Pharmacophore aided hit identification, structural optimization and biological evaluation of benzothiazole derivatives as new potent and selective non-steroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1

Dissertation

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Abbreviations

17β-HSD1	17β-hydroxysteroid dehydrogenase type 1
17β-HSD2	17β-hydroxysteroid dehydrogenase type 2
A	androstenedione
Å	Ångström
ACTH	adrenocorticotropic hormone
AD	acceptor and/or donor
ADME	absorption, distribution, metabolism, and excretion
AI	aromatase inhibitor
AKR	aldo-keto reductase
AP-1	alternative responsive element
Arg (A)	arginine
Asn (N)	asparagine
Asp (D)	aspartic acid
AUC	area under the curve
CC	column chromatography
$CDCl_3$	deuterated chloroform
CD_3COCD_3	aceton
CD_3OD	deuterated methanol
CD_3SOCD_3	deuterated dimethylsulfoxyde
Clint	intrinsic clearance
CNS	central nervous system
COF	cofactor binding site
COX	cyclooxygenase
CRH	corticotrophin releasing hormone
CT	X-ray computed tomography
CYP	cytochrome P450 superfamily
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone-sulfate
DHT	5-a-dihydrotestosterone
DME	di-methoxyethane
DMEM	Dulbecco's modified eagle medium
DMF	dimethylformamide
E1	estrone
E1-S	estrone sulfate
E2	17β-estradiol
E2B	3-(((8R,9S,13S,14S,16R,17S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthren-16-yl)methyl)benzamide
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDD	Estrogen-dependent disease
EDTA	ethylene diaminetetraacetate
EGF	epidermal growth factor
EQI	equiline
ER	estrogen receptor
ERE	estrogen responsive element
ESI	Electrospray ionization
ESP	electrostatic potential

Eq	equivalent
Et	ethyl
Et_3N	triethylamine
FCS	fetal calf serum
GF	growth factor
Glu (E)	glutamic acid
Gly (G)	glycine
GnRH	gonatropin releasing hormone
GPER1	G protein-coupled estrogen receptor 1
h	human
His (H)	histidine
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HSD	hydroxysteroid dehydrogenase
HY	hydrophobic
HYC	hybrid inhibitor O5'-[9-(3,17β-dihydroxy-1,3,5(10)-estratrien-16β-yl)-nonanoyl]adenosine
Hz	hertz
IC_{50}	half maximal inhibitory concentration
IGF-1	insulin-like growth factor-1
IL-1β	Interleukines
Leu (L)	leucine
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
Lys (K)	lysine
m	marmoset
μM	micromolar
MAPK	mitogen-activated protein kinase
Me	methyl
MEP	molecular electrostatic potential
MES	2-(morpholino)ethanesulfonic acid
Met	methionine
MHz	megahertz
mM	millimolar
MOE	molecular operating environment
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MW	molecular weight
NADP(H)	nicotinamide adenine dinucleotide phosphate
NAD(H)	nicotinamide adenine dinucleotide
nBuLi	*n*-Butyllithium
nM	nanomolar
NMR	nuclear magnetic resonance
P	projection
P_{app}	apparent permeability coefficient
PDB	protein data bank
PGE	prostaglandine
Ph	phenyl
PK	pharmacokinetic
ppm	parts per million
RBA	relative binding affinity

RMSD	root mean square deviation
RPMI	Roswell Park Memorial Institute
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 (S)	serine
SERM	selective estrogen receptor modulator
SF	selectivity factor
SIBX	stabilized 2-iodoxybenzoic acid
SRE	steroid response element
StAR	steroid acute regulatory protein
SUB	substrate binding site
TBAF	tetra-*n*-butylammonium fluoride
TBDMSiCl	*tert*-butyldimethylsilyl chloride
TG	transgenic
THF	tetrahydrofurane
TE	tris-EDTA
Thr (T)	threonine
TMSiCl	trimethylsilyl chloride
TSQ	triple stage quadrupole
Tyr (Y)	tyrosine
VEGF	vascular endothelial growth factor

Abstract

17β-Hydroxysteroid dehydrogenase 1 (17β-HSD1) catalyzes the conversion of the weakly active estrone (E1) to the highly active estradiol (E2). Recently, 17β-HSD1 came into the focus of interest as a novel therapeutic target for the treatment of estrogen dependent diseases like breast cancer (BC) and endometriosis. Two new classes of non-steroidal 17β-HSD1 inhibitors were designed and synthesized as potential therapeutics. The search started from the pharmacophore screening of *in house* library (molecules with MW < 350). The virtual hits were experimentally validated, and, finally, a new core structure ([5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-2-yl](3-hydroxyphenyl)methanone) with a moderate inhibitory activity for 17β-HSD1 was identified. Rigidification of the flexible hydroxy ethyl chain led to a benzothiazole derivative which already showed high inhibitory potency towards the target enzyme. Further structural modifications - OH substitution pattern, addition and variation of small substituents on different position of the phenyl moiety – via the synthesis of 70 new compounds led the discovery of two new classes of potent 17β-HSD1 inhibitors with IC_{50} values in the low nanomolar range. In order not to counteract the therapeutic efficacy of 17β-HSD1 inhibitors it is important that the compounds are selective towards 17β-HSD2 since this enzyme catalyses the reverse reaction (oxidation of E2 to E1) and furthermore to avoid intrinsic estrogenic and systemic effects, the inhibitors should not show affinity to the estrogen receptors α and β. Besides an excellent selectivity over 17β-HSD2 and the estrogen receptors (ERs) α and β, the most promising compounds of this study showed good cell permeability (T47-D), fair metabolic stability in human liver microsomes and moderate hepatic CYP inhibition. High inhibitory potency and selectivity was also found towards marmoset monkey 17β-HSD1 and 17β-HSD2 indicating that these compounds are suitable for in vivo evaluation in this animal endometriosis model. In conclusion, the present thesis provides an extensive structure-activity study regarding 17β-HSD1 inhibition which might be useful for the developement of a clinically applicable therapeutic for the treatment of estrogen-dependent diseases.

Zusammenfassung

17β-Hydroxysteroid Dehydrogenase 1 (17β-HSD1) katalysiert die Biosynthese vom schwach wirksamen Estron (E1) zum hoch potenten Estradiol (E2). In den letzten Jahren ist 17β-HSD1 als neuartiges therapeutisches Target zur Behandlung von Estrogen-abhängigen Krankheiten, wie Burstkrebs und Endometriose in den Fokus gekommen.
In dieser Arbeit wurden zwei neue Klassen nicht-steroidaler 17β-HSD1 Hemmstoffe designet, synthetisiert und biochemisch evaluiert. Zum Einstieg wurde basierend auf ko-krystallisierte steroidale Inhibitoren ein Pharmakophor-Modell erstellt. Dieses wurde dann zum Screening einer in In-house Substanzbibliothek (MW < 350) genutzt. Die besten virtuellen Hits wurden experimentell validiert und ([5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-2-yl](3-hydroxyphenyl)methanone) wurde als neue Core-Struktur mit moderater Hemmwirkung identifiziert.
Rigidisierung der flexiblen Hydroxyethyl-Seitenkette führte zur Synthese eines Benzylimidazol-Derivates, welches eine beachtliche Steigerung der Aktivität zeigte. Weitere strukturelle Variationen, wie z.B. Veränderung der OH-Position, Einfügen bzw. Ersetzen neuer Substituenten an unterschiedlichen Positionen des Phenyl-Ringes, führten zur Synthese von mehr als siebzig Verbindungen, die in zwei Klassen potenter HSD1-Hemmstoffe unterteilt werden können. IC50-Werte im niedrigen nanomolaren Bereich wurden erzielt.
Um der therapeutischen Effizienz dieser 17β-HSD1 Inhibitoren nicht entgegenzuwirken ist es wichtig, dass diese Verbindungen selektiv gegen 17β-HSD2 sind, da dieses Enzym die Rückreaktion (Umwandlung von E2 wird in E1) katalysiert. Des weiteren sollen potentielle 17β-HSD1 Hemmstoffe auch keine Affinität zu den Estrogen-Rezeptoren α und β aufweisen, um systemische Wirkungen zu vermeiden. Abgesehen von einer exzellenten Selektivität gegen 17β-HSD2 sowie beider Estrogenrezeptoren zeigten die vielversprechendsten der synthetisierten Substanzen auch eine gute Permeabilität in T47-D Zellen, eine akzeptable metabolische Stabilität in humanen Lebermikrosomen und nur eine moderate Hemmung einiger hepatischer CYP Enzyme. Beide neuen Substanzklassen wiesen eine hohe Hemmung von 17β-HSD1 sowie Selektivität gegen 17β-HSD2 von Callithrix jacchus auf, was sie für eine in vivo Testung an diesem Endometriose-Model in Frage kommen läßt.
Zusammenfassend ist festzustellen, daß in dieser Doktorarbeit eine ausführliche Struktur-Wirkungs-Studie von 17β-HSD1 Inhibitoren beschrieben ist, die eine wichtige Rolle für die weitere Entwicklung von Therapeutika zur Behandlung von Estrogen-abhängigen Erkrankungen spielen kann.

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1. Introduction

1.1 Estrogenic hormones

Estrogens are important steroidal hormones which bear different physiological functions. The main beneficial effects include their role in programming the breast and uterus for sexual reproduction [1]: in the ovary, E2 stimulates the proliferation of granulosa cells and the growth of follicles [2-3], in the uterus, E2 in combination with progesterone regulates the cyclic growth of the endometrium [4] and is also crucial for the maintenance of pregnancy and fetal development [5-6]. Moreover, estrogens control cholesterol production in ways that limit the build-up of plaque in the coronary arteries [7], and preserve bone strength by helping to maintain the proper balance between bone build-up and breakdown [8-9]. Moreover, a large number of studies have pointed out the influence of estrogens on the cardiovascular system [10], central nervous system [11-12] and immune system [13].
In addition to its important beneficial effects, however, estrogens can also cause serious problems arising from its ability to promote the cell proliferation in breast and uterus.

1.1.1 Estradiol and Estrogen Receptors (ERs) signaling pathways

The most important estrogenic hormones in the human body are E1 and E2. The most physiologically active estrogen is E2 which has an affinity to ERs higher than E1 and differently from the short-acting estrogens like E1 causes nuclear binding of the ER complex for a long period of time [14]. The biological effects of E2 are mediated through at least three (A and C-D in Figure 1) of the four ER signaling pathways [15].

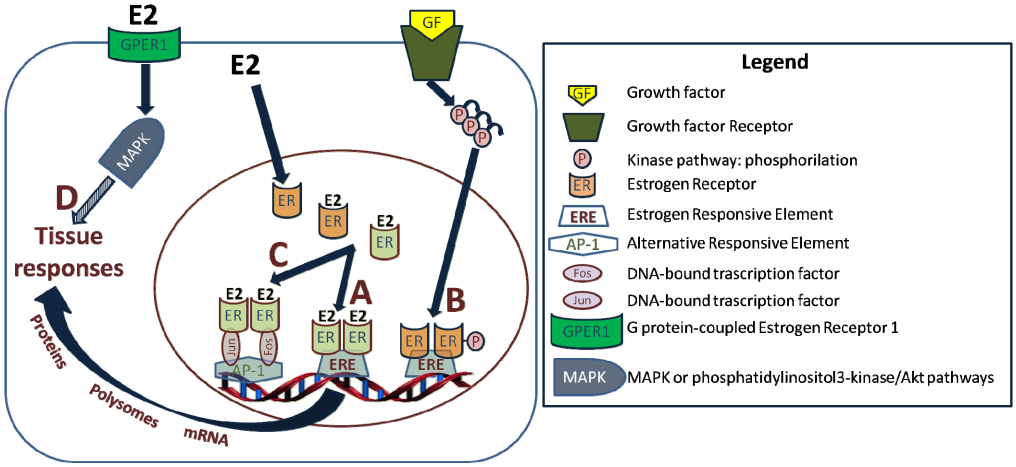


Figure 1: The four main mechanisms of E2 and Estrogen Receptor signaling: A) Classical ligand-dependent; B) Ligand-independent; C) ERE-independent; D) Cell-surface (nongenomic) signaling (figure revised from [16]).

The four main mechanisms of E2 and ER signaling are the following:

A) Classical ligand-dependent mechanism: The binding of E2 induces ER to conformational change, homodimerization and high affinity binding to specific DNA response elements (EREs), which are located within the regulatory regions of target genes. Depending on the cell and the presence of co-activators [17] this mechanism exerts either a positive or negative effect on expression of the down-stream target gene.

B) Ligand-independent mechanism: In the absence of E2 polypeptide growth factors [18] such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) are able to activate ER (via phosphorilation cascate) and increase the expression of ER target genes.

C) ERE independent mechanism: The complex E2-ER may activate alternative responsive elements such as AP-1 through association with other DNA-bound transcription factors (Fos/Jun) [19], which tether the activated ER to DNA, resulting in an up-regulation of gene expression.

D) Cell-surface (non genomic) signaling: The observed rapid biological effects of E2 in the bone, breast, vasculature and nervous system suggests that estrogens may also elicit nongenomic effects, possibly through cell surface ER form (i.e. GPER1 [20-21]) that are linked to intracellular signal transduction proteins (e.i. MAPK or PI3-kinase/Akt pathways).

Two estrogen receptors, ERα and ERβ, have been described as nuclear transcription factors that are activated by estrogens. Upon E2 binding the two receptors form homodimers or heterodimers and play different roles in estrogen signalling: whereas ERα has been usually described as proliferative mediator [22], ERβ was found modulating the action of ERα in reproductive tissue (like uterus [23]) and having features of tumor soppressor in prostate cancer [24]. However, recently ERβ was found to be the principal receptor involved in the progression of human endometriosis [25]. Tissue localization studies have revealed distinct expression patterns for the two ERs: whereas ERα is the predominant subtype expressed in the breast, uterus, cervix, vagina, and several additional target organs, ERβ exhibits a more limited expression pattern and is primarily detected in the ovary, prostate, testis, spleen, endometrium, lung, hypothalamus, and thymus [16]. In recent years, a third estrogen receptor has emerged, GPR30 or G protein-coupled estrogen receptor 1 (GPER1). This is a membrane-bound protein and mediates estrogenic responses in cardiovascular and metabolic regulation. Both GPER1 knock-out models and pharmacological agents have revealed that GPER1 activation may have several beneficial effects in the cardiovascular system including vasorelaxation, inhibition of smooth muscle cell proliferation, and protection of the myocardium against ischemia/reperfusion injury, and in the metabolic system including stimulation of insulin release and protection against pancreatic β-cell apoptosis [20].

1.1.2 Production of estrogen in women

In man and higher primates (i.e. monkeys) a large part (before menopause) or entirely (after menopause) of sex steroids are synthesized locally in peripheral target tissues from the inactive adrenal precursors: dehydroepiandrosterone (DHEA) and its sulfate derivative (DHEA-S) [26] (Figure 2).

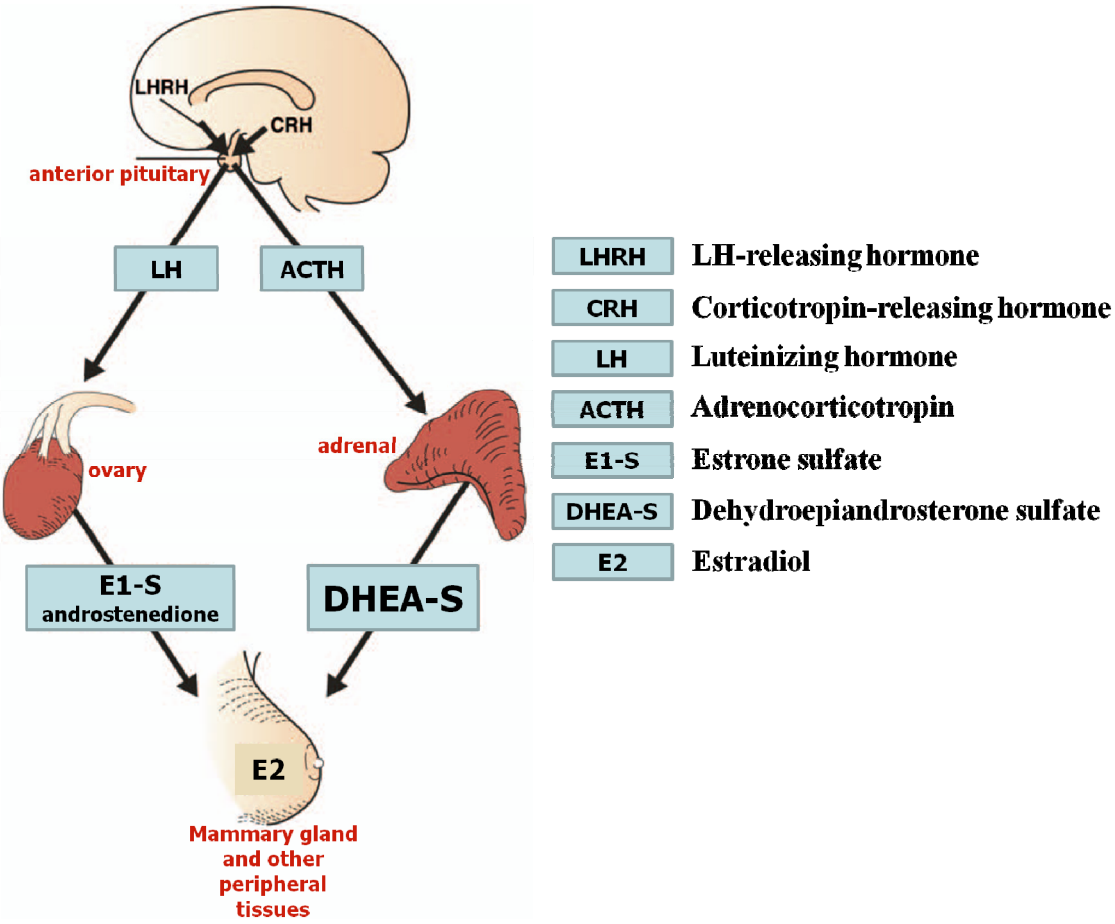


Figure 2: Schematic representation of the role of ovarian and adrenal sources of sex steroids in women. (figure revised from [27]).

In human at the age of 6-8 years the production of DHEA and DHEA-S by the adrenals increases and plasma levels of DHEA-S in adult women are usually 1000 to 10000 times higher than those of estrogens produced by the ovaries. This indicates that in human the highly active estrogenic hormone E2 is mainly synthesized in in the peripheral cells where it exerts its action (intracrine concept).

The membrana granulosa of dominant follicles of ovaries is the main source of circulating estrogens in premenopausal women [28-29]. After menopause, androgens of both adrenal (DHEA-S) and ovarian (androstenedione) origin are converted in E2 in peripheral tissues [30-33]. The great majority of estrogen form in circulation, in both premenopausal and postmenopausal women, is estrone sulfate (E1-S) which is hydrolyzed to E1 by estrone sulfatase in various human tissues [34-35]. E1 is reduced to E2 by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), which is also widely distributed in various peripheral tissues [36-38]. Nowadays, the peripheral estrogen production is considered to play very important roles in the pathogenesis and development of hormone dependent diseases (like breast carcinoma) (Figure 3).

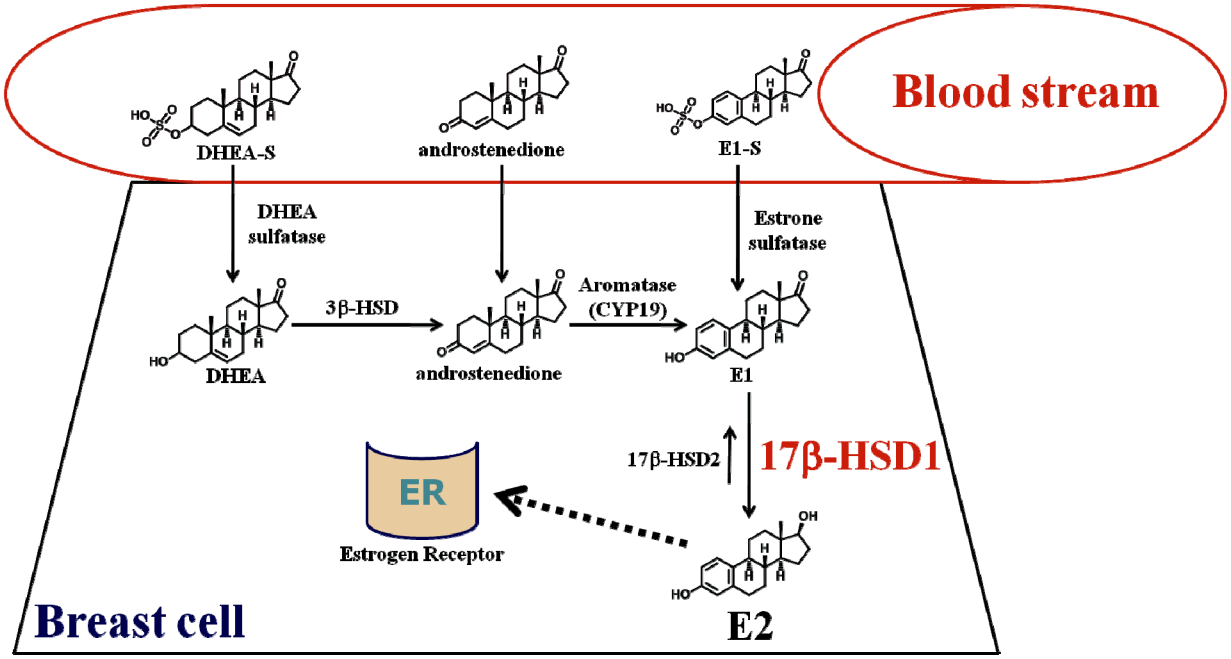


Figure 3: Illustration of estrogen production in breast cells. (figure revised from [39]).

Dehydroepiandrosterone (DHEA) sulfatase, Estrone sulfatase, Aromatase, 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) and 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) modulate the transactivaction of estrogen receptors in the target cell [40-41]. This implicate that via the intracrine mechanism where the active steroid is directly synthesized in the target cell without being released in the blood system these steroidogenic enzymes act as molecular switches allowing for pre-receptor modulation of E2 action [42].

1.2 Estrogen-dependent diseases (EDDs)

E2 is involved in the initiation and progression of estrogen dependent diseases (EDDs) like breast cancer, ovarian tumor [43], endometriosis, endometrial hyperplasia [44], and uterine leiomyoma [45]. The E2 action is mediated via activation of ERs [46-50].

1.2.1 Breast cancer

Breast cancer is a malignant neoplasia that forms in tissues of the breast, usually the ducts and lobules. Different types of breast cancer exist and they are classified depending on stage, aggressiveness, and genetic makeup. In 2008, 460,000 females died from breast cancer which is the most frequent cancer type among women; the risk factors to contract this disease are still poorly understood but it is well known that they increase with age.

More than 70% of patients affected by breast cancer have an estrogen-receptor positive (ER^+) tumor indicating that estradiol stimulates the cancer growth [51]. In these cases in addition to the standard cancer therapies like surgery, chemotherapy and radiotherapy, different endocrine therapies are well established in the clinics [52-55]: SERMs (Selective Estrogen Receptor Modulators) and antiestrogen constrain the estrogen action at the receptor level, aromatase inhibitors (AIs) suppress the estrogen formation by inhibiting the last step of E1 biosynthesis and Gonadotropin Releasing Hormone (GnRH) analogues completely cut the ovarian steroid formation via direct blockage of the GnRH receptors in the pituitary gland or via stimulating the negative feedback GnRH response. (Figure 4).

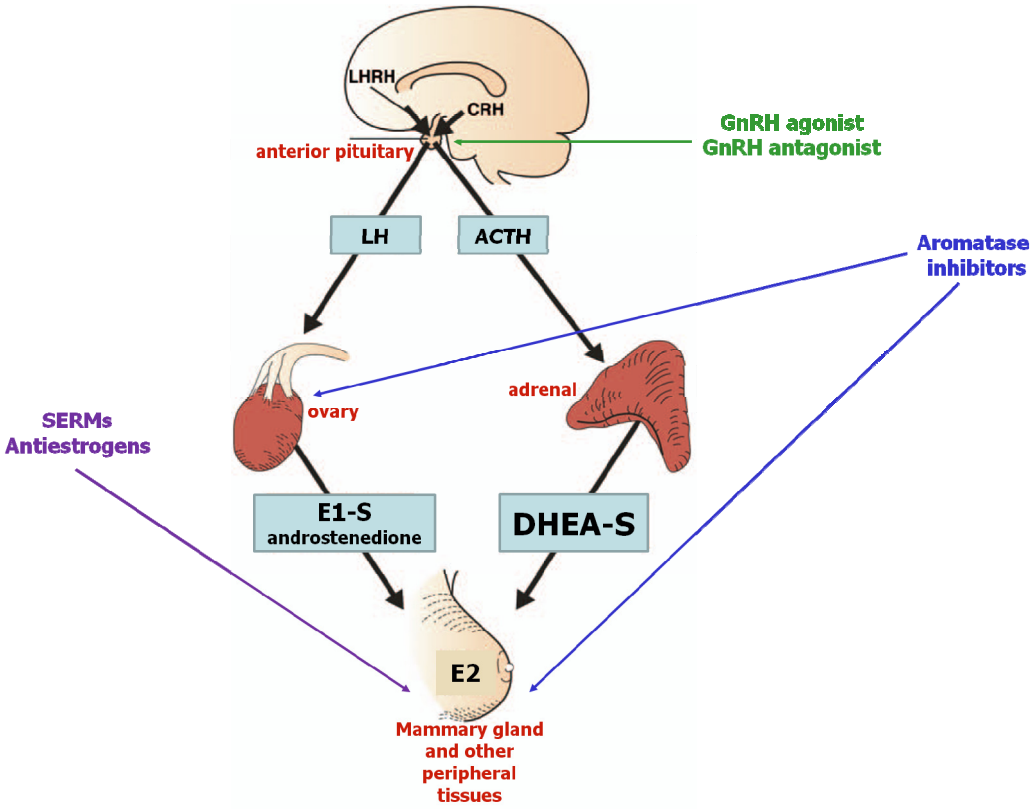


Figure 4: Schematic representation of the site of action of the existing endocrine therapies (figure revised from [27]).

All these hormone therapies result in a systemical reduction of estrogenic effects throughout the body leading to severe side-effects. For example, SERMs are also known to induce carcinoma in endometrium while aromatase inhibitors induce osteoporosis.

Furthermore, AIs are restricted to postmenopausal women since in premenopausal women they cause an increase of gonadotropin secretion by hypothalamus and pituitary axis counteracting the effect of the AIs itself via indirect stimulation of the ovaries to increase androgen production. On the contrary, GnRH analogues are only restricted to premenopausal women in the adjuvant treatment of breast cancer.

1.2.2 Endometriosis

Endometriosis is a condition in which the cells that line inside of the uterus grow outside of this organ in other areas, such as the ovaries, fallopian tubes and abdominal cavity. Up to 10% of all women may have endometriosis (most of all without symptoms). It causes adhesions and scarring, pain, dysmenorrhea (nausea, vomiting and diarrhea), fatigue, heavy bleeding and it may be found in 24 % to 50 % of women who experience infertility (American Society For Reproductive Medicine). Usually is diagnosed during pelvic exam, like laparoscopy, MRI scans, CT scans, gynecological examinations or surgical treatment [56].

The etiology of this disease is still poorly understood. The most widely accepted theory is that the disorder originates from retrograde menstruation of endometrial tissue through the fallopian tubes into the peritoneal cavity [57-58]. In endometriosis cell proliferation and inflammation are the symptoms responsible for chronic pelvic pain and infertility. Based on these symptomatic evidences a positive-feedback cycle for E2 and prostaglandin formation in endometriotic tissues has been postulated (Figure 4 [59]).

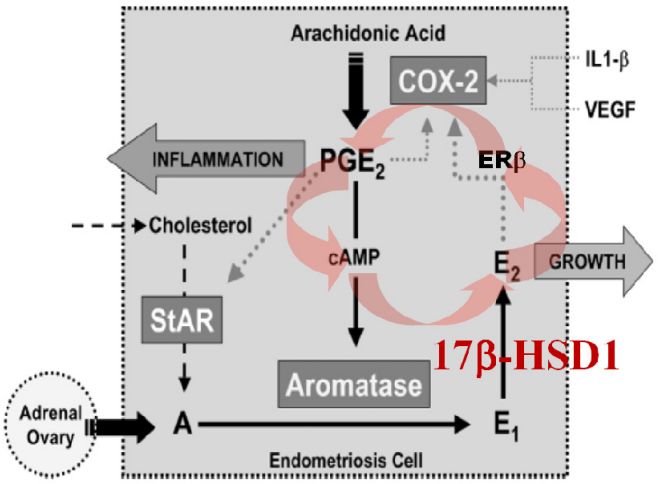


Figure 5: Schematic representation of the positive-feedback cycle for estrogen and prostaglandin formation (figure revised from [59]).

E2 enhances the cell proliferation and invasion of endometriotic tissue (growth) while both prostaglandins and cytokines mediate pain (inflammation) and infertility. E2 is produced locally in the endometriotic tissue in both ovulatory and postmenopausal women: the precursor androstenedione (A) coming from ovaries or adrenals is first converted to E1 by aromatase and subsequently reduced to E2 by 17β-HSD1 in the peripheral tissues and endometriotic implants. Endometriotic tissue is also capable to synthesize A from cholesterol via the activity of steroid acute regulatory (StAR) proteins and other steroidogenic enzymes. E2 induces cyclooxygenase (COX-2) which synthesize prostaglandins (PGE_2) in endometriosis. Interleukines (IL-1β), vascular endothelial growth factor (VEGF), and PGE_2 itself are also potent inducers of COX-2. PGE_2 in turn is the most potent known stimulator of StAR and aromatase in endometriotic stromal cells. This establishes a positive-feedback loop in favor of continuous estrogen and prostaglandin formation in endometriosis.

Beside the surgery (endometriotic tissue removal) and the usage of palliative therapies focused on sympthomes (non-steroidal anti-inflammatory drugs)**,** clinical hormone therapies are designed to suppress the estrogen biosynthesis: Oral contraceptive (which inhibits the menstruation), GnRH-analogues and recently AIs are used to restrain the growth of endometriotic tissue [53]. Due to the alteration of the hormone balance, it leads also to several side effects such as weight gain, acne, depression, osteoporosis [60].

1.3 17β-HSDs

1.3.1 General features

17β-HSDs are NADPH/NAD^+-dependent oxidoreductive enzymes, characterized by cofactor folding pattern known as Rossmann fold, which stereospecifically interconvert ketones and the corresponding secondary alcohols. They belong to the short chain dehydrogenase/reductase (SDR) family [61]. The only exception is 17β-HSD5 (AKR1C3) which is an aldo-ketoreductase (AKR) [62]. Up to date twelve 17β-HSDs of the fourteen mammalian subtypes identified [63-65] have been found in human tissues [63]. The tissue distribution, the sub-cellular localization, the substrate specifity (i.e. steroids, lipids or retinoids), and the catalytic preference (oxidation or reduction) are different for these enzymes. Nine subtypes of 17β-HSDs are involved in diseases, mainly steroid dependent diseases like EDD, while for the other five the physiological function is not clear yet (Table 1) [66].

For the sake of clarity the nomenclature of 17β-HSD enzymes that is followed here is based on the chronological order of descovery [67]. Recently, a new gene-based nomenclature for SDRs has been proposed by the international SDR-initiative [68].

Table 1: Human 17β-hydroxysteroid dehydrogenases (table revised [66]).

Type	Gene	SDR or AKR nomenclature	Function	Disease-association
1	HSD17B1	SDR28C1	Steroid (estrogen) synthesis	Breast & prostate cancer, endometriosis
2	HSD17B2	SDR9C2	Steroid (estrogen, androgen, progestin) inactivation	Osteoporosis
3	HSD17B3	SDR12C2	Steroid (androgen) synthesis	Pseudohermaphroditism in males associated with obesity
4	HSD17B4	SDR8C1	Fatty acid β-oxidation, steroid (estrogen, androgen) inactivation	D-specific Bifunctional Protein-deficiency, prostate cancer
5	AKR1C3	AKR1C3	Steroid (androgen, estrogen, prostaglandin) synthesis	Breast & prostate cancer
6	HSD17B6	SDR9C6	retinoid metabolism, 3α-3β–epimerase, steroid (androgen) inactivation?	
7	HSD17B7	SDR37C1	Cholesterol biosynthesis, steroid (estrogen) synthesis	Breast cancer
8	HSD17B8	SDR30C1	Fatty acid elongation, steroid inactivation, estrogens, androgens	Polycystic kidney disease
9	HSD17B9	SDR9C5-2	Retinoid metabolism	
10	HSD17B10	SDR5C1	Isoleucine, fatty acid, bile acid metabolism, steroid (estrogen, androgen) inactivation	X-linked mental retardation MHBD deficiency Alzheimer's disease
11	HSD17B11	SDR16C2	Steroid (estrogen, androgen) inactivation, lipids?	
12	HSD17B12	SDR12C1	Fatty acid elongation, steroid (estrogen) synthesis	
13	HSD17B13	SDR16C3	Not demonstrated	
14	HSD17B14	SDR47C1	Steroid (estrogen, androgen?) inactivation, fatty acid metabolism	Breast cancer, prognostic marker

1.3.2 Cell metabolism and unidirectional steroid flux in living cells

In intact cells 17β-HSDs catalyze unidirectional reactions: either a hydride transfer from reduced cofactor (NADPH) to a ketosubstrate or a hydride transfer from a hydroxysubstrate to oxidized cofactor (NAD^+), which occur with a proton shift for charge equalization. The different 17β-HSDs bind either NADP(H) or NAD(H), which act as soluble electron carriers [69]. Structural and mutagenesis studies showed that in the Rossmann fold [70] of 17β-HSD1 and 17β-HSD3, a positively charged amino acid forms a ionic bond with the 2'-phosphate group of NADP(H) (Arg37 in 17β-HSD1, [71]; Arg80 in 17β-HSD3, [72]). These enzymes preferably bind the phosphorylated cofactors rather than the non-phosphorilated one. On the contrary in 17β-HSD2 and 17β-HSD4 a negatively charged amino acid residue (Glu116 in 17β-HSD2, [73]; Asp41 in 17β-HSD4, [74]) is considered to be the reason for NAD(H) specificity (Figure 6).

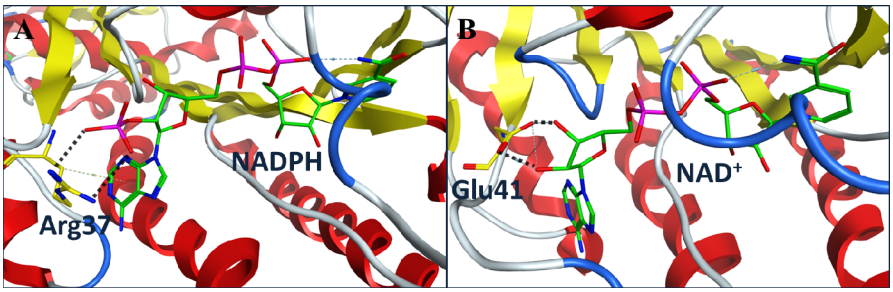


Figure 6: Important 17β-HSDs residues for cofactor recognition. (A) Reductive 17β-HSD1: interaction of Arg37with NADPH (PDB file: 1A27); (B) Oxidative 17β-HSD4: interaction of Glu41with NAD^+ (PDB file: 1ZBQ); with NADPH, NAD^+, Arg37 and Asp41 rendered as sticks and labelled. Figure rendered with MOE (www.chemcomp.com) (figure revised from [66]).

In living cells, the catalysis is unidirectional due to the fact that concentrations of cofactors (NADP(H) and NAD(H)) are much higher (micromolar range) than the ones of substrates (i.e. ketosteroids and hydroxysteroids) which are in the nanomolar range. Furthermore, NADP(H) is abundant in its reduced form ($NADPH/NADP^+ > 500$), whereas NAD(H) is mainly present in its oxidized form ($NAD^+/NADH > 700$) and these ratios are homeostatically maintained by glucose metabolism and O_2 supply (Figure 7).

