Development of 17β-Hydroxysteroid Dehydrogenase Type 2 and Type 1 Inhibitors for the Treatment of Osteoporosis and Estrogen Dependent Diseases

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Papers included in this thesis

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

I. **Metabolic stability optimization and metabolite identification of 2,5-thiophene amide 17b-hydroxysteroid dehydrogenase type 2 inhibitors**
Emanuele M. Gargano, Enrico Perspicace, Nina Hanke, Angelo Carotti, Sandrine Marchais-Oberwinkler, Rolf W. Hartmann
Eur. J. Med. Chem., **2014**, *87*, 203-219

II **17β-Hydroxysteroid Dehydrogenase Type 2 Inhibition: Discovery of Selective and Metabolically Stable Compounds Inhibiting Both the Human Enzyme and Its Murine Ortholog**
Emanuele M. Gargano, Giuseppe Allegretta, Enrico Perspicace, Angelo Carotti, Chris Van Koppen, Martin Frotscher, Sandrine Marchais-Oberwinkler, Rolf W. Hartmann.
PLoS ONE, **2015**, *10*(7): e0134754. doi: 10.1371/journal.pone.0134754

III **Addressing cytotoxicity of 1,4-biphenyl amide derivatives: Discovery of new potent and selective 17β-hydroxysteroid dehydrogenase type 2 inhibitors**
Emanuele M. Gargano, Enrico Perspicace, Angelo Carotti, Sandrine Marchis-Oberwinkler, Rolf W. Hartmann
Accepted in *Biorg. Med. Chem. Lett.*

IV **Monograph: Inhibition of 17β-Hydroxysteroid Dehydrogenase Type 1 for the Treatment of NSCLC: Discovery of Potent, Selective and Metabolically Stable Compounds for an in vivo Proof of Concept Study.**

Contribution Report

The author wishes to clarify his contributions to the papers **I**-**III** in the thesis:

I. Planned and characterized all new compounds. Synthesized most of the new molecular entities. The author performed all the analytical method developments for the metabolic stability assay. The author planned and executed the metabolite identification experiments. Further the author significantly contributed to the interpretation of the results and concepted and wrote the manuscript.

II Planned and characterized all new compounds. The synthesis of all compounds were carried on in collaboration with Giuseppe Allegretta, as part of his master thesis. The author performed all the analytical method developments for the metabolic stability assay and performed the estrogen receptor assay and the metabolic stability assay. Further the author significantly contributed to the interpretation of the results and concepted and wrote the manuscript.

III Planned and characterized all new compounds. Compounds **1-8** and compounds **9-15** were synthesized with the contribution of Mateusz Piontek and Arcangela Mazzini, respectively, as part of their diploma thesis. Further the author significantly contributed to the interpretation of the results and concepted and wrote the manuscript.

Furthers publications of the Author, not included in this thesis

- Marie Wetzel, Emanuele M. Gargano, Stefan Hinsberger, Sandrine Marchais-Oberwinkler, Rolf W. Hartmann, Discovery of a new class of bicyclic substituted hydroxyphenylmethanones as 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) inhibitors for the treatment of osteoporosis, *Eur. J. Med. Chem.***2012**, *47*, 1-17.

- Enrico Perspicace, Liliana Cozzoli, Emanuele M. Gargano, Nina Hanke, Angelo Carotti, Rolf W. Hartmann, Sandrine Marchais-Oberwinkler, Novel, potent and selective 17β-hydroxysteroid dehydrogenase type 2 inhibitors as potential therapeutics for osteoporosis with dual human and mouse activities, *Eur. J. Med. Chem.***2014**, *83*, 317-337.

- Ahmed S. Abdelsamie, Emmanuel Bey, Emanuele M. Gargano, Chris J. van Koppen, Martin Empting, Martin Frotscher, Towards the evaluation in an animal disease model: Fluorinated 17β-HSD1 inhibitors showing strong activity towards both the human and the rat enzyme, *Eur. J. Med. Chem.***2015**, *103*, 56-68.

