M. J.; Potter, B. V. *J. Med. Chem.* **2006**, *49*, 1325.
29. Poirier, D.; Chang, H. J.; Azzi, A.; Boivin, R. P.; Lin, S.-X. *Mol. Cell. Endocrinol.* **2006**, *248*, 236.
30. Ciobanu, L. C.; Poirier, D. *ChemMedChem* **2006**, *1*, 1249.
31. Allan, G. M.; Vicker, N.; Lawrence, H. R.; Tutill, H. J.; Day, J. M.; Huchet, M.; Ferrandis, E.; Reed, M. J.; Purohit, A.; Potter, B. V. *Bioorg. Med. Chem.* **2008**, *16*, 4438.
32. Messinger, J.; Hirvela, L.; Husen, B.; Kangas, L.; Koskimies, P.; Pentikainen, O.; Saarenketo, P.; Thole, H. *Mol. Cell. Endocrinol*. **2006**, *248*, 192.
33. Karkola, S.; Lilienkampf, A.; Wahala, K. *ChemMedChem* **2008**, *3*, 461.
34. Frotscher, M.; Ziegler, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Neugebauer, A.; Fetzer, L.; Scherer, C.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W. *J. Med. Chem.* **2008**, *51*, 2158.
35. Breton, R.; Housset, D.; Mazza, C.; Fontecilla-Camps, J. C. *Structure* **1996**, *4*, 905.
36. Ghosh, D.; Pletnev, V. Z.; Zhu, D. W.; Wawrzak, Z.; Duax, W. L.; Pangborn, W.; Labrie, F.; Lin, S.-X. *Structure* **1995**, *3*, 503.
37. Han, Q.; Campbell, R. L.; Gangloff, A.; Huang, Y. W.; Lin, S.-X. *J. Biol. Chem.* **2000**, *275*, 1105.
38. Sawicki, M. W.; Erman, M.; Puranen, T.; Vihko, P.; Ghosh, D. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 840.
39. Mazza, C.; Breton, R.; Housset, D.; Fontecilla-Camps, J. C. *J. Biol. Chem.* **1998**, *273*, 8145.
40. Qiu, W.; Campbell, R. L.; Gangloff, A.; Dupuis, P.; Boivin, R. P.; Tremblay, M. R.; Poirier, D.; Lin, S.-X. *FASEB J.* **2002**, *16*, 1829.
41. Azzi, A.; Rehse, P. H.; Zhu, D. W.; Campbell, R. L.; Labrie, F.; Lin, S.-X. *Nat. Struct. Biol*. **1996**, *3*, 665.
42. Gangloff, A.; Shi, R.; Nahoum, V.; Lin, S.-X. *FASEB J*. **2003**, *17*, 274.

43. Shi, R.; Lin, S.-X. *J. Biol. Chem.* **2004**, *279*, 16778.
44. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. *Nucleic Acids Res.* **2000**, *28*, 235.
45. Filling, C.; Berndt, K. D.; Benach, J.; Knapp, S.; Prozorovski, T.; Nordling, E.; Ladenstein, R.; Jornvall, H.; Oppermann, U. *J. Biol. Chem.* **2002**, *277*, 25677.
46. Fink, B. E.; Mortensen, D. S.; Stauffer, S. R.; Aron, Z. D.; Katzenellenbogen, J. A. *Chem. Biol.* **1999**, *6*, 205.
47. Prakash, O.; Ranjani, S. N.; Goyal, S.; Tomar, R. K.; Singh, S. P. *Indian J. Chem. B: Org. Chem. Incl. Med. Chem.* **1994**, *33*, 116.
48. Dodson, R. M.; Ross, F. *J. Am. Chem. Soc.* **1950**, *72*, 1478.
49. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2001**, *40*, 2004.
50. Suzuki, M.; Maeda, S.; Matsumoto, K.; Ishizuka, T.; Iwasawa, Y. *Chem. Pharm. Bull.* **1986**, *34*, 3111.
51. Nicolaou, K. C.; Hao, J.; Reddy, M. V.; Rao, P. B.; Rassias, G.; Snyder, S. A.; Huang, X.; Chen, D. Y. K.; Brenzovich, W. E.; Giuseppone, N.; Giannakakou, P.; O'Brate, A. *J. Am. Chem. Soc.* **2004**, *126*, 12897.
52. Simon, D.; Lafont, O.; Farnoux, C.; Combet-Miocque, M. *J. Het. Chem.* **1985**, *22*, 1551.
53. Lee, J. C.; Kim, S.; Lee, Y. C. *Synth. Commun.* **2003**, *33*, 1611.
54. Lin, S.-X.; Yang, F.; Jin, J. Z.; Breton, R.; Zhu, D. W.; Luu-The, V.; Labrie, F. *J. Biol. Chem.* **1992**, *267*, 16182.
55. Hidalgo, I. J. *Curr. Top. Med. Chem.* **2001**, *1*, 385.
56. Mewshaw, R. E.; Edsall, R. J. J.; Yang, C.; Manas, E. S.; Xu, Z. B.; Henderson, R. A.; Keith, J. C., Jr.; Harris, H. A. *J. Med. Chem.* **2005**, *48*, 3953.
57. Nobeli, I.; Price, S. L.; Lommerse, J. P. M.; Taylor, R. *J. Comp. Chem.* **1997**, *18*, 2060.
58. Stauffer, S. R.; Coletta, C. J.; Tedesco, R.; Nishiguchi, G.; Carlson, K.; Sun, J.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A. *J. Med. Chem.* **2000**, *43*, 4934.
59. Stauffer, S. R.; Huang, Y.; Coletta, C. J.; Tedesco, R.; Katzenellenbogen, J. A. *Bioorg. Med. Chem.* **2001**, *9*, 141.
60. Stauffer, S. R.; Huang, Y. R.; Aron, Z. D.; Coletta, C. J.; Sun, J.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A. *Bioorg. Med. Chem.* **2001**, *9*, 151.
61. Coskun, N.; Tat, F. T.; Guven, O. O. *Tet. Assym.* **2001**, *12*, 1463.
62. Coskun, N.; Tat, F. T. *Turkish J. Chem.* **2004**, *28*, 1.
63. Pirali, T.; Gatti, S.; Di Brisco, R.; Tacchi, S.; Zaninetti, R.; Brunelli, E.; Massarotti, A.; Sorba, G.; Canonico, P. L.; Moro, L.; Genazzani, A. A.; Tron, G. C.; Billington, R. A. *ChemMedChem* **2007**, *2*, 437.
64. Pulici, M.; Quartieri, F.; Felder, E. R. *J. Comb. Chem.* **2005**, *7*, 463.
65. Shvaika, O. P.; Korzhenevskaya, N. G.; Snagoshchenko, L. P. *KhGS [Chem. Het. Comp.]* **1985**, *2*, 193.
66. Butler, R. N.; Cloonan, M. O.; McMahon, J. M.; Burke, L. A. *J. Chem. Soc., Perkin Trans. 1* **1999**, *12*, 1709.
67. Widler, L.; Green, J.; Missbach, M.; Susa, M.; Altmann, E. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 849.
68. Bowman, M. D.; Jacobson, M. M.; Blackwell, H. E. *Org. Lett.* **2006**, *8*, 1645.
69. Bhat, B. A.; Puri, S. C.; Qurishi, M. A.; Dhar, K. L.; Qazi, G. N. *Synth. Commun.* **2005**, *35*, 1135.
70. Babar, L.; Tomar, S. S. *Pest. Res. J.* **2005**, *17*, 1.
71. Huebner, V. D.; Lin, X.; James, I.; Chen, L.; Desai, M.; Moore, J. C.; Krywult, B.; Navaratnam, T.; Singh, R.; Trainor, R.; Wang, L. WO1999US17798, **2000**.
72. Schwartz, M. A.; Rose, B. F.; Holton, R. A.; Scott, S. W.; Vishnuvajjala, B. *J. Am. Chem. Soc.* **1977**, *99*, 2571.
73. Zhu, D. W.; Lee, X.; Breton, R.; Ghosh, D.; Pangborn, W.; Daux, W. L.; Lin, S.-X. *J. Mol. Biol.* **1993**, *234*, 242.
74. Sam, K. M.; Auger, S.; Luu-The, V.; Poirier, D. *J. Med. Chem.* **1995**, *38*, 4518.
75. Sam, K. M.; Boivin, R. P.; Tremblay, M. R.; Auger, S.; Poirier, D. *Drug. Des. Discov.* **1998**, *15*, 157.
76. Zimmermann, J.; Liebl, R.; von Angerer, E. *J. Steroid Biochem. Mol. Biol.* **2005**, *94*, 57.

77. Denizot, F.; Lang, R. *J. Immunol. Methods* **1986**, *89*, 271.
78. Yee, S. *Pharm. Res.* **1997**, *14*, 763.
79. Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. *J. Mol. Biol.* **1997**, *267*, 727.

3.II The Role of the Heterocycle in Bis(hydroxyphenyl)triazoles for Inhibition of 17β-Hydroxysteroid Dehydrogenase (17β-HSD) Type 1 and Type 2

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Paper II

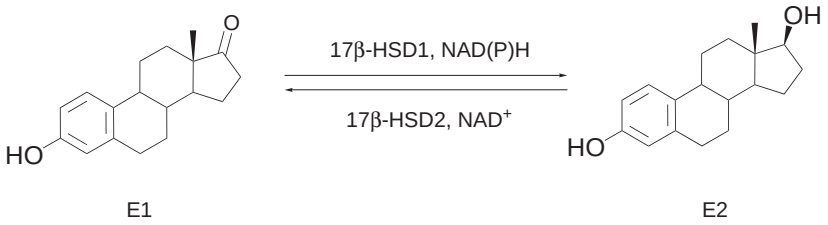
abstract: 17β Hydroxysteroid dehydrogenase type 1 (17β-HSD1) is responsible for the catalytic reduction of the weak estrogen estrone (E1) into the highly potent 17β-estradiol (E2). As 17β-HSD1 is often overexpressed in mammary tumors and endometriosis, the selective inhibition of this enzyme is discussed as a promising approach for the treatment of estrogen-dependent diseases. Recently, we reported on bis(hydroxyphenyl)azoles as a new class of potent inhibitors of 17β-HSD1. In this paper, we focused on bis(hydroxyphenyl)triazoles. The influence of nitrogens on the potency as well as the space available around the heterocycle was investigated. Substituents were introduced on the triazole core in order to establish additional interactions with the enzyme active site. The compounds were evaluated for activity towards 17β-HSD1 and selectivity with regard to 17β-HSD2, the enzyme which is responsible for the deactivation of E2 into E1. 3-[4-(4-Hydroxyphenyl)-1*H*-1,2,3-triazol-1-yl]phenol (**3**) was the most active compound discovered in this study with an IC_{50} value of 840 nM and a reasonable selectivity toward 17β-HSD2.

Introduction

Estrogens are responsible for the development and differentiation of estrogen-sensitive tissues. Furthermore, it is well known that 17β-estradiol (E2), the most active estrogen, also plays a crucial role in the growth of estrogen-dependent mammary tumors[1] and is involved in the pathophysiology of endometriosis.[2]

The potency of steroidal sex hormones like E2 is modulated by enzymes of the 17β-hydroxysteroid dehydrogenase (17β-HSD) family,[3] especially 17β-HSD1 and 17β-HSD2, which catalyze the activation of E2 from estrone (E1) or the deactivation of E2 into E1, respectively (Chart 1). 17β-HSD1 is a cytosolic enzyme present in different organs like ovaries, placenta, breast, endometrium[4] which is often overexpressed in breast cancer tissues[5-8] and endometriosis.[9] Consequently, selective inhibition of 17β-HSD1 is discussed to be a good strategy to reduce the intracellular E2 levels and regarded as a new therapeutic approach for the treatment of estrogen-dependent diseases. As a biological counterpart, 17β-HSD2 should not be affected by potential inhibitors of 17β-HSD1, as it protects the cell from excessively high concentrations of active estrogens.[10]

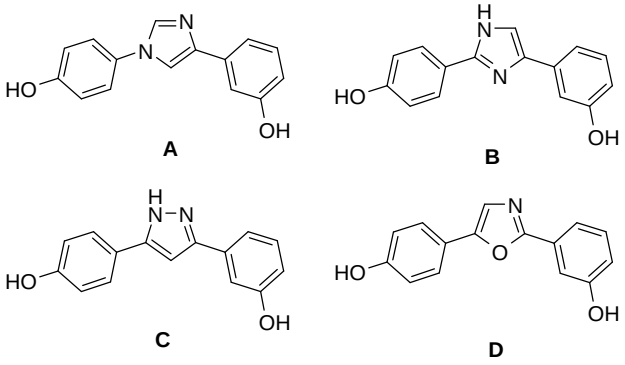
Chart 1: interconversion of estrone (E1) to 17β-estradiol (E2)



E1 E2

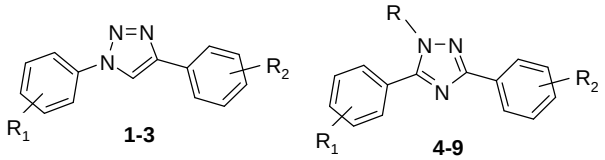
Until now, several groups have reported on 17β-HSD1 inhibitors, most of them showing steroidal structures.[11,12] Concerning the non-steroidal cores, only four compound classes have been described so far: thienopyrimidinones,[13, 14] biphenyl ethanones,[15] from our group 6-(hydroxyphenyl) naphthols[16,17] and recently, we reported on bis(hydroxyphenyl) azoles[19] as potent and selective inhibitors of 17β-HSD1. Different core structures were investigated (Chart 2): imidazoles like compounds **A** and **B**, pyrazoles like compound **C** and oxazoles like compound **D**. The most active was compound **D** with an IC_{50} value of 310 nM and a selectivity factor of 56 against 17β-HSD2.

Chart 2: described bis(hydroxyphenyl) azoles



In the following, we will report on the design, synthesis and biological evaluation of a new family of azoles: bis(hydroxyphenyl) triazoles (Chart 3).

Chart 3: title compounds



compound	R	R_1	R_2
1		3-OH	3-OH
2		4-OH	3-OH
3		3-OH	4-OH
4	CH_3	3-OH	3-OH
5	CH_3	4-OH	3-OH
6	CH_3	3-OH	4-OH
7	Ph	3-OH	3-OH
8	Ph	4-OH	3-OH
9	Ph	3-OH	4-OH

Design of the inhibitors

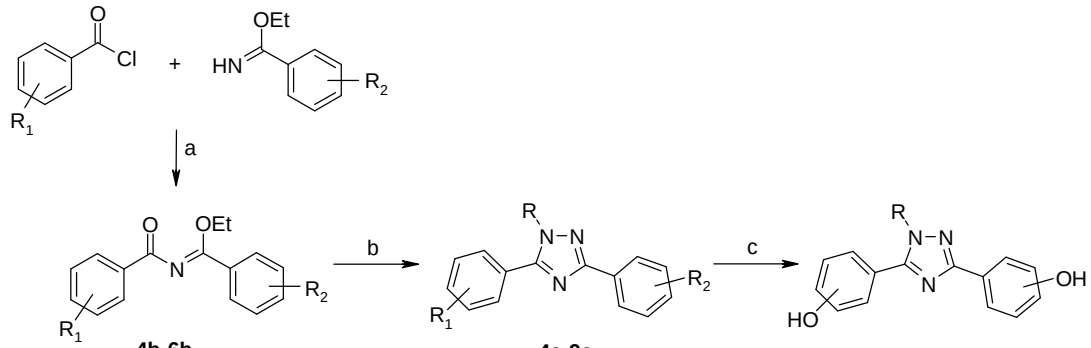
The analysis of the available ternary complex of 17β-HSD1 with E2 and $NADP^+$ (PDB-ID: 1FDT;[18] provided useful informations about the architecture of the active site of the enzyme. A substrate binding site and a cofactor binding pocket can be defined. The former is a narrow hydrophobic tunnel containing two polar areas at each end. His221/Glu282 are located on the one side and Ser142/Tyr155 on the other side, corresponding to the binding oxygens in 3- and 17-hydroxy group of E2. Interestingly, close to the hydrophobic B/C region of the estrogen two polar amino acids, Tyr218 and Ser222, can be found which do not interact with the steroid.
In our design concept, we focused on non-steroidal structures to avoid binding to other steroid receptors. The compounds should be able to mimic the substrate of the enzyme. We have previously reported on the importance of the two hydroxyphenyl moieties present in our steroidomimetics.[19] The positions of the OH substituents on the phenyl groups is decisive for the inhibitory potency of the compounds: only inhibitors bearing a *meta-para* di-OH substitution pattern (O-O distance in the same range as observed for the steroid, d= 11 Å) were active probably due to the establishment of hydrogen bond interactions with His221/Glu282 and Ser142/Tyr155. *Meta-meta* disubstituted compounds which were not investigated so far might also show a favorable geometry to establish these interactions.
Furthermore, the nature of the heterocycle present between the two hydroxyphenyl moieties also influences the activity of the compounds. Heterocycles like oxazole **D** which can act as hydrogen bond acceptors were active while inhibitors bearing a H-bond donator function (NH) like 2,4-disubstituted imidazoles (e.g. compound **B**) and 3,5-disubstituted pyrazoles (e.g. compound **C**) were devoid of activity. It was hypothesized that the activity of compound **D** was linked to its ability to establish hydrogen bond interaction with Tyr218 and/or Ser222.[19]
It is striking that 1,4-disubstituted imidazoles like compound **A**, although pure hydrogen bond acceptors, were inactive. To obtain an insight into the impact of an additional nitrogen atom and on distribution of the nitrogen atoms in the central ring on inhibitory activity, 1,2,3-triazoles **1**-**3** and 1,2,4-triazoles **4**-**9** were designed.
In addition, a non polar substituent was introduced on the heterocycle, anticipating supplementary hydrophobic interactions with amino acid residues of the substrate binding site. Compounds **4**-**9** bearing a methyl or a phenyl on the triazole core could give additional informations on the feasibility of these interactions (π-stacking interactions with Tyr218 for example in case of compounds **7**-**9**) as well as on the space available around the heterocycle.

Chemistry

1,2,3-Triazoles **1-3** were obtained according to the synthetic pathway previously described by Bey et al.[19]

The synthesis of 1,2,4-triazoles **4**-**9** is presented in Scheme 1. Starting from the *N*-acylimidates **4b**-**6b** prepared according to the method described by Kelarev et al.,[21] the dimethoxylated 1,2,4-triazoles **4a**-**9a** were synthesized by nucleophilic addition of methylhydrazine for compounds **4a-6a** and phenylhydrazine for compounds **7a-9a**. The deprotection of the methoxy groups of compounds **4a-9a** was performed with borontrifluoride dimethyl sulfide complex.[19]

Scheme 1[a]: synthesis of compounds **4-9**



4b-6b **4a-9a**

4-6 R= CH_3
7-9 R= Ph

4a: R_1= 3-OMe, R_2= 3-OMe
5a: R_1= 4-OMe, R_2= 3-OMe
6a: R_1= 3-OMe, R_2= 4-OMe
7a: R_1= 3-OMe, R_2= 3-OMe
8a: R_1= 4-OMe, R_2= 3-OMe
9a: R_1= 3-OMe, R_2= 4-OMe

[a]**Reagents and conditions**: (a) CH_2Cl_2, NEt_3, 30-40 °C, 6 h; (b) for compounds **4a-6a** H_3CNHNH_2 and for compounds **7a-9a** $PhNHNH_2$, CH_2Cl_2, 30-40 °C, 4 h; (c) $BF_3 \cdot SMe_2$, CH_2Cl_2, rt , 20 h.

Inhibition of human 17β-HSD1 and 17β-HSD2

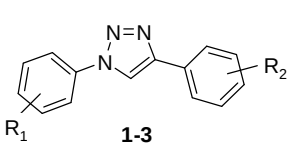
The 17β-HSD1 and 17β-HSD2 inhibition assays were carried out following a previously described procedure.[19] Briefly, the cytosolic (17β-HSD1) or microsomal (17β-HSD2) fraction of human placental was incubated with tritiated substrate, cofactor and inhibitor at 37 °C. After HPLC separation of substrate and product, the amount of radiolabeled E2 (17β-HSD1) or E1 (17β-HSD2) formed was quantified. The inhibition values of compounds **1**-**9** are shown in Table 1.

The *meta-meta* disubstituted 1,2,3-triazole **1** is a weak inhibitor of 17β-HSD1 (17 % inhibition at 1 μM). Shifting one group from the *meta* to the *para* position (compounds **2** and **3**) leads to an increase in activity, compound **3** being the most active one (IC_{50}= 0.84 μM and 1.32 μM for **3** and **2**, respectively). Concerning selectivity, the 1,2,3-triazoles **2** and **3** are very weak inhibitors of the type 2 enzyme (IC_{50}= 8.12 μM and 7.28 μM, respectively).

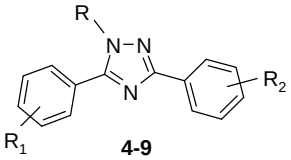
None of the 1,2,4-triazoles (compounds **4**-**9**) shows inhibition of 17β-HSD1. Surprisingly, the *N*-1 phenyl substituted 1,2,4-triazoles (compounds **7** and **8**) are selective inhibitors of 17β-HSD2 (44 % and 41 % inhibition at 1 μM, respectively).

The introduction of an additional nitrogen into the inactive imidazole core structure (compound **A**) enhances inhibition of 17β-HSD1 strongly (compound **2**, IC_{50}= 1.32 μM) whereas a supplementary substituent (methyl for compounds **4**-**6** and phenyl for compounds **7**-**9**) is obviously not tolerated by the enzyme.

Table 1: inhibition of human 17β-HSD1 and 17β-HSD2 by compounds **1**-**9**



1-3



4-9

compound	R	R_1	R_2	IC_{50} value (μM)[a]	
				17β-HSD1[b]	17β-HSD2[c]
1		3-OH	3-OH	17[d]	ni
2		4-OH	3-OH	1.32	8.12
3		3-OH	4-OH	0.84	7.28
4	CH_3	3-OH	3-OH	ni	ni
5	CH_3	4-OH	3-OH	ni	20[d]
6	CH_3	3-OH	4-OH	ni	21[d]
7	Ph	3-OH	3-OH	ni	41[d]
8	Ph	4-OH	3-OH	ni	44[d]
9	Ph	3-OH	4-OH	ni	ni

[a]Mean values of three determinations, standard deviation less than 18 %; [b]Human placental, cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], cofactor NADH [500 μM]; [c]Human placental, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM]; [d]% inhibition at 1 μM; ni: no inhibition (<10 % inhibition at 1 μM).

Discussion and conclusion

As already mentioned in a previous report,[19] the OH substitution pattern on the phenyl ring is a decisive criterion for inhibitory activity. The distance between the *meta-meta* hydroxy group (compound **1**) which is shorter compared to the *para-meta* (compound **2**) and the *meta-para* (compound **3**) might be one explanation for the lower activity of compound **1**.

Varying the heterocycle, two active 1,2,3-triazoles **2** and **3** have been identified. They differ from the inactive 1,4-disubstituted imidazole (compound **A**) by the presence of an additional nitrogen in position 2. It is interesting that the introduction of this supplementary heteroatom leads to a strong increase in activity (**A**: inactive, IC_{50}= 1.32 μM for **2** and 0.84 μM for **3**, respectively). It can be concluded that this nitrogen in position 2 is stabilizing the molecule in the active site via additional interactions. A hydrogen bond interaction between the nitrogen of the heterocycle and the OH of Tyr218 might be responsible for this gain in activity.

We also wanted to investigate whether an additional substituent on the heterocycle might be able to increase the potency of the inhibitor by establishing supplementary interactions. 1,2,4-Triazole was chosen as core structure as none of the nitrogen of the 1,2,3-triazoles can be substituted in an appropriate position where space is available.

The 1,2,4-triazoles are structurally related to the 2,4-disubstituted imidazoles like compound **B** and to the 3,5-disubstituted pyrazoles like compound **C** which are both inactive. It was previously hypothesized that this lack of activity was due to the presence of a hydrogen bond donator (NH). Introduction of a methyl or phenyl group in this position will remove the hydrogen bond donating function of the heteroatom and was hypothesized to increase the ability of the inhibitors to induce hydrophobic interactions with the enzyme active site. The fact that all 1,2,4-triazoles (compounds **4**-**9**) are inactive indicates that either the nitrogen in the vertex position on the heterocycle and/or the supplementary substituent are not tolerated by the enzyme. The introduction of a phenyl moiety into the 1,2,4-triazole core (compounds **7**-**9**) induces conformational constraints: to adopt a stable conformation the hydroxyphenyl moiety close to the phenyl substituent and/or the phenyl itself will rotate out of the heterocyclic plane. This conformational change disturbs the flatness of the inhibitor considerably. The

hydroxyphenyl moieties might therefore not be able to adopt an appropriate conformation to establish hydrogen bond interactions with His221/Glu282 and Ser142/Tyr155, thus leading to compounds devoid of 17β-HSD1 inhibitory activity. The loss of planarity, however, is correlated with an increase of 17β-HSD2 inhibition. This finding is in agreement with the structures of previously described inhibitors of this enzyme[21] which also lack planarity.
In this study, the 1,2,3-triazoles (compounds **2** and **3**) have been identified as a new class of potent and selective inhibitors of 17β-HSD1. The hydroxy substitution pattern (*meta-para* and *para-meta*) of the most active compounds confirms the reduced flexibility of the enzyme active site already observed. The introduction of an additional nitrogen in the inactive 1,4-disubstituted imidazole core structure leads to a highly active inhibitor of 17β-HSD1. In contrast introduction of a substituent on the heterocycle is not tolerated by this enzyme but in case of a phenyl moiety results in fairly potent 17β-HSD2 inhibitors. They might be considered as leads for further structural optimizations in the development of selective 17β-HSD2 inhibitors.

Acknowledgments

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Supplementary material

Synthetic procedures, spectroscopic data of compounds **4-9**. This material is available via internet at http://www.sciencedirect.com.

References

1. Travis, R. C.; Key, T. J. Oestrogen exposure and breast cancer risk. *Breast Cancer Res.* **2003**, *5*, 239-247.
2. Dizerega, G. S.; Barber, D. L.; Hodgen, G. D. Endometriosis: role of ovarian steroids in initiation, maintenance and suppression. *Fertil. Steril.* **1980**, *33*, 649-653.
3. Lukacik, P.; Kavanagh, K. L.; Oppermann, U. Structure and function of human 17beta-hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* **2006**, *248*, 61-71.
4. Vihko, P.; Isomaa, V.; Ghosh, D. Structure and function of 17beta-hydroxysteroid dehydrogenase type 1 and type 2. *Mol. Cell. Endocrinol.* **2001**, *171*, 71-76.
5. Suzuki, T.; Moriya, T.; Ariga, N.; Kaneko, C.; Kanazawa, M.; Sasano, H. 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br. J. Cancer* **2000**, *82*, 518-523.
6. Miyoshi, Y.; Ando, A.; Shiba, E.; Taguchi, T.; Tamaki, Y.; Noguchi, S. Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* **2001**, *94*, 685-689.
7. Gunnarsson, C.; Olsson, B. M.; Stål, O. Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.* **2001**, *61*, 8448-8451.
8. Gunnarsson, C.; Hellqvist, E.; Stål, O. 17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer* **2005**, *92*, 547-552.
9. Šmuc, T.; Pucelj Ribič , M.; Šinkovec, J.; Husen, B.; Thole, H.; Lanišnik Rižner , T. Expression analysis of the genes involved in estradiol and progesterone action in human ovarian endometriosis. *Gynecol. Endocrinol.* **2007**, *23*, 105-111.

10. Vihko, P.; Härkönen, P.; Soronen, P.; Törn, S.; Herrala, A.; Kurkela, R.; Pulkka, A.; Oduwole, O.; Isomaa, V. 17beta-hydroxysteroid dehydrogenases- their role in pathophysiology. *Mol. Cell. Endocrinol.* **2004**, *215*, 83-88.
11. Brožic, P.; Lanišnik Rižner, T.; Gobec, S. Inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Curr. Med. Chem.* **2008**, *15*, 137-150 and references therein cited.
12. Poirier, D. Inhibitors of 17 beta-hydroxysteroid dehydrogenases. *Curr. Med. Chem.* **2003**, *10*, 453-477.
13. Messinger, J.; Hirvelä, L.; Husen, B.; Kangas, L.; Koskimies, P.; Pentikäinen, O.; Saarenketo, P.; Thole, H. New inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* **2006**, *248*, 192-198.
14. Karkola, S.; Lilienkampf, A.; Wähälä, K. A 3D QSAR model of 17beta-HSD1 inhibitors based on a thieno[2,3-d]pyrimidin-4(3H)-one core applying molecular dynamics simulations and ligand-protein docking. *ChemMedChem* **2008**, *3*, 461-472.
15. Allan, G. M.; Vicker, N.; Lawrence, H. R.; Tutill, H. J.; Day, J. M.; Huchet, M.; Ferrandis, E.; Reed, M. J.; Purohit, A.; Potter, B. V. Novel inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: templates for design. *Bioorg. Med. Chem.* **2008**, *16*, 4438-4456.
16. Frotscher, M.; Ziegler, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Neugebauer, A.; Fetzer, L.; Scherer, C.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W. Design, synthesis and biological evaluation of (hydroxyphenyl)-naphthalene and quinoline derivatives: potent and selective non steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* **2008**, *51*, 2158-2169.
17. Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Ziegler, E.; Neugebauer, A.; Bhoga, U. D.; Bey, E.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W. Substituted 6-phenyl-2-naphthols. Potent and selective non-steroidal inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): design, synthesis, biological evaluation and pharmacokinetics. *J. Med. Chem.* **2008**, *51*, 4685-4698.
18. Breton, R.; Housset, D.; Mazza, C.; Fontecilla-Camps, J. C. The structure of a complex of human 17beta-hydroxysteroid dehydrogenase with estradiol and $NADP^+$ identifies two principal targets for the design of inhibitors. *Structure* **1996**, *4*, 905-915.
19. Bey, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Werth, R.; Oster, A.; Algül, O.; Neugebauer, A.; Hartmann, R. W. Design, synthesis and biological evaluation of bis(hydroxyphenyl) azoles as potent and selective non-steroidal inhibitors of 17beta- hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *Bioorg. Med. Chem.* **2008**, *16*, 6423-6435.
20. Pirali, T.; Gatti, S.; Di Brisco, R.; Tacchi, S.; Zaninetti, R.; Brunelli, E.; Massarotti, A.; Sorba, G.; Canonico, P. L.; Moro, L.; Genazzani, A. A.; Tron, G. C.; Billington, R. A. Estrogenic analogues synthesized by click chemistry. *ChemMedChem* **2007**, *2*, 437-440.
21. Kelarev, V. I.; Silin, M. A.; Kobrakov, K. I.; Rybina, I. I.; Korolev, V. K. Synthesis of 1,3,5-trisubstituted 1*H*-1,2,4-triazoles containing hetaryl fragments. *Chem. Heterocycl. Comp.* **2003**, *39*, 736-743.
21. Bydal, P.; Auger, S.; Poirier, D. Inhibition of type 2 17beta-hydroxysteroid dehydrogenase by estradiol derivatives bearing a lactone on the D-ring: structure-activity relationships. *Steroids* **2004**, *69*, 325-342.

3.III Design, Synthesis, Biological Evaluation and Pharmacokinetics of Bis(hydroxyphenyl) substituted Azoles, Thiophenes, Benzenes and Aza-Benzenes as Potent and Selective Non-Steroidal Inhibitors of 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1)

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Paper III

abstract: 17β-Estradiol (E2), the most potent female sex hormone, stimulates the growth of mammary tumours and endometriosis via activation of the estrogen receptor α (ERα). 17β–Hydroxysteroid dehydrogenase type 1 (17β-HSD1), which is responsible for the catalytic reduction of the weakly active estrogen estrone (E1) into E2, is therefore discussed as a novel drug target. Recently, we have discovered a 2,5-bis(hydroxyphenyl) oxazole to be a potent inhibitor of 17β-HSD1. In this paper, further structural optimizations were performed: 39 bis(hydroxyphenyl) azoles, thiophenes, benzenes and aza-benzenes were synthesized and their biological properties were evaluated. The most promising compounds of this study show enhanced IC_{50} values in the low nanomolar range, a high selectivity toward 17β-HSD2, a low binding affinity to ERα, a good metabolic stability in rat liver microsomes and a reasonable pharmacokinetic profile after peroral application. Calculation of the molecular electrostatic potentials revealed a correlation between 17β-HSD1 inhibition and the electron density distribution.

Introduction

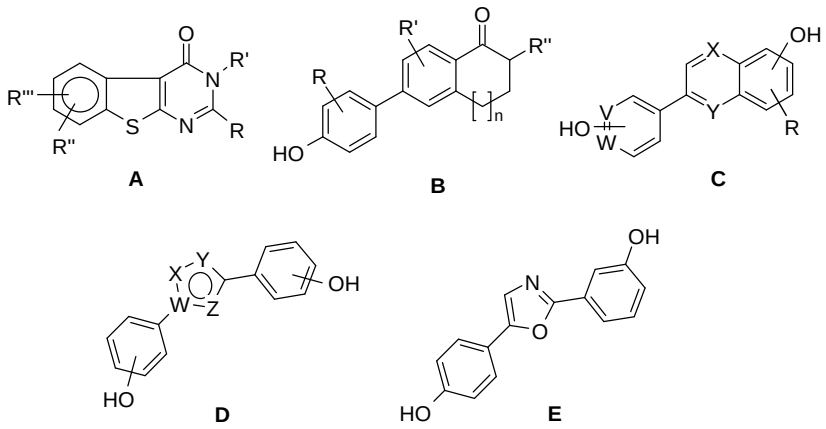
Estrogens, the most potent one being 17β-estradiol (E2), act as female sex hormones and are predominantly produced before menopause by the ovaries. They unfold their activity by stimulation of the estrogen receptors (ERs) α and β. Besides their physiological effects, they are, however, also involved in the initiation and progression of estrogen-dependent diseases like mammary tumor[1] and endometriosis.[2] Presently, the three main endocrine therapies for the treatment of breast cancer are:[3,4] inhibition of estrogen biosynthesis by aromatase inhibitors or GnRH agonists or antagonists and interference with the estrogen action at the receptor level by selective estrogen receptor modulators (SERMs) or pure antiestrogens.[5] Besides specific disadvantages of each therapeutic concept, all of these strategies have in common that they reduce estrogen levels systemically leading to the corresponding side effects.

A softer approach could be inhibition of the enzyme involved in the last step of the E2 biosynthesis: 17β-hydroxysteroid dehydrogenase (17β-HSD) which is able to convert estrone (E1) into E2. There are three subtypes (1, 7 and 12) described, the most important of which is 17β-HSD1. The primary function of 17β-HSD7 and 17β-HSD12 is supposed to be in the cholesterol synthesis[6] and in the regulation of the lipid biosynthesis,[7] respectively. Moreover,

Day et al.[8] showed that 17β-HSD12, although highly expressed in breast cancer cell lines, is inefficient in E2 formation.
17β-HSD1 is NAD(P)H-dependent and intracellularly converts the weak estrogen E1 into the strong estrogen E2. As it is often over-expressed in breast cancer cells[9-12] and endometriosis,[13] 17β-HSD1 is regarded as a promising novel target for the treatment of estrogen-dependent diseases. Appropriate inhibitors of this enzyme should exhibit less side effects compared to the current treatments as they should selectively reduce the concentration of active E2 in the diseased tissues.[14]
As a biological counterpart, 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) catalyzes the deactivation of E2 into E1. It protects the cell from excessively high concentrations of active estrogens[15] and should therefore not be affected by inhibitors of 17β-HSD1. In addition 17β-HSD1 inhibitors should not show affinity to the ERs to avoid intrinsic estrogenic effects.

17β-HSD1 was crystallized with different steroidal ligands.[16-24] The published X-ray structures provide insight into the active site, a narrow hydrophobic tunnel with polar contacts at each end. On one side His221/Glu282 are located, on the other Ser142/Tyr155 (two members of the catalytic tetrad).[25] Surprisingly, close to the hydrophobic B/C region of the steroid two polar amino acids, Tyr218 and Ser222, can be found which do not interact with E2.
Several steroidal and non-steroidal inhibitors of 17β-HSD1 have been described. The first report on steroidal compounds by Penning[26] was as early as 1996. In the last few years several articles of other groups followed.[27] Regarding the non-steroidal inhibitors, only four compound classes have been described so far, all of them very recently: thienopyrimidinones **A**,[28,29] biphenyl ethanones **B**,[30] and from our group 6-(hydroxyphenyl) naphthols **C**[31,32] and bis(hydroxyphenyl) azoles **D**[33] (Chart 1).

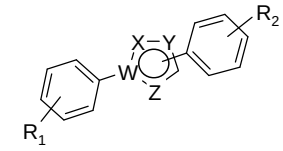
Chart 1: non-steroidal 17β-HSD1 inhibitors



The most promising compound in the latter series was the 2,5-bis(hydroxyphenyl) oxazole **E** with an IC_{50} of 310 nM and a selectivity factor of 56 against 17β-HSD2. In general it was discovered that the inhibitory activity of these compounds depends on the existence of hydroxy rather than methoxy groups on the phenyl substituents and on the OH substitution pattern, *meta-para* and *para-meta* substituted compounds being more active than *para-para* substituted ones. Furthermore it became apparent that inhibition also depends on the nature of the heterocycle. Hydrogen bond donor functions turned out to be inappropriate, whereas in several compounds H-bond acceptor atoms were favorable. This finding led to the hypothesis that active compounds are capable of interacting with Tyr218 or Ser222 via H-bonds.[33]
In order to enhance activity and selectivity and to get a better insight into the interaction of these compounds with the active site of 17β-HSD1, the significance of the OH groups will be

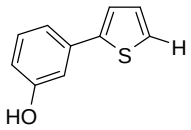
further evaluated and other five-membered heterocycles, especially sulfur containing ones, will be investigated. Furthermore it will be evaluated, whether six-membered rings are also appropriate to connect the two hydroxyphenyl moieties.
In the following we describe the synthesis of 39 bis(hydroxyphenyl) azoles, thiophenes, benzenes and aza-benzenes (Chart 2) as well as the determination of their 17β-HSD1 inhibitory activity and selectivity toward 17β-HSD2, ERα and ERβ. Furthermore, cell permeability using CaCo-2 cells, metabolic stability in rat liver microsomes, inhibition of the most important hepatic CYP enzymes and pharmacokinetic properties in the rat of selected compounds are determined. For a better understanding of the SARs molecular electrostatic potentials (MEPs) were calculated.

Chart 2: title compounds

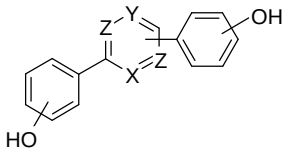


W, X, Y, Z = N, S, Se, C, CH, CCH_3
R_1, R_2 = H, OH

1-17, 19-32



18



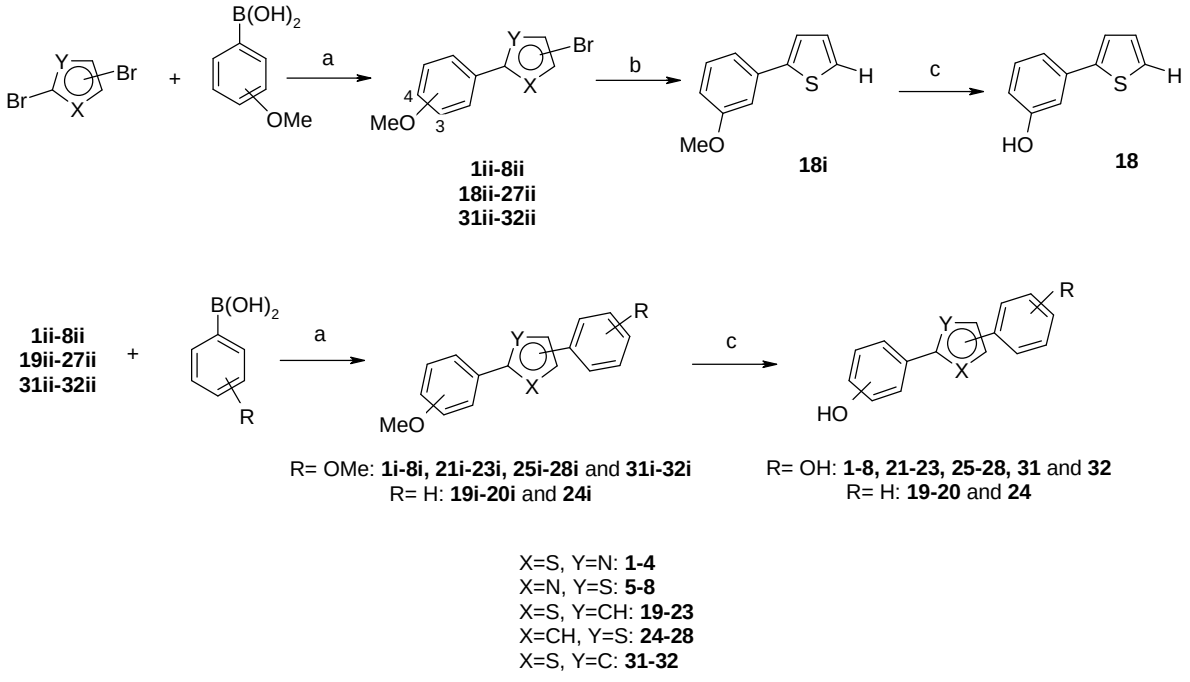
X, Y, Z = N, CH

33-39

Chemistry

Starting from the commercially available dibrominated heterocycles and methoxylated benzene boronic acids, compounds **1** to **8, 19** to **28**, **31** and **32** were prepared via two successive Suzuki reactions[34] followed by a demethylation step using borontribromide[35] as reagent. The Suzuki cross-coupling was carried out using three different methods. Intermediates **1ii** to **8ii, 18ii** to **27ii** and **1i** to **8i** were prepared following Method A (aq. sodium carbonate, toluene, $Pd(PPh_3)_4$, reflux, 4 h) and compounds **31ii**, **32ii**, **19i** to **28i, 31i** and **32i** were synthesized using Method B (sodium carbonate, THF/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h). During the first cross coupling reaction leading to the mono(methoxyphenyl) substituted derivatives **1ii-8ii**, **18ii-27ii**, **31ii**, **32ii** no disubstituted compounds were obtained indicating that the (methoxyphenyl)bromo azoles and thiophenes are less reactive than their dibromo aryl precursors. The bromo intermediate **18ii** was treated with *n*-BuLi in anhydrous THF and subsequently hydrolyzed with water to yield the mono methoxylated thiophene **18i**. The methoxy groups of **1i** to **8i**, **18i** to **28i**, **31i** and **32i** were cleaved with boron tribromide (Method E: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h, Scheme 1).