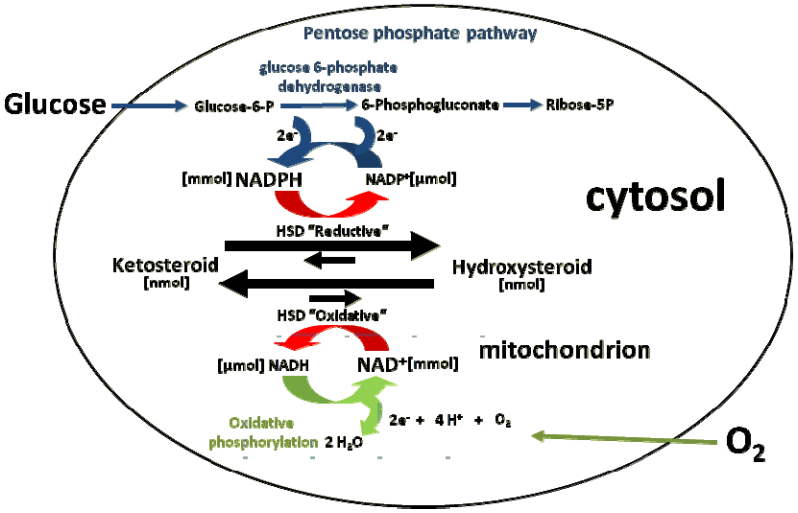


Figure 7: Interplay between intermediary metabolism and 17β-HSDs reactions (figure revised from [69]).

1.4 17β-HSD1

1.4.1 Potential drug target

17β-HSD type 1 catalyzes the final step of the estrogen biosynthesis: the activation of the weak estrogen, E1, to its potent homologue E2 which has a crucial role in supporting the growth of hormone-dependent breast cancer and endometriosis in women (Figure 8).

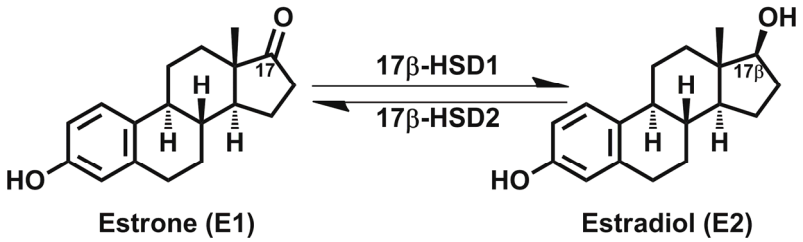


Figure 8: Conversion of E1 into E2 catalyzed by 17β-HSD1 and the reverse reaction (from E2 to E1) catalyzed by 17β-HSD2.

In estrogen-dependent diseases an increased E2/E1 ratio as well as high levels of 17β-HSD1 expression at mRNA and protein level, make the selective inhibition of 17β-HSD1 an attractive therapeutic target for their treatment. Different studies have shown that the ratio 17β-HSD1/17β-HSD2 is increased in the tumors of patients with ER^+ breast cancer [75-77]. Furthermore, several studies indicate that patients with high 17β-HSD1 expression have significantly shortened disease free and overall survival [78-79].

A change in the expression pattern of the steroidogenic enzymes is responsible for a high concentration of E2 in endometriotic tissues as well [80]. Recent studies showed that there is down regulation of 17β-HSD2 at mRNA level in endometriotic lesions, while both aromatase and 17β-HSD1 are up regulated, in comparison to normal tissues [81-82].

Therapies aiming the inhibition of 17β-HSD1, consequently, would give an innovative approach to the treatment of this disorder. 17β-HSD1 inhibitors must be selective towards 17β-HSD2, since inhibition of this enzyme may have opposing action (increase the E2/E1 ratio) and towards ER to avoid systemical estrogenic response.

1.4.2 Sequence and structural analysis of human 17β-HSD1

17β-HSD1, as already mentioned above, belongs to short-chain dehydrogenase/reductase (SDR) family. It is active as a soluble cytosolic homodimer, it has characteristical cofactor (NADPH) folding pattern known as Rossmann fold and catalyzes stereospecifically the transfer of a hydride (pro-S from nicotinamide C4 of cofactor) to estrone [83]. 17β-HSD1 has characteristic amino acid motifs which are conserved in all SDR enzymes (Figure 9).

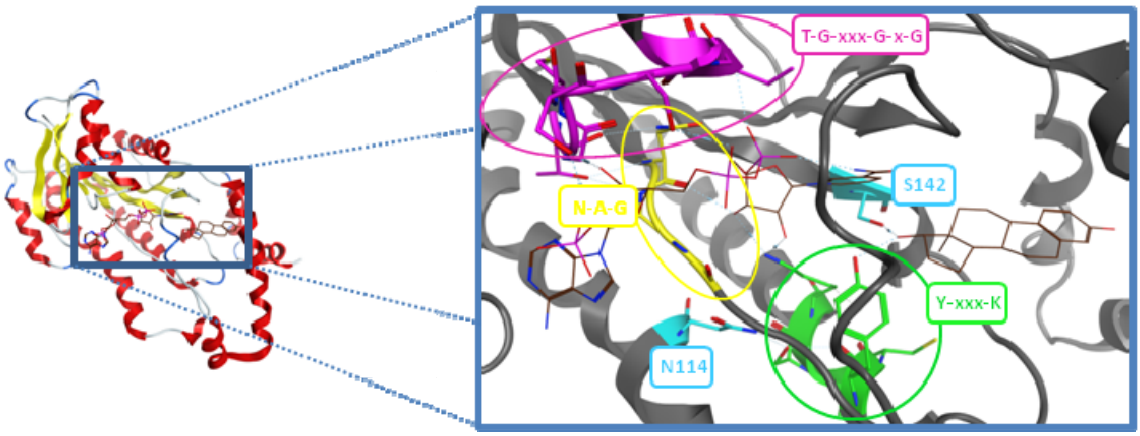


Figure 9: SDR characteristic amino acid motifs of 17β-HSD1: Tertiary structure of 17β-HSD1 rendered as cartoon, with a zoom into SDR conserved regions T-G-xxx-G-x-G, N-A-G, Y-xxx-K as well as S142 and N114 are highlighted. Figure rendered with MOE (http://www.chemcomp.com/).

The SDR conserved motifs are T-G-xxx-G-x-G in the cofactor binding cleft, N-A-G and Y-xxx-K in the active center where Y (Tyr155) and K (Lys159) together with Asn114 and Ser142 are fundamental for catalysis (catalytic tetrad) [84-85]. The N-A-G motif is responsible for stabilization of protein 3D-structure. Differently from other SDR enzymes, 17β-HSD1 displays high substrate selectivity mainly due to the ordered C-terminal region which forms a specific binding cavity. Furthermore, important amino acids of the substrate binding pocket are Leu149 which responsible for C18/C19-steroid discrimination [86-87] and Gly145 which plays a key role in steroid/retinoid discrimination [88]. The cofactor binding cleft is constituted by the Rossmann fold that consists of a seven-stranded, parallel β-sheet core structure surrounded by six parallel α-helics. Differently from the substrate binding cleft (i.e. the C-terminal part), the Rossmann fold together with the T-G-xxx-G-x-G motif is the most rigid and conserved part of 17β-HSD1.

No 17β-HSD1 crystal structure complexed with E1 (substrate) exists. The published crystal structures are available in the protein data bank (PDB) as: apoform, holoform, binary complex with E2, androgens or steroidal inhibitors and ternary complex with cofactor, E2 or inhibitors. If present E2 (product) is stabilized by hydrogen bonds between its O3 and His221/Glu282, as well as between its O17 and Tyr155/Ser142 [89]. Furthermore, a highly flexible βFαG'-loop (not always resolved) has been identified between cofactor and substrate. The data reveal the importance of this loop for ligand binding. Two small apolar subpockets could be identified in proximity of substrate binding cleft, the first next to the catalytic tetrade (next to Tyr155) while the second next to C-terminal region (next to Glu282) (Figure 10).

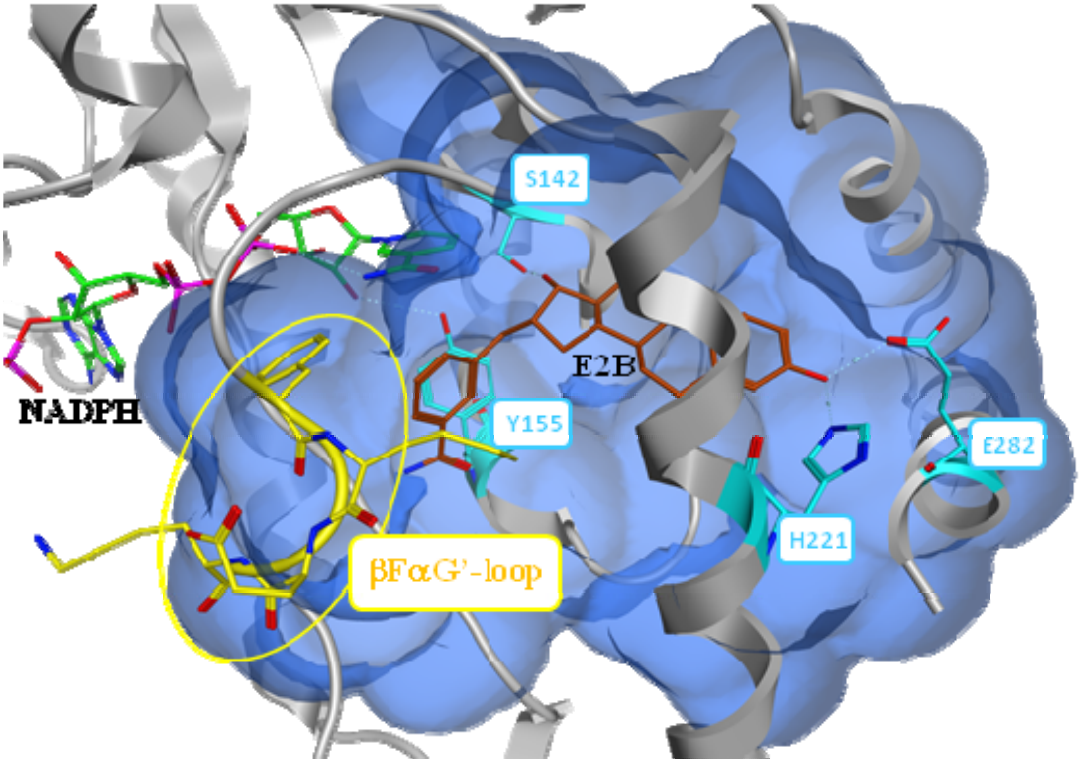


Figure 10: 17β-HSD1 complexed with E2B inhibitor: interactions between steroidal inhibitor and S142, Y155, H221 and E282 are highlighted. βFαG'-loop is depicted in yellow between substrate and cofactor (NADPH). Figure rendered with MOE (http://www.chemcomp.com/).

1.4.3 Catalytic mechanism of 17β-HSD1

In the postulated catalytic mechanism of 17β-HSD1, the *pro*-S hydride on C4 of pyridine moiety of NADPH is transferred to the α-face of the estrone at the C17 carbon. It has not been clarified yet wether the hydride transfer occurs simultaneously (concerted mechanism) or stepwise. Structure analysis, mutagenesis studies and sequence alignment evidenced the amino acid residues important for the catalysis: Asn114, Ser142, Tyr155 and Lys159 (catalytic tetrad) and a water molecule play a critical role in the enzymatic process stybilizing the substrate and forming a H-bond network which is responsible for the proton transfer (Figure 11) [90-93].

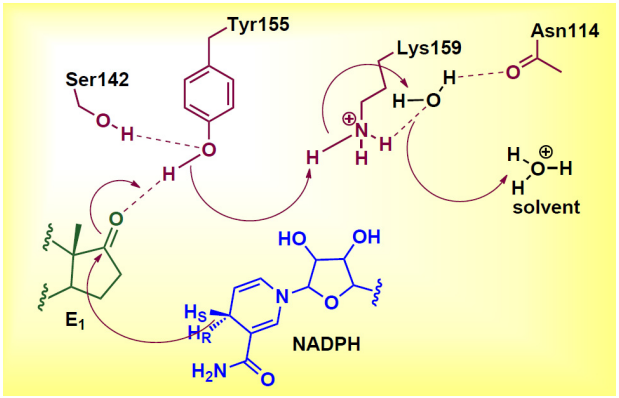


Figure 11: scheme of the possible catalytic mechanism for 17β-HSD1. Hydrogen bonds are represented in dashed lines.

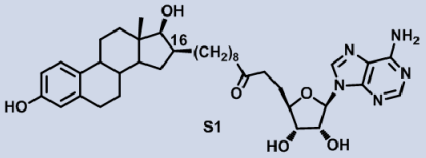
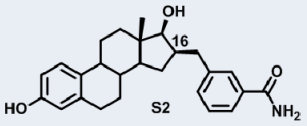
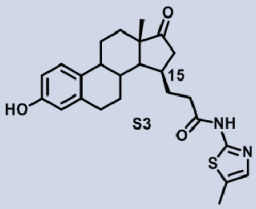
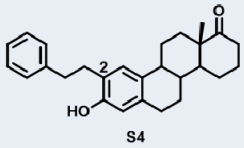
1.5 Inhibitors of 17β-HSD1

17β-HSD1 inhibitors should not inhibit 17β-HSD2 and, of course, should not be estrogenic. Inhibitory activity towards aromatase or E1-sulfatase might be considered not deleterious for the terapeutical goal (Figure 3). Beside biological inhibitory activity towards the target enzyme and selectivity, 17β-HSD1 inhibitors should show properties related to "druggability" (i.e., ADME/toxicity) defined by Lipinski [94]. During the last fifteen years several patents and publications concerning 17β-HSD1 inhibitors have been reported [66 and 95-100]. Although clear structure-activity relationships have been shown, no clinical candidate has been reported yet.

Steroidal 17β-HSD1 inhibitors as well as non-steroidal ones are described to bind to the steroid binding cleft and/or the pocket where usually the cofactor is situated. An exception is given by phytoestrogens which interact with the dimer interface of 17β-HSD1 as suggested by competitive NMR-experiments and docking studies [101]. Recently, new non-steroidal potent coumarin derivatives as inhibitors of 17β-HSD type 1 were published [102-103].

The steroidal compounds are based on E1 and E2 cores and the best biological results were exhibited by derivatives with substitution at the C2, C15 and C16 positions [104-112] (Table 2).

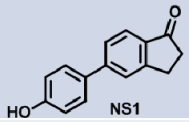
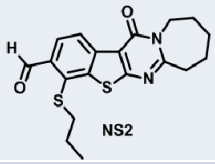
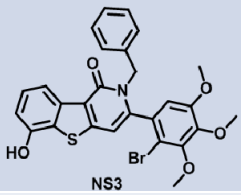
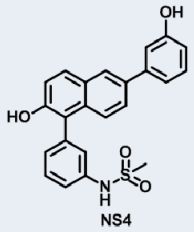
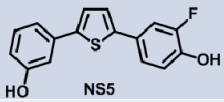
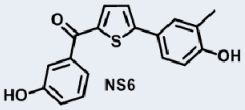
Table 2: Best steroidal 17β-HSD1 inhibitors described.

17β-HSD1 steroidal inhibitor	17β-HSD1 IC_{50} (nM)	Assay
 S1	4	Cell-free [E1] = 0.1 μM [NADH] = 1 mM
 S2	44	Cellular assay (T-47D) [E1] = 0.06 μM
 S3	4	Cell-free [E1] = 0.03 μM
 S4	15	Cell-free [E1] = 0.015 μM

Beside their high inhibitory potency toward the target enzyme drawbacks were reported for steroidal inhibitors: i.e. the hybrid inhibitor (**S1** in Table 2) designed to bind to both the steroid binding cleft and the cofactor binding site has been described as not able to penetrate the cell membrane. Compound **S2** showed to increase cancer T-47D cell proliferation (estrogenicity).

Concerning the non-steroidal inhibitors different classes have been published in the last four years [113-126]. These compounds show advantages compared to steroidal structures such as synthetic accessibility, drug-likeness, selectivity and non-estrogenicity.

Table 3: Best non-steroidal 17β-HSD1 inhibitors described.

17β-HSD1 non-steroidal inhibitor	17β-HSD1 Inhibitory activity	Cellular assay
 NS1	IC_{50} = 1.7μM	T-47D [E1] = 0.002 μM
 NS2	74 % at 1 μM	MCF-7 expressing h17β-HSD1 [E1] = 0.002 μM
 NS3	67 % at 1 μM	MCF-7 expressing h17β-HSD1 [E1] = 0.002 μM
 NS4	IC_{50} = 71 nM	T-47D [E1] = 0.050 μM
 NS5	IC_{50} = 282 nM	T-47D [E1] = 0.050 μM
 NS6	IC_{50} = 26 nM	T-47D [E1] = 0.050 μM

The biphenyl mimetics of E1, like **NS1** in Table 3, showed very weak inhibitory activity in cell-based assay. Thiophenepyrimidinones (i.e. **NS2**) showed high 17β-HSD1 inhibitory activity (73 % at 0.1 μM) in a cell-free recombinant human assay at low E1 concentration (30 nM) but only 74 % at 1 μM in human MCF-7 breast cancer cells transfected with cDNA encoding for human 17β-HSD1 and using a low substrate concentration (2 nM). The best non-steroidal 17β-HSD1 inhibitors were described so far by the Hartmann group. Three classes have been developed via pharmacophore model: a) (Hydroxyphenyl)naphthols (i.e. **NS4**), b) Bis(hydroxyphenyl)substituted arenes (i.e. **NS5**) and c) Bicyclic substituted hydroxyphenylmethanones (i.e. **NS6**). The best results were obtained by structures like **NS6**, which showed very good 17β-HSD1 inhibitory activity (in non-cellular as well as cellular assays) and selectivity towards 17β-HSD2 and ER.

1.6 Animal models for *in vivo* evaluation of 17β-HSD1 inhibitors.

In lower mammals unlike man and higher primates (i.e. monkeys), the ovaries and not the peripheral target tissues are the primary sources of estrogens. In fact in rat and mouse the secretion of sex steroids takes place exclusively in the gonads. When animal models are developed to study hormone dependent diseases, this difference is usually overtaken by the usage of animal´s ovariectomy followed by h17β-HSD1 source implatation or of xenograft

imunodeficient animals. With aim of testing 17β-HSD1 inhibitors *in vivo* as well as gaining insight into physiopathological role of this enzyme in the development of EDDs, several animal models were recently established. 17β-HSD1 of different species differs regarding sequence-identity in a range from 51 % (zebrafish) to 99 % (chimpanzee) and similarity from 70 % to 100 %, respectively. 17β-HSD1 of mouse and rat has 74 % and 75 % sequence identity, respectively, and both share 83 % similarity comparing with human one. Due to this moderate homology/identity and due to the fact that 17β-HSD1 tissue distribution considerably varies between lower species and human, xenograft models using nude mice were developed to overcome these problems (Table 4 [44 and 127-133]). A marmoset (Callithrix jacchus) model was used to reproduce endometriosis [134]. 17β-HSD1 of marmoset has 80 % sequence identity and 83 % homology with human one. Although several potent human 17β-HSD1 inhibitors showing strong activity towards the marmoset enzyme could be identified, in vivo application has not been reported yet. The phylogenetic similarity between marmoset and human makes this model interesting and more significant than rodent models [66].

Table 4: Summary of the different 17β-HSD1 animal models described.

	Species	Background features	Disease	17β-HSD1
1	mice	immunodeficient, ovariectomized	breast cancer	inoculated MCF-7 cells stably expressing recombinant human 17β-HSD1
2	mice	immunodeficient, ovariectomized	breast cancer	inoculated T47-D tumor cells
3	mice	immunodeficient, ovariectomized	endometriosis	human endometrium (proliferative phase) implanted into rodent peritoneal cavity
4	mice	transgenic	breast cancer	transgenic (TG) mice expressing human (h) HSD17B1 under mouse mammary tumor virus (MMTV) promoter (MMTV-hHSD17B1TG mice)
5	mice	transgenic	endometrial hyperplasia	transgenic (TG) mice expressing human (h) HSD17B1 under mouse mammary tumor virus (MMTV) promoter (MMTV-hHSD17B1TG mice)
6	marmoset (Callithrix jacchus)	non-menstruating primate	endometriosis	non-invasive or invasive intrapelvic placement of endometrial cells

2. Outline of this thesis

2.1 Scientific goal

In both premenopausal and postmenopausal women, the inhibition of peripheral E2 production is nowadays regarded as a better therapeutical strategy compared to the existing endocrine therapies in the treatment of EDDs. Most of all fewer side effects are supposed since the systemic estrogen should not be affected.

17β-HSD1 and 17β-HSD2, expressed in different amounts in estrogen target cells (usually expressing also ERα and/or β), play a pivotal role in regulating the growth of hormone-dependent breast cancer and endometriosis in women. Due to the fact that 17β-HSD1 catalyzes the last step of the synthesis of the most potent estrogen (E2) and has been often found overexpressed compared to 17β-HSD2 in diseased tissues of patients affected by EDDs, this enzyme is considered to be the key-target to stop pheripheral estradiol production.

Several animal models published in the last years proved the *in vivo* efficacy of 17β-HSD1 inhibitors to reduce estrogen stimulated growth in diseases like breast cancer, endometriosis and endometrial hyperplasia. Although up to date no 17β-HSD1 inhibitor has entered clinical trials, the inhibition of 17β-HSD1 is considered a promising new therapeutical approach for the treatment of estrogen-dependent pathologies.

Thereby the main goal of the present thesis was to develop new potent and selective non-steroidal inhibitors of 17β-HSD1 structurally different from the previously described ones as suitable tools for *in vivo* application in order to afford new chemical entities able to improve the hormone-dependent diseases treatment.

To achieve this purpose, novel 17β-HSD1 inhibitors have been designed and synthesized via a combination of computer-aided methodology (hit identification by pharmacophore screening) and subsequent structural optimization. In addition to their ability to inhibit 17β-HSD1 in a cell free assay (placental preparate), the selectivity of the compounds here described towards 17β-HSD2 (cell free inhibition assay) and ERs (binding affinity test) was evaluated. Furthermore, the most potent and selective 17β-HSD1 inhibitors were screened according to their ability to inhibit E2 formation in intact T47-D tumour cells (intracellular potency) to have insights regarding cellular membrane permeation and to exclude possible intracellular metabolism. Finally, the inhibitory activity of the most interesting compounds was determined towards 17β-HSD1 and 17β-HSD2 enzymes of *Callithrix jacchus* (placental cell preparation) to select the most suitable inhibitors for in vivo evaluation in marmoset monkey model.

2.2 Working strategy

Derivation of pharmacophore model and hit identification (chapter 3.I):

Although up to now more than twenty crystallographic structures of human 17β-HSD1 are available in the Protein Data Bank (PDB) as apoform, binary or ternary complex (with steroidal ligand and/or cosubstrate), X-ray based information about protein-ligand interactions of the enzyme with nonsteroidal inhibitors is not available. For this reason, in our search for new non-steroidal hits structurally different from those previously described, in the first project of the present work it was considered unsuitable an outwardly simple virtual screening strategy based on docking studies performed on a randomly selected 17β-HSD1 crystal structure available in the literature. Alternatively, a ligand-based approach was followed and a new pharmacophore model for 17β-HSD1 was built, as *in silico* screening tool, based on

cocrystallized ligands with some additional protein structure information. This pharmacophore model was used to screen a small *in house* library (approximately forty thiazole derivatives with MW in the range of 150-350). The virtual hits were experimentally validated regarding their inhibitory activity towards 17β-HSD1, and, finally, [5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-2-yl](3-hydroxyphenyl)methanone covering six features in the pharmacophore and showing weak activity in 17β-HSD1 cell free inhibition assay was selected as new core structure for further optimization. (Figure 12).

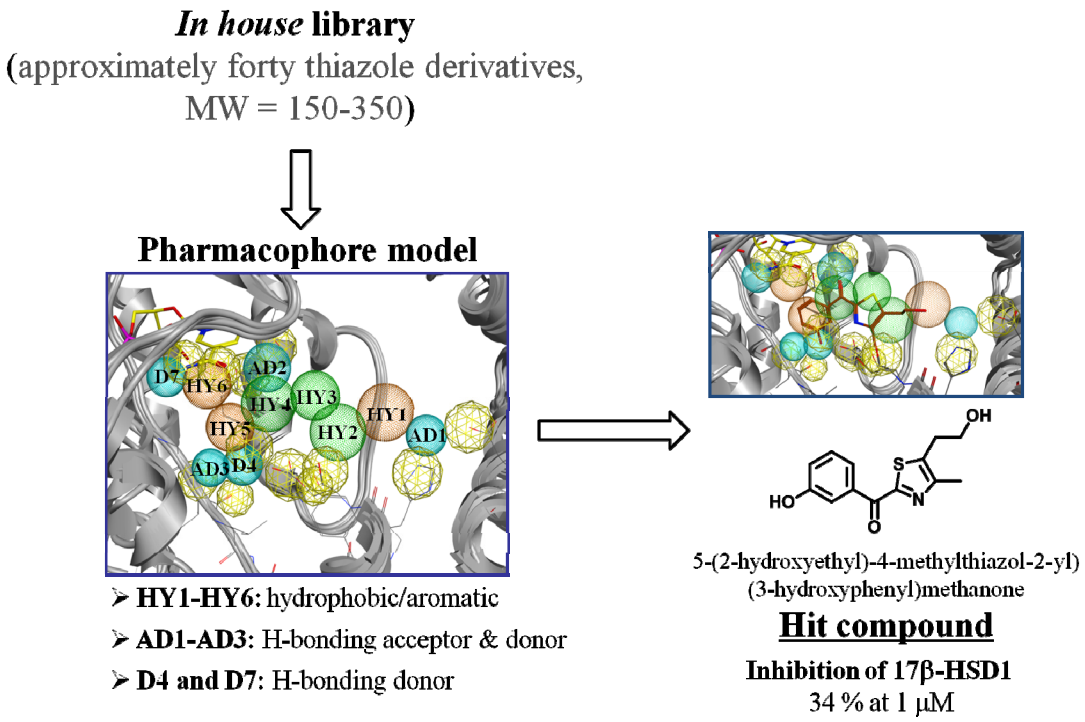


Figure 12: pharmacophore aided hit descovery

Hit optimization (chapter 3.I):

The hit optimization started from the rigidification (decrease of freedom conformational degrees) of the flexible hydroxy ethyl chain of the hit, leading to a benzothiazole derivative linked via a carbonyl bridge in the 2 position to 3-hydroxy-phenyl ring (Figure 13).

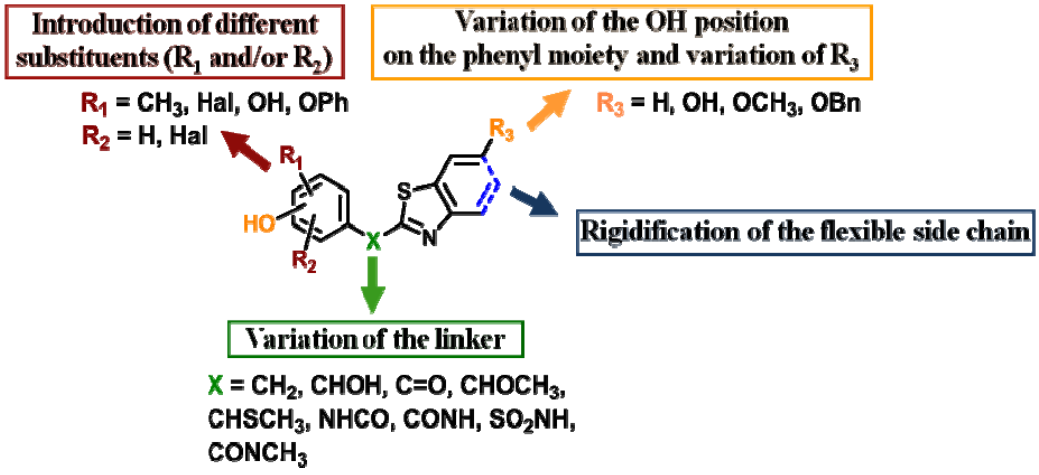


Figure 13: hit optimization overview

Furthermore the carbonyl bridge between the two aromatic rings bearing a sp^2 trigonal geometry which allows for electronic delocalization (conjugation) and the oxygen atom which may accept two H-bonds was exchanged with different linkers bearing a sp^3 tetrahedral bridge and/or H-bond donating groups to investigate the influence of these features on the 17βHSD1 inhibitory activity. The carbonyl bridge was also replaced by longer functional groups, such as amide, retro amide, sulphonamide, urea, and thiourea and benzyl amide to inquire whether a linker, bearing a non-constrained angled geometry, could allow the phenyl ring to exploit different regions of the binding cleft (Figure 13).

Further optimization in the class of benzothiazole derivatives (chapter 3.II):

In the second project of the present work (1,3-benzothiazol-2-yl)-(3-hydroxy-phenyl)methanone and 3-hydroxy-*N*-(6-hydroxy-benzothiazol-2-yl)benzamide as the best 17β-HSD1 inhibitors from the first study were optimized. The influence of electronic, lipophilic, steric and H-bonding properties on biological activity have been investigated by introducing different substituents on the phenyl moiety, exchanging the hydroxy group on the benzothiazole moiety with hydrogen, methoxy and benzyloxy as well as N-methylating the amide function (Figure 13).

3. Results

3.I Hydroxybenzothiazoles as New Nonsteroidal Inhibitors of 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1).

Major part of this chapter will be published in *PloSOne*.

Abstract: Beside its physiological role, 17β-estradiol (E2), the most potent estrogen is also known to be involved in the development of estrogen-dependent diseases (EDD) like breast cancer and endometriosis. Existing endocrine therapies for these indications – treatments with aromatase inhibitors, GnRH-analogues or anti-estrogens - result in a radical and unspecific reduction of estrogenic effects throughout the body leading to severe side-effects like osteoporosis or induction of endometrial carcinoma.
17β-HSD1 catalyses the reduction of the weak estrogen estrone (E1) to the highly potent E2 and is often overexpressed in breast cancer tissue. An inhibition of 17β-HSD1 could selectively reduce the E2-level in specific tissues thus allowing for a novel, targeted approach in the treatment of EDD resulting in fewer side-effects compared to the established treatments.
In continuation of our search for new nonsteroidal 17β-HSD1 inhibitors a pharmacophore screening of a compound library was conducted which led to the identification of the moderately active hit (**5**). Rigidification and further modifications resulted in the discovery of novel inhibitors with benzothiazole-scaffold. Their putative binding modes were investigated by correlating their biological data with features of the pharmacophore model. The most active compounds **6** and **21** show IC_{50}-values in the nanomolar range and reasonable selectivity against 17β-HSD2 and the estrogen receptors, respectively. They can thus be regarded as first benzothiazole-type lead compounds for the development of potential therapeutics.

Introduction

Estrogens are important steroidal hormones which bear different physiological functions. The main beneficial effects include their role in programming the breast and uterus for sexual reproduction [1], controlling cholesterol production in ways that limit the build-up of plaque in the coronary arteries [2], and preserving bone strength by helping to maintain the proper balance between bone build-up and breakdown [3-4]. Among female sex hormones, 17β-estradiol (E2) is the most potent estrogen carrying out its action either via transactivation of estrogen receptors (ERs) [5] or by stimulating nongenomic effects via the MAPK signaling pathway [6]. In addition to its important beneficial effects, however, E2 can also cause serious problems arising from its ability to promote the cell proliferation in breast and uterus. Although this is one of normal function of estrogen in the body, it can also increase the risk of estrogen dependent diseases (EDD), like breast cancer, endometriosis and endometrial hyperplasia [7-10]. Suppression of estrogenic effects is consequently a major therapeutic approach. This is proved by routine clinic use of different endocrine therapies, for instance with GnRH analogues, SERMs (Selective estrogen receptor modulators), antiestrogens, and aromatase inhibitors [11-13] for the prevention, as well as the adjuvant treatment of breast cancer. However, all these therapeutics systemically lower estrogen hormone action, therefore causing significant side effects such as osteoporosis, thrombosis, stroke and endometrial cancer [14-16]. Thus, a new approach which aims at affecting predominantly the intracellular E2

production in the diseased tissues (intracrine approach), would consequently be a very beneficial improvement for the treatment of EDD. Such a therapeutic strategy has already been shown to be effective in androgen dependent diseases like benign prostate hyperplasia by using 5α- reductase inhibitors [17-21].

The type 1 enzyme of the 17β-hydroxysteroid dehydrogenase (17β-HSD) family, which is responsible for the intracellular NADPH-dependent conversion of the weak E1 into the highly potent estrogen E2, was found overexpressed at mRNA level in breast cancer cells [22-24] and endometriosis [25]. Inhibition of this enzyme is therefore regarded as a novel intracrine strategy in EDD treatment with the prospect of avoiding the systemic side effects of the existing endocrine therapies. Although to date, no candidate has entered clinical trials, the ability of 17β-HSD1 inhibitors to reduce the E1 induced tumor growth has been shown using different animal models, indicating that the 17β-HSD1 enzyme is a suitable target for the treatment of breast cancer [26-28]. The same effect was also demonstrated by Day *et al.* [28], Laplante *et al.* [29] and Kruchten *et al.* [30] using *in vitro* proliferation assays.

In order not to counteract the therapeutic efficacy of 17β-HSD1 inhibitors it is important that the compounds are selective against 17β-HSD2. This enzyme catalyses the reverse reaction (oxidation of E2 to E1), thus playing a protective role against enhanced E2 formation in the diseased estrogen dependent tissues. Interestingly, potent and selective 17β-HSD2 inhibitors were recently reported for the treatment of osteoporosis [31-32]. Additionally, to avoid intrinsic estrogenic and systemic effects, the inhibitors should not show affinity to the estrogen receptors α and β.

Several classes of 17β-HSD1 inhibitors have been described in the last years [33-40], most of them having a steroidal structure. During the past decade, our group reported on four different classes of nonsteroidal 17β-HSD1 inhibitors [41-51]. Compounds **1-4** (Figure 1) exhibit IC_{50} values toward 17β-HSD1 in the nanomolar range and high selectivity against 17β-HSD2 and the ERs in our biological screening system [52].

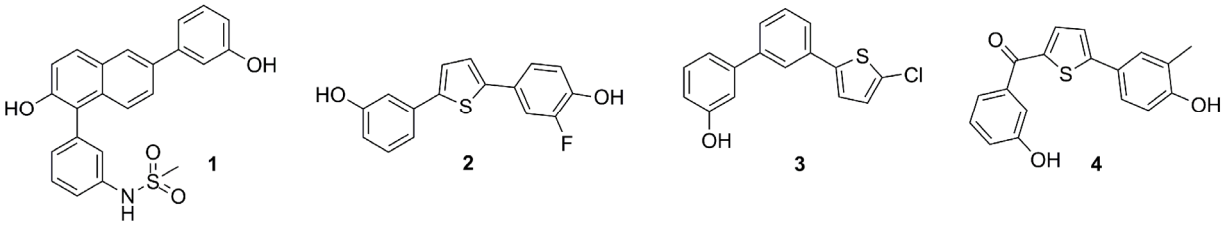


Figure 1. Non-steroidal 17β-HSD1 inhibitors published by our group.

In our search for new nonsteroidal leads that are structurally different from those previously described, an *in silico* screening of our in-house compound library was performed using a pharmacophore model derived from crystallographic data. A virtual hit was identified with a moderate inhibitory activity for 17β-HSD1, structural optimization led to the discovery of benzothiazoles as novel, potent inhibitors of the target enzyme with good *in vitro* biological activitiy. Further computational studies were performed to better understand the favourable interactions achieved by these inhibitors in the active site.

1) Pharmacophore and hit identification. Although up to now more than twenty crystallographic structures of human 17β-HSD1 are available in the Protein Data Bank (PDB) as apoform, binary or ternary complex (with steroidal ligand and/or cosubstrate), X-ray based information about protein-ligand interactions of the enzyme with nonsteroidal inhibitors is not available. Further, the steroidal binding pocket in these crystal structures evidenced differences in terms of size and geometry due to a pronounced flexibility of some parts of its surrounding [53]. This fact might affect an outwardly simple virtual screening strategy such as random

selection of one crystal structure to perform docking studies and was considered unsuitable to the search of new hits. To overcome this, we chose to follow a different strategy: a new pharmacophore model for 17β-HSD1 was built, as *in silico* screening tool, based on cocrystallized ligands and with some additional protein structure information, and a ligand-based approach was followed.
This pharmacophore model was used to screen a small *in house* library (approximately forty thiazole derivatives with MW in the range of 150-350), virtual hits were experimentally validated, and, finally, a new core structure (compound **5 -** [5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-2-yl](3-hydroxyphenyl)methanone) was selected. This was used as a new scaffold for further optimization.