Abbreviations

3β-HSD	3β-Hydroxysteroid dehydrogenase
17β-HSD	17β-Hydroxysteroid dehydrogenase
h17β-HSD1	Human 17β-hydroxysteroid dehydrogenase type 1
h17β-HSD2	Human 17β-hydroxysteroid dehydrogenase type 2
m17β-HSD1	Mouse 17β-hydroxysteroid dehydrogenase type 1
m17β-HSD2	Mouse 17β-hydroxysteroid dehydrogenase type 2
r17β-HSD1	Rat 17β-hydroxysteroid dehydrogenase type 1
r17β-HSD2	Rat 17β-hydroxysteroid dehydrogenase type 2
A-dione	4-Androstene-3,17-dione
AKR	Aldo-keto reductase
AR	Androgen receptor
CDCl3	Deuterated chloroform
COX	Cyclooxygenase
CYP	Cytochrome P450
CYP11A1	Cholesterol desmolase
CYP17	17α-Hydroxylase-17,20-lyase
CYP19	Aromatase
DHT	5α-Dihydrotestosterone
DME	Dimethoxyethane
DMEM	Dulbecco's modified eagle medium
DMF	Dimethylformamide
E1	Estrone
E2	17β-Estradiol
EDD	Estrogen-dependent disease
EGF	Epidermal Growth Factor
ER	Estrogen receptor
equiv	Equivalent
Et	Ethyl

FCS	Fetal calf serum
GnRH	Gonadotropin-releasing hormone
Hz	Hertz
KO	Knock-out
Me	Methyl
MEP	Molecular electrostatic potential
MHz	Megahertz
nM	Nanomolar
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NSAID	Non-steroidal anti-inflammatory drugs
NSCLC	Non-small cell lung cancer
PDB	Protein data bank
Ph	Phenyl
PPB	Plasma protein binding
ppm	Parts per million
PSA	Polar surface area
PTH	Parathyroid hormone
RANK(L)	Receptor activator of nuclear factor κβ (ligand)
RBA	Relative binding affinity
rt	Room temperature
SAR	Structure activity relationship
SDR	Short dehydrogenase reductase
SERM	Selective estrogen receptor modulator
s.f.	Selectivity factor
T	Testosterone
THF	Tetrahydrofurane
VEGF	Vascular endothelial growth factor
µM	Micromolar

Abstract

17β-HSD2 is a new promising target for the treatment of osteoporosis. In the first part of this thesis, a rational approach to overcome metabolic instability of the 2,5-thiophene amide class of 17β-HSD2 inhibitors is described, as well as a general overview of the occurring biotransformation in this series of molecules (Chapter 2.I). This information was then used to design and synthesize a new class of compounds, bearing a 1,4-phenyl amide core, which proved to be metabolically stable, potent inhibitors of both human and mouse 17β-HSD2 and selective over human and mouse 17β-HSD1 and ERs. The best compound in the series, **II.17a** is therefore a potential candidate for pre-clinical animal studies, prior to human studies (Chapter 2.II).
Cell viability experiments, using an MTT assay, indicated that cell toxicity might constitute a drawback for **II.17a**. A preliminary study to address cell toxicity and maintain good inhibitory activity is also presented (Chapter 2.III).

In the second part of this thesis (Chapter 2.IV) the theoretical basis for the treatment of NSCLC, through inhibition of 17β-HSD1, is given. The knowledge gained about metabolic stability of 2,5-thiophene amides is exploited to design and synthesize new potent, selective and metabolically stable 17β-HSD1 inhibitors. Compound **IV.15** completely inhibits the growth of NSCLC Calu-1 cell at low nanomolar concentration, suggesting new treatment option for patients affected with NSCLC.

Zusammenfassung

17β-HSD2 ist ein neues vielversprechendes Target für die Behandlung von Osteoporose. Im ersten Teil dieser Arbeit wird ein rationaler Ansatz, die metabolische Instabilität der 2,5-Thiophenamid Klasse zu überwinden, beschrieben (Kapitel 2.I). Die erlangten Erkenntnisse wurden genutzt um eine neue Verbindungklasse mit 1,4-Phenylamid Core zu entwerfen und zu synthetisieren. Diese Verbindungen sind metabolisch stabile potente Hemmstoffe von humanem und murinem 17β-HSD2 und selektiv gegenüber den entsprechenden 17β-HSD1 Isoformen und ERs. Mit der besten Verbindung dieser Serie **II.17a** ist es gelungen einen potentiellen Kandidaten für präklinische Tierstudien zu synthetisieren (Kapitel 2.II).Toxizitätsstudien an menschlichen Zellen zeigten jedoch, dass diese Verbindung nicht frei von toxischen Effekten ist. Eine vorläufige Studie mit dem Ziel dieses Handikap zu überwinden und gleichzeitig die Hemmaktivität zu erhalten, wird in Kapitel 2.III vorgestellt.Im zweiten Teil dieser Arbeit (Kapitel 2.IV) wird die theoretische Basis für die Behandlungvon NSCLC durch Hemmung von 17β-HSD1 vorgestellt. Das in dieser Arbeit erlangte Wissen über die metabolische Stabilität der 2,5-Thiophenamide wurden beim Design und der Synthese neuer, potenter, selektiver und metabolisch stabiler 17β-HSD1 Hemmstoffe angewandt. Verbindung **IV.15** hemmt bereits bei nanomolaren Konzentrationen vollständig das Wachstum von NSCLC Calu-1 Zellen. Diese Erkenntnisse eröffnen neue Behandlungswege für Patienten mit NSCLC.