Scheme 1[a]: synthesis of compounds **1** to **8, 18** to **28**, **31** and **32**

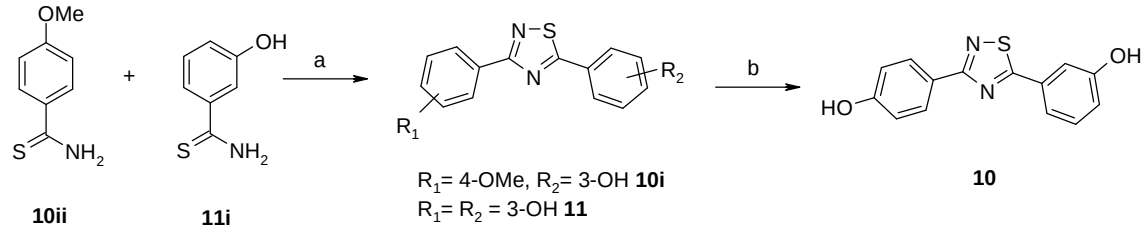


[a]**Reagents and conditions**: a. for compounds **1ii** to **8ii**, **18ii** to **27ii** and **1i** to **8i**: Method A: aq. Na_2CO_3, toluene, $Pd(PPh_3)_4$, reflux, 4 h; for compounds **31ii**, **31i**, **32ii**, **32i** and **19i** to **28i**: Method B: Na_2CO_3, THF/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h; b. 1. *n*-BuLi, THF dry, -78 °C, 15 min, 2. water; c. Method E: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

The 1,3,4-thiadiazole **9** was prepared in a three step synthetic pathway based on the method described by Gierczyk and Zalas.[36] The 3-methoxybenzoyl chloride was treated with hydrazine monohydrate to give the resulting 3-methoxy-*N*'-(3-methoxybenzoyl)benzohydrazide, which was cyclized into the corresponding thiadiazole using Lawesson reagent in DME under microwave assisted conditions. In a last step, the methoxy substituents were cleaved with boron tribromide (Method E: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h).

The synthesis of the 1,2,4-thiadiazoles **10** and **11** is shown in Scheme 2. The commercially available 4-methoxybenzonitrile and 3-hydroxybenzonitrile were converted into the thioamide intermediates **10ii** and **11i**, respectively, using aqueous ammonium sulfide under microwave assisted reaction.[37] These thioamides were submitted to strong acidic conditions resulting in a mixture of the thiadiazoles **10i** and **11** which were separated by column chromatography. The bis(methoxyphenyl) compound could not be isolated. Compound **10i** was demethylated with boron tribromide (Method E: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h, Scheme 2).

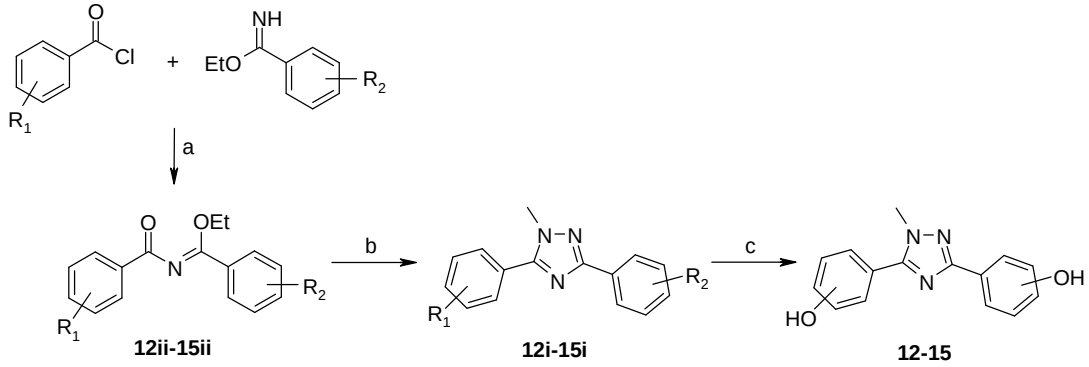
Scheme 2[a]: synthesis of compounds **10** and **11**



[a]**Reagents and conditions**: a. conc. HCl, 38 °C, 8 h; b. Method E: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

The synthesis of compounds **12** to **15** is presented in Scheme 3. The dimethoxylated-1,2,4-triazoles **12i** to **15i** were synthesized by reaction of the *N*-acylimidates[38] **12ii** to **15ii** with methylhydrazine (Method D: $MeNHNH_2$, CH_2Cl_2, 30-40 °C, 4 h). The methoxy groups of compounds **12i** to **15i** were cleaved with borontrifluoride dimethyl sulfide complex[35] (Method F: $BF_3{\cdot}SMe_2$, CH_2Cl_2, rt, 20 h, Scheme 3).

Scheme 3[a]: synthesis of compounds **12** to **15**



12i: R_1= 4-OMe, R_2= 3-OMe
13i: R_1= 4-OMe, R_2= 4-OMe
14i: R_1= 3-OMe, R_2= 4-OMe
15i: R_1= 3-OMe, R_2= 3-OMe

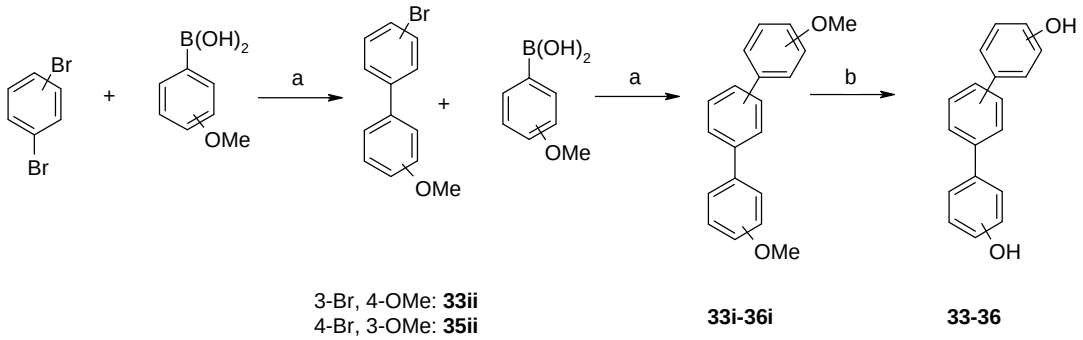
[a]**Reagents and conditions**: a. CH_2Cl_2, NEt_3, 30-40 °C, 6 h; b. Method D: $MeNHNH_2$, CH_2Cl_2, 30-40 °C, 4 h; c. Method F: $BF_3{\cdot}SMe_2$, CH_2Cl_2, rt, 20 h.

Compound **16** was prepared according to Sharpless[39] using 3-azidophenol and 3-hydroxyphenyl acetylene.

Compounds **17**, **29**, **30**, **37** and **38** were obtained, under microwave assisted conditions, in a one pot synthesis (Method C: DME/EtOH/water (1:1:1), Cs_2CO_3, $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bar, 15 min)) with benzene boronic acid for **17**, hydroxylated benzene boronic acid for **29**, **30**, **37** and **38** and the corresponding dibrominated heterocycle.

The synthesis of compounds **33** to **36** is depicted in Scheme 4. Starting from the commercially available dibrominated benzene and methoxylated benzene boronic acids, compounds **33** to **36** were prepared via two successive Suzuki reactions following the conditions of Method A. In the first reaction only the mono substituted compounds **33ii** and **35ii** were obtained due to the fact that the (methoxyphenyl)bromobenzenes are less reactive than the dibromobenzenes. Consequently, longer reaction times were required for the second cross coupling reaction (20 versus 4 h). The methoxy groups of compounds **33i-36i** were cleaved using boron tribromide (Method E: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h, Scheme 4).

Scheme 4[a]: synthesis of compounds **33** to **36**



[a]**Reagents and conditions**: a. Method A: Na_2CO_3 10 % in water, toluene, $Pd(PPh_3)_4$, reflux, 4 h; b. Method E: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

1,2,4,5-Tetrazine **39** was synthesized following the procedure described by Guither et al.[40] Briefly, 3-hydroxybenzonitrile was refluxed with hydrazine monohydrate and sulfur. Treatment with sodium nitrite led to cyclization resulting in 3,3'-(1,2,4,5-tetrazine-3,6-diyl)diphenol **39**.

Biological results

Activity: inhibition of human 17β-HSD1

Placental enzyme was partially purified following a described procedure.[41] Tritiated E1 was incubated with 17β-HSD1, cofactor and inhibitor. The amount of labelled E2 formed was quantified by HPLC. Compounds showing less than 10 % inhibition at 1 μM were considered to be inactive.

The inhibition values of the test compounds are shown in Table 1. It becomes apparent that eleven compounds are more active than the previously described oxazole **E**[98] (IC_{50}= 310 nM).

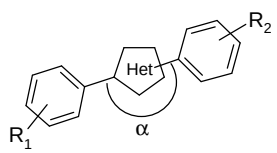
All methoxy compounds (data not shown) and *para-para* dihydroxylated derivatives are inactive except phenylene **34** which is a weak inhibitor (IC_{50}> 1000 nM). The shift of one hydroxy substituent from the *para*- into the *meta*- position leads to highly active compounds except for thiazoles **1, 7** (IC_{50} values> 1000 nM) and **5** (IC_{50}> 5000 nM) which have a weak inhibitory activity and triazoles **12** and **14** which are inactive. Moving the second OH substituent also in *meta* position (*meta-meta* derivatives) results in potent compounds except for triazole **16** which is a weak inhibitor (IC_{50}> 5000 nM) and selenophene **30** and triazole **15** which are inactive.

The exchange of the *para*-OH group of the highly active thiophene **22** (IC_{50}= 69 nM) with hydrogen reduces activity (compound **19**, IC_{50}= 342 nM). More dramatically, the replacement of the *meta*-OH function of compound **22** with hydrogen results in the inactive compound **20**. Similarly, the exchange of the *meta*-hydroxyphenyl moiety as well as the two hydroxy groups of **22** with hydrogens leads to the inactive compounds **17** and **18**. This exemplifies the importance of the existence of two OH substituents and their positions at the phenyl moieties with at least one being in the *meta* position.

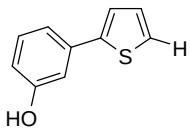
The synthesized thiazoles **1** to **8** show lower activities than the thiophene analogues **17** to **28** except compound **3** (IC_{50}= 50 nM) which exhibits similar inhibition as **22** (IC_{50}= 69 nM) and **27** (IC_{50}= 77 nM). The introduction of a second nitrogen (compounds **9** to **11**) in the heterocyclic scaffold of these potent inhibitors does not strongly reduce activity (**9**, IC_{50}= 336 nM; **11**, IC_{50}= 169 nM). For *meta-meta* disubstituted compounds the introduction of nitrogen in compound **4** decreases activity slightly (**9**, IC_{50}= 336 vs 243 nM), whereas introduction in

compound **8** increases activity (**11**, IC_{50}= 169 vs 455 nM), as it is observed for the *meta-para* disubstituted compound **7** (**10**, IC_{50}= 413 vs >1000 nM).
Moving the hydroxyphenyl moiety of the highly active thiophene **22** (IC_{50}= 69 nM) from position 5 to the 3 position decreases activity dramatically (**32**, IC_{50}> 1000 nM). Exchange of the sulfur atom of the potent thiophene **23** (IC_{50}= 173 nM) with selenium results in the inactive compound **30**.
In case of the six-membered rings, benzene and some of its aza-analogues were investigated. The 1,3-bis(hydroxyphenyl) substituted phenylenes were not active, whereas the 1,4-disubstituted compounds showed some activity, the most active benzene compound being the *meta-meta* derivative **35** (IC_{50}= 173 nM). The introduction of one or more nitrogens into the central benzene ring of **35** (compounds **37** to **39**) results in an increase (pyridine **37**, IC_{50}= 101 nM), no change (tetrazine **39**, IC_{50}= 201 nM) and a decrease of inhibitory activity (pyrazine **38**, IC_{50}= 1000 nM).
Furthermore in Table 1, the angles between the two phenyl moieties are presented as a structural parameter. No correlation to the activities of the corresponding compounds can be observed.

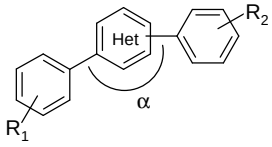
Table 1: inhibition of human 17β-HSD1 and 17β-HSD2 by compounds **1**-**39**, O-O distances and phenyl-het-phenyl angles



1-17
19-32



18



33-39

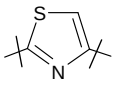
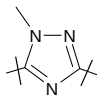
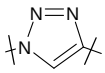
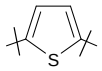
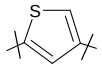
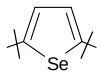
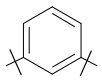
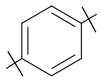
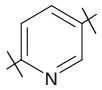
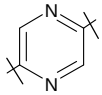
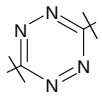
compound	Het	R_1	R_2	d(Å)[a]	α[b]	IC_{50} (nM)[c] 17β-HSD1[d]	IC_{50} (nM)[c] 17β-HSD2[e]	selectivity factor[f]
1		4-OH	3-OH	11.9< d< 12.7	153	>1000	nt	
2		4-OH	4-OH	13.6		ni	nt	
3		3-OH	4-OH	11.9< d< 12.7		50	4004	80
4		3-OH	3-OH	10.1< d< 11.7		243	2500	10
5		4-OH	3-OH	10.5< d< 12.0	130	>5000	nt	
6		4-OH	4-OH	12.5		ni	nt	
7		3-OH	4-OH	10.5< d< 12.0		>1000	nt	
8		3-OH	3-OH	9.0< d< 11.2		455	2220	5
9		3-OH	3-OH	9.9< d< 11.5	157	336	nt	
10		3-OH	4-OH	9.7< d< 11.9	128	413	2194	5
11		3-OH	3-OH	8.5< d< 11.2		169	602	3

Table 1 continued

compound	Het	R_1	R_2	d(Å)[a]	α[b]	IC_{50} (nM)[c] 17β-HSD1[d]	IC_{50} (nM)[c] 17β-HSD2[e]	selectivity factor[f]
12		4-OH	3-OH	10.2< d< 11.9	145	ni	nt	
13		4-OH	4-OH	12.4		ni	nt	
14		3-OH	4-OH	10.2< d< 11.9		ni	nt	
15		3-OH	3-OH	8.7< d< 11.4		ni	nt	
16		3-OH	3-OH	8.7< d< 11.2	147	>5000	nt	
17		H	H			ni	nt	
18						ni	nt	
19		3-OH	H			342	2337	7
20		4-OH	H		147	ni	nt	
21		4-OH	4-OH	13.5		ni	nt	
22		3-OH	4-OH	11.8< d< 12.8		69	1953	28
23		3-OH	3-OH	9.8< d< 11.8		173	745	4
24		3-OH	H			>5000	nt	
25		4-OH	3-OH	10.4< d< 12.0		151	1690	11
26		4-OH	4-OH	12.3	136	ni	nt	
27		3-OH	4-OH	10.4< d< 12.0		77	1271	16
28		3-OH	3-OH	8.5< d< 11.4		185	559	3
29		4-OH	4-OH	13.8	162	ni	nt	
30		3-OH	3-OH	10.4< d< 11.3		ni	nt	
31		4-OH	4-OH	8.1	69	ni	nt	
32		3-OH	4-OH	6.2< d< 8.9		>1000	nt	
33		4-OH	3-OH	9.3< d< 12.1	120	>1000	nt	
34		4-OH	4-OH	12.3		>1000	nt	
35		3-OH	3-OH	11.4< d< 12.4	180	173	2259	13
36		3-OH	4-OH	13.1		471	4509	9
37		3-OH	3-OH	11.6< d< 12.3	180	101	3399	34
38		3-OH	3-OH	11.4< d< 12.2	180	1000	5502	5
39		3-OH	3-OH	11.3< d< 12.2	180	201	5102	25

[a]O-O distance between the hydroxy substituents, for E2 d= 11.0 Å; [b]Angle between the two phenyl moieties in °; [c]Mean values of three determinations, standard deviation less than 12 % except **3**: 18 % for 17β-HSD1; [d]Human placenta, cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], cofactor NADH [500 µM]; [e]Human placenta, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [f]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested.

Selectivity: inhibition of human 17β-HSD2 and affinities for ERα and ERβ

Since 17β-HSD2 catalyzes the inactivation of E2 to E1, inhibitory activity toward this enzyme must be avoided. The 17β-HSD2 inhibition was determined using an assay similar to the 17β-HSD1 test. Placental microsomes were incubated with tritiated E2 in the presence of NAD^+ and inhibitor. Separation and quantification of labelled product (E1) was performed by HPLC using radio detection. A selection of the most potent 17β-HSD1 inhibitors was tested for inhibition of 17β-HSD2. IC_{50} values and selectivity factors (IC_{50} HSD2 / IC_{50} HSD1) are presented in Table 1.
It is striking that most *meta-meta* bis(hydroxyphenyl) substituted inhibitors present only poor selectivity except for compounds **37** and **39** which show high selectivity factors of 34 and 25, respectively. The *meta-para/para-meta* disubstituted inhibitors mostly show higher selectivity with compound **3** exhibiting the highest selectivity factor of 80.

A further prerequisite for 17β-HSD1 inhibitors to be used as potential drugs is that these compounds do not show affinity for ERα and ERβ, or only a marginal one, since binding to these receptors could counteract the therapeutic efficacy. The binding affinities of selected compounds were determined using recombinant human protein in a competition assay applying [^{3}H]-E2 and hydroxyapatite (Table 2). All tested compounds show very marginal or marginal affinity to the ERs.

Table 2: binding affinities for the human estrogen receptors α and β of selected compounds

compound	RBA[a] (%)	
	ERα[b]	ERβ[b]
3	<0.01	0.01< RBA< 0.1
22	0.1< RBA< 1	1.5
25	0.01< RBA< 0.1	0.1< RBA< 1
27	0.01< RBA< 0.1	0.1< RBA< 1
35	<0.001	0.01< RBA< 0.1
37	0.01< RBA< 0.1	<0.01

[a]RBA (relative binding affinity), E2: 100 %, mean values of three determinations, standard deviations less than 10 %; [b]Human recombinant protein, incubation with 10 nM [^{3}H]-E2 and inhibitor for 1 h.

Further biological evaluation

The intrinsic estrogenic activity of a representative compound of each class was determined using the ER-positive mammary tumor T-47D cell line. No agonistic, i.e. no stimulatory effect was observed after application of the inhibitors even at a concentration 1000 fold higher than E2 (data not shown).

A selection of active and selective compounds was investigated for permeation of CaCo-2 cells. These cells exhibit morphological and physiological properties of the human small intestine[42] and are a generally accepted model for the prediction of peroral absorption. Depending on the P_{app} data obtained, compounds can be classified as low (P_{app} ($\cdot 10^{-6}$ cm/sec) <

1), medium (1 < P_{app} < 10) or highly permeable (P_{app} > 10)). Thiazole **3** shows medium cell permeation while thiophenes **22**, **25** and phenylene **35** exhibit high cell permeability (Table 3).

Table 3: CaCo-2 cell permeation of highly active 17β-HSD1 inhibitors

compound	P_{app}(·10^{-6} cm/s)[a,b]	classification
3	7.8	medium
22	22.0	high
25	14.4	high
35	12.5	high
atenolol	0.1	low
testosterone	9.4	medium
ketoprofene	25.7	high

[a]Permeability of reference compounds similar to the values described [atenolol,[71] testosterone,[67] ketoprofene[72]]; [b]P_{app}: apparent permeability coefficient, mean values of three determinations, standard deviations less than 10 %.

A representative compound of the thiazole, thiophene and phenylene class (compounds **4**, **25** and **35**) was evaluated for their phase 1 metabolic stability using rat liver microsomes. Samples were taken at defined time points and the remaining percentage of parent compound was determined by LC-MS/MS. Half-life and intrinsic clearance were evaluated and compared to the two reference compounds diazepam and diphenhydramine (Table 4). All tested compounds show longer half lives than the antihistaminic drug diphenhydramine (values in the range between 12.6 and 22.7 min vs 6.8 min).

Table 4: half-lives and intrinsic clearances of compounds **4**, **25** and **35** in rat liver microsomes[a]

compound	half-life (min)	CL_{int}[b] (µL /min /mg protein)
4	12.6	367.2
25	18.6	248.5
35	22.7	203.4
diazepam[c]	40.8	113.3
diphenhydramine[c]	6.8	679.6

[a]0.33 mg/mL protein, $NADP^+$-regenerating system, [inhibitor]: 1 µM, incubation at 37 °C, samples taken at 0, 15, 30 and 60 min, determination of parent compound by MS; [b]CL_{int}: intrinsic body clearance; [c]values of reference compounds similar to described values.

The same compounds (**4**, **25** and **35**) were further investigated for inhibition of the six most important human hepatic enzymes: CYP1A2, 2B6, 2C9, 2C19, 2D6 and 3A4. All compounds show very low inhibition of CYP1A2, 2B6, 2C19 and 2D6 (IC_{50}> 4 µM). In case of CYP2C9 and CYP3A4 (**4**: 2.1 and 0.8, **25**: 0.8 and 1.9, **35**: 1.9 and 2.1 µM, respectively) inhibition was higher, but still clearly below the IC_{50} values of 17β-HSD1 inhibition. These results indicate a low risk of drug-drug interaction caused by CYP-inhibition.

The pharmacokinetic profiles of the most active and selective azole compound **3** and one of the six-membered ring compounds (**39**) were determined in rats after oral administration in a cassette. The most potent six-membered ring compound **37** was not chosen as it was unstable in buffer over 24 h. Each group consisted of 4 male rats and the compounds were administered in doses of 10 mg/kg. Plasma samples were collected over 24 h and plasma concentrations were determined by HPLC-MS/MS. The pharmacokinetic parameters are presented in Table 5. Maximal plasma concentration (C_{max}) and AUC value are much higher for compound **39** compared to compound **3**.

Table 5: pharmacokinetic parameters of compound **3** and **39** in male rats after oral application of 10 mg/kg

parameters	compound	
	3	**39**
$\mathbf{C_{max\ obs}}$ **(ng/mL)**[a]	7.8	106.0
$\mathbf{C_z}$ **(ng/mL)**[b]	6.6	54.0
$\mathbf{t_{max\ obs}}$ **(h)**[c]	8.0	3.0
$\mathbf{t_z}$ **(h)**[d]	10.0	10.0
$\mathbf{t_{1/2z}}$ **(h)**[e]	1.5	1.2
$\mathbf{AUC_{0\text{-}tz}}$ **(ng/mL)**[f]	99.2	1204.0

[a]$C_{max\ obs}$: maximal measured concentration; [b]C_z: last analytical quantifiable concentration; [c]$t_{max\ obs}$: time to reach the maximum measured concentration; [d]t_z: time of the last sample which has an analytically quantifiable concentration; [e]$t_{1/2z}$: half-life of the terminal slope of a concentration time curve; [f]$AUC_{0\text{-}tz}$: area under the concentration time curve up to the time tz of the last sample.

Computational Chemistry

To obtain an insight which physicochemical parameter might influence biological activity, the charge density distribution was considered and the molecular electrostatic potentials (MEPs) of selected compounds were determined. The geometry of the compounds had been fully optimized in the gas phase at the B3LYP/6-311++G (d,p) level of density functional theory (DFT). MEPs were plotted for every compound on its electron density with GaussView 3.09. The electrostatic potential distribution of the charge density is presented by a color code ranging from $-3.1 \cdot 10^{-2}$ to $4.5 \cdot 10^{-2}$ Hartree (Figures 1 and 2 and Supporting Information). For better comparison, the MEPs of different compounds were divided into 3 regions corresponding to each aromatic system.

In Figure 1, the MEPS of five-membered heterocyclic compounds are arranged based on their increasing inhibitory potency. While the positions of the hydroxyphenyl moieties are fixed, the nature of the heterocycle is varied. It becomes apparent that the heterocycle influences the ESP distribution of the whole molecule. In order to rationalise these MEPs, the ESP distribution ranges ($\mathbf{C^1}$), the mean values of the distribution ranges ($\mathbf{C^2}$) and the Δ of ESP ($\mathbf{C^3}$) were analyzed: negative ESP values (red/orange/yellow) in region I and II and less negative to almost neutral ESP values (green/yellow) in region III are obviously an important factor for high inhibitory potency. Trying to establish a semi-quantitative MEP-activity relationship ("semi-QMAR") optimal ESP ranges for areas I, II and III for potent inhibition were identified (hydrogens and the OH groups were not considered): for region I ESP from –1.7 to $-1.2 \cdot 10^{-2}$, for region II –1.6 to $-0.9 \cdot 10^{-2}$ and for region III –1.2 to $-0.5 \cdot 10^{-2}$ Hartree. Similarly, the optimal Δ values of ESP for each region were determined: 0.5, 0.7 and 0.7 Hartree, respectively. Both the shift of a certain ESP distribution range on the scale and the change of

the Δ value result in a decrease of inhibitory activity. The combination of these two criteria is substantiated with +, – and 0 in Figure 1.**D** indicating favorable, unfavorable and neutral impact on activity.

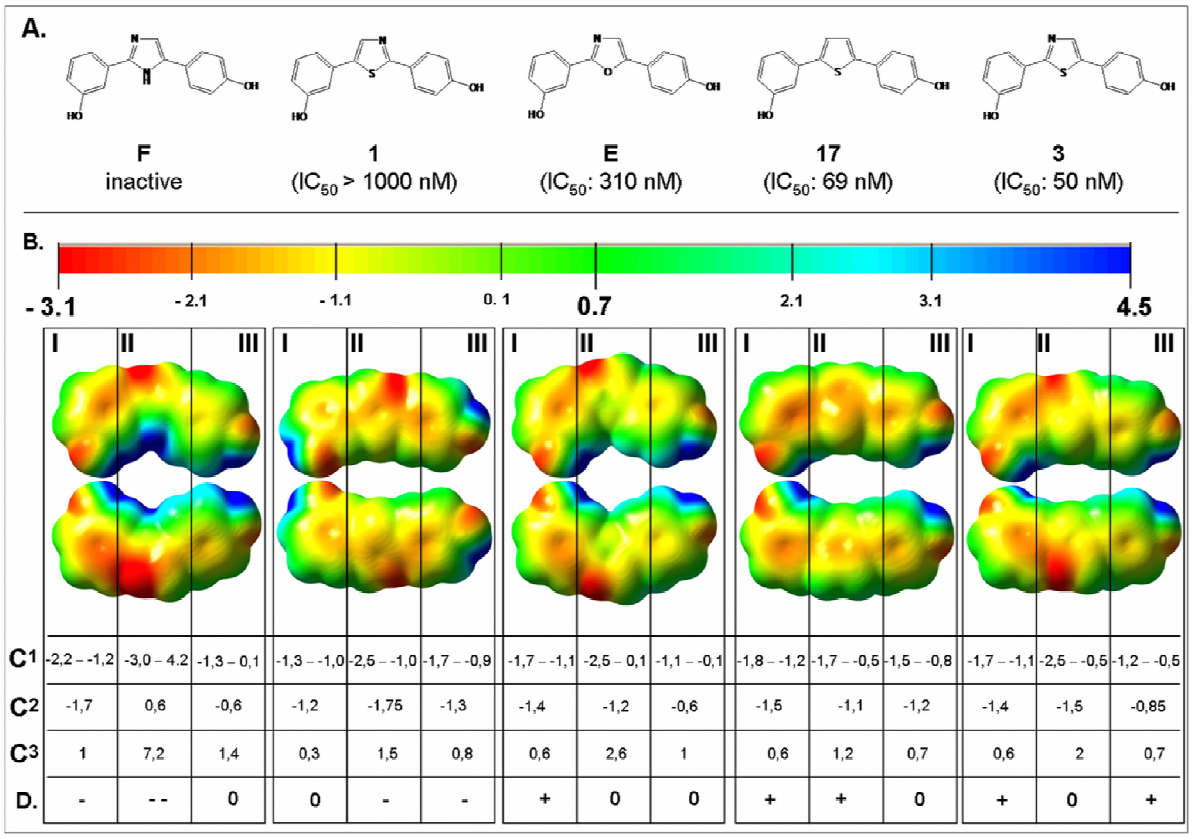


Figure 1. (**A**) structure and 17β-HSD1 inhibitory activity of **F**,[33] **E**,[33] and compounds **1**, **3**, **17** ordered by increasing activity; (**B**) MEP maps, dorsal and ventral view; (**C**^{1}) ESP distribution range (Hartree; $\cdot 10^{-2}$); (**C**^{2}) mean value of distribution range (Hartree, $\cdot 10^{-2}$); (**C**^{3}) Δ of ESP; (**D**) "semi-QMAR". MEP surfaces were plotted with GaussView 3.09.

Compound **F**[33] exhibits a polarization between top and bottom sides of the molecule: the vertex-side (NH) has positive ESP values, while on the opposite side (N-C) negative to neutral values are predominant. This polarization (Δ= 7.2 $\cdot 10^{-2}$ Hartree) results in an inactive compound, indicating that the ESP of the central heterocycle is crucial for 17β-HSD1 inhibitory activity.
A good example for the change in activity is well demonstrated by the ESP distribution in region III for thiazoles **1** and **3** (Figure 1 **C**^{1} and **C**^{2}). The dramatic loss of activity of compound **1** (IC_{50}> 1000 nM) in comparison to **3** (IC_{50}= 50 nM) is due to a non optimal electron density distribution.

The MEP maps of the six-membered ring compounds are depicted in Figure 2. A similar charge density distribution was observed except for the only planar compound of this series, tetrazine **33**, which showed no polarization between top and bottom side of the molecule. It remains to be clarified whether this is the reason for the reasonable activity of compound **33**.

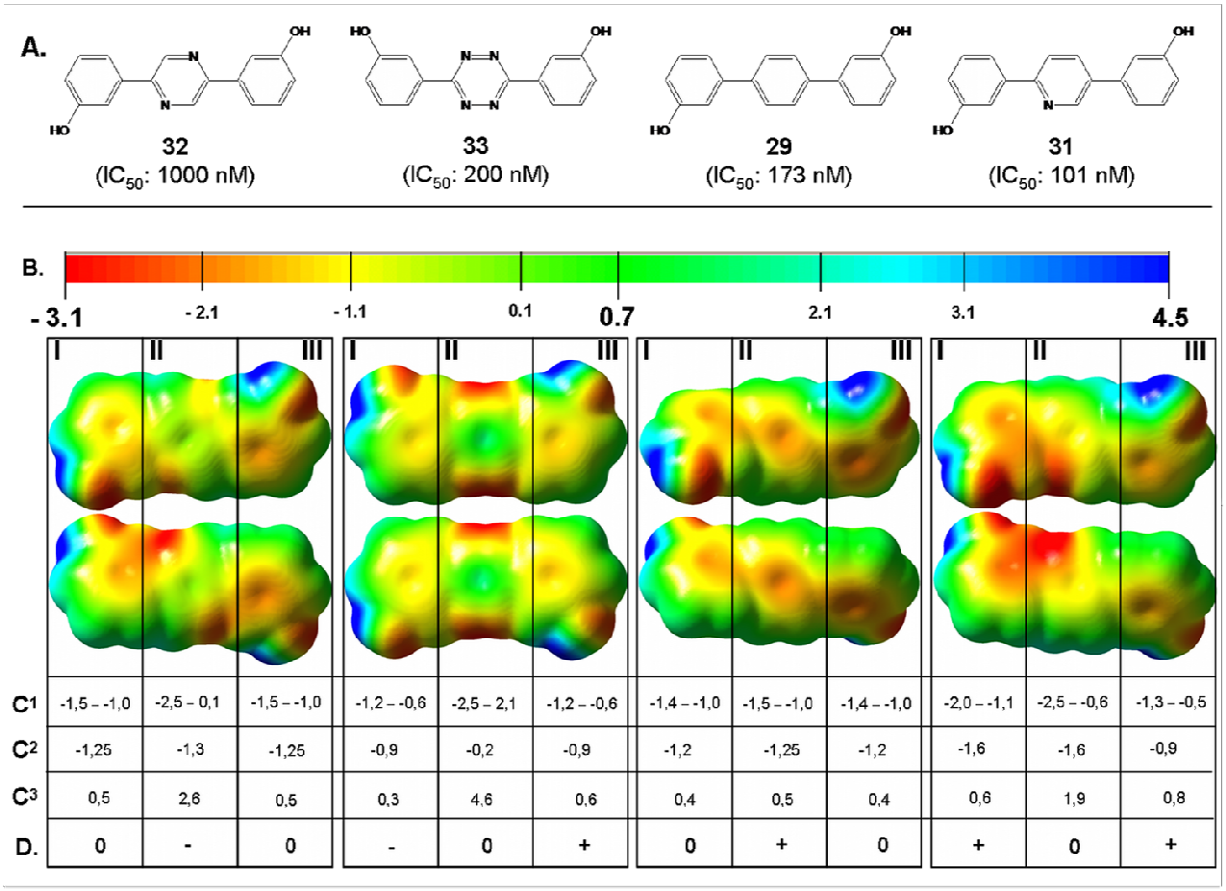


Figure 2. (**A)** structure and 17β-HSD1 inhibitory activity of compounds **29** and **31** to **33** ordered by increasing activity; (**B**) MEP maps, dorsal and ventral view; (**C**^{1}) ESP distribution range (Hartree;·10^{-2}); (**C**^{2}) mean value of distribution range (Hartree,·10^{-2}); (**C**^{3}) Δ of ESP; (**D**) "semi-QMAR". MEP surfaces were plotted with GaussView 3.09.

Discussion and conclusion

The present paper shows that compound **E** from a previous study[33] could be optimized. The most active and selective thiazole **3** shows an IC_{50} value of 50 nM and a selectivity factor of 80 (compound **E**, IC_{50}= 310 nM, selectivity factor: 56).

The biological results obtained confirm similar findings described in our previous article:[33] the OH substitution pattern of our compounds is decisive for inhibitory activity. Comparison of the mono hydroxylated thiophenes **19** and **20** (*meta*- and *para*-, respectively) shows that the *meta*-hydroxy group is crucial for activity. The inactivity of compound **18** indicates that the phenyl moiety of the *meta*-hydroxyphenyl thiophene **19** is also important for potency. The replacement of the *meta*-hydroxy group of **19** with hydrogen leading to the inactive compound **17** demonstrates the importance of the hydrogen bond interaction for activity.

As observed for the previously described bis(hydroxyphenyl) derivatives,[33] the distance between the two oxygens obviously has to be close to the value observed for the substrate (*d*= 11 Å). The *para-para* disubstituted compounds show distances longer than 12.5 Å. They are all inactive. Concerning the *meta-para* and *meta-meta* disubstituted compounds, which have O-O distances between 8.5 and 12.8 Å, medium to high inhibitory activities are observed for most compounds. They could be able to establish hydrogen bond interactions with His221/Glu282 and Ser142/Tyr155. However, the inactivity of thiazoles **1**, **5**, **7** and triazoles **12** to **16**, which are all *meta-para* and *meta-meta* disubstituted, indicates that this distance is not the only

decisive criterion for activity. The heterocycle also influences the inhibitory potencies of the compounds.
In the five-membered ring series, the potency of 1,2,3-triazoles described previously[98] led us to further investigate this class of compounds. The inactivity of compounds **12** to **15** shows that either the nitrogen distribution in the ring or the methyl group are not tolerated by the enzyme. The replacement of the N-Me moiety of **14** with sulfur (compound **10**) leading to a fairly potent compound indicates that S in this position has a positive influence. To further investigate the role of the nitrogen in the ring, thiazoles **1** to **8** and thiophenes **21** to **28**, **31** and **32** were investigated. The fact that thiazole **3** and thiophene **22** exhibit almost identical potencies shows that the nitrogen does not contribute to binding, i.e. that there is obviously no hydrogen bond interaction.
In this report, we could also show that six-membered rings are appropriate for the design of highly active 17β-HSD1 inhibitors. Comparison of the almost equipotent phenylene **35**, pyridine **37** and tetrazine **39** confirms the hypothesis that nitrogens are tolerated in the ring but do not contribute to a specific interaction.
The role of the angle between the two hydroxyphenyl moieties was also investigated in order to find out whether there is a correlation between this parameter and the inhibitory potency. The broad range of angles calculated (128° for **11** to 180° for **37**, two highly active compounds) could not be correlated with high or low inhibitory potency indicating that the enzyme presents some flexibility for ligand binding. Interestingly, for smaller angles between the two hydroxyphenyl groups, as in case of compounds **10** and **11** (N atom at the vertex), the *meta-meta* substitution results in a higher activity, while for larger angles, in presence of a sulfur atom at the vertex of the five-membered heterocycle (2,5-disubstituted thiazoles and 2,5-disubstituted thiophenes), the optimal substitution pattern is *meta-para/para-meta*. This phenomenon will be further investigated.
Our finding that there exists a correlation between the MEPs and the biological activities of the compounds (semi-QMAR) might be exploited for further structure optimization underlining the relevance of this descriptor for biological activity. Furthermore, the MEPs could be used to investigate how these inhibitors approach and bind to the enzyme as it is described for genistein and the estrogen receptor.[99]

Interestingly the exchange of a CH in the thiophene **22** by a N leading to the thiazole **3** increases selectivity toward 17β-HSD2 dramatically (IC_{50} 17β-HSD2 / IC_{50} 17β-HSD1, 28 vs 80). In general, the most potent compounds exhibit a really low affinity for the ERα and ERβ and show no stimulation of cell proliferation (agonistic effect) in the ER-positive T-47D cell line. It is worth mentioning that **3** in spite of its good CaCo-2 permeability shows a really low bioavailability compared to **39**. Glucuronidation and/or sulfatation might be responsible for the low plasma levels of the parent compounds.
In the present report, we described the synthesis of bis(hydroxyphenyl) azoles, thiophenes, benzenes, aza-benzenes and the evaluation of their biological properties. The most promising compounds of this study, **3**, **22** and **39**, show a high selectivity toward 17β-HSD2, a low binding affinity to the ERα, a high CaCo-2 permeability and a reasonable pharmacokinetic profile after peroral application. These new compounds should be useful tools to further investigate *in vivo* 17β-HSD1 as a target for the treatment of estrogen-dependent diseases.

Experimental section

Chemical methods

Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Lancaster, Roth, Merck or Fluka and were used without purification.
Column chromatography (CC) was performed on silica gel (70-200 μm) coated with silica, preparative thin layer chromatography (TLC) on 1 mm SIL G-100 UV_{254} glass plates (Macherey-Nagel) and reaction progress was monitored by TLC on Alugram SIL G UV_{254} (Macherey-Nagel).
Melting points were measured on a Mettler FP1 melting point apparatus and are uncorrected.
IR spectra were recorded on a Bruker Vector 33 spectrometer (neat sample).
^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker AM500 spectrometer (500 MHz) at 300 K. Chemical shifts are reported in δ (parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard ($CDCl_3$: δ = 7.24 ppm (^{1}H NMR) and δ = 77 ppm (^{13}C NMR), CD_3OD: δ = 3.35 ppm (^{1}H NMR) and δ = 49.3 ppm (^{13}C NMR), CD_3COCD_3: δ = 2.05 ppm (^{1}H NMR) and δ = 29.9 ppm (^{13}C NMR), CD_3SOCD_3: δ = 2.50 ppm (^{1}H NMR) and δ = 39.5 ppm (^{13}C NMR). Signals are described as s, d, t, dd, m, dt for singlet, doublet, triplet, doublet of doublets, multiplet and doublet of triplets, respectively. All coupling constants (*J*) are given in hertz (Hz).
Mass spectra (ESI) were recorded on a TSQ Quantum (Thermo/Fischer) instrument. Elemental analyses were performed at the Department of Instrumental Analysis and Bioanalysis, Saarland University.

5-Bromo-2-(4-methoxyphenyl)-1,3-thiazole (**1ii**),[10044] 4-methoxythiobenzamide (**10ii**),[45] 3-bromo-2-(4-methoxyphenyl)thiophene (**31ii**),[46] 3-bromo-4'-methoxybiphenyl (**33ii**),[47] 4'-bromo-3-methoxybiphenyl (**35ii**),[48] 2-(4-methoxyphenyl)-5-(3-methoxyphenyl)-1,3-thiazole (**1i**),[49] 2,5-bis(4-methoxyphenyl)-1,3-thiazole (**2i**),[50] 2,4-bis(4-methoxyphenyl)-1,3-thiazole (**6i**),[35] 3-hydroxythiobenzamide (**11i**),[16] 2,5-bis(4-methoxyphenyl)thiophene (**21i**),[51] 2,4-bis(4-methoxyphenyl)thiophene (**26i**),[52] 2,3-bis(4-methoxyphenyl)thiophene (**31i**),[53] 3,4"-dimethoxy-1,1':3',1"-terphenyl (**33i**),[54] 4,4"-dimethoxy-1,1':3',1"-terphenyl (**34i**),[54] 4,4'-(1,3-thiazole-2,5-diyl)diphenol (**2**),[55] 4,4'-(1,3-thiazole-2,4-diyl)diphenol (**6**),[35] 3,3'-(1,2,4-thiadiazole-3,5-diyl)diphenol (**11**),[56] 3,3'-(1*H*-1,2,3-triazole-1,4-diyl)diphenol (**16**)[101],[57] 2,5-diphenylthiophene (**17**),[58] 4,4'-thiene-2,5-diyldiphenol (**21**),[51] 4,4'-thiene-2,4-diyldiphenol (**26**),[55] 1,1':3',1"-terphenyl-3,4"-diol (**33**),[59] 1,1':3',1"-terphenyl-4,4"-diol (**34**),[60] 1,1':4',1"-terphenyl-3,3"-diol (**35**)[102],[59] 1,1':4',1"-terphenyl-3,4"-diol (**36**)[102],[59] 3,3'-pyrazine-2,5-diyldiphenol (**38**),[61] 3,3'-(1,2,4,5-tetrazine-3,6-diyl)diphenol (**39**)[40] were prepared following described procedures.

General procedure for Suzuki coupling
Method A:
A mixture of arylbromide (1 eq), methoxybenzene boronic acid (1 eq), sodium carbonate (2 eq) and tetrakis(triphenylphosphine) palladium (0.005 eq) in an oxygen free toluene/water (1:1) solution was stirred at 100 °C for 4 h under nitrogen. The reaction mixture was cooled to rt. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC.

Method B:
A mixture of arylbromide (1 eq), methoxybenzene boronic acid (1.2 eq), sodium carbonate (2 eq) and tetrakis(triphenylphosphine) palladium (0.005 eq) in an oxygen free tetrahydrofurane/water (1:1) solution was stirred at 100 °C for 20 h under nitrogen. The reaction mixture was cooled to rt. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC.

Method C:
A mixture of aryl dibromide (1 eq), methoxybenzene boronic acid (2.4 eq), caesium carbonate (4 eq) and tetrakis(triphenylphosphine) palladium (0.001 eq) was suspended in an oxygen free DME/EtOH/water (1:1:1) solution. The reaction mixture was exposed to microwave irradiation (15 min, 150 W, 150 °C, 15 bar). After reaching rt, water was added and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated to dryness. The product was purified by preparative TLC.

Method D: **General procedure for synthesis of 1,2,4-triazoles:**
A solution of acyl chloride (1 eq) in dichloromethane was added dropwise to a mixture of ethyl imino ester (1 eq) and dry triethylamine (1 eq) in 20 mL dichloromethane and heated to 30-35 °C for 6 h. After cooling to rt, the mixture was poured into 3 % $NaHCO_3$ solution (25 mL). The layers were separated and the organic layer was washed with water, dried over sodium sulfate and evaporated to dryness under reduced pressure. The resulting *N*-acylimino esters **12ii** to **15ii** were heated to 30-35°C with methyl hydrazine (2 eq) in CH_2Cl_2 for 4 h. The solvent was removed under reduced pressure and the 1,2,4-triazoles **12i** to **15i** were crystallized from CH_2Cl_2/ Et_2O.