2) Hit optimization.
a) rigidification. Due to the fact that rigidification (decrease of freedom conformational degrees) can often increase potency, compound **6** was designed freezing the flexible hydroxy ethyl chain of compound **5** leading to a benzothiazole derivative linked via a carbonyl bridge in the 2 position to 3-hydroxy-phenyl ring (**6**) (see Figure 2).

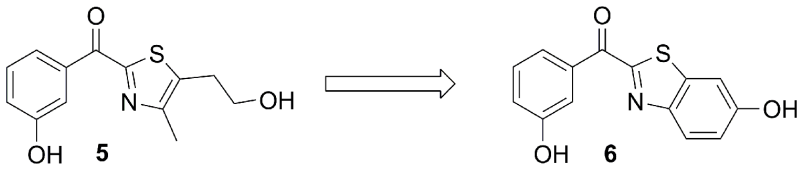
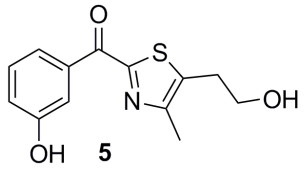


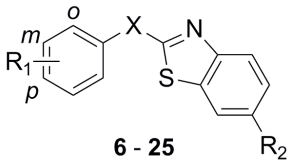
Figure 2. Rigidification strategy.

b) Bridge: length, geometry and H-bonding properties.
The carbonyl bridge between the two aromatic rings in compound **6** bearing a sp^2 trigonal geometry allows for electronic delocalization (conjugation) and the oxygen atom may accept two H-bonds. To investigate the influence of these features on the 17βHSD1 inhibitory activity, compounds **7**-**11** (Table 1), bearing a sp^3 tetrahedral bridge, were synthesized.

Table 1. 17β-HSD1 inhibitory activity for compounds, 5 and 6-25.



5



6 - 25

Compound	X	R_1	R_2	HSD1[a]	
				% inhib. (1μM)	IC_{50}[b] (nM)
5				34	nd
6	CO	*m*-OH	OH	91	44
7	CHOH	*m*-OH	OH	28	nd
8	$CHOCH_3$	*m*-OH	OH	13	nd
9	$CHSCH_3$	*p*-OH	OH	ni	nd
10	CH_2	*p*-OH	OCH_3	ni	nd
11	CH_2	*p*-OH	OH	24	nd
12	CO	*m*-OH	H	75	365
6i	CO	*m*-OCH_3	OCH_3	ni.	nd
13	CO	*p*-OCH_3	OH	27	nd
14	CO	*p*-OH	OH	85	243
15	NHCO	*o*-OH	OCH_3	ni	nd
16	NHCO	*o*-OH	OH	ni	nd
17	NHCO	*m*-OH	OCH_3	31	nd
18	NHCO	*m*-OH	OH	40	nd
19	CONH	*p*-OCH_3	OH	ni.	nd
20	CONH	*p*-OH	OH	39	nd
21	CONH	*m*-OH	OH	83	243
22	SO_2NH	*m*-OH	OH	ni	nd
23	NHCONH	*m*-OH	OH	ni.	nd
24	NHCSNH	*m*-OH	OH	62	nd
25	CH_2CONH	*m*-OH	OH	ni	nd

[a] Human placenta, cytosolic fraction, substrate [^{3}H]E1 + E1 [500 nM], cofactor NADH [500 μM]. [b] Mean values of three determinations, standard deviation less than 10 %. [c] nd: not determined. [d] ni: no inhibition.

The carbonyl bridge was also replaced by longer functional groups, such as amide (i.e. **18**), retro amide (i.e. **21**), sulphonamide (**22**), urea (**23**), thiourea (**24**) and benzyl amide (**25**) to inquire whether a linker, bearing a non-constrained angled geometry, could allow the phenyl ring to exploit different regions of the binding cleft.

Results

1) Pharmacophore generation.

Five diverse 17β-HSD1 crystal structures (PDB-ID: 1equ, 1i5r, 3hb5, 1a27, 1dht) [53-57] were superimposed (backbone root mean square deviation (RMSD) of 0.7 Å) and the cocrystallized steroidal ligands EQI, HYC, E2B, E2, and DHT, respectively, (Figure 3) were used to build the pharmacophore model using the Molecular Operating Environment (MOE; www.chemcomp.com) software.

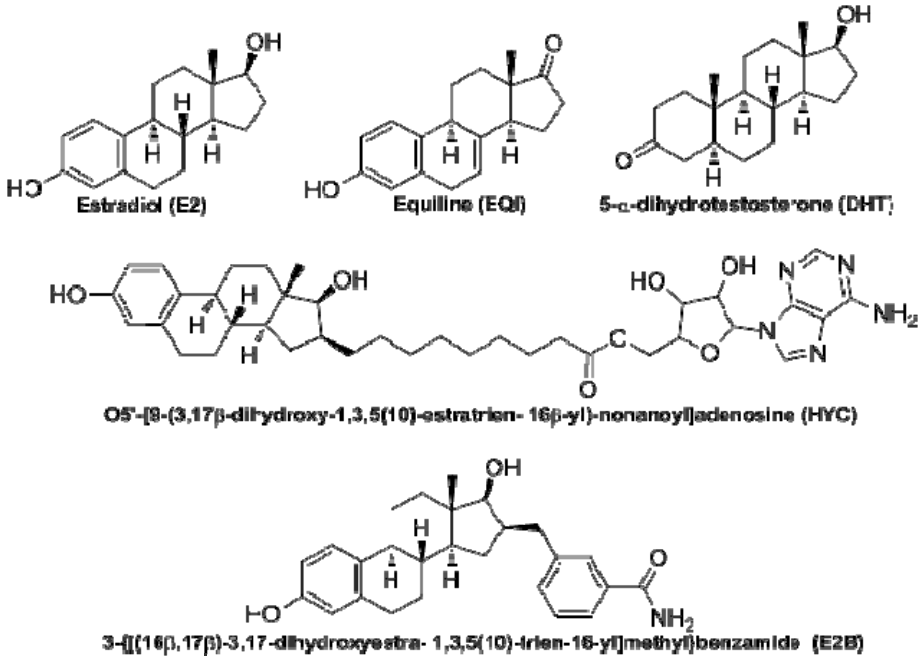


Figure 3: 17β-HSD1 co-crystallized steroidal ligands. Five 17β-HSD1 cocrystalized steroidal ligands (EQI, HYC, E2B, E2, DHT) available as PDB (respectively: 1equ, 1i5r, 3hb5, 1a27 and 1dht) used to build the pharmacophore model.

The five crystal structures were chosen to cover most of the chemical space occupied by their 17βHSD1 ligands, and both the presence of the cosubstrate ($NADP^+$/NADPH) as well as a good resolution was considered important for their selection. This because our pharmacophore model should integrate both ligand- and protein-derived information, gained from the analysis of different crystal structures.
Indeed, superimposition of the mentioned 3D complexes (ligands and proteins) enabled us to define the pharmacophore features of both the ligands and of the constant regions of the protein, involved in ligand-protein interaction. While the selection of the ligand-derived features was focused on the slightly different chemical properties of substrates and inhibitors, the protein-derived features were chosen considering "rigid" active site residues (all atom RMSD for all crystal structures < 0.5 Å) as well as amino acids important for the enzymatic activity. Additionally, the flexible βFαG'-loop (residues 187-196) [53] lining the active site was excluded, and additional Don/Acc features were retained from the cofactor ($NADP^+$).
Thus, on the ligand side the pharmacophore model (Figure 4) consists of five hydrophobic/aromatic features **HY1**-**HY5** (four from steroid scaffold and one from E2B/HYC, respectively), one aromatic ring projection **P5** (associated to **HY5;** used to direct the ligand placement in the pharmacophore screen), three H-bond acceptor-and-donor **AD1**-**AD3** (two from the steroid scaffold and one from E2B) and one H-bond donor **D4** (corresponding to the NH_2 of the amide in E2B).

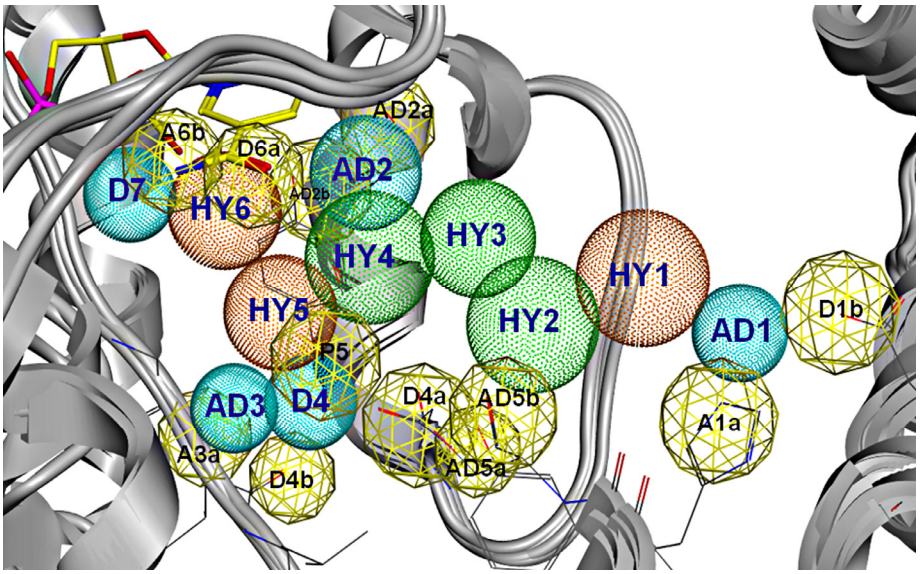


Figure 4. Pharmacophore model. The pharmacophoric features derived from the ligands are rendered as dotted spheres and are color-coded: dark orange for aromatic ring and aromatic ring projection (**HY1** and **HY5**), green for hydrophobic regions (**HY2-HY4**) and magenta for acceptor and donor atom features (**AD1-AD3** and **D4**). The identified aromatic ring projection **HY6** as well as the donor projection feature **D7** is not exploited by inhibitors with steroidal molecular scaffold. The protein-derived acceptor or donor features (**A1a**, **D1b**, **AD2a**, **AD2b**, **A3a**, **D4a**, **D4b**, **AD5a**, **AD5b**, **D6a** and **A6b**) and the aromatic ring projection **P5** are depicted as yellow, meshed spheres.

Nine acceptor (A) or donor (D) feature projections were derived from the protein and used to direct the ligand orientation in the pharmacophore screening (projections indicate putative protein binding partners; the number indicate the ligand feature, while the small letters a and b the inverse H-bonding properties of residues involved in a common network, e.g. A1a is a donor and D1b is an acceptor, and both interact with AD1): **A1a** - His221, **D1b** - Glu282, **AD2a** - Ser142, **AD2b** - Tyr155, **A3a** - Leu96, **D4a** - Asn152, **D4b** - Leu95, **AD5a** - Ser222, **AD5b** - Tyr218. In addition, four features were also retained from the cofactor NADP(H): **A6b** and **D6a** from the amide moiety and **HY6** as aromatic ring projections from the nicotinamide ring (potential interaction site of the ligand with Tyr155 and cofactor), as well as **D7** as a acceptor projection (phosphate group of the cofactor). More geometric properties of the pharmacophore are attached as supporting information in TableS1.
This pharmacophore model, comprising 23 features, was now used to screen our small molecule *in house* library and the virtual hits were tested experimentally. A partial match strategy was adopted for the screening, in which the molecules are left free to be placed into the pharmacophore and only virtual hits are retained that cover at least six features.
Compound **5**, which resulted to be the most potent hit, covers the following six features: **HY2-HY5**, **AD2** and **D4** (Figure 5).

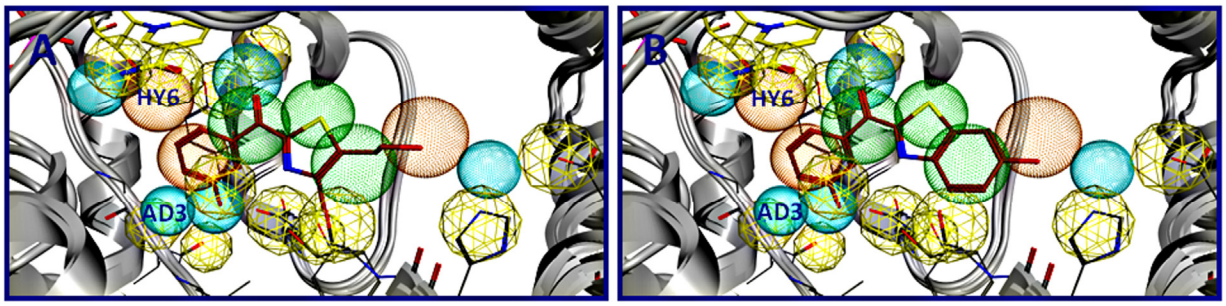


Figure 5. Rigidification. Compound **5** (A) and compound **6** (B) mapped to the pharmacophore model.

Interestingly, in a subsequent screening, also compound **6**, the newly synthesized, rigidified homologue of **5**, was found to match six features. Overlaying it with equilin, one of the steroidal ligands used to build the pharmacophore model, shows that the phenyl ring of the benzothiazole moiety can mimic the steroidal B ring (see figure S1 in supporting information).

2) Chemistry.

The synthesis of the thiazolyl derivative **5** was performed as shown in Figure 6 starting from the commercially available 2-(4-methyl-1,3-thiazol-5-yl)ethanol and 3-hydroxybenzaldehyde.

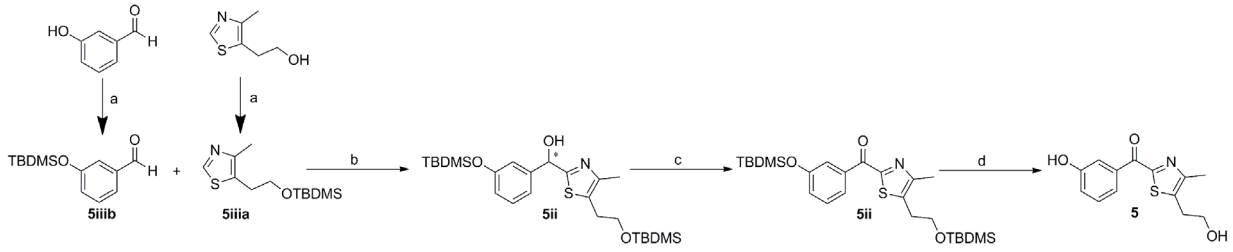
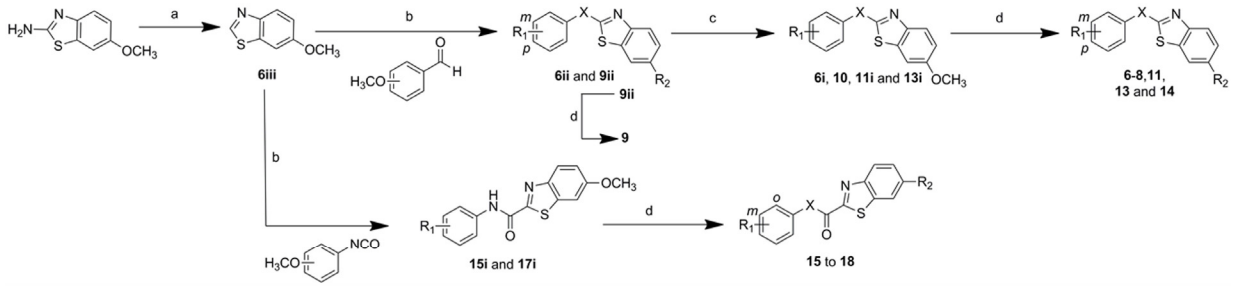


Figure 6. Synthesis of compound 5. Reagents and conditions: (a) TBDMSiCl, imidazole, DMF, rt, 20 h; (b) Method A: 1) nBuLi, anhydrous THF, - 70 °C, 1 h; 2) anhydrous THF, - 15 °C, 90 min; (c) Method C: SIBX, anhydrous THF, 0 °C to 60 °C, 20 h; (d) TBAF, THF, rt, 2 h.

To avoid side reactions due to the presence of the free hydroxy groups, we decided to protect both groups using tert-butyldimethylsilyl chloride and imidazole in DMF at room temperature, overnight [59], to afford **5iiia** and **5iiib**, respectively. Then nucleophilic addition (method A) [60] of in situ 2-lithiated **5iiia** to **5iiib** in THF at -15°C for 90 minutes yielded the secondary alcohol **5ii**. The latter was oxidized to the carbonyl derivative **5i** using stabilized 2-iodoxybenzoic acid (SIBX) as oxidative reagent in THF at 60°C (method C) [61] prior to the deprotection of both silyl protecting groups under mild basic conditions (TBAF in THF at room temperature for 2 hours) [62] to afford compound **5**.

The benzothiazolyl derivatives **6-11** and **13-18** were synthesized as shown in Figure 7.

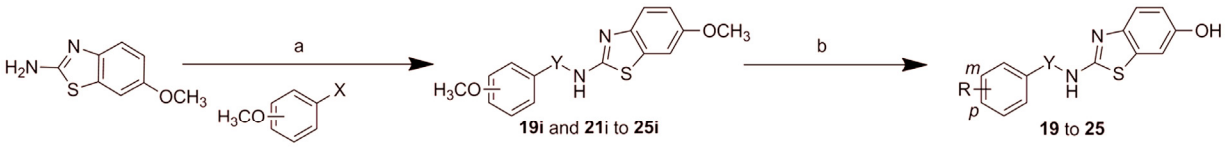


compound	X	R_1	R_2	compound	X	R_1	R_2
6ii	CHOH	*m*-OCH_3	OCH_3	**6**	CO	*m*-OH	OH
9ii	CHOH	*p*-OCH_3	OCH_3	**7**	CHOH	*m*-OH	OH
6i	CO	*m*-OCH_3		**8**	$CHOCH_3$	*m*-OH	OH
11i	CH_2	*p*-OCH_3		**9**	$CHSCH_3$	*p*-OH	OH
13i	CO	*p*-OCH_3		**10**	CH_2	*p*-OH	
15i		*o*-OCH_3		**11**	CH_2	*p*-OH	OH
17i		*m*-OCH_3		**13**	CO	*p*-OCH_3	OH
				14	CO	*p*-OH	OH
				15	NH	*o*-OH	OCH_3
				16	NH	*o*-OH	OH
				17	NH	*m*-OH	OCH_3
				18	NH	*m*-OH	OH

Figure 7. Synthesis of compounds 6-11 and 13-18. Reagents and conditions: (a) 1) $NaNO_2$, H_3PO_4 (85 %), - 10 °C, 20 min, 2) H_3PO_2, H_3PO_4 (85 %), - 10 °C to rt, 20 h; (b) Method A: 1) nBuLi, anhydrous THF, - 70 °C to - 20 °C, 1 h, 2) for **6ii** and **9ii**: methoxy benzaldehyde, for **15i** and **17i**: methoxy benzoisocyanate, anhydrous THF, - 15 °C, 90 min; (c) for **6i** and **13i,** Method C: SIBX, anhydrous THF, 0 °C to 60 °C, 20 h; for **10** and **11i**: TMSiCl, NaI, CH_3CN, reflux, 20 h; (d) Method D: for **6-9** and **15-18**: $BF_3S(CH_3)_2$, anhydrous CH_2Cl_2, rt, 20 h; for compounds **11** and **13-14,** Method E: pyridinium hydrochloride, 220 °C, 4 h.

The commercially available 6-methoxy-benzothiazol-2-yl amine was reduced to **6iii** in the first step via a previously described diazotation followed by reductive elimination of nitrogen [63]: 6-methoxy-benzothiazol-2-yl amine was first dissolved in 85 % phosphoric acid, heated up until complete solubilisation and then cooled to -10°C, prior the slowly addition of aqueous solution of $NaNO_2$ to yield the diazonium salt. The resulting instable intermediate was subsequently transformed in **6iii** adding the former solution to a 0°C pre-chilled 50 % aqueous phosphonic acid solution and let the temperature rise to room temperature overnight. Then nucleophilic addition (method A) of in situ 2-lithiated **6iii** to 3-methoxybenzaldehyde or 4-methoxybenzaldehyde, respectively, in THF at -15°C for 90 minutes, allowed us to afford the secondary alcohols **6ii** and **9ii**, respectively. The same method was used for the synthesis of **12ii**, starting from the commercially available benzothiazole and 3-methoxabenzothiazole, and the preparation of amides **15i** and **17i**, starting from **6iii** and 2-methoxybenzoisocyanate or 3-methoxybenzoisocyanate, respectively. The secondary alcohols **6ii**, **9ii** and **12ii** were oxidized (method C) to the carbonyl derivatives, **6i**, **12i** and **13i**. The ether cleavage on the methoxy groups of **9ii** with $BF_3S(CH_3)_2$ in anhydrous CH_2Cl_2 at room temperature for 20 hours (method D) [42] took place with concomitant formation of the thioether derivative **9**. The reduction of **9ii** using trimethyl silyl chloride and NaI in acetonitrile [64] led to the formation of the desired compound **11i** and an additional product (**10**), where one of the methoxy group was retained. The reaction of **6i** with $BF_3S(CH_3)_2$ (method D) led to the formation of the desired compound **6** and two additional reduced products (**7** and **8**). The ether cleavage of the methoxy groups of **11i** and **13i** was carried out by using pyridinium hydrochloride at 220°C for 4 hours (method E) [42] to afford compounds **11**, **13** and **14**. The ether cleavage on the methoxy groups of **15i** and **17i** with $BF_3S(CH_3)_2$ (method D) allowed us to obtain compounds **15-18.**

The benzamides (**19-21**), benzenesulfonamide (**22**), urea (**23**), thiourea (**24**) and acetamide (**25**) derivatives were afforded in a common 2-steps synthetic pattern (Figure 8).



compound	Y	compound	Y	R
19i	CO	**19**	CO	*p*-OCH_3
21i	CO	**20**	CO	*p*-OH
22i	SO_2	**21**	CO	*m*-OH
23i	NHCO	**22**	SO_2	*m*-OH
24i	NHCS	**23**	NHCO	*m*-OH
25i	CH_2CO	**24**	NHCS	*m*-OH
		25	CH_2CO	*m*-OH

Figure 8. Synthesis of compounds 19-25. Reagents and conditions: (a) Method B: pyridine, 100°C, 20 h, for **19i** to **21i**: methoxy benzoylchloride, for **22i**: methoxy phenylsulfochloride, for compound **23i**: methoxy benzoisocyanate, for **24i**: methoxy benzoisothiocyanate, for compound **25i**: methoxy benzylchloride; (b) for **19-21**, Method D: $BF_3S(CH_3)_2$, anhydrous CH_2Cl_2, rt, 20 h; for **22-25**, Method F: BBr_3, CH_2Cl_2, -78 °C to rt, 20 h.

Compounds **19i** and **21i-25i** were synthesised via amide coupling (method B) [65] starting from the commercial available 6-methoxy-benzothiazol-2-ylamine and 4-methoxy-benzoyl chloride, 3-methoxy-benzoyl chloride, 3-methoxy-sulphonyl chloride, 3-methoxy-benzoisocyanate, 3-methoxy-benzoisothiocyanate and 3-methoxy-phenyl acetyl chloride, respectively. The ether cleavage of the methoxy groups was carried out with pyridinium hydrochloride (method E) or BBr_3 (method F) [44] yielding compounds **19-21** and **22-25**, respectively.

3) Biological results.

a) Activity: Inhibition of human 17β-HSD1. In the cell free assay, the placental cytosolic enzyme was used. Tritiated E1 (final concentration: 500 nM) was incubated with 17β-HSD1, cofactor (500 µM), and inhibitor as previously described [52]. After HPLC separation of substrate and product, the amount of labeled E2 formed was quantified. The hybrid inhibitor (**EM-1745**) evaluated using recombinant protein in a cell free assay as described by Poirier *et al*. [55] was used as external reference and gave similar values as described (IC_{50} = 52 nM). Compounds showing less than 10 % inhibition at 1µM were considered to be inactive. IC_{50} values were determined for compounds showing more than 70 % inhibition at 1 µM. The inhibition values of the test compounds are shown in Table 1. Compound **2** (Figure 1), identified in our previous study [44] was used as internal reference (IC_{50} = 8 nM).

The rigidified benzothiazole derivative **6** was far more potent towards the target enzyme (91 % at 1 µM) than **5** (34 % at 1 µM) and thus turned out to be a very promising scaffold (IC_{50} = 44 nM), taking into account also its low molecular weight (271 g/mol).

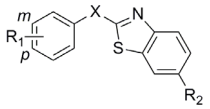
The inhibitory potency of the compounds is strongly dependent on length and type of the bridge connecting the aromatic moieties. The replacement of the flat sp^2 bridge (methanone in compound **6**) by a tetrahedral sp^3 one like alcohol (**7**), methoxy (**8**), methylsulfanyl (**9**) and methylene (**11**) turned out to be deleterious for the inhibitory activity at the target enzyme. Compounds **7-9** showed activities of 28 %, 13 % and 7 %, respectively at 1 µM concentration. The loss of potency obtained from those compounds as well as from compounds **10** and **11** seems to be influenced by the bridge geometry and not by the H-bonding properties, since an alcohol (acceptor and donor), a methoxy methyl (acceptor only) and a methylene (neither acceptor nor donor) were all less potent than the carbonyl group.

As the keto bridge (**6**) appeared to be the most appropriate moiety, it was taken as starting point to perform further modifications on the influence of the H-bonding groups. Compound **12**, without hydroxy group in the 6 position of the benzothiazole moiety, was designed to evaluate the importance of this functional group (**6i**) and showed 8 fold lower inhibitory activity than **6** (see Table 1). Furthermore, to investigate whether either methoxy group or hydroxy group in para on the phenyl moiety of compound **6** could exploit the pharmacophoric

feature AD3 (see Figure 4 and 5) compounds **13** and **14** were synthesized. The lower inhibitory activity of the para methoxy derivative **13** (27 % at 1 μM) compared to the hydroxy compound **14** (85 % at 1 μM) proves the importance of an H-bonding donator in this position; there is however a decrease of activity when the hydroxy group is shifted from meta to para.
Derivatives with two bridging atoms between the hydroxy phenyl and the hydroxyl benzothiazole moieties, like amide **18**, retroamide **21** and sulphonamide **22**, were also synthesized. Unlike the one unit bridge class, in the amide series, both the H-bonding properties as well as the sp^2 geometry turned out to be discriminating factors for 17β-HSD1 inhibitory activity. The introduction of an amide bridge in the place of the carbonyl function (compound **18**: 40 % at 1 μM *vs* compound **6**: 91 % at 1 μM) results in a significantly decreased inhibitory potency as well as in the case of sulphonamide derivative **22** (no inhibition at 1 μM). Interestingly, replacement with retro amide (**21**) gives only a slight decrease of inhibitory activity (**21**: IC_{50} = 243 nM *vs* **6**: IC_{50} = 44 nM).
Independently on the nature of the bridge (amide or retroamide), changing the hydroxyl substitution pattern (**16** and **20**) is detrimental for activity as well as protection as methoxy group (**15**, **17** and **19**).
The extension of the bridge to three units resulted in the inactive urea **23** and benzylamide **25**. Interestingly the thiourea **24** showed a moderate inhibitory activity.

b) 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 for compounds showing 17β-HSD1 inhibitory activity of 70 % at 1 μM using an established assay [52] similar to the 17β-HSD1 test and a selectivity factor (SF = IC_{50}(HSD2)/IC_{50}(HSD1)) was determined. Placental microsomal 17β-HSD2 was incubated with tritiated E2 (final concentration: 500 nM) in the presence of NAD^+ (1500 μM) and inhibitor. Separation and quantification of labeled substrate (E2) and product (E1) was performed by HPLC using radiodetection. IC_{50} values and selectivity factors are presented in Table 2.

Table 2. Influence of the bridge and hydroxy group on the inhibition of the human 17β-HSD1 and 17β-HSD2.



Compound	X	R_1	R_2	HSD1[a] [IC_{50} (nM)[c]]	HSD2[b] [IC_{50} (nM)[c]]	SF[d]
6	CO	*m*-OH	OH	44	1035	24
12	CO	*m*-OH	H	365	1356	4
14	CO	*p*-OH	OH	243	2471	10
18	NHCO	*m*-OH	OH	1307	3813	3
21	CONH	*m*-OH	OH	243	9264	38

[a] Human placenta, cytosolic fraction, substrate [^{3}H]E1 + E1 [500 nM], cofactor NADH [500 μM]. [b] Human placenta, microsomal fraction, substrate [^{3}H]E2 + E2 [500 nM], cofactor NAD^+ [1500 μM]. [c] Mean values of three determinations, standard deviation less than 10 %. [d] Selectivity factor = IC_{50} (HSD2)/IC_{50}(HSD1).

Among the series bearing the carbonyl function as bridge, compound **6** showed the best selectivity factor (24 fold more active on HSD1 than HSD2). The absence of hydroxyl on the benzothiazole (**12**) or moving the meta hydroxyl of **6** to para hydroxyl of **14** leads to a drop in selectivity. Furthermore, compound **21**, bearing the retro amide function as bridge, shows higher selectivity factor (SF = 38) than compound **18** (amide bridge, SF = 3) as well as compound **6** (carbonyl bridge, SF = 24).
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, either agonistically or antagonistically, could counteract the therapeutic concept of mainly local action. The binding affinities of the most interesting compounds of this study (**6** and **21**) were determined using recombinant human protein (0.25 pmol of ERα or ERβ, respectively) in a known competition assay [52] applying [^{3}H]E2 (10 nM) and hydroxyapatite (data are shown in Table 3).

Table 3. Binding affinities for the human estrogen receptors α and β of lead compounds.

	RBA[a] (%)	
Compound	**ERα[b]**	**ERβ[b]**
6	1.000 < RBA < 10.000	0.100 < RBA < 1.000
21	0.010 < RBA < 0.100	RBA < 0.001

[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.

A compound exhibiting less than 0.1 % relative binding affinity towards ERs was considered not to bind to the ERs under *in vivo* conditions (using T47-D [30] cell line obtained from ECACC, Salisbury). This assumption is based on the comparison of the compound's binding affinity with the one of E1. E1 itself indeed is a ligand of the ERs with a relative binding affinity of about 10% [66-67]. Due to its RBA value of less than 0.1%, a selective inhibitor will be displaced by E1 from the ER binding site and is therefore unlikely to exert an ER mediated effect *in vivo*.
Compound **6** turned out to considerably bind to both estrogen receptors, whereas compound **21** shows very marginal affinity towards them (RBA lower than 0.1 % for ERα and lower than 0.001 % in the case of ERβ).

c) Further Biological Evaluation. Additionally, the intracellular potency of the most selective compound (**21**) on E2 formation was evaluated as previously described [52] using the T47-D cell line that expresses 17β-HSD1 and - to a much lesser extent -17β-HSD2 [68]. The compound inhibited the formation of E2 after incubation with labelled E1 showing an IC_{50} value of 245 nM. This result indicates that the compound is able to permeate the cell membrane, and to inhibit the transformation of E1 into E2 intracellularly.

4) Binding mode analysis and SAR
In order to rationalize the influence of the different bridges in this new set of 17β-HSD1 inhibitors, and to clarify the position of the phenyl moiety, all synthesized compounds reported herein were screened with our pharmacophore model.
The carbonyl compound **6** matched six pharmacophore features (**HY2**-**HY5**, **AD2** and **D4**), but it does not exploit the feature **HY1**, which corresponds to the steroidal A-ring moiety (see Figure 9A).

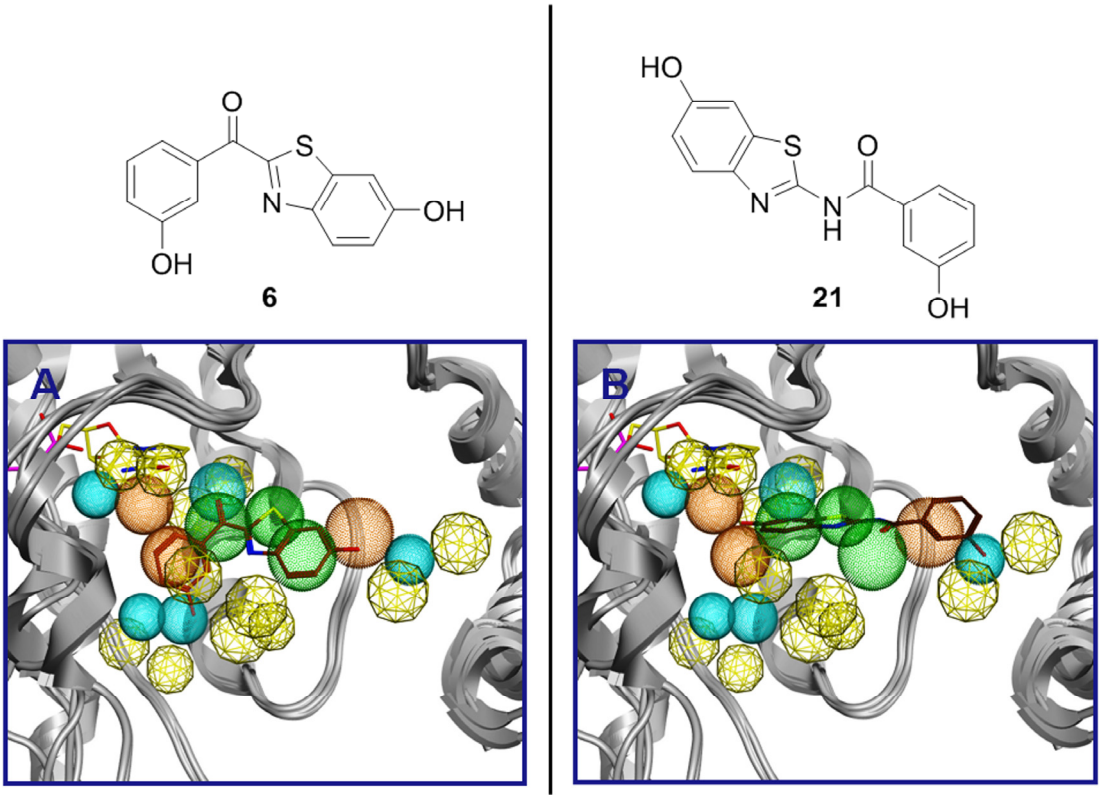


Figure 9. Pharmacophoric features exploited by the two lead compounds. Six for compound **6** (showed in A) and five for compound **21** (showed in B).

The retroamide **21** was found in a different orientation in the pharmacophore with respect to **6**, without occupying the feature **HY6**. It might adopt two preferred isomeric forms (cis and trans) which cannot be separated at room temperature because they interconvert readily. The energetically favorable [69] linear (trans) isomer exploits five pharmacophoric features (**HY1-HY4** and **AD1**). In addition the hydroxy group on the benzothiazole in proximity of aromatic features **HY5** and **HY6**, as depicted in Figure 9B, while the cis isomer could not match the required six features. Binding of **21** in the trans-form may thus be assumed. Interestingly, the retro amide bridge exploits the pharmacophoric feature **HY2**, in the case of compound **6** exploited by the benzothiazole phenyl ring, suggesting these two compounds to bind differently. Thus, both compound **6** and **21** were considered as two new lead structures.
To better understand the favourable interactions achieved by compounds **6** and **21** in the five different crystal structures used to build up the pharmacophore, a thorough analysis of the respective surrounding residues was performed. Herefore also the flexible amino acid residues, formerly excluded in the pharmacophore generation process, were considered. The cocrystallized steroidal ligands were replaced by either **6** or **21** (via the pharmacophore) and the resultant complexes were optimized with the ligX module of MOE. This module optimize the protein-ligand complex by first adjusting the protonation state of the residues, tethering the active site heavy atoms and, finally, energy minimizing the complex. The results highlighting the interactions between our 17β-HSD1 inhibitors and the five crystal structures are shown in Table 4.

Table 4. Interactions found in the complexes between the lead compounds (6 and 21) and the five 17β-HSD1 crystal structures used to build up the pharmacophore.