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1. Introduction

1.1 Endocrine system and steroidal hormones

1.1.1 Hormones and sexual hormones

Hormones are signal transmitter, produced by endocrine cellsand released in the circulatory system to regulate the physiology of target organs.
Several physiological processes, such as regulation of temperature and blood pressure, metabolism, control of the reproductive cycle, stimulation or inhibition of growth and other activities are under the control of these chemical substances named hormones.
Hormones mainly act on the target cells by modulating the kinetic of enzymatic reactions, by controlling the transport of molecules through the cell membrane and by controlling the expression of genes and the synthesis of proteins.
Hormones production is regulated through a complex system, which ensures a fine modulation of the molecules release (Figure 1).
The production of a hormone from a peripheral gland, such as adrenal or thyroid, starts with the production from the hypothalamus of a releasing hormone (RH). The RH stimulates the pituitary to release a glandotropic hormone, which in turn stimulates the target gland to secrete the specific hormonal molecule.
The hormones produced from the peripheral gland exert a negative feedback, by inhibiting the hormonal production from the hypothalamus and the pituitary. This mechanism of regulation is known as the hypothalamus-pituitary axis.(Monticelli, 2013)

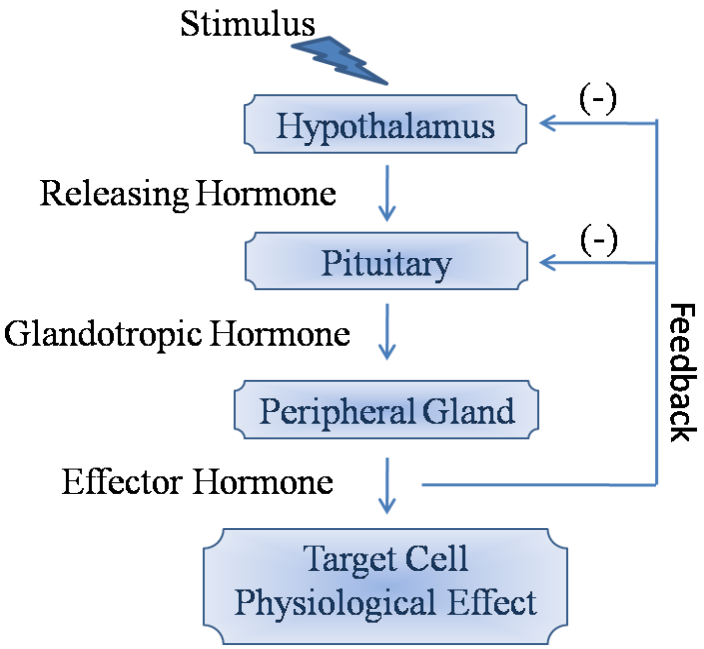


Figure 1.Mechanism of regulation of hormones release. The concentration of the effector hormone regulates the activity of the hypothalamic-pituitary axis.

From a chemical point of view hormones can fall into four main classes: amino acid derivatives (i.a. melatonine and thyroxine), polypeptides and proteins (i.a. vasopressin), eicosanoids (i.a. arachidonic acid) and steroids (i.a. sex hormones).
At the same time signals of chemical nature released from a cell can influence (Figure 2) (Voet and Voet, 2011; Labrie, 1991):

- The cell itself (autocrine activity)
- Neighboring cells (paracrine activity)
- Distant cells, after release in the circulation (classical endocrine activity)

Complementary to the well-known autocrine, paracrine and endocrine activities a so called intracrine activity (Figure 2) has been described in 1988 by Labrie *et al.* (Labrie et al., 1988). The concept of intracrine activity was coined to describe "a system, where locally produced androgens and/or estrogens exert their action in the cells where synthesis took place, without release in the extracellular space".