General procedure for ether cleavage
Method E:
To a solution of methoxybenzene derivative (1 eq) in dry dichloromethane at -78 °C (dry ice/acetone bath), boron tribromide in dichloromethane (1 M, 3 eq per methoxy function) was added dropwise. The reaction mixture was stirred for 20 h at rt under nitrogen. Water was added to quench the reaction, and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, evaporated to dryness under reduced pressure and purified by preparative TLC.

Method F:
To a solution of bis(methoxyphenyl) derivative (1 eq) in dry dichloromethane, borontrifluoride dimethyl sulfide complex (75 eq) was added dropwise at rt. The reaction mixture was stirred for 20 h. Water was added to quench the reaction and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, evaporated to dryness under reduced pressure and purified by preparative TLC.

3-[2-(4-Hydroxyphenyl)-1,3-thiazol-5-yl]phenol (1). The title compound was prepared by reaction of 5-(3-methoxyphenyl)-2-(4-methoxyphenyl)-1,3-thiazole (**1i**) (40 mg, 0.13 mmol) and boron tribromide (0.81 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 77 % (28 mg); MS (ESI): 270 $(M+H)^+$; Anal. ($C_{15}H_{11}NO_2S$) C, H, N.

5-Bromo-2-(3-methoxyphenyl)-1,3-thiazole (**3ii**). The title compound was prepared by reaction of 2,5-dibromo-1,3-thiazole (500 mg, 2.06 mmol), 3-methoxybenzeneboronic acid (376 mg, 2.47 mmol), sodium carbonate (437 mg, 4.12 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 10 µmol) according to method A. The product was purified by CC (dichloromethane/methanol 95:5); yield: 50 % (278 mg).

2-(3-Methoxyphenyl)-5-(4-methoxyphenyl)-1,3-thiazole (**3i**). The title compound was prepared by reaction of 5-bromo-2-(3-methoxyphenyl)-1,3-thiazole (**3ii**) (250 mg, 0.93 mmol), 4-methoxybenzeneboronic acid (170 mg, 1.11 mmol), sodium carbonate (197 mg, 1.86 mmol) and tetrakis(triphenylphosphine) palladium (5.4 mg, 4.6 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 58 % (160 mg).

3-[5-(4-Hydroxyphenyl)-1,3-thiazol-2-yl]phenol (**3**). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-5-(4-methoxyphenyl)-1,3-thiazole (**3i**) (40 mg, 0.13 mmol) and boron tribromide (0.81 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 80 % (50 mg); MS (ESI): 270 $(M+H)^+$; Anal. ($C_{15}H_{11}NO_2S$) C, H, N.

2,5-Bis(3-methoxyphenyl)-1,3-thiazole (**4i**). The title compound was prepared by reaction of 5-bromo-2-(3-methoxyphenyl)-1,3-thiazole (**3ii**) (250 mg, 0.93 mmol), 3-methoxybenzeneboronic acid (170 mg, 1.11 mmol), sodium carbonate (197 mg, 1.86 mmol) and tetrakis(triphenylphosphine) palladium (5.4 mg, 4.6 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 40 % (111 mg).

3,3'-(1,3-Thiazole-2,5-diyl)diphenol (**4**). The title compound was prepared by reaction of 2,5-bis(3-methoxyphenyl)-1,3-thiazole (**4i**) (100 mg, 0.36 mmol) and boron tribromide (2.02 mmol) according to

method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 85 % (82 mg); MS (ESI): 270 $(M+H)^+$; Anal. ($C_{15}H_{11}NO_2S$) C, H, N.

4-Bromo-2-(4-methoxyphenyl)-1,3-thiazole (**5ii**). The title compound was prepared by reaction of 2,4-dibromo-1,3-thiazole (500 mg, 2.06 mmol), 4-methoxybenzeneboronic acid (376 mg, 2.47 mmol), sodium carbonate (437 mg, 4.12 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 10 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 55 % (305 mg).

2-(4-Methoxyphenyl)-4-(3-methoxyphenyl)-1,3-thiazole (**5i**). The title compound was prepared by reaction of 4-bromo-2-(4-methoxyphenyl)-1,3-thiazole (**5ii**) (250 mg, 0.93 mmol), 3-methoxybenzeneboronic acid (170 mg, 1.11 mmol), sodium carbonate (197 mg, 1.86 mmol) and tetrakis(triphenylphosphine) palladium (5.4 mg, 4.6 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 52 % (143 mg).

3-[2-(4-Hydroxyphenyl)-1,3-thiazol-4-yl]phenol (**5**). The title compound was prepared by reaction of 2-(4-methoxyphenyl)-4-(3-methoxyphenyl)-1,3-thiazole (**5i**) (70 mg, 0.24 mmol) and boron tribromide (1.44 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 78 % (50 mg); MS (ESI): 268 $(M-H)^-$; Anal. ($C_{15}H_{11}NO_2S$) C, H, N.

4-Bromo-2-(3-methoxyphenyl)-1,3-thiazole (**7ii**). The title compound was prepared by reaction of 2,4-dibromo-1,3-thiazole (500 mg, 2.06 mmol), 3-methoxybenzeneboronic acid (376 mg, 2.47 mmol), sodium carbonate (437 mg, 4.12 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 10 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 50 % (270 mg).

2-(3-Methoxyphenyl)-4-(4-methoxyphenyl)-1,3-thiazole (**7i**). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)-1,3-thiazole (**7ii**) (250 mg, 0.93 mmol), 4-methoxybenzeneboronic acid (170 mg, 1.11 mmol), sodium carbonate (197 mg, 1.86 mmol) and tetrakis(triphenylphosphine) palladium (5.4 mg, 4.6 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 79 % (218 mg).

3-[4-(4-Hydroxyphenyl)-1,3-thiazol-2-yl]phenol (7). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-4-(4-methoxyphenyl)-1,3-thiazole (**7i**) (70 mg, 0.24 mmol) and boron tribromide (1.44 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 80 % (52 mg); MS (ESI): 268 $(M-H)^-$; Anal. ($C_{15}H_{11}NO_2S$) C, H, N.

2,4-Bis(3-methoxyphenyl)-1,3-thiazole (**8i**). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)-1,3-thiazole (**7ii**) (250 mg, 0.93 mmol), 3-methoxybenzeneboronic acid (170 mg, 1.11 mmol), sodium carbonate (197 mg, 1.86 mmol) and tetrakis(triphenylphosphine) palladium (5.4 mg, 4.6 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 18 % (50 mg).

3,3'-(1,3-Thiazol-2,4-diyl)diphenol (**8**). The title compound was prepared by reaction of 2,4-bis(3-methoxyphenyl)-1,3-thiazole (**8i**) (70 mg, 0.24 mmol) and boron tribromide (1.44 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 78 % (50 mg); MS (ESI): 268 $(M-H)^-$; Anal. ($C_{15}H_{11}NO_2S$) C, H, N.

3,3'-(1,2,4-Thiadiazol-2,5-diyl)diphenol (**9**). A solution of 3-hydroxythiobenzamide (100 mg, 0.17 mmol, 2 eq) in DMSO (10 ml) was stirred for 5 h at rt with 3 ml concentrated chlorhydric acid. The crude mixture was poured into water and the resulting precipitate was filtered, washed with water and dried overnight in a desiccator; yield: 92 % (41 mg); MS (ESI): 269 $(M-H)^-$. Anal. ($C_{14}H_{10}N_2O_2S$) C, H, N.

3-[3-(4-Methoxyphenyl)-1,2,4-thiadiazol-5-yl]phenol (**10i**). A solution of 4-methoxythiobenzamide (**10ii**) (318 mg, 1.90 mmol, 1 eq) in DMSO was heated for 8 h at 38 °C with 3-hydroxythiobenzamide

(**11i**) (290 mg, 1.90 mmol, 1 eq) and concentrated hydrochloric acid (193 µL, 1.90 mmol, 1 eq). After cooling to rt, the crude mixture was poured into water and the resulting precipitate was collected by filtration. The mixture of **10i** and **11** was separated by CC (hexane/ethyl acetate 8:2); yield: 30 % (70 mg) for **10i** and 42 % (215 mg) for **11**.

3-[3-(4-Hydroxyphenyl)-1,2,4-thiadiazol-5-yl]phenol (**10**). The title compound was prepared by reaction of 3-[3-(4-methoxyphenyl)-[1,2,4]-thiadiazol-5-yl]-phenol (**10i**) (150 mg, 0.53 mmol) and boron tribromide (1.59 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 91 % (130 mg); MS (ESI): 271 $(M+H)^+$; Anal. ($C_{14}H_{10}N_2O_2S$) C, H, N.

3-(3-Methoxyphenyl)-5-(4-methoxyphenyl)-1-methyl-1*H*-1,2,4-triazole (**12i**). The title compound was prepared from *para*-anisoyl chloride (170 mg, 1.0 mmol), ethyl 3-methoxybenzimidate (179 mg, 1.0 mmol) and methyl hydrazine (92 mg, 2.0 mmol) according to method D; yield: 85 % (250 mg); m.p. 108-110 °C (CH_2Cl_2/ Et_2O); MS (ESI): 296 $(M+H)^+$.

3-[5-(4-Hydroxyphenyl)-1-methyl-1*H*-1,2,4-triazol-3-yl]phenol (**12**). The title compound was prepared by reaction of 3-(3-methoxyphenyl)-5-(4-methoxyphenyl)-1-methyl-1*H*-1,2,4-triazole (**12i**) (100 mg, 0.37 mmol) and borontrifluoride dimethyl sulfide complex (27.75 mmol) according to method F. The product was purified by preparative TLC (ethyl acetate); yield: 46 % (42 mg); MS (ESI): 268 $(M+H)^+$.

3,5-Bis(4-methoxyphenyl)-1-methyl-1*H*-1,2,4-triazole (**13i**). The title compound was prepared from *para*-anisoyl chloride (170 mg, 1.0 mmol), ethyl 4-methoxybenzimidate (179 mg, 1.0 mmol) and methyl hydrazine (92 mg, 2.0 mmol) according to method D; yield: 78 % (230 mg); m.p. 141-143 °C (CH_2Cl_2/ Et_2O); MS (ESI): 296 $(M+H)^+$.

4-[5-(4-Hydroxyphenyl)-1-methyl-1*H*-1,2,4-triazol-3-yl]phenol (**13**). The title compound was prepared by reaction of 3,5-bis(4-methoxyphenyl)-1-methyl-1*H*-1,2,4-triazole (**13i**) (100 mg, 0.37 mmol) and borontrifluoride dimethyl sulfide complex (27.7 mmol) according to method F. The product was purified by preparative TLC (ethyl acetate); yield: 63 % (57 mg); MS (ESI): 268 $(M+H)^+$.

3-(4-Methoxyphenyl)-5-(3-methoxyphenyl)-1-methyl-1*H*-1,2,4-triazole (**14i**). The title compound was prepared from *meta*-anisoyl chloride (170 mg, 1.0 mmol), ethyl 4-methoxybenzimidate (179 mg, 1.0 mmol) and methyl hydrazine (92 mg, 2.0 mmol) according to method D; yield: 77 % (227 mg); m.p. 114-116 °C (CH_2Cl_2/ Et_2O); MS (ESI): 296 $(M+H)^+$.

4-[5-(3-Hydroxyphenyl)-1-methyl-1*H*-1,2,4-triazol-3-yl]phenol (**14**). The title compound was prepared by reaction of 3-(4-methoxyphenyl)-5-(3-methoxyphenyl)-1-methyl-1*H*-1,2,4-triazole (**14i**) (100 mg, 0.37 mmol) and borontrifluoride dimethyl sulfide complex (27.7 mmol) according to method F. The product was purified by preparative TLC (ethyl acetate); yield: 53 % (48 mg); MS (ESI): 268 $(M+H)^+$.

3,5-Bis(3-methoxyphenyl)-1-methyl-1*H*-1,2,4-triazole (**15i**). The title compound was prepared from *meta*-anisoyl chloride (170 mg, 1.0 mmol), ethyl 3-methoxybenzimidate (179 mg, 1.0 mmol) and methyl hydrazine (92 mg, 2.0 mmol) according to method D; yield: 67 % (198 mg); m.p. 67-69 °C (CH_2Cl_2/ Et_2O); MS (ESI): 296 $(M+H)^+$.

3-[5-(3-Hydroxyphenyl)-1-methyl-1*H*-1,2,4-triazol-3-yl]phenol (**15**). The title compound was prepared by reaction of 3,5-bis(3-methoxyphenyl)-1-methyl-1*H*-1,2,4-triazole (**15i**) (100 mg, 0.37 mmol) and borontrifluoride dimethyl sulfide complex (27.7 mmol) according to method F. The product was purified by preparative TLC (ethyl acetate); yield: 64 % (58 mg); MS (ESI): 268 $(M+H)^+$.

2-Bromo-5-(3-methoxyphenyl)thiophene (**18ii**). The title compound was prepared by reaction of 2,5-dibromothiophene (465 µL, 4.13 mmol), 3-methoxybenzeneboronic acid (753 mg, 4.95 mmol), sodium

carbonate (876 mg, 8.26 mmol) and tetrakis(triphenylphosphine) palladium (24 mg, 20 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 41 % (445 mg).

2-(3-Methoxyphenyl)thiophene (**18i**). To a solution of 2-bromo-5-(3-methoxyphenyl)thiophene **(18ii**) (100 mg, 0.37 mmol, 1 eq) in dry THF cooled to -78 °C for 5 min, *n*-BuLi (1.6 M in hexane, 0.28 mL, 0.44 mmol, 1.2 eq) was added dropwise and stirred for 15 min at -78 °C. The crude mixture was carefully hydrolyzed by addition of water (10 ml) and layers were separated. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate and evaporated to dryness under reduced pressure; yield: 98 % (69 mg).

3-(2-Thienyl)phenol (**18**). The title compound was prepared by reaction of 2-(3-methoxyphenyl)thiophene (**18i**) (80 mg, 0.42 mmol) and boron tribromide (1.26 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 85 % (63 mg); MS (ESI): 177 $(M+H)^+$; Anal. ($C_{10}H_8OS$) C, H, N.

2-(3-Methoxyphenyl)-5-phenylthiophene (**19i**). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**18ii**) (400 mg, 1.52 mmol), benzeneboronic acid (223 mg, 1.82 mmol), sodium carbonate (322 mg, 3.04 mmol) and tetrakis(triphenylphosphine) palladium (8.8 mg, 7.6 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 70 % (283 mg).

3-(5-Phenyl-2-thienyl)phenol (**19**). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-5-phenylthiophene (**19i**) (100 mg, 0.37 mmol) and boron tribromide (2.22 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 81 % (75 mg); MS (ESI): 253 $(M+H)^+$; Anal. ($C_{16}H_{12}OS$) C, H, N.

2-Bromo-5-(4-methoxyphenyl)thiophene (**20ii**). The title compound was prepared by reaction of 2,5-dibromothiophene (V= 465 μL, 4.13 mmol), 4-methoxybenzeneboronic acid (753 mg, 4.95 mmol), sodium carbonate (876 mg, 8.26 mmol) and tetrakis(triphenylphosphine) palladium (24 mg, 20 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 75 % (815 mg).

2-(4-Methoxyphenyl)-5-phenylthiophene (**20i**). The title compound was prepared by reaction of 2-bromo-5-(4-methoxyphenyl)thiophene (**20ii**) (400 mg, 1.52 mmol), benzeneboronic acid (223 mg, 1.82 mmol), sodium carbonate (322 mg, 3.04 mmol) and tetrakis(triphenylphosphine) palladium (8.8 mg, 7.6 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 75 % (303 mg).

4-(5-Phenyl-2-thienyl)phenol (**20**). The title compound was prepared by reaction of 2-(4-methoxyphenyl)-5-phenylthiophene (**20i**) (100 mg, 0.37 mmol) and boron tribromide (2.22 mmol) according to method E. The product was purified by preparative TLC (dichloromethane/methanol 99:1); yield: 95 % (89 mg); MS (ESI): 253 $(M+H)^+$; Anal. ($C_{16}H_{12}OS$) C, H, N.

2-(3-Methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**22i**). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**18ii**) (150 mg, 0.57 mmol), 4-methoxybenzeneboronic acid (223 mg, 0.68 mmol), sodium carbonate (120 mg, 1.14 mmol) and tetrakis(triphenylphosphine) palladium (3.2 mg, 2.8 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 75 % (126 mg).

3-[5-(4-Hydroxyphenyl)-2-thienyl]phenol (**22**). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**22i**) (150 mg, 0.51 mmol) and boron tribromide (3.06 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 93 % (127 mg); MS (ESI): 269 $(M+H)^+$; Anal. ($C_{16}H_{12}O_2S$) C, H, N.

2,5-Bis(3-methoxyphenyl)thiophene (**23i**). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**18ii**) (150 mg, 0.57 mmol), 3-methoxybenzeneboronic acid (223 mg,

0.68 mmol), sodium carbonate (120 mg, 1.14 mmol) and tetrakis(triphenylphosphine) palladium (3.2 mg, 2.8 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 78 % (132 mg).

3,3'-Thiene-2,5-diyldiphenol (**23**). The title compound was prepared by reaction of 2,5-bis(3-methoxyphenyl)thiophene (**23i**) (150 mg, 0.51 mmol) and boron tribromide (3.06 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 95 % (130 mg); MS (ESI): 269 $(M+H)^+$; Anal. ($C_{16}H_{12}O_2S$) C, H, N.

4-Bromo-2-(3-methoxyphenyl)thiophene (**24ii**). The title compound was prepared by reaction of 2,4-dibromothiophene (1.00 g, 4.13 mmol), 3-methoxybenzeneboronic acid (753 mg, 4.95 mmol), sodium carbonate (876 mg, 8.26 mmol) and tetrakis(triphenylphosphine) palladium (24 mg, 20 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 72 % (782 mg).

2-(3-Methoxyphenyl)-4-phenylthiophene (**24i**). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)thiophene (**24ii**) (250 mg, 0.93 mmol), benzeneboronic acid (137 mg, 1.12 mmol), sodium carbonate (197 mg, 1.86 mmol) and tetrakis(triphenylphosphine) palladium (6 mg, 4.64 μmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 7:3); yield: 91% (225 mg).

3-(4-Phenyl-2-thienyl)phenol (**24**). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-4-phenylthiophene (**24i**) (225 mg, 0.85 mmol) and boron tribromide (3.6 mmol) according to method E. The product was purified by preparative TLC (dichloromethane/methanol 99:1); yield: 69 % (147 mg); MS (ESI): 253 $(M+H)^+$.

4-Bromo-2-(4-methoxyphenyl)thiophene (**25ii**). The title compound was prepared by reaction of 2,4-dibromothiophene (1.00 g, 4.13 mmol), 4-methoxybenzeneboronic acid (753 mg, 4.95 mmol), sodium carbonate (876 mg, 8.26 mmol) and tetrakis(triphenylphosphine) palladium (24 mg, 20 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 78 % (847 mg).

2-(4-Methoxyphenyl)-4-(3-methoxyphenyl)thiophene (**25i**). The title compound was prepared by reaction of 4-bromo-2-(4-methoxyphenyl)thiophene (**25ii**) (150 mg, 0.57 mmol), 3-methoxybenzeneboronic acid (223 mg, 0.68 mmol), sodium carbonate (120 mg, 1.14 mmol) and tetrakis(triphenylphosphine) palladium (3.2 mg, 2.8 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 70 % (118 mg).

3-[5-(4-Hydroxyphenyl)-3-thienyl]phenol (**25**). The title compound was prepared by reaction of 2-(4-methoxyphenyl)-4-(3-methoxyphenyl)thiophene (**25i**) (150 mg, 0.51 mmol) and boron tribromide (3.06 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 80 % (109 mg); MS (ESI): 267 $(M-H)^-$; Anal. ($C_{16}H_{12}O_2S$) C, H, N.

2-(3-Methoxyphenyl)-4-(4-methoxyphenyl)thiophene (**27i**). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)thiophene (**24ii**) (150 mg, 0.57 mmol), 4-methoxybenzeneboronic acid (223 mg, 0.68 mmol), sodium carbonate (120 mg, 1.14 mmol) and tetrakis(triphenylphosphine) palladium (3.2 mg, 2.8 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 70 % (118 mg).

3-[4-(4-Hydroxyphenyl)-2-thienyl]phenol (**27**). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-4-(4-methoxyphenyl)thiophene (**27i**) (150 mg, 0.51 mmol) and boron tribromide solution (3.06 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 85 % (116 mg); MS (ESI): 267 $(M-H)^-$; Anal. ($C_{16}H_{12}O_2S$) C, H, N.

2,4-Bis(3-methoxyphenyl)thiophene (**28i**). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)thiophene (**24ii**) (150 mg, 0.57 mmol), 3-methoxybenzeneboronic acid (223 mg, 0.68 mmol), sodium carbonate (120 mg, 1.14 mmol) and tetrakis(triphenylphosphine) palladium (3.2

mg, 2.8 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 72 % (121 mg).

3,3'-Thiene-2,4-diyldiphenol (**28**). The title compound was prepared by reaction of 2,4-bis(3-methoxyphenyl)thiophene (**28i**) (150 mg, 0.51 mmol) and boron tribromide (3.06 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 88 % (120 mg); MS (ESI): 267 $(M-H)^-$; Anal. ($C_{16}H_{12}O_2S$) C, H, N.

4,4'-(Seleniene-2,5-diyl)diphenol (**29**). The title compound was prepared by reaction of 2,5-dibromoselenophene (150 mg, 0.52 mmol), 4-hydroxybenzeneboronic acid (172 mg, 1.25 mmol), caesium carbonate (679 mg, 2.08 mmol) and tetrakis(triphenylphosphine) palladium (6.0 mg, 5.2 μmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 49 % (81 mg); MS (ESI): 317 $(M+H)^+$; Anal. ($C_{16}H_{12}O_2Se$) C, H, N.

3,3'-(Seleniene-2,5-diyl)diphenol (**30**). The title compound was prepared by reaction of 2,5-dibromoselenophene (150 mg, 0.52 mmol), 3-hydroxybenzeneboronic acid (172 mg, 1.25 mmol), caesium carbonate (679 mg, 2.08 mmol) and tetrakis(triphenylphosphine) palladium (6.0 mg, 5.2 μmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 42 % (69 mg); MS (ESI): 317 $(M+H)^+$; Anal. ($C_{16}H_{12}O_2Se$) C, H, N.

3-Bromo-2-(4-methoxyphenyl)thiophene (**31ii**). The title compound was prepared by reaction of 2,3-dibromothiophene (234 μL, 2.1 mmol), 4-methoxybenzeneboronic acid (383 mg, 2.52 mmol), sodium carbonate (403 mg, 4.2 mmol) and tetrakis(triphenylphosphine) palladium (12 mg, 10 μmol) according to method B. The product was purified by CC (hexane); yield: 70 % (387 mg).

4,4'-(Thiene-2,3-diyl)diphenol (**31**). The title compound was prepared by reaction of 2,3-bis(4-methoxyphenyl)thiophene (**31i**) (150 mg, 0.51 mmol) and boron tribromide (3.06 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 70 % (95 mg); MS (ESI): 269 $(M+H)^+$; Anal. ($C_{16}H_{12}O_2S$) C, H, N.

3-Bromo-2-(3-methoxyphenyl)thiophene (**32ii**). The title compound was prepared by reaction of 2,3-dibromothiophene (234 μL, 2.1 mmol), 3-methoxybenzeneboronic acid (383 mg, 2.52 mmol), sodium carbonate (403 mg, 4.2 mmol) and tetrakis(triphenylphosphine) palladium (12 mg, 10 μmol) according to method B. The product was purified by CC (hexane); yield: 58 % (320 mg).

2-(3-Methoxyphenyl)-3-(4-methoxyphenyl)-thiophene (**32i**). The title compound was prepared by reaction of 3-bromo-2-(3-methoxyphenyl)thiophene (**32ii**) (150 mg, 0.57 mmol), 4-methoxybenzeneboronic acid (223 mg, 0.68 mmol), sodium carbonate (120 mg, 1.14 mmol) and tetrakis(triphenylphosphine) palladium (3.2 mg, 2.8 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 7:3); yield 40 % (68 mg).

3-[3-(4-Hydroxyphenyl)-2-thienyl]phenol (**32**). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-3-(4-methoxyphenyl)thiophene (**32i**) (150 mg, 0.51 mmol) and boron tribromide (3.06 mmol) according to method E. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 56 % (77 mg); MS (ESI): 269 $(M+H)^+$; Anal. ($C_{16}H_{12}O_2S$) C, H, N.

3,3''-Dimethoxy-1,1':4',1''-terphenyl (**35i**). The title compound was prepared by reaction of 4'-bromo-3-methoxybiphenyl (**35ii**) (500 mg, 1.90 mmol), 3-methoxybenzeneboronic acid (346 mg, 2.28 mmol), sodium carbonate (403 mg, 3.80 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 9.5 μmol) according to an adaptation of method A (heating the mixture 20 h instead of 4 h). The product was purified by CC (hexane/ethyl acetate 95:5); yield: 14 % (77 mg).

3,4''-Dimethoxy-1,1':4',1''-terphenyl (**36i**). The title compound was prepared by reaction of 4'-bromo-3-methoxybiphenyl (**35ii**) (500 mg, 1.90 mmol), 4-methoxybenzeneboronic acid (346 mg, 2.28 mmol), sodium carbonate (403 mg, 3.80 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 9.5 μmol)

according to an adaptation of method A (heating the mixture 20 h instead of 4 h). The product was purified by CC (hexane/ethyl acetate 95:5); yield: 90 % (496 mg).

3,3'-Pyridine-2,5-diyldiphenol (**37**). The title compound was prepared by reaction of 2,5- dibromo pyridine (150 mg, 0.63 mmol), 3-hydroxybenzeneboronic acid (231 mg, 1.52 mmol), caesium carbonate (821 mg, 2.52 mmol) and tetrakis(triphenylphosphine) palladium (7.3 mg, 6.3 μmol) according to method C. The product was purified by preparative TLC (dichloromethane/methanol 98:2); yield: 67 % (111 mg); MS (ESI): 262 $(M-H)^-$; Anal. ($C_{17}H_{13}NO_2$) C, H, N.

Biological Methods

[2, 4, 6, 7-^{3}H]-E2 and [2, 4, 6, 7-^{3}H]-E1 were bought from Perkin Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt.
17β-HSD1 and 17β-HSD2 were obtained from human placenta according to previously described procedures.[22,62] Fresh human placenta was homogenized and centrifuged. The pellet fraction contains the microsomal 17β-HSD2, while 17β-HSD1 was obtained after precipitation with ammonium sulfate from the cytosolic fraction.

1. Inhibition of 17β-HSD1

Inhibitory activities were evaluated by a well established method with minor modifications.[41, 63, 64] Briefly, the enzyme preparation was incubated with NADH [500 μM] in the presence of potential inhibitors at 37 °C in a phosphate buffer (50 mM) supplemented with 20 % of glycerol and EDTA 1mM**.** Inhibitor stock solutions were prepared in DMSO. Final concentration of DMSO was adjusted to 1 % in all samples. The enzymatic reaction was started by addition of a mixture of unlabelled- and [2, 4, 6, 7-^{3}H]- E1 (final concentration: 500 nM, 0.15 μCi). After 10 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with ether. After evaporation, the steroids were dissolved in acetonitrile. E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 rp chromatography column (Nucleodur C18 Gravity, 3 μm, Macherey-Nagel, Düren) connected to a HPLC-system (Agilent 1100 Series, Agilent Technologies, Waldbronn). Detection and quantification of the steroids were performed using a radioflow detector (Berthold Technologies, Bad Wildbad). The conversion rate was calculated according to following equation: $\%conversion = \frac{\%E2}{\%E2 + \%E1} \cdot 100$.
Each value was calculated from at least three independent experiments.

2. Inhibition of 17β-HSD2

The 17β-HSD2 inhibition assay was performed similarly to the 17β-HSD1 procedure. The microsomal fraction was incubated with NAD^+ [1500 μM], test compound and a mixture of unlabelled- and [2, 4, 6, 7-^{3}H]-E2 (final concentration: 500 nM, 0.11 μCi) for 20 min at 37 °C. Further treatment of the samples and HPLC separation was carried out as mentioned above.

3. ER affinity

The binding affinity of select compounds to the ERα and ERβ was determined according to Zimmermann et al.[65] Briefly, 0.25 pmol of ERα or ERβ, respectively, were incubated with [2, 4, 6, 7-^{3}H]-E2 (10 nM) and test compound for 1 h at rt. The potential inhibitors were dissolved in DMSO (5 % final concentration). Non-specific-binding was performed with diethylstilbestrol (10 μM). After incubation, ligand-receptor complexes were selectively bound to hydroxyapatite (5 g/ 60 mL TE-buffer). The formed complex was separated, washed and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (Rack Beta Primo 1209, Wallac, Turku). For determination of the relative binding affinity (RBA), inhibitor and E2 concentrations required to displace 50 % of the

receptor bound labelled E2 were determined. RBA values were calculated according to the following equation: $RBA[\%] = \frac{IC_{50}(E2)}{IC_{50}(compound)} \cdot 100$. The RBA value for E2 was arbitrarily set at 100 %.

4. Evaluation of the estrogenic activity using T-47D cells

Phenol red-free medium was supplemented with sodium bicarbonate (2 g/L), streptomycin (100 µg/mL), insuline zinc salt (10 µg/mL), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/mL) and DCC-FCS 5% (v/v). RPMI 1640 (without phenol red) was used for the experiments. Cells were grown for 48 h in phenol red-free medium. Compounds **4**, **10**, **22**, **25**, **35** and **36** were added at a final concentration of 100 nM. Inhibitors and E2 were diluted in ethanol (final ethanol concentration was adjusted to 1 %). As a positive control E2 was added at a final concentration of 0.1 nM. Ethanol was used as negative control. Medium was changed every two to three days and supplemented with the respective additive. After eight days of incubation, the cell viability was evaluated measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT). The cleavage of MTT to a blue formazan by mitochondrial succinat-dehydrogenase was quantified spectrophotometrically at 590 nm as described by Denizot and Lang[66] with minor modifications. The control proliferation was arbitrarily set at 1 and the stimulation induced by the inhibitor was calculated according to following equation: $\%stimulation = \frac{[proliferation(compound-induced)-1]}{[proliferation(E2-induced)-1]} \cdot 100\%$.
Each value is calculated as a mean value of at least three independent experiments

5. Caco-2 transport experiments

Caco-2 cell culture and transport experiments were performed according to Yee[67] with small modifications. Cell culture time was reduced from 21 to 10 days by increasing seeding density from $6.3 \cdot 10^4$ to $1.65 \cdot 10^5$ cells per well. Four reference compounds (atenolol, testosterone, ketoprofene, erythromycin) were used in each assay for validation. The compounds were applied to the cells as a mixture (cassette dosing) to increase the throughput. The initial concentration of the compounds in the donor compartment was 50 µM (0.2 M MES, pH: 6.5, containing either 1 % ethanol or DMSO). Samples were taken from the acceptor side after 0 min, 60, 120 and 180 min and from the donor side after 0 and 180 min. Each experiment was run in triplicate. The integrity of the monolayers was checked by measuring the transepithelial electrical resistance (TEER) before the transport experiments and by measuring lucifer yellow permeability after each assay. All samples of the CaCo-2 transport experiments were analyzed by LC/MS/MS after dilution with buffer of the opposite transwell chamber (1:1, containing 2 % acetic acid). The apparent permeability coefficients (P_{app}) were calculated using equation $P_{app} = \frac{dQ}{dtAc_0}$, where $\frac{dQ}{dt}$ is the appearance rate of mass in the acceptor compartment, A the surface area of the transwell membrane, and c_0 the initial concentration in the donor compartment.

6. Metabolic stability assay

The assay was performed with liver microsomes from male Sprague Dawley rats (BD Gentest, USA). Stock solutions (10 mM in acetonitrile) were diluted to give working solutions in 20 % acetonitrile. The incubation solutions consisted of a microsomal suspension of 0.33 mg/mL of protein in phosphate buffer 100 mM pH 7.4 and 90 µL $NADP^+$-regenerating system ($NADP^+$ 1 mM, glucose-6-phosphate 5 mM, glucose–6-phosphate dehydrogenase 5 U/mL, $MgCl_2$ 5 mM).
The reaction was initiated by the addition of test compound (final concentration 1µM) to the pre-incubated microsomes/buffer mix at 37 °C. The samples were removed from the incubations after 0, 15, 30, and 60 min and processed for acetonitrile precipitation. The samples were analyzed by LC-MS/MS. Two control groups were run in parallel: positive controls (PC; n= 1) using 7-ethoxycoumarin as reference compound to prove the quality of the microsomal enzymatic activity and negative controls (NC; n= 1), using boiled microsomes (boiling water bath, 25 min) without regenerating system to ensure that the potential apparent loss of parent compound in the assay incubation is due to metabolism. The amount of compound in the samples was expressed in percentage of remaining compound

compared to time point zero (= 100 %). These percentages were plotted against the corresponding time points and the half-life time was derived by a standard fit of the data.
Intrinsic clearance (Cl_{int}) estimates were determined using the rate of parent disappearance. The slope (-k) of the linear regression from log [test compound] versus time plot was determined as well as the elimination rate constant: $k= \ln 2/t_{1/2.}$ The equation expressing the microsomal Cl_{int} can be derived: $Cl_{int}=k*V*f_u$ [µl/min/mg protein], where f_u is the unbound fraction. V gives a term for the volume of the incubation expressed in µL / mg protein. As f_u is not known for the tested compound, the calculation was performed with f_u =1 (V= incubation volume [µL]/microsomal protein[mg] = 6667).

7. Inhibition of human hepatic CYPs

The commercially available P450 inhibition kits from BD Gentest™ (Heidelberg, Germany) were used according to the instructions of the manufacturer. Compounds **4, 25** and **35** were tested for inhibition of the following enzymes: CYP1A2, 2B6, 2C9, 2C19, 2D6 and 3A4. Inhibitory potencies were determined as IC_{50}-values.

8. *In-Vivo* Pharmacokinetics

Male Wistar rats weighing 300-330 g (Janvier France) were housed in a temperature- controlled room (20-22 °C) and maintained in a 12h light/12h dark cycle. Food and water were available *ad libitum*. They were anesthetized with a ketamine (135 mg/kg)/ xylyzine (10 mg/kg) mixture and cannulated with silicone tubing via the right jugular vein and attached to the skull with dental cement.[68] Prior to the first blood sampling, animals were connected to a counterbalanced system and tubing to perform blood sampling in the freely moving rat.
Compounds **3** and **39** were applied orally in a cassette dosing in 4 rats at the dose of 10 mg/kg body weight by using a feeding needle. The compounds were dissolved in a mixture labrasol/water (1:1) and given at a volume of 5mL/kg. Blood samples (0.2 mL) were taken at 0, 1, 2, 3, 4, 6, 8, 10 and 24 h postdose and collected in heparinised tubes. They were centrifuged at 3000g for 10 min, and plasma was harvested and kept at -20 °C until analyzed.
HPLC-MS/MS analysis and quantification of the samples was carried out on a Surveyor-HPLC-system coupled with a TSQ Quantum (Thermo/Fisher) triple quadrupole mass spectrometer equipped with an electrospray interface (ESI).

Computational chemistry

1. Distance and angle calculations

All distances and angles were calculated after energy minimisation using Hyperchem v. 6.0.

2. MEP

For selected compounds *ab initio* geometry optimisations were performed gas phase at the B3LYP/6-311**G (d,p) level of density functional theory (DFT) by means of the Gaussian03 software and the molecular electrostatics potential map (MEP) was plotted using GaussView 3.09, the 3D molecular graphics package of Gaussian.[69] These electrostatic potential surfaces were generated by mapping 6-311G** electrostatic potentials onto surfaces of molecular electron density (isovalue = 0.002e/Å). The MEP maps are color coded, where red stands for negative values ($3.1*10^{-2}$ Hartree) and blue for positive ones ($4.5*10^{-2}$ Hartree).[103]

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Supporting information

Spectroscopic data of all compounds (^{1}H-NMR, ^{13}C-NMR, IR), purity data of final compounds and MEP maps of compounds **7**, **10**, **14**, **27** and **G**. This material is available free of charge via internet at http://pubs.acs.org

References

1. Travis, R. C.; Key, T. J. Oestrogen exposure and breast cancer risk. *Breast Cancer Res.* **2003**, *5*, 239-247.
2. Dizerega, G. S.; Barber, D. L.; Hodgen, G. D. Endometriosis: role of ovarian steroids in initiation, maintenance and suppression. *Fertil. Steril.* **1980**, *33*, 649-653.
3. Miller, W. R.; Bartlett, J. M.; Canney, P.; Verrill, M. Hormonal therapy for postmenopausal breast cancer: the science of sequencing. *Breast Cancer Res. Treat.* **2007**, *103*, 149-160.
4. Bush, N. J. Advances in hormonal therapy for breast cancer. *Semin. Oncol. Nurs.* **2007**, *23*, 46-54.
5. Adamo, V.; Iorfida, M.; Montalto, E.; Festa, V.; Garipoli, C.; Scimone, A.; Zanghi, M.; Caristi, N. Overview and new strategies in metastatic breast cancer (MBC) for treatment of tamoxifen-resistant patients. *Ann. Oncol.* **2007**, *18 Suppl 6*, vi53-57.
6. Ohnesorg, T.; Keller, B.; Hrabe de Angelis, M.; Adamski, J. Transcriptional regulation of human and murine 17beta-hydroxysteroid dehydrogenase type-7 confers its participation in cholesterol biosynthesis. *J. Mol. Endocrinol.* **2006**, *37*, 185-197.
7. Sakurai, N.; Miki, Y.; Suzuki, T.; Watanabe, K.; Narita, T.; Ando, K.; Yung, T. M.; Aoki, D.; Sasano, H.; Handa, H. Systemic distribution and tissue localizations of human 17beta-hydroxysteroid dehydrogenase type 12. *J. Steroid Biochem. Mol. Biol.* **2006**, *99*, 174-181.
8. Day, J. M.; Foster, P. A.; Tutill, H. J.; Parsons, M. F.; Newman, S. P.; Chander, S. K.; Allan, G. M.; Lawrence, H. R.; Vicker, N.; Potter, B. V.; Reed, M. J.; Purohit, A. 17beta-hydroxysteroid dehydrogenase Type 1, and not Type 12, is a target for endocrine therapy of hormone-dependent breast cancer. *Int. J. Cancer* **2008**, *122*, 1931-1940.
9. Gunnarsson, C.; Hellqvist, E.; Stål, O. 17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer* **2005**, *92*, 547-552.
10. Gunnarsson, C.; Olsson, B. M.; Stål, O. Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.* **2001**, *61*, 8448-8451.
11. Miyoshi, Y.; Ando, A.; Shiba, E.; Taguchi, T.; Tamaki, Y.; Noguchi, S. Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* **2001**, *94*, 685-689.
12. Suzuki, T.; Moriya, T.; Ariga, N.; Kaneko, C.; Kanazawa, M.; Sasano, H. 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br. J. Cancer* **2000**, *82*, 518-523.
13. Šmuc, T.; Pucelj Ribič, M.; Šinkovec, J.; Husen, B.; Thole, H.; Lanisnik Rižner, T. Expression analysis of the genes involved in estradiol and progesterone action in human ovarian endometriosis. *Gynecol. Endocrinol.* **2007**, *23*, 105-111.
14. Labrie, F. Intracrinology. *Mol. Cell. Endocrinol.* **1991**, *78*, C113-C118.
15. Vihko, P.; Härkönen, P.; Soronen, P.; Törn, S.; Herrala, A.; Kurkela, R.; Pulkka, A.; Oduwole, O.; Isomaa, V. 17beta-hydroxysteroid dehydrogenases- their role in pathophysiology. *Mol. Cell. Endocrinol.* **2004**, *215*, 83-88.
16. Azzi, A.; Rehse, P. H.; Zhu, D. W.; Campbell, R. L.; Labrie, F.; Lin, S.-X. Crystal structure of human estrogenic 17beta-hydroxysteroid dehydrogenase complexed with 17 beta-estradiol. *Nat. Struct. Biol.* **1996**, *3*, 665-668.
17. Breton, R.; Housset, D.; Mazza, C.; Fontecilla-Camps, J. C. The structure of a complex of human 17beta-hydroxysteroid dehydrogenase with estradiol and $NADP^{+}$ identifies two principal targets for the design of inhibitors. *Structure* **1996**, *4*, 905-915.