Compound	interactions	amino acid residues	1equ	1i5r	3hb5	1a27	1dht
6	**H**	Ser142 (donor)		3.31	3.38	3.70	3.26
		Asn152 (acceptor)	2.53[a]	2.45	2.56		2.97
		Asn152 (donor)				2.49	2.55
		Tyr155 (donor)	3.90		3.80	3.71	
		His221 (d donor)	3.13	3.33			
	π	Tyr 155	6.71	3.98	3.82	4.06	4.16
		Arg258	4.89				
21	**H**	Tyr155 (acceptor)	2.91	3.13	3.03	2.75	3.03
		Tyr218 (donor)		3.82			
		His221 (donor)	2.47	2.45		2.65	2.51
		Glu282 (acceptor)	2.43		2.52		2.43
	π	Arg258	4.34				

[a] Distance (Å) between the heteroatoms for H-bonds (H) and between centroids or centroid and cation for π-interactions (π).

For compound **6** five hydrogen bond interactions could be observed: between its meta hydroxy group of the phenyl ring and Asn152 (d_{O-O} = 2.45-2.97 Å and d_{O-N} = 2.49-2.55 Å), between the carbonyl oxygen of the bridge and both Ser142 and Tyr155 (d_{O-O} = 3.26-3.70 Å and d_{O-O} = 3.71-3.90 Å), and between the hydroxy group of the benzothiazol and His221 (d_{O-N} = 3.13-3.33 Å). In addition, a cation-π interaction between the phenyl ring of benzothiazole and Arg258 ($d_{N-centroid}$ = 4.89 Å) as well as a π-π interaction between the phenyl ring and Tyr155 ($d_{centroid-centroid}$ = 3.98-6.71 Å) were found.

For compound **21** also five hydrogen bond interactions were identified: between the meta hydroxy group of the phenyl ring and both His221 and Glu282 (d_{O-N} = 2.45-2.65 Å and d_{O-O} = 2.43-2.52 Å, respectively), between the carbonyl oxygen of the amide bridge and Tyr218 (d_{O-O} 3.82 Å), and between the hydroxy group of the benzothiazole and Tyr155 (d_{O-O} = 2.75-3.13 Å). Again, a cation-π interaction between the phenyl ring and Arg258 was found ($d_{N-centroid}$ = 4.34 Å).

It is noteworthy that only the interactions with Tyr155 (π-π stacking for **6** and hydrogen bond for **21**) could be observed for all five crystal structure-inhibitor complexes optimized with ligX, whereas all the other were present depending on which crystal structure was used. This further substantiated the importance of Tyr155 for the stabilization of a ligand.

The differences in inhibitory activity between **6** (IC_{50} 43 nM) and **21** (IC_{50} 243 nM) could not be thoroughly explained by considering only the hydrogen bonds, since only in 3hb5 and 1a27 more interactions were found for **6** compared to **21**. On contrary, the π-π stacking interaction of **6** with Tyr155, missing for **21**, as well as the cation-π (charge transfer) interaction between Arg258 and the phenyl ring of benzothiazole seem to be particularly important for the 17β-HSD1 inhibition. Compound **21** was found to be involved only in π-stacking with Arg258, and no H-bond interactions with Ser142 were found in any of the complexes (exemplificative shown for 1equ, Figure 10 and 11).

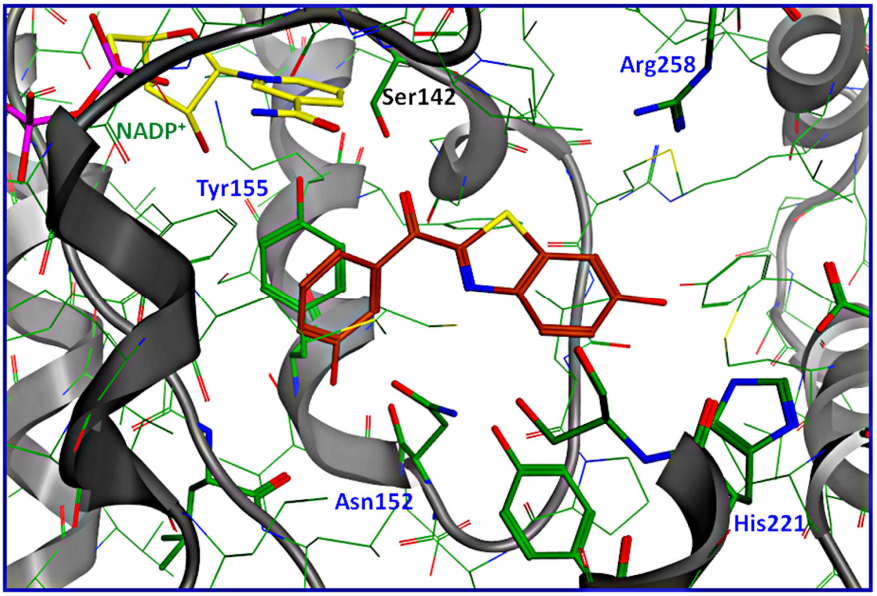


Figure 10. Pharmacophore derived complex between 17β-HSD1 (X-ray 1equ) and compound 6 (dark orange). $NADP^+$ (green), interacting residues (blue), potential interacting residues (black) and ribbon rendered tertiary structure of the active site are shown.

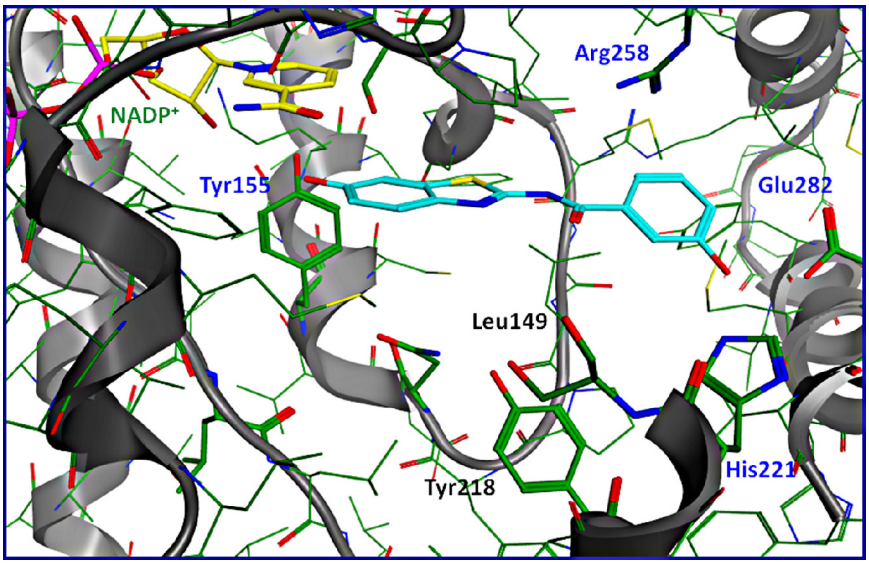


Figure 11. Pharmacophore derived complex between 17β-HSD1 (X-ray 1equ) and compound 21 (magenta). $NADP^+$ (green), interacting residues (blue), potential interacting residues (black) and ribbon rendered tertiary structure of the active site are shown.

In addition, the interactions between the hydroxy group in HY1 of compound **6** and His221, as found in different crystal structures (1equ and 1i5r), is in agreement with the postulated binding mode.
Summarizing, the two lead compounds **6** and **21** bind very differently: they are flipped horizontally by 180° and with the planes of their benzothiazole moieties form an angle of 90°.

Discussion

The inhibitor design concept of the present study triggered the synthesis of compound **6** and **21** as promising new lead structures by optimizing a novel, *in silico* identified, core scaffold (**5**).
The classical medicinal chemistry approach of rigidification was successfully applied to compound **5** and led to the discovery of the highly potent benzothiazole **6**. The introduction of the aromatic benzothiazole freezes the position of hydroxyl moiety in an ideal position to establish an H-bond with H221. In addition, this aromatic benzothiazole can undergo a cation-π interaction with Arg258, explaining the high gain in potency of **6** compared to **5**.
In the optimization process the carbonyl bridge of **6** was varied using several linkers with different lengths, geometries and H-bonding properties. From the biological results as well as from the performed *in silico* studies it became apparent, that the 17β-HSD1 inhibitory activity is highly influenced by the nature of the linker: the comparison of inactive tetrahedral alcohol (**7**), methoxy (**8**), thiomethyl (**9**), methylene (**10**), and sulphonamide (**22**) with the active, planar carbonyl (**6**) and amide derivatives (**18** and **21**) led us to conclude that a flat geometry of the linker is required for the activity. The fact that the retroamide **21** is five times more active than the amide **18** can be explained by a steric clash observed between the carbonyl of amide bridge and Leu149. Furthermore, the carbonyl group of **21** was found to establish a H-bond interaction with Tyr218 which is not possible for **18**.
Comparing the binding modes of **6** and **21**, it becomes clear that the hydroxyl-phenyl moieties of the two compounds do not interact with the same area of the enzyme. In case of compound **6, HY5** and **D4** are plausible features covered by the hydroxyphenyl moiety. The meta hydroxy-phenyl moiety of **21**, on the other hand, exploits **HY1** and **AD1**. The difference in activity between **6** and **21** is in agreement with the number of features covered by each compound (6 versus 5).
It is striking that the newly discovered class of benzothiazole derivatives shows structural characteristics which are similar to those of other classes of 17β-HSD1 inhibitors: two phenolic hydroxy-groups separated by a rather unpolar scaffold structure [39, 44, 51]. The necessity for the lipophilicity of the scaffold is reflected by the gain in potency observed with the thiourea (**24**: 62 % inhibition at 1 μM) compared to the less lipophilic urea (**23**: no inhibition at 1 μM).
Analysis of the amino acid residues which surround compound **6** in its pharmacophore binding pose indicates that two hydrogen bonds with Asn152 and one π-π interaction with Tyr155 are established. Recently published docking studies in the case of bicyclic substituted hydroxyphenylmethanones suggest similar interactions [51]. Interestingly, there is a decrease of activity in both compound classes when the hydroxy group is shifted from the *meta*- to the *para* position. This similarity in SAR supports the hypothesis that the hydroxyphenyl moieties of both compound classes bind in the same area of the enzyme.
In order to evaluate the protein-ligand interaction, the ligands of the different X-ray structures studied were replaced by compound **6** and **21** according to their pharmacophoric binding modes and the interactions between the inhibitors **6** and **21** and each of the crystal structures were considered. The maximum number of interactions was observed with the crystal structure 1equ, originally containing the inhibitor equiline. This because Arg258 of 1equ is turned into the active site. Notably, the importance of this amino acid residue was already postulated by Alho-Richmond S et al. [70], who proposed to target it in the inhibitor design process.
The selectivity against 17β-HSD2 should be achieved to mainly avoid systemic effects since the expression of this enzyme is downregulated in EDD tissues but it is present in several organs (i.e. liver, small intestine, bones). However, it is difficult to estimate how high the SF should be to minimize potential side effects due to the lack of respective *in vivo* data. For our drug development program, an SF of approximately 20 is considered sufficient to justify further biological evaluation. In this study the retroamide **21** is the most 17βHSD2 selective compound identified. It is striking that the amide **18** shows a complete loss in selectivity

against 17βHSD2. As no 3D-structure of this enzyme is known, it is not possible to figure out the structural differences between the two enzymes at protein level. These results indicate that the orientation of the amide group is an important feature to gain activity for 17βHSD1 and selectivity against 17βHSD2.

Conclusions

In the present study two new classes of 17β-HSD1 inhibitors were identified. As no X-ray structure of 17βHSD1 complexed with non-steroidal compound exists the followed pharmacophoric approach resulted in being a powerful tool since it combines the knowledge of crystal structure-steroidal ligand complexes with structure analysis of active nonsteroidal inhibitors. In this study, **6** is the most active compound in term of 17β-HSD1 inhibition (IC_{50} = 44 nM), showing fair selectivity against 17β-HSD2 (SF = 24) but pronounced affinity to ERs (RBA < 10 for Erα and RBA < 1 for ERβ).
Compound **21** on the other hand showed medium inhibitory activity at the target enzyme (IC_{50} = 243 nM) as well as fair selectivity (SF = 38) against 17β-HSD2 and ERs (RBA < 0.1 for Erα and RBA < 0.001 for ERβ). Furthermore, **21** inhibits the formation of E2 intracellularly with an IC_{50} value in the nanomolar range (245 nM). Thus shows that it is able to enter the cell, and to be active at the target enzyme. Further optimizations of these first benzothiazole-type lead compounds are underway in order to develop potential candidates for *in vivo* application.

Supporing information

Experimental section

Chemical Methods. Chemical names follow IUPAC nomenclature. Starting materials were purchased from Acros, Aldrich, Alfa Aesar, Maybridge, 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).
^{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 = 3.32 ppm (^{1}H-NMR) and δ = 49.05 ppm (^{13}C-NMR), CD_3COCD_3: δ = 2.05 ppm (^{1}H-NMR) and δ = 29.9 ppm (^{13}C-NMR) and CD_3SOCD_3: δ = 2.50 ppm (^{1}H-NMR) and δ = 39.43 ppm (^{13}C-NMR). Signals are described as s, d, t, dd, ddd, m, dt, q for singlet, doublet, triplet, doublet of doublets, doublet of doublets of doublets, multiplet, doublet of triplets and quadruplet respectively. All coupling constants (*J*) are given in hertz (Hz).
Mass spectra (ESI) were recorded on a TSQ Quantum (Thermo Finnigan) instrument.
Tested compounds are > 95 % chemical purity as measured by HPLC. The methods for HPLC analysis and a table of data for all tested compounds are provided in the supporting information.
The following compounds were prepared according to previously described procedures: 3-(tert-butyl-dimethyl-silanyloxy)-benzaldehyde (**5iiib**) [1], 6-methoxy-1,3-benzothiazole (**6iii**) [2]**,** (6-hydroxy-1,3-benzothiazol-2-yl)(3-hydroxyphenyl)methanone (**6**) [3], (6-methoxy-1,3-benzothiazol-2-yl)(4-methoxyphenyl)methanone (**13i**) [3], 1,3-benzothiazol-2-yl(3-hydroxyphenyl)methanone (**12**) [4], (6-hydroxy-1,3-benzothiazol-2-yl)(4-methoxyphenyl)methanone (**13**) [3], (6-hydroxy-1,3-benzothiazol-2-yl)(4-hydroxyphenyl)methanone (**14**) [3], 4-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**19i**) [5], 3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**21i**) [5].

Method A, general procedure for nucleophilic addition: To a solution of thiazole derivative unsubstituted in position 2 (1 eq) in anhydrous THF a 2.5 M solution of n-BuLi (1 eq) in hexane was added dropwise at -78 °C (dry ice/acetone bath) under anhydrous conditions. The reaction mixture was stirred for 1 h at –78 °C to form the formyl anion in situ. Then a solution of electrophile (0.8 eq) in

anhydrous THF was added dropwise at –78 °C and additionally stirred for 30 min. The temperature was then risen to –20 °C (NaCl/ice/dry ice/acetone bath) and the stirring was continued for 90 min. Saturated NH_4Cl solution was added to quench the reaction, and the aqueous layer was extracted three times 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 or recrystallisation.

Method B, general procedure for nucleophilic substitution: To a solution of 2-amino-6-methoxybenzothiazole (1 eq) in dry pyridine the respective benzoyl chloride, isocyanate or isothiocianate (1 eq.) was added dropwise at room temperature. The reaction mixture was refluxed for 4 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, filtered, and concentrated to dryness. The product was purified by CC or recrystallisation.

Method C, general procedure for oxidation: A mixture of aliphatic alcohol (1 eq) and 2-iodoxybenzoic acid (2 eq) in anhydrous THF was stirred at 0 °C for 10 min. Then, the reaction mixture was heated to 60 °C for 18 h. After cooling to room temperature, saturated sodium thiosulfate solution 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 magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC or recrystallisation.

Method D, general procedure for ether cleavage: To a solution of methoxybenzene derivative (1 eq) in dry dichloromethane at room temperature, boron trifluoride methyl sulfide complex in dichloromethane (1 M, 75 eq per methoxy function) was added dropwise. The reaction mixture was stirred for 20 h at room temperature 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 magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC, preparative TLC, preparative HPLC or recrystallisation, respectively.

Method E, general procedure for ether cleavage: A mixture of methoxybenzene derivative (1 eq) and pyridinium hydrochloride (50 eq per methoxy function) was heated to 220 °C for 18 h. After cooling to room temperature, water, 1 M HCl and ethyl acetate were added. The aqueous layer was separated and 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 CC followed by preparative TLC or preparative HPLC, respectively.

Method F, general procedure for ether cleavage: To a solution of methoxybenzene derivative (1 eq) in anhydrous dichloromethane at -78 °C (dry ice/acetone bath), boron tribromide in dichloromethane (1 M, 5 eq per methoxy function) was added dropwise. The reaction mixture was stirred for 20 h at room temperature 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 CC followed by preparative TLC or preparative HPLC, respectively.

General procedure for purification using preparative HPLC. All declared final compounds were purified via an Agilent Technologies Series 1200-preparative HPLC using a linear gradient run (solvents: acetonitrile, water) from 20 % acetonitrile to 100 % in 36 min.

5-[2-(tert-butyl-dimethyl-silanyloxy)-ethyl]-4-methyl-thiazole (**5iiia**). To a solution of 5-(2-hydroxyethyl)-4-methyl-thiazole (8.0 g, 55.86 mmol) and *tert*-butyldimethylsilyl chloride (12.63 g, 83.79 mmol) in DMF, imidazole (6.46 g, 94.97 mmol) was added in portion for 1 hour at 0 °C. The reaction mixture was stirred for 6 h at room temperature. Water was added to quench the reaction and the aqueous solution was then extracted three times with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated to dryness. The product was used in the next synthetic step without purification; yield: quant. (14.38 g); ^{1}H NMR ($CDCl_3$): 0.06 (s, 6H), 0.80 (s, 9H), 2.32 (s, 3H), 2.88 (t, J = 6.0 Hz, 2H), 3.70 (t, J = 6.0 Hz, 2H), 8.47 (s, 1H); ^{13}C NMR ($CDCl_3$): -2.3, 15.3, 24.9, 32.0, 34.6, 62.2, 127.3, 149.2, 150.0;

{5-[2-(tert-butyl-dimethyl-silanyloxy)-ethyl]-4-methyl-thiazol-2-yl}-[3-(tert-butyl-dimethyl-silaniloxy)-phenyl]-methanol (**5i**). The title compound was prepared by reaction of 5-[2-(tert-butyl-dimethyl-silanyloxy)-ethyl]-4-methyl-thiazole (**5iiia**) (5 g, 19.42 mmol), n-BuLi (2,5 M in hexane; 7.8 mL, 19.42 mmol) and 3-(tert-butyl-dimethyl-silanyloxy)-benzaldehyde (**5iiib**) (3.53 g, 14.94 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 80:20); yield: 64 % (4.06 g); ^{1}H NMR ($CDCl_3$): -0.03 (s, 6H), 0.18 (s, 6H), 0.84 (s, 9H), 0.97 (s, 9H), 2.30 (s, 3H), 2.86 (t, *J* = 6.5 Hz, 2H), 3.70 (t, *J* = 6.5 Hz, 2H), 5.89 (s, 1H), 6.77 (dd, *J* = 8.2 Hz, *J* = 2.5 Hz, 1H), 6.96 (s, 1H), 7.03-7.05 (m, 1H), 7.20 (t, *J* = 7.9 Hz, 1H); ^{13}C NMR ($CDCl_3$): -5.5, -4.4, 18.2, 18.2, 21.0, 25.7, 25.8, 30.0, 63.2, 73.2, 118.2, 119.4, 119.8, 129.3, 129.5, 143.2, 147.6, 155.8, 171.1;

{5-[2-(tert-butyl-dimethyl-silanyloxy)-ethyl]-4-methyl-thiazol-2-yl}-[3-(tert-butyl-dimethyl-silaniloxy)-phenyl]-methanone (**5i**). The title compound was prepared by reaction of {5-[2-(tert-butyl-dimethyl-silanyloxy)-ethyl]-4-methyl-thiazol-2-yl}-[3-(tert-butyl-dimethyl-silaniloxy)-phenyl]-methanol (**5ii**) (0.5 g, 1.01 mmol) and SIBX (0.57 g, 2.02 mmol) according to method C. The product was purified by CC (hexane/ethyl acetate 95:5); yield: 94 % (0.47 g); ^{1}H NMR (CD_3OD) 2.33 (s, 3H), 2.91 (t, *J* = 6.0 Hz, 2H), 3.68 (t, *J* = 6.0 Hz, 2H), 6.94 (d, *J* = 7.9 Hz, 1H), 7.20 (t, *J* = 8.0 Hz, 1H), 7.59 (s, 1H), 7.72 (d, *J* = 7.9 Hz, 1H); ^{13}C NMR (CD_3DO): 15.1, 30.3, 62.0, 117.3, 121.1, 122.8, 129.6, 136.7, 138.2, 152.0, 152.1, 157.1, 163.3;

[5-(2-hydroxyethyl)-4-methyl-thiazol-2-yl]-(3-hydroxy-phenyl)-methanone (**5**). To a solution of {5-[2-(tert-butyl-dimethyl-silanyloxy)-ethyl]-4-methyl-thiazol-2-yl}-[3-(tert-butyl-dimethyl-silaniloxy)-phenyl]-methanone (**5i**) (0.47 g, 0.96 mmol) in THF at 0 °C (dry ice) tetra-n-butylammonium fluoride (0.62 g, 2.38 mmol) in THF was added dropwise. The reaction mixture was stirred for 2 h at room temperature 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 magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC (hexane/ethyl acetate 50:50); yield: 80 % (0.20 g); ^{1}H NMR (CD_3OD): 2.33 (s, 3H), 2.91 (t, *J* = 6.4 Hz, 2H), 3.68 (t, *J* = 6.4 Hz, 2H), 6.94 (d, *J* = 7.9 Hz, 1H), 7.20 (t, *J* = 8.0 Hz, 1H), 7.59 (s, 1H), 7.72 (d, *J* = 7.9 Hz, 1H); ^{13}C NMR (CD_3DO): 15.1, 30.3, 62.0, 117.3, 121.1, 122.8, 129.6, 136.7, 138.2, 152.0, 152.1, 157.1, 163.3; MS (ESI): 264.0 $(M+H)^+$;

(6-methoxy-1,3-benzothiazol-2-yl)(3-methoxyphenyl)methanol (**6ii**)**.** The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazole (**6iii**) (0.5 g, 3.03 mmol), n-BuLi (2,5 M in hexane; 1.2 mL, 3.03 mmol) and 3-methoxy-benzaldehyde (0.27 mL, 2.32 mmol) according to method A. The product was purified first by CC (chloroform/ethyl acetate 70:30); yield: quant. (0.61 g); ^{1}H NMR (CD_3OD): 3.72 (s, 3H), 3.79 (s, 3H), 5.95 (s, 1H), 6.83 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H) 7.02-7.05 (m, 3H), 7.25 (t, *J* = 8.2 Hz, 1H), 7. 6 (d, *J* = 2.5 Hz, 1H), 7.76 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (CD_3DO): 55.0, 55.6, 72.5, 104.8, 112.1, 112.9, 115.2, 118.7, 123.0, 129.4, 135.8, 143.9, 147.2, 147.3, 156.9, 159.2;

(6-methoxy-1,3-benzothiazol-2-yl)(4-methoxyphenyl)methanol (**9ii**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazole (**6iii**) (0.5 g, 3.03 mmol), n-BuLi (2,5 M in hexane; 1.2 mL, 3.03 mmol) and 4-methoxy-benzaldehyde (0.3 mL, 2.32 mmol) according to method A. The product was purified by CC (chloroform/ethyl acetate 70:30); yield: quant. (0.61 g); ^{1}H NMR ($CDCl_3$): 3.79 (s, 3H), 3.85 (s, 3H), 6.06 (s, 1H), 6.88-6.90 (m, 2H), 7.04 (dd, *J* = 9.1 Hz, *J* = 2.5 Hz, 1H), 7.26 (d, *J* = 2.5 Hz, 1H), 7.42 (d, *J* = 8.8 Hz, 2H), 7.84 (d, *J* = 9.1 Hz, 1H); ^{13}C NMR ($CDCl_3$): 55.3, 55.8, 73.9, 104.3, 114.2, 115.5, 116.5, 117.0, 123.4, 128.1, 133.2, 136.6, 146.9, 157.6, 159.8, 172.6;

1,3-benzothiazol-2-yl(3-methoxyphenyl)methanol (**12ii**)**.** The title compound was prepared by reaction of benzothiazole (0.20 mL, 1.85 mmol), n-BuLi (2,5 M in hexane; 0.74 mL, 1.85 mmol) and 3-methoxy-benzaldehyde (0.2 mL, 1.42 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 80:20); yield: 91 % (0.35 g); ^{1}H NMR $CDCl_3$: 3.92 (s, 3H), 6.26 (s, 1H), 6.97-7.03 (m, 1H), 7.24-7.26 (m, 2H), 7.42 (t, *J* = 8.2 Hz, 1H), 7.48-7.51 (m, 1H), 7.57-7.60 (m, 1H), 7.96 (d,

J = 8.5 Hz, 1H), 8.10 (d, *J* = 8.2 Hz, 1H); ^{13}C NMR ($CDCl_3$): 55.2, 74.1, 112.0, 114.3, 119.0, 121.7, 123.0, 125.1, 126.1, 129.8, 135.2, 142.4, 152.4, 159.9, 175.1;

6-methoxy-benzothiazole-2-carboxylic acid (2-methoxy-phenyl)-amide (**15i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazole (**6iii**) (0.20 g, 1.21 mmol), n-BuLi (2,5 M in hexane; 0.5 mL, 1.21 mmol) and 1-isocyanato-2-methoxy-benzene (0.1 mL, 0.93 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 80:20); yield: quant. (0.25 g); ^{1}H NMR (CD_3OD): 3.92 (s, 3H), 4.0 (s, 3H), 6.95 (dd, *J* = 8.2 Hz, *J* = 1.3 Hz, 1H), 7.03 (dd, *J* = 8.2 Hz, *J* = 1.2 Hz, 1H), 7.10 (dq, *J* = 8.2 Hz, *J* = 1.6 Hz, *J* = 0.6 Hz, 1H), 7.17 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H), 7.40 (d, *J* = 2.5 Hz, 1H), 8.03 (d, *J* = 9.1 Hz, 1H), 8.51 (dd, *J* = 7.9 Hz, *J* = 1.6 Hz, 1H), 9.76 (s, 1H); ^{13}C NMR (CD_3OD): 55.9, 56.0, 104.3, 110.5, 117.1, 120.1, 120.3, 121.3, 124.5, 125.2, 127.3, 139.3, 147.7, 148.9, 159.3, 162.0;

6-methoxy-benzothiazole-2-carboxylic acid (3-methoxy-phenyl)-amide (**17i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazole (**6iii**) (0.25 g, 1.51 mmol), n-BuLi (2,5 M in hexane; 0.6 mL, 1.51 mmol) and 1-isocyanato-3-methoxy-benzene (0.2 mL, 1.16 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 80:20); yield: quant. (0.25 g); the compound was used for the next step without characterization.

(6-methoxy-1,3-benzothiazol-2-yl)(3-methoxyphenyl)methanone (**6i**)**.** The title compound was prepared by reaction of (6-methoxy-1,3-benzothiazol-2-yl)(3-methoxyphenyl)methanol (**6ii**) (0.35 g, 1.16 mmol) and SIBX (0.65 g, 2.32 mmol) according to method C. The product was purified by CC (hexane/ethyl acetate 95:5); yield: quant. (0.35 g); ^{1}H NMR (CD_3OD): 3.91 (s, 3H), 3.93 (s, 3H), 7.17-7.22 (m, 2H), 7.41 (d, *J* = 2.5 Hz, 1H), 7.46 (t, *J* = 7.8 Hz, 1H), 8.03-8.04 (m, 1H), 8.10 (d, *J* = 9.1 Hz, 1H), 8.19-8.22 (m, 1H); ^{13}C NMR (CD_3DO): 55.5, 55.9, 103.4, 115.2, 117.6, 120.3, 124.1, 126.5, 129.5, 136.4, 139.1, 148.5, 159.6, 159.8, 164.6, 184.9; MS (ESI): 299.9 $(M+H)^+$;

2-[(4-hydroxyphenyl)(methylsulfanyl)methyl]-1,3-benzothiazol-6-ol (**9**). The title compound was prepared by reaction of (6-methoxy-1,3-benzothiazol-2-yl)(4-methoxyphenyl)methanol (**9ii**) (0.30 g, 1.00 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 15.8 mL, 150.00 mmol) according to method D. The product was purified by CC (hexane/ethyl acetate 50:50); yield: 86 % (0.26 g); ^{1}H NMR (CD_3OD): 2.10 (s, 3H), 5.36 (s, 1H), 6.77-6.79 (m, 2H), 6.97 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H), 7.27 (d, *J* = 1.9 Hz, 1H), 7.31-7.33 (m, 2H), 7.72 (d, *J* = 8.5 Hz, 1H); ^{13}C NMR (CD_3OD): 14.4, 56.7, 107.5, 114.9, 115.5, 116.8, 123.0, 131.1, 131.3, 131.5, 136.6, 145.4, 155.1, 156.9, 168.7; MS (ESI): 302.6 $(M+H)^+$;

6-methoxy-2-(4-hydroxybenzyl)benzothiazole (**10**)**.** A solution of NaI (0.89 g, 5.97 mmol) and Trimethylsilyl chloride (0.8 mL, 5.97 mmol) in acetonitrile was stirred at room temperature for 15 min. The reaction mixture was cooled to 0 °C, and before a solution of (6-methoxy-1,3-benzothiazol-2-yl)(4-methoxyphenyl)methanol (**9ii**) (0.20 g, 0.66 mmol) in acetonitrile was added drop-wise during 15 min. The reaction mixture was then refluxed overnight. To quench the reaction a solution of $NaHCO_3$ was slowly added at room temperature, followed by a solution of $Na_2S_2O_3.5H_2O$. The aqueous solution was then extracted three times 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 (hexane/ethyl acetate 80:20); yield: 28 % (0.05 g); ^{1}H NMR ($CDCl_3$): 3.84 (s, 3H), 4.31 (s, 2H), 6.76-6.79 (m, 2H), 7.04 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H), 7.16-7.19 (m, 2H), 7.24 (d, *J* = 2.5 Hz, 1H), 7.86 (d, *J* = 9.1 Hz, 1H); ^{13}C NMR ($CDCl_3$): 39.3, 55.8, 104.3, 114.3, 115.3, 115.9, 122.8, 128.7, 130.2, 130.3, 136.5, 147.0, 155.5, 157.5, 170.2; MS (ESI): 272.5 $(M+H)^+$;

6-methoxy-2-(4-methoxybenzyl)benzothiazole (**11i**)**.** A solution of NaI (0.89 g, 5.97 mmol) and Trimethylsilyl chloride (0.8 mL, 5.97 mmol) in acetonitrile was stirred at room temperature for 15 min. The reaction mixture was cooled at 0 °C, and a solution of (6-methoxy-1,3-benzothiazol-2-yl)(4-methoxyphenyl)methanol (**9ii**) (0.20 g, 0.66 mmol) in acetonitrile was added drop-wise during 15 min. The reaction mixture was then refluxed overnight. To quench the reaction a solution of $NaHCO_3$ was slowly added at room temperature, followed by a solution of $Na_2S_2O_3 \cdot 5H_2O$. The aqueous solution was then extracted three times 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 (hexane/ethyl acetate 80:20); yield: 56 % (0.11 g); ^{1}H NMR ($CDCl_3$): 3.79 (s, 3H), 3.83 (s, 3H), 4.33 (s, 2H), 6.86-6.89 (m, 2H), 7.04 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H), 7.23 (d, *J* = 2.5 Hz, 1H), 7.26-7.29 (m, 2H), 7.86 (d, *J* = 9.1 Hz, 1H); ^{13}C NMR ($CDCl_3$): 39.6, 55.2, 55.7, 104.2, 114.2, 115.0, 123.1, 125.7, 129.4, 130.2, 136.8, 147.6, 157.4, 158.8, 163.8, 169.3;

1,3-benzothiazol-2-yl(3-methoxyphenyl)methanone (**12i**). The title compound was prepared by reaction of 1,3-benzothiazol-2-yl(3-methoxyphenyl)methanol (**12ii**) (0.35 g, 1.29 mmol) and SIBX (0.72 g, 2.58 mmol) according to method C. The product was purified by CC (hexane/ethyl acetate 90:10); yield: 61 % (0.215 g); ^{1}H NMR $CDCl_3$: 3.92 (s, 3H), 7.23 (dd, *J* = 8.2 Hz, *J* = 2.8 Hz, 1H), 7.48 (t, *J* = 8.2 Hz, 1H), 7.54-7.61 (m, 2H), 8.02-8.06 (m, 2H), 8.24 (t, *J* = 8.2 Hz, 2H); ^{13}C NMR ($CDCl_3$): 55.5, 115.3, 120.6, 122.2, 124.2, 125.8, 126.9, 127.6, 129.5, 136.2, 137.0, 153.9, 159.6, 167.1, 185.1;

2-[(3-hydroxyphenyl)(hydroxy)methyl]-1,3-benzothiazol-6-ol. (**7**). The title compound was prepared by reaction of (6-methoxy-1,3-benzothiazol-2-yl)(3-methoxyphenyl)methanone (**6i**) (0.17 g, 0.57 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 9.0 mL, 85.55 mmol) according to method D. The product was purified by preparative HPLC; yield: 16 % (0.02 g). ^{1}H NMR (CD_3OD): 5.84 (s, 1H), 6.61 (dd, *J* = 2.5 Hz, *J* = 0.9 Hz, 1H), 6.84-6.87 (m, 3H), 7.06 (t, *J* = 7.9 Hz, 1H), 7.17 (d, *J* = 2.5 Hz, 1H), 7.59 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (CD_3OD): 66.8, 75.0, 107.6, 114.6, 116.1, 116.8, 119.0, 123.8, 130.6, 144.6, 147.6, 156.9, 158.7, 175.6; MS (ESI): 274.0 $(M+H)^+$;

2-[(3-hydroxyphenyl)(methoxy)methyl]-1,3-benzothiazol-6-ol. (**8**). The title compound was prepared by reaction of (6-methoxy-1,3-benzothiazol-2-yl)(3-methoxyphenyl)methanone (**6i**) (0.17 g, 0.57 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 9.0 mL, 85.55 mmol) according to method D. The product was purified by preparative HPLC; yield: 16 % (0.02 g). ^{1}H NMR (CD_3OD): 3.35 (s, 3H), 5.42 (s, 1H), 6.63-6.65 (m, 1H), 6.80-6.87 (m, 3H), 7.08 (t, *J* = 7.8 Hz, 1H), 7.17 (d, *J* = 2.2 Hz, 1H), 7.61 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (CD_3OD): 57.8, 84.3, 107.6, 114.9, 116.5, 116.9, 119.4, 124.0, 130.8, 137.6, 141.9, 147.5, 157.1, 158.9, 172.5; MS (ESI): 288.0 $(M+H)^+$;

6-hydroxy-2-(4-hydroxybenzyl)benzothiazole (**11**)**.** The title compound was prepared by reaction of 6-methoxy-2-(4-methoxybenzyl)benzothiazole (**11i**) (0.11 g, 0.37 mmol) and pyridinium hydrochloride (4.29 g, 37.10 mmol) according to method E. The product was purified by recrystallisation (hexane/ethyl acetate); yield: 47 % (0.05 g); ^{1}H NMR (CD_3OD): 4.27 (s, 2H), 6.76-6.80 (m, 2H), 6.96 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H), 7.16-7.18 (m, 2H), 7.21 (d, *J* = 2.5 Hz, 1H), 7.71 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (CD_3OD): 39.9, 107.6, 116.6, 116.7, 118.5, 118.8, 123.4, 129.5, 131.2, 137.6, 137.9, 157.3, 163.7, 171.6; MS (ESI): 258.5 $(M+H)^+$;

6-methoxy-benzothiazole-2-carboxylic acid (2-hydroxy-phenyl)-amide (**15**). The title compound was prepared by reaction of 6-methoxy-benzothiazole-2-carboxylic acid (2-methoxy-phenyl)-amide (**15i**) (0.10 g, 0.32 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 5.0 mL, 47.72 mmol) according to method D. The product was purified by recrystallisation (ethyl acetate); yield: 21 % (0.02 g). ^{1}H NMR (CD_3COCD_3): 3.94 (s, 3H), 6.90-6.95 (m, 1H), 7.02-7.03 (m, 2H), 7.24 (dd, *J* = 9.1 Hz, *J* = 2.7 Hz, 1H), 7.73 (d, *J* = 2.4 Hz, 1H), 8.05 (d, *J* = 9.1 Hz, 1H), 8.36 (d, *J* = 8.2, 1H), 9.38 (s, 1H), 9.87 (s, 1H); ^{13}C NMR (CD_3COCD_3): 56.8, 105.7, 116.5, 118.9, 121.4, 121.4, 126.2, 126.4, 127.5, 140.4, 148.0, 148.7, 158.8, 160.8, 162.9; MS (ESI): 301.1 $(M+H)^+$;