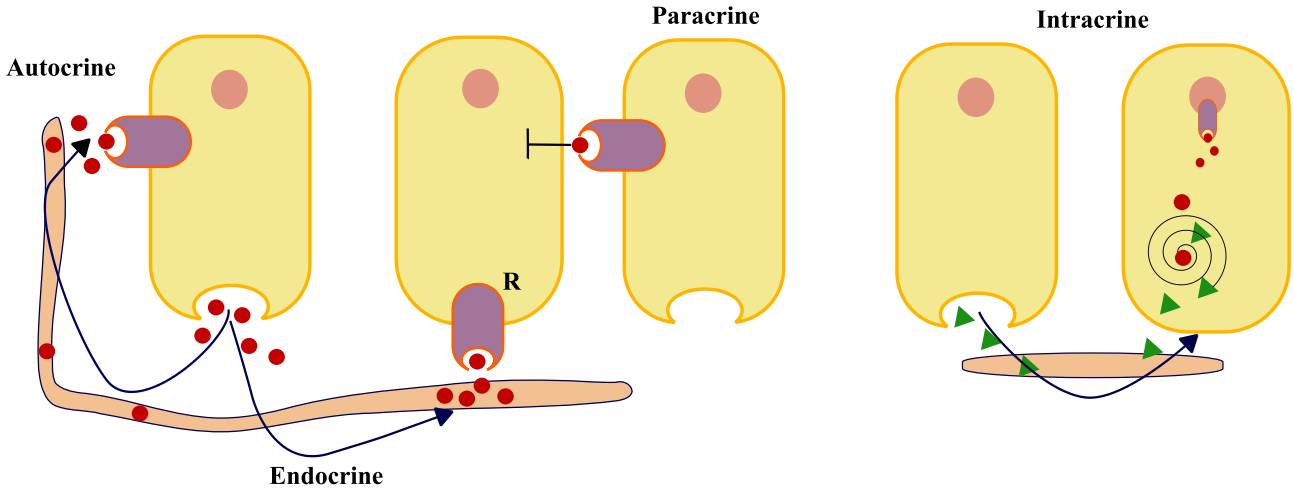


Figure 2.A cell can secrete hormones, which can influence the cell itself, a neighbouring cell, a distant cell or after modification the cell where the modification itself takes place. Red points: active hormons; Green triangles: inactive hormons.

Steroidal hormones are lipophilic molecules, which are derived from cholesterol. Sex hormones belong to the class of the steroidal hormones. Two main classes have been identified: 1) Androgens, which are the male sex hormones, such as androstenedione (A-dione), testosterone (T) are mainly produced in the testes and dihydrotestosterone (DHT) mainly in the prostate, although a small but physiologically important amount is produced in the adrenal cortex. 2) Estrogens, which are the female sex hormones, such as estradiol (E2) and estrone (E1) are mainly produced in the ovary and in the placenta.
Estrogens and androgens have a variety of effects on both the sexual organs and diverse target tissues.Estrogens promote the development of female sexual characteristics, are associated with energy homeostasis and metabolism, prevent the loss of bone in male and female and play a role in vasoprotection (Koos, 2011; Simpson et al., 2005; Gruber et al., 2002).

Testosterone, along with dihydrotestosterone is responsible for the development of male sexual characteristics as well as for mood, sexual drive and desire. They are also linked to bone formation, metabolism and erythropoiesis (Ferrucci et al., 2006; Snyder et al., 2000; Burger, 2006).

1.1.2 Biosynthesis of androgens and estrogens

In the endocrine tissues, cholesterol is the deposit steroid and it is converted to estrogens, progesterone or androgens when the tissue is stimulated by the glandotropic hormone. A simplified view of the biosynthesis pathway is depicted in Figure 3.

The cholesterol is converted into pregnenolone, via cleavage of the lateral chain and is in turn transformed either in progesterone by the 3β-hydroxysteroid dehydrogenase (3β-HSD) or in 17α-hydroxypregnenoloneby the 17α-hydroxylase (CYP17) (Ryan and Smith, 1965).

The next step involves the cleavage of the bond between C-17 and C-18, which leads to dehydroepiandrosterone(DHEA).It is catalyzed by the CYP17 itself (Nakajin and Hall, 1981).

The DHEA is then converted by 3β-HSD into 4-androsten-3,17-dione (A-dione)which can be converted into testosterone by 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3), whereas 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) catalyses the opposite reaction.

Aromatase (CYP19) through aromatisation of the A ring and cleavage of the methyl C-19 catalyses the conversion of androstenedione and testosterone into estrone and estradiol, respectively.The principal substrate for extra-ovarian aromatase activity in women is androstenedione (Bulun et al., 2000).

17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) and 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) catalyse the inter-conversion between estrone and estradiol.

It is of interest to notice that whereas in pre-menopausal women estrogens are mainly produced in the ovaries, after menopause occurrence, the estrogen production moves to peripheral tissues, such as adipose tissue or skin(Bulun et al., 2001) and the precursors of almost all of the sex steroids are of adrenal origin (Jansson, 2009).

Testosterone, which is in males the main circulating hormone is synthesized in testes and adrenals, starting from cholesterol. The main routes of the biosynthesis of testosterone have already been described and are depicted in Figure 3.