18. Gangloff, A.; Shi, R.; Nahoum, V.; Lin, S.-X. Pseudo-symmetry of C19 steroids, alternative binding orientations, and multispecificity in human estrogenic 17beta-hydroxysteroid dehydrogenase. *FASEB J.* **2003**, *17*, 274-276.
19. Ghosh, D.; Pletnev, V. Z.; Zhu, D. W.; Wawrzak, Z.; Duax, W. L.; Pangborn, W.; Labrie, F.; Lin, S.-X. Structure of human estrogenic 17beta- hydroxysteroid dehydrogenase at 2.20 Å resolution. *Structure* **1995**, *3*, 503-513.
20. Han, Q.; Campbell, R. L.; Gangloff, A.; Huang, Y. W.; Lin, S.-X. Dehydroepiandrosterone and dihydrotestosterone recognition by human estrogenic 17beta-hydroxysteroid dehydrogenase. C-18/C-19 steroid discrimination and enzyme-induced strain. *J. Biol. Chem.* **2000**, *275*, 1105-1111.
21. Mazza, C.; Breton, R.; Housset, D.; Fontecilla-Camps, J. C. Unusual charge stabilization of $NADP^+$ in 17beta-hydroxysteroid dehydrogenase. *J. Biol. Chem.* **1998**, *273*, 8145-8152.
22. Qiu, W.; Campbell, R. L.; Gangloff, A.; Dupuis, P.; Boivin, R. P.; Tremblay, M. R.; Poirier, D.; Lin, S.-X. A concerted, rational design of type 1 17beta-hydroxysteroid dehydrogenase inhibitors: estradiol-adenosine hybrids with high affinity. *FASEB J.* **2002**, *16*, 1829-1831.
23. Sawicki, M. W.; Erman, M.; Puranen, T.; Vihko, P.; Ghosh, D. Structure of the ternary complex of human 17beta-hydroxysteroid dehydrogenase type 1 with 3-hydroxyestra-1,3,5,7-tetraen-17-one (equilin) and $NADP^+$. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 840-845.
24. Shi, R.; Lin, S.-X. Cofactor hydrogen bonding onto the protein main chain is conserved in the short chain dehydrogenase/reductase family and contributes to nicotinamide orientation. *J. Biol. Chem.* **2004**, *279*, 16778-16785.
25. Filling, C.; Berndt, K. D.; Benach, J.; Knapp, S.; Prozorovski, T.; Nordling, E.; Ladenstein, R.; Jornvall, H.; Oppermann, U. Critical residues for structure and catalysis in short-chain dehydrogenases/reductases. *J. Biol. Chem.* **2002**, *277*, 25677-25684.
26. Penning, T. M. 17beta-hydroxysteroid dehydrogenase: inhibitors and inhibitor design. *Endocr. Relat. Cancer* **1996**, *3*, 41-56.
27. Brožic, P.; Lanišnik Rižner, T.; Gobec, S. Inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Curr. Med. Chem.* **2008**, *15*, 137-150 and references therein cited.
28. Messinger, J.; Hirvelä, L.; Husen, B.; Kangas, L.; Koskimies, P.; Pentikäinen, O.; Saarenketo, P.; Thole, H. New inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* **2006**, *248*, 192-198.
29. Karkola, S.; Lilienkampf, A.; Wähälä, K. A 3D QSAR model of 17beta-HSD1 inhibitors based on a thieno[2,3-d]pyrimidin-4(3H)-one core applying molecular dynamics simulations and ligand-protein docking. *ChemMedChem* **2008**, *3*, 461-472.
30. Allan, G. M.; Vicker, N.; Lawrence, H. R.; Tutill, H. J.; Day, J. M.; Huchet, M.; Ferrandis, E.; Reed, M. J.; Purohit, A.; Potter, B. V. Novel inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: templates for design. *Bioorg. Med. Chem.* **2008**, *16*, 4438-4456.
31. Frotscher, M.; Ziegler, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Neugebauer, A.; Fetzer, L.; Scherer, C.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W. Design, synthesis and biological evaluation of (hydroxyphenyl)-naphthalene and quinoline derivatives: potent and selective non steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* **2008**, *51*, 2158-2169.
32. Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Ziegler, E.; Neugebauer, A.; Bhoga, U. D.; Bey, E.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W. Substituted 6-phenyl-2-naphthols. Potent and selective non-steroidal inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): design, synthesis, biological evaluation and pharmacokinetics. *J. Med. Chem.* **2008**, *51*, 4685-4698.
33. Bey, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Werth, R.; Oster, A.; Algül, O.; Neugebauer, A.; Hartmann, R. W. Design, synthesis and biological evaluation of bis(hydroxyphenyl) azoles as potent and selective non-steroidal inhibitors of 17beta- hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *Bioorg. Med. Chem.* **2008**, *16*, 6423-6435.
34. Miyaura, N.; Suzuki, A. The palladium catalysed cross-coupling reaction of phenylboronic acid with haloarenes in the presence of bases. *Synth. commun.* **1995**, *11*, 513-519.
35. Fink, B. E.; Mortensen, D. S.; Stauffer, S. R.; Aron, Z. D.; Katzenellenbogen, J. A. Novel structural templates for estrogen-receptor ligands and prospects for combinatorial synthesis of estrogens. *Chem. Biol.* **1999**, *6*, 205-219.

36. Gierczyk, B.; Zalas, M. Synthesis of substituted 1,3,4-thiadiazoles using Lawesson reagents. *Org. Prep. proc. int.* **2005**, *37*, 213-222.
37. Bagley, M. C.; Chapaneri, K.; Glover, C.; Merrit, E. A. Simple microwave-assisted method for the synthesis of primary thioamides from nitriles. *Synlett* **2004**, *14*, 2615-2617.
38. Kelarev, V. I.; Silin, M. A.; Kobrakov, K. I.; Rybina, I. I.; Korolev, V. K. Synthesis of 1,3,5-trisubstituted 1*H*-1,2,4-triazoles containing hetaryl fragments. *Chem. Heterocycl. Comp.* **2003**, *39*, 736-743.
39. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. Click chemistry: Diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004-2021.
40. Guither, W. D.; Coburn, M. D.; Castle, R. N. 3,6-Bis-substituted s-tetrazines. *Heterocycles* **1979**, *12*, 745-749.
41. Lin, S.-X.; Yang, F.; Jin, J. Z.; Breton, R.; Zhu, D. W.; Luu-The, V.; Labrie, F. Subunit identity of the dimeric 17beta-hydroxysteroid dehydrogenase from human placenta. *J. Biol. Chem.* **1992**, *267*, 16182-16187.
42. Hidalgo, I. J. Assessing the absorption of new pharmaceuticals. *Curr. Top. Med. Chem.* **2001**, *1*, 385-401.
43. Yearley, E. J.; Zhurova, E. A.; Zhurov, V. V.; Pinkerton, A. A. Binding of genistein to the estrogen receptor based on an experimental electron density study. *J. Am. Chem. Soc.* **2007**, *129*, 15013-15021.
44. White, E. H.; Bursey, M. M.; Roswel, D. F.; Hill, J. H. M. Chemiluminescence of some monoacylhydrazides. *J. Org. Chem.* **1967**, *32*, 1198-1202.
45. Manaka, A.; Sato, M. Synthesis of aromatic thioamide from nitrile without handling of hydrogen sulfide. *Synth. commun.* **2005**, *11*, 761-764.
46. Ando, K.; Kato, T.; Kawai, A.; Nonomura, T. Preparation of heterocyclyl sulfonylbenzene compounds as anti-inflammatory/analgesic agents. WO9964415A1, **1999**.
47. Uozumi, Y.; Kikuchi, M. Controlled monoarylation of dibromoarenes in water with a polymeric palladium catalyst. *Synlett* **2005**, *11*, 1775-1778.
48. Ewing, W. R.; Becker, M. R.; Pauls, H. W.; Cheney, D. L.; Mason, J. S.; Spada, A. P.; Choi-Sledeski; Mi., Y. Preparation of substituted (sulfinic acid, sulfonic acid, sulfonylamino or sulfinylamino) *N*-[(aminoiminomethyl)phenylalkyl]azaheterocyclamide compounds as factor Xa inhibitors. WO9640679A1, **1996**.
49. Vachal, P.; Toth, L. M. General facile synthesis of 2,5-diarylheteropentalenes. *Tetrahedron Lett.* **2004**, *45*, 7157-7161.
50. Yokooji, A.; Okazawa, T.; Satoh, T.; Miura, M.; Nomura, M. Palladium-catalyzed direct arylation of thiazoles with aryl bromides. *Tetrahedron* **2003**, *59*, 5685-5689.
51. Chandra, R.; Kung, M.-P.; Kung, H. F. Design, synthesis, and structure-activity relationship of novel thiophene derivatives for beta-amyloid plaque imaging. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1350-1352.
52. Sone, T.; Sato, K.; Umetsu, Y.; Yoshino, A.; Takahashi, K. Acid-catalyzed reactions of thiophene nuclei. VIII. Synthesis of unsymmetrical 2,3-diaryl- and 2,4-diarylthiophenes starting from 2,5-dichlorothiophene. *Bull. Chem. Soc. Jpn.* **1994**, *67*, 2187-2194.
53. Nakano, M.; Satoh, T.; Miura, M. Palladium-catalyzed selective 2,3-diarylation of alpha, alpha-disubstituted 3-thiophenemethanols via cleavage of C-H and C-C bonds. *J. Org. Chem.* **2006**, *71*, 8309-8311.
54. Liu, L.; Zhang, Y.; Xin, B. Synthesis of biaryls and polyaryls by ligand-free Suzuki reaction in aqueous phase. *J. Org. Chem.* **2006**, *71*, 3994-3997.
55. Demerseman, P.; Buu-Hoi, N. P.; Royer, R. Thiophene derivatives of biological interest. VII. The synthesis of 2,4-diarylthiophenes and 2,4-diarylselenophenes from anils of alkyl aryl ketones. *J. Chem. Soc.* **1954**, 2720-2722.
56. Lebrini, M.; Bentiss, F.; Lagrenee, M. Rapid synthesis of 2,5-disubstituted 1,3,4-thiadiazoles under microwave irradiation. *J. Het. Chem.* **2005**, *42*, 991-994.
57. Pirali, T.; Gatti, S.; Di Brisco, R.; Tacchi, S.; Zaninetti, R.; Brunelli, E.; Massarotti, A.; Sorba, G.; Canonico, P. L.; Moro, L.; Genazzani, A. A.; Tron, G. C.; Billington, R. A. Estrogenic analogues synthesized by click chemistry. *ChemMedChem* **2007**, *2*, 437-440.

58. Yas'ko, S. V.; Korchevin, N. A.; Gusarova, N. K.; Kazantseva, T. I.; Chernysheva, N. A.; Klyba, L. V.; Trofimov, B. A. Microwave induced synthesis of diphenylthiophenes from elemental sulfur and styrene. *Chem. Het. Comp.* **2006**, *42*, 1486-1487.
59. Lahti, P. M.; Ichimura, A. S. Semiempirical study of electron exchange interaction in organic high-spin pi-systems. Classifying structural effects in organic magnetic molecules. *J. Org. Chem.* **1991**, *56*, 3030-3042.
60. Tagat, J. R.; McCombie, S. W.; Barton, B. E.; Jackson, J.; Shortall, J. Synthetic inhibitors of interleukin-6. II. 3,5-Diarylpyridines and m-terphenyls. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2143-2146.
61. Muller, G.; Amiard, G.; Mathieu, J. Dihydropyrazines and pyrazines obtained by condensation of some phenacylamines. *Bull. Soc. Chim. Fr.* **1949**, 533-535.
62. Zhu, D. W.; Lee, X.; Breton, R.; Ghosh, D.; Pangborn, W.; Duax, W. L.; Lin, S.-X. Crystallization and preliminary X-ray diffraction analysis of the complex of human placental 17beta-hydroxysteroid dehydrogenase with $NADP^+$. *J. Mol. Biol.* **1993**, *234*, 242-244.
63. Sam, K. M.; Auger, S.; Luu-The, V.; Poirier, D. Steroidal spiro-gamma-lactones that inhibit 17beta-hydroxysteroid dehydrogenase activity in human placental microsomes. *J. Med. Chem.* **1995**, *38*, 4518-4528.
64. Sam, K. M.; Boivin, R. P.; Tremblay, M. R.; Auger, S.; Poirier, D. C16 and C17 derivatives of estradiol as inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: chemical synthesis and structure-activity relationships. *Drug. Des. Discov.* **1998**, *15*, 157-180.
65. Zimmermann, J.; Liebl, R.; von Angerer, E. 2,5-Diphenylfuran-based pure antiestrogens with selectivity for the estrogen receptor alpha. *J. Steroid Biochem. Mol. Biol.* **2005**, *94*, 57-66.
66. Denizot, F.; Lang, R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **1986**, *89*, 271-277.
67. Yee, S. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man - fact or myth. *Pharm. Res.* **1997**, *14*, 763-766.
68. Van Dongen, J. J.; Remie, R.; Rensema, J. W.; Van Wunnik, G. H. J. Manual of microsurgery on the laboratory rat. In Huston, J. P., Ed., Ed. 1990; p 159.
69. Dennington, I.; Roy, K. T.; Millam, J.; Eppinnett, K.; Howell, W. L.; Gilliland, R. *GaussView*, 3.0; Semichem Inc., Shawnee Mission: 2003.
70. Petti, M. A.; Shepodd, T. J.; Barrans, R. E.; Dougherty, D. A. "Hydrophobic" binding of water-soluble guests by high-symmetry, chiral hosts. An electron-rich receptor site with a general affinity for quaternary ammonium compounds and electron-deficient pi-systems. *J. Am. Chem. Soc.* **1988**, *110*, 6825-6840.
71. Tannergren, C.; Langguth, P.; Hoffmann, K. J. Compound mixtures in CaCo-2 cell permeability screens as a means to increase screening capacity. *Pharmazie* **2001**, *56*, 337-342.
72. Laitinen, L.; Kangas, H.; Kaukonen, A. M.; Hakala, K.; Kotiaho, T.; Kostiainen, R.; Hirvonen, J. N-in-one permeability studies of heterogeneous sets of compounds across Caco-2 cell monolayers. *Pharm. Res.* **2003**, *20*, 187-197.

3.IV New Insights into the SAR and Binding Modes of Bis(hydroxyphenyl)thiophenes and Benzenes: Influence of Additional Substituents on 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1) Inhibitory Activity and Selectivity.

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Paper IV

abstract: 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) is responsible for the catalytic reduction of weakly active E1 to highly potent E2. E2 stimulates the proliferation of hormone-dependent diseases via activation of the estrogen receptor α (ERα). Due to the overexpression of 17β-HSD1 in mammary tumors, this enzyme should be an attractive target for the treatment of estrogen-dependent pathologies. Recently, we have reported on a series of potent 17β-HSD1 inhibitors: bis(hydroxyphenyl) azoles, thiophenes and benzenes. In this paper, different substituents were introduced into the core structure and the biological properties of the corresponding inhibitors were evaluated. Computational methods and analysis of different X-rays of 17β-HSD1 lead to identification of two different binding modes for these inhibitors. The fluorine compound **23** exhibits an IC_{50} values of 8 nM and is the most potent non-steroidal inhibitor described so far. It also shows a high selectivity (17β-HSD2, ERα) and excellent pharmacokinetic properties after peroral application to rats.

Introduction

Estrogens are involved in the regulation of the female reproduction system. However, it is also well known that 17β-estradiol (E2), the natural ligand of the estrogen receptors (ERs) α and β, plays a critical role in the development of several estrogen-dependent pathologies like breast cancer[1] and endometriosis.[2]

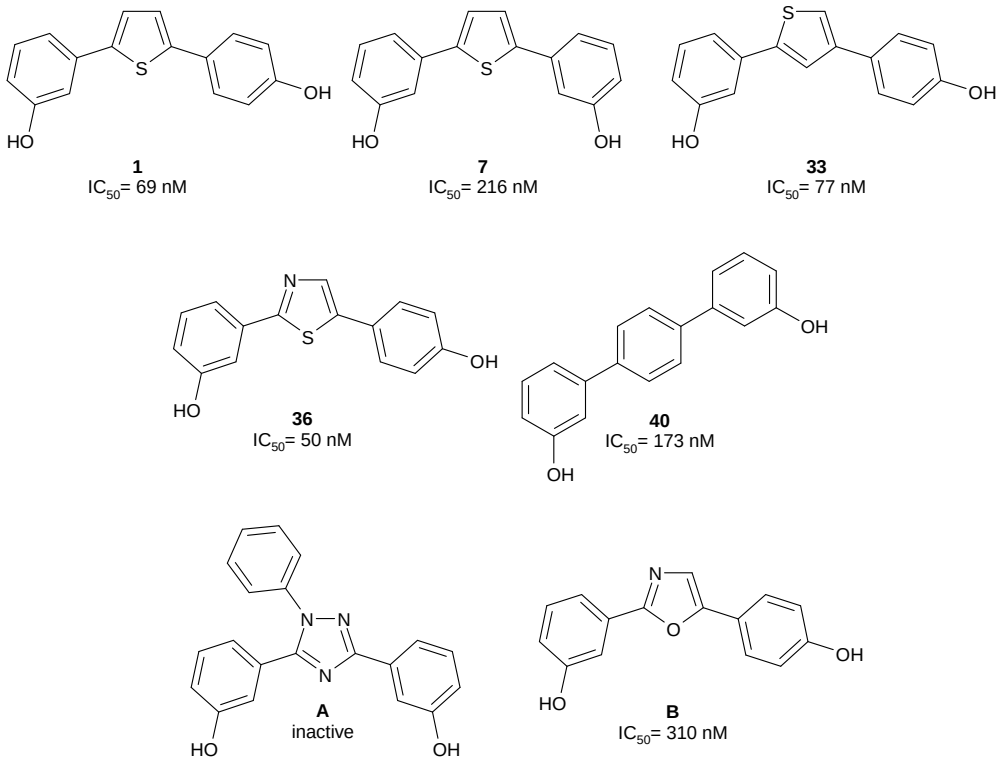
Until now, hormone-dependent breast cancers are treated using three different endocrine therapies:[3,4] aromatase inhibitors and GnRH analogues disrupt the estrogen biosynthesis while selective estrogen receptor modulators (SERMs) or pure antiestrogens[5] prevent E2 to unfold its action at the receptor level. Besides specific disadvantages of each therapeutic approach, all of these strategies have in common a rather radical reduction of estrogen levels in the whole body leading to significant side effects.

A softer approach could be the inhibition of an enzyme of the 17β-hydroxysteroid dehydrogenase (17β-HSD) family, especially one which is responsible for the E2 formation from estrone (E1). Until now, three subtypes (1, 7 and 12) are able to catalyze this reaction, the most important being 17β-HSD1. The primary physiological role of 17β-HSD7 and 17β-HSD12 is supposed to be in the cholesterol synthesis[6,7] and in the regulation of the lipid biosynthesis,[8] respectively. In addition, Day et al.[9] recently, showed that 17β-HSD12, although highly expressed in breast cancer cell lines, is inefficient in E2 formation.

17β-HSD1, which is responsible for the intracellular NAD(P)H-dependent conversion of the weak E1 into the highly potent estrogen E2, is often overexpressed in breast cancer cells[10-13]

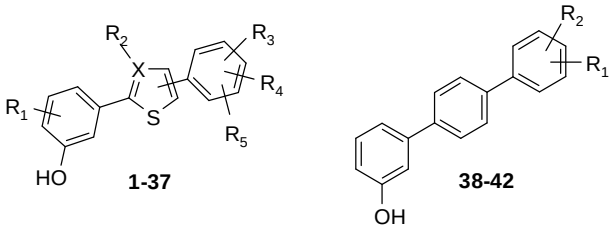
and endometriosis.[14] Inhibition of this enzyme is therefore regarded as a promising novel target for the treatment of estrogen-dependent diseases.
Recently, two groups[9, 15, 16] reported about the *in-vivo* efficacy of 17β-HSD1 inhibitors to reduce E1 induced tumour growth using two different mouse models and indicating that the 17β-HSD1 enzyme is a suitable target for the treatment of breast cancer.
In order to not counteract the therapeutic efficacy of 17β-HSD1 inhibitors, it is very important that the compounds are selective toward 17β-HSD2, the enzyme which catalyzes the deactivation of E2 into E1. Additionally, to avoid intrinsic estrogenic effects, the inhibitors should not show affinity to the estrogen receptors α and β.
During the last decade, several groups reported on 17β-HSD1 inhibitors, most of them having steroidal structures.[17-19] Recently non-steroidal cores have been published too. Until today four classes of compounds are described: thienopyrimidinones,[20-21] biphenyl ethanones[22] and from our group (hydroxyphenyl)naphthalenes[23-25] and bis(hydroxyphenyl)azoles, thiophenes, benzenes and aza-benzenes.[26-30] The most promising compounds of the latest series are thiophenes **1**, **7** and **33**, thiazole **36** and phenylene **40** exhibiting IC_{50} values toward 17β-HSD1 in the nanomolar range and high selectivity toward 17β-HSD2 and the ERs (Chart 1).

Chart 1: described bis(hydrophenyl)azoles, thiophenes, benzenes and aza-benzenes



In the following, we will report on structural optimizations which led to the discovery of new highly potent and selective 17β-HSD1 inhibitors.

Chart 2: title compounds



Design

Up to now, several crystal structures of human 17β-HSD1 were resolved: as apoenzyme (i.e. PDB code: 1BHS[31]), as binary complex (enzyme-E2, i.e. PDB code: 1IOL[32]) or as ternary complex (enzyme-E2-$NADP^+$: i.e. PDB code 1FDT;[33] 1A27;[34] enzyme-HYC (hybride inhibitor): PDB code: 1I5R[35]).

The analysis of the ternary complexes available from 17β-HSD1 provides useful knowledge about the architecture of the enzyme and important hints for structure based drug design: a substrate binding site (SUB) and a cofactor binding pocket (COF) can be identified as well as the most important amino acids responsible for substrate and cofactor anchoring. The SUB is a narrow hydrophobic tunnel containing two polar regions at each end: His221/Glu282 on the one side and Ser142/Tyr155 on the other side, corresponding to the binding oxygens in 3- and 17-hydroxy group of E2. Additionally a flexible loop can be identified which is not well resolved in almost all the structures.

From previous results obtained in the class of bis(hydroxyphenyl)azoles, thiophenes, benzenes and aza-benzenes,[26, 27] a SAR study highlighted four structural features which are important for high 17β-HSD1 inhibitory activity: 1. one hydroxyphenyl moiety on the core structure is not sufficient for a high potency, 2. only the *meta-para* and *meta-meta* dihydroxy substitution pattern (O-O distance in the same range as observed for the steroid, d = 11 Å) are active, 3. the presence of the *meta*-hydroxy group is more important for inhibitory activity than the *para*-, 4. only central aromatic rings without hydrogen bond donor function like thiophene, thiazole, benzene exhibit inhibitory activity. It was also shown that a correlation seems to exist between the activity of the compounds and the electrostatic potential distribution of the molecules:[27] to be active the ESP values of the different regions of the inhibitor has to be in an appropriate range.

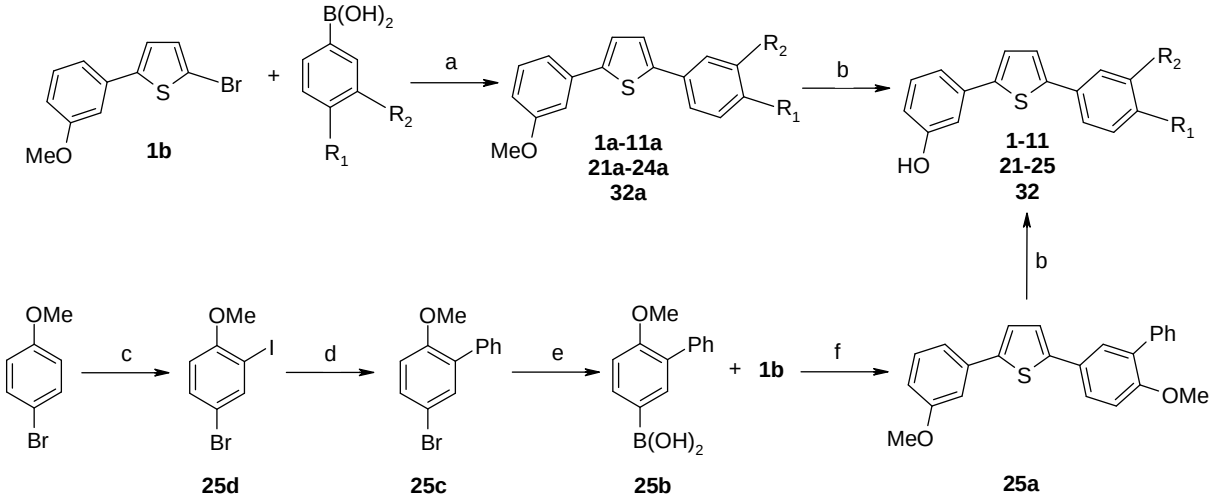
In the present report, we will present the structure optimization of this class of compounds leading to an increase in activity and in selectivity of these inhibitors. First, the influence of the bioisosteric exchange of one OH group on the enzyme activity will be determined. Secondly, the space availability around the inhibitors and the nature of the most appropriate substituent will be investigated by substitutions, either on the heterocycle, or on the hydroxyphenyl moieties. The nature of the substituent will be varied in order to investigate the possible interactions between the inhibitor and the enzyme. Thirdly, computational studies (docking studies and ESP calculations) will be performed in order to identify the most plausible binding mode for this class of compounds. Furthermore the selectivity toward 17β-HSD2 and the ERs α and β will be determined as well as the potency of the compounds in T-47D cells and inhibition of the two most important hepatic CYP enzymes. Finally, the pharmacokinetic profile of the two most promising candidates will be evaluated in rats after oral administration.

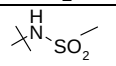
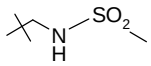
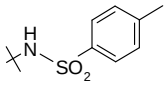
Chemistry

The synthesis of compounds **1** to **11**, **21** to **25** and **32** is presented in Scheme 1. Starting from the mono-brominated key intermediate **1b** and the appropriate commercially available boronic

acids, the preparation of compounds **1a** to **11a**, **21a** to **25a** and **32a** was accomplished via Suzuki cross coupling reaction[36] under microwave assisted conditions (Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min). The resulting disubstituted thiophenes were subsequently submitted to ether cleavage with borontribromide[27] (Method C: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h) leading to compounds **1** to **11**, **21** to **25** and **32** (Scheme 1). In case of intermediate **25a**, the boronic acid **25b** was prepared in a three step synthesis pathway: first, an iodine substituent was selectively introduced in position 2 of the *para*-bromoanisole (compound **25d**) using (diacetoxyiodo) benzene.[37] Then, a selective Suzuki reaction on the iodo-position of **25d** under Method B (Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h) led to the intermediate **25c** and the corresponding boronic acid **25b** was prepared using *n*-butyl lithium and triethyl borate followed by hydrolysis with diluted hydrochloric acid.

Scheme 1[a]: synthesis of compounds **1** to **11, 21** to **25** and **32**



cmpd	R_1	R_2	cmpd	R_1	R_2
1	OH	H	**9**	H	
2	H	H	**10**	H	
3	F	H	**11**	H	
4	NH_2	H	**21**	OH	CH_3
5	SH	H	**22**	OH	OH
6	CN	H	**23**	OH	F
7	H	OH	**24**	OH	CF_3
8	H	F	**25**	OH	Ph
			32	F	F

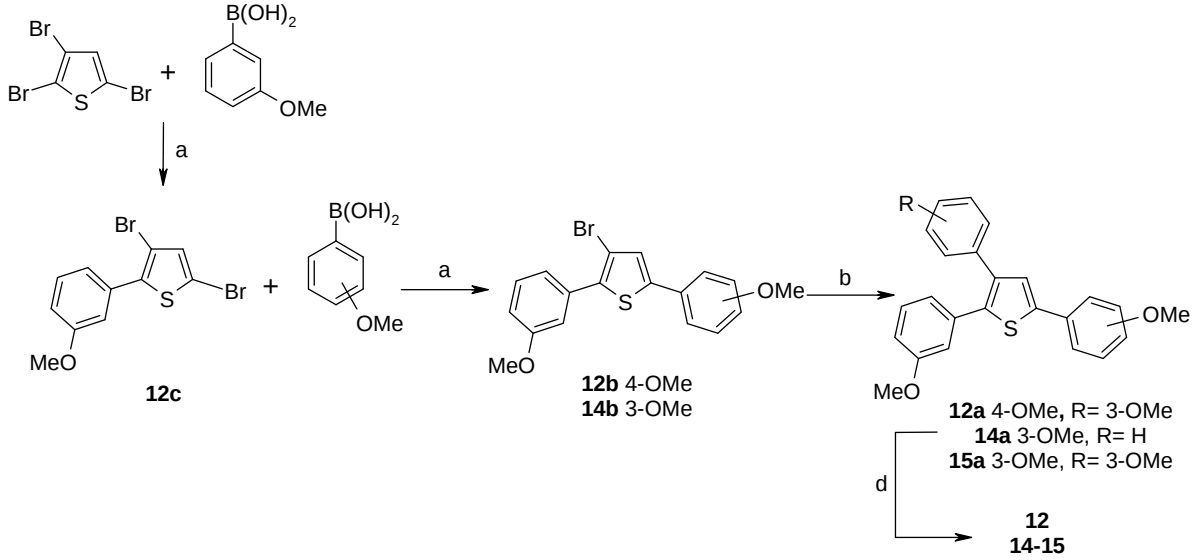
[a]**Reagents and conditions**: (a) Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min; (b) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h; (c) $PhI(OAc)_2$, I_2, AcOEt, 60 °C, 5 h; (d) Ph-$B(OH)_2$, Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h; (e) 1. *n*-BuLi, dry THF, 5 min, -78 °C, 2. $B(OEt)_3$, 2 h -78°C to rt, 3. HCl 1N, rt; (f) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h.

The preparation of compounds **31** and **33** to **42** is similar to the synthetic pathway presented in Scheme 1 for compounds **1** to **11**. The first Suzuki coupling was carried out according to Method B with the corresponding dibrominated heterocycle and the methoxylated benzene boronic acid. The resulting mono substituted compounds **31b** and **33b** to **42b** were submitted to a second cross coupling reaction under microwave assisted conditions following Method A.

The compounds were subsequently demethylated with boron tribromide to yield compounds **31** and **33** to **42**.

The synthesis of compounds **12**, **14** and **15** is depicted in Scheme 2. The key intermediate mono methoxylated dibromothiophene **12b** was prepared following two successive Suzuki coupling reactions according to Method B (Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 4 h) from 2,3,5-tribromothiophene and methoxybenzene boronic acid. The reaction time of both cross couplings was carefully controlled (restricted to 4 h) in order to get a selective bromine replacement each time. Intermediates **12a**, **14a**, and **15a** were obtained via a third Suzuki coupling using Method B. The methoxy substituents were cleaved in a last step, using boron tribromide (Method C: BBr_3, CH_2Cl_2, -78 °C to rt, 18 h).

Scheme 2[a]: synthesis of compounds **12, 14** and **15**

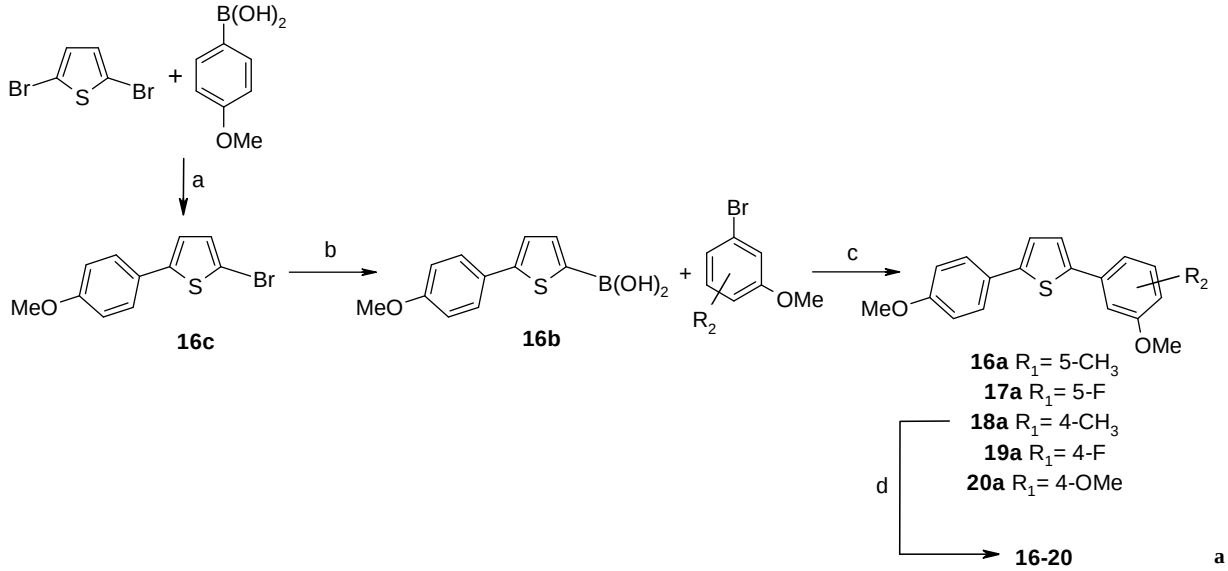


[a]**Reagents and conditions**: (a) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 4 h; (h) boronic acid, Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h; (d) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

Compound **13** was synthesized under microwave assisted conditions in a one pot reaction using 2,5-dibromo-3-methylthiophene and 3-hydroxyphenyl boronic acid following Method A (Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars)) for 15 min.

The synthesis of the molecules bearing an additional substituent on the *meta*-hydroxyphenyl moiety of thiophene **1** (compounds **16** to **20**) is shown in Scheme 3. Intermediate **16c** was prepared via Suzuki reaction from the *para*-methoxylated benzene boronic acid and the 2,5-dibromothiophene following Method B heating the reaction 4 h instead of 20 h in order to avoid any dicoupling reaction. Treatment of **16c** with *n*-butyl lithium and triethyl borate afforded after hydrolysis with diluted hydrochloric acid the corresponding boronic acid **16b**. The resulting compound was subjected to an additional cross coupling reaction which was carried out with the appropriate bromine derivative following Method A for compounds **17a** to **20a** and Method B for compound **16a**. The hydrolysis of the methoxy groups with boron tribromide (Method C) led to compounds **16** to **20**.

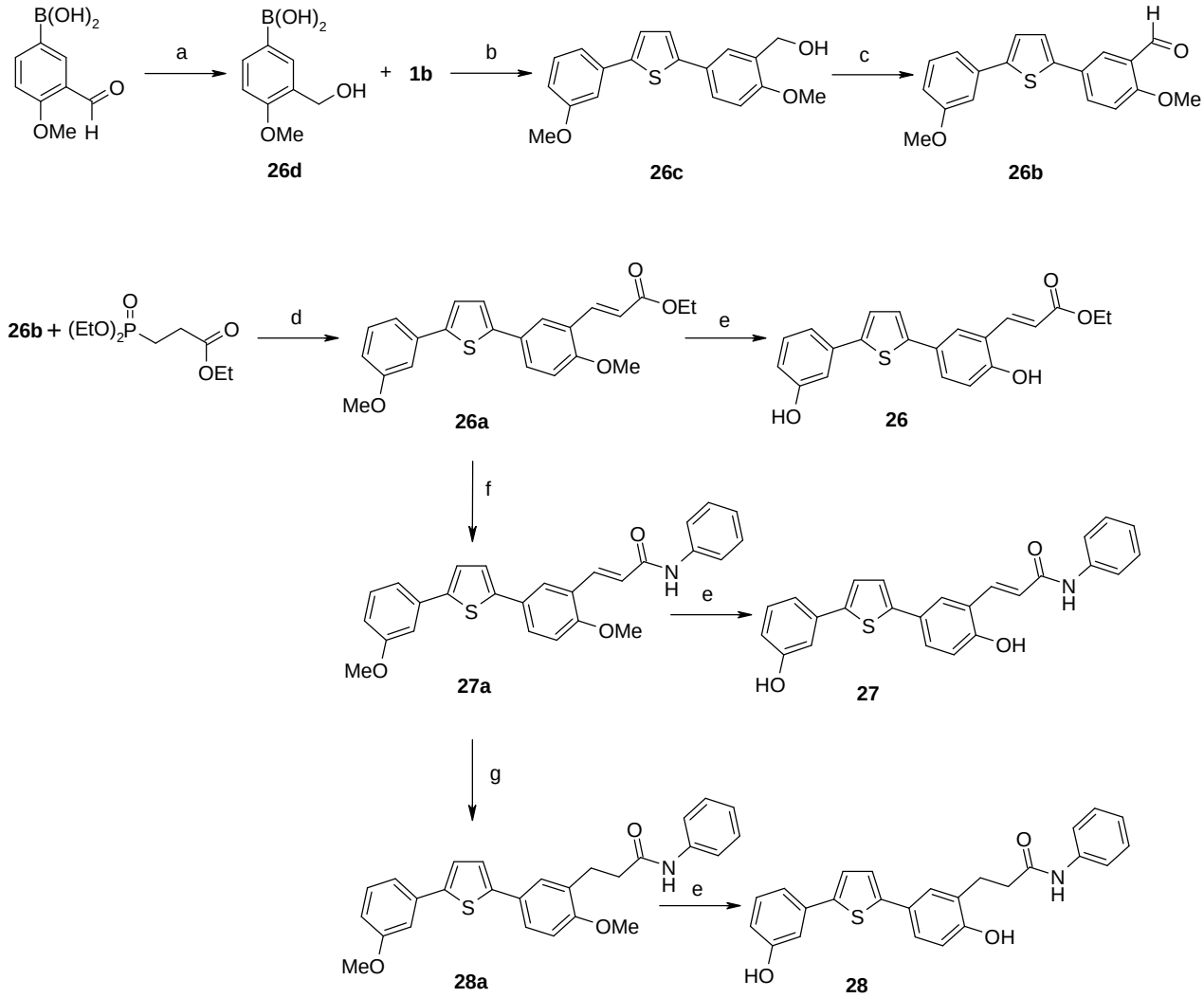
Scheme 3[a]: synthesis of compounds **16** to **20**



Reagents and conditions: (a) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 4 h; (b) 1.*n*-BuLi, anhydrous THF, -78 °C, 15 min, 2. $B(OEt)_3$, THF, -78 °C to rt, 2 h, 3. HCl 1N; (c) Method A for **17a**-**20a** (Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min); Method B for **16a** (Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h); (d) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

The synthesis of compounds **26** to **28** substituted in *ortho*-position of the *para*-OH group is depicted in Scheme 4. The preparation of the key intermediate **26b** started from the commercially available 3-formyl-4-methoxyphenyl boronic acid. Reduction of the aldehyde function using sodium borohydride followed by a cross coupling reaction with **1b** under microwave irradiation according to Method A (Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min) afforded the disubstituted thiophene **26c**. The alcohol function of **26c** was subsequently oxidized with pyridinium chlorochromate to yield to the key aldehyde **26b**. It was subjected to the Horner-Wadworths-Emmons conditions[38] to introduce the acrylic ester moiety (intermediate **26a**). Hydrolysis of the ester function using lithium hydroxide,[24] amide bond formation with aniline, EDCI and HOBt[39] afforded compound **27a** The catalytic double bond hydrogenation of **27a** was performed using Perlman's catalyst.[40] The ether functions of **26a**, **27a** and **28a** were deprotected using boron tribromide (Method C) to give the desired compounds **26** to **28**.

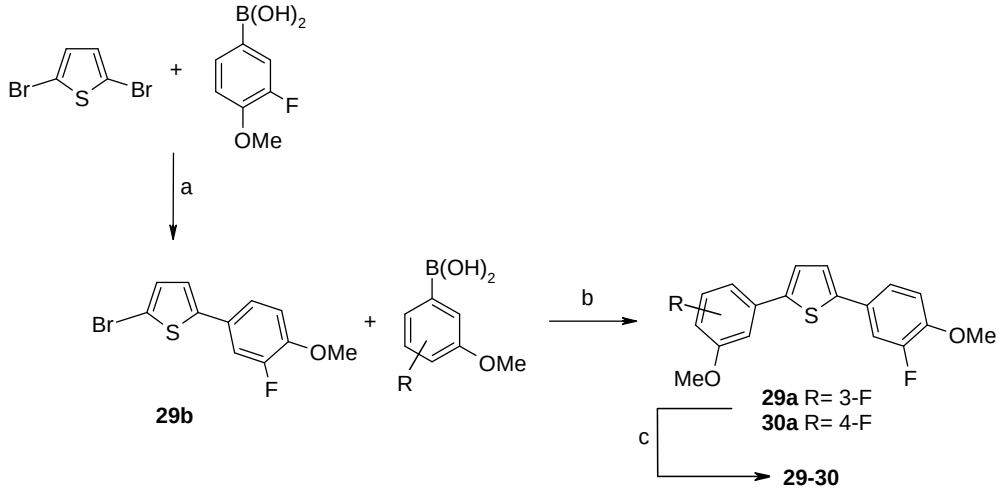
Scheme 4[a]: synthesis of compounds **26** to **28**



[a]**Reagents and conditions**: (a) $NaBH_4$, THF/EtOH (1:1), 0 °C to rt, 2 h; (b) Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min; (c) PCC, CH_2Cl_2, rt, 30 min; (d) NaH, THF dry, rt, 4 h; (e) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h; (f) 1. LiOH, THF/H_2O (2:1), reflux, 20 h, 2. aniline, EDCI, HOBt, CH_2Cl_2, reflux, 20 h; (g) $Pd(OH)_2$, THF/EtOH (1:1), H_2, rt, 20 h.

The synthesis of the difluorinated thiophenes **29** and **30** is presented in Scheme 5. These compounds were obtained after two successive cross coupling reactions: in a first step 2,5-dibromothiophene reacted with 3-fluoro-4-methoxyphenyl boronic acid following Method B (Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h). In a second step, the resulting mono substituted thiophene **29b** was subsequently submitted to a second cross coupling reaction under microwave irradiation (Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min) to yield the intermediates **29a** and **30a**. Ether cleavage with boron tribromide led to the final compounds **29** and **30**.

Scheme 5[a]: synthesis of compounds **29** to **30**



[a]**Reagents and conditions**: (a) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h ; (b) Method A: Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bars), 15 min; (c) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

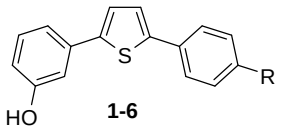
Biological Results

Activity: inhibition of human 17β-HSD1

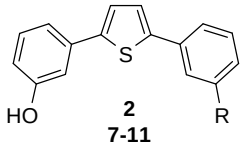
Placental enzyme was partially purified following a described procedure.[26, 27] Tritiated E1 was incubated with 17β-HSD1, cofactor and inhibitor. After HPLC separation of substrate and product, the amount of labelled E2 formed was quantified. The inhibition values of the test compounds are shown in Tables 1 to 5. Thiophenes **1, 2**, **7** and **29**, thiazole **33** and phenylenes **35** and **37**, identified in our previous article,[27] were used as reference compounds.

It was first investigated whether one of the two hydrophenyl moieties could be exchanged by another functional group having similar properties. Previous results[27] showed that the *meta*-hydroxy group is highly important for activity and was therefore maintained in the core structure. The exchange of the *para*-hydroxy group on the *meta-para* disubstituted thiophene (**1**, IC_{50}= 69 nM) by a bioisosteric function (F, NH_2, SH) resulted in moderate (**3**, IC_{50}= 717 nM) or weak inhibitors (**4** and **5**, IC_{50}> 5000 nM) of 17β-HSD1 (Table 1). Moving the F atom from the *para*- (compound **3**) to the *meta*-position (compound **8**) led to a small increase in activity (**8**, IC_{50}= 535 nM vs. **3**, IC_{50}= 717 nM). Replacement of the *meta*-fluorine for a methylsulfonamide moiety (**9**) did not improve the activity (**9**, IC_{50}= 523 nM vs. **8**, IC_{50}= 535 nM), while a compound bearing a bulky substituent like tolylsulfonamide (**11**, IC_{50}= 350 nM) showed comparable activity to the mono hydroxylated thiophene (**2**, IC_{50}= 342 nM) indicating that there is some space in this region of the enzyme for substitution but it is unlikely that specific interactions between the tolylsulfonamide moiety and amino acids of the active site take place. The insertion of a C1-linker between the phenyl moiety and the methylsulfonamide group was detrimental for the activity (**9**, IC_{50}= 523 nM vs. **10**, IC_{50}> 1000 nM). It can be therefore concluded that the two hydroxy functions are necessary for high activity and the *para*-hydroxy group can not be replaced by a bioisoteric group.

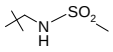
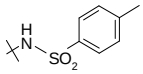
Table 1: effect of the exchange of one OH substituent for other functional groups on human 17β-HSD1 and 17β-HSD2 inhibitory activities.