6-hydroxy-benzothiazole-2-carboxylic acid (2-hydroxy-phenyl)-amide (**16**). The title compound was prepared by reaction of 6-methoxy-benzothiazole-2-carboxylic acid (2-methoxy-phenyl)-amide (**15i**) (0.1 g, 0.32 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 5.0 mL, 47.72 mmol) according to method D. The product was purified by recrystallisation (ethyl acetate); yield: 11 % (0.01 g). ^{1}H NMR (CD_3COCD_3): 6.90-6.95 (m, 1H), 7.02-7.03 (m, 2H), 7.20 (dd, *J* = 8.8 Hz, *J* = 2.2 Hz, 1H), 7.54 (d, *J* = 2.5 Hz, 1H), 7.99 (d, *J* = 8.8 Hz, 1H), 8.30 (m, 1H); ^{13}C NMR (CD_3COCD_3): 107.7, 115.9, 118.3, 120.8, 120.9, 125.6, 126.1, 126.9, 139.9, 147.3, 147.7, 158.1, 158.3, 161.6; MS (ESI): 287.0 $(M+H)^+$;

6-methoxy-benzothiazole-2-carboxylic acid (3-hydroxy-phenyl)-amide (**17**). The title compound was prepared by reaction of 6-methoxy-benzothiazole-2-carboxylic acid (3-methoxy-phenyl)-amide (**17i**) (0.10 g, 0.32 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 5.0 mL, 47.72 mmol) according to method D. The product was purified by recrystallisation (ethyl acetate); yield: 21 % (0.02 g). ^{1}H NMR (CD_3COCD_3): 3.94 (s, 3H), 6.67 (dd, *J* = 2.1 Hz, *J* = 0.9, Hz, 1H), 7.2 (m, 2H), 7.36 (m, 1H), 7.36 (t, *J* = 2.1 Hz, 1H), 7.59-7.61 (m, 1H), 7.99 (d, *J* = 8.8 Hz, 1H), 8.83 (s, 1H), 9.86 (s, 1H); ^{13}C NMR (CD_3COCD_3): 57.3, 106.2, 109.2, 113.2, 113.5, 119.3, 126.8, 131.5, 140.8, 141.0, 149.3, 159.8, 159.8, 161.2, 163.6; MS (ESI): 301.0 $(M+H)^+$;

6-hydroxy-benzothiazole-2-carboxylic acid (3-hydroxy-phenyl)-amide (**18**). The title compound was prepared by reaction of 6-methoxy-benzothiazole-2-carboxylic acid (3-methoxy-phenyl)-amide (**17i**) (0.10 g, 0.32 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 5.0 mL, 47.72 mmol) according to method D. The product was purified by recrystallisation (ethyl acetate); yield: 11 % (0.01 g). ^{1}H NMR (CD_3COCD_3): 6.67 (dd, *J* = 2.1 Hz, *J* = 0.9, Hz, 1H), 7.19-7.21 (m, 2H), 7.35-7.37 (m, 1H), 7.55 (d, *J* = 2.1 Hz, 1H), 7.59 (t, *J* = 2.1 Hz, 1H), 7.95 (d, *J* = 8.8 Hz, 1H), 8.83 (s, 2H), 9.83 (s, 1H); ^{13}C NMR (CD_3COCD_3): 108.7, 109.1, 113.2, 113.4, 119.2, 127.0, 131.5, 140.9, 141.1, 148.7, 159.1, 159.8, 159.9, 162.9; MS (ESI): 287.0 $(M+H)^+$;

3-methoxy-N-(6-methoxy-benzothiazol-2-yl)-benzenesulfonamide (**22i**). The title compound was prepared by reaction of 6-methoxy-benzothiazol-2-ylamine (0.3 g, 1.66 mmol) and 3-methoxy-benzenesulfonyl chloride (0.3 mL, 1.66 mmol) according to method B. The product was purified by recrystallisation (methanol); yield: 89 % (0.66 g); ^{1}H NMR (CD_3COCD_3): 3.84 (s, 3H), 3.85 (s, 3H), 7.00 (dd, *J* = 2.5 Hz, *J* = 8.8 Hz, 1H), 7.13 (ddd, *J* = 7.9 Hz, *J* = 2.5 Hz, *J* = 0.9 Hz, 1H), 7.34 (d, *J* = 8.8 Hz, 1H), 7.38 (d, *J* = 2.5 Hz, 1H), 7.41-7.43 (m, 1H), 7.45 (d, *J* = 8.2 Hz, 1H), 7.48 (dt, *J* = 7.9 Hz, *J* = 1.3 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 56,0; 56,2; 107,5; 112,3; 114,4; 115,6; 118,6; 119,1; 130,7; 130,8; 133,2; 134,3; 136,1; 145,1; 157,8;

1-(6-methoxy-benzothiazol-2-yl)-3-(3-methoxy-phenyl)-urea (**23i**). The title compound was prepared by reaction of 6-methoxy-benzothiazol-2-ylamine (0.33 g, 1.85 mmol) and 1-isocyanato-3-methoxy-benzene (0.2 mL, 1.85 mmol) according to method B. The product was purified by recrystallisation (methanol); yield: quant. (0.61 g); ^{1}H NMR (CD_3COCD_3): 3.78 (s, 3H), 3.82 (s, 3H), 6.64 (dd, *J* = 8.2 Hz, *J* = 2.2 Hz, 1H), 6.99 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H), 7.03-7.05 (m, 1H), 7.21-7.25 (m, 2H), 7.50 (d, *J* = 2.5 Hz, 1H), 7.56 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 56,2; 56,8; 105,9; 106,1; 109,5; 112,3; 115,6; 121,5; 121,5; 131,0; 134,0; 134,0; 141,2; 157,2; 161,3; 206,2;

1-(6-methoxy-benzothiazol-2-yl)-3-(3-methoxy-phenyl)-thiourea (**24i**). The title compound was prepared by reaction of 6-methoxy-benzothiazol-2-ylamine (0.30 g, 1.66 mmol) and 1-isothiocyanato-3-methoxy-benzene (0.23 mL, 1.66 mmol) according to method B. The product was purified by CC (chloroform/methanol 95:05); yield: 30 % (0.17 g); MS (ESI): 346 $(M+H)^+$;

N-(6-methoxy-benzothiazol-2-yl)-2-(3-methoxy-phenyl)-acetamide (**25i**). The title compound was prepared by reaction of 6-methoxy-benzothiazol-2-ylamine (0.3 g, 1.66 mmol) and (3-methoxy-phenyl)-acetyl chloride (0.26 mL, 1.66 mmol) according to method B. The product was purified by CC (chloroform/methanol 95:05); yield: 82 % (0.45 g); MS (ESI): 329 $(M+H)^+$;

4-methoxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**19**). The title compound was prepared by reaction of 4-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**19i**) (0.5 g, 1.59 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 25.1 mL, 239.00 mmol) according to method D. The product was purified by preparative HPLC; yield: 23 % (0.11 g). ^{1}H NMR (CD_3OD): 3.94 (s, 3H), 7.12-7.16 (m, 3H), 7.40 (d, *J* = 2.5 Hz, 1H), 7.78 (d, *J* = 9.1 Hz, 1H), 8.30-8.32 (m, 2H); ^{13}C NMR (CD_3OD): 56.4, 109.2, 115.5, 118.4, 118.5, 118.6, 119.8, 124.4, 130.2, 134.2, 133.4, 158.0, 166.4, 168.2, 173.3; MS (ESI): 300.1 $(M+H)^+$;

4-hydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**20**). The title compound was prepared by reaction of 4-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**19i**) (0.5 g, 1.59 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 25.1 mL, 239.00 mmol) according

to method D. The product was purified by preparative HPLC; yield: 13 % (0.06 g). ^{1}H NMR (CD_3OD): 6.80-6.83 (m, 2H), 6.98 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H), 7.22 (d, *J* = 2.2 Hz, 1H), 7.64 (d, *J* = 9.1 Hz, 1H), 8.10-8.12 (m, 2H); ^{13}C NMR (CD_3OD): 107.7, 115.9, 118.3, 120.8, 120.9, 125.6, 126.1, 126.9, 139.9, 147.3, 147.7, 158.1, 158.3, 161.6; MS (ESI): 287.6 $(M+H)^+$;

3-hydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**21**). The title compound was prepared by reaction of 3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**21i**) (0.5 g, 1.59 mmol) and boron trifluoride methyl sulfide complex in dichloromethane (1 M, 25.0 mL, 239.00 mmol) according to method D. The product was purified by recrystallisation (ethyl acetate); yield: quant. (0.46 g). ^{1}H NMR (CD_3OD): 6.83 (dd, *J* = 8.5 Hz, *J* = 2.5 Hz, 1H), 6.95 (dd, *J* = 2.5 Hz, *J* = 0.9 Hz, 1H), 7.14 (d, *J* = 2.2 Hz, 1H), 7.26 (t, *J* = 8.2 Hz, 1H), 7.32 (t, *J* = 2.2 Hz, 1H), 7.37-7.39 (m, 1H), 7.48 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (CD_3OD): 107.2, 112.3, 115.9, 116.5, 119.9, 121.0, 122.4, 127.5, 131.0, 134.7, 135.2, 146.6, 155.9, 159.2; MS (ESI): 286.9 $(M+H)^+$;

3-hydroxy-N-(6-hydroxy-benzothiazol-2-yl)-benzenesulfonamide (**22**). The title compound was prepared by reaction of 3-methoxy-N-(6-methoxy-benzothiazol-2-yl)-benzenesulfonamide (**22i**) (0.52 g, 1.48 mmol) and boron tribromide in dichloromethane (1 M, 14.8 mL, 14.80 mmol) according to method F. The product was purified by recrystallisation (H_2O:methanol); yield: 88 % (0.42 g); ^{1}H NMR (CD_3SOCD_3): 6.82 (dd, *J* = 2.5 Hz, *J* = 8.8 Hz, 1H), 6.96 (dd, *J* = 8.2 Hz, *J* = 2.2 Hz, 1H), 7.12 (d, *J* = 8.5 Hz, 1H), 7.18 (d, *J* = 2.5 Hz, 1H), 7.21-7.26 (m, 2H), 7.33 (t, *J* = 8.0 Hz, 1H), 9.63 (s, 1H), 10.00 (s, 1H), 12.9 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 108,4; 112,2; 113,4; 115,2; 116,2; 119,1; 124,8; 125,8; 130,0; 139,1; 143,2; 154,1; 157,5; MS (ESI): 323.1 $(M+H)^+$;

1-(6-hydroxy-benzothiazol-2-yl)-3-(3-hydroxy-phenyl)-urea (**23**). The title compound was prepared by reaction of 1-(6-methoxy-benzothiazol-2-yl)-3-(3-methoxy-phenyl)-urea (**23i**). (0.60 g, 1.82 mmol) and boron tribromide in dichloromethane (1 M, 18.2 mL, 18.20 mmol) according to method F. The product was purified by CC (chloroform/methanol 90:10); yield: 64 % (0.35 g); ^{1}H NMR (CD_3SOCD_3): 6.46 (dd, *J* = 1.5 Hz, *J* = 8.2 Hz, 1H), 6.84-6.87 (m, 2H), 7.08 (t, *J* = 7.9 Hz, 1H), 7.14 (t, *J* = 2.1 Hz, 1H), 7.24 (d, *J* = 2.4 Hz, 1H), 7.46 (d, *J* = 8.8 Hz, 1H), 9.01 (s, 1H), 9.39 (s, 1H), 9.41 (s, 1H), 10.48 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 105,8; 106,5; 109,3; 110,0; 114,8; 120,1; 120,2; 129,6; 132,6; 139,7; 140,9; 153,9; 158,0; 206,5; MS (ESI): 302.1 $(M+H)^+$;

1-(6-hydroxy-benzothiazol-2-yl)-3-(3-hydroxy-phenyl)- thiourea (**24**). The title compound was prepared by reaction of 1-(6-methoxy-benzothiazol-2-yl)-3-(3-methoxy-phenyl)-thiourea (**24i**). (0.17 g, 0.49 mmol) and boron tribromide in dichloromethane (1 M, 4.9 mL, 4.90 mmol) according to method F. The product was purified by CC (chloroform/methanol 90:10); yield: 26 % (0.04 mg); ^{1}H NMR (CD_3SOCD_3): 6.53-6.56 (m, 1H), 6.87 (dd, *J* = 8.5 Hz, *J* = 2.3, 1H), 7.09-7.22 (m, 4H), 7.39-7.40 (m, 1H), 8.30 (s, 1H), 9.41 (s, 1H), 9.48 (s, 1H), 9.58 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 115,2; 115,8; 116,1; 116,23; 116,7; 116,8; 116,9; 117,0; 117,4; 117,6; 117,7; 125,2; 125,6; 157,3; MS (ESI): 318.1 $(M+H)^+$;

N-(6-Hydroxy-benzothiazol-2-yl)-2-(3-hydroxy-phenyl)-acetamide (**25**). The title compound was prepared by reaction of N-(6-methoxy-benzothiazol-2-yl)-2-(3-methoxy-phenyl)-acetamide (**25i**). (0.45 g, 1.37 mmol) and boron tribromide in dichloromethane (1 M, 13.7 mL, 13.70 mmol) according to method F. The product was purified by preparative HPLC; yield: 36 % (0.15 g); ^{1}H NMR (CD_3SOCD_3): 3.69 (s, 2H), 6.66 (dd, *J* = 8.2 Hz, *J* = 2.2, *J* = 0.9, 1H), 6.74-6.77 (m, 2H), 6.88 (dd, *J* = 8.8 Hz, *J* = 2.5, 1H), 7.11 (t, *J* = 7.7, 1H), 7.26 (d, *J* = 2.2, 1H), 7.54 (d, *J* = 8.8 Hz, 1H), 12.3 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 41,8; 106,4; 113,8; 115,2; 116,0; 119,8; 121,0; 129,3; 132,7; 136,0; 141,5; 154,1; 154,9; 157,3; 169,7; MS (ESI): 301.1 $(M+H)^+$;

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. Fresh human placenta was homogenized and cytosolic fraction and microsomes were separated by centrifugation. For the partial purification of 17β-HSD1, the cytosolic fraction was precipitated with ammonium sulfate. 17β-HSD2 was obtained from the microsomal fraction.

Inhibition of 17β-HSD1. Inhibitory activities were evaluated by an established method with minor modifications. 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. 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 the following equation: $\%conversion = \frac{\%E2}{\%E2 + \%E1} \times 100$. Each value was calculated from at least three independent experiments.

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 were carried out as mentioned above.
The conversion rate was calculated after analysis of the resulting chromatograms according to the following equation: $\%conversion = \frac{\%E1}{\%E1 + \%E2} \times 100$.

ER affinity. The binding affinity of selected compounds to the ERα and ERβ was determined according to Zimmermann et al. 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). Evaluation of 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 %.

Inhibition of 17β-HSD1 in T47-D cells. T47-D cells were obtained from ECACC, Salisbury. Stripped FCS and cell culture media were purchased from CCpro, Oberdorla. A stock culture of T47-D 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 0.5 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.

Table S1. Geometrical properties of the extended pharmacophore model.

distances (Å)		angles (°)		radii of spheres (Å)	
A1a-D1b	4.0	A6b-HY6-D6a	~42	A1a	1.5
A3a-D4b	2.8	AD1-HY1-HY2	~129	A3a	1.1
A6b-D6a	2.2	AD2-AD2a-AD2b	~67	A6b	1.4
AD1-A1a	2.8	AD2-AD2b-AD2a	~59	AD1	1.2
AD1-D1b	2.6	AD3-A3a-D4b	~82	AD2	1.5
AD1-HY1	2.8	AD5b-HY2-AD5a	~54	AD2a	1.4
AD2a-AD2b	2.6	D4-A3a-D4b	~54	AD2b	1.5
AD2-AD2a	2.7	D4b-AD3-A3a	~50	AD3	1.2
AD2-AD2b	2.9	D4-D4a-D4b	~41	AD5a	1.2
AD3-D4	2.2	D4-D4b-A3a	~77	AD5b	1.5
AD5a-AD5b	3.3	D4-D4b-D4a	~43	D1b	1.3
AD5a-D4a	3.9	D6a-A6b-HY6	~78	D4	1.3
D4a-D4b	4.7	D7-AD3-D4	~115	D4a	1.7
D4-D4a	3.2	HY1-A1a-D1b	~75	D4b	1.3
D4-D4b	3.0	HY1-AD1-HY2	~27	D6a	1.4
D7-A6b	2.8	HY1-D1b-A1a	~55	D7	1.2
HY1-HY2	3.0	HY1-HY2-A1a	~52	HY1	1.7
HY2-AD5a	4.7	HY1-HY3-HY2	~41	HY2	1.7
HY2-AD5b	5.8	HY2-AD5a-AD5b	~75	HY3	1.5
HY2-D4a	3.9	HY2-AD5b-AD5a	~51	HY4	1.7
HY2-HY3	2.7	HY2-HY3-HY4	~115	HY5	1.5
HY3-HY4	2.9	HY3-AD2-HY4	~47	HY6	1.5
HY4-AD2	2.6	HY3-HY2-HY4	~34		
HY4-HY5	3.0	HY3-HY4-HY2	~31		
HY4-HY6	4.1	HY4-AD2-HY5	~40		
HY5-AD3	3.5	HY4-AD3-D4	~60		
HY5-D4	3.7	HY4-D4-AD3	~102		
HY5-P5	2.1	HY4-D7-AD2	~23		
HY6-A6a	3.2	HY4-D7-AD3	~56		
HY6-A6b	2.9	HY4-HY3-AD2	~40		
HY6-D7	2.5	HY4-HY5-AD3	~146		
P5-HY4	2.9	HY4-HY5-D4	~110		
		HY4-HY5-P5	~70		
		HY4-HY6-AD2	~37		
		HY4-HY6-D7	~167		
		HY4-HY6-HY5	~45		
		HY4-P5-HY5	~66		

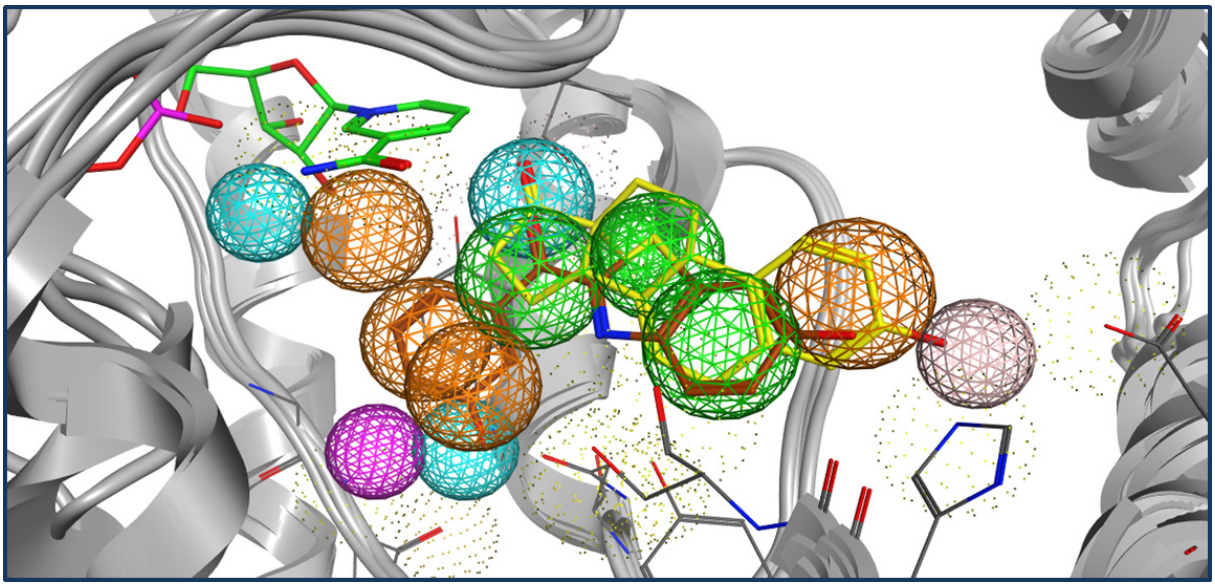


Figure S1. Compound **6** (dark orange) mapped to the pharmacophore model and overlaid with equiline (yellow).

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3.II Novel Nonsteroidal Benzothiazole Type 17β-Hydroxysteroid Dehydrogenase 1 (17β-HSD1) Inhibitors as new Candidates for in Vivo Evaluation.

Major part of this chapter will be published in *Journal of Medicinal Chemistry*.

Abstract: 17β-HSD1 is a novel target for the treatment of estrogen-dependent diseases as it catalyzes intracellular estradiol formation. Starting from two recently described compounds, highly active 17β-HSD1 inhibitors with strong selectivity regarding 17β-HSD2 and the estrogen receptors α and β were developed. **4** and **15** are the most selective compounds toward 17β-HSD2 described so far. They showed strong inhibition of the marmoset target enzyme. Thus, they are ideal candidates to be tested in vivo in a marmoset model for endometriosis.

Introduction

For the treatment of steroid hormone-dependent diseases biosynthesis inhibitors blocking hormone formation in the corresponding endocrine gland provide an excellent strategy as has been demonstrated by the use of CYP19 (aromatase) inhibitors for the treatment of breast cancer (BC). [1] Three further cytochrome P450 (CYP) enzymes are presently in the focus of drug development: CYP17, [2] CYP11B2 [3] and CYP11B1 [4] for the treatment of prostate cancer, congestive heart failure and myocardial fibrosis and Cushing`s syndrome, respectively. As these therapeutic options result in systemic reductions of steroid hormone levels which often are associated with side effects, a softer option could be the inhibition of hormone activation in the diseased (target) cell. For the treatment of benign prostatic hyperplasia, this strategy has already been proven to be successful with inhibitors of 5α-reductase, [5] which catalyzes the conversion of testosterone to the most potent androgen dihydrotestosterone. More recently 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1) came into the focus of interest as a novel therapeutic target for the treatment of estrogen dependent diseases like BC and endometriosis. [6] 17β-HSD1 catalyzes the conversion of the weakly active estrone (E1) to the highly active estradiol (E2) and is overexpressed in some BC tissues. [6] Efficacy of 17β-HSD1 inhibitors against human breast cancer cell lines has already been reported *in vitro* and *in vivo* (nude mouse), [7] whereas a proof of concept for endometriosis is still missing. Besides some approaches to develop steroidal inhibitors, [8] there have also been attempts by our and other groups to develop nonsteroidal inhibitors which should be advantageous with respect to side effects. [9] Recently we reported about two new 17β-HSD1 inhibitors with different activity/selectivity profiles, the benzothiazol-2-yl-phenylmethanone **A** and the *N*-benzothiazol-2-ylbenzamide **B** (Chart 1). [10]

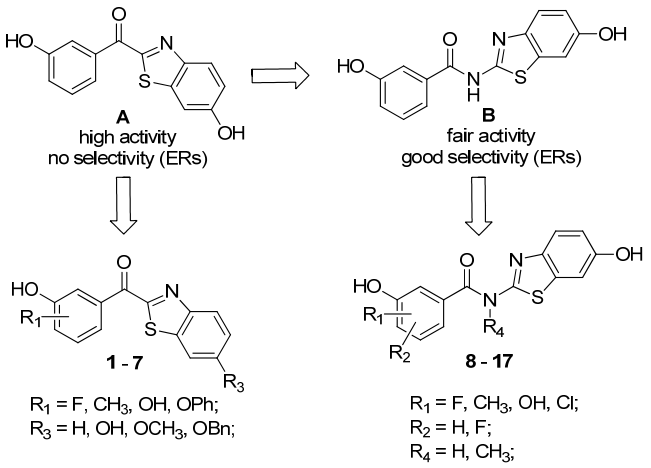
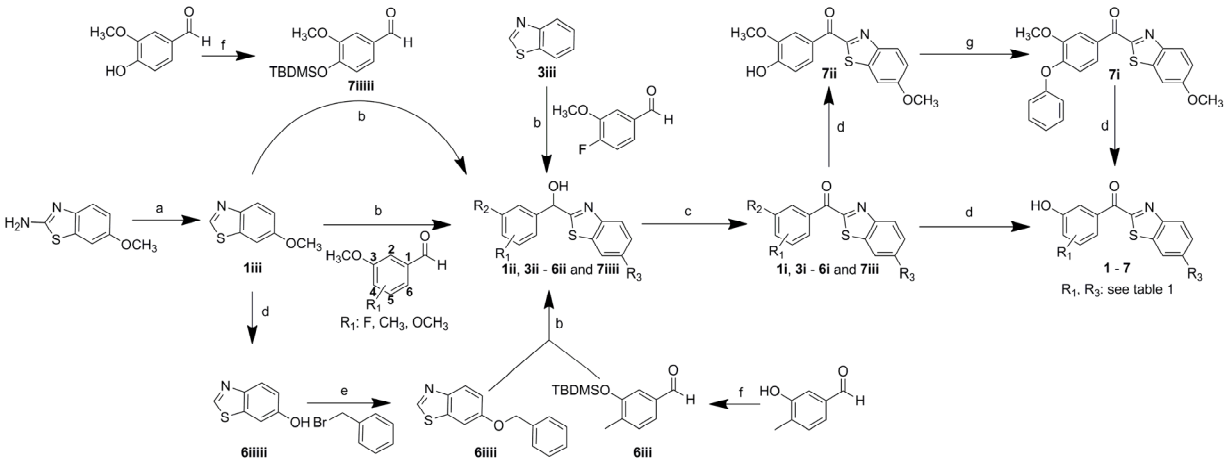


Chart 1: Structures` optimization

Herein we report about the structure optimization of these two lead compounds with regard to 17β-HSD1 inhibitory activity as well as selectivity towards 17β-HSD2 (the enzyme catalysing the reverse reaction) and the estrogen receptors (ERs) α and β. For selected compounds, 17β-HSD1 inhibitory potency in human T47-D breast cancer cells, inhibitory potency toward 17β-HSD1 and 17β-HSD2 from *Callithrix jacchus* (animal model for endometriosis), [11] and inhibition of human hepatic CYP enzymes were determined.

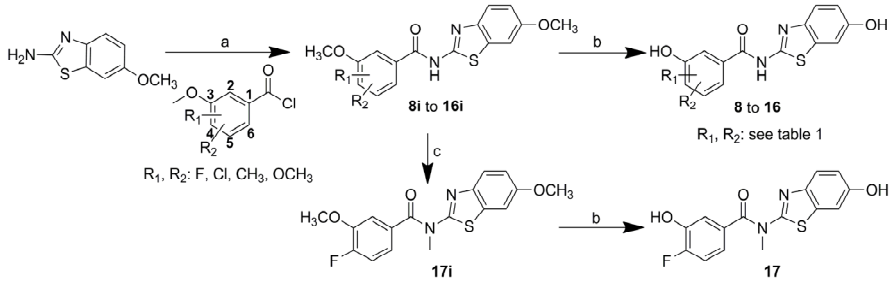
Results

Chemistry: The synthesis of compounds **1-7** started from 6-methoxy-1,3-benzothiazol-2-yl amine or 1,3-benzothiazole. **1-2** and **4-5** were prepared in a four-step synthesis previously described [10] (a-d in Scheme 1).



Scheme 1. Synthesis of Compounds **1-7**.. Reagents and conditions: (a) 1) $NaNO_2$, H_3PO_4 (85%), -10 °C, 20 min, 2) H_3PO_2, H_3PO_4 (85%), -10 °C to rt, 20 h; (b) Method A: 1) nBuLi, anhydrous THF, -70 °C to -20 °C, 1 h, 2) methoxy benzaldehyde, anhydrous THF, -15 °C, 90 min; (c) Method C: SIBX, anhydrous THF, 0 °C to 60 °C, 20 h; (d) for compounds **1-4**, Method D: pyridinium hydrochloride, 220 °C, 4 h, for compounds **5**, **6iiiii** and **7**, Method E: BBr_3, CH_2Cl_2, -78 °C to rt, 20 h; (e) benzyl bromide, K_2CO_3, acetone, 100 °C, 20 h, for compounds **7ii** and **6**, TBAF, THF, rt, 2 h; (f) TBDMSiCl, imidazole, DMF, rt, 20 h; (g) benzeneboronic acid, $Cu(AcO)_2$, Et_3N, anhydrous CH_2Cl_2, rt, 18 h.

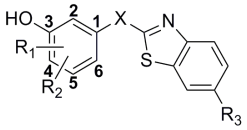
3 was synthesized in three steps starting with the nucleophilic addition of 1,3-benzothiazole (**3iii**) to 4-fluoro-3-methoxy-benzaldehyde. For compound **6**, 6-benzyloxy-1,3-benzothiazole (**6iiii**) and 3-(*tert*-butyl-dimethyl-silanyloxy)-4-methyl-benzaldehyde (**6iii**) were synthesized and coupled via nucleophilic addition. Oxidation of the resulting alcohol and ether cleavage of the silanyl ether using tetra-*n*-butylammonium fluoride (TBAF) gave the final compound. **7** was synthesized coupling **1iii** and 4-(*tert*-butyl-silanyloxy)-3-methoxy-benzaldehyde (**7iiiii**), followed by oxidation, cleavage with TBAF, boronic acid-phenol cross-coupling reaction mediated by copper(II) acetate and cleavage of the two methoxy groups with boron tribromide (BBr_3). **8**-**16** were prepared via two steps as described [10] (a-b in Scheme 2): (a) amide coupling between 6-methoxy-benzothiazol-2-yl amine and the corresponding benzoyl chloride and (b) ether cleavage. **17** was synthesized by N-methylation of **9i** with methyl iodide and sodium hydride in DMF and subsequent ether cleavage.



Scheme 2. Synthesis of Compounds **8**-**17.** Reagents and conditions: (a) Method B: pyridine, 100°C, 4 h; (b) Method E: BBr_3, CH_2Cl_2, -78 °C to rt, 20.

Inhibition of human 17β-HSD1 and 17β-HSD2: For the determination of 17β-HSD1 and 17β-HSD2 inhibition, tritiated substrates E1 or E2 were incubated with placental enzymes 17β-HSD1 or 17β-HSD2, respectively, cofactor, and inhibitor. The labeled products formed were quantified by HPLC using radiodetection. The inhibition values (IC_{50}) and the selectivity factors (SF = IC_{50} (HSD2)/IC_{50} (HSD1)) are shown in Table 1. Compounds **A** and **B** were used as references. In the series of the ketone compounds, **1**-**5** strongly inhibited 17β-HSD1. Also, in the series of the amide derivatives stronger 17β-HSD1 inhibition than the one shown by **B** was observed, with **8** and **15** exhibiting IC_{50} values in the low nanomolar range. Furthermore, **4** and **15** displayed strong selectivity for 17β-HSD1 over 17β-HSD2, exhibiting the highest selectivity factors (>130) reported in the literature.
factors (>130) reported in the literature.

Table 1: 17β-HSD1 inhibitory activities and selectivities of compounds **1-17**.



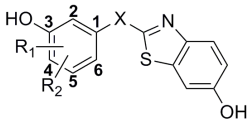
Cpd	X	R_1	R_2	R_3	HSD1[a]	HSD2[b]	SF[d]
					IC_{50} (nM)[c]		
A	CO	H	H	OH	44	1035	23
1		4-F	H	OH	13	121	9
2		4-F	H	OCH_3	38	59	2
3		4-F	H	H	136	104	1
4		4-CH_3	H	OH	27	4003	148
5		6-OH	H	OH	78	1538	20
6		4-CH_3	H	OBn	ni	ni	nd
7		4-OPh	H	OH	863	1457	2
B	CONH	H	H	OH	243	9264	38
8		2-F	H	OH	29	1000[f]	<40
9		4-F	H	OH	171	1480	9
10		6-F	H	OH	112	3804	34
11		4-CH_3	H	OH	ni	nd	nd
12		5-OH	H	OH	3000[e]	nd	nd
13		6-OH	H	OH	ni	nd	nd
14		2-Cl	6-F	OH	221	469	2
15		2-F	6-F	OH	13	1774	136
16		2-F	4-F	OH	99	541	5
17	$CONCH_3$	4-F	H	OH	670[e]	2700[e]	4

[a]Human placenta, cytosolic fraction, substrate [^{3}H]E1 + E1 [500 nM], cofactor NADH [500 μM]; [b]Human placenta, microsomal fraction, substrate [^{3}H]E2 + E2 [500 nM], cofactor NAD^+ [1500 μM]; [c]Mean values of three determinations, standard deviation less than 10%; [d]Selectivity factor: IC_{50} 17β-HSD2/ IC_{50} 17β-HSD1; [e]Calculated with LOGIT transformation; [f]standard deviation less than 25 %; nd: not determined, ni: no inhibition (less than 10 % inhibition at 1μM).

Affinities to ER α and β: The relative binding affinities (RBA) of the most selective compounds of this study were determined using recombinant human ER α and ER β in a competition assay [12] with [^{3}H]E2 (E2 was used as reference and its RBA value was set to 100 %). Only **1** showed a marked affinity to the ERs, while all other compounds were more than 1000-fold less potent compared to E2.

Further biological evaluation: Additionally, the inhibition of intracellular E2 formation for compounds **1** (IC_{50} = 11 nM), **4** (258 nM), **5** (365 nM), **8** (73 nM) and **15** (37 nM) in T47-D cells was determined (Table 2).

Table 2: Binding affinities for the ERs α and β, 17β-HSD1 inhibitory activity in T47-D cells and inhibition of *Callithrix jacchus* 17β-HSD1 and 17β-HSD2 by selected compounds.



Cpd	X	R_1	R_2	ERα[a]	ERβ[a]	T47-D	mHSD1[d]		mHSD2[e]
				RBA (%)[b]		IC_{50} (nM)[c]	% inhibition		
							5 nM	50 nM	50 nM
A	CO	H	H	< 10	< 1				
1		4-F	H	< 1	< 1	11	80		34
4		4-CH_3	H	< 0.1	< 0.1	258		83	40
5		6-OH	H	< 0.1	< 0.1	365[f]			
B	CONH	H	H	< 0.1	< 0.001	245			
8		2-F	H	< 0.1	< 0.1	73[g]	67		48
10		6-F	H	< 0.1	< 0.1	152[g]			
15		2-F	6-F	< 0.1	< 0.1	37[g]	87		51

[a]Human recombinant protein, incubation with 10 nM [^{3}H]E2 and inhibitor for 1 h. [b]RBA: relative binding affinity in percent (E2=100%), mean value of three determinations, standard deviation less than 10%. [c]Mean value of three determinations, standard deviation less than 10%; [d]Marmoset placenta, cytosolic fraction, substrate [^{3}H]E1 + E1 [500 nM], cofactor NADH [500 μM]; [e]Marmoset placenta, microsomal fraction, substrate [^{3}H]E2 + E2 [500 nM], cofactor NAD^+ [1500 μM]; [f]Standard deviation less than 15 %; [g]Calculated with LOGIT transformation at 50 nM inhibitor concentration.

The high cellular activities of compounds **1**, **8**, and **15** indicate that they easily permeate the cell membrane and are not metabolized quickly. Furthermore, compounds **1**, **4**, **8** and **15** were tested for inhibitory activity towards *Callithrix jacchus* 17β-HSD1 and 17β-HSD2 (Table 2). The compounds showed stronger inhibition of marmoset 17β-HSD1 compared to the human enzyme and a fair selectivity toward 17β-HSD2. Compounds **1** and **4** were further investigated for inhibition of the most important human hepatic CYP enzymes: 3A4, 2D6, 2C9, 2C19, 1A2 and 2B6. Both compounds showed very little inhibition (54 μM > IC_{50} > 3 μM) except for 1A2 which was inhibited by **1** and **4** moderately (IC_{50} = 1.8 and 0.57 μM) indicating that there should be no major problems regarding CYP inhibition in this compound class.