In target tissues, like in the prostate, testosterone can be converted to 5α-dihydrotestosterone by the 5α-reductase (Figure 3). It is the most active androgen (Dorfman and Ungar, 1965).

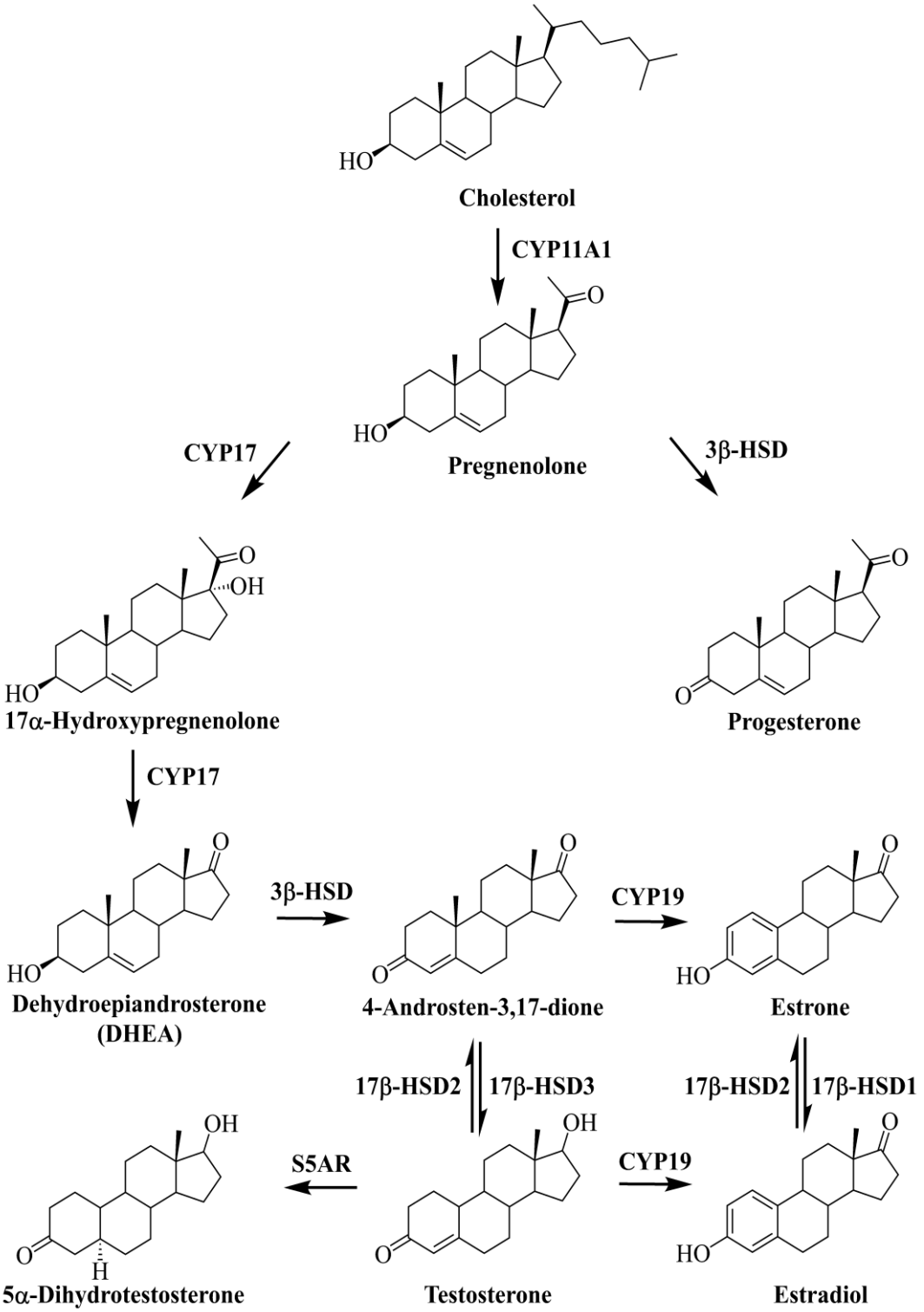


Figure 3.Simplified view of estrogens and androgens synthetic pathway. CYP11A1: cholesterol desmolase; CYP17: 17α-hydroxylase/17,20-lyase;3β-HSD: 3β-hydroxysteroid dehydrogenase/Δ5-Δ4isomerase; CYP19: aromatase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; S5AR: 5α-reductase.

The sex hormones achieve their effects via the sex hormone receptors, intracellular receptors, which function as transcription regulators, by altering the transcription of specific target genes.