1-6



2
7-11

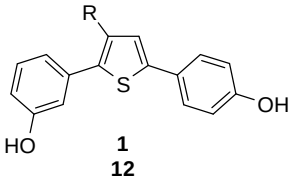
cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]	cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]
1	OH	69	1950	28	**7**	OH	173	745	4
2	H	342	2337	7	**2**	H	342	2337	7
3	F	717	3655	5	**8**	F	535	1824	3
4	NH_2	>5000	nt		**9**		523	1575	3
5	SH	>5000	nt		**10**		>1000	nt	
6	CN	>1000	nt		**11**		350	276	1

[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested

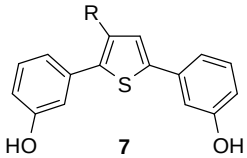
In order to improve the activity and the selectivity of our inhibitors, substituents capable to establish further interactions with the enzyme were added either on the heterocycle or on the hydroxyphenyl moieties. Additional functional groups were introduced in both of the *meta-para* 2,5-bis(hydroxyphenyl)thiophene **1** (IC_{50} = 69 nM) and the *meta-meta* 2,5-disubstituted derivative **7** (IC_{50} = 216 nM).

Concerning substitution on the heterocycle, two kinds of hydrophobic substituents (Me, Ph) were introduced in position 3 on the thiophene ring to investigate the space availability around the core (Table 2). The *meta-meta* thiophenes bearing a methyl (compound **13**) or phenyl (compound **14**) as well as the *meta-para* thiophene bearing a hydroxyphenyl substituent (compound **12**) showed a drop of activity compared to the reference compound **7** (IC_{50}= 216 nM vs. IC_{50}> 1000 nM, 567 nM and 493 nM for **12**, **13** and **14**, respectively). It is striking that only in case of the *meta-meta* disubstituted series the insertion of a polar *meta*-hydroxyphenyl substituents leads to an increase in activity (**15**, IC_{50}= 119 nM vs. **12**, IC_{50}> 1000 nM). This exemplifies that there is space available for further substitution around the heterocycle only in case of the *meta-meta* bis(hydroxyphenyl) substitution pattern and that the third *meta*-OH group is certainly at an appropriate distance to establish supplementary hydrogen bond interactions with the active site.

Table 2: inhibition of human 17β-HSD1 and 17β-HSD2 by compounds bearing a supplementary substituent on the thiophene core structure



1
12



7
13-15

cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]
1	H	69	1950	28
12		>1000	nt	

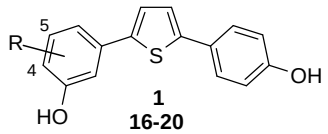
cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]
7	H	173	745	4
13	CH_3	567	856	1
14		493[e]	nt	
15		119	188	2

[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); [e]calculated value, obtained with the LOGIT transformed; ni: no inhibition, nt: not tested

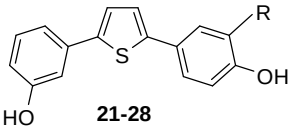
Concerning the substitution of the hydroxyphenyl rings, different groups were introduced either on the *meta*-hydroxyphenyl ring (compounds **16** to **20**) or on the *para*-hydroxyphenyl moiety (compounds **21** to **28**, Table 3). The *ortho*-position was not considered, as these compounds would not be planar any more.

Introduction of a substituent in position 5 on the *meta*-hydroxyphenyl moiety resulted in case of a methyl group in a drop of activity (**16**, IC_{50}= 629 nM vs. **1**, IC_{50}= 69 nM). The introduction of a fluorine atom led to slight increase in activity in comparison to the unsubstituted compound **1** (**17**, IC_{50}= 42 nM vs. **1**, IC_{50}= 69 nM). Moving these functional groups to position 4 gave a highly active fluorinated compound **19** (IC_{50}= 113 nM) and a very weak methylated inhibitor **18** (IC_{50}> 5000 nM). Substituents have also been introduced in position 5 on the *para*-hydroxyphenyl ring: a polar group like a hydroxy (compound **22**) or a bulky substituent like a phenyl (compound **25**) in *ortho*- of the *para*-OH induced a decrease in activity compared to thiophene **1** (IC_{50}= 69 nM vs. IC_{50}= 402 nM and >5000 nM for **22** and **25**, respectively). The introduction of a fluorine substituent into the same position led to the highly potent compound **23** (IC_{50}= 8 nM) while substituents like methyl or trifluoromethyl showed equal or slightly better activities compared to the reference compound **1** (IC_{50}= 69 nM vs. IC_{50}= 46 nM and 38 nM for **21** and **24**, respectively). Other functional groups showing a higher flexibility like ethylacrylate (compound **26)**, phenylacrylamide (compound **27**) or phenylpropaneamide (compound **28**) were also synthesized and the resulting compounds **26**, **27** and **28** turned out to have weaker inhibitory activity compared to the unsubstituted thiophene **1** (IC_{50}= 69 nM vs. 130, 427 and 620 nM for **26**, **27** and **28,** respectively). The low activity of the unconjugated compound **28** indicates that an overall distributed electronic density is an important parameter for activity. These results indicate that there is space available in this area for substituents but the nature of the substituents are probably not yet optimal (Table 3).

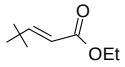
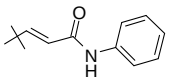
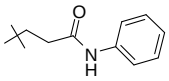
Table 3: effect of a supplementary substituent on the hydroxyphenyl moieties on the inhibition of the human 17β-HSD1 and 17β-HSD2



1
16-20



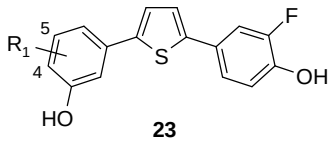
21-28

cmpd	R	IC_{50} (nM)[a]		selectivity factor[d]
		17β-HSD1[b]	17β-HSD2[c]	
1	H	69	1950	28
16	5-CH_3	629	2584	4
17	5-F	42	463	11
18	4-CH_3	>5000	nt	
19	4-F	113	183	2
20	4-OH	>5000	nt	
21	CH_3	46	1971	49
22	OH	402	1636	4
23	F	8	940	118
24	CF_3	38	97	3
25	Ph	>5000	nt	
26		130	502	4
27		427	468	1
28		620	982	2

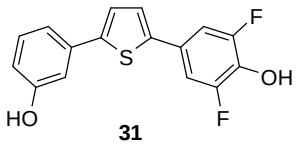
[a]Mean values of three determinations, standard deviation less than 15 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested

The influence of the introduction of a second fluorine on the highly active thiophene **23** (IC_{50}= 8 nM), either one F on each hydroxyphenyl ring or two F on the same hydroxyphenyl moiety, was also examined (Table 4). When the two F were located on each hydroxyphenyl moieties, the 4-substituted fluoro derivative (compound **30**) is slightly more potent that the one with the fluorine in 5-position (**29**, IC_{50}= 29 nM, vs. **30**, IC_{50}= 17 nM). A slight decrease in activity was observed when the two fluorine substituents were present at the same hydroxyphenyl ring (compound **31**, IC_{50}= 56 nM). The exchange of the *para*-OH function of **23** by a fluorine atom (compound **32**) confirmed the essential role of this *para*-hydroxy moiety as previously observed.

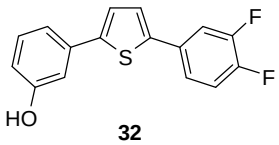
Table 4: effect of two additional fluorine atoms on the 17β-HSD1 and 17β-HSD2 inhibitory activity



23
29-30



31



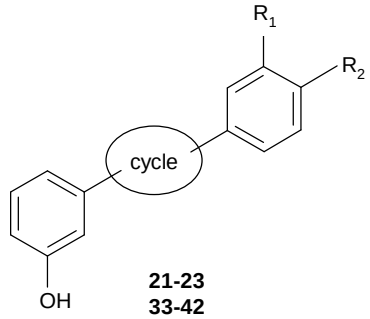
32

cmpd	R_1	IC_{50} (nM)[a]		selectivity factor[d]
		17β-HSD1[b]	17β-HSD2[c]	
23	H	8	940	118
29	5-F	29	227	8
30	4-F	17	218	13
31		56	312	6
32		780	2640	3

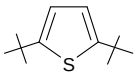
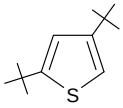
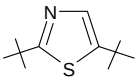
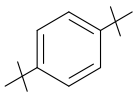
[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 μM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); nt: not tested.

Methyl and fluorine substituents have been identified as functional groups able to increase the inhibitory activity of the 2,5-bis(hydroxyphenyl) thiophene **1**. Previously[27] we reported that other central core structures like 2,4-thiophene, 2,5-thiazole and 1,4-benzene lead to highly active compounds. The influence of an additional methyl or fluorine substituent at these structures was therefore also investigated (Table 5). Introduction of CH_3 or F into the *para*-hydroxyphenyl ring of **33, 36, 38** and **40** resulting in compounds **34, 35**, **37**, **39** and **42** led to equally active derivatives in case of **34** and **35** (IC_{50}= 64 nM vs. **21**, IC_{50}= 46 nM). A decrease in inhibitory activity in the thiazole and in the benzene classes of compounds was observed compared to the thiophene family (**37**, IC_{50}= 143 nM vs. **21**, IC_{50}= 46 nM; **39** and **42**, IC_{50}= 123 nM and 51 nM, respectively vs. **23**, IC_{50}= 8 nM). Amongst the investigated molecules, introduction of a methyl or fluorine substituent led only in the class of the bis(hydroxyphenyl) thiophenes to an increase in activity.

Table 5: influence of the core and a supplementary substituent on the inhibition of the human 17β-HSD1 and 17β-HSD2



21-23
33-42

cmpd	cycle	R_1	R_2	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]
21		CH_3	OH	46	1971	49
23		F	OH	8	940	118
33		H	OH	77	1270	16
34		CH_3	OH	64	869	14
35		F	OH	64	510	8
36		H	OH	50	4000	80
37		CH_3	OH	143	2023	14
38		H	OH	471	4509	10
39		F	OH	123	872	7
40		OH	H	173	2259	21
41		OH	CH_3	171	1248	7
42		OH	F	51	239	5

[a]Mean values of three determinations, standard deviation less than 13 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested

Selectivity: inhibition of 17β-HSD2 and affinities to the estrogen receptors α and β

In order to gain insight into the selectivity of the most active compounds, inhibition of 17β-HSD2 and the relative binding affinities to the estrogen receptors α and β were determined.
Since 17β-HSD2 catalyzes the inactivation of E2 into E1, inhibitory activity toward this enzyme must be avoided. The 17β-HSD2 inhibition was determined using an assay similar to the 17β-HSD1 test. Placental microsomes were incubated with tritiated E2 in the presence of NAD^+ and inhibitor. Separation and quantification of labelled product (E1) was performed by HPLC using radio detection. A selection of the most potent 17β-HSD1 inhibitors was tested for inhibition of 17β-HSD2. IC_{50} values and selectivity factors (IC_{50} HSD2 / IC_{50} HSD1) are presented in Tables 1 to 5.
Mono-hydroxylated compounds (Table 1) exhibited a poor selectivity regarding 17β-HSD2, the most selective one being compound **5** with a selectivity factor of 5. This finding suggests that the *para*-OH is important for activity as well as for selectivity (selectivity of the *para-meta* derivative **1**: 28). Introduction of further substituents (Tables 2 to 5) into the highly active

bis(hydroxyphenyl) scaffold induced a loss of selectivity against 17β-HSD2 except in case of compounds **21** and **23**, which exhibit excellent selectivity factors of 49 and 118, respectively toward 17β-HSD2.
A further prerequisite for 17β-HSD1 inhibitors to be used as potential drugs is that they do not show affinity for ERα and ERβ, since binding to these receptors could counteract the therapeutic concept of selective 17β-HSD1 inhibition. The binding affinities of the most selective compounds of this study were determined using recombinant human protein in a competition assay applying [^{3}H]-E2 and hydroxyapatite (Table 6). All tested compounds show very marginal to marginal affinity to the ERs except compound **23**, which binds weakly to ERβ (RBA= 1 %). Compound **21** was evaluated for estrogenic effects on the ER-positive, mammary tumor T-47D cell line. No agonistic, i.e. stimulatory effect was observed after application of compound **21** even at a concentration 1000 fold higher compared to E2.

Table 6: binding affinities for the human estrogen receptors α and β of selected compounds

cmpd	RBA[a] (%)	
	ERα[b]	**ERβ**[b]
1	0.1< RBA <1	1.5
17	0.1< RBA <1	0.1< RBA <1
21	< 0.01	< 0.01
23	0.01< RBA <0.1	1
30	0.1	0.01< RBA <0.1
34	0.01< RBA <0.1	0.01< RBA <0.1
37	0.01< RBA <0.1	< 0.01

[a]RBA (relative binding affinity), E2: 100 %, mean values of three determinations, standard deviations less than 10 %; [b]Human recombinant protein, incubation with 10 nM ^{3}H-E2 and inhibitor for 1 h.

Further biological evaluations

Additionally, the intracellular potency of compounds **21** and **23** on E2 formation was evaluated using a cell line which expresses both 17β-HSD1 and 17β-HSD2 (T47D cells). Compound **21** and **23** inhibited the formation of E2 after incubation with labelled E1 showing IC_{50} values of 426 nM and 282 nM, respectively. These results indicate that both compounds are able to permeate the cell membrane and inhibit the transformation of E1 into E2.
Compounds **21** and **23** were further investigated for inhibition of the two most important human hepatic enzymes: CYP3A4 and CYP2D6, which are responsible for 75 % of drug metabolism. At a concentration of 2 μM, both compounds turned out to be equally active inhibiting the CYP3A4 by 80 (**21**) and 71 % (**23**), respectively and CYP2D6 by 55 (**21**) and 56 % (**23**), respectively. The relatively high inhibition of these enzymes has to be taken into consideration in the process of further developing these compounds but should not have an impact on the proof of concept *in-vitro*.
The pharmacokinetic profiles of compound **21** and **23** were determined in rats after oral administration in a cassette dosing approach. Each group consisted of 4 male rats and the compounds were administered in doses of 10 mg/kg. Plasma samples were collected over 24 h

and plasma concentrations were determined by HPLC-MS/MS. The pharmacokinetic parameters are presented in Table 7. The maximal concentration ($C_{max\ obs}$) as well as the AUC-value is higher for compound **23** (C_{max}= 1388.2 ng/mL, AUC= 19407 ng/mL) than for compound **21** (C_{max}= 905.0 ng/mL, AUC= 12275 ng/mL). The maximal plasma concentration ($t_{max\ obs}$) for compounds **21** and **23** was reached after 4.0 and 8.0 h, respectively, after oral administration. These data show that both compounds exhibit excellent pharmacokinetic properties in the rat and might therefore be good candidates for further experiments in disease-oriented rat models.

Table 7: pharmacokinetic parameters of compounds **21** and **23** in male rats after oral application (10 mg/kg)

parameters[a]	cmpd	
	21	**23**
$C_{max\ obs}$ (ng/mL)	905.0	1388.2
C_z (ng/mL)	43.3	24.9
$t_{max\ obs}$ (h)	4.0	8.0
t_z (h)	24.0	24.0
$t_{1/2z}$ (h)	3.8	2.7
$AUC_{0-\infty}$ (ng/mL)	12275	19407

[a] $C_{max\ obs}$, maximal measured concentration; C_z, last analytical quantifiable concentration; $t_{max\ obs}$, time to reach the maximum measured concentration; t_z, time of the last sample which has an analytical quantifiable concentration; $t_{1/2z}$, half-life of the terminal slope of a concentration time curve; $AUC_{0-\infty}$, area under the concentration- time curve extrapolated to infinity.

Computational chemistry

Molecular modelling

From the biological results it became apparent that introduction of a fluorine atom in *ortho*-position to the *para*-OH phenyl thiophene structure (compound **23**) led to a significant increase in the 17β-HSD1 inhibitory activity. To get an insight into the binding mode of this compound and to better understand the favourable interactions achieved by this inhibitor in the active site, computational studies were performed by means of the docking software GOLDv3.2 and Autodock 4.1.

The choice of the 3D-structure of the enzyme, i.e. crystal structure, used for the docking studies is crucial for obtaining reliable results. It was decided to focus on X-ray structures of 17β-HSD1 having a high resolution and showing a ternary complex (to get closer to the in vivo 3D-enzyme structure). Three structures appeared to fulfil the criteria: 1FDT and 1A27 both describing the ternary complex: enzyme-E2-$NADP^+$ and 1I5R, describing the binary complex: enzyme-steroidal hybride inhibitor (HYC), the latter being an adenosine moiety linked to an E2 core via a C9-linker. These three crystal structures differ mainly in the location of the amino acids belonging to the flexible loop αG'βF (Pro187-Pro200). Since this loop borders both the SUB and the COF, its conformational variations strongly influence the size of both binding cavities. It is therefore important to take care of the position of this loop in the structures used for the docking studies.

In the X-ray structure 1FDT, the residues 187-200 are not well resolved, but two plausible conformations for the loop (noted 1FDT-A and 1FDT-B) have been described[33] The backbones of these two loops are similar (RMSD of ~ 1 Å), while the main difference is given by the orientation of the sidechains, mainly concerning the four amino acids Phe192, Met193, Glu194 and Lys195. In 1FDT-A, Phe192 and Met193 are turned toward the outer part of the enzyme

while Glu194 and Lys195 are oriented toward the substrate and the cofactor (extending the substrate binding site = open conformation). On the other hand, in 1FDT-B these two couples of residues show a reversed orientation limiting length and volume of the steroid binding site compared to 1FDT-A (= closed conformation). Although others[21] have only considered 1FDT-B, we decided to investigate both conformations of this loop.
Interestingly, the flexible loop in 1A27 shows a comparable geometry as observed in 1FDT-B, with Phe192 and Met193 oriented toward the nicotinamide moiety, also restricting the space in the substrate binding site. In case of 1I5R, the loop is shifted in direction of the cofactor, resulting in a different conformation compared to both 1FDT-A and 1FDT-B. Although, like for 1FDT-A, it extends the SUB.
Compound **23** was docked with NADPH into four different X-ray structures: 1FDT-A, 1FDT-B, 1A27 and 1I5R. Two different binding modes were observed for compound **23**: in case of 1FDT-B and 1A27, the inhibitor is located exclusively in the steroid binding site (Figure 1) adopting a similar orientation as previously described for the bis(hydroxyphenyl) oxazole **B**[26] (chart 1) while for 1FDT-A and 1I5R, the inhibitor is located in between the steroid and the cofactor binding sites, interacting with the nicotinamide moiety. In the following, this binding mode will be named as alternative binding mode (Figure 2).

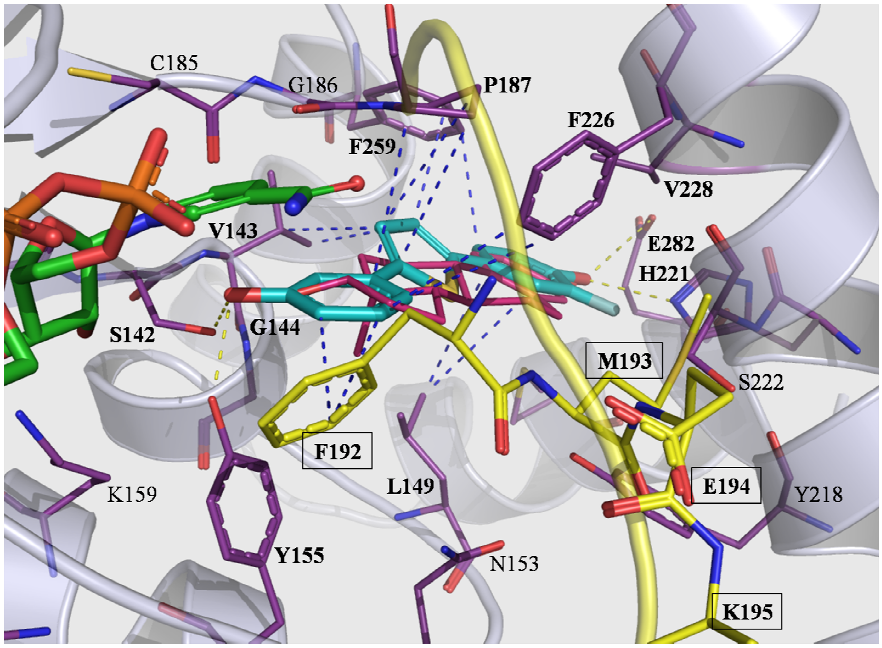


Figure 1. docking complex between 17β-HSD1 (X-ray 1FDT-B) and compound **23** (blue; SUB binding mode). NADPH, interacting residues and ribbon rendered tertiary structure of the active site are shown. Residues of the flexible loop are rendered in sticks and colored in yellow. Hydrogen bonds and π-π stackings (and hydrophobic interactions) are drawn in yellow and blue dashed lines, respectively. For comparison, E2 is depicted in magenta lines. Figures were generated with Pymol (http://www.pymol.org).

In case of the steroidal binding mode (1FDT-B and 1A27, Figure 1) the following specific interactions can be observed: hydrogen bond interactions between the *meta*-hydroxy group of **23** and Ser142/Tyr155 ($d_{O\text{-}O}$= 2.6 Å for both amino acids) and between the *para*-OH group and His221/Glu282 ($d_{O\text{-}N}$= 2.8 Å and $d_{O\text{-}O}$= 3.8 Å, Figure 1). Additionally, hydrophobic interactions and π-π stackings (Phe226, Phe259) are also involved.

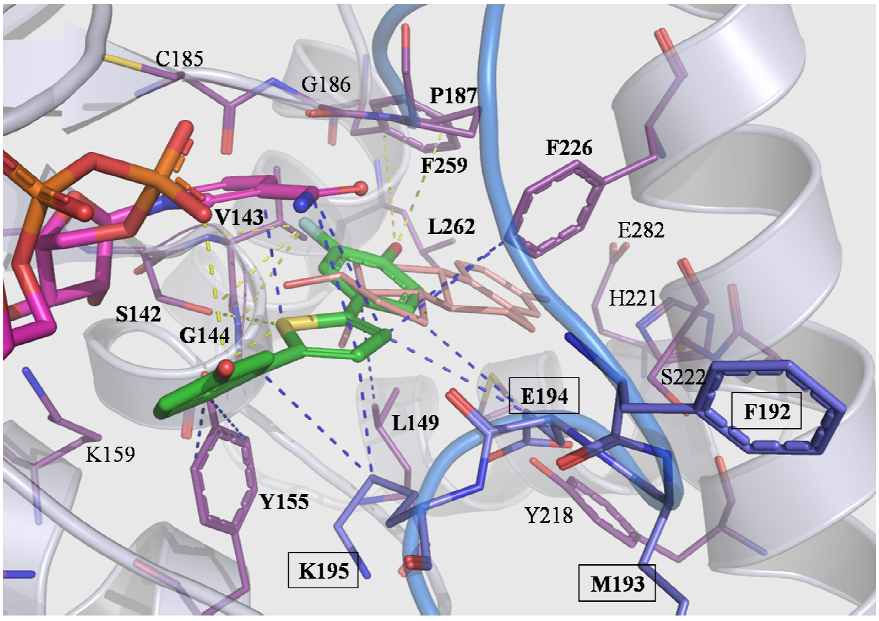


Figure 2. docking complex between 17β-HSD1 (X-ray 1FDT-A) and compound **23** (green; alternative binding mode). NADPH, interacting residues and ribbon rendered tertiary structure of the active site are shown. Residues of the flexible loop are rendered in sticks and colored in blue. Hydrogen bonds and π-π stackings (and hydrophobic interactions) are drawn in yellow and blue dashed lines, respectively. For comparison, E2 is depicted in magenta lines.

In the alternative binding mode obtained using 1FDT-A and 1I5R (Figure 2), compound **23** is also stabilized by hydrogen bond interactions: the *meta*-OH group forms a strong H-bond with the phosphate group of the cofactor ($d_{O\text{-}O}$= 2.9 Å). The fluorine atom could establish halogen bonds with the backbone -NH- of Val143 and Gly144 ($d_{F\text{-}N}$= 3.2 Å and 3.8 Å, respectively), in addition to a halogen bond with the OH-group of Ser142 ($d_{F\text{-}O}$= 3.5 Å) which is involved in the catalytic process. Further, the *para*-OH points perpendicular toward Phe259 ($d_{O\text{-centroid}}$= 4.5 Å) indicating a possible OH-π interaction. This could explain the importance of this group observed in the SARs. Moreover, strong π-π stacking interactions seem to stabilize the inhibitor in this binding mode: between the *meta*-OHphenyl-thiophene moiety and the nicotineamide part of the cofactor (parallel-displaced configuration; distance between the two ring centers, 4.3 Å) and between the *para*-OH-phenyl-thiophene moiety and Phe226 (T-shape conformation; closest C-C contact distance 3.7 Å). Moreover, electrostatic interactions between the sulfur atom of the heterocycle with the surrounding amino acids like Tyr155 and Ser142 might also play a role as described.[41]

The results presented so far suggest that both binding modes have to be considered as possible for this class of inhibitors. They depend mainly on the orientation of the flexible loop. There is only one conformation of the loop leading to a steroidal binding mode (1FDT-B/1A27). In case of 1FDT-A/1I5R the pose showing the alternative binding mode is obtained using two X-ray structures having two different conformations of the loop. Unfortunately, due to the almost identical scoring function values observed for both poses with the docking programs (Gold and Autodock), it was not possible to determine which model (1FDT-A/1I5R or 1FDT-B/1A27) is the most appropriate to describe the interactions between the inhibitor and the enzyme and therefore which is the most plausible binding mode.

Comparing both poses obtained by docking of **23** in 1FDT-A and 1FDT-B shows that there is a common area in the neighbourhood of the catalytic tetrade which corresponds to the D-ring of the enzyme-substrate complex (Figure in Supporting Information).

Molecular Electrostatic Potential (MEP)

Recently we reported on the influence of the electronic density (MEP maps, "semi-QMAR") on the potency of the inhibitors in this class of compounds.[27] The 3D-structures of the inhibitors were virtually divided into three areas and a given optimal range of ESP values (in Hartree) was determined for each region (-1.7 to -1.2 × 10^{-2} for I, -1.6 to -0.9 × 10^{-2} for II and -1.2 to -0.5 × 10^{-2} for III). The MEPs of compound **23** were calculated as shown in Figure 3. The molecular ESP distribution observed (-1.8 to -1.2 × 10^{-2} for I, -1.6 to -0.8 × 10^{-2} for II and -1.1 to -0.4 × 10^{-2} for III) fitted well to the optimal ranges identified previously, confirming the correlation between the ESP range and the potency of the compounds. The MEP maps of the natural substrate E1 and of E2 were also calculated (Figure 3) and compared to the one of **23**. The finding that the ESP distribution of **23** and E2/E1 is very different might be an indication that compound **23** does not bind in the same way as the steroid.

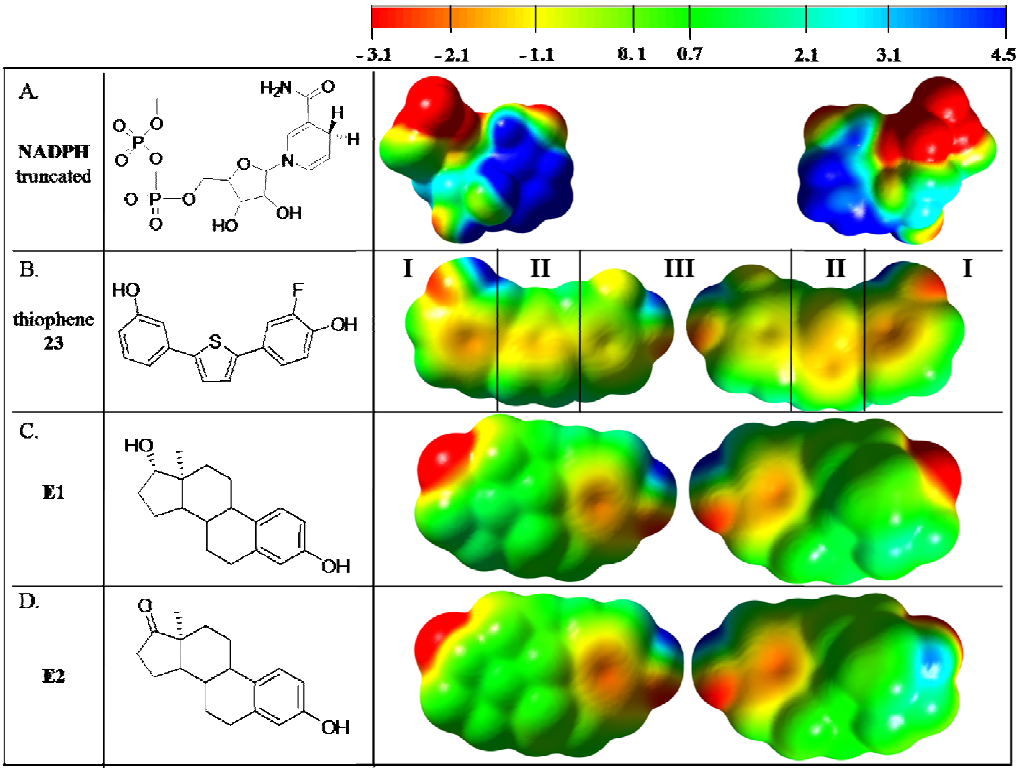


Figure 3. structures and MEP maps of both ventral (steroidal α-side) and dorsal (steroidal β-side) views of truncated NADPH (**A**), thiophene **23** (**B**), E1 (**C**) and E2 (**D**). MEP surfaces were plotted with GaussView 3.09.

According to the alternative binding mode, the *meta*-hydroxyphenyl thiophene part of **23** overlaps with the nicotinamide part of the cofactor and forms stabilizing π-π interactions. The ESP distribution of these two entities should therefore show complementarity. To get an insight into this, the MEP map of a truncated NADPH –the counterpart of the *meta*-OHphenyl-thiophene moiety- was calculated by *ab initio* methods. As it can be seen in Figure 3 a certain complementarity was observed. The NADPH MEP maps give the explanation for the observation[27] that a strong polarization between the vertex and the base of the central ring of the inhibitors is negative for binding. Positive ESP values on the vertex side lead to repulsion effects with the nicotinamide and therefore reduce the inhibitory activity. This finding indicates that this class of compound might bind according to the alternative binding mode (Figure 2). However, this hypothesis needs to be further investigated.

Discussion and Conclusion

Structural optimizations of compound **1** led to the discovery of new substituted 2,5-bis(hydroxyphenyl)thiophene derivatives: the fluorinated **23** and the methylated **21** being the most active and selective inhibitors identified.
From a previous work in this class of compounds[27] it was demonstrated that removal of one of the two hydroxyphenyl moieties is detrimental for the activity. In this paper it was shown that replacement of the *para*-OH function by a bioisoteric group like F, NH_2, SH, CN leads to a drop of activity. The lack of hydrogen donating properties of the fluoro and cyano substituents might not be the only reason for this decrease in activity as the amino and the thiol derivatives are also less active than the parent compound **1**. Interestingly, the omission of a C1-linker between the methylsulfonamide moiety and the phenyl ring (compound **9**) resulted in an increase of potency. Deprivation of electrons from the phenyl ring, obviously is necessary for a good inhibition. The relatively high activity observed for compound **11** (IC_{50}= 350 nM) especially compared to compound **9** (IC_{50}= 523 nM) demonstrates that in the protein there is some space available in this position for a bulky substituent. Furthermore, the tolyl group might also be involved in the stabilization of the inhibitor in the binding site, establishing π-π stacking interactions with appropriate amino acid residues present in this region.
With the aim to increase the activity and the selectivity in this class of compound, substituents were introduced on the 2,5-bis(hydroxyphenyl)thiophenes **1** and **7**. This was successful for compound **15** (IC_{50}= 119 nM vs. **7**, IC_{50}= 216 nM). Apparently, the formation of an additional hydrogen bond is responsible for this increase in inhibitory activity, while a pure π-π stacking interaction as supposed for compound **14** is not sufficient. The 2,5-disubstituted thiophenes **12** and **15** differ only in the position of one hydroxy group (*para:* compound **12,** *meta:* compound **15**). The fact that compound **15** shows a much higher activity (IC_{50}= 119 nM vs. **12,** IC_{50}> 1000 nM) indicates that only in case of **15** the geometry of the OH groups is acceptable for a reasonable interaction. It demonstrates, as observed already,[23, 26] a sharp SAR and a reduced flexibility in this region of the active site.
The trisubstituted compound **14** bearing a phenyl substituent at the thiophene differs from the triazole **A**[28] (Chart 2) only in the nature of the heterocycle. The following comparisons highlight the importance of the heterocycle for the potency of the molecules: inactive compound **A** vs. thiophene **14** (IC_{50}= 493 nM), thiophene **21** (IC_{50}= 46 nM) vs. thiazole **37** (IC_{50}= 143 nM) and thiophene **23** (IC_{50}= 8 nM) vs. benzene **39** (IC_{50}= 123 nM). It becomes apparent that the thiophene ring is the most appropriate heterocycle for high inhibitory activity. Provided that all compounds bind according to the same binding mode, there are different explanations for these results: 1. the presence of one or several nitrogens in this area of the enzyme is not well tolerated, 2. the absence of the sulfur leads to an inadequate repartition of the electron density in the molecule, 3. a reduced flexibility in the binding site is responsible that the enzyme can not adjust its geometry to the different hydroxyphenyl moieties (depending on the heterocycle, the angles between the phenyl-OHs are different).
A high increase in activity and selectivity could be reached by introduction of substituents into the hydroxyphenyl moiety, especially when the substituent is located *ortho*- of the *para*-hydroxyphenyl group (compounds **21** to **24**). Not all substituents are equally well tolerated: there is no space available for a phenyl group (compound **25**). An additional OH group (compound **22**) is obviously not able to establish specific interactions while small lipophilic substituents (methyl, compounds **21**; fluorine, compound **23** and trifluoromethyl, compound **24**) are enhancing the activity. There is enough space in this region of the enzyme to introduce a flexible chain (**26**) but conjugation seems to be necessary to achieve a higher activity as already observed with the tolylsulfonamide substituent (compound **11**).
The positive influence of the fluorine atom has often been demonstrated in medicinal chemistry[42-44] and was also proven in this study with compound **23** (IC_{50} = 8 nM). The position

of the fluorine is decisive for an increase in activity: it has to be in *meta*-position (**17** and **23**). Highest activity was achieved in *ortho*- of the hydroxy group (compound **23**). This indicates that either direct interactions of the fluorine with amino acid residues in this region of the active site or the increase of acidity of the neighbouring OH groups might be responsible for this effect.

Introduction of a second fluorine atom into this fluorohydroxyphenyl ring (compound **31**) does not enhance the activity suggesting that the effects of the fluorine are not additional. A second fluorine was also added to the other hydroxyphenyl moiety (in *ortho*- and *meta*- of the *meta*-OH group) leading to compounds **29** and **30**. However, no enhancement of the activity compared to the monofluorinated **23** was observed indicating that there are no specific interactions of the second fluoro substituent.

A close look at the X-ray structures of 17β-HSD1 crystallized in presence of different steroidal ligands showed that the flexible loop (amino acids 187-200) can adopt different geometries depending on the nature of the ligand and on the absence or presence of the cofactor in the catalytic region. It indicates that some parts of the enzyme can adapt their geometry to the molecule present in the active site in order to stabilize it. However, other parts are rigid, explaining the sharp SAR observed in this paper and previously.[26, 27]

Two plausible conformations of the loop in the ternary complex enzyme-E2-$NADP^+$, PDB code: 1FDT, have been described (1FDT-A, 1FDT-B). We have shown that both can be used for docking studies. In case of 1FDT-A the substrate binding site is extended, enhancing the volume of the active site. It is therefore a good model to evaluate an alternative binding mode for inhibitors which are larger than the steroid. A binding mode as observed for steroids in the X-ray structures was found when the loop closes the SUB (1FDT-B). Surprisingly, when the inhibitors were docked to the protein with the loop in the open conformation, they interact with the nicotinamide part of the cofactor. MEP calculations showed a certain complementarity between the electronic density of **23** and of the nicotinamide moiety of the cofactor indicating that this alternative binding mode is not only plausible, it might be the one which is more likely.

Up to now, designing compounds as potential 17β-HSD1 inhibitors, several groups[35, 45-47] tried to mimic the cofactor. Our finding of the above mentioned alternative binding mode makes another strategy very promising: the cofactor, which is likely to be present in the active site when the inhibitor is entering, could be used as partner to achieve additional interactions rather than trying to displace it.

The most potent 17β-HSD1 inhibitors **21** and **23** exhibit a higher selectivity toward 17β-HSD2 compared to parent compound, (selectivity factors 49 and 118, respectively vs. 28 for **1**). This indicates that the amino acids close to the CH_3 or F substituents must have different properties in the two 17β-HSD enzymes, which could be further exploited to increase selectivity.

The most potent inhibitors show only marginal to very little affinity to the ERα and no stimulation of cell proliferation (agonistic effect) in the ER-positive T-47D cell line could be observed. The weak affinity of compound **23** for ERβ may not be critical as it is reported that ERβ exhibits anti-proliferative effects in breast cancer cells.[48]

Compound **23** might be used in an appropriate animal model to prove the concept of 17β-HSD1 inhibition with non-steroidal inhibitors. This compound shows a good pharmacokinetic profile in rats.

In this paper, we described the synthesis of substituted bis(hydroxyphenyl)thiophenes, thiazoles and benzenes as inhibitors of 17β-HSD1 and the evaluation of their biological properties. The most promising compounds of this study, **21** and **23**, exhibit high selectivity toward 17β-HSD2, marginal binding to ERα and excellent pharmacokinetic profiles in rats after peroral application. These new compounds provide useful tools to validate 17β-HSD1 as a target for the treatment of estrogen-dependent diseases.

Experimental section

Chemical methods

Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Lancaster, Roth, Merck or Fluka and were used without purification.
Column chromatography (CC) was performed on silica gel (70-200 □m) coated with silica, preparative thin layer chromatography (TLC) on 1 mm SIL G-100 UV_{254} glass plates (Macherey-Nagel) and reaction progress was monitored by TLC on Alugram SIL G UV_{254} (Macherey-Nagel).
IR spectra were recorded on a Bruker Vector 33 spectrometer (neat sample).
^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker AM500 spectrometer (500 MHz) at 300 K. Chemical shifts are reported in δ (parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard ($CDCl_3$: δ = 7.24 ppm (^{1}H NMR) and δ = 77 ppm (^{13}C NMR), CD_3OD: δ = 3.35 ppm (^{1}H NMR) and δ = 49.3 ppm (^{13}C NMR), CD_3COCD_3: δ = 2.05 ppm (^{1}H NMR) and δ = 29.9 ppm (^{13}C NMR), CD_3SOCD_3: δ = 2.50 ppm (^{1}H NMR) and δ = 39.5 ppm (^{13}C NMR)). Signals are described as s, d, t, q, dd, m, dt for singlet, doublet, triplet, quadruplet, doublet of doublets, multiplet and doublet of triplets, respectively. All coupling constants (*J*) are given in hertz (Hz).
Mass spectra (ESI) were recorded on a TSQ Quantum (Thermofischer) instrument. Elemental analyses were performed at the Department of Instrumental Analysis and Bioanalysis, Saarland University.

Compounds 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**),[27] 2-(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**1a**),[27] 3-[5-(4-hydroxyphenyl)-2-thienyl]phenol (**1**),[27] 2-(3-methoxyphenyl)-5-phenylthiophene (**2a**),[27] 3-(5-phenyl-2-thienyl)phenol (**2**),[27] 2,5-bis(3-methoxyphenyl)thiophene (**7a**),[27] 3,3'-thiene-2,5-diyldiphenol (**7**),[27] 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**),[27] 4-bromo-2-iodo-1-methoxy-benzene (**25d**),□□ 5-bromo-2-methoxybiphenyl (**25c**),[50] [6-methoxy-1,1'-biphenyl-3-yl]boronic acid (**25b**),[50] [3-(hydroxymethyl)-4-methoxyphenyl]-boronic acid (**26d**),[51] 4-bromo-2-(3-methoxyphenyl)thiophene (**33b**),[27] 2-(3-methoxyphenyl)-4-(4-methoxyphenyl)thiophene (**33a**),[27] 3-[4-(4-hydroxyphenyl)-2-thienyl]phenol (**29**),[27] 5-bromo-2-(3-methoxyphenyl)-1,3-thiazole (**36b**),[27] 2-(3-methoxyphenyl)-5-(4-methoxyphenyl)-1,3-thiazole (**36a**),[27] 3-[5-(4-hydroxyphenyl)-1,3-thiazol-2-yl]phenol (**36**),[27] 4'-bromo-3-methoxybiphenyl (**38b**),[27] 3,4"-dimethoxy-1,1':4',1"-terphenyl (**38a**),[27] 1,1':4',1"-terphenyl-3,4"-diol (**38**),[27] 3,3"-dimethoxy-1,1':4',1"-terphenyl (**40a**)[27] and 1,1':4',1"-terphenyl-3,3"-diol (**40**)[27] were prepared following described procedures.

General procedure for Suzuki coupling
Method A
A mixture of aryl bromide (1 eq), aryl boronic acid (1.2 eq), caesium carbonate (2.2 eq) and tetrakis(triphenylphosphine) palladium (0.01 eq) was suspended in an oxygen free DME/EtOH/water (1:1:1) solution. The reaction mixture was exposed to microwave irradiation (15 min, 150 W, 150 °C, 15 bars). After cooling to rt, water was added and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated to dryness. The product was purified by column chromatography (CC).

Method B:
A mixture of arylbromide (1 eq), aryl boronic acid (1 eq), sodium carbonate (2 eq) and tetrakis(triphenylphosphine) palladium (0.05 eq) in an oxygen free toluene/water (1:1) solution was stirred at 100 °C for 20 h under nitrogen atmosphere. The reaction mixture was cooled to rt. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC.

General procedure for ether cleavage
Method C:
To a solution of methoxyphenyl derivative (1 eq) in dry dichloromethane at -78 °C (dry ice/acetone bath), boron tribromide in dichloromethane (1 M, 3 eq per methoxy function) was added dropwise. The reaction mixture was stirred for 20 h at rt under nitrogen atmosphere. Water was added to quench the reaction, and the aqueous layer was extracted with ethyl acetate. The combined organic layers were

washed with brine, dried over sodium sulfate, filtered and concentrated to dryness. The product was purified by preparative thin layer chromatography (TLC).

2-(4-Fluorophenyl)-5-(3-methoxyphenyl)thiophene (3a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 4-fluorophenylboronic acid (94 mg, 0.67 mmol), caesium carbonate (383 mg, 1.24 mmol) and tetrakis(triphenylphosphine) palladium (6.4 mg, 5.6 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 75 % (120 mg).

3-[5-(4-Fluorophenyl)-2-thienyl]phenol (3). The title compound was prepared by reaction of 2-(4-fluorophenyl)-5-(3-methoxyphenyl)thiophene (**3a**) (80 mg, 0.28 mmol) and boron tribromide (0.84 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 68 % (52 mg); MS (ESI): 270 $(M+H)^+$; Anal. ($C_{16}H_{11}FOS$) C, H, N.

4-[5-(3-Methoxyphenyl)-2-thienyl]aniline (4a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 4-aminophenylboronic acid (92 mg, 0.67 mmol), caesium carbonate (383 mg, 1.24 mmol) and tetrakis(triphenylphosphine) palladium (6.4 mg, 5.6 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 63 % (100 mg).

3-[5-(4-Aminophenyl)-2-thienyl]phenol (4). The title compound was prepared by reaction of 4-[5-(3-methoxyphenyl)-2-thienyl]aniline (**4a**) (100 mg, 0.37 mmol) and boron tribromide (1.11 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 82 % (82 mg); MS (ESI): 268 $(M+H)^+$; Anal. ($C_{16}H_{13}NOS$) C, H, N.