Discussion and conclusion

Recently we discovered two new 17β-HSD1 inhibitors, compounds **A** and **B** (Chart 1). [10] **A** had shown high inhibitory activity, medium selectivity toward 17β-HSD2 and very weak selectivity toward the ERs. On the contrary, **B** had shown only medium inhibitory activity and good selectivity toward 17β-HSD2 and the ERs. In the present study **A** and **B** should be optimised regarding activity and selectivity in order to obtain compounds suitable for in vivo use. Introduction of various substituents with different electronic, lipophilic, steric and H-bonding properties into the benzoyl moiety of **A** and **B** led to the discovery of two new lead structures, compounds **4** and **15**. As suggested in our previous study [10] where **A** and **B** were hypothesised to interact differently with the binding site, the introduction of a wide variety of substituents into the benzoyl moiety of **A** and **B** had different influence on inhibitory activity: comparing **1** with **9**, **4** with **11** and **5** with **13** shows that only the fluorine in 4-position increased inhibitory activity in both classes. The introduction of OH in 2-position and methyl in 4-position turned out to be beneficial in case of the ketones (IC_{50} = 27 nM for **4** and 78 nM for **5**) but was deleterious for the amides (**11**, **13**: no inhibition at 1 μM). The introduction of a bulky

substituent like the phenoxy group in 4-position (**7**), benzylation of the hydroxy group of **4** (**6**) and methylation of the amide function (**17**) decreased inhibitory activity. Interestingly, methylation of the OH group of **1** (**2**) or replacement by hydrogen (**3**) on the benzothiazole moiety resulted only in a slight decrease of 17β-HSD1 inhibition but in a complete loss of selectivity toward 17β-HSD2. Changing the F position as well as adding a second fluorine in the amide series increased 17β-HSD1 inhibitory activity: **15** showed the same IC_{50} value of 13 nM as the best ketone compound **1**. This may be due to favourable electronic effects exerted by the fluorines on the H-bonding property of the OH, to the increased lipophilicity or to the change in π-interacting properties of the aromatic ring as suggested by SAR studies. [10] Regarding selectivity, in the ketone series introduction of the methyl group in 4-position (**4**) not only increased activity but also decreased inhibition of 17β-HSD2 and affinity to the ERs. Thus, compound **4** shows the highest selectivity toward 17β-HSD2 described so far (SF 148) and only little ER affinity. It has been known for a long time that a substituent in o-position to the 3-OH group of E2 or its nonsteroidal analog hexestrol [13] reduces ERα affinity of the parent compound. Therefore it can be assumed that the ketone **A** binds to the ERα like the natural substrate. In the amide series introduction of fluorine in 4-position (**9**) led to a decrease in selectivity towards 17β-HSD2, like it has been observed for **1** in the ketone series, while introducing a fluorine in 2-position (**8**) and 6-position (**10**) did not alter selectivity. The difluoro-substituted compound **15** showed the best selectivity in this series toward 17β-HSD2 and the ERs with values similar to **4**. Summarising, we have discovered two highly potent 17β-HSD1 inhibitors, which are the most selective compounds described so far. Compounds **4** and **15** also show very good intracellular activity and a high potency and selectivity towards the marmoset enzymes. Accordingly, they should be suitable candidates for being further evaluated in a marmoset model of endometriosis.

Experimental section

The following compounds were prepared according to previously described procedures: 6-methoxy-1,3-benzothiazole (**1iii**), [14] 6-hydroxy-1,3-benzothiazole (**6iiiii**), [14] [3-(*tert*-butyl-dimethyl-silanyloxy)-4-methyl]benzaldehyde (**6iii**), [15] 3-methoxy-4-(*tert*-butyl-dimethyl-silanyloxy) benzaldehyde (**7iiiii**). [16] Synthetic details and characterization of compounds can be found in supporting information. Method A: Formation of methanols; Method B: Amide formation; Method C: Oxidation with SIBX; Method D: Ether cleavage with PyHCl; Method E: Ether cleavage with BBr_3; Compounds **4ii**, **15i**, **4i**, **4** and **15** are presented as examples:

(6-Methoxy-1,3-benzothiazol-2-yl)(4-methyl-3-methoxyphenyl)methanol (**4ii**). The title compound was prepared by reaction of 6-methoxy-benzothiazole (**1iii**) (0.25 g, 1.5 mmol), nBuLi (0.61 ml, 1.5 mmol) and 4-methyl-3-methoxybenzaldehyde (0.18 g, 1.2 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 60:40); yield: 89 % (0.42 g); ^{1}H NMR ($CDCl_3$): 2.2 (s, 3H), 3.79 (s, 3H), 3.84 (s, 3H), 6.07 (s, 1H), 6.98-7.05 (m, 2H), 7.03 (dd, J = 9.1 Hz and J = 2.7 Hz, 1H), 7.11 (d, J = 7.6 Hz, 1H), 7.24 (d, J= 2.4 Hz, 1H), 7.82 (d, J = 9.1 Hz, 1H); ^{13}C NMR ($CDCl_3$): 16.0, 55.3, 55.7, 74.2, 104.2, 115.4, 118.1, 118.4, 123.4, 127.1, 130.7, 136.5, 139.8, 146.8, 157.6, 158.0, 172.

2,6-Difluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**15i**). The title compound was prepared by reaction of 6-methoxybenzothiazol-2ylamine (0.3 g, 1.79 mmol) and 2,6-difluoro-3-methoxy-benzoyl chloride (0.4 g, 1.79 mmol) according to method B. The product was purified by CC (dichloromethane/methanol 95:05); yield: 29 % (0.18 g); ^{1}H NMR ($CDCl_3$): 3.83 (s, 3H), 3.89 (s, 3H), 7.07 (dd, J = 2.5 Hz, J = 8.8 Hz, 1H), 7.21 (dt, J = 1.9 Hz, J = 8.8 Hz, J = 9.1, Hz 1H), 7.36-7.40 (m, 1H), 7.63 (d, J = 2.5 Hz, 1H), 7.70 (d, J = 8.8 Hz, 1H), 13.07 (s, 1H); ^{13}C NMR ($CDCl_3$): 55,6; 56,8; 104,8; 111,4; 115,3; 116,1; 116,2; 117,6; 130,6; 143,9; 144,0; 150.7; 150.8; 150,8; 156,5; 164,6.

(4-Methyl-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)methanone (**4i**). The title compound was prepared by reaction of (4-methyl-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)-methanol (**4ii**) (0.33 g, 1.03 mmol) and 2-iodoxybenzoic acid (0.58 g, 2.06 mmol) according to method C. The

product was purified by CC (hexane/ethyl acetate 85:15); yield: quantitative (0.33 g); ^{1}H NMR ($CDCl_3$): 2.2 (s, 3H), 3.79 (s, 3H), 3.82 (s, 3H), 7.04 (dd, *J* = 9.1 Hz and *J* = 2.5 Hz, 1H), 7.13-7.15 (m, 1H), 7.27 (d, *J* = 2.5 Hz, 1H), 7.83 (d, *J* = 1.3 Hz, 1H), 7.96 (d, *J* = 9.1 Hz, 1H), 8.12 (dd, *J* = 7.8 Hz and *J* = 1.6 Hz, 1H); ^{13}C NMR ($CDCl_3$): 16.6, 55.3, 55.7, 103.3, 111.3, 117.4, 124.4, 126.3, 130.3, 133.9, 138.9, 148.4, 159.6, 165.0, 184.3.

(4-Methyl-3-hydroxyphenyl)(6-hydroxy-1,3-benzothiazol-2-yl)methanone (**4**). The title compound was prepared by reaction of (4-methyl-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)-methanone (**4i**) (0.33 g, 1.05 mmol) and pyridinium hydrochloride (12.13 g, 105 mmol) according to method D. The product was purified by preparative HPLC; yield: 42 % (0.12 g); ^{1}H NMR (CD_3COCD_3): 2.32 (s, 3H), 7.21 (dd, *J* = 8.8 Hz and *J* = 2.4 Hz, 1H), 7.32 (d, *J* = 7.6 Hz, 1H), 7.55 (d, *J* = 2.1 Hz, 1H), 8.02 (d, *J* = 1.5 Hz, 1H), 8.06 (d, *J* = 8.8 Hz, 1H), 8.10 (dd, *J* = 7.6 Hz and *J* = 1.5 Hz, 1H), 8.70 (s, 1H), 9.22 (s, 1H); ^{13}C NMR (CD_3COCD_3): 17.1, 107.9, 113.75, 117.7, 117.7, 119.0, 124.3, 127.8, 132.1, 135.5, 140.3, 142.7, 156.7, 159.3, 185.2; MS (ESI): 287.0 $(M+H)^+$.

2,6-Difluoro-3-hydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**15**). The title compound was prepared by reaction of 2,6-difluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)-benzamide (**15i**) (0.18 g, 0.51 mmol) and boron tribromide (5.1 mmol) according to method E. The product was purified by CC (dichloromethane/methanol 90:10); yield: 30 % (0.05 g); ^{1}H NMR (CD_3SOCD_3): 6.97-7.01 (m, 2H), 7.17-7.22 (m, 1H); 7.41 (d, *J* = 2.5 Hz, 1H), 7.59 (d, *J* = 8.2 Hz, 1H); ^{13}C NMR (CD_3SOCD_3): 107,2; 112,2; 112,3; 116,4; 120,6; 120,7; 122,7; 134,4; 142,6; 142,7; 142,7; 142,8; 143,3; 147,9; 147,9; 151,9; 151,9; 155,3; 155,6; 159,8; 167,1; MS (ESI): 323.1 $(M+H)^+$.

Supporting information

Contents:

a) Chemical synthesis and spectroscopic data for compounds **1**-**17**.

b) Purity (HPLC determination)

c) Biological Methods

a) Chemical synthesis and spectroscopic data for compounds **1**-**17**.

Chemical names follow IUPAC nomenclature. Starting materials were purchased from Acros, Aldrich, Alfa Aesar, Maybridge, 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).
^{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 = 3.32 ppm (^{1}H-NMR) and δ = 49.05 ppm (^{13}C-NMR), CD_3COCD_3: δ = 2.05 ppm (^{1}H-NMR) and δ = 29.9 ppm (^{13}C-NMR) and CD_3SOCD_3: δ = 2.50 ppm (^{1}H-NMR) and δ = 39.43 ppm (^{13}C-NMR). Signals are described as s, d, t, dd, ddd, m, dt, q for singlet, doublet, triplet, doublet of doublets, doublet of doublets of doublets, multiplet, doublet of triplets and quadruplet respectively. All coupling constants (*J*) are given in hertz (Hz).
Mass spectra (ESI) were recorded on an MSQ® electro spray mass spectrometer (ThermoFisher, Dreieich, Germany).
Tested compounds are ≥ 95 % chemical purity as measured by HPLC. The methods for HPLC analysis and a table of data for all tested compounds are provided (see section b).

Method A, general procedure for formation of methanols:
To a solution of thiazole derivative unsubstituted in position 2 (1 eq) in anhydrous THF a 2.5 M solution of nBuLi (1 eq) in hexane was added dropwise at -78 °C (dry ice/acetone bath) under

anhydrous conditions. The reaction mixture was stirred for 1 h at –78 °C to form the formyl anion in situ. Subsequently, a solution of electrophile (0.8 eq) in anhydrous THF was added dropwise at –78 °C (dry ice/acetone bath) and stirring was continued for 30 min. The temperature was then raised to –20 °C (NaCl/ice/dry ice/acetone bath) and the reaction mixture was stirred for 90 min. A mixture of water and saturated aqueous NH_4Cl solution (1:2) was added to quench the reaction at – 20 ° C, and the aqueous layer was extracted (3x) 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 or recrystallisation.

Method B, general procedure for amide formation:
To a solution of 2-amino-6-methoxy-1,3-benzothiazole (1 eq) in dry pyridine methoxy-benzoyl chloride (1 eq.) was added dropwise at room temperature. The reaction mixture was refluxed for 4 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, filtered, and concentrated to dryness. The product was purified by CC or recrystallisation.

Method C, general procedure for oxidation:
A mixture of aliphatic alcohol-derivative (1 eq) and stabilized 2-iodoxybenzoic acid (SIBX; 2 eq) in anhydrous THF was stirred at 0 °C. After 10 min, the reaction mixture was heated to 60 °C for 18 h. After cooling to room temperature, saturated sodium thiosulfate solution 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 magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC or recrystallisation.

Method D, general procedure for ether cleavage:
A mixture of methoxybenzene derivative (1 eq) and pyridinium hydrochloride (50 eq per methoxy function) was heated to 220 °C for 18 h. After cooling to room temperature, water, 1 M HCl and ethyl acetate were added. 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 CC followed by preparative TLC or preparative HPLC, respectively.

Method E, general procedure for ether cleavage:
To a solution of methoxybenzene derivative (1 eq) in anhydrous dichloromethane at -78 °C (dry ice/acetone bath), boron tribromide in dichloromethane (1 M; 5 eq per methoxy function) was added dropwise. The reaction mixture was stirred for 20 h at room temperature 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 CC followed by preparative TLC or preparative HPLC, respectively.
General procedure for purification using preparative HPLC.

All declared final compounds were purified via an Agilent Technologies Series 1200–preparative HPLC using a linear gradient run (solvents: acetonitrile, water) starting from 20% acetonitrile up to 100% in 36 min.

(4-Fluoro-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)-methanol (**1ii**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazole (**1iii**) (0.30 g, 1.51 mmol), 2.5 M solution of nBuLi (0.6 mL, 1.51 mmol) in hexane and 4-fluoro-3-methoxy-benzaldehyde (0.2 g, 1.16 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 80:20); yield: 24 % (0.09 g); ^{1}H NMR ($CDCl_3$): 3.85 (s, 3H), 3.86 (s, 3H), 6.07 (s, 1H), 7.02-7.07 (m, 3H), 7.15 (dd, J = 8.2 Hz and J = 1.9 Hz, 1H), 7.24-7.26 (m, 1H), 7.83 (d, J = 8.8 Hz, 1H); ^{13}C NMR ($CDCl_3$): 55.8, 56.2, 73.6, 104.2, 111.7, 115.6, 116.0, 116.1, 119.1, 123.4, 136.5, 137.2, 146.7, 147.9, 148.0, 151.3, 151.4, 153.4, 157.8, 172.1.

(4-Fluoro-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)methanone (**1i**). The title compound was prepared by reaction of (4-fluoro-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)methanol (**1ii**) (0.09 g, 0.28 mmol) and 2-iodoxybenzoic acid (0.16 g, 0.56 mmol) according to method C. The product was purified by CC (chloroform); yield: quantitative (0.09 g); ^{1}H NMR ($CDCl_3$): 3.93 (s, 3H),

4.02 (s, 3H), 7.30-7.32 (m, 1H), 7.35-7.39 (m, 1H), 8.00-8.04 (m, 1H), 8.06-8.09 (m, 1H), 8.12-8.16 (m, 1H); ^{13}C NMR ($CDCl_3$): 56.0, 56.2, 104.4, 115.8, 115.9, 118.4, 120.6, 125.2, 125.5, 125.9, 126.5, 132.1, 139.1, 142.4, 149.2, 160.9, 184.4.

(4-Fluoro-3-hydroxyphenyl)(6-hydroxy-1,3-benzothiazol-2-yl)methanone (**1**). The title compound was prepared by reaction of (4-fluoro-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)methanone (**1i**) (0.10 g, 0.32 mmol) and pyridinium hydrochloride (3.64 g, 31.5 mmol) according to method D. The product was directly purified by preparative HPLC; yield: 22 % (0.02 g); ^{1}H NMR (CD_3COCD_3) 7.11 (dd, J = 8.8 Hz and J = 2.2 Hz, 1H), 7.23 (dd, J = 8.5 Hz and J = 2.2 Hz, 1H), 7.38 (d, J = 2.2 Hz, 1H), 8.02 (d, J = 8.8 Hz, 1H), 7.97-8.00 (m, 1H), 8.15 (dd, J = 8.5 Hz and J = 2.2 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 107.2, 116.9, 117.1, 118.8, 121.6, 124.8, 124.9, 127.5, 133.2, 146.4, 146.5, 149.0, 155.8, 157.8, 159.7, 164.9, 184.7; MS (ESI): 290.1 $(M+H)^+$.

(4-Fluoro-3-hydroxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)methanone (**2**). The title compound was prepared by reaction of (4-fluoro-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)methanone (**1i**) (0.10 g, 0.32 mmol) and pyridinium hydrochloride (3.64 g, 31.5 mmol) according to method D. The product was directly purified by preparative HPLC; yield: 21 % (0.02 g); ^{1}H NMR ($CDCl_3$): 3.93 (s, 3H), 7.16-7.24 (m, 2H), 7.31 (d, J = 2.7 Hz, 1H), 8.10 (d, J = 9.1 Hz, 1H), 8.15-8.18 (m, 1H), 8.29 (dd, J = 8.5 Hz and J = 2.1 Hz, 1H); ^{13}C NMR ($CDCl_3$): 55.9, 103.4, 115.6, 115.8, 117.8, 120.5, 124.9, 125.0, 125.6, 126.5, 132.1, 139.1, 143.4, 148.4, 159.9, 184.4; MS (ESI): 304.0 $(M+H)^+$.

1,3-Benzothiazol-2-yl-(4-fluoro-3-methoxyphenyl)-methanol (**3ii**). The title compound was prepared by reaction of 1,3-benzothiazole (0.20 ml, 1.85 mmol), 2.5 M solution of nBuLi (0.74 ml, 1.85 mmol) in hexane and 4-fluoro-3-methoxybenzaldehyde (0.22 g, 1.42 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 70:30); yield: 61 % (0.25 g); ^{1}H NMR ($CDCl_3$): 4.04 (s, 3H), 6.33 (s, 1H), 7.20-7.26 (m, 2H), 7.35-7.41 (m, 1H), 7.53-7.58 (m, 1H), 7.61-7.65 (m, 1H), 7.99-8.04 (m, 1H), 8.08-8.13 (m, 1H); ^{13}C NMR ($CDCl_3$): 56.1, 73.5, 111.7, 115.9, 116.0, 119.0, 121.7, 122.8, 125.2, 126.1, 134.9, 137.1, 137.2, 147.7, 147.8, 151.3, 152.3, 153.2, 175.5.

1,3-Benzothiazol-2-yl-(4-fluoro-3-methoxyphenyl)methanone (**3i**). The title compound was prepared by reaction of 1,3-benzothiazol-2-yl-(4-fluoro-3-methoxyphenyl)methanol (**3ii**) (0.25 g, 0.86 mmol) and 2-iodoxybenzoic acid (0.48 g, 1.72 mmol) according to method C. The product was purified by CC (hexane/ethyl acetate 90:10); yield: 73 % (0.18 g); ^{1}H NMR ($CDCl_3$): 4.01 (s, 3H), 7.23-7.27 (m, 2H), 7.55-7.62 (m, 2H), 8.02-8.04 (m, 1H), 8.20 (dd, J = 8.2 Hz and J = 2.2 Hz, 1H), 8.23-8.25 (m, 1H); ^{13}C NMR ($CDCl_3$): 56.4, 115.7, 115.8, 116.0, 116.1, 122.2, 125.7, 126.0, 126.1, 126.6, 127.0, 127.7, 131.4, 136.9, 137.0, 147.8, 166.4, 167.1, 183.4.

1,3-Benzothiazol-2-yl-(4-fluoro-3-hydroxyphenyl)methanone (**3**). The title compound was prepared by reaction of 1,3-benzothiazol-2-yl-(4-fluoro-3-methoxyphenyl)-methanone (**3i**). (0.25 g, 0.87 mmol) and pyridinium hydrochloride (5.03 g, 43.5 mmol) according to method D. The product was directly purified by preparative HPLC; yield: 78 % (0.18 g); ^{1}H NMR (CD_3COCD_3): 7.36 (dd, J = 8.5 Hz and J = 2.2 Hz, 1H), 7.64-7.70 (m, 2H), 8.22-8.30 (m, 4H), 9.19 (s, 1H); ^{13}C NMR (CD_3COCD_3): 118.0, 118.1, 122.5, 124.4, 126.1, 126.2, 127.3, 129.1, 129.8, 133.6, 138.7, 147.0, 155.7, 158.3, 169.1, 184.9. MS (ESI): 273.9 $(M+H)^+$.

(6-Methoxy-1,3-benzothiazol-2-yl)(4-methyl-3-methoxyphenyl)methanol (**4ii**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazole (**1iii**) (0.25 g, 1.5 mmol), nBuLi (0.61 ml, 1.5 mmol) and 4-methyl-3-methoxybenzaldehyde (0.18 g, 1.2 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 60:40); yield: 89 % (0.42 g); ^{1}H NMR ($CDCl_3$): 2.2 (s, 3H), 3.79 (s, 3H), 3.84 (s, 3H), 6.07 (s, 1H), 6.98-7.05 (m, 2H), 7.03 (dd, J = 9.1 Hz and J = 2.7 Hz, 1H), 7.11 (d, J = 7.6 Hz, 1H), 7.24 (d, J= 2.4 Hz, 1H), 7.82 (d, J = 9.1 Hz, 1H); ^{13}C NMR ($CDCl_3$): 16.0, 55.3, 55.7, 74.2, 104.2, 115.4, 118.1, 118.4, 123.4, 127.1, 130.7, 136.5, 139.8, 146.8, 157.6, 158.0, 172.7.

(4-Methyl-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)methanone (**4i**). The title compound was prepared by reaction of (4-methyl-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)methanol (**4ii**) (0.33 g, 1.03 mmol) and 2-iodoxybenzoic acid (0.58 g, 2.06 mmol) according to method C. The product was purified by CC (hexane/ethyl acetate 85:15); yield: quantitative (0.33 g); ^{1}H NMR ($CDCl_3$): 2.2 (s, 3H), 3.79 (s, 3H), 3.82 (s, 3H), 7.04 (dd, J = 9.1 Hz and J = 2.5 Hz, 1H), 7.13-7.15 (m, 1H), 7.27 (d, J = 2.5 Hz, 1H), 7.83 (d, J = 1.3 Hz, 1H), 7.96 (d, J = 9.1 Hz, 1H), 8.12 (dd, J = 7.8 Hz

and J = 1.6 Hz, 1H); ^{13}C NMR ($CDCl_3$): 16.6, 55.3, 55.7, 103.3, 111.3, 117.4, 124.4, 126.3, 130.3, 133.9, 138.9, 148.4, 159.6, 165.0, 184.3.

(4-Methyl-3-hydroxyphenyl)(6-hydroxy-1,3-benzothiazol-2-yl)methanone (**4**). The title compound was prepared by reaction of (4-methyl-3-methoxyphenyl)(6-methoxy-1,3-benzothiazol-2-yl)methanone (**4i**) (0.33 g, 1.05 mmol) and pyridinium hydrochloride (12.13 g, 105 mmol) according to method D. The product was purified by preparative HPLC; yield: 42 % (0.12 g); ^{1}H NMR (CD_3COCD_3): 2.32 (s, 3H), 7.21 (dd, J = 8.8 Hz and J = 2.4 Hz, 1H), 7.32 (d, J = 7.6 Hz, 1H), 7.55 (d, J = 2.1 Hz, 1H), 8.02 (d, J = 1.5 Hz, 1H), 8.06 (d, J = 8.8 Hz, 1H), 8.10 (dd, J = 7.6 Hz and J = 1.5 Hz, 1H), 8.70 (s, 1H), 9.22 (s, 1H); ^{13}C NMR (CD_3COCD_3): 17.1, 107.9, 113.75, 117.7, 117.7, 119.0, 124.3, 127.8, 132.1, 135.5, 140.3, 142.7, 156.7, 159.3, 185.2; MS (ESI): 287.0 $(M+H)^+$.

(2,5-Dimethoxyphenyl)-(6-methoxy-1,3-benzothiazol-2-yl)-methanol (**5ii**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazole (**1iii**) (3.00 g, 18.2 mmol), nBuLi (7.26 ml, 18.2 mmol) and 2,5-dimethoxybenzaldehyde (2.32 g, 14.0 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 80:20); yield: 87 % (5.2 g); ^{1}H NMR (CD_3COCD_3): 3.76 (s, 3H), 3.80 (s, 3H), 3.87 (s, 3H), 6.50 (s, 1H), 6.89 (dd, J = 8.8 Hz and J = 3.1 Hz, 1H), 6.96 (d, J = 8.8 Hz, 1H), 7.09 (dd, J = 8.8 Hz and J = 2.5 Hz, 1H), 7.25 (d, J= 3.1 Hz, 1H), 7.54 (d, J = 2.5 Hz, 1H), 7.83 (d, J = 9.1 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 56.8, 57.0, 57.5, 69.9, 106.1, 114.0, 115.2, 115.5, 117.0, 125.0, 133.3, 138.3, 149.4, 152.6, 155.7, 159.4, 175.2.

(2,5-dimethoxyphenyl)-(6-methoxy-1,3-benzothiazol-2-yl)methanone (**5i**). The title compound was prepared by reaction of (2,5-dimethoxy-phenyl)-(6-methoxy-1,3-benzothiazol-2-yl)-methanol (**5ii**) (5.20 g, 15.7 mmol) and 2-iodoxybenzoic acid (8.79 g, 31.4 mmol) according to method C. The product was purified by CC (hexane/ethyl acetate 80:20); yield: 63 % (3.2 g); the compound was used in the next step without characterization.

(2,5-dihydroxyphenyl)(6-hydroxy-1,3-benzothiazol-2-yl)methanone (**5**). The title compound was prepared by reaction of (2,5-dimethoxyphenyl)-(6-methoxy-1,3-benzothiazol-2-yl)methanone (**5i**) (5.2 g, 15.8 mmol) and boron tribromide (78.9 mmol) according to method E. The product was purified by CC (hexane/ethyl acetate 80:20); yield: 59 % (4.5 g); ^{1}H NMR (CD_3COCD_3): 3.03 (s, 2H), 6.92 (d, J = 9.1 Hz, 1H), 7.21-7.25 (m, 2H), 7.57 (d, J = 2.5 Hz, 1H), 8.09 (d, J = 8.8 Hz, 1H), 8.82 (d, J = 3.2 Hz, 1H), 11.67 (s, 1H); ^{13}C NMR (CD_3COCD_3): 108.2, 119.5, 119.8, 119.9, 120.6, 128.1, 128.4, 140.8, 149.5, 151.5, 159.4, 160.3, 165.8, 188.7; MS (ESI): 287.6 $(M+H)^+$.

6-(Benzyloxy)-1,3-benzothiazole (**6iiii**). To a solution of 6-hydroxy-1,3-benzothiazole (**6iiiii**) (0.82 g, 5.42 mmol) and benzyl bromide (0.71 ml, 5.97 mmol) in acetone, potassium carbonate (3.25 g, 38.0 mmol) was added. The reaction mixture was refluxed overnight. A mixture of water and saturated ammonium chloride solution (1:2) was added to quench the reaction, and the aqueous solution was then extracted (3x) 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 (hexane/ethyl acetate 80:20); yield: quantitative (1.31 g); ^{1}H NMR ($CDCl_3$): 5.15 (s, 2H), 7.21 (dd, J = 8.8 Hz and J = 2.4 Hz, 1H), 7.33-7.36 (m, 1H), 7.39-7.42 (m, 2H), 7.45-7.47 (m, 3H), 8.03 (d, J = 9.1 Hz, 1H), 8.84 (s, 1H); ^{13}C NMR ($CDCl_3$): 70.7, 105.4, 116.5, 124.0, 127.5, 128.2, 128.7, 136.5, 138.1, 139.1, 151.6, 157.2, 176.3, 180.0.

[6-(Benzyloxy)-1,3-benzothiazol-2-yl][3-(*tert*-butyldimethylsilanyloxy)-4-methylphenyl]methanol (**6ii**). The title compound was prepared by reaction of 6-benzyloxy-1,3-benzothiazole (**6iiii**) (0.3 g, 1.24 mmol), nBuLi (0.5 ml, 1.24 mmol) and [3-(*tert*-butyldimethylsilanyloxy)-4-methyl]benzaldehyde (**6iii**) (0.23 g, 0.96 mmol) according to method A. The product was purified by CC (hexane/ethyl acetate 80:20); yield: 74 % (0.35 g); ^{1}H NMR ($CDCl_3$): 0.15 (s, 6H), 0.96 (s, 9H), 2.35 (s, 3H), 5.42 (s, 2H), 6.04 (s, 1H), 7.38-7.40 (m, 3H), 7.40-7.44 (m, 2H), 7.50-7.53 (m, 1H), 7.8 (d, J = 2.5 Hz, 1H), 8.11 (d, J = 1.6 Hz, 1H), 8.12 (dd, J = 7.5 Hz and J = 1.6 Hz, 1H), 8.15 (d, J = 8.8 Hz, 1H), 8.75 (s, 1H); ^{13}C NMR ($CDCl_3$): -4.6, 17.5, 18.2, 25.6, 72.2, 74.4, 106.3, 117.5, 119.3, 124.0, 128.0, 128.1, 128.3, 129.0, 130.9, 131.0, 132.2, 133.5, 136.0, 138.7, 141.3, 150.5, 158.0, 161.0, 167.5.

[6-(Benzyloxy)-1,3-benzothiazol-2-yl][3-(*tert*-butyl-dimethyl-silanyloxy)-4-methylphenyl]methanone (**6i**). The title compound was prepared by reaction of [6-(benzyloxy)-1,3-benzothiazol-2-yl][3-(*tert*-butyldimethylsilanyloxy)-4-methylphenyl]methanol (**6ii**) (0.35 g, 0.71 mmol) and 2-iodoxybenzoic acid (0.4 g, 1.42 mmol) according to method C. The product was purified by CC

(hexane/ethyl acetate 85:15); yield: 72 % (0.25 g); the compound was used in the next step without characterization.

[6-(Benzyloxy)-1,3-benzothiazol-2-yl](3-hydroxy-4-methylphenyl)methanone (**6**). To a solution of [6-(benzyloxy)-1,3-benzothiazol-2-yl][3-(*tert*-butyldimethylsilanyloxy)-4-methylphenyl]methanone (**6i**) (0.26 g, 0.53 mmol) in THF at 0 °C tetra-*n*-butylammonium fluoride (TBAF) (0.68 ml, 0.68 mmol) was added dropwise. The reaction mixture was stirred for 2 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 magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC (hexane/ethyl acetate 80:20) followed by preparative HPLC; yield: 50 % (0.10 g); ^{1}H NMR (CD_3COCD_3) 2.32 (s, 3H), 5.28 (s, 2H), 7.32-7.37 (m, 3H), 7.41-7.44 (m, 2H), 7.53-7.54 (m, 2H), 7.81 (d, J = 2.5 Hz, 1H), 8.03 (d, J = 1.6 Hz, 1H), 8.10 (dd, J = 7.5 Hz and J = 1.6 Hz, 1H), 8.12 (d, J = 8.8 Hz, 1H), 8.76 (s, 1H); ^{13}C NMR (CD_3COCD_3): 17.5, 72.2, 107.0, 118.2, 120.0, 124.8, 127.7, 128.1, 129.6, 129.9, 130.4, 130.6, 132.6, 133.6, 135.9, 138.7, 140.7, 150.4, 157.3, 160.9, 167.0, 185.6; MS (ESI): 376.1 $(M+H)^+$.

(6-Methoxy-1,3-benzothiazol-2-yl)[3-methoxy-4-(*tert*-butyldimethylsilanyloxy)phenyl]methanol (**7iiii**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazole (**1iii**) (0.20 g, 1.21 mmol), nBuLi (0.5 ml, 1.21 mmol) and 3-methoxy-4-*(tert*-butyldimethylsilanyloxy)benzaldehyde (**7iiiii**) (0.25 g, 0.93 mmol) according to method A. The product was purified by CC (hexane/ ethyl acetate 90:10); yield: 65 % (0.34 g); ^{1}H NMR ($CDCl_3$): 0.14 (s, 6H), 0.99 (s, 9H), 3.77 (s, 3H), 3.84 (s, 3H), 6.03 (s, 1H), 6.83 (d, J = 7.9 Hz, 1H), 6.94 (dd, J = 7.9 Hz and J = 1.8 Hz, 1H), 7.02-7.04 (m, 2H), 7.26 (t, J = 1.8 Hz, 1H), 7.83 (d, J = 9.2 Hz, 1H); ^{13}C NMR ($CDCl_3$): -4.7, 18.4, 25.6, 55.4, 55.7, 74.0, 104.2, 110.4, 115.4, 119.3, 120.8, 123.4, 134.4, 136.6, 145.3, 146.8, 151.2, 157.6, 172.9.

(6-Methoxy-1,3-benzothiazol-2-yl)[3-methoxy-4-(*tert*-butyl-dimethyl-silanyloxy) phenyl]methanone (**7iii**). The title compound was prepared by reaction of (6-methoxy-1,3-benzothiazol-2-yl)[3-methoxy-4-(*tert*-butyl-dimethyl-silanyloxy) phenyl]methanol (**7iiii**) (0.34 g, 0.78 mmol) and 2-iodoxybenzoic acid (0.44 g, 1.56 mmol) according to method C. The product was purified by CC (hexane/ ethyl acetate 95:05); yield: quantitative (0.34 g); ^{1}H NMR ($CDCl_3$): 0.22 (s, 6H), 1.00 (s, 9H), 3.92 (s, 6H), 6.98 (d, J = 8.5 Hz, 1H), 7.17 (dd, J = 8.8 Hz and J = 2.5 Hz, 1H), 7.40 (d, J = 2.5 Hz, 1H), 8.06 (d, J = 2.2 Hz, 1H), 8.08 (d, J = 8.8 Hz, 1H), 8.38 (dd, J = 8.2 Hz and J = 1.9 Hz, 1H); ^{13}C NMR ($CDCl_3$): -4.5, 18.5, 25.6, 55.5, 55.8, 103.4, 114.0, 117.3, 120.4, 126.2, 126.5, 128.8, 130.2, 148.5, 150.9, 151.1, 159.6, 165.4, 183.2.

(6-Methoxy-1,3-benzothiazol-2-yl)(3-methoxy-4-hydroxyphenyl)methanone (**7ii**). To a solution of (6-methoxy-1,3-benzothiazol-2-yl)[3-methoxy-4-(*tert*-butyl-dimethyl-silanyloxy)phenyl] methanone (**7iii**) (0.34 g, 0.78 mmol) in THF at 0 °C tetra-n-butylammonium fluoride (TBAF) (0.82 g, 3.14 mmol) was added dropwise. The reaction mixture was stirred for 2 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 magnesium sulfate, filtered and concentrated to dryness. The product was purified by CC (hexane/ethyl acetate 80:20); yield: 81 % (0.2 g); the compound was used for the next step without characterisation.

(6-Methoxy-1,3-benzothiazol-2-yl)(3-methoxy-4-phenoxyphenyl)methanone (**7i**). A mixture of (6-methoxy-1,3-benzothiazol-2-yl)(3-methoxy-4-hydroxyphenyl)methanone (**7ii**) (0.2 g, 0.63 mmol), molecular sives (4 Å), benzeneboronic acid (0.23 g, 1.9 mmol) and copper(II) acetate (0.12 g, 0.63 mmol) in anhydrous dichloromethane was stirred for 5 min. Triethylamine (0.22 ml, 1.58 mmol) was added dropwise and the reaction mixture was stirred for 18 h at room temperature 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 magnesium sulfate, filtered and concentrated to dryness. The product was purified by preparative CC (hexane/ethyl acetate 80:20); yield: quantitative (0.25 g); ^{1}H NMR ($CDCl_3$): 3.92 (s, 3H), 4.00 (s, 3H), 6.95 (d, J = 8.5 Hz, 1H), 7.08-7.10 (m, 2H), 7.16-7.19 (m, 2H), 7.36-7.41 (m, 3H), 8.06 (d, J = 9.1 Hz, 1H), 8.17 (d, J = 1.9 Hz, 1H), 8.36 (dd, J = 8.5 Hz and J = 1.9 Hz, 1H); ^{13}C NMR ($CDCl_3$): 55.8, 56.2, 103.4, 114.5, 117.5, 117.6, 118.2, 119.3, 119.4, 124.1, 126.0, 126.3, 129.8, 130.0, 139.0, 148.5, 150.3, 151.6, 156.0, 159.7, 165.0, 183.2.