Estrogens interact with the estrogen receptors (ERs) ERα(Walter et al., 1985) and ERβ(Kuiper et al., 1996) and androgens interact with the androgen receptor (AR)(Trapman et al., 1988).
Estrogens and androgens are also produced in men and women, respectively (Simpson et al., 2005; Burger, 2002; Miller and Auchus, 2011; Stocco, 2011).
Androgens are formedin females from circulating precursors (such as DHEA and androstenedione). They have been found to be beneficial for bone mineral density and decreased risk of anemia.
In males certain peripheral target tissues express aromatase, which facilitates the conversion of circulating testosterone to estradiol and androstenedione to estrone. In men, estrogen insensitivity has been associated to a loss of bone mineral density and premature atherogenesis (Koos, 2011; Simpson et al., 2005; Gruber et al., 2002).

1.2 17β-HSD2 and 1 in the steroidal hormone synthesis

1.2.1 17β-hydroxysteroid dehydrogenases (17β-HSDs)

In general HSDs are oxidoreductases, which interconvert ketones to the corresponding secondary alcohols, using NADPH/NAD^+ as cofactor. Steroid hormones are the main substrate, but some HSDs are involved in the metabolism of different non-steroidal compounds (Hoffmann and Maser, 2007; Maser, 1995; Matsunaga et al., 2006).

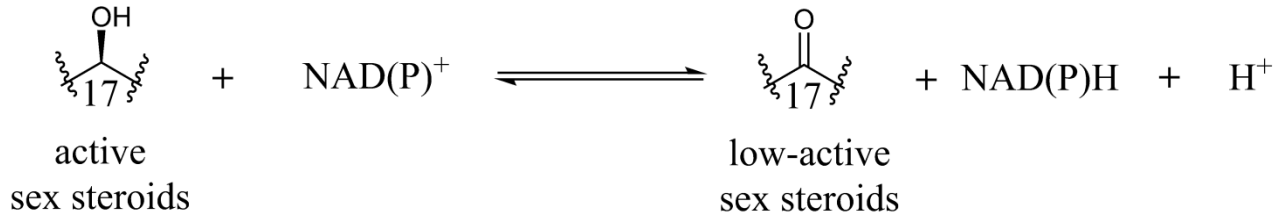


Figure 4. General reactions catalysed by 17β-HSDs.

With the exception of 17β-HSD5, belonging to the aldo-reductase family (AKR) (Penning and Byrns, 2009), the remaining 17β-HSDs are members of the short chain dehydrogenase/reductase (SDR) protein family (Prehn et al., 2009).
17β-HSDs play a key role in the final steps of estrogen and androgen biosynthesis and are tissue specifically expressed. Therefore in recent years they gained major interest as potential drug targets for the treatment of sex steroid hormone-related diseases.
Fourteen different mammalian 17β-HSDs have been to date identified (Möller and Adamski, 2009; Meier et al., 2009; Luu-The, 2001), but only twelve have been characterised in human (17β-HSD6 and 17β-HSD9 only in rodents).
All 17β-HSDs are able to transform steroid hormones *in vitro* and actually, the name 17β-HSD has been in the past routinely assigned to many enzymes converting 17-ketosteroids to 17-hydroxysteroids, regardless of the fact they can have diverse substrate specificities *in vivo*.

Table 1. Human 17β-HSDs. With the coloured background "reductive" 17β-HSDs. With the white background "oxidative" 17β-HSDs (modified and updated from (Marchais-Oberwinkleret al., 2011))