4-[5-(3-Methoxyphenyl)-2-thienyl]benzenethiol (5a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (250 mg, 0.93 mmol), 4-mercaptophenylboronic acid (172 mg, 1.12 mmol), caesium carbonate (636 mg, 2.05 mmol) and tetrakis(triphenylphosphine) palladium (10.8 mg, 9.3 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 61 % (160 mg).

3-[5-(4-Sulfanylphenyl)-2-thienyl]phenol (5). The title compound was prepared by reaction of 4-[5-(3-methoxyphenyl)-2-thienyl]benzenethiol (**5a**) (150 mg, 0.50 mmol) and boron tribromide (1.50 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 81 % (115 mg); MS (ESI): 285 $(M+H)^+$; Anal. ($C_{16}H_{12}OS_2$) C, H, N.

4-[5-(3-Methoxyphenyl)-2-thienyl]benzonitrile (6a). The title compound was prepared by reaction by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (200 mg, 0.74 mmol), 4-cyanophenylboronic acid (131 mg, 0.89 mmol), caesium carbonate (508 mg, 1.64 mmol) and tetrakis(triphenylphosphine) palladium (8.5 mg, 7.4 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 27 % (60 mg).

4-[5-(3-Hydroxyphenyl)-2-thienyl]benzonitrile (6). The title compound was prepared by reaction of 4-[5-(3-methoxyphenyl)-2-thienyl]benzonitrile (**6a**) (42 mg, 0.14 mmol) and boron tribromide (0.42 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 62 % (25 mg); MS (APCI): 277 $(M)^+$; Anal. ($C_{17}H_{11}NOS$) C, H, N.

2-(3-Fluorophenyl)-5-(3-methoxyphenyl)thiophene (8a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 3-fluorophenylboronic acid (94 mg, 0.67 mmol), caesium carbonate (381 mg, 1.22 mmol) and tetrakis(triphenylphosphine) palladium (6.5 mg, 5.6 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 82 % (130 mg).

3-[5-(3-Fluorophenyl)-2-thienyl]phenol (8). The title compound was prepared by reaction of 2-(3-fluorophenyl)-5-(3-methoxyphenyl)thiophene (**8a**) (130 mg, 0.45 mmol) and boron tribromide (1.35 mmol) according to method C. The product was purified by preparative TLC

(dichloromethane/methanol 99:1); yield: 66 % (82 mg); MS (ESI): 271 $(M+H)^+$; Anal. ($C_{16}H_{11}FOS$) C, H, N.

***N*-(3-[5-(3-Methoxyphenyl)-2-thienyl]phenyl)methanesulfonamide (9a).** The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (160 mg, 0.60 mmol), 3-methylsulfonylaminophenylboronic acid (155 mg, 0.72 mmol), caesium carbonate (410 mg, 1.32 mmol) and tetrakis(triphenylphosphine) palladium (6.9 mg, 6.0 µmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 6:4); yield: 75 % (150 mg).

***N*-(3-[5-(3-Hydroxyphenyl)-2-thienyl]phenyl)methanesulfonamide (9).** The title compound was prepared by reaction of *N*-(3-[5-(3-methoxyphenyl)-2-thienyl]phenyl)methanesulfonamide (**9a**) (150 mg, 0.44 mmol) and boron tribromide (1.32 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 61 % (92 mg); MS (ESI): 346 $(M+H)^+$; Anal. ($C_{17}H_{15}NO_3S_2$) C, H, N

***N*-(3-[5-(3-Methoxyphenyl)-2-thienyl]benzyl)methanesulfonamide (10a).** The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 3-[(methylsulphonylamino)methyl]benzeneboronic acid (153 mg, 0.67 mmol), caesium carbonate (382 mg, 1.23 mmol) and tetrakis(triphenylphosphine) palladium (6.5 mg, 5.6 µmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 8:2); yield: 58 % (122 mg).

***N*-(3-[5-(3-Hydroxyphenyl)-2-thienyl]benzyl)methanesulfonamide (10).** The title compound was prepared by reaction of *N*-(3-[5-(3-methoxyphenyl)-2-thienyl]benzyl)methanesulfonamide (**10a**) (122 mg, 0.37 mmol) and boron tribromide (1.11 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 33 % (44 mg); MS (ESI): 360 $(M+H)^+$; Anal. ($C_{18}H_{17}NO_3S_2$) C, H, N.

***N*-(3-[5-(3-Methoxyphenyl)-2-thienyl]phenyl)-4-methylbenzenesulfonamide (11a).** The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), [3-[[(4-methylphenyl)sulfonyl]amino]phenyl]-boronic acid (195 mg, 0.67 mmol), caesium carbonate (383 mg, 1.23 mmol) and tetrakis(triphenylphosphine) palladium (6.5 mg, 5.6 µmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 88 % (214 mg).

***N*-(3-[5-(3-Hydroxyphenyl)-2-thienyl]phenyl)-4-methylbenzenesulfonamide (11).** The title compound was prepared by reaction of *N*-(3-[5-(3-methoxyphenyl)-2-thienyl]phenyl)-4-methylbenzenesulfonamide (**11a**) (214 mg, 0.49 mmol) and boron tribromide (1.47 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 75 % (156 mg); MS (APCI): 421 $(M)^+$; Anal. ($C_{23}H_{19}NO_3S_2$) C, H, N.

3,5-Dibromo-2-(3-methoxyphenyl)thiophene (12c). The title compound was prepared by reaction of 2,3,5-tribromothiophene (100 mg, 0.31 mmol), 3-methoxybenzeneboronic acid (46 mg, 0.31 mmol), sodium carbonate (67 mg, 0.62 mmol) and tetrakis(triphenylphosphine) palladium (17.9 mg, 15.5 µmol) according to method B heating the reaction 4 h instead of 20 h. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 23 % (25 mg).

3-Bromo-2-(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (12b). The title compound was prepared by reaction of 3,5-dibromo-2-(3-methoxyphenyl)thiophene (**12c**) (500 mg, 1.43 mmol), 4-methoxybenzeneboronic acid (268 mg, 1.72 mmol), sodium carbonate (333 mg, 3.15 mmol) and tetrakis(triphenylphosphine) palladium (82.6 mg, 71.5 µmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 52 % (278 mg).

2,3-Bis(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (12a). The title compound was prepared by reaction of 3-bromo-2-(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**12b**) (250 mg, 0.67 mmol), 3-methoxybenzeneboronic acid (124 mg, 0.80 mmol), sodium carbonate (142 mg, 1.34 mmol)

and tetrakis(triphenylphosphine) palladium (38.7 mg, 33.5 μmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 72 % (194 mg).

3,3'-[5-(4-Hydroxyphenyl)thiene-2,3-diyl]diphenol (12). The title compound was prepared by reaction of 2,3-bis(3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**12a**) (100 mg, 0.24 mmol) and boron tribromide (2.16 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 92 % (79 mg); MS (ESI): 359 $(M-H)^+$; Anal. ($C_{22}H_{16}O_3S$) C, H, N.

3,3'-(3-Methylthiene-2,5-diyl]diphenol (13). The title compound was prepared by reaction of 2,5-dibromo-3-methylthiophene (150 mg, 0.58 mmol), 3-hydroxybenzeneboronic acid (179 mg, 1.27 mmol), caesium carbonate (868 mg, 2.79 mmol) and tetrakis(triphenylphosphine) palladium (6.7 mg, 5.8 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 4:6); yield: 45 % (73 mg); MS (ESI): 281 $(M-H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

3-Bromo-2,5-bis(3-methoxyphenyl)thiophene (14b). The title compound was prepared by reaction of 3,5-dibromo-2-(3-methoxyphenyl)thiophene (**12c**) (250 mg, 0.72 mmol), 3-methoxybenzeneboronic acid (134 mg, 0.86 mmol), sodium carbonate (148 mg, 1.44 mmol) and tetrakis(triphenylphosphine) palladium (41.6 mg, 36.0 μmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 72 % (194 mg).

2,5-Bis(3-methoxyphenyl)-3-phenylthiophene (14a). The title compound was prepared by reaction of 3-bromo-2,5-bis(3-methoxyphenyl)thiophene (**14b**) (102 mg, 0.27 mmol), benzeneboronic acid (38 mg, 0.27 mmol), sodium carbonate (58 mg, 0.54 mmol) and tetrakis(triphenylphosphine) palladium (15.6 mg, 13.5 μmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 54 % (51 mg).

3,3'-(3-Phenylthiene-2,5-diyl)diphenol (14). The title compound was prepared by reaction of 2,5-bis(3-methoxyphenyl)-3-phenylthiophene (**14a**) (50 mg, 0.13 mmol) and boron tribromide (0.78 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 53 % (49 mg); MS (ESI): 345 $(M+H)^+$; Anal. ($C_{22}H_{16}O_2S$) C, H, N.

2,3,5-Tris(3-methoxyphenyl)thiophene (**15a**). The title compound was prepared by reaction of 3-bromo-2,5-bis(3-methoxyphenyl)thiophene (**14b**) (102 mg, 0.27 mmol), 3-methoxybenzene boronic acid (42 mg, 0.27 mmol), sodium carbonate (58 mg, 0.54 mmol) and tetrakis(triphenylphosphine) palladium (15.6 mg, 13.5 μmol) according to method B. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 34 % (37 mg).

3,3',3''-Thiene-2,3,5-triyltriphenol (15). The title compound was prepared by reaction of 2,3,5-tris(3-methoxyphenyl)thiophene (**15a**) (37 mg, 0.09 mmol) and boron tribromide (0.81 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 67 % (21 mg); MS (ESI): 361 $(M+H)^+$; Anal. ($C_{22}H_{16}O_3S$) C, H, N.

5-(4-Methoxyphenyl)-2-(boronic acid)thiophene (16b). To a solution of 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**) (100 mg, 0.37 mmol, 1 eq) in anhydrous THF cooled to -78 °C for 5 min, *n*-BuLi (1.6 M in hexane, 0.28 mL, 0.44 mmol, 1.2 eq) was added dropwise and stirred at -78 °C. After 15 min, triethyl borate (0.37 mL, 2.22 mmol, 6 eq) was added at -78°C and the mixture was stirred for 2 h. After warming to rt, the crude material was acidified with 20 mL of a 1N hydrochloric acid solution. The aqueous layer was washed with ethyl acetate. The combined organic layers were dried over sodium sulfate, filtered and evaporated under reduced pressure. The title compound was not characterized and used without further purification.

2-(3-Methoxyphenyl-5-methylphenyl)-5-(4-methoxyphenyl)thiophene (16a). The title compound was prepared by reaction of 1-bromo-3-methoxy-5-methylbenzene (150 mg, 0.74 mmol), [5-(4-methoxyphenyl)-2-thienyl]-boronic acid (**16b**) (206 mg, 0.88 mmol), sodium carbonate (181 mg, 1.76 mmol) and tetrakis(triphenylphosphine) palladium (42.7 mg, 37.0 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 22 % (50 mg).

3-[5-(4-Hydroxyphenyl)-2-thienyl]-5-methylphenol (16). The title compound was prepared by reaction of 2-(3-methoxyphenyl-5-methylphenyl)-5-(4-methoxyphenyl)thiophene (**16a**) (50 mg, 0.16 mmol) and boron tribromide (0.96 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 90 % (41 mg); MS (ESI): 281 $(M-H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

2-(3-Fluoro-5-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (17a). The title compound was prepared by reaction of 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**) (200 mg, 0.75 mmol), 3-fluoro-5-methoxybenzeneboronic acid (152 mg, 0.89 mmol), caesium carbonate (513 mg, 1.65 mmol) and tetrakis(triphenylphosphine) palladium (8.7 mg, 7.5 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 43 % (122 mg).

3-Fluoro-5-[5-(4-hydroxyphenyl)-2-thienyl]phenol (17). The title compound was prepared by reaction of 2-(3-fluoro-5-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**17a**) (100 mg, 0.32 mmol) and boron tribromide (1.92 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 88 % (80 mg); MS (APCI): 286 M^+; Anal. ($C_{16}H_{11}FO_2S$) C, H, N.

5-[5-(4-Methoxyphenyl)-2-thienyl]-2-methylphenol (18a). The title compound was prepared by reaction of 5-bromo-2-methylphenol (250 mg, 1.34 mmol), [5-(4-methoxyphenyl)-2-thienyl]-boronic acid (**16b**) (690 mg, 2.95 mmol), caesium carbonate (914 mg, 2.94 mmol) and tetrakis(triphenylphosphine) palladium (15.5 mg, 13.4 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 47 % (193 mg).

5-[5-(4-Hydroxyphenyl)-2-thienyl]-2-methylphenol (18). The title compound was prepared by reaction of 5-[5-(4-methoxyphenyl)-2-thienyl]-2-methylphenol (**18a**) (161 mg, 0.54 mmol) and boron tribromide (3.24 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 27 % (42 mg); MS (ESI): 283 $(M+H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

2-(4-Fluoro-3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (19a). The title compound was prepared by reaction of 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**) (200 mg, 0.75 mmol), 4-fluoro-3-methoxybenzeneboronic acid (152 mg, 0.89 mmol), caesium carbonate (553 mg, 1.78 mmol) and tetrakis(triphenylphosphine) palladium (8.7 mg, 7.5 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 49 % (149 mg).

2-Fluoro-5-[5-(4-hydroxyphenyl)-2-thienyl]phenol (19). The title compound was prepared by reaction of 2-(4-fluoro-3-methoxyphenyl)-5-(4-methoxyphenyl)thiophene (**19a**) (100 mg, 0.32 mmol) and boron tribromide (1.92 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 88 % (80 mg); MS (ESI): 287 $(M+H)^+$; Anal. ($C_{16}H_{11}FO_2S$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-(4-methoxyphenyl)thiophene (20a). The title compound was prepared by reaction of 2-bromo-5-(4-methoxyphenyl)thiophene (**16c**) (195 mg, 0.73 mmol), 3,4-dimethoxybenzene boronic acid (160 mg, 0.88 mmol), caesium carbonate (500 mg, 1.61 mmol) and tetrakis(triphenylphosphine) palladium (8.4 mg, 7.3 μmol) according to method A. The product was purified by CC (dichloromethane/methanol 99:1); yield: 46 % (119 mg).

4-[5-(4-Hydroxyphenyl)-2-thienyl]benzene-1,2-diol (20). The title compound was prepared by reaction of 2-(3,4-dimethoxyphenyl)-5-(4-methoxyphenyl)thiophene (**20a**) (100 mg, 0.31 mmol) and boron tribromide (2.79 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 17 % (49 mg); MS (ESI): 285 $(M+H)^+$; Anal. ($C_{16}H_{12}O_3S$) C, H, N.

2-(4-Methoxy-3-methylphenyl)-5-(3-methoxyphenyl)thiophene (21a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (250 mg, 0.92 mmol), 3-methyl-4-methoxybenzeneboronic acid (152.8 mg, 0.92 mmol), sodium carbonate (243 mg, 2.36 mmol) and

tetrakis(triphenylphosphine) palladium (53.1 mg, 46.0 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 54 % (154 mg).

4-[5-(3-Hydroxyphenyl)-2-thienyl)-2-methyl]phenol (21). The title compound was prepared by reaction of 2-(4-methoxy-3-methylphenyl)-5-(3-methoxyphenyl)thiophene (**21a**) (100 mg, 0.32 mmol) and boron tribromide (1.92 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 79 % (72 mg); MS (ESI): 281 $(M-H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

2-(3,4-Dimethoxyphenyl)-5-(3-methoxyphenyl)thiophene (22a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (206 mg, 1.14 mmol), 3,4-dimethoxybenzeneboronic acid (247 mg, 1.36 mmol), caesium carbonate (779 mg, 2.51 mmol) and tetrakis(triphenylphosphine) palladium (13.2 mg, 11.4 μmol) according to method A. The product was purified by CC (dichloromethane/methanol 99:1); yield: 34 % (126 mg).

4-[5-(3-Hydroxyphenyl)-2-thienyl]benzene-1,2-diol (22). The title compound was prepared by reaction of 2-(3,4-dimethoxyphenyl)-5-(3-methoxyphenyl)thiophene (**22a**) (100 mg, 0.32 mmol) and boron tribromide (2.88 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 61 % (56 mg); MS (ESI): 283 $(M-H)^+$; Anal. ($C_{16}H_{12}O_3S$) C, H, N.

2-(3-Fluoro-4-methoxyphenyl)-5-(3-methoxyphenyl)thiophene (23a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (370 mg, 1.37 mmol), 3-fluoro-4-methoxybenzeneboronic acid (255 mg, 1.50 mmol), caesium carbonate (717 mg, 3.01 mmol) and tetrakis(triphenylphosphine) palladium (15.8 mg, 13.7 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 98 % (421 mg).

2-Fluoro-4-[5-(3-hydroxyphenyl)-2-thienyl]phenol (23). The title compound was prepared by reaction of 2-(3-fluoro-4-methoxyphenyl)-5-(3-methoxyphenyl)thiophene (**23a**) (240 mg, 0.76 mmol) and boron tribromide (4.56 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 90 % (195 mg); MS (ESI): 285 $(M-H)^+$; Anal. ($C_{16}H_{11}FO_2S$) C, H, N.

2-(3-Methoxyphenyl)-5-[4-methoxy-3-(trifluoromethyl)phenyl]thiophene (24a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (408 mg, 1.51 mmol), 3-trifluoromethyl-4-methoxybenzeneboronic acid (398 mg, 1.81 mmol), caesium carbonate (1033 mg, 3.32 mmol) and tetrakis(triphenylphosphine) palladium (17.5 mg, 15.1 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 75 % (412 mg).

4-[5-(3-Hydroxyphenyl)-2-thienyl]-2-(trifluoromethyl)phenol (24). The title compound was prepared by reaction of 2-(3-methoxyphenyl)-5-[4-methoxy-3-(trifluoromethyl)phenyl]thiophene (**24a**) (300 mg, 0.82 mmol) and boron tribromide (4.95 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 98 % (272 mg); MS (ESI): 285 $(M-H)^+$; Anal. ($C_{17}H_{11}F_3O_2S$) C, H, N.

2-(6-Methoxybiphenyl-3-yl)-5-(3-methoxyphenyl)thiophene (25a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (287 mg, 1.07 mmol), [6-methoxy-1,1'-biphenyl-3-yl]boronic acid (**25b**) (338 mg, 1.29 mmol), sodium carbonate (250 mg, 2.35 mmol) and tetrakis(triphenylphosphine) palladium (61.8 mg, 53.5 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 35 % (135 mg).

5-[5-(3-Hydroxyphenyl)-2-thienyl]biphenyl-2-ol (25). The title compound was prepared by reaction of 2-(6-methoxybiphenyl-3-yl)-5-(3-methoxyphenyl)thiophene (**25a**) (100 mg, 0.26 mmol) and boron tribromide (1.56 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 88 % (81 mg); MS (ESI): 343 $(M-H)^+$; Anal. ($C_{22}H_{16}O_2S$) C, H, N.

[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]methanol (26c). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (408 mg, 1.51 mmol), [3-(hydroxymethyl)-4-methoxyphenyl]-boronic acid (**26d**) (329 mg, 1.81 mmol), caesium carbonate (1032 mg, 3.32 mmol) and tetrakis(triphenylphosphine) palladium (17.5 mg, 15.1 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 8:2); yield: 12 % (59 mg).

2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]benzaldehyde (26b). To a solution of [2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]methanol (**26c**) (100 mg, 0.31 mmol, 1 eq) in dichloromethane, pyridium chlorochromate (66 mg, 0.31 mmol, 1 eq) was added in small portions over 5 min and stirred at rt. After 30 min, the reaction was quenched with water. The resulting organic layer was dried over sodium sulfate, filtered and concentrated to dryness. The title compound was not characterized and used in the next step without purification.

Ethyl (2*E*)-3-[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]acrylate (26a). To a solution of sodium hydride (10.4 mg, 0.43 mmol, 1 eq) in anhydrous THF triethyl phosphonate (93 μL, 0.46 mmol, 1.1 eq) was added dropwise and stirred at rt. After 15 min, 2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]benzaldehyde (**26b**) (100 mg, 0.31 mmol, 0.6 eq) was added and stirred for 4 h at rt. To quench the reaction water was added and the resulting organic layer was washed with brine, dried over sodium sulfate, filtered, evaporated and purified by CC (hexane/ethyl acetate 7:3); yield: 98 % (120 mg).

Ethyl (2*E*)-3-[2-hydroxy-5-[5-(3-hydroxyphenyl)-2-thienyl]phenyl]acrylate (26). The title compound was prepared by reaction of ethyl (2*E*)-3-[2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]acrylate (**26a**) (60 mg, 0.15 mmol) and boron tribromide (0.90 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 17 % (10 mg); MS (APCI): 366 $(M)^+$; Anal. ($C_{21}H_{18}O_4S$) C, H, N.

(2*E*)-3-[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylacrylamide (27a). Ethyl (2*E*)-3-[2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]acrylate (**26a**) (720 mg, 2.22 mmol, 1 eq) in a solution of THF/water (2:1), was refluxed for 20 h together with lithium hydroxide (320 mg, 13.33 mmol, 6 eq). After cooling to rt, ether was added, the aqueous layer was acidified with hydrochloric acid 1N and washed with dichloromethane. The combined organic layers were dried over sodium sulfate, filtered and evaporated under reduced pressure. The resulting carboxylic acid was solubilized in dichloromethane (180 mg, 0.53 mmol, 1 eq) and refluxed for 20 h with EDCI (102 mg, 0.53 mmol, 1 eq) and HOBt (72 mg, 0.53 mmol, 1 eq). After cooling to rt, the organic layer was washed with a 1.5 M sodium hydrogenocarbonate solution, brine, dried over sodium sulfate, evaporated under reduced pressure and purified by CC (hexane/ethyl acetate 7:3); yield: 51 % (120 mg); MS (ESI): 442 $(M+H)^+$.

(2*E*)-3-[2-Hydroxy-5-[5-(3-hydroxyphenyl)-2-thienyl]phenyl]-*N*-phenylacrylamide (27).
The title compound was prepared by reaction of **(**2*E*)-3-[2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylacrylamide (**27a**) (55 mg, 0.13 mmol) and boron tribromide (0.78 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 4:6); yield: 31 % (17 mg); MS (ESI): 414 $(M+H)^+$; Anal. ($C_{25}H_{19}NO_3S$) C, H, N.

3-[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylpropanamide (28a). (2*E*)-3-[2-Methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylacrylamide (**27a**) (50 mg, 0.11 mmol, 1 eq) was solubilized in thr mixture of THF/EtOH (1:1). After addition of palladium hydroxide (1.7 mg, 0.01 mmol, 0.1 eq) the reaction was stirred at rt under nitrogen atmosphere for 20 h. The crude mixture was filtered and the organic layer was evaporated under reduced pressure; yield: quantitative.

3-[2-Hydroxy-5-[5-(3-hydroxyphenyl)-2-thienyl]phenyl]-*N*-phenylpropanamide (28). The title compound was prepared by reaction of 3-[2-methoxy-5-[5-(3-methoxyphenyl)-2-thienyl]phenyl]-*N*-phenylpropanamide (**28a**) (55 mg, 0.13 mmol) and boron tribromide (0.78 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 20 % (10 mg); MS (ESI): 416 $(M+H)^+$; Anal. ($C_{25}H_{21}NO_3S$) C, H, N.

2-Bromo-5-(3-fluoro-4-methoxyphenyl)thiophene (29b). The title compound was prepared by reaction of 2,5-dibromothiophene (500 mg, 2.10 mmol), 3-fluoro-4-methoxybenzeneboronic acid (357 mg, 2.10 mmol), sodium carbonate (432 mg, 4.20 mmol) and tetrakis(triphenylphosphine) palladium (121 mg, 1.05 mmol) according to method B. The product was purified by CC (hexane/ethyl acetate 95:5); yield: 85 % (427 mg).

2-(3-Fluoro-4-methoxyphenyl)-5-(3-fluoro-5-methoxyphenyl)thiophene (29a). The title compound was prepared by reaction of 2-bromo-5-(3-fluoro-4-methoxyphenyl)thiophene (**29b**) (100 mg, 0.41 mmol), 3-fluoro-5-methoxybenzeneboronic acid (85 mg, 0.50 mmol), caesium carbonate (280 mg, 0.90 mmol) and tetrakis(triphenylphosphine) palladium (4.7 mg, 4.1 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 82 % (113 mg).

2-Fluoro-4-[5-(3-fluoro-5-hydroxyphenyl)thien-2-yl]phenol (29). The title compound was prepared by reaction of 2-(3-fluoro-4-methoxyphenyl)-5-(3-fluoro-5-methoxyphenyl)thiophene (**30a**) (100 mg, 0.30 mmol) and boron tribromide (1.80 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 38 % (35 mg); MS (APCI): 304 $(M)^+$; Anal. ($C_{16}H_{10}F_2O_2S$) C, H, N.

2-(3-Fluoro-4-methoxyphenyl)-5-(4-fluoro-3-methoxyphenyl)thiophene (30a). The title compound was prepared by reaction of 2-bromo-5-(3-fluoro-4-methoxyphenyl)thiophene (**29b**) (100 mg, 0.41 mmol), 4-fluoro-3-methoxybenzeneboronic acid (85 mg, 0.50 mmol), caesium carbonate (279 mg, 0.90 mmol) and tetrakis(triphenylphosphine) palladium (4.7 mg, 4.1 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 72 % (100 mg).

2-Fluoro-4-[5-(4-fluoro-3-hydroxyphenyl)thien-2-yl]phenol (30). The title compound was prepared by reaction of 2-(3-fluoro-4-methoxyphenyl)-5-(3-fluoro-5-methoxyphenyl)thiophene (**30a**) (100 mg, 0.30 mmol) and boron tribromide (1.80 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 6:4); yield: 75 % (69 mg); MS (APCI): 304 $(M)^+$; Anal. ($C_{16}H_{10}F_2O_2S$) C, H, N.

2-(3,5-Difluoro-4-methoxyphenyl)-5-(3-methoxyphenyl)thiophene (31a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (430 mg, 1.60 mmol), 3,5-difluoro-4-methoxybenzeneboronic acid (357 mg, 1.92 mmol), caesium carbonate (1094 mg, 3.52 mmol) and tetrakis(triphenylphosphine) palladium (18.5 mg, 16.0 μmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 42 % (223 mg).

2,6-Difluoro-4-[5-(3-hydroxyphenyl)-2-thienyl]phenol (31). The title compound was prepared by reaction of 2-(3,5-difluoro-4-methoxyphenyl)-5-(3-methoxyphenyl)thiophene (**31a**) (220 mg, 0.62 mmol) and boron tribromide (3.72 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 5:5); yield: 10 % (18 mg); MS (ESI): 305 $(M+H)^+$; Anal. ($C_{16}H_{10}F_2O_2S$) C, H, N.

2-(3,4-Difluorophenyl)-5-(3-methoxyphenyl)thiophene (32a). The title compound was prepared by reaction of 2-bromo-5-(3-methoxyphenyl)thiophene (**1b**) (150 mg, 0.56 mmol), 3,4-difluorobenzeneboronic acid (105 mg, 0.67 mmol), caesium carbonate (383 mg, 1.23 mmol) and tetrakis(triphenylphosphine) palladium (6.5 mg, 5.6 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 90 % (152 mg).

3-[5-(3,4-Difluorophenyl)-2-thienyl]phenol (32). The title compound was prepared by reaction of 2-(3,4-difluorophenyl)-5-(3-methoxyphenyl)thiophene (**32a**) (120 mg, 0.40 mmol) and boron tribromide (1.20 mmol) according to method C. The product was purified by CC (dichloromethane/methanol 99:1); yield: 85 % (98 mg); MS (ESI): 289 $(M+H)^+$; Anal. ($C_{16}H_9F_2OS$) C, H, N.

4-(4-Methoxy-3-methylphenyl)-2-(3-methoxyphenyl)thiophene (34a). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)thiophene (**33b**) (400 mg, 1.49 mmol), 3-methyl-4-methoxybenzeneboronic acid (296 mg, 1.79 mmol), caesium carbonate (1019 mg, 3.27 mmol) and

tetrakis(triphenylphosphine) palladium (17.2 mg, 14.9 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 69 % (320 mg).

4-[5-(3-Hydroxyphenyl)-3-thienyl]-2-methylphenol (34). The title compound was prepared by reaction of 4-(4-methoxy-3-methylphenyl)-2-(3-methoxyphenyl)thiophene (**34a**) (180 mg, 0.58 mmol) and boron tribromide (3.48 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 54 % (88 mg); MS (ESI): 281 $(M-H)^+$; Anal. ($C_{17}H_{14}O_2S$) C, H, N.

4-(3-Fluoro-4-methoxyphenyl)-2-(3-methoxyphenyl)thiophene (35a). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)thiophene (**33b**) (400 mg, 1.49 mmol), 3-fluoro-4-methoxybenzeneboronic acid (303 mg, 1.78 mmol), caesium carbonate (1019 mg, 3.30 mmol) and tetrakis(triphenylphosphine) palladium (17.2 mg, 14.9 µmol) according to method A. The product was purified by CC (petroleum ether/ethyl acetate 9:1); yield: 80 % (403 mg).

2-Fluoro-4-[5-(3-hydroxyphenyl)-3-thienyl]phenol (35). The title compound was prepared by reaction of 4-(3-fluoro-4-methoxyphenyl)-2-(3-methoxyphenyl)thiophene (**35a**) (400 mg, 1.27 mmol) and boron tribromide (7.63 mmol) according to method C. The product was purified by CC (dichloromethane/methanol 98:2); yield: 22 % (88 mg); MS (ESI): 287 $(M-H)^-$

4-(4-Methoxy-3-methylphenyl)-2-(3-methoxyphenyl)-1,3-thiazole (37a). The title compound was prepared by reaction of 5-bromo-2-(3-methoxyphenyl)-1,3-thiazole (**36b**) (402 mg, 1.49 mmol), 3-methyl-4-methoxybenzeneboronic acid (247 mg, 1.79 mmol), caesium carbonate (1019 mg, 3.27 mmol) and tetrakis(triphenylphosphine) palladium (17.2 mg, 14.9 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 69 % (320 mg).

4-[2-(3-Hydroxyphenyl)-1,3-thiazol-5-yl]-2-methylphenol (37). The title compound was prepared by reaction of 4-(4-methoxy-3-methylphenyl)-2-(3-methoxyphenyl)-1,3-thiazole (**37a**) (80 mg, 0.26 mmol) and boron tribromide (1.56 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 3:7); yield: 16 % (11 mg), MS (ESI): 274 $(M+H)^+$; Anal. ($C_{16}H_{13}NO_2S$) C, H, N.

3-Fluoro-3'',4-dimethoxy-1,1':4',1''-terphenyl (39a). The title compound was prepared by reaction of 4'-bromo-3-methoxybiphenyl (**38b**) (175 mg, 0.67 mmol), 3-fluoro-4-methoxybenzeneboronic acid (136.7 mg, 0.88 mmol), caesium carbonate (457 mg, 1.47 mmol) and tetrakis(triphenylphosphine) palladium (7.7 mg, 6.7 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 98:2); yield: 58 % (117 mg).

3''-Fluoro-1,1':4',1''-terphenyl-3,4''-diol (**39**). The title compound was prepared by reaction of 3-fluoro-3",4-dimethoxy-1,1':4',1"-terphenyl (**39a**) (115 mg, 0.37 mmol) and boron tribromide (2.22 mmol) according to method C. The product was purified by preparative TLC (dichloromethane/methanol 99:1); yield: 62 % (65 mg); MS (ESI): 281 $(M+H)^+$.

3,3''-Dimethoxy-4-methyl-1,1':4',1''-terphenyl (**41a**). The title compound was prepared by reaction of 4'-bromo-3-methoxybiphenyl (**38b**) (230 mg, 0.87 mmol), 4-methoxy-3-methylbenzeneboronic acid (172 mg, 1.04 mmol), caesium carbonate (595 mg, 1.91 mmol) and tetrakis(triphenylphosphine) palladium (10.1 mg, 8.7 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 98:2); yield: 53 % (140 mg).

4-Methyl-1,1':4',1''-terphenyl-3,3''-diol (**41**). The title compound was prepared by reaction of 3,3"-dimethoxy-4-methyl-1,1':4',1"-terphenyl (**41a**) (120 mg, 0.39 mmol) and boron tribromide (2.34 mmol) according to method C. The product was purified by preparative TLC (dichloromethane/methanol 97:3); yield: 38 % (42 mg); MS (ESI): 277 $(M+H)^+$.

4-Fluoro-3,3''-dimethoxy-1,1':4',1''-terphenyl (**42a**). The title compound was prepared by reaction of 4'-bromo-3-methoxybiphenyl (**38b**) (200 mg, 0.76 mmol), 4-fluoro-3-methoxybenzeneboronic acid

(154 mg, 0.91 mmol), caesium carbonate (520 mg, 1.67 mmol) and tetrakis(triphenylphosphine) palladium (8.8 mg, 7.6 µmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 75 % (175 mg).

4-Fluoro-1,1':4',1''-terphenyl-3,3''-diol (42). The title compound was prepared by reaction of 4-fluoro-3,3''-dimethoxy-1,1':4',1''-terphenyl (**42a**) (175 mg, 0.57 mmol) and boron tribromide solution (3.42 mmol) according to method C. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 63 % (100 mg); MS (ESI): 281 $(M+H)^+$.

Biological Methods

[2, 4, 6, 7-^{3}H]-E2 and [2, 4, 6, 7-^{3}H]-E1 were bought from Perkin Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt.
17β-HSD1 and 17β-HSD2 were obtained from human placenta according to previously described procedures.[35, 52, 53] Fresh human placenta was homogenized and centrifuged. The pellet fraction contains the microsomal 17β-HSD2, while 17β-HSD1 was obtained after precipitation with ammonium sulfate from the cytosolic fraction.

1. Inhibition of 17β-HSD1

Inhibitory activities were evaluated by a well established method with minor modifications.[54-56] Briefly, the enzyme preparation was incubated with NADH [500 µM] in the presence of potential inhibitors at 37 °C in a phosphate buffer (50 mM) supplemented with 20 % of glycerol and EDTA (1mM)**.** Inhibitor stock solutions were prepared in DMSO. Final concentration of DMSO was adjusted to 1 % in all samples. The enzymatic reaction was started by addition of a mixture of unlabelled- and [2, 4, 6, 7-^{3}H]-E1 (final concentration: 500 nM, 0.15 µCi). After 10 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with ether. After evaporation, the steroids were dissolved in acetonitrile. E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 rp chromatography column (Nucleodur C18 Gravity, 3 µm, Macherey-Nagel, Düren) connected to a HPLC-system (Agilent 1100 Series, Agilent Technologies, Waldbronn). Detection and quantification of the steroids were performed using a radioflow detector (Berthold Technologies, Bad Wildbad). The conversion rate was calculated according to following equation: $\%conversion = \frac{\%E2}{\%E2 + \%E1} \cdot 100$. Each value was calculated from at least three independent experiments.

2. Inhibition of 17β-HSD2

The 17β-HSD2 inhibition assay was performed similarly to the 17β-HSD1 procedure. The microsomal fraction was incubated with NAD^+ [1500 µM], test compound and a mixture of unlabelled- and [2, 4, 6, 7-^{3}H]-E2 (final concentration: 500 nM, 0.11 µCi) for 20 min at 37 °C. Further treatment of the samples and HPLC separation was carried out as mentioned above.

3. ER affinity

The binding affinity of select compounds to the ERα and ERβ was determined according to Zimmermann et al.[57] Briefly, 0.25 pmol of ERα or ERβ, respectively, were incubated with [2, 4, 6, 7-^{3}H]-E2 (10 nM) and test compound for 1 h at rt. The potential inhibitors were dissolved in DMSO (5 % final concentration). Non-specific-binding was performed with diethylstilbestrol (10 µM). After incubation, ligand-receptor complexes were selectively bound to hydroxyapatite (5 g/ 60 mL TE-buffer). The formed complex was separated, washed and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (Rack Beta Primo 1209, Wallac, Turku). For determination of the relative binding affinity (RBA), inhibitor and E2 concentrations required to displace 50 % of the

receptor bound labelled E2 were determined. RBA values were calculated according to the following equation: $RBA[\%] = \frac{IC_{50}(E2)}{IC_{50}(compound)} \cdot 100$. The RBA value for E2 was arbitrarily set at 100 %.

4.Evaluation of the estrogenic activity using T-47D cells

Phenol red-free medium was supplemented with sodium bicarbonate (2 g/L), streptomycin (100 µg/mL), insuline zinc salt (10 µg/mL), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 U/mL) and DCC-FCS 5% (v/v). RPMI 1640 (without phenol red) was used for the experiments. Cells were grown for 48 h in phenol red-free medium. Compound **21** wasadded at a final concentration of 100 nM. Inhibitors and E2 were diluted in ethanol (final ethanol concentration was adjusted to 1%). As a positive control E2 was added at a final concentration of 0.1 nM. Ethanol was used as negative control. Medium was changed every two to three days and supplemented with the respective additive. After eight days of incubation, the cell viability was evaluated measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT). The cleavage of MTT to a blue formazan by mitochondrial succinat-dehydrogenase was quantified spectrophotometrically at 590 nm as described by Denizot and Lang[58] with minor modifications. The control proliferation was arbitrarily set at 1 and the stimulation induced by the inhibitor was calculated according to following equation: $\%stimulation = \frac{[proliferation(compound-induced)-1]}{[proliferation(E2-induced)-1]} \cdot 100\%$. Each value is calculated as a mean value of at least three independent experiments

5. Inhibition of human hepatic CYPs

The commercially available P450 inhibition kits from BD Gentest™ (Heidelberg, Germany) were used according to the instructions of the manufacturer. Compounds **21** and **23** were tested for inhibition of the following enzymes: CYP2D6 and 3A4. Percentage of inhibition at 2 µM for compounds **21** and **23** were determined.

6. *In-Vivo* Pharmacokinetics

Male Wistar rats weighing 300-330 g (Janvier France) were housed in a temperature- controlled room (20-22 °C) and maintained in a 12 h light/12 h dark cycle. Food and water were available *ad libitum.* They were anesthetized with a ketamine (135 mg/kg)/ xylyzine (10 mg/kg) mixture and cannulated with silicone tubing via the right jugular vein and attached to the skull with dental cement. Prior to the first blood sampling, animals were connected to a counterbalanced system and tubing to perform blood sampling in the freely moving rat.
Compounds **21** and **23** were applied orally in a cassette dosing in 4 rats at the dose of 10 mg/kg body weight by using a feeding needle. The compounds were dissolved in a mixture labrasol/water (1:1) and given at a volume of 5mL/kg. Blood samples (0.2 mL) were taken at 0, 1, 2, 3, 4, 6, 8, 10 and 24 h postdose and collected in heparinised tubes. They were centrifuged at 3000g for 10 min, and plasma was harvested and kept at -20 °C until analyzed.
HPLC-MS/MS analysis and quantification of the samples was carried out on a Surveyor-HPLC-system coupled with a TSQ Quantum (Thermo/Fisher) triple quadrupole mass spectrometer equipped with an electrospray interface (ESI).

Computational Chemistry

1. Molecular Modelling

All molecular modelling studies were performed on Intel(R) P4 CPU 3.00 GHz running Linux CentOS 5.2. The X-ray structures of 17β-HSD1 (PDB-ID: 1A27, 1FDT and 1I5R) were obtained from the Protein Databank[59] and further prepared using the BIOPOLYMER module of SYBYL v8.0 (Sybyl, Tripos Inc., St. Louis, Missouri, USA). Water molecules, E2 (or HYC for 1I5R) and sulfate ions were stripped from the PDB files and missing protein atoms were added and correct atom types set. Finally

hydrogen atoms and neutral end groups were added. All basic and acidic residues were considered protonated and deprotonated, respectively. Since almost all histidines are oriented toward the outer part of the enzyme, accessible for the surface, they were considered as protonated (HIP) after a prediction run made by MolProbity.[60] For 1I5R the cofactor NADPH was merged into the enzyme after an accurate overlay with the hybrid inhibitor HYC and the X-rays 1A27 and 1FDT. Further, every crystal structure was minimized for 500 steps with the steepest descent minimizer as implemented in SYBYL with the backbone atoms kept at fixed positions in order to fix close contacts, followed by 2000 steps conjugate gradient minimization requested for an overall better starting structure.
Inhibitor **23** was built with SYBYL and energy-minimized in MMFF94s force-field as implemented in Sybyl. Subsequently an *ab-initio* geometry optimizations was performed gas phase at the B3LYP/6-311**G (d,p) level of density functional theory (DFT) by means of the Gaussian03 software,[61, 62] in order to obtain the RESP charges of compound **23**, thought to better perform in Autodock4.
Two different softwares were used for docking studies: GOLDv3.2[63] and Autodock4,[64, 65] using the graphical user interface AutoDockTools (ADT 1.5.2). Since both allow flexible docking of ligands, no conformational search was employed to the ligand structure. For both programs the compound **23** was docked in 50 independent genetic algorithm (GA) runs.
GOLDv3.2: Active-site origin was set at the center of the steroid binding site, while the radius was set equal to 13 Å. The automatic active-site detection was switched on. Further, a slightly modified GOLDSCORE fitness function (increased scaling for hydrophobic contacts) was used and genetic algorithm default parameters were set as suggested by the GOLD authors.
Autodock4: The docking area has been defined by a box, centered on the mass center of the CD-rings of the cocrystallized E2. Grids points of 60 × 70 × 74 with 0.375 Å spacing were calculated around the docking area for all the ligand atom types using AutoGrid4. The Lamarckian genetic algorithm local search (GALS) method was used. Each docking run was performed with a population size of 200. A mutation rate of 0.02 and a crossover rate of 0.8 were used to generate new docking trials for subsequent generations. The GALS method evaluates a population of possible docking solutions and propagates the most successful individuals from each generation into the next one.
Both programs performed in a similar way, supporting the herein suggested binding modes. The quality of the docked poses was evaluated based mainly on visual inspection of the putative binding modes of the ligand, and secondly on the scoring functions, which give a good measure to discriminate between the found binding modes for one single X-ray conformation, but do not help us to compare the poses of different X-rays.

2. MEP

For selected compounds *ab-initio* geometry optimisations were performed gas phase at the B3LYP/6-311**G (d,p) level of density functional theory (DFT) by means of the Gaussian03 software and the molecular electrostatics potential map (MEP) was plotted using GaussView3, the 3D molecular graphics package of Gaussian.[66] These electrostatic potential surfaces were generated by mapping 6-311G** electrostatic potentials onto surfaces of molecular electron density (isovalue = 0.0002e/Å). The MEP maps are color coded, where red stands for negative values ($3.1*10^{-2}$ Hartree) and blue for positive ones ($4.5*10^{-2}$ Hartree).