(6-Hydroxy-1,3-benzothiazol-2-yl)(3-hydroxy-4-phenoxyphenyl)methanone (**7**). The title compound was prepared by reaction of (6-methoxy-1,3-benzothiazol-2-yl)(3-methoxy-4-phenoxyphenyl)methanone (**7i**) (0.1 g, 0.26 mmol) and boron tribromide (1.56 mmol) according to method E. The product was directly purified by preparative HPLC; yield: 84 % (0.08 g); ^{1}H NMR (CD_3COCD_3): 7.02 (d, J = 8.5 Hz, 1H), 7.09 (d, J = 8.5 Hz, 2H), 7.17-7.22 (m, 2H), 7.42 (t, J = 7.5 Hz, 2H), 7.55 (d, J = 2.5 Hz, 1H), 8.08 (d, J = 9.1 Hz, 1H), 8.19 (dd, J = 8.5 Hz and J = 2.2 Hz, 1H), 8.28 (d, J = 1.9 Hz, 1H), 8.71 (s, 1H), 9.21 (s, 1H); ^{13}C NMR (CD_3COCD_3): 108.3, 119.5, 119.7, 119.8, 119.9, 120.4, 121.3, 125.7, 125.9, 128.4, 131.8, 133.1, 140.8, 149.8, 150.1, 151.4, 158.4, 159.9, 184.7; MS (ESI): 363.9 $(M+H)^+$.

2-Fluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**8i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazol-2ylamine (0.4 g, 1.96 mmol) and 2-fluoro-3-methoxy-benzoyl chloride (0.4 g, 1.96 mmol) according to method B. The product was recrystallized in ethyl acetate; yield: 30 % (0.18 g); ^{1}H NMR ($CDCl_3$): 3.83 (s, 3H), 3.89 (s, 3H), 7.07 (dd, J = 2.5 Hz, J = 8.8 Hz, 1H), 7.31 (dt, J = 1.9 Hz, J = 8.8 Hz, J = 9.1, Hz 1H), 7.50-7.55 (m, 2H), 7.62 (d, J = 2.5 Hz, 1H), 7.68 (d, J = 8.8 Hz, 1H), 12.69 (s, 1H).

2-Fluoro-3-hydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**8**). The title compound was prepared by reaction of 2-fluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**8i**) (0.13 g, 0.40 mmol) and boron tribromide (4.0 mmol) according to method E. The product was purified by CC (dichloromethane/methanol 90:10); yield: 33 % (0.04 g); ^{1}H NMR (CD_3SOCD_3): 6.91 (dd, J = 2.5 Hz, J = 8.8 Hz, 1H), 7.09-7.18 (m, 3H); 7.32 (d, J = 2.5 Hz, 1H), 7.58 (d, J = 8.5 Hz, 1H), 9.58 (s, 1H), 10.21 (s, 1H), 12.51 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 106,5; 110,1; 115,4; 119,2; 120,7; 120,7; 121,2; 123,2; 123,3; 124,4; 124,4; 132,8; 145,4; 145,4; 147,5; 149,5; 154,3; 163,3; MS (ESI): 305.2 $(M+H)^+$.

4-Fluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**9i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazol-2ylamine (0.3 g, 1.66 mmol) and 4-fluoro-3-methoxy-benzoyl chloride (0.3 g, 1.66 mmol) according to method B. The product was recrystalized in ethyl acetate; yield: 100 % (0.55 g); ^{1}H NMR (CD_3COCD_3): 3.88 (s, 3H), 4.03 (s, 3H), 7.05 (dd, J = 8.8 Hz, J = 2.5 Hz, 1H), 7.33-7.37 (m, 1H), 7.54 (d, J = 2.5 Hz, 1H), 7.65 (d, J = 8.8 Hz, 1H), 7.83-7.86 (m, 1H), 8.00 (dd, J = 8.2 Hz, J = 1.9 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 56,2; 56,8; 105,1; 109,0; 114,3; 116.0; 116,9; 117,1; 117,4; 117,7; 117,9; 122,2; 122,4; 127,3; 157,9; 165,2.

4-Fluoro-3-hydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**9**). The title compound was prepared by reaction of 4-fluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**9i**) (0.25 g, 0.75 mmol) and boron tribromide (7.5 mmol) according to method E. The product was directly purified by preparative HPLC; yield: 22 % (0.05 g); ^{1}H NMR (CD_3SOCD_3): 6.92 (dd, J = 2.5 Hz, J = 8.5 Hz, 1H), 7.28-7.32 (m, 2H); 7.58 (d, J = 8.8 Hz, 1H), 7.61-7.64 (m, 1H), 7.67 (dd, J = 2.2 Hz, J = 8.5 Hz, 1H), 9.55 (s, 1H), 10.31 (s, 1H), 12.56 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 106,3; 115,3; 116,2; 118,1; 119,8; 119,9; 122,3; 125,8; 132,7; 137,2; 144,5; 144,9, 145,0; 145,0; 145,2; 145,3; 152,1; 154,3; 164,3; MS (ESI): 305.1 $(M+H)^+$.

2-Fluoro-5-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**10i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazol-2ylamine (0.4 g, 1.96 mmol) and 2-fluoro-5-methoxy-benzoyl chloride (0.4 g, 1.96 mmol) according to method B. The product was purified by CC (dichloromethane/methanol 95:05); yield: 35 % (0.23 g); ^{1}H NMR ($CDCl_3$): 3.36 (s, 3H), 3.38 (s, 3H), 6.62 (dd, J = 2.7 Hz, J = 8.8 Hz, 1H), 6.71-6.74 (m, 2H), 6.85-6.87 (m, 2H), 7.25 (d, J = 8.8 Hz, 1H).

2-Fluoro-5-hydroxy-N-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**10**). The title compound was prepared by reaction of 2-fluoro-5-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**10i**) (0.23 g, 0.68 mmol) and boron tribromide (6.8 mmol) according to method E. The product was purified by CC (dichloromethane/methanol 90:10); yield: 30 % (0.06 g); ^{1}H NMR (CD_3SOCD_3): 6.92 (dd, J = 2.5 Hz, J = 8.5 Hz, 1H), 6.95-6.99 (m, 1H); 7.06-7.07 (m, 1H), 7.17 (t, J = 8.5 Hz, 1H), 7.33 (d, J = 2.5 Hz, 1H), 7.58 (d, J = 8.8 Hz, 1H), 9.62 (s, 1H), 9.83 (s, 1H), 12.44 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 106,5; 115,4; 115,5; 116,7; 117,2; 119,8; 119,9; 121,2; 122,3; 122,3; 122,5; 132,8; 132,8; 151,6; 153,5; 153,5; 154,4; 154,8; 166,1; MS (ESI): 305.1 $(M+H)^+$.

3-Methoxy-4-methyl-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**11i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazol-2ylamine (0.3 g, 1.66 mmol) and 3-methoxy-4-methylbenzoyl chloride (0.3 g, 1.66 mmol) according to method B. The product was recrystallized in

ethyl acetate; yield: 100 % (0.55 g); ^{1}H NMR ($CDCl_3$): 2.27 (s, 3H), 3.87 (s, 3H), 3.88 (s, 3H), 7.00 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H), 7.22 (d, *J* = 8.5 Hz, 1H), 7.31 (d, *J* = 2.2 Hz, 1H), 7.49 (dd, *J* = 7.6 Hz, *J* = 1.6 Hz, 1H), 7.52-7.54 (m, 2H); ^{13}C NMR ($CDCl_3$): 16,5; 55,5; 55,9; 104,2; 109,2; 110,7; 113,6; 115,4; 119,4; 121,2; 130,5; 130,9; 132,9; 133,0; 157,0; 158,2; 165,2.

3-Hydroxy-4-methyl-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**11**). The title compound was prepared by reaction of 3-methoxy-4-methyl-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**11i**) (0.25 g, 0.76 mmol) and boron tribromide (7.6 mmol) according to method E. The product was purified by CC (dichloromethane/methanol 90:10); yield: 13 % (0.03 g); ^{1}H NMR (CD_3SOCD_3): 2.20 (s, 3H), 6.91 (dd, *J* = 2.5 Hz, *J* = 8.8 Hz, 1H), 7.22 (d, *J* = 8.5 Hz, 1H), 7.31 (d, *J* = 2.5 Hz, 1H), 7.46 (d, *J* = 1.6 Hz, 1H), 7.53 (dd, *J* = 1.6 Hz, *J* = 7.6 Hz, 1H), 7.57 (d, *J* = 8.5 Hz, 1H), 9.73 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 15,9; 106,3; 114,2; 115,2; 118,5; 120,7; 120,8; 129,5; 130,4; 132,7; 138,3; 141,4; 146,3; 154,1; 165,5; MS (ESI): 301.1 $(M+H)^+$.

3,5-Dimethoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**12i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazol-2ylamine (0.3 g, 1.66 mmol) and 3,5-dimethoxy-benzoyl chloride (0.3 g, 1.66 mmol) according to method B. The product was recrystalized in ethyl acetate; yield: 100 % (0.57 g); ^{1}H NMR (CD_3OD): 3.88 (s, 3H), 3.90 (s, 6H), 6.75 (t, *J* = 2.5 Hz, *J* = 2.2 Hz, 1H), 7.05 (dd, *J* = 8.8 Hz, *J* = 2.5 Hz, 1H), 7.18 (d, *J* = 2.2 Hz, 1H), 7.38 (d, *J* = 2.2 Hz, 1H), 7.53 (d, *J* = 2.5 Hz, 1H), 7.65 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (CD_3OD): 56,8; 57,1; 57,1; 106,1; 107,0; 107,6; 109,1; 117,0; 123,3; 133,4; 135,4; 136,2; 144,5; 158,2; 158,9; 163,1; 166,9.

3,5-Dihydroxy-N-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**12**). The title compound was prepared by reaction of 3,5-dimethoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**12i**) (0.57 g, 1.66 mmol) and boron tribromide (16.6 mmol) according to method E. The product was recrystalized in methanol/water; yield: 80 % (0.40 g); ^{1}H NMR (CD_3SOCD_3): 6.47-6.49 (m, 2H), 6.90-6.92 (m, 2H), 7.30 (d, *J* = 1.9 Hz, 1H), 7.56 (d, *J* = 8.8 Hz, 1H), 9.55 (s, 1H); 9.64 (s, 2H), 12.4 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 106,3; 115,3; 116.0; 116,6; 117,1; 117,2; 121,0; 130,1; 130,4; 133,9; 135,9; 154,2; 158,4; 165,7; MS (ESI): 303.1 $(M+H)^+$.

2,5-Dimethoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**13i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazol-2ylamine (0.3 g, 1.66 mmol) and 2,5-dimethoxy-benzoyl chloride (0.3 g, 1.66 mmol) according to method B. The product was recrystalized in ethyl acetate; yield: 100 % (0.55 g); The product was purified by CC (dichloromethane/methanol 95:05); yield: 100 % (0.57 g); the compound was used in the next step without characterisation.

2,5-Dihydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**13**). The title compound was prepared by reaction of 2,5-dimethoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**13i**) (0.48 g, 1.39 mmol) and boron tribromide (13.9 mmol) according to method E. The product was purified by CC (dichloromethane/methanol 90:10); yield: 25 % (0.15 g); ^{1}H NMR (CD_3SOCD_3): 6.74 (dd, *J* = 2.5 Hz, *J* = 8.8 Hz, 1H), 7.07 (s, 1H), 7.12 (d, *J* = 2.2 Hz, 1H), 9.22 (s, 1H), 9.61 (s, 1H), 11.29 (s, 1H), 11.91 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 107,7; 114,3; 115,2; 116,6; 117,1; 117,6; 118,1, 122,2; 137,8; 141,0; 145,7; 154,4; 155,3; 164,5; MS (ESI): 303.1 $(M+H)^+$.

2-Chloro-6-fluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**14i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazol-2ylamine (0.3 g, 1.66 mmol) and 2-chloro-6-fluoro-3-methoxy-benzoyl chloride (0.4 g, 1.66 mmol) according to method B. The product was purified by CC (hexan/ethyl acetate 60:40); yield: 83 % (0.51 g); ^{1}H NMR ($CDCl_3$): 3.72 (s, 3H), 3.85 (s, 3H), 6.73 (dd, *J* = 4.4 Hz, *J* = 9.1 Hz, 1H), 6.79 (dd, *J* = 2.5 Hz, *J* = 8.8 Hz, 1H), 6.84 (t, *J* = 8.5 Hz, 1H), 7.06 (d, *J* = 8.8 Hz, 1H), 7.25 (d, *J* = 2.5 Hz, 1H); ^{13}C NMR ($CDCl_3$): 55,8; 56,6; 108,4; 103,8; 112,0; 114,0; 114,1; 114,4; 114,6; 115,4; 120,7; 133,1; 141.8; 143.4; 156,8; 160,5.

2-Chloro-6-fluoro-3-hydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**14**). The title compound was prepared by reaction of 2-chloro-6-fluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**14i**) (0.25 g, 0.68 mmol) and boron tribromide (6.8 mmol) according to method E. The product was purified by CC (dichloromethane/methanol 90:10); yield: 43 % (0.10 g); ^{1}H NMR (CD_3SOCD_3): 6.14 (dd, *J* = 2.5 Hz, *J* = 8.8 Hz, 1H), 6.22-6.28 (m, 2H); 6.46 (d, *J* = 2.5 Hz, 1H), 6.78 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (CD_3SOCD_3): 104,6; 110,1; 113,2; 113,3; 114,0; 116,6; 116,6; 119,9; 122,7; 122,9; 132,0; 140,5; 149,0; 149,0; 150,0; 151,9; 153,4; 153,4; 153,4; 153,9;160,6; MS (ESI): 339.2 $(M+H)^+$.

2,6-Difluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**15i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazol-2ylamine (0.3 g, 1.79 mmol) and 2,6-difluoro-3-methoxy-benzoyl chloride (0.4 g, 1.79 mmol) according to method B. The product was purified by CC (dichloromethane/methanol 95:05); yield: 29 % (0.18 g); ^{1}H NMR ($CDCl_3$): 3.83 (s, 3H), 3.89 (s, 3H), 7.07 (dd, *J* = 2.5 Hz, *J* = 8.8 Hz, 1H), 7.21 (dt, *J* = 1.9 Hz, *J* = 8.8 Hz, *J* = 9.1, Hz 1H), 7.36-7.40 (m, 1H), 7.63 (d, *J* = 2.5 Hz, 1H), 7.70 (d, *J* = 8.8 Hz, 1H), 13.07 (s, 1H); ^{13}C NMR ($CDCl_3$): 55,6; 56,8; 104,8; 111,4; 115,3; 116,1; 116,2; 117,6; 130,6; 143,9; 144,0; 150.7; 150.8; 150,8; 156,5; 164,6.

2,6-Difluoro-3-hydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**15**). The title compound was prepared by reaction of 2,6-difluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**15i**) (0.18 g, 0.51 mmol) and boron tribromide (5.1 mmol) according to method E. The product was purified by CC (dichloromethane/methanol 90:10); yield: 30 % (0.05 g); ^{1}H NMR (CD_3SOCD_3): 6.97-7.01 (m, 2H), 7.17-7.22 (m, 1H); 7.41 (d, *J* = 2.5 Hz, 1H), 7.59 (d, *J* = 8.2 Hz, 1H); ^{13}C NMR (CD_3SOCD_3): 107,2; 112,2; 112,3; 116,4; 120,6; 120,7; 122,7; 134,4; 142,6; 142,7; 142,7; 142,8; 143,3; 147,9; 147,9; 151,9; 151,9; 155,3; 155,6; 159,8; 167,1; MS (ESI): 323.1 $(M+H)^+$.

2,4-Difluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**16i**). The title compound was prepared by reaction of 6-methoxy-1,3-benzothiazol-2ylamine (0.3 g, 1.79 mmol) and 2,4-difluoro-3-methoxy-benzoyl chloride (0.4 g, 1.79 mmol) according to method B. The product was purified by CC (dichloromethane/methanol 95:05); yield: 20 % (0.13 g); the compound was used in the next step without characterisation.

2,4-Difluoro-3-hydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)benzamide (**16**). The title compound was prepared by reaction of 2,4-difluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**16i**) (0.13 g, 0.36 mmol) and boron tribromide (3.6 mmol) according to method E. The product was purified by CC (dichloromethane/methanol 90:10); yield: 30 % (0.04 g); ^{1}H NMR (CD_3SOCD_3): 6.92 (dd, *J* = 2.5 Hz, *J* = 8.8 Hz, 1H), 7.15-7.23 (m, 2H); 7.32 (d, *J* = 2.5 Hz, 1H), 7.59 (d, *J* = 8.8 Hz, 1H), 9.61 (s, 1H), 10.61 (s, 1H), 12.54 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 106,5; 111,7; 111,8; 115,4; 118,8; 118,9; 121,2; 132,7; 134,2; 134,4; 135,2; 138,6; 139,0; 141,0; 149,4; 154,4; 166,5; MS (ESI): 323.1 $(M+H)^+$.

4-Fluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)-N-methyl-benzamide (**17i**). To a solution of 4-fluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)benzamide (**9i**) (0.28 g, 0.83 mmol) in DMF, NaH (0.02 g, 0.91 mmol) was added and the mixture cooled to 0 ° C for 30 min. Methyl iodide (0.1 mL, 1.66 mmol) was added and the reaction mixture was stirred for 3 h at room temperature. Water was added to quench the reaction and the aqueous solution was then extracted (3x) 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 (hexane/ethyl acetate 50:50); yield: 70 % (0.20 g); ^{1}H NMR (CD_3COCD_3): 3.88 (s, 3H), 3.99 (s, 3H), 4.00 (s, 3H), 7.11-7.13 (m, 1H), 7.20-7.23 (m, 1H), 7.45-7.54 (m, 2H), 8.00-8.04 (m, 2H); ^{13}C NMR (CD_3COCD_3): 32,8; 56,3; 56,5; 107,5; 113,6; 115,2; 115,7; 116,1; 116,2; 123,4; 123,5; 128,6; 132,3; 134,8; 154,5; 156,5; 158,0; 167,8.

4-Fluoro-3-hydroxy-*N*-(6-hydroxy-1,3-benzothiazol-2-yl)-N-methyl-benzamide (**17**). The title compound was prepared by reaction of 4-fluoro-3-methoxy-*N*-(6-methoxy-1,3-benzothiazol-2-yl)-N-methyl-benzamide (**17i**) (0.20 g, 0.58 mmol) and boron tribromide (5.8 mmol) according to method E. The product was purified by CC (dichloromethane/methanol 90:10); yield: 22 % (0.04 g); ^{1}H NMR (CD_3SOCD_3): 3.91 (s, 3H), 6.92 (dd, *J* = 2.4 Hz, *J* = 8.8 Hz, 1H), 7.19-7.28 (m, 2H); 7.49 (d, *J* = 8.8 Hz, 1H), 7.71-7.74 (m, 1H), 7.89 (dd, *J* = 2.1 Hz, *J* = 9.1 Hz, 1H), 9.81 (s, 1H), 10.14 (s, 1H); ^{13}C NMR (CD_3SOCD_3): 32.3; 108,5; 113,1; 115,4; 115,7; 115,8; 118,4; 118,4; 120,7; 120,8; 126,7; 129,8; 133,2; 144,5; 144,6; 152,4; 154,4; 154,6; 165,8; MS (ESI): 319.1 $(M+H)^+$.

b) Purity data of compounds **1** to **17** were determined using LC/MS as follows and are indicated in Table 1:

A SpectraSystems® LC system consisting of a pump, an autosampler, and a PDA detector was employed.
Mass spectrometry was performed on an MSQ® electro spray mass spectrometer (ThermoFisher, Dreieich, Germany).
The system was operated by the standard software Xcalibur®.
An RP-C18 NUCLEODUR® 100-5 (125x3 mm) column (Macherey-Nagel GmbH, Düren, Germany) was used as stationary phase. All solvents were HPLC grade.
In a gradient run the percentage of acetonitrile (containing 0.1 % trifluoroacetic acid) in 0.1 % trifluoroacetic acid was increased from an initial concentration of 0 % at 0 min to 100 % at 15 min and kept at 100 % for 5 min. The injection volume was 15 µL and flow rate was set to 800 µL/min. MS analysis was carried out at a spray voltage of 3800 V, a capillary temperature of 350 °C, and a source CID of 10 V. Spectra were acquired in positive mode from 100 to 1000 m/z at 254 nm for the UV trace.

Table S1: Purity

Cmpd	R_t (min)	HPLC purity (≥ %)	MS (ESI) m/z
1	12.15	95	290.1 $(M+H)^+$
2	12.66	95	304.0 $(M+H)^+$
3	12.30	96	273.9 $(M+H)^+$
4	9.64	95	287.0 $(M+H)^+$
5	11.37	97	287.6 $(M+H)^+$
6	16.59	95	376.1 $(M+H)^+$
7	11.20	98	363.9 $(M+H)^+$
8	8.10	97	305.2 $(M+H)^+$
9	8.39	99	305.1 $(M+H)^+$
10	8.38	96	305.1 $(M+H)^+$
11	9.24	99	301.1 $(M+H)^+$
12	7.36	95	303.1 $(M+H)^+$
13	7.81	99	303.1 $(M+H)^+$
14	8.32	95	339.2 $(M+H)^+$
15	8.04	98	323.1 $(M+H)^+$
16	8.46	98	323.1 $(M+H)^+$
17	9.09	99	319.1 $(M+H)^+$

c) 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. Fresh human placenta was homogenized and cytosolic fraction and microsomes were separated by centrifugation. For the partial purification of 17β-HSD1, the cytosolic fraction was precipitated with ammonium sulfate. 17β-HSD2 was obtained from the microsomal fraction.

Inhibition of human 17β-HSD1

Inhibitory activities were evaluated by an established method with minor modifications. 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. 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 the following equation: $\%conversion = \frac{\%E2}{\%E2 + \%E1} \times 100$. Each value was calculated from at least three independent experiments.

Inhibition of human 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 the following equation: $\%conversion = \frac{\%E1}{\%E1 + \%E2} \times 100$.

ER affinity

The binding affinity of selected compounds to the ERα and ERβ was determined according to Zimmermann et al. 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). Evaluation of 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 %.

Inhibition of 17β-HSD1 in T47-D cells

T47-D cells were obtained from ECACC, Salisbury. Stripped FCS and cell culture media were purchased from CCpro, Oberdorla. A stock culture of T47-D 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 0.5 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.

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 **1** and **4** were tested for inhibition of the following enzymes: CYP3A4, 2D6, 2C9, 2C19, 1A2 and 2B6. Inhibitory potencies were determined as IC50 values.

Inhibition of marmoset *(Callithrix jacchus)* 17β-HSD1 and 17β-HSD2

17β-HSD1 and 17β-HSD2 were obtained from marmoset placenta. The inhibition assays were performed similarly to the ones carried out using human enzymes.

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3.III 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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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).

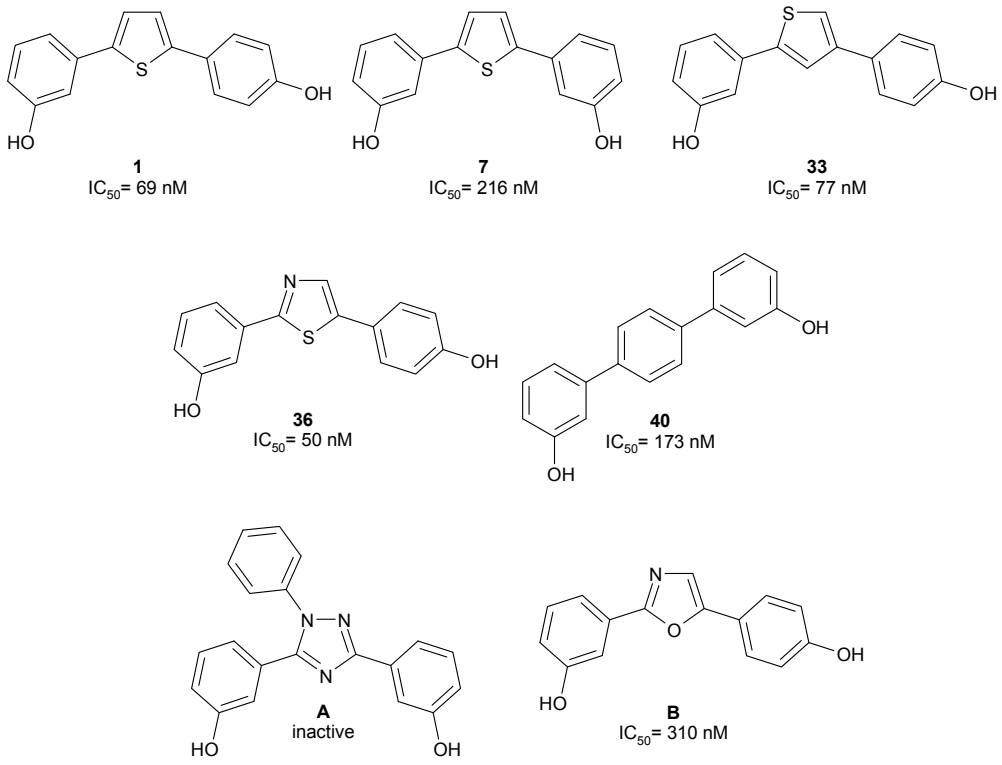


Chart 1: described bis(hydrophenyl)azoles, thiophenes, benzenes and aza-benzenes

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

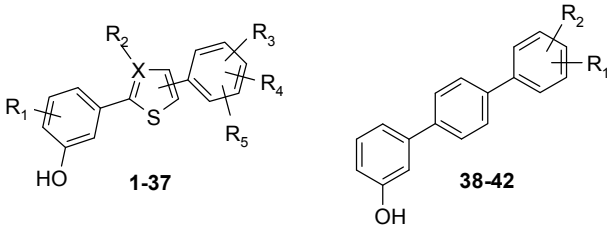


Chart 2: title compounds

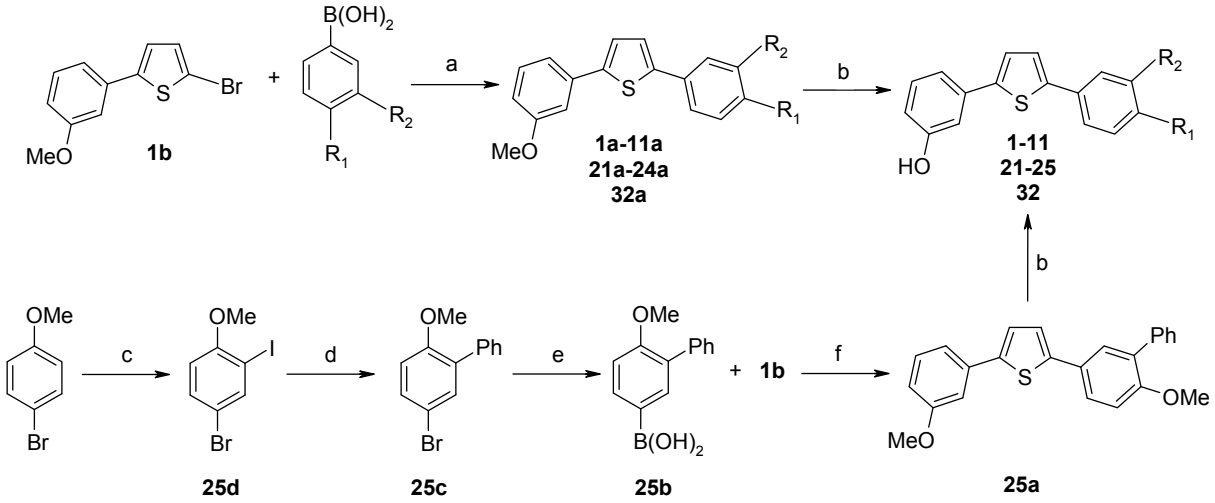
Design

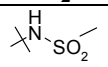
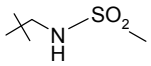
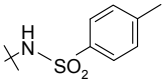
Up to now, several crystal structures of human 17β-HSD1 were resolved: as apoenzyme (i.e. PDB code: 1BHS[31]), as binary complex (enzyme-E2, i.e. PDB code: 1IOL[32]) or as ternary complex (enzyme-E2-$NADP^+$: i.e. PDB code 1FDT;[33] 1A27;[34] enzyme-HYC (hybride inhibitor): PDB code: 1I5R[35]).
The analysis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

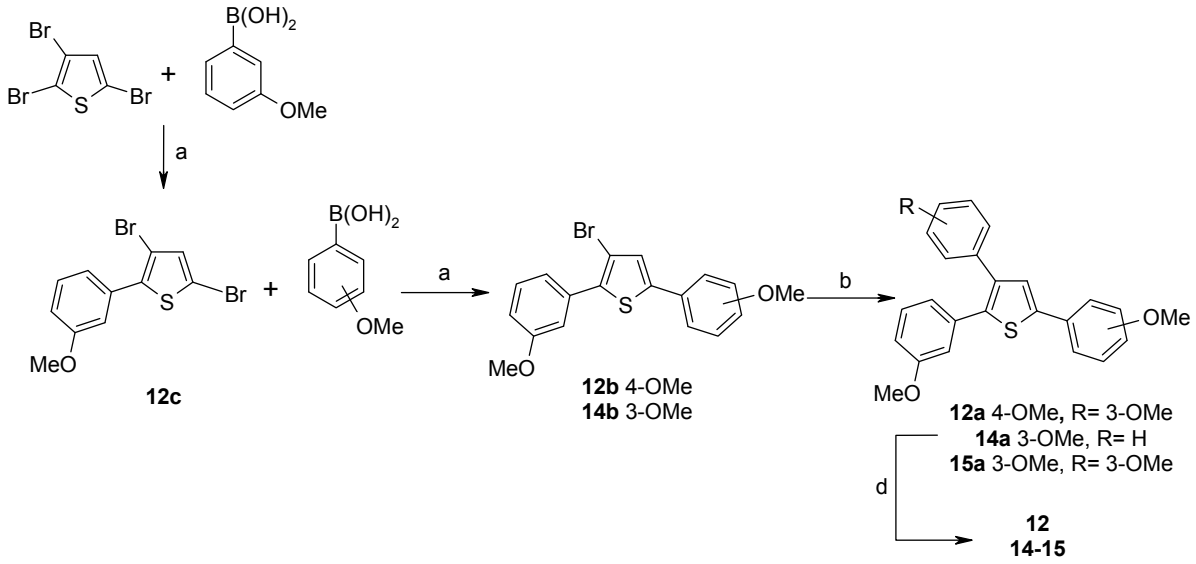


cmpd	R_1	R_2	cmpd	R_1	R_2
1	OH	H	**9**	H	
2	H	H	**10**	H	
3	F	H	**11**	H	
4	NH_2	H	**21**	OH	CH_3
5	SH	H	**22**	OH	OH
6	CN	H	**23**	OH	F
7	H	OH	**24**	OH	CF_3
8	H	F	**25**	OH	Ph
			32	F	F

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

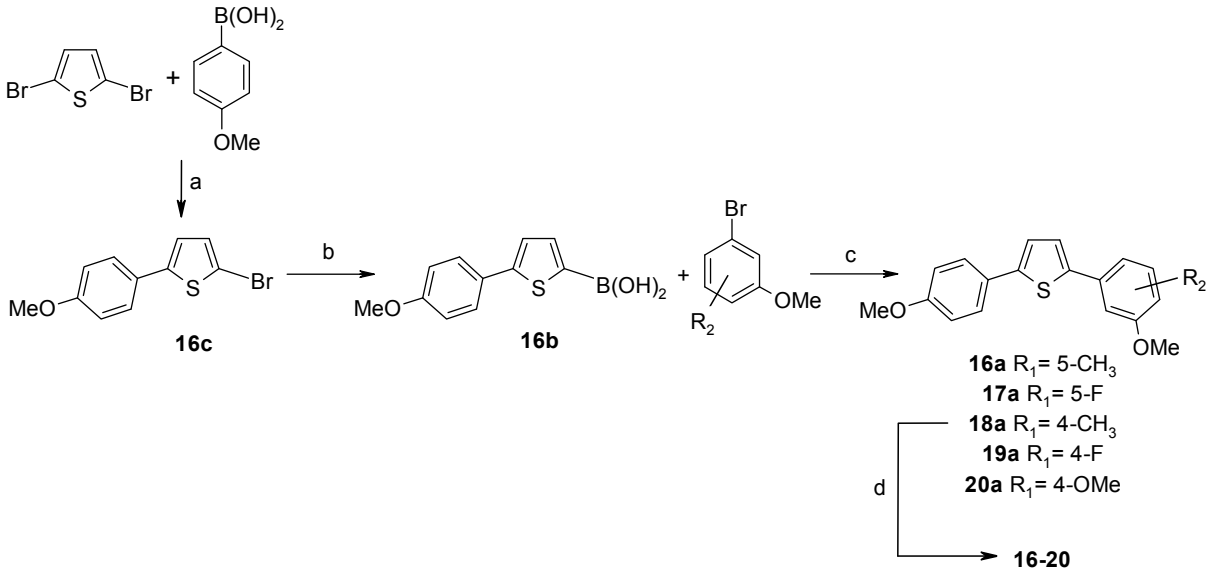
The preparation of compounds **31** and **33** to **42** is similar to the synthetic pathway presented in Scheme 1 for compounds **1** to **11**. The first Suzuki coupling was carried out according to Method B with the corresponding dibrominated heterocycle and the methoxylated benzene boronic acid. 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).



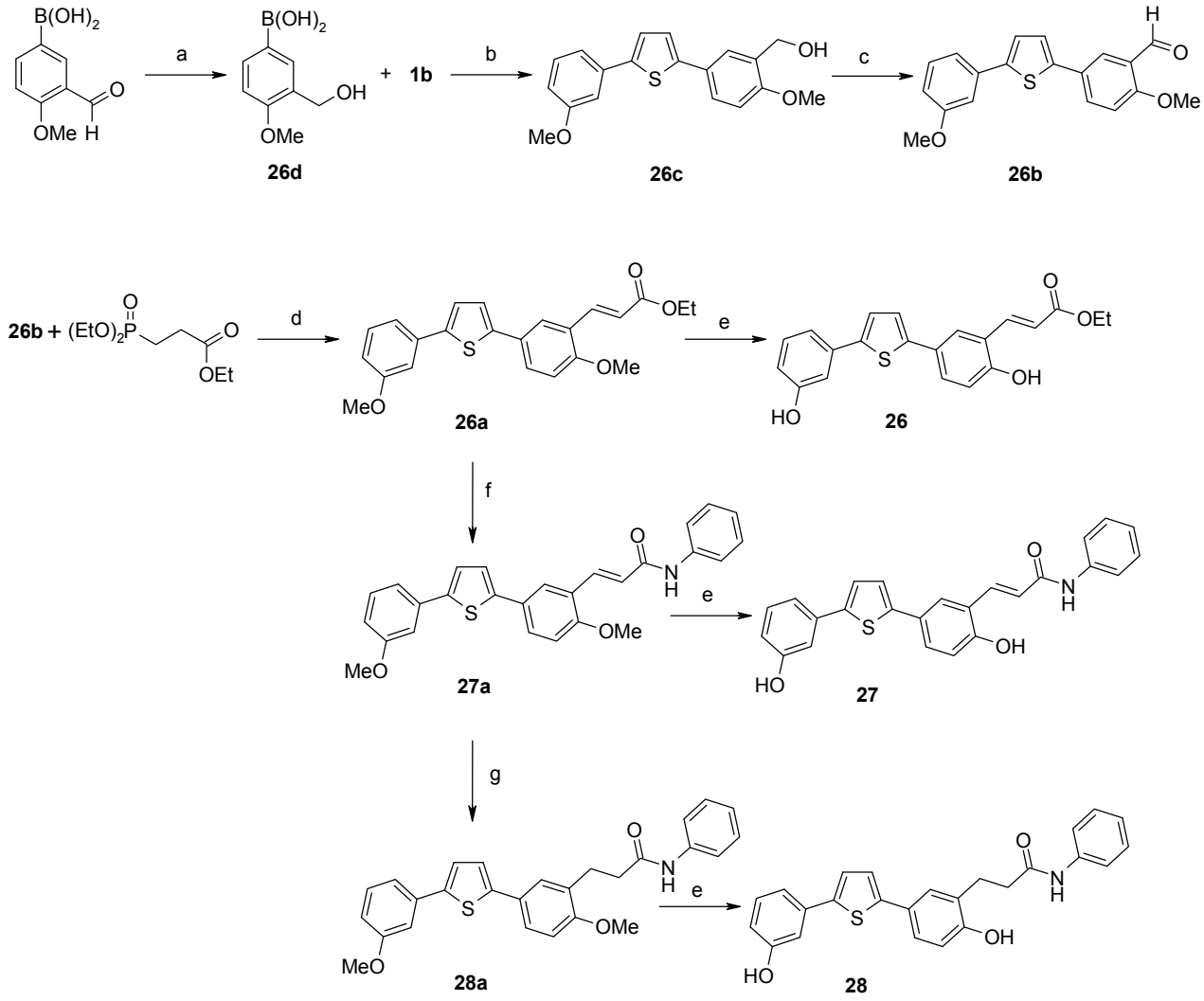
Scheme 2[a]: synthesis of compounds **12, 14** and **15.** **Reagents and conditions**: (a) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 4 h; (h) boronic acid, Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 20 h; (d) BBr_3, CH_2Cl_2, -78 °C to rt, 18 h.