Name	Localisation	Function	Disease-association	References
17β-HSD1	Breast, Ovary, Endometrium, Placenta, Lung	Estrogen synthesis	Breast, prostate and lung cancer, endometriosis (when overexpressed)	(P. Vihko et al., 2006; Vihko et al., 2005; Verma et al., 2013)
17β-HSD2	Liver, Breast, Endometrium, Placenta, Prostate, Bones, Lungs, Kidney GI tract	Estrogen, androgen, progestin inactivation	Osteoporosis, breast, prostate and lung cancer, endometriosis	(Vihkoet al., 2006; Vihkoet al., 2005; Drzewiecka and Jagodzinski, 2012)
17β-HSD3	Testis	Androgen synthesis	Pseudohermaphroditism in males associated with obesity when missing	(Geissler, et al., 1994)
17β-HSD4	ubiquitous	Fatty acid β-oxydation, estrogen and androgen inactivation	D-specific bifunctional protein-deficiency, prostate cancer Zellweger syndrome when deficiency	(Rasiah, et al., 2009)
17β-HSD5	Prostate, Liver	Androgen, estrogenandprostaglandinesynthesis	Prostatecancer	(Stanbrough, et al., 2006)
17β-HSD6	Not Characterised in Human	Retinoid metabolism, 3α-3β-epimerase		(Biswas and Russell, 1997)
17β-HSD7	Liver, Lung, Thymus, breast, ovary, placenta	Cholesterol biosynthesis, estrogen synthesis	Breast cancer CHILD syndrome when deficiency	(Haynes et al., 2010)
17β-HSD8	Liver, Placenta, Kidney	Fatty acid elongation, estrogens and androgens inactivation	Polycistic kidney diease	(Fomitcheva et al., 1998; Maxwell et al., 1995)
17β-HSD9	Not Characterised in Human	Retinoid metabolism		(Su et al., 1999)
17β-HSD10	Central Nervous System, Brain	Isoleucine, fatty acid, bile acid metabolism, estrogen and androgens inactivation	X-linked mental retardation MHBD deficiency, pathogenesis of Alzheimer disease	(Froyen et al., 2008 ; Yang et al. 2007)
17β-HSD11	Liver Kidney, Lung, Placenta	Estrogen and androgens inactivation, lipid metabolism		(Breton et al., 2001)
17β-HSD12	Breast, Liver, Placenta, Kidney, Ovary, Uterus	Fatty acid elongation, estrogen synthesis	Breast cancer	(Luu-The et al., 2006; Day et al., 2008)
17β-HSD13	Liver	Not demonstrated		(Horiguchi et al., 2008)
17β-HSD14	Liver, Placenta, Brain	Estrogenand androgen inactivation, fatty acid metabolism	Breast cancer, prognostic marker	(Jansson et al., 2006 ; Lukacik et al., 2007)

A summary of the different function and disease associations for the fourteen 17β-HSDs is given in Table 1.
The numbering follows the chronological order of discovery of the different 17β-HSDs. The one belonging to the SDR family share low sequence identity (25% - 30%) but they conserved all characteristics of the family, *i.e.* the Rossmann fold implicated in cofactor binding.
Although 17β-HSDs are able to catalyse both oxidation and reduction reactions *in vitro*, they catalyse only one type of reaction *in vivo*, depending on the concentration of cofactors in cells and the preference of each 17β-HSD for a specific cofactor (Figure 5).

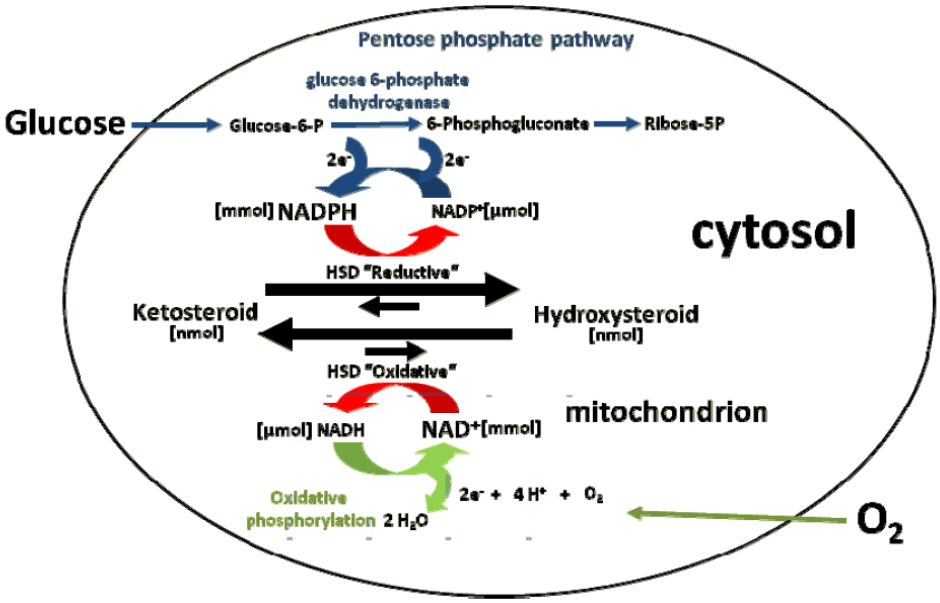


Figure 5. Interplay between metabolism and 17β-HSDs catalysed reactions. (Figure taken from PhD Thesis, Spadaro, 2011)