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References

1. Travis, R. C.; Key, T. J. Oestrogen exposure and breast cancer risk. *Breast Cancer Res*. **2003**, *5*, 239-247.
2. Dizerega, G. S.; Barber, D. L.; Hodgen, G. D. Endometriosis: role of ovarian steroids in initiation, maintenance and suppression. *Fertil. Steril.* **1980**, *33*, 649-653.
3. Miller, W. R.; Bartlett, J. M.; Canney, P.; Verrill, M. Hormonal therapy for postmenopausal breast cancer: the science of sequencing. *Breast Cancer Res. Treat.* **2007**, *103*, 149-160.
4. Bush, N. J. Advances in hormonal therapy for breast cancer. *Semin. Oncol. Nurs.* **2007**, *23*, 46-54.
5. Adamo, V.; Iorfida, M.; Montalto, E.; Festa, V.; Garipoli, C.; Scimone, A.; Zanghi, M.; Caristi, N. Overview and new strategies in metastatic breast cancer (MBC) for treatment of tamoxifen-resistant patients. *Ann. Oncol.* **2007**, 18 Suppl 6, vi53-57.
6. Ohnesorg, T.; Keller, B.; Hrabe de Angelis, M.; Adamski, J. Transcriptional regulation of human and murine 17beta-hydroxysteroid dehydrogenase type-7 confers its participation in cholesterol biosynthesis. *J. Mol. Endocrinol.* **2006**, *37*, 185-197.
7. Shehu, A.; Mao, J.; Gibori, G. B.; Halperin, J.; Le, J.; Devi, Y. S.; Merrill, B.; Kiyokawa, H.; Gibori, G. Prolactin receptor-associated protein/17beta-hydroxysteroid dehydrogenase type 7 gene (Hsd17b7) plays a crucial role in embryonic development and fetal survival. *Mol. Endocrinol.* **2008**, *22*, 2268-2277.
8. Sakurai, N.; Miki, Y.; Suzuki, T.; Watanabe, K.; Narita, T.; Ando, K.; Yung, T. M.; Aoki, D.; Sasano, H.; Handa, H. Systemic distribution and tissue localizations of human 17beta-hydroxysteroid dehydrogenase type 12. *J. Steroid Biochem. Mol. Biol*. **2006**, *99*, 174-181.
9. Day, J. M.; Foster, P. A.; Tutill, H. J.; Parsons, M. F.; Newman, S. P.; Chander, S. K.; Allan, G. M.; Lawrence, H. R.; Vicker, N.; Potter, B. V.; Reed, M. J.; Purohit, A. 17beta-hydroxysteroid dehydrogenase Type 1, and not Type 12, is a target for endocrine therapy of hormone-dependent breast cancer. *Int. J. Cancer* **2008**, *122*, 1931-1940.
10. Gunnarsson, C.; Hellqvist, E.; Stål, O. 17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer* **2005**, *92*, 547-552.
11. Gunnarsson, C.; Olsson, B. M.; Stål, O. Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.* **2001**, *61*, 8448-8451.
12. Miyoshi, Y.; Ando, A.; Shiba, E.; Taguchi, T.; Tamaki, Y.; Noguchi, S. Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* **2001**, *94*, 685-689.
13. Suzuki, T.; Moriya, T.; Ariga, N.; Kaneko, C.; Kanazawa, M.; Sasano, H. 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br. J. Cancer* **2000**, *82*, 518-523.
14. Šmuc, T.; Pucelj Ribič , M.; Šinkovec, J.; Husen, B.; Thole, H.; Lanišnik Rižner , T. Expression analysis of the genes involved in estradiol and progesterone action in human ovarian endometriosis. *Gynecol. Endocrinol.* **2007**, *23*, 105-111.
15. Husen, B.; Huhtinen, K.; Poutanen, M.; Kangas, L.; Messinger, J.; Thole, H. Evaluation of inhibitors for 17beta-hydroxysteroid dehydrogenase type 1 in vivo in immunodeficient mice inoculated with MCF-7 cells stably expressing the recombinant human enzyme. *Mol. Cell. Endocrinol.* **2006**, *248*, 109-113.
16. Husen, B.; Huhtinen, K.; Saloniemi, T.; Messinger, J.; Thole, H. H.; Poutanen, M. Human hydroxysteroid (17-beta) dehydrogenase 1 expression enhances estrogen sensitivity of MCF-7 breast cancer cell xenografts. *Endocrinology* **2006**, *147*, 5333-5339.
17. Penning, T. M. 17beta-hydroxysteroid dehydrogenase: inhibitors and inhibitor design. *Endocr. Relat. Cancer* **1996**, *3*, 41-56.
18. Poirier, D. Inhibitors of 17 beta-hydroxysteroid dehydrogenases. *Curr. Med. Chem.* **2003**, *10*, 453-477.
19. Brožic, P.; Lanišnik Rižner, T.; Gobec, S. Inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Curr. Med. Chem.* **2008**, *15*, 137-150 and references therein cited.
20. Messinger, J.; Hirvelä, L.; Husen, B.; Kangas, L.; Koskimies, P.; Pentikäinen, O.; Saarenketo, P.; Thole, H. New inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* **2006**, *248*, 192-198.

21. Karkola, S.; Lilienkampf, A.; Wähälä, K. A 3D QSAR model of 17beta-HSD1 inhibitors based on a thieno[2,3-d]pyrimidin-4(3H)-one core applying molecular dynamics simulations and ligand-protein docking. *ChemMedChem* **2008**, *3*, 461-472.
22. Allan, G. M.; Vicker, N.; Lawrence, H. R.; Tutill, H. J.; Day, J. M.; Huchet, M.; Ferrandis, E.; Reed, M. J.; Purohit, A.; Potter, B. V. Novel inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: templates for design. *Bioorg. Med. Chem*. **2008**, *16*, 4438-4456.
23. Frotscher, M.; Ziegler, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Neugebauer, A.; Fetzer, L.; Scherer, C.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W. Design, synthesis and biological evaluation of (hydroxyphenyl)-naphthalene and quinoline derivatives: potent and selective non steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* **2008**, *51*, 2158-2169.
24. Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Ziegler, E.; Neugebauer, A.; Bhoga, U. D.; Bey, E.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W. Substituted 6-phenyl-2-naphthols. Potent and selective non-steroidal inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): design, synthesis, biological evaluation and pharmacokinetics. *J. Med. Chem.* **2008**, *51*, 4685-4698.
25. Marchais-Oberwinkler, S.; Frotscher, M.; Ziegler, E.; Werth, R.; Kruchten, P.; Messinger, J.; Thole, H.; Hartmann, R. W. Investigation of a new class of potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases: 6-(3'-hydroxyphenyl) naphthalenes. *Mol. Cell. Endocrinol.* **2008**, doi:10.1016/j.mce.2008.09.024.
26. Bey, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Werth, R.; Oster, A.; Algül, O.; Neugebauer, A.; Hartmann, R. W. Design, synthesis and biological evaluation of bis(hydroxyphenyl) azoles as potent and selective non-steroidal inhibitors of 17beta- hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *Bioorg. Med. Chem.* **2008**, *16*, 6423-6435.
27. Bey, E.; Marchais-Oberwinkler, S.; Werth, R.; Negri, M.; Al-Soud, Y. A.; Kruchten, P.; Oster, A.; Frotscher, M.; Birk, B.; Hartmann, R. W. Design, synthesis, biological evaluation and pharmacokinetics of bis(hydroxyphenyl) substituted azoles, thiophenes, benzenes and aza-benzenes as potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1). *J. Med. Chem.* **2008**, *51*, 6725-6739.
28. Al-Soud, Y. A.; Bey, E.; Oster, A.; Marchais-Oberwinkler, S.; Werth, R.; Kruchten, P.; Frotscher, M.; Hartmann, R. W. The role of the heterocycle in bis(hydroxyphenyl) triazoles for inhibition of 17beta-hydroxysteroid dehydrogenase (17beta-HSD) type 1 and type 2. *Mol. Cell. Endocrinol.* **2008**, doi:10.1016/j.mce.2008.09.012.
29. Kruchten, P.; Werth, R.; Bey, E.; Oster, A.; Marchais-Oberwinkler, S.; Frotscher, M.; Hartmann, R. W. Selective inhibition of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) reduces estrogen responsive cell growth of T-47D breast cancer cells. *J. Steroid Biochem. Mol. Biol.* **2008**, submitted.
30. Kruchten, P.; Werth, R.; Marchais-Oberwinkler, S.; Bey, E.; Ziegler, E.; Oster, A.; Frotscher, M.; Hartmann, R. W. Development of biological assays for the identification of selective inhibitors of estradiol formation from estrone in rat liver preparations. *C. R. Chim*. **2008**, submitted.
31. Ghosh, D.; Pletnev, V. Z.; Zhu, D. W.; Wawrzak, Z.; Duax, W. L.; Pangborn, W.; Labrie, F.; Lin, S.-X. Structure of human estrogenic 17beta- hydroxysteroid dehydrogenase at 2.20 Å resolution. *Structure* **1995**, *3*, 503-513.
32. Azzi, A.; Rehse, P. H.; Zhu, D. W.; Campbell, R. L.; Labrie, F.; Lin, S.-X. Crystal structure of human estrogenic 17beta-hydroxysteroid dehydrogenase complexed with 17 beta-estradiol. *Nat. Struct. Biol*. **1996**, *3*, 665-668.
33. Breton, R.; Housset, D.; Mazza, C.; Fontecilla-Camps, J. C. The structure of a complex of human 17beta-hydroxysteroid dehydrogenase with estradiol and NADP+ identifies two principal targets for the design of inhibitors. *Structure* **1996**, *4*, 905-915.
34. Mazza, C. Human type I 17beta-hydroxysteroid dehydrogenase: site directed mutagenesis and X-Ray crystallography structure-function analysis PhD Thesis, Université Joseph Fourier, Grenoble, 1997.

35. Qiu, W.; Campbell, R. L.; Gangloff, A.; Dupuis, P.; Boivin, R. P.; Tremblay, M. R.; Poirier, D.; Lin, S.-X. A concerted, rational design of type 1 17beta-hydroxysteroid dehydrogenase inhibitors: estradiol-adenosine hybrids with high affinity. *FASEB J.* **2002**, *16*, 1829-1831.
36. Miyaura, N.; Suzuki, A. The palladium catalysed cross-coupling reaction of phenylboronic acid with haloarenes in the presence of bases. *Synth. Commun.* **1995**, *11*, 513-519.
37. Togo, H.; Nogami, G.; Yokoyama, M. Synthetic application of poly[styrene(iodoso diacetate)]. *Synlett* **1998**, *5*, 534-536.
38. Emmons, W. D. The utility of phosphonate carbanions in olefin synthesis. *J. Am. Chem. Soc.* **1961**, *83*, 1733-1738.
39. Pascal, C.; Dubois, J.; Guénard, D.; Guéritte, F. Synthesis of biphenyls mimicking the structure of the antimitotic rhazinilam. *J. Org. Chem.* **1998**, *63*, 6414-6420.
40. Hwang, K.-J.; O'Neil, J. P.; Katzenellenbogen, J. A. 5,6,11,12-Tetrahydrochrysenes: synthesis of rigid stilbene systems designed to be fluorescent ligands for the estrogen receptor. *J. Org. Chem.* **1992**, *57*, 1262-1271.
41. Bruno, I. J.; Cole, J. C.; Lommerse, J. P. M.; Rowland, R. S.; Verdonk, M. L. Isostar: A library of information about non-bonded interactions. *J. Comput. Chem.* **1997**, *11*, 523-537.
42. Hagmann, W. K. The many roles for fluorine in medicinal chemistry. *J. Med. Chem.* **2008**, *51*, 4359-4369.
43. Bohm, H. J.; Banner, D.; Bendels, S.; Kansy, M.; Kuhn, B.; Muller, K.; Obst-Sander, U.; Stahl, M. Fluorine in medicinal chemistry. *ChemBioChem* **2004**, *5*, 637-643.
44. Muller, K.; Faeh, C.; Diederich, F. Fluorine in pharmaceuticals: looking beyond intuition. *Science* **2007**, *317*, 1881-1886.
45. Poirier, D.; Boivin, R. P.; Tremblay, M. R.; Bérubé, M.; Qiu, W.; Lin, S.-X. Estradiol-adenosine hybrid compounds designed to inhibit type 1 17beta-hydroxysteroid dehydrogenase. *J. Med. Chem.* **2005**, *48*, 8134-8147.
46. Bérubé, M.; Poirier, D. Synthesis of simplified hybrid inhibitors of type 1 17beta-hydroxysteroid dehydrogenase via cross-metathesis and sonogashira coupling Reactions. *Org. Lett.* **2004**, *6*, 3127-3130.
47. Fournier, D.; Poirier, D.; Mazumdar, M.; Lin, S.-X. Design and synthesis of bisubstrate inhibitors of type 1 17beta-hydroxysteroid dehydrogenase: Overview and perspectives. *Eur. J. Med. Chem.* **2008**, *43*, 2298-2306.
48. Hartman, J.; Lindberg, K.; Morani, A.; Inzunza, J.; Strom, A.; Gustafsson, J. A. Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts. *Cancer Res.* **2006**, *66*, 11207-11213.
49. Smith, G.; Mikkelsen, G.; Eskildsen, J.; Bundgaard, C. The synthesis and SAR of 2-arylsulfanylphenyl-1-oxyalkylamino acids as GlyT-1 inhibitors. *Bioorg. Med. Chem.* **2006**, *16*, 3981-3884.
50. Pfahl, M.; Al-Shamma, H. A.; Fanjul, A.; Pleynet, D. P. M.; Spruce, L. W.; Wiemann, T. R.; Ibarra, J. B.; Tachdjian, C. Preparation of benzylidenethiazolidenediones and analogs as antitumor agents. WO 2002072009 A2 20020919 2002.
51. Duggan, H. M. E.; Leroux, F. G. M.; Malagu, K.; Martin, N. M. B.; Menear, K. A.; Smith, G. C. M. 2-Methylmorpholine-substituted pyrido-, pyrazo- and pyrimidopyrimidine derivs. as mTOR inhibitors and their preparation, pharmaceutical compositions and use in the treatment of cancer. WO 2008023161 A1 20080228, **2008**.
52. Zhu, D. W.; Lee, X.; Breton, R.; Ghosh, D.; Pangborn, W.; Duax, W. L.; Lin, S.-X. Crystallization and preliminary X-ray diffraction analysis of the complex of human placental 17beta-hydroxysteroid dehydrogenase with NADP+. *J. Mol. Biol.* **1993**, *234*, 242-244.
53. Kruchten, P.; Werth, R.; Marchais-Oberwinkler, S.; Frotscher, M.; Hartmann, R. W. Development of a biological screening system for the evaluation of highly active and selective 17beta-HSD1-inhibitors as potential therapeutic agents. *Mol. Cell. Endocrinol.* **2008**. doi: 10.1016/j.mce.2008.09.035.
54. Lin, S.-X.; Yang, F.; Jin, J. Z.; Breton, R.; Zhu, D. W.; Luu-The, V.; Labrie, F. Subunit identity of the dimeric 17beta-hydroxysteroid dehydrogenase from human placenta. *J. Biol. Chem.* **1992**, *267*, 16182-16187.

55. Sam, K. M.; Auger, S.; Luu-The, V.; Poirier, D. Steroidal spiro-gamma-lactones that inhibit 17beta-hydroxysteroid dehydrogenase activity in human placental microsomes. *J. Med. Chem.* **1995**, *38*, 4518-4528.
56. Sam, K. M.; Boivin, R. P.; Tremblay, M. R.; Auger, S.; Poirier, D. C16 and C17 derivatives of estradiol as inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: chemical synthesis and structure-activity relationships. *Drug. Des. Discov.* **1998**, *15*, 157-180.
57. Zimmermann, J.; Liebl, R.; von Angerer, E. 2,5-Diphenylfuran-based pure antiestrogens with selectivity for the estrogen receptor alpha. *J. Steroid Biochem. Mol. Biol.* **2005**, *94*, 57-66.
58. Denizot, F.; Lang, R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **1986**, *89*, 271-277.
59. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28*, 235-242.
60. Davis, I. W.; Leaver-Fay, A.; Chen, V. B.; Block, J. N.; Kapral, G. J.; Wang, X.; Murray, L. W.; Arendall, W. B., 3rd; Snoeyink, J.; Richardson, J. S.; Richardson, D. C. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res.* **2007**, *35*, W375-383.
61. Frisch, M. J.; Trucks, G. W. S., H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A. Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen,; W.; Wong, M. W. G., C.; Pople, J. A. . Gaussian, Inc.: Pittsburgh, PA: 2003.
62. Bayly, C. I.; Cieplak, P.; Cornell, W. D.; Kollman, P. A. A well behaved electrostatic potential based method using charge restraints for determining atom-centered charges: the RESP model. *J. Phys. Chem.* **1993**, *97*, 10269-10280.
63. Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.* **1997**, *267*, 727-748.
64. Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. Automated Docking Using a Lamarckian Genetic Algorithm and Empirical Binding Free Energy Function. *J. Comput. Chem.* **1998**, *19*, 1639-1662.
65. Huey, R.; Morris, G. M.; Olson, A. J.; Goodsell, D. S. A Semiempirical Free Energy Force Field with Charge-Based Desolvation. *J. Comput. Chem.* **2007**, *28*, 1145-1152.
66. Dennington, I.; Roy, K. T.; Millam, J.; Eppinnett, K.; Howell, W. L.; Gilliland, R. GaussView, 3.0; Semichem Inc., Shawnee Mission: 2003.

4. Summary and Conclusion

The aim of the present thesis was the development of a class of non-steroidal highly potent and selective inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) as potential therapeutics for the treatment of estrogen-dependent pathologies. Following the pharmacophore model established in our group, bis(hydroxyphenyl)substituted azoles and arenes were designed with the goal to reach high 17β-HSD1 inhibitory activity as well as selectivity versus 17β-HSD2, the enzyme which catalyzes the intracellular deactivation of E2 into E1, and the estrogen receptors α and β. In order to deliver a suitable candidate which could be applied as a drug, the biological properties of the most potent inhibitors were further investigated aiming at metabolic stability in rat liver microsomes, inhibition of hepatic CYP enzymes and intestinal absorption using a CaCo2-permeability assay. In a final step, the pharmacokinetic profile of the most promising candidates was evaluated in rats after oral administration in a cassette dosing approach.

Chapter **3.I** deals with the design, synthesis and biological evaluation of a series of 19 bis(hydroxyphenyl) azoles. As the compounds have to mimic the steroidal substrate, different substitutions patterns concerning the phenolic OH groups (*meta* and *para*), in which the O-O distance is the same range as observed for E1 (*d*= 11 Å), were investigated to find out the one which fits best to the active site. Additionally, the influence of the central ring for 17β-HSD1 inhibition was also investigated. Heterocycles containing nitrogen and/or oxygen were selected as their heteroatoms might be able to establish supplementary interactions with the amino acids Tyr218/Ser222 which are located close to the C6 position of the steroid. It was established that the inhibitory activity of the di-substituted azole derivatives depends on the presence of hydroxy rather than methoxy groups on the phenyl substituents and on the OH substitution pattern: *meta-para* and *para-meta* substituted compounds being more active than *para-para* substituted ones. The fact that only several *meta-para*, *para-meta* di-substituted azoles showed inhibitory activity, indicates that also the heterocycle is involved in the stabilization of the inhibitor in the active site of the enzyme. The presence of a hydrogen bond donor function on the heterocycle seemed to be detrimental for activity (2,4-disubstituted imidazoles **I.9-11**,[‡] 1,3-disubstituted pyrazoles **I.12-13**), whereas several compounds bearing H-bond acceptor atoms in the central aromatic ring (1,4-disubstituted 1,2,3-triazoles **I.7-8**, 1,3-disubstituted isoxazole **I.16**, 2,4- and 2,5-disubstituted 1,3-oxazoles **I.18**, **I.19**) are active (Table 4). It is hypothesized that the increase of inhibitory activity is due to the establishment of an additional H-bond interaction between the heteroatoms of the azole moiety and Tyr218/Ser222.

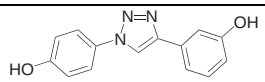
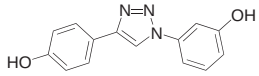
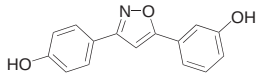
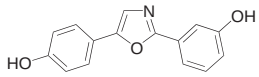
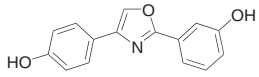
Interestingly, aiming the N, O containing heterocycles, it becomes evident that the location of the heteroatoms in the aromatic scaffold has a decisive effect on the 17β-HSD inhibitory action: the 3,5-disubstituted isoxazole **I.16** and the 2,4-disubstituted oxazole **I.19** turned out to be selective 17β-HSD2 inhibitors while their 2,5-disubstituted oxazole isomer **I.18** shows higher 17β-HSD1 inhibitory activity (Table 4). Consistent with these findings, it can be assumed that besides a high amino acid similarity, both enzymes present also important structural differences in the area of the binding site where the heterocycle is anchored.

Aiming at the selectivity over the ERs, all the tested compounds showed very marginal to weak binding affinities to the receptors (Table 4). In the literature, it has been described that the high binding affinity of similar ER ligands like tris(hydroxyphenyl) pyrazoles[104-109] depends only on the presence of the three hydroxyphenyl moieties, the heterocycle playing

[‡]For the sake of clarity, all compounds that are referred to in chapter 4 are presented as a combination of a Roman numeral (**I-IV**) and an Arabic numeral. The roman numeral indicates in which paper they are published and the Arabic numeral corresponds to the compound number of the publication. (e.g. **I.9** is compound 9 from paper I)

only a passive role, orienting the peripheral substituents in an appropriate topology. Contrariwise, in the case of our bis(hydroxyphenyl) substituted azoles, it becomes evident that the combination of at least two parameters is necessary to reach activity and selectivity: an adequate O-O substitution pattern and a suitable heterocycle. Therefore, the concept of varying the heterocyclic moiety as well as the position of the OH groups on the hydroxyphenyl moieties is further investigated in the following subprojects of this work (Chapter **3.II** and **3.III**) to get on insight into the impact of these two criteria on activity and selectivity.

Table 4: inhibition of human 17β-HSD1, 17β-HSD2 and binding affinities for the estrogen receptors α and β by selected azole derivative.

Cmpd	Structure	IC_{50} (nM)[a]		Selectivity factor[d]	RBA[e] (%)	
		17β-HSD1[b]	17β-HSD2[c]		ERα	ERβ
I.7		1320	8120	6	<0.01	<0.01
I.8		840	7280	9	0.01< RBA < 0.1	0.01< RBA < 0.1
I.16		1610	270	0.2	0.01< RBA < 0.1	<0.01
I.18		310	17500	56	0.01< RBA < 0.1	0.5
I.19		1850	250	0.1	0.01< RBA < 0.1	0.01< RBA < 0.1

[a]Mean value of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate E1, 500 nM, cofactor NADH, 500 µM; [c]Human placenta, microsomal fraction, substrate E2, 500 nM, cofactor NAD^+, 1500 µM; [d]IC_{50} HSD2/IC_{50} HSD1; [e]RBA: relative binding affinity, E2: 100 %, mean value of three determinations, standard deviation less than 10 %.

It is striking that compounds sharing the same *meta-para* O-O substitution pattern and bearing a heterocycle without hydrogen bond donating function like the 1,4-disubstituted imidazole **I.6** are inactive while the corresponding 1,2,3-triazole isomer **I.8** is a fairly potent inhibitor of the enzyme. Therefore we focussed in the second part of this thesis (chapter **3.II**) on triazole core structures and especially on 1,3,4-triazoles (Figure 17). Thereby, we gained further insights into the impact of an additional nitrogen atom and on the influence of the nitrogen distribution in the central ring on inhibitory activity.

In addition, a non polar substituent was introduced on the heterocycle, with the aim, on the one side to tackle the hydrogen bond donating function of the heterocycle. On the other side, these additional substituents should be able to increase the potency of compounds by the induction of supplementary hydrophobic interactions with amino acids of the binding site (e.g. π-π stacking with Tyr218 in case of compounds **II.7-9**). Moreover, it would also deliver important knowledges about the space availability around the heterocycle.

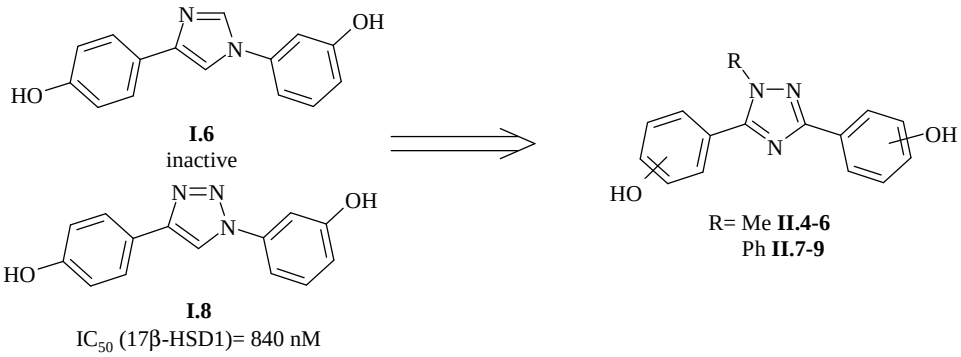
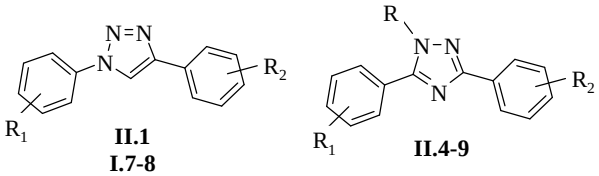


Figure 17: design strategy followed in chapter **3.II**

Analysis of the biological data of these molecules presented in Table 5, revealed interesting structure-activity relationships regarding 17β-HSD type 1 and 2 inhibitory potencies. As a general rule, molecules bearing a 1,2,3-triazole moiety show higher inhibitory activities toward 17β-HSD1 than their corresponding 1,2,4-triazole isomers. Obviously, the nitrogen in the vertex position on the heterocycle and/or the supplementary substituent is not tolerated by the type 1 enzyme. The introduction in *N*-1 position of a methyl or phenyl moiety on the triazole core (compounds **II.4-9**) leads to a strong limitation of the free rotatability around the C-C bonds between each aromatic ring. Thus, a coplanar conformation becomes energetically disfavoured compared to the di-substituted derivatives. The hydroxyphenyl moieties might therefore not be able to adopt the required geometry to establish polar interactions with the enzyme active site.

It is also noteworthy that the loss of planarity is correlated with an increase of 17β-HSD2 activity. This observation is in accordance with the almost published 17β-HSD2 inhibitors which also lack planarity.[110-112] These important structural features should be of high relevance for the future design of selective non-steroidal 17β-HSD2 inhibitors.

Table 5: Inhibition of human 17β-HSD1 and 17β-HSD2 by bis(hydroxyphenyl) triazoles



cmpd	R	R_1	R_2	IC_{50} value (μM)[a]	
				17β-HSD1[b]	17β-HSD2[c]
II.1		3-OH	3-OH	17[d]	ni
I.7		4-OH	3-OH	1.32	8.12
I.8		3-OH	4-OH	0.84	7.28
II.4	CH_3	3-OH	3-OH	ni	ni
II.5	CH_3	4-OH	3-OH	ni	20[d]
II.6	CH_3	3-OH	4-OH	ni	21[d]
II.7	Ph	3-OH	3-OH	ni	41[d]
II.8	Ph	4-OH	3-OH	ni	44[d]
II.9	Ph	3-OH	4-OH	ni	ni

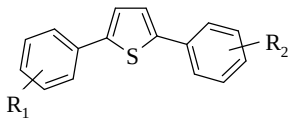
[a]Mean values of three determinations, standard deviation less than 18 %; [b]Human placenta, cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], cofactor NADH [500 μM]; [c]Human placenta, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM]; [d]% inhibition at 1 μM; ni: no inhibition (<10 % inhibition at 1 μM).

In Chapters **3.I** and **3.II**, it has been identified that the inhibitory activity of this class of compounds depends on the O-O substitution pattern and on the nature and location of the heteroatoms present in the central aromatic ring. Regarding the properties of the heterocycle, hydrogen bond donor functions turned out to be detrimental, while hydrogen bond acceptor atoms are favourable. Chapter **3.III** is principally concerned with the design and the synthesis of bis(hydroxyphenyl) azoles and arenes. Besides the investigation of molecules bearing 5-membered sulfur containing heterocycles, it is also evaluated whether 6-membered rings are suitable cores to link the two hydroxyphenyl moieties.

Most of the compounds described in Chapter **3.III** turned out to be even more active than the previously identified 2,5-disubstituted oxazole **I.18**. Regarding the OH-OH substitution pattern of the inhibitors similar findings, as seen in Chapter **3.I**., can be observed: to reach 17β-HSD1 activity, the distance between the two oxygens of the OH groups of the molecule has to be close to the value observed for E1 (d= 11 Å). Therefore only compound with a *meta-para*, *para-meta* and *meta-meta* substitution pattern (8.5< d< 12.8 Å) shows activity while the *para-para* analogues are inactive (d> 12.5 Å).

In order to obtain insights in the role of the OH-groups on inhibitory activity, several thiophenes bearing only one hydroxy substituent (**III.19-20**) were synthesized and their biological activities compared to the di-substituted (**III.22**) analogues. The slightly decrease in case of the replacement of the *para*-OH with a hydrogen and the dramatical loss of activity in case of the exchange of the *meta*-OH by a H atom, indicates that both hydroxy groups are important for activity, with the *meta*-OH being, obviously, more important than the *para*-hydroxy (Table 6).

Table 6: influence of the number and location of the OH groups on 17β-HSD1 and 17β-HSD2 inhibitory activity



cmpd	R_1	R_2	IC_{50} (nM)[a]		selectivity factor[d]
			17β-HSD1[b]	17β-HSD2[c]	
III.17	H	H	ni	nt	--
III.19	3-OH	H	342	2337	7
III.20	4-OH	H	ni	nt	--
III.22	4-OH	3-OH	69	1953	28

[a]Mean values of three determinations, standard deviation less than 12 %; [b]Human placenta, cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], cofactor NADH [500 μM]; [c]Human placenta, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested

Considering the influence of the heterocycle, the exchange of the N-Me moiety of the inactive 1,2,4-triazole **II.6** by a sulfur leads to a fairly potent compound **III.10** (IC_{50}= 413 nM, Table 7). This finding indicates that a sulfur atom in this position has a positive influence on the binding affinity of the enzyme. The role of the nitrogen and the sulfur was further investigated by varying the position as well as the number of heteroatoms in the central ring. Regarding the biological data of the 1,3-thiazole derivatives, it becomes apparent that the location of the sulfur and the nitrogen is decisive for the activity of the compounds: the 2,4-disubstituted thiazole **III.22** turned out to be a very weak inhibitor of the enzyme (IC_{50}> 1000 nM) while its 2,5-disubstituted isomer **III.3** is the most potent compound (IC_{50}= 50 nM). In contrary, in the thiophene class of compounds the variation of the position of the sulfur atom

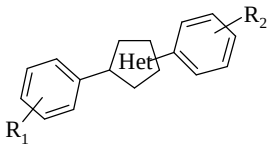
seems not to be relevant: both compounds exhibited similar inhibition values (IC_{50}= 77 nM and 69 nM for **III.27** and **III.22**, respectively).

Surprisingly, the 1,3-thiazole **III.3** (IC_{50}= 50 nM) and thiophene **III.22** (IC_{50}= 69 nM) show almost similar inhibition values. The nitrogen of **III.3** is obviously not contributing to the binding, i.e. no hydrogen bond interaction can be formed between this heteroatom and the binding site of the enzyme.

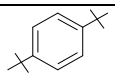
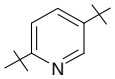
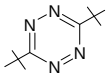
In the six-membered ring class of compounds, phenylene **III.35**, pyridine **III.37** and tetrazine **III.39** show similar inhibitory activities suggesting that the nitrogen of the different ring are tolerated by the enzyme active site but do not contribute to specific interactions (H-bond, Table 7), as it is observed for the 5-membered heterocycles.

Looking at the selectivity toward 17β-HSD2, the biological results show that most of the *meta-meta* di-substituted inhibitors present only a poor selectivity toward the type 2 enzyme except for two members of the six-membered ring class of compounds (compounds **III.37** and **III.39**, selectivity factor of 34 and 25, respectively). Considering the *meta-para* disubstituted inhibitors, compound **III.3** is the most potent molecule with a selectivity factor of 80.

Table 7: inhibition of human 17β-HSD1 and 17β-HSD2 by selected bis(hydrophenyl) substituted azoles and arenes



cmpd	Het	R_1	R_2	IC_{50} (nM)[a]		selectivity factor[d]
				17β-HSD1[b]	17β-HSD2[c]	
II.6		3-OH	4-OH	ni	nt	
III.10		3-OH	4-OH	413	2194	5
III.7		3-OH	4-OH	>1000	nt	
III.27		3-OH	4-OH	77	1271	16
III.22		3-OH	4-OH	69	1953	28
III.3		3-OH	4-OH	50	4004	80
III.4		3-OH	3-OH	243	2500	10
III.8		3-OH	3-OH	455	2220	5
III.20		3-OH	3-OH	173	745	4
III.28		3-OH	3-OH	185	559	3

cmpd	Het	R_1	R_2	IC_{50} (nM)[a] 17β-HSD1	IC_{50} (nM)[a] 17β-HSD2	selectivity factor[d]
III.35		3-OH	3-OH	173	2259	13
III.37		3-OH	3-OH	101	3399	34
III.39		3-OH	3-OH	201	5102	25

[a]Mean values of three determinations, standard deviation less than 12 % except **III.3**: 18 % for 17β-HSD1; [b]Human placenta, cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], cofactor NADH [500 μM]; [c]Human placenta, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested

As it is shown in Table 7, the nature, the number as well as the location of the heteroatoms in the central aromatic ring plays a crucial role for the potency of this class of compounds. Besides their ability to establish polar interactions (e.g. H-bond in case of the nitrogen), they have also an important impact on the electron density of the whole molecule.

Therefore, the charge density distribution of a sample of molecules was calculated and their corresponding MEP maps plotted. For a better comparison, the MEP maps were divided into three different regions corresponding to the three aromatic rings of the compounds (Figure 19).

Trying to establish a semiquantitative MEP-activity relationship ("semi-QMAR"), optimal ESP ranges for area I, II and III for high inhibition activity were identified: for region I ESP from –1.7 to –1.2 $\cdot 10^{-2}$, for region II –1.6 to –0.9 $\cdot 10^{-2}$ and for region III –1.2 to –0.5 $\cdot 10^{-2}$ Hartree. Similarly, the optimal Δ values of ESP for each region were determined: 0.5, 0.7 and 0.7 Hartree, respectively. The shift of a certain ESP distribution range on the scale or the change of the Δ value results in a decrease of inhibitory activity.

The high importance of the location of the heteroatoms on the central aromatic ring on the MEP maps is well demonstrated with the 1,3-thiazole **III.2** and **III.3** isomers, which only differ by the position of the nitrogen in the thiazole moiety. The change of the position of the nitrogen (compound **III.3** vs. compound **III.2**) in the thiazole ring has a decisive influence on the electronic distribution in each area making compound **III.2** inactive.

Regarding the 2,4-disubstituted imidazole **I.12**, a polarization between top and bottom sides of the molecule resulting in a high Δ for area II (Δ= 7.2 $\cdot 10^{-2}$ Hartree) might be the main reason for the inactivity of this compound.

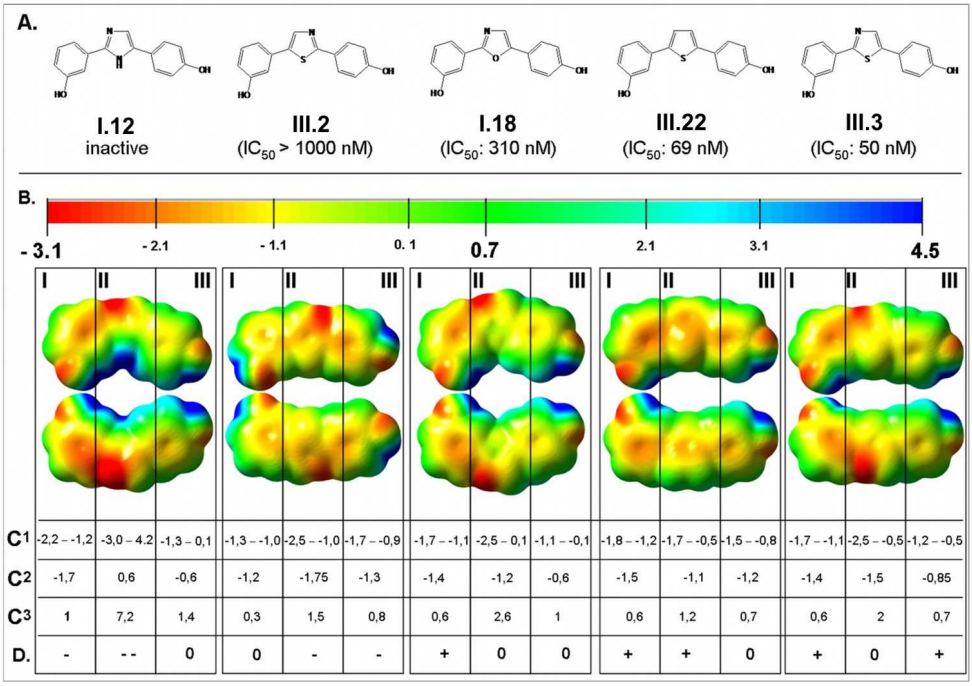


Figure 19: (**A**) structure and 17β-HSD1 inhibitory activity of compounds **I.12**, **III.2**, **I.18**, **III.22** and **III.3** ordered by increasing activity; (**B**) MEP maps, dorsal and ventral view; (**C**[1]) ESP distribution range (Hartree; $\cdot 10^{-2}$); (**C**[2]) mean value of distribution range (Hartree, $\cdot 10^{-2}$); (**C**[3]) Δ of ESP; (**D**) "semi-QMAR". MEP surfaces were plotted with GaussView 3.09.

The selectivity profile of the compounds was further investigated. The binding affinities of molecules with fair to high selectivity toward 17β-HD2 were therefore examined on ERα and ERβ (Table 8). All tested compounds show very marginal to weak affinity to the ERs. Since binding affinity does not explore intrinsic activity at the receptor, the compounds were further analyzed on the estrogen dependent mammary tumor cell-line T-47D. Cell proliferation was monitored after incubation with the test compounds. No agonistic, i.e. stimulatory effect was observed even at a concentration 1000-fold higher than E2 indicating that this class of compounds is devoid of estrogenic activity.

Table 8: binding affinities for the human estrogen receptor α and β of selected compounds

cmpd	RBA (%)[a]	
	ERα[b]	ERβ[b]
III.3	<0.01	0.01< RBA< 0.1
III.22	0.1< RBA< 1	1.5
III.27	0.01< RBA< 0.1	0.1< RBA< 1
III.35	< 0.001	0.01< RBA< 0.1
III.37	0.01< RBA< 0.1	< 0.01

[a]RBA (relative binding affinity), E2: 100 %, mean values of three determinations, standard deviations less than 10 %; [b]Human recombinant protein, incubation with 10 nM [^{3}H]-E2 and inhibitor for 1 h.

In order to get an insight into the pharmacokinetic properties of this class of compounds, a representative compound of the thiazoles **III.4**, thiophenes **III.25** and of the phenylenes **III.35** compound classes were evaluated for its phase 1 metabolic stability using rat liver microsomes.

The tested compounds show longer half-lives ($t_{1/2}$= 12.6, 18.6 and 22.7 min for **III.4**, **III.25** and **III.35**, respectively) than the antihistaminic drug diphenhydramine ($t_{1/2}$= 6.8 min).

The pharmacokinetic profiles of the most active and selective azole **III.3** and aza-benzenes **III.39** were determined in rats after oral administration in a cassette dosing approach. The examination of the availability in the plasma reveals that both compounds show relative short half-lives ($t_{1/2}$= 1.5 and 1.2 h for **III.3** and **III.39**, respectively) and reasonable plasma levels in case of **III.39** ($AUC_{0\text{-}tz}$= 1204.0 ng/mL following a dose of 10 mg/kg). The low plasma levels observed for **III.3** might be due to glucuronidation or sulfatation of the parent compound.

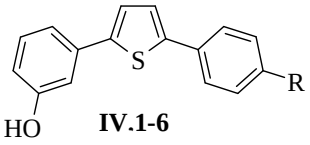
In conclusion, non-steroidal 17β-HSD1 inhibitors with a bis(hydroxyphenyl) thiophene, thiazole and benzene molecular scaffold proved to be significantly superior to the previously identified bis(hydroxyphenyl) oxazole **I.19**. These molecules are highly selective toward 17β-HSD2 and the ERs, show good metabolic stability in rat liver microsomes and a reasonable PK profile. The structural motives obtained in this part were pursued and refined in Chapter **3.IV**.

Chapter **3.IV** describes the efforts of improving the activity and selectivity of bis(hydroxyphenyl) thiophenes, thiazoles and benzenes by the introduction of an additional substituent on the inhibitor backbone. Therefore, based on the molecular scaffolds of **III.3**, **III.22** and **III.35**, several structurally modified derivatives were synthesized and their biological properties were evaluated.

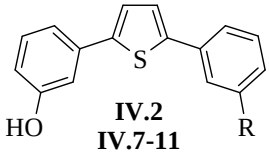
In chapter **3.III**, it was found that removing one of the two hydroxyphenyl moieties of compound **IV.1** is detrimental for 17β-HSD1 inhibitory activity. The replacement of the *para*-OH function by one of its bioisoteric groups like F, NH_2, SH, CN leads to a drop of activity (compounds **IV.2-6**). The lack of hydrogen donating function of the fluoro and cyano substituents might not be the only reason for this decrease in activity as the amino and the thiol derivatives are also less active than the reference compound **IV.1**. Interestingly, the insertion of a C1-linker between the methylsulfonamide moiety and the phenyl ring resulted also in a loss of potency (compound **IV.11**). This finding supports the fact that the delocalization of the electronic density, resulting in a deprivation of electrons in the phenyl ring is necessary for a good inhibition (Table 9).

Looking at the selectivity of these compounds, the biological data presented in Table 9 shows that mono-hydroxylated molecules exhibit a poor selectivity over 17β-HSD2, the most selective one being compound **IV.5** with a selectivity factor of 5. This finding suggests that the *para*-OH is important for activity but is also influencing the selectivity of this class of compounds.

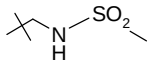
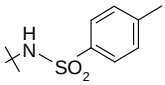
Table 9: effect of the exchange of one OH group on the inhibitory activity of bis(hydroxyphenyl) thiophene



IV.1-6



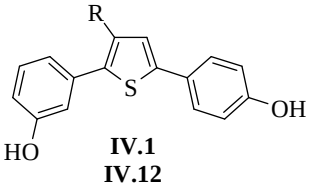
IV.2
IV.7-11

cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]	cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]
IV.1	OH	69	1950	28	**IV.7**	OH	173	745	4
IV.2	H	342	2337	7	**IV.2**	H	342	2337	7
IV.3	F	717	3655	5	**IV.8**	F	535	1824	3
IV.4	NH_2	>5000	nt		**IV.9**		523	1575	3
IV.5	SH	>5000	nt		**IV.10**		>1000	nt	
IV.6	CN	>1000	nt		**IV.11**		350	276	1

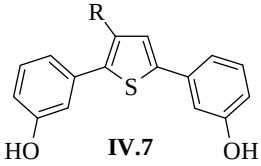
[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 µM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 µM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); ni: no inhibition, nt: not tested.