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**.



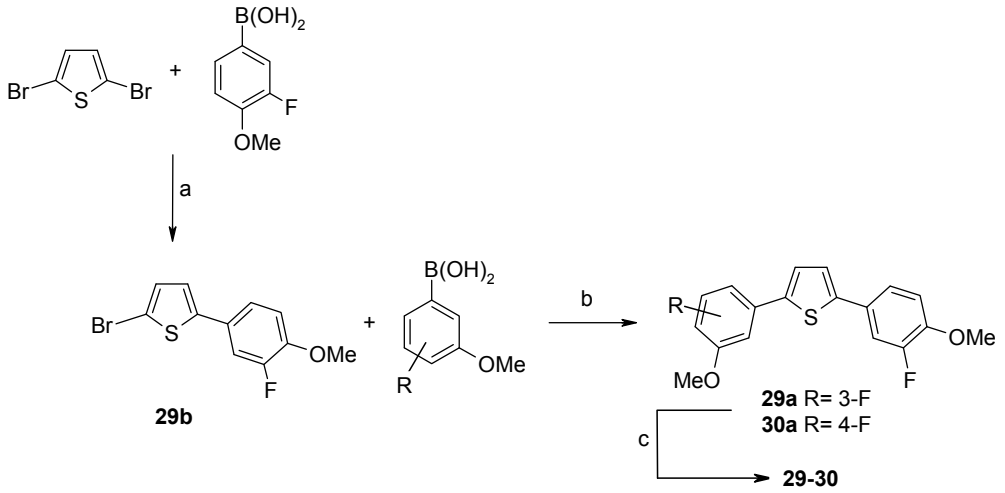
Scheme 3: synthesis of compounds **16** to **20.** **Reagents and conditions**: (a) Method B: Na_2CO_3, toluene/water (1:1), $Pd(PPh_3)_4$, reflux, 4 h; (b) 1.*n*-BuLi, anhydrous THF, -78 °C, 15 min, 2. $B(OEt)_3$, THF, -78 °C to rt, 2 h, 3. HCl 1N; (c) Method A for **17a-20a** (Cs_2CO_3, DME/EtOH/water (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: synthesis of compounds **26** to **28.** **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: synthesis of compounds **29** to **30.** **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.

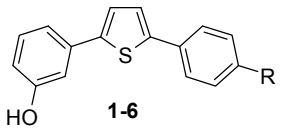
Biological Results

Activity: inhibition of human 17β-HSD1

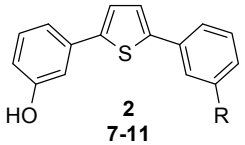
Placental enzyme was partially purified following a described procedure.[26, 27] Tritiated E1 was incubated with 17β-HSD1, cofactor and inhibitor. After HPLC separation of substrate and product, the amount of labelled E2 formed was quantified. The inhibition values of the test compounds are shown in 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.

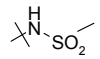
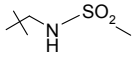
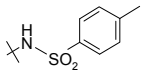
Table 1: effect of the exchange of one OH substituent for other functional groups on human 17β-HSD1 and 17β-HSD2 inhibitory activities.



1-6



2
7-11

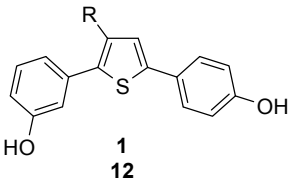
cmpd	R	IC_{50} (nM)[a]		selectivity factor[d]	cmpd	R	IC_{50} (nM)[a]		selectivity factor[d]
		17β-HSD1[b]	17β-HSD2[c]				17β-HSD1[b]	17β-HSD2[c]	
1	OH	69	1950	28	**7**	OH	173	745	4
2	H	342	2337	7	**2**	H	342	2337	7
3	F	717	3655	5	**8**	F	535	1824	3
4	NH_2	>5000	nt		**9**		523	1575	3
5	SH	>5000	nt		**10**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

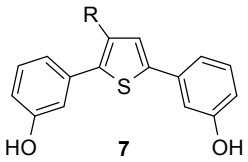
In order to improve the activity and the selectivity of our inhibitors, substituents capable to establish further interactions with the enzyme were added either on the heterocycle or on the hydroxyphenyl moieties. Additional functional groups were introduced in both of the *meta-para* 2,5-bis(hydroxyphenyl)thiophene **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.

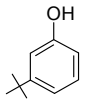
Table 2: inhibition of human 17β-HSD1 and 17β-HSD2 by compounds bearing a supplementary substituent on the thiophene core structure



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	

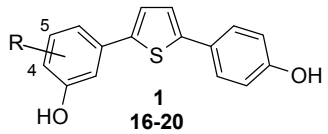
cmpd	R	IC_{50} (nM)[a] 17β-HSD1[b]	IC_{50} (nM)[a] 17β-HSD2[c]	selectivity factor[d]
7	H	173	745	4
13	CH_3	567	856	1
14		493[e]	nt	
15		119	188	2

[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

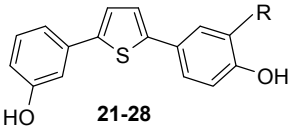
Concerning the substitution of the hydroxyphenyl rings, different groups were introduced either on the *meta*-hydroxyphenyl ring (compounds **16** to **20**) or on the *para*-hydroxyphenyl moiety (compounds **21** to **28**, Table 3). The *ortho*-position was not considered, as these compounds would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

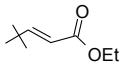
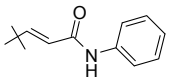
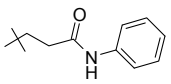
Table 3: effect of a supplementary substituent on the hydroxyphenyl moieties on the inhibition of the human 17β-HSD1 and 17β-HSD2



1
16-20



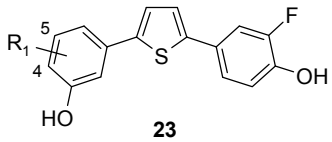
21-28

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

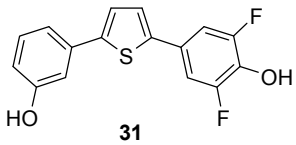
[a]Mean values of three determinations, standard deviation less than 15 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 μM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM], [d]IC_{50} (17β-HSD2)/ IC_{50} (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.

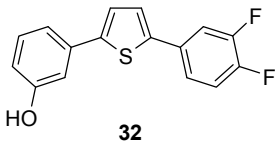
Table 4: effect of two additional fluorine atoms on the 17β-HSD1 and 17β-HSD2 inhibitory activity



23
29-30



31



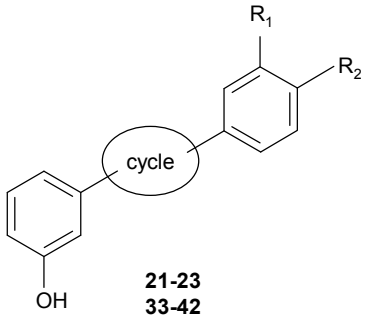
32

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

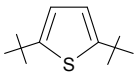
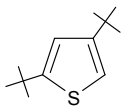
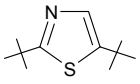
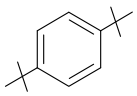
[a]Mean values of three determinations, standard deviation less than 10 %; [b]Human placenta, cytosolic fraction, substrate ^{3}H-E1 + E1 [500 nM], cofactor NADH [500 μM], [c]Human placenta, microsomal fraction, substrate ^{3}H-E2 + E2 [500 nM], cofactor NAD^+ [1500 μM], [d]IC_{50} (17β-HSD2)/ IC_{50} (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).

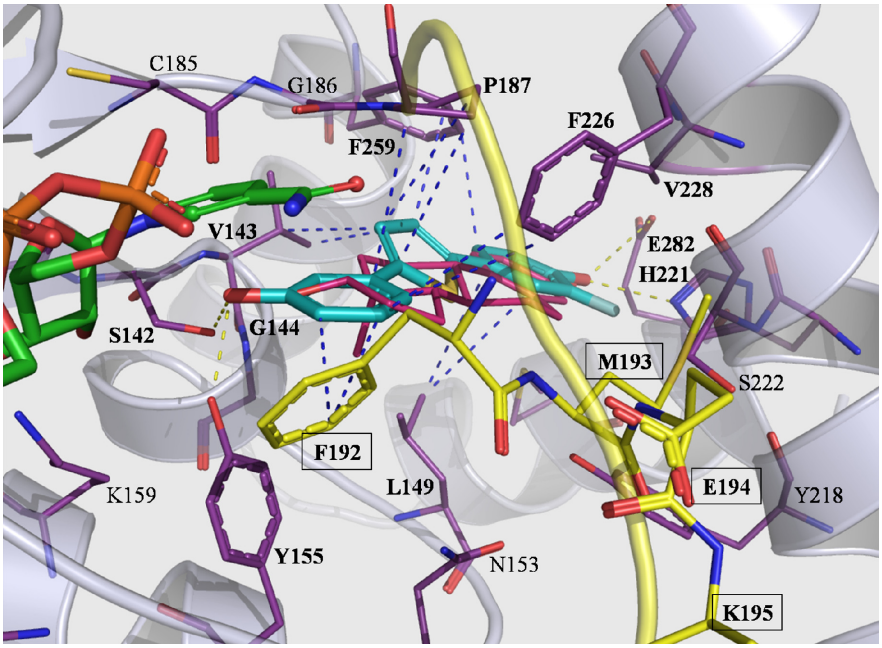


Figure 1. docking complex between 17β-HSD1 (X-ray 1FDT-B) and compound **23** (blue; SUB binding mode). NADPH, interacting residues and ribbon rendered tertiary structure of the active site are shown. Residues of the flexible loop are rendered in sticks and colored in yellow. Hydrogen bonds and π-π stackings (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.

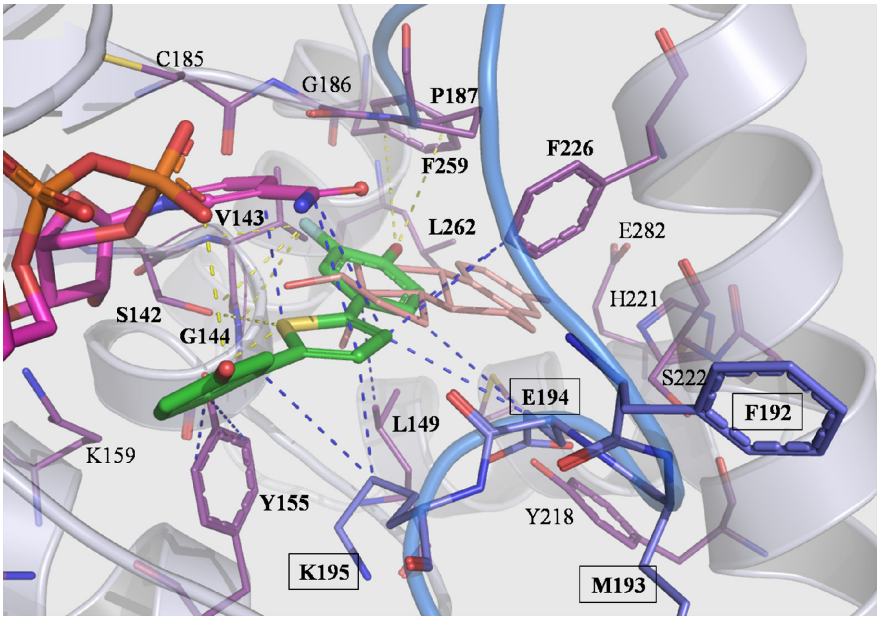


Figure 2. docking complex between 17β-HSD1 (X-ray 1FDT-A) and compound **23** (green; alternative binding mode). NADPH, interacting residues and ribbon rendered tertiary structure of the active site are shown. Residues of the flexible loop are rendered in sticks and colored in blue. Hydrogen bonds and 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.

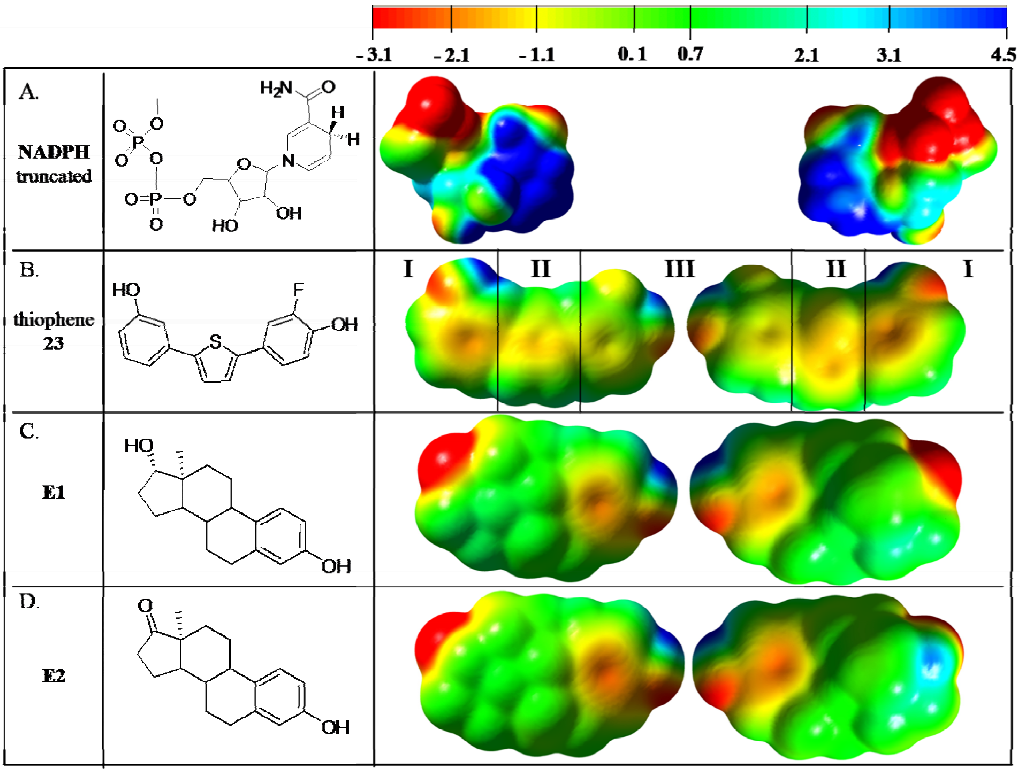


Figure 3. structures and MEP maps of both ventral (steroidal α-side) and dorsal (steroidal β-side) views of truncated NADPH (**A**), thiophene **23** (**B**), E1 (**C**) and E2 (**D**). MEP surfaces were plotted with GaussView 3.09.

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.

1. *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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4. Discussion and Conclusion

The main goal of this thesis was to develop a new class of non-steroidal highly potent and selective inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) as suitable tools for *in vivo* application in order to afford new chemical entities able to improve the treatment of estrogen-dependent pathologies.

Chapter **3.I** reports about the discovery of compound **I.6** and **I.21** by optimizing a novel, *in silico* identified, core scaffold (**I.5**). Pharmacophore model derivation, hit identification, and optimization via the synthesis and biological evaluation of 21 benzothiazole derivatives were successfully performed. The pharmacophore model was generated by superimposing five diverse 17β-HSD1 crystal structures (PDB-ID: 1equ, 1i5r, 3hb5, 1a27, 1dht) available in the Protein Data Bank and their corresponding cocrystallized steroidal ligands EQI, HYC, E2B, E2, and DHT, respectively. This new pharmacophore model enabled us to define 23 features of both the ligands and of the constant regions of the protein, involved in ligand-protein interaction. A small molecule *in house* library was screened through a partial match strategy: the molecules were left free to be placed into the pharmacophore and only virtual hits that cover at least six features were retained. The virtual hits were tested experimentally and [5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-2-yl](3-hydroxyphenyl)methanone) (**I.5** ‡), which resulted to be the most potent one (17β-HSD1 inhibition = 34 % at 1 μM), was selected as starting point for further optimization. Interestingly, based on pharmacophore results, it was observed that the ethyl group in 5 position of the thiazole moiety of **I.5** is placed where the steroidal B ring is located (corresponding to the feature **HY2** as depicted in Figure 14A).

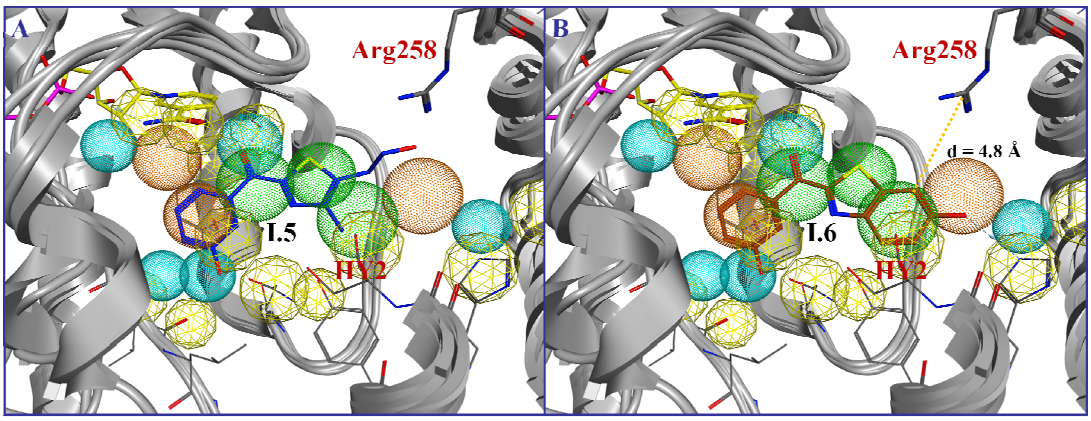


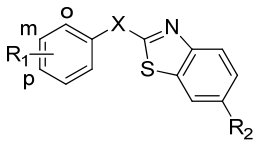
Figure 14: **Rigidification.** Compound **I.5** (A) and compound **I.6** (B) mapped to the pharmacophore model (figure revised from Figure 5 of Chapter 3.I).

Consequently, the rigidification was successfully applied to compound **I.5** and led to the discovery of the highly potent benzothiazole **I.6** (17β-HSD1 IC_{50} = 44 nM). In addition to the fact that the decrease of freedom conformational degrees given by rigidification might be sufficient to explain the much higher potency of **I.6** compared to **I.5**, further analysis of the surrounding aminoacid residues demonstrated that the phenyl ring of the benzothiazole moiety is at optimal distance (d = 4.8 Å) to undergo a cation-π interaction with Arg258, which is not possible in the case of **I.5** (see Figure 14).

‡For the sake of clarity, all compounds that are referred to chapter 3 are presented as a combination of a Roman numeral (**I-III**) and an Arabic numeral. The roman numeral indicates in which result part they are enclosed and the Arabic numeral corresponds to the compound number of the chapter. (e.g. **I.9** is compound 9 described in chapter I)

In the optimization process the carbonyl bridge of **I.6** was varied using several linkers with different lengths, geometries and H-bonding properties. The 17β-HSD1 inhibitory activity was found highly influenced by the nature of the linker: the comparison of inactive tetrahedral alcohol (**I.7**), methoxy (**I.8**), thiomethyl (**I.9**), methylene (**I.10**), and sulphonamide (**I.22**) with the active, planar carbonyl (**I.6**) and amide derivatives (**I.18** and **I.21**) led us to conclude that a flat geometry of the linker is required for the activity (see Table 5).

Table 5. Influence of linker´s geometry on 17β-HSD1 inhibitory activity.



Compound	X	R_1	R_2	HSD1[a]	
				% inhib. (1µM)	IC_{50}[b] (nM)
I.6	CO	*m*-OH	OH	91	44
I.7	CHOH	*m*-OH	OH	28	nd
I.8	$CHOCH_3$	*m*-OH	OH	13	nd
I.9	$CHSCH_3$	*p*-OH	OH	ni	nd
I.10	CH_2	*p*-OH	OCH_3	ni	nd
I.18	NHCO	*m*-OH	OH	40	nd
I.21	CONH	*m*-OH	OH	83	243
I.22	SO_2NH	*m*-OH	OH	ni	nd

[a] Human placenta, cytosolic fraction, substrate [^{3}H]E1 + E1 [500 nM], cofactor NADH [500 µM]. [b] Mean values of three determinations, standard deviation less than 10 %. [c] nd: not determined. [d] ni: no inhibition.

Based on pharmacophore results it was observed that the hydroxy-phenyl moieties of the two most active 17β-HSD1 inhibitors described in chapter **3.I** (**I.6** and **I.21**) do not interact with the same area of the enzyme (see Figure 15).

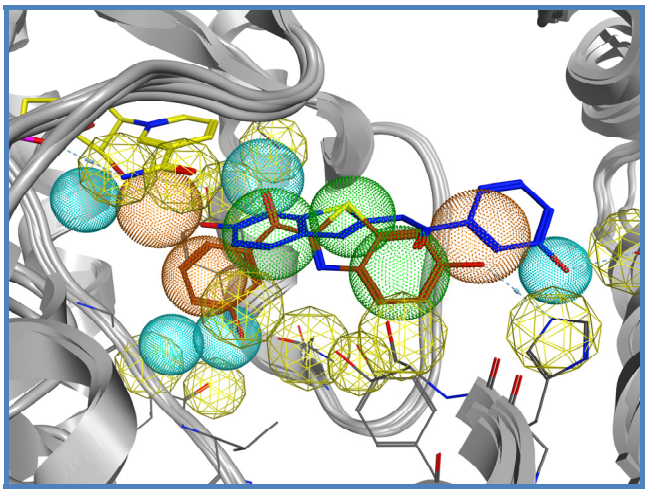


Figure 15. I.6 (dark orange) and **I.21** (blue) mapped to the pharmacophore.

The difference in activity between **I.6** and **I.21** is in agreement with the number of features covered by each compound (6 versus 5). Furthermore, concerning the binding modes similarities were observed between the previously described bicyclic substituted hydroxyphenylmethanone [126] 17β-HSD1 inhibitors and **I.6**. These results were taken into account in the further optimization process (described in chapter **3.II**). In order to evaluate the

protein-ligand interaction, the ligands of the different X-ray structures studied were replaced by compound **I.6** and **I.21** according to their pharmacophoric binding modes and the interactions between the inhibitors **I.6** and **I.21** and each of the crystal structures were considered. The maximum number of interactions was observed with the crystal structure 1equ, originally containing the inhibitor equiline (see Table 6).

Table 6. Interactions found in the complexes between **I.6** and 1equ and **I.21** and 1equ.

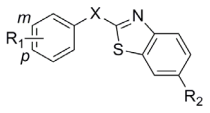
Compound	interactions	amino acid residues	1equ
6	**H**	Ser142 (donor)	
		Asn152 (acceptor)	2.53[a]
		Asn152 (donor)	
		Tyr155 (donor)	3.90
		His221 (d donor)	3.13
	π	Tyr 155	6.71
		Arg258	4.89
21	**H**	Tyr155 (acceptor)	2.91
		Tyr218 (donor)	
		His221 (donor)	2.47
		Glu282 (acceptor)	2.43
	π	Arg258	4.34

[a] Distance (Å) between the heteroatoms for H-bonds (H) and between centroids or centroid and cation for π-interactions (π).

Interestingly 1equ is the unique 17β-HSD1 crystal structure where the aminoacid residue Arg258 is turned into the active site. Notably, the importance of this amino acid residue was already postulated by Alho-Richmond S et al. [137], who proposed to target it in the inhibitor design process.

Concerning the selectivity against 17β-HSD2, it should be achieved to mainly avoid systemic effects since the expression of this enzyme is downregulated in EDD tissues but it is present in several organs (i.e. liver, small intestine, bones). However, it is difficult to estimate how high the SF should be to minimize potential side effects due to the lack of respective *in vivo* data. For our drug development program, an SF of approximately 20 is considered sufficient to justify further biological evaluation. In this study the retroamide **I.21** is the most 17βHSD2 selective compound identified. It is striking that the amide **I.18** shows a complete loss in selectivity against 17βHSD2 (see Table 7).

Table 7: Inhibition of human 17β-HSD1 and 17β-HSD2 by the best 17β-HSD1 inhibitors described in chapter **3.I**.



Compound	X	R_1	R_2	HSD1[a] [IC_{50} (nM)[c]]	HSD2[b] [IC_{50} (nM)[c]]	SF[d]
I.6	CO	*m*-OH	OH	44	1035	24
I.12	CO	*m*-OH	H	365	1356	4
I.14	CO	*p*-OH	OH	243	2471	10
I.18	NHCO	*m*-OH	OH	1307	3813	3
I.21	CONH	*m*-OH	OH	243	9264	38

[a] Human placenta, cytosolic fraction, substrate [^{3}H]E1 + E1 [500 nM], cofactor NADH [500 µM]. [b] Human placenta, microsomal fraction, substrate [^{3}H]E2 + E2 [500 nM], cofactor NAD^+ [1500 µM]. [c] Mean values of three determinations, standard deviation less than 10 %. [d] Selectivity factor = IC_{50} (HSD2)/IC_{50}(HSD1).

As no 3D-structure of 17β-HSD2 is known, it is not possible to figure out the structural differences between the two enzymes at protein level. These results indicate that the orientation of the amide group is an important feature to gain activity for 17βHSD1 and selectivity against 17βHSD2.

In the first part of our search, **I.6** resulted as the most active compound in terms of 17β-HSD1 inhibition, showing fair selectivity against 17β-HSD2 but pronounced affinity to ERs (see table 8).

Table 8. Binding affinities for the human estrogen receptors α and β of **I.6** and **I.21**.

	RBA[a] (%)	
Compound	**ERα[b]**	**ERβ[b]**
I.6	1.000 < RBA < 10.000	0.100 < RBA < 1.000
I.21	0.010 < RBA < 0.100	RBA < 0.001

[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.

Compound **I.21** on the other hand showed medium inhibitory activity at the target enzyme as well as fair selectivity against 17β-HSD2 and ERs. Furthermore, **I.21** inhibits the formation of E2 intracellularly with an IC_{50} value in the nanomolar range (245 nM). This shows that it is able to enter the cell, and that it can be active at the target enzyme. Further optimizations of these first benzothiazole-type lead compounds were necessary in order to develop potential candidates for *in vivo* application.
Chapter **3.II** deals with the optimization of **I.6** (= **II.A**) and **I.21** (= **II.B**).

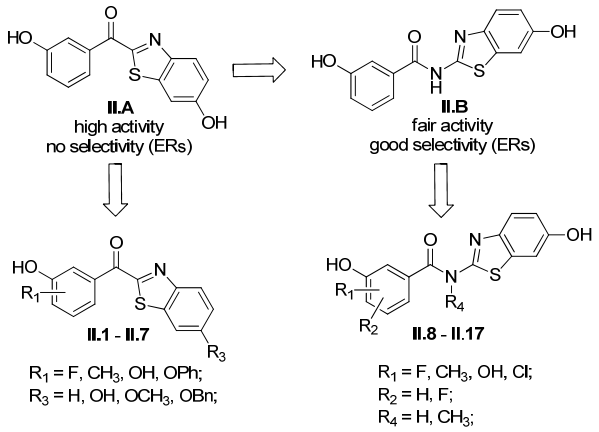


Figure 14: Structures´modifications of **II.A** and **II.B**.

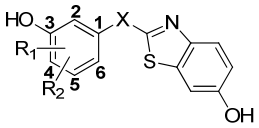
Introduction of various substituents with different electronic, lipophilic, steric and H-bonding properties into the benzoyl moiety of these compounds successfully led to the discovery of two new lead structures, compounds **II.4** and **II.15** (see following).

As suggested in our previous study where **II.A** and **II.B** were hypothesised to interact differently with the binding site, the introduction of a wide variety of substituents into the benzoyl moiety of **II.A** and **II.B** had different influence on inhibitory activity: comparing **II.1** with **II.9**, **II.4** with **II.11** and **II.5** with **II.13** shows that only the fluorine in 4-position increased inhibitory activity in both classes (IC_{50} = 13 nM for **II.1** and 171 nM for **II.9**). The introduction of OH in 2-position and methyl in 4-position turned out to be beneficial in case of the ketones (IC_{50} = 27 nM for **II.4** and 78 nM for **II.5**) but was deleterious for the amides (**II.11**, **II.13**: no inhibition at 1 μM). The introduction of a bulky substituent like the phenoxy group in 4-position (**II.7**), benzylation of the hydroxy group of **II.4** (**II.6**) and methylation of

the amide function (**II.17**) decreased inhibitory activity. Interestingly, methylation of the OH group of **II.1** (**II.2**) or replacement by hydrogen (**II.3**) on the benzothiazole moiety resulted only in a slight decrease of 17β-HSD1 inhibition (IC_{50} = 38 nM) but in a complete loss of selectivity toward 17β-HSD2 (SF = 2). Changing the F position as well as adding a second fluorine in the amide series increased 17β-HSD1 inhibitory activity: **II.15** showed the same IC_{50} value of 13 nM as the best ketone compound **II.1**. This may be due to favourable electronic effects exerted by the fluorines on the H-bonding property of the OH, to the increased lipophilicity or to the change in π-interacting properties of the aromatic ring.

Regarding selectivity, in the ketone series introduction of the methyl group in 4-position (**II.4**) not only increased activity but also decreased inhibition of 17β-HSD2 and affinity to the ERs. Thus, compound **II.4** shows the highest selectivity toward 17β-HSD2 described so far (SF 148) and only little ER affinity. It has been known for a long time that a substituent in o-position to the 3-OH group of E2 or its nonsteroidal analog hexestrol [138] reduces ERα affinity of the parent compound. Therefore it can be assumed that the ketone **II.A** binds to the ERα like the natural substrate. In the amide series introduction of fluorine in 4-position (**II.9**) led to a decrease in selectivity towards 17β-HSD2, like it has been observed for **II.1** in the ketone series, while introducing a fluorine in 2-position (**II.8**) and 6-position (**II.10**) did not alter selectivity. The difluoro-substituted compound **II.15** showed the best selectivity in this series toward 17β-HSD2 and the ERs with values similar to **II.4** (see Table 9).

Table 9: 17β-HSD1 inhibitory activity, selectivity against 17β-HSD2, binding affinities for the human estrogen receptors α and β and intacellular activity (T47-D) for the best 17β-HSD1 inhibitors compared to **II.A** and **II.B**.



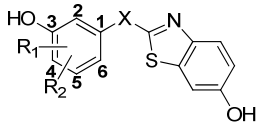
Cpd	X	R_1	R_2	HSD1[a] [IC_{50} (nM)[c]]	HSD2[b] [IC_{50} (nM)[c]]	SF[d]	ERα[e] RBA[f] (%)	ERβ[e] RBA[f] (%)	T47-D IC_{50} (nM)[c]
II.A[g]	CO	H	H	44	1035	23	< 10	< 1	
II.1		4-F	H	13	121	9	< 1	< 1	11
II.4		4-CH_3	H	27	403	148	< 0.1	< 0.1	258
II.5		6-OH	H	78	1538	20	< 0.1	< 0.1	365[i]
II.B[g]	CONH	H	H	243	9264	38	< 0.1	< 0.001	245
II.8		2-F	H	29	1000[h]	<	< 0.1	< 0.1	73[l]
II.10		6-F	H	112	3804	34	< 0.1	< 0.1	152[l]
II.15		2-F	6-	13	1774	136	< 0.1	< 0.1	37[l]

[a] Human placental, cytosolic fraction, substrate [^{3}H]E1 + E1 [500 nM], cofactor NADH [500 μM]; [b] Human placental, microsomal fraction, substrate [^{3}H]E2 + E2 [500 nM], cofactor NAD^+ [1500 μM]; [c] Mean values of three determinations, standard deviation less than 10%; [d] Selectivity factor: IC_{50} 17β-HSD2/ IC_{50} 17β-HSD1; [e] Human recombinant protein, incubation with 10 nM [^{3}H]E2 and inhibitor for 1 h. [f] RBA (relative binding affinity). E2: 100%, mean values of three determinations, standard deviations less than 10%. [g] Compounds **II.A** and **II.B**: internal standard; [h] standard deviation less than 25 %. [i] standard deviation less than 15 %; [l] Calculated with LOGIT transformation at 50 nM inhibitor concentration.

Summarising, we have discovered two highly potent 17β-HSD1 inhibitors (**II.4** and **II.15**), which are the most selective (against 17β-HSD2) compounds described so far. The amide derivative **II.15** showed better intracellular activity than **II.4** in T47-D cell line. The high cellular activity of compound **II.15** indicates that it easily permeates the cell membrane and is not metabolized quickly.

Furthermore, compounds **II.1**, **II.4**, **II.8**, and **II.15** were tested for inhibitory activity towards *Callithrix jacchus* 17β-HSD1 and 17β-HSD2 (Table 10).

Table 10: Inhibition of *Callithrix jacchus* 17β–HSD1 and 17β–HSD2.



Cpd	X	R_1	R_2	m17β-HSD1[a] % inhibition		m17β-HSD2[b] % inhibition
				5 nM	50 nM	50 nM
II.1	CO	4-F	H	80		34
II.4		4-CH_3	H		83	40
II.8	CONH	2-F	H	67		48
II.15		2-F	6-F	87		51

[a] Marmoset placenta, cytosolic fraction, substrate [^{3}H]E1 + E1 [500 nM], cofactor NADH [500 μM]; [b] Marmoset placenta, microsomal fraction, substrate [^{3}H]E2 + E2 [500 nM], cofactor NAD^+ [1500 μM];

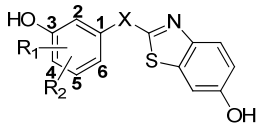
The compounds showed stronger inhibition of marmoset 17β-HSD1 compared to the human enzyme and a fair selectivity against 17β-HSD2. Accordingly, they should be suitable candidates for being further evaluated in a marmoset model of endometriosis.

Compounds **II.1** and **II.4** were further investigated for inhibition of the most important human hepatic CYP enzymes: 3A4, 2D6, 2C9, 2C19, 1A2 and 2B6. Both compounds showed very little inhibition (54 μM > IC_{50} > 3 μM) except for 1A2 which was inhibited by **1** and **4** moderately (IC_{50} = 1.8 and 0.57 μM) indicating that there should be no major problems regarding CYP inhibition in this compound class.

Furthermore, *in vitro* metabolic stability for the best inhibitors described here was evaluated using human hepatic microsomes (**II.4** and **II.5**), and human hepatic S9-fraction (**II.1**, **II.8** and **II.15**). The hepatic microsomes contain a wide variety of phase I enzymes while the hepatic S9 fraction (microsomes and cytosol of hepatocytes) contain a wide variety of both phase I and phase II enzymes. The two carbonyl derivatives **II.4** and **II.5** showed intrinsic clearance (Clint) values of 94 and 108 μL/min/mg protein, respectively, and thus, they can be arranged in the high clearance category (through phase I metabolism) although the often prescribed calcium channel blocker verapamil, used here as reference, revealed similar value (Clint = 105 μL/min/mg protein) defining a high but not too high liability to metabolic transformation.

Concerning the metabolic stability which resulted from the S9 assay (see Table 11), two compounds were used as controls: Midazolam as marker for phase I metabolism (phase I metabolism is prerequisite for phase II metabolism) and 7-hydroxycoumarin as marker for phase II metabolism (predominantly metabolised by glucuronidation and sulphatation).

Table 11: Metabolic stability in human hepatic S9-fraction.



Cpd	X	R_1	R_2	S9[a] Clint (mL/min/mg/protein)
II.1	CO	4-F	H	279
II.8	CONH	2-F	H	128
II.15		2-F	6-F	102
Midazolam[b]				35
7-Hydroxycoumarin[c]				65

[a] Human hepatic S9 fraction (1 mg/mL mixture of microsomes and cytosol of hepatocytes) supplemented with NADPH, UDPGA and PAPS; [b] Reference (phase I metabolism); [c] Reference (phase II metabolism);

Although the amide derivatives **II.8** and **II.15** showed more than two-fold better overall metabolic stability than the keto derivative **II.1** , they can be arranged in the high clearance category (through phase I and phase II metabolism) and this should be taken into account for *in vivo* evaluation of these newly discovered potent and selective 17β-HSD1 inhibitors.

5. References

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