With the aim to increase the activity and the selectivity, substituents were added on the 2,5-bis(hydroxyphenyl) thiophene **IV.1** and **IV.7**. The introduction of a substituent into the thiophene core (compounds **IV.12** to **IV.15**) seems to be detrimental for activity, except for compound **IV.15** (IC_{50}= 119 nM vs. **IV.7**, IC_{50}= 216 nM). Obviously, a supplementary hydrogen bond between the additional hydroxyphenyl moiety of **IV.15** with polar amino acid residues might be responsible for the increase in inhibitory activity. A pure π-π stacking interaction as observed in compound **IV.14** is obviously not enough to achieve an enhancement of activity. The 2,5-disubstituted thiophenes **IV.12** and **IV.15** differ only in the shift of one hydroxy group from the *para*- (compound **IV.12**) into the *meta*-position (compound **IV.15**). The gain in activity observed for compound **IV.15** (IC_{50}= 119 nM vs. **IV.12**, IC_{50}> 1000 nM) indicates that only in case of **IV.15** the geometry of the OH moiety allows additional hydrogen bond interactions.

Table 10: influence of an additional substituent on 17β-HSD1 and 17β-HSD2 inhibitory activity



IV.1
IV.12



IV.7
IV.13-15

cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]
IV.1	H	69	1950	28
IV.12		>1000	nt	

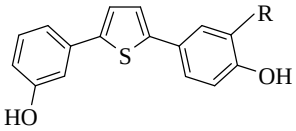
cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]
IV.7	H	173	745	4
IV.13	CH_3	567	856	1
IV.14		493[e]	nt	
IV.15		119	188	2

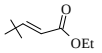
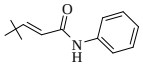
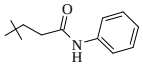
[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 μM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); [e]caculated value, obtained with the LOGIT transformation; ni: no inhibition, nt: not tested.

The trisubstituted compound **IV.14** bearing a phenyl substituent at the thiophene differs from the triazole **II.7** only in the nature of the heterocycle. The inactivity observed for compound **II.7** supports the important role exerted by the central aromatic ring on inhibitory activity, the thiophene being always the best core. Taking the hypothesis that all the compounds bind according to the same binding mode, different reasons could explain these results: 1. the presence of one or several nitrogens or the absence of the sulfur in the heterocycle leads to an inadequate repartition of the electron density in the molecule, 2. a reduced flexibility of the enzyme which cannot adjust its geometry to the different hydroxyphenyl moieties (the position of each hydroxyphenyl moiety being different in the active site as the heterocycles and especially the location of the heteroatoms in this ring induces different angles between the phenyl-OH).

An abrupt rise in activity and selectivity could be reached by insertion of substituents on the hydroxyphenyl moieties, especially when the substituent is located in *ortho*- of the *para*-hydroxyphenyl (compounds **IV.21-24**). The inhibitory data of some representative compounds out of this series are shown in Table 11. All substituents are not equally well tolerated: no space is available for introduction of a bulky phenyl group (compound **IV.25**), the polar additional OH (compound **IV.22**) is not able to establish specific interactions while small lipophilic substituents (methyl, compounds **IV.21**; fluorine, compound **IV.23** and trifluoromethyl, compound **IV.24**) are enhancing the activity compared to the core structure **1** (IC_{50}= 69 nM vs. IC_{50}= 46 nM, 8 nM, 38 nM for **IV.21**, **IV.23** and **IV.24**, respectively). Furthermore, space to introduce a flexible chain (compounds **IV.26-28**) is available in this region of the enzyme but conjugation (compound **26**) seems to be necessary to achieve a higher activity.

Table 11: effect of an additional substituent on the hydroxyphenyl moieties on the inhibition of human 17β-HSD1 and 17β-HSD2

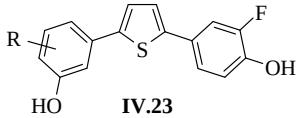


cmpd	R	IC$_{50}$ (nM)[a]		selectivity factor[d]
		17β-HSD1[b]	17β-HSD2[c]	
IV.1	H	69	1950	28
IV.21	CH_3	46	1971	49
IV.22	OH	402	1636	4
IV.23	F	8	940	118
IV.24	CF_3	38	97	3
IV.25	Ph	>5000	nt	
IV.26		130	502	4
IV.27		427	468	1
IV.28		620	982	2

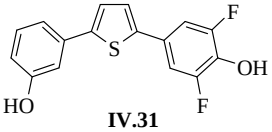
[a]Mean values of three determinations, standard deviation less than 15 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 μM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM], [d]IC$_{50}$ (17β-HSD2)/ IC$_{50}$ (17β-HSD1); ni: no inhibition, nt: not tested

The insertion of a fluorine atom induces a significant increase of the activity and selectivity. Introduction of a second fluorine atom on the fluorohydroxyphenyl ring (compounds **IV.31**) does not enhance the activity anymore suggesting that the effects of these atoms are not additional. The presence of two fluorohydroxyphenyl moieties on the thiophene (compounds **IV.29** and **IV.30**) is slightly better tolerated when the fluorine is located in *ortho*- to the hydroxy moiety (compound **IV.30**) leading to a derivative equally active as the monofluorinated **IV.23**. A plausible explanation could be that in case of compound **IV.29**, the fluorine is located to far away from the hydroxy group to have any influence on its pKa-value leading therefore to a slightly decrease in activity compared to compound **IV.30** (Table 12).

Table 12: effect of two additional fluorine atoms on the 17β-HSD1 and 17β-HSD2 inhibitory activity



IV.23
IV.29-30



IV.31

cmpd	R	IC_{50} (nM)[a]		selectivity factor[d]
		17β-HSD1[b]	17β-HSD2[c]	
IV.23	H	8	940	118
IV.29	5-F	29	227	8
IV.30	4-F	17	218	13
IV.31		56	312	6

[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 μM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM], [d]IC_{50} (17β-HSD2)/ IC_{50} (17β-HSD1); nt: not tested.

The close look at the X-ray structures of 17β-HSD1 crystallized in presence of different ligands showed that the flexible loop (amino acids 187-200) can adopt different geometry depending on the nature of the ligand and on the absence or presence of the cofactor in the catalytic region. It indicates that some parts of the enzyme are able to adapt their geometries to the molecule present in the active site in order to stabilize it.

Two plausible conformations of the loop in the ternary complex enzyme, E2 and cofactor (PDB-ID: 1FDT) have been described (1FDT-A, 1FDT-B). In case of 1FDT-A, the loop is in the open conformation extending the substrate binding site. It is therefore expected that it is a good model to evaluate the binding mode of non-steroidal inhibitors which might have a geometry different to the one of the steroid. Two different binding modes have been identified for this class of inhibitors depending on the position of the flexible loop. A steroidal binding mode was found when the loop closes the SUB (1FDT-B). Surprisingly, an alternative binding mode was also identified when the loop is in the open conformation: the inhibitor interacts with the nicotinamide part of the cofactor (Figure 20).

MEPs calculations can show a certain complementarity between the electronic density of the inhibitor **IV.23** and the nicotinamide moiety of the cofactor indicating that this alternative binding mode is plausible and might be the favourite one.

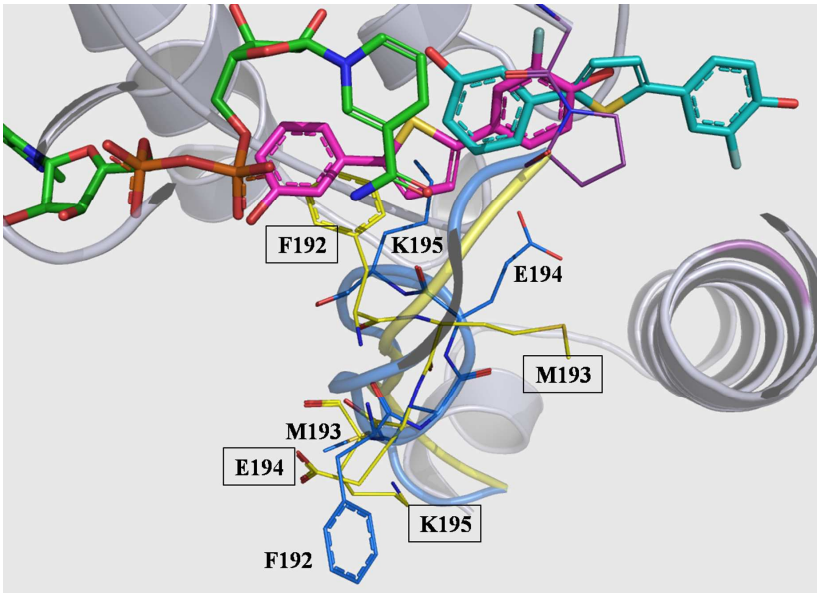


Figure 20: overlay of the binding poses of compound **IV.23** obtained with 1FDT-A (presented in lilac) and with 1FDT-B (marked in light blue). For the sake of clarity, the amino acids of the flexible loop are drawn in blue and yellow for 1FDT-A and 1FDT-B, respectively.

The most potent 17β-HSD1 inhibitors **IV.21** and **IV.23** exhibit a high selectivity toward 17β-HSD2, the fluorinated derivative **IV.23** being the best one (selectivity factors of 49 and 118, respectively). This result indicates that inspite of a high active site similarity between the type 1 and type 2 enzymes, the amino acids close to the CH_3 (compound **IV.21**) and the F substituents (compound **IV.23**) must exhibit different properties, which could be further exploited to increase selectivity. Moreover, the molecules show marginal to very little affinity to ERα (RBA< 1 %) and no stimulation of cell proliferation (agonistic effect) in the ER-positive T-47D cell line could be observed. The weak affinity measured, in case of compound **IV.23**, for ERβ (RBA = 1 %) may not be critical as it is reported that ERβ exhibits anti-proliferative effects in breast cancer cells.[113]

The selectivity over CYP3A4 and CYP2D6, the two most hepatic CYP enzymes which are responsible for 75 % of drug metabolism was investigated. At a concentration of 2 µM, compounds **IV.21** and **IV.23** turned out to be equally active on both enzymes inhibiting the CYP3A4 activity by 80 and 71 % and CYP2D6 by 55 % and 56 %, respectively. The high affinity observed for both isoenzymes has to be taken into consideration in the process of drug development but might not be relevant for the proof of concept. In addition, the PK-profile of these two molecules was determined in a cassette dosing experiment and compared to the results obtained for the most potent compounds of chapter **III** (Figure 21). Compounds **IV.21** and **IV.23** reach higher plasma concentrations after peroral application in rats (following a 10 mg/kg dose, $AUC_{0-\infty}$= 19407 and 12275 ng×h/mL for **IV.21** and **IV.23**, respectively) than the parent compounds **III.3** and **III.39** (Figure 21) which is indicative of an excellent bioavailability.

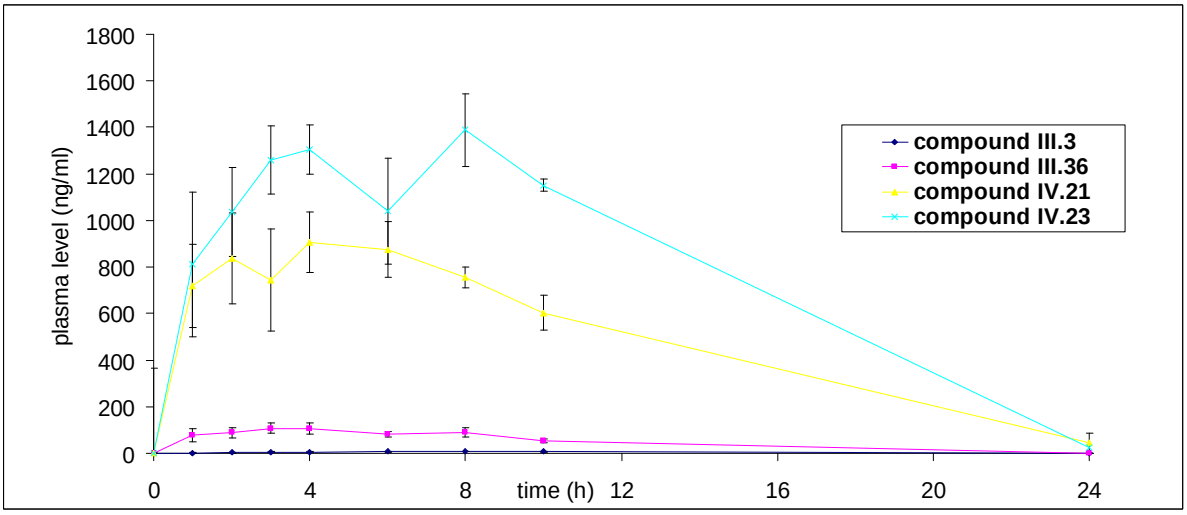


Figure 21: mean profile (±SEM of plasma levels (ng/mL) in rat versus time after oral application (10 mg/kg) of compounds **III.3**, **III.36**, **IV.21** and **IV.23** determined in a cassette dosing experiment.

In summary, the present work describes the design, synthesis and biological evaluation which led to the identification of a new class of non-steroidal 17β-HSD1 inhibitors: bis(hydroxyphenyl) azoles and arenes. Beside a strong 17β-HSD1 inhibition with IC_{50} values in the low nanomolar range and an excellent 17β-HSD2 selectivity (up to 100-fold), the compounds show also low to marginal binding affinity to the estrogen receptors and good cell permeability. In view of the pharmacokinetic properties, two of the investigated compounds reach excellent plasma-levels indicative of a high bioavailability and might be therefore excellent candidates to validate the concept of selective 17β-HSD1 inhibition as treatment for estrogen dependent diseases. In addition, the investigations of the present work provide a concise structure-activity study for 17β-HSD1 inhibition which can be used in the future development of new inhibitors interacting with 17β-HSD1. Moreover, this work delivers also information about SARs which are important for selective 17β-HSD2 inhibition. These findings might be of particular interest for the design of compounds for the treatment of estrogen deprivation disorders such as osteoporosis.

5. References

1. Bulun, S. E.; Yang, S.; Fang, Z.; Gurates, B.; Tamura, M.; Zhou, J.; Sebastian, S. Role of aromatase in endometrial disease. *J. Steroid Biochem. Mol. Biol.* **2001**, *79*, 19-25.
2. Gangloff, A.; Garneau, A.; Huang, Y. W.; Yang, F.; Lin, S.-X. Human oestrogenic 17beta-hydroxysteroid dehydrogenase specificity: enzyme regulation through an NADPH-dependent substrate inhibition towards the highly specific oestrone reduction. *Biochem. J.* **2001**, *356*, 269-276.
3. Labrie, F. Intracrinology. *Mol. Cell. Endocrinol.* **1991**, *78*, C113-C118.
4. Labrie, F.; Luu-The, V.; Lin, S. X.; Simard, J.; Labrie, C. Role of 17 beta-hydroxysteroid dehydrogenases in sex steroid formation in peripheral intracrine tissues. *Trends Endocrinol. Metab.* **2000**, *11*, 421-427.
5. Suzuki, T.; Miki, Y.; Nakamura, Y.; Moriya, T.; Ito, K.; Ohuchi, N.; Sasano, H. Sex steroid-producing enzymes in human breast cancer. *Endocr. Relat. Cancer* **2005**, *12*, 701-720.
6. Robker, R. L.; Richards, J. S. Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Mol. Endocrinol.* **1998**, *12*, 924-940.
7. Adashi, E. Y. Immune modulators in the context of the ovulatory process: a role for interleukin-1. *Am. J. Reprod. Immunol.* **1996**, *35*, 190-194.
8. Mertens, H. J.; Heineman, M. J.; Theunissen, P. H.; de Jong, F. H.; Evers, J. L. Androgen, estrogen and progesterone receptor expression in the human uterus during the menstrual cycle. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2001**, *98*, 58-65.
9. Pepe, G. J.; Albrecht, E. D. Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr. Rev.* **1995**, *16*, 608-648.
10. Albrecht, E. D.; Aberdeen, G. W.; Pepe, G. J. The role of estrogen in the maintenance of primate pregnancy. *Am. J. Obstet. Gynecol.* **2000**, *182*, 432-438.
11. Turner, R. T.; Riggs, B. L.; Spelsberg, T. C. Skeletal effects of estrogen. *Endocr. Rev.* **1994**, *15*, 275-300.
12. Mendelsohn, M. E.; Karas, R. H. The protective effects of estrogen on the cardiovascular system. *N. Engl. J. Med.* **1999**, *340*, 1801-1811.
13. Fink, G.; Sumner, B. E.; Rosie, R.; Grace, O.; Quinn, J. P. Estrogen control of central neurotransmission: effect on mood, mental state, and memory. *Cell. Mol. Neurobiol.* **1996**, *16*, 325-344.
14. Stahl, S. M. Effects of estrogen on the central nervous system. *J. Clin. Psychiatry* **2001**, *62*, 317-318.
15. Olsen, N. J.; Kovacs, W. J. Gonadal steroids and immunity. *Endocr. Rev.* **1996**, *17*, 369-384.
16. Simpson, E. R.; Mahendroo, M. S.; Means, G. D.; Kilgore, M. W.; Hinshelwood, M. M.; Graham-Lorence, S.; Amarneh, B.; Ito, Y.; Fisher, C. R.; Michael, M. D.; et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr Rev* **1994**, *15*, 342-55.
17. Labrie, F.; Luu-The, V.; Lin, S. X.; Simard, J.; Labrie, C.; El-Alfy, M.; Pelletier, G.; Belanger, A. Intracrinology: role of the family of 17 beta-hydroxysteroid dehydrogenases in human physiology and disease. *J. Mol. Endocrinol.* **2000**, *25*, 1-16.
18. Sasano, H.; Suzuki, T.; Takeyama, J.; Utsunomiya, H.; Ito, K.; Ariga, N.; Moriya, T. 17-beta-hydroxysteroid dehydrogenase in human breast and endometrial carcinoma. A new development in intracrinology. *Oncology* **2000**, *59 Suppl 1*, 5-12.
19. Lukacik, P.; Kavanagh, K. L.; Oppermann, U. Structure and function of human 17beta-hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* **2006**, *248*, 61-71.

20. Penning, T. M. 17beta-hydroxysteroid dehydrogenase: inhibitors and inhibitor design. *Endocr. Relat. Cancer* **1996**, *3*, 41-56.
21. Jornvall, H.; Persson, B.; Krook, M.; Atrian, S.; Gonzalez-Duarte, R.; Jeffery, J.; Ghosh, D. Short-chain dehydrogenases/reductases (SDR). *Biochemistry* **1995**, *34*, 6003-6013.
22. Puranen, T. J.; Poutanen, M. H.; Peltoketo, H. E.; Vihko, P. T.; Vihko, R. K. Site-directed mutagenesis of the putative active site of human 17 beta-hydroxysteroid dehydrogenase type 1. *Biochem. J.* **1994**, *304*, 289-293.
23. Penning, T. M. Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocr. Rev.* **1997**, *18*, 281-305.
24. Penning, T. M. Hydroxysteroid dehydrogenases and pre-receptor regulation of steroid hormone action. *Hum Reprod Update* **2003**, *9*, 193-205.
25. Moeller, G.; Adamski, J. Multifunctionality of human 17beta-hydroxysteroid dehydrogenases. *Mol Cell Endocrinol* **2006**, *248*, 47-55.
26. Peltoketo, H.; Luu-The, V.; Simard, J.; Adamski, J. 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. *J Mol Endocrinol* **1999**, *23*, 1-11.
27. Adamski, J.; Jakob, F. J. A guide to 17beta-hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* **2001**, *171*, 1-4.
28. Sherbet, D. P.; Papari-Zareei, M.; Khan, N.; Sharma, K. K.; Brandmaier, A.; Rambally, S.; Chattopadhyay, A.; Andersson, S.; Agarwal, A. K.; Auchus, R. J. Cofactors, redox state, and directional preferences of hydroxysteroid dehydrogenases. *Mol. Cell. Endocrinol.* **2007**, *265-266*, 83-88.
29. Williamson, D. H.; Lund, P.; Krebs, H. A. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.* **1967**, *103*, 514-527.
30. Voet, D.; Voet, J. G. *Biochemistry*. New York, 1990.
31. Jin, J. Z.; Lin, S. X. Human estrogenic 17beta-hydroxysteroid dehydrogenase: predominance of estrone reduction and its induction by NADPH. *Biochem. Biophys. Res. Commun.* **1999**, *259*, 489-493.
32. Duax, W. L.; Pletnev, V.; Addlagatta, A.; Bruenn, J.; Weeks, C. M. Rational proteomics I. Fingerprint identification and cofactor specificity in the short-chain oxidoreductase (SCOR) enzyme family. *Proteins* **2003**, *53*, 931-943.
33. Pletnev, V. Z.; Weeks, C. M.; Duax, W. L. Rational proteomics II: electrostatic nature of cofactor preference in the short-chain oxidoreductase (SCOR) enzyme family. *Proteins* **2004**, *57*, 294-301.
34. Mazza, C.; Breton, R.; Housset, D.; Fontecilla-Camps, J. C. Unusual charge stabilization of $NADP^+$ in 17beta-hydroxysteroid dehydrogenase. *J. Biol. Chem.* **1998**, *273*, 8145-8152.
35. Sawicki, M. W.; Erman, M.; Puranen, T.; Vihko, P.; Ghosh, D. Structure of the ternary complex of human 17beta-hydroxysteroid dehydrogenase type 1 with 3-hydroxyestra-1,3,5,7-tetraen-17-one (equilin) and $NADP^+$. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 840-845.
36. Chen, Z.; Lee, W. R.; Chang, S. H. Role of aspartic acid 38 in the cofactor specificity of Drosophila alcohol dehydrogenase. *Eur. J. Biochem.* **1991**, *202*, 263-267.
37. Scrutton, N. S.; Berry, A.; Perham, R. N. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. *Nature* **1990**, *343*, 38-43.
38. Huang, Y. W.; Pineau, I.; Chang, H. J.; Azzi, A.; Bellemare, V.; Laberge, S.; Lin, S. X. Critical residues for the specificity of cofactors and substrates in human estrogenic 17beta-hydroxysteroid dehydrogenase 1: variants designed from the three-dimensional structure of the enzyme. *Mol. Endocrinol.* **2001**, *15*, 2010-2020.
39. Ohnesorg, T.; Keller, B.; Hrabe de Angelis, M.; Adamski, J. Transcriptional regulation of human and murine 17beta-hydroxysteroid dehydrogenase type-7 confers its participation in cholesterol biosynthesis. *J. Mol. Endocrinol.* **2006**, *37*, 185-197.

40. Sakurai, N.; Miki, Y.; Suzuki, T.; Watanabe, K.; Narita, T.; Ando, K.; Yung, T. M.; Aoki, D.; Sasano, H.; Handa, H. Systemic distribution and tissue localizations of human 17beta-hydroxysteroid dehydrogenase type 12. *J. Steroid Biochem. Mol. Biol.* **2006**, *99*, 174-181.
41. Day, J. M.; Foster, P. A.; Tutill, H. J.; Parsons, M. F.; Newman, S. P.; Chander, S. K.; Allan, G. M.; Lawrence, H. R.; Vicker, N.; Potter, B. V.; Reed, M. J.; Purohit, A. 17beta-hydroxysteroid dehydrogenase Type 1, and not Type 12, is a target for endocrine therapy of hormone-dependent breast cancer. *Int. J. Cancer* **2008**, *122*, 1931-1940.
42. Langer, L. J.; Engel, L. L. Human placental estradiol-17 beta dehydrogenase. I. Concentration, characterization and assay. *J. Biol. Chem.* **1958**, *233*, 583-588.
43. Lin, S.-X.; Yang, F.; Jin, J. Z.; Breton, R.; Zhu, D. W.; Luu-The, V.; Labrie, F. Subunit identity of the dimeric 17beta-hydroxysteroid dehydrogenase from human placenta. *J. Biol. Chem.* **1992**, *267*, 16182-16187.
44. Peltoketo, H.; Isomaa, V.; Maentausta, O.; Vihko, R. Complete amino acid sequence of human placental 17 beta-hydroxysteroid dehydrogenase deduced from cDNA. *FEBS Lett.* **1988**, *239*, 73-77.
45. Luu-The, V.; Zhang, Y.; Poirier, D.; Labrie, F. Characteristics of human types 1, 2 and 3 17 beta-hydroxysteroid dehydrogenase activities: oxidation/reduction and inhibition. *J. Steroid Biochem. Mol. Biol.* **1995**, *55*, 581-587.
46. Poutanen, M.; Miettinen, M.; Vihko, R. Differential estrogen substrate specificities for transiently expressed human placental 17 beta-hydroxysteroid dehydrogenase and an endogenous enzyme expressed in cultured COS-m6 cells. *Endocrinology* **1993**, *133*, 2639-2644.
47. Han, Q.; Campbell, R. L.; Gangloff, A.; Huang, Y. W.; Lin, S.-X. Dehydroepiandrosterone and dihydrotestosterone recognition by human estrogenic 17beta-hydroxysteroid dehydrogenase. C-18/C-19 steroid discrimination and enzyme-induced strain. *J. Biol. Chem.* **2000**, *275*, 1105-1111.
48. Puranen, T. J.; Poutanen, M. H.; Peltoketo, H. E.; Vihko, P. T.; Vihko, R. K. Site-directed mutagenesis of the putative active site of human 17 beta-hydroxysteroid dehydrogenase type 1. *Biochem J* **1994**, *304*, 289-293.
49. Puranen, T.; Poutanen, M.; Ghosh, D.; Vihko, P.; Vihko, R. Characterization of structural and functional properties of human 17 beta-hydroxysteroid dehydrogenase type 1 using recombinant enzymes and site-directed mutagenesis. *Mol Endocrinol* **1997**, *11*, 77-86.
50. Ghosh, D.; Pletnev, V. Z.; Zhu, D. W.; Wawrzak, Z.; Duax, W. L.; Pangborn, W.; Labrie, F.; Lin, S.-X. Structure of human estrogenic 17beta- hydroxysteroid dehydrogenase at 2.20 Å resolution. *Structure* **1995**, *3*, 503-513.
51. Breton, R.; Housset, D.; Mazza, C.; Fontecilla-Camps, J. C. The structure of a complex of human 17beta-hydroxysteroid dehydrogenase with estradiol and $NADP^+$ identifies two principal targets for the design of inhibitors. *Structure* **1996**, *4*, 905-915.
52. Azzi, A.; Rehse, P. H.; Zhu, D. W.; Campbell, R. L.; Labrie, F.; Lin, S.-X. Crystal structure of human estrogenic 17beta-hydroxysteroid dehydrogenase complexed with 17 beta-estradiol. *Nat. Struct. Biol.* **1996**, *3*, 665-668.
53. Gangloff, A.; Shi, R.; Nahoum, V.; Lin, S.-X. Pseudo-symmetry of C19 steroids, alternative binding orientations, and multispecificity in human estrogenic 17beta-hydroxysteroid dehydrogenase. *FASEB J.* **2003**, *17*, 274-276.
54. Shi, R.; Lin, S.-X. Cofactor hydrogen bonding onto the protein main chain is conserved in the short chain dehydrogenase/reductase family and contributes to nicotinamide orientation. *J. Biol. Chem.* **2004**, *279*, 16778-16785.
55. Qiu, W.; Campbell, R. L.; Gangloff, A.; Dupuis, P.; Boivin, R. P.; Tremblay, M. R.; Poirier, D.; Lin, S.-X. A concerted, rational design of type 1 17beta-hydroxysteroid dehydrogenase inhibitors: estradiol-adenosine hybrids with high affinity. *FASEB J.* **2002**, *16*, 1829-1831.

56. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28*, 235-242.
57. Filling, C.; Berndt, K. D.; Benach, J.; Knapp, S.; Prozorovski, T.; Nordling, E.; Ladenstein, R.; Jornvall, H.; Oppermann, U. Critical residues for structure and catalysis in short-chain dehydrogenases/reductases. *J. Biol. Chem.* **2002**, *277*, 25677-25684.
58. Travis, R. C.; Key, T. J. Oestrogen exposure and breast cancer risk. *Breast Cancer Res.* **2003**, *5*, 239-247.
59. Lower, E. E.; Blau, R.; Gazder, P.; Stahl, D. L. The effect of estrogen usage on the subsequent hormone receptor status of primary breast cancer. *Breast Cancer Res. Treat.* **1999**, *58*, 205-211.
60. Miller, W. R.; Bartlett, J. M.; Canney, P.; Verrill, M. Hormonal therapy for postmenopausal breast cancer: the science of sequencing. *Breast Cancer Res. Treat.* **2007**, *103*, 149-160.
61. Bush, N. J. Advances in hormonal therapy for breast cancer. *Semin. Oncol. Nurs.* **2007**, *23*, 46-54.
62. Adamo, V.; Iorfida, M.; Montalto, E.; Festa, V.; Garipoli, C.; Scimone, A.; Zanghi, M.; Caristi, N. Overview and new strategies in metastatic breast cancer (MBC) for treatment of tamoxifen-resistant patients. *Ann. Oncol.* **2007**, *18 Suppl 6*, vi53-57.
63. Giudice, L. C.; Kao, L. C. Endometriosis. *Lancet* **2004**, *364*, 1789-1799.
64. Ferrero, S.; Ragni, N.; Remorgida, V. Antiangiogenic therapies in endometriosis. *Br. J. Pharmacol.* **2006**, *149*, 133-135.
65. Berkley, K. J.; Rapkin, A. J.; Papka, R. E. The pains of endometriosis. *Science* **2005**, *308*, 1587-1589.
66. Miyoshi, Y.; Ando, A.; Shiba, E.; Taguchi, T.; Tamaki, Y.; Noguchi, S. Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int. J. Cancer* **2001**, *94*, 685-689.
67. Gunnarsson, C.; Hellqvist, E.; Stål, O. 17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *Br. J. Cancer* **2005**, *92*, 547-552.
68. Vihko, P.; Herrala, A.; Harkonen, P.; Isomaa, V.; Kaija, H.; Kurkela, R.; Li, Y.; Patrikainen, L.; Pulkka, A.; Soronen, P.; Torn, S. Enzymes as modulators in malignant transformation. *J. Steroid Biochem. Mol. Biol.* **2005**, *93*, 277-283.
69. Husen, B.; Huhtinen, K.; Poutanen, M.; Kangas, L.; Messinger, J.; Thole, H. Evaluation of inhibitors for 17beta-hydroxysteroid dehydrogenase type 1 in vivo in immunodeficient mice inoculated with MCF-7 cells stably expressing the recombinant human enzyme. *Mol. Cell. Endocrinol.* **2006**, *248*, 109-113.
70. Husen, B.; Huhtinen, K.; Saloniemi, T.; Messinger, J.; Thole, H. H.; Poutanen, M. Human hydroxysteroid (17-beta) dehydrogenase 1 expression enhances estrogen sensitivity of MCF-7 breast cancer cell xenografts. *Endocrinology* **2006**, *147*, 5333-5339.
71. Bulun, S. E.; Zeitoun, K. M.; Takayama, K.; Sasano, H. Estrogen biosynthesis in endometriosis: molecular basis and clinical relevance. *J. Mol. Endocrinol.* **2000**, *25*, 35-42.
72. Bulun, S. E.; Zeitoun, K.; Takayama, K.; Noble, L.; Michael, D.; Simpson, E.; Johns, A.; Putman, M.; Sasano, H. Estrogen production in endometriosis and use of aromatase inhibitors to treat endometriosis. *Endocr. Relat. Cancer* **1999**, *6*, 293-301.
73. Dassen, H.; Punyadeera, C.; Kamps, R.; Delvoux, B.; Van Langendonckt, A.; Donnez, J.; Husen, B.; Thole, H.; Dunselman, G.; Groothuis, P. Estrogen metabolizing enzymes in endometrium and endometriosis. *Hum. Reprod.* **2007**, *22*, 3148-3158.
74. Solvay pharmaceuticals. WO2004110459, **2004**.
75. Solvay pharmaceuticals. US2005038053, **2005**.
76. Solvay pharmaceuticals. WO2006125800, **2006**.
77. Solvay pharmaceuticals. WO2008034796, **2008**.
78. Schering AG. WO2006003012, **2006**.

79. Schering AG. WO2006003013, **2006**.
80. Brožic, P.; Lanišnik Rižner, T.; Gobec, S. Inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Curr. Med. Chem.* **2008**, *15*, 137-150 and references therein cited.
81. Poirier, D. Inhibitors of 17 beta-hydroxysteroid dehydrogenases. *Curr. Med. Chem.* **2003**, *10*, 453-477.
82. Lipinski, C. A. Drug-like properties and the causes of poor solubility and poor permeability. *J. Pharmacol. Toxicol. Methods* **2000**, *44*, 235-249.
83. Allan, G. M.; Lawrence, H. R.; Cornet, J.; Bubert, C.; Fischer, D. S.; Vicker, N.; Smith, A.; Tutill, H. J.; Purohit, A.; Day, J. M.; Mahon, M. F.; Reed, M. J.; Potter, B. V. Modification of estrone at the 6, 16, and 17 positions: novel potent inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *J. Med. Chem.* **2006**, *49*, 1325-1345.
84. Makela, S.; Poutanen, M.; Lehtimaki, J.; Kostian, M. L.; Santti, R.; Vihko, R. Estrogen-specific 17 beta-hydroxysteroid oxidoreductase type 1 (E.C. 1.1.1.62) as a possible target for the action of phytoestrogens. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 51-59.
85. Brown, W. M.; Metzger, L. E.; Barlow, J. P.; Hunsaker, L. A.; Deck, L. M.; Royer, R. E.; Vander Jagt, D. L. 17-beta-Hydroxysteroid dehydrogenase type 1: computational design of active site inhibitors targeted to the Rossmann fold. *Chem. Biol. Interact.* **2003**, *143-144*, 481-491.
86. Deluca, D.; Krazeisen, A.; Breitling, R.; Prehn, C.; Moller, G.; Adamski, J. Inhibition of 17beta-hydroxysteroid dehydrogenases by phytoestrogens: comparison with other steroid metabolizing enzymes. *J. Steroid Biochem. Mol. Biol.* **2005**, *93*, 285-292.
87. Allan, G. M.; Bubert, C.; Vicker, N.; Smith, A.; Tutill, H. J.; Purohit, A.; Reed, M. J.; Potter, B. V. Novel, potent inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* **2006**, *248*, 204-207.
88. Lawrence, H. R.; Vicker, N.; Allan, G. M.; Smith, A.; Mahon, M. F.; Tutill, H. J.; Purohit, A.; Reed, M. J.; Potter, B. V. Novel and potent 17beta-hydroxysteroid dehydrogenase type 1 inhibitors. *J. Med. Chem.* **2005**, *48*, 2759-2762.
89. Vicker, N.; Lawrence, H. R.; Allan, G. M.; Bubert, C.; Smith, A.; Tutill, H. J.; Purohit, A.; Day, J. M.; Mahon, M. F.; Reed, M. J.; Potter, B. V. Focused libraries of 16-substituted estrone derivatives and modified E-ring steroids: inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *ChemMedChem* **2006**, *1*, 464-481.
90. Allan, G. M.; Vicker, N.; Lawrence, H. R.; Tutill, H. J.; Day, J. M.; Huchet, M.; Ferrandis, E.; Reed, M. J.; Purohit, A.; Potter, B. V. Novel inhibitors of 17beta-hydroxysteroid dehydrogenase type 1: templates for design. *Bioorg. Med. Chem.* **2008**, *16*, 4438-4456.
91. Messinger, J.; Hirvelä, L.; Husen, B.; Kangas, L.; Koskimies, P.; Pentikäinen, O.; Saarenketo, P.; Thole, H. New inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. *Mol. Cell. Endocrinol.* **2006**, *248*, 192-198.
92. Karkola, S.; Lilienkampf, A.; Wähälä, K. A 3D QSAR model of 17beta-HSD1 inhibitors based on a thieno[2,3-d]pyrimidin-4(3H)-one core applying molecular dynamics simulations and ligand-protein docking. *ChemMedChem* **2008**, *3*, 461-472.
93. Frotscher, M.; Ziegler, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Neugebauer, A.; Fetzer, L.; Scherer, C.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W. Design, synthesis and biological evaluation of (hydroxyphenyl)-naphthalene and quinoline derivatives: potent and selective non steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *J. Med. Chem.* **2008**, *51*, 2158-2169.
94. Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Ziegler, E.; Neugebauer, A.; Bhoga, U. D.; Bey, E.; Müller-Vieira, U.; Messinger, J.; Thole, H.; Hartmann, R. W. Substituted 6-phenyl-2-naphthols. Potent and selective non-steroidal inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17beta-HSD1): design, synthesis, biological evaluation and pharmacokinetics. *J. Med. Chem.* **2008**, *51*, 4685-4698.

95. Marchais-Oberwinkler, S.; Frotscher, M.; Ziegler, E.; Werth, R.; Kruchten, P.; Messinger, J.; Thole, H.; Hartmann, R. W. Investigation of a new class of potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases: 6-(3'-hydroxyphenyl) naphthalenes. *Mol. Cell. Endocrinol.* **2008**, doi:10.1016/j.mce.2008.09.024.
96. Suzuki, T.; Moriya, T.; Ariga, N.; Kaneko, C.; Kanazawa, M.; Sasano, H. 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br. J. Cancer* **2000**, *82*, 518-523.
97. Gunnarsson, C.; Olsson, B. M.; Stål, O. Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.* **2001**, *61*, 8448-8451.
98. Bey, E.; Marchais-Oberwinkler, S.; Kruchten, P.; Frotscher, M.; Werth, R.; Oster, A.; Algül, O.; Neugebauer, A.; Hartmann, R. W. Design, synthesis and biological evaluation of bis(hydroxyphenyl) azoles as potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. *Bioorg. Med. Chem.* **2008**, *16*, 6423-6435.
99. Yearley, E. J.; Zhurova, E. A.; Zhurov, V. V.; Pinkerton, A. A. Binding of genistein to the estrogen receptor based on an experimental electron density study. *J. Am. Chem. Soc.* **2007**, *129*, 15013-15021.
100. White, E. H.; Bursey, M. M.; Roswel, D. F.; Hill, J. H. M. Chemiluminescence of some monoacylhydrazides. *J. Org. Chem.* **1967**, *32*, 1198-1202.
101. Pirali, T.; Gatti, S.; Di Brisco, R.; Tacchi, S.; Zaninetti, R.; Brunelli, E.; Massarotti, A.; Sorba, G.; Canonico, P. L.; Moro, L.; Genazzani, A. A.; Tron, G. C.; Billington, R. A. Estrogenic analogues synthesized by click chemistry. *ChemMedChem* **2007**, *2*, 437-440.
102. Lahti, P. M.; Ichimura, A. S. Semiempirical study of electron exchange interaction in organic high-spin pi-systems. Classifying structural effects in organic magnetic molecules. *J. Org. Chem.* **1991**, *56*, 3030-3042.
103. Petti, M. A.; Shepodd, T. J.; Barrans, R. E.; Dougherty, D. A. "Hydrophobic" binding of water-soluble guests by high-symmetry, chiral hosts. An electron-rich receptor site with a general affinity for quaternary ammonium compounds and electron-deficient pi-systems. *J. Am. Chem. Soc.* **1988**, *110*, 6825-6840.
104. Fink, B. E.; Mortensen, D. S.; Stauffer, S. R.; Aron, Z. D.; Katzenellenbogen, J. A. Novel structural templates for estrogen-receptor ligands and prospects for combinatorial synthesis of estrogens. *Chem. Biol.* **1999**, *6*, 205-219.
105. Stauffer, S. R.; Coletta, C. J.; Tedesco, R.; Nishiguchi, G.; Carlson, K.; Sun, J.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A. Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *J. Med. Chem.* **2000**, *43*, 4934-4947.
106. Stauffer, S. R.; Huang, Y.; Coletta, C. J.; Tedesco, R.; Katzenellenbogen, J. A. Estrogen pyrazoles: defining the pyrazole core structure and the orientation of substituents in the ligand binding pocket of the estrogen receptor. *Bioorg. Med. Chem.* **2001**, *9*, 141-150.
107. Stauffer, S. R.; Huang, Y. R.; Aron, Z. D.; Coletta, C. J.; Sun, J.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A. Triarylpyrazoles with basic side chains: development of pyrazole-based estrogen receptor antagonists. *Bioorg. Med. Chem.* **2001**, *9*, 151-161.
108. Stauffer, S. R.; Katzenellenbogen, J. A. Solid-phase synthesis of tetrasubstituted pyrazoles, novel ligands for the estrogen receptor. *J. Comb. Chem.* **2000**, *2*, 318-329.
109. Stauffer, S. R.; Sun, J.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A. Acyclic amides as estrogen receptor ligands: synthesis, binding, activity and receptor interaction. *Bioorg. Med. Chem.* **2000**, *8*, 1293-1316.
110. Bydal, P.; Auger, S.; Poirier, D. Inhibition of type 2 17beta-hydroxysteroid dehydrogenase by estradiol derivatives bearing a lactone on the D-ring: structure-activity relationships. *Steroids* **2004**, *69*, 325-42.

111. Gunn, D.; Akuche, C.; Baryza, J.; Blue, M. L.; Brennan, C.; Campbell, A. M.; Choi, S.; Cook, J.; Conrad, P.; Dixon, B.; Dumas, J.; Ehrlich, P.; Gane, T.; Joe, T.; Johnson, J.; Jordan, J.; Kramss, R.; Liu, P.; Levy, J.; Lowe, D.; McAlexander, I.; Natero, R.; Redman, A. M.; Scott, W.; Seng, T.; Sibley, R.; Wang, M.; Wang, Y.; Wood, J.; Zhang, Z. 4,5-Disubstituted cis-pyrrolidinones as inhibitors of type II 17beta-hydroxysteroid dehydrogenase. Part 2. SAR. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3053-3057.

112. Wood, J.; Bagi, C. M.; Akuche, C.; Bacchiocchi, A.; Baryza, J.; Blue, M. L.; Brennan, C.; Campbell, A. M.; Choi, S.; Cook, J. H.; Conrad, P.; Dixon, B. R.; Ehrlich, P. P.; Gane, T.; Gunn, D.; Joe, T.; Johnson, J. S.; Jordan, J.; Kramss, R.; Liu, P.; Levy, J.; Lowe, D. B.; McAlexander, I.; Natero, R.; Redman, A. M.; Scott, W. J.; Town, C.; Wang, M.; Wang, Y.; Zhang, Z. 4,5-Disubstituted cis-pyrrolidinones as inhibitors of type II 17beta-hydroxysteroid dehydrogenase. Part 3. Identification of lead candidate. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4965-4968.

113. Hartman, J.; Lindberg, K.; Morani, A.; Inzunza, J.; Strom, A.; Gustafsson, J. A. Estrogen receptor beta inhibits angiogenesis and growth of T47D breast cancer xenografts. *Cancer Res.* **2006**, *66*, 11207-11213.