1.2.1.1 17β-HSD2

Human 17β-hydroxysteroid dehydrogenase type 2 is composed of 387 amino acids and possesses a molecular weight of 42.8 kDa. It is an endoplasmic reticulum bound enzyme, which converts primarily the highly active 17β-hydroxysteroids (i.a. estradiol, testosterone,dihydrotestosterone, etc.) to their inactive keto forms (Puranen et al., 1999; Wu et al., 1993). No 3D-structure of the protein is available yet.
Although 17β-HSD2 were thought to be exclusively converting sex steroids, new evidences indicate that this enzyme is also involved in retinoic acid metabolism (Haller et al., 2010; Zhongyi et al., 2007).
17β-HSD2, as the others 17β-HSDs, is able to catalyze both oxidation and reduction reactions in cells, though it catalyses one reaction preferentially, based on two factors: the concentration of cofactor present in cells and the higher affinity for one specific cofactor. NAD^+/NADH is the principal system in cells for oxidative reactions. NAD^+function as

electron acceptor and the fact that its concentration is 700 times higher than its reduced state NADH, assures the direction of the reaction to be oxidative (Sherbet et al., 2007; Agarwal and Auchus, 2005). On the other hand NADPH is the main source of electrons for catalysis of reduction reactions and its concentration is 500 times higher than the one of the corresponding state $NADP^+$. A part from the cofactor concentration, the different affinity of 17β-HSD2 for different cofactors explains why it preferably catalyses the oxidative reaction in the cell. In fact it has been shown that the Km of NAD^+ for 17β-HSD2 is 110 ± 10 µM, whereas that of $NADP^+$ is 9600 ± 100 µM (Lu et al., 2002).As a member of the SDR-family, 17β-HSD2 shares some common amino acid motifs, which are highly conserved, despite a very low sequence identity across the family: the T-G-xxx-G-x-G motif ("cofactor binding site"), the Y-xxx-K sequence ("active center") and the N-A-G motif for the stabilization of the 3D structure (Puranen et al., 1994; Hwang et al., 2005; Zhongyi et al., 2007).

1.2.1.2 17β-HSD1

Human 17β-hydroxysteroid dehydrogenase type 1 is composed of 327 amino acid residues and possesses a molecular weight of 34.9 kDa. It is a cytosolic enzyme, which mainly catalyses reactions between the low-active female sex steroid, estrone, and the more potent estradiol (Poutanen et al., 1995), though like 17β-HSD2, seems to be involved in the metabolism of retinoic acid (Haller et al., 2010). To date 22 crystal structures of 17β-HSD1 are available in the protein data bank (PDB), as apoenzyme, holoenzyme or substrate cofactor complexes.
17β-HSD1, which catalyses only estrogen reduction,shows a higheraffinity for NADPH, which is used as cofactor in the catalysis of the reductive reaction, with a Km value of 0.03±0.01 µM (Jin and Lin, 1999) and it shares the same amino acids motifs common to the SDR family and described above.

1.3 17β-HSD2 and 17β-HSD1 as drug target

1.3.1 Osteoporosis and 17β-HSD2

Osteoporosis (Cree et al., 2000) is a systemic disease characterised by a decline in bone density and quality, determined by an unmatched activity of osteoclasts and osteoblasts. The impairment of bone mass and microarchitecture increases bone fragility and risk of fractures, leading to higher risk of death and to huge costs for the healthcare system (Blume and Curtis, 2011).
Different therapies are already available for the treatment of this disease (Silva and Bilezikian, 2011) and they include: Calcium, Vitamin D, Parathyroid hormone (PTH), Bisphosphonates, Selective estrogen receptor modulators (SERMs) and RANKL inhibitors (Borba and Mañas, 2010; Jansen et al., 2011; Pazianas et al., 2010; Johnston et al., 2000; McClung, 2007). Despite the large number of therapies available, none of them present a satisfying profile in terms of safety and efficacy. For example, bisphosphonates, with

alendronate as the principal drug used in the treatment of osteoporosis, are able to reduce by only 50% the risk of fractures of both women and men, but are associated with adverse effects such as osteonecrosis of the jaw and SERMs, if effective are associated with an increased risk of venous thromboembolism.

The drop in E2 and T levels, naturally occurring with ageing, is the main factor driving the onset and progression of osteoporosis (Vanderschueren et al., 2008; Bodine and komm, 2002).“Physiologically normal” levelsof E2 induce bone formation and repress bone resorption (Bodine and Komm, 2002).In fact estrogen replacement therapy (ERT) was successfully used in the past, for reducing the risk of fractures. Unfortunately the ERT was found to be associated with severe adverse effects, such as cardiovascular diseases and breast cancer (Chen et al., 2002).

Since 17β-HSD2 is expressed in osteoblastic cells (Dong et al., 1998), it represents a promising target for the treatment of osteoporosis. In fact its inhibition might lead to a local increase of E2 and T levels,whose beneficial effect on bone quality has already been proved by ERT, and potentially leads to less adverse effects in comparison to the ERT (Figure 6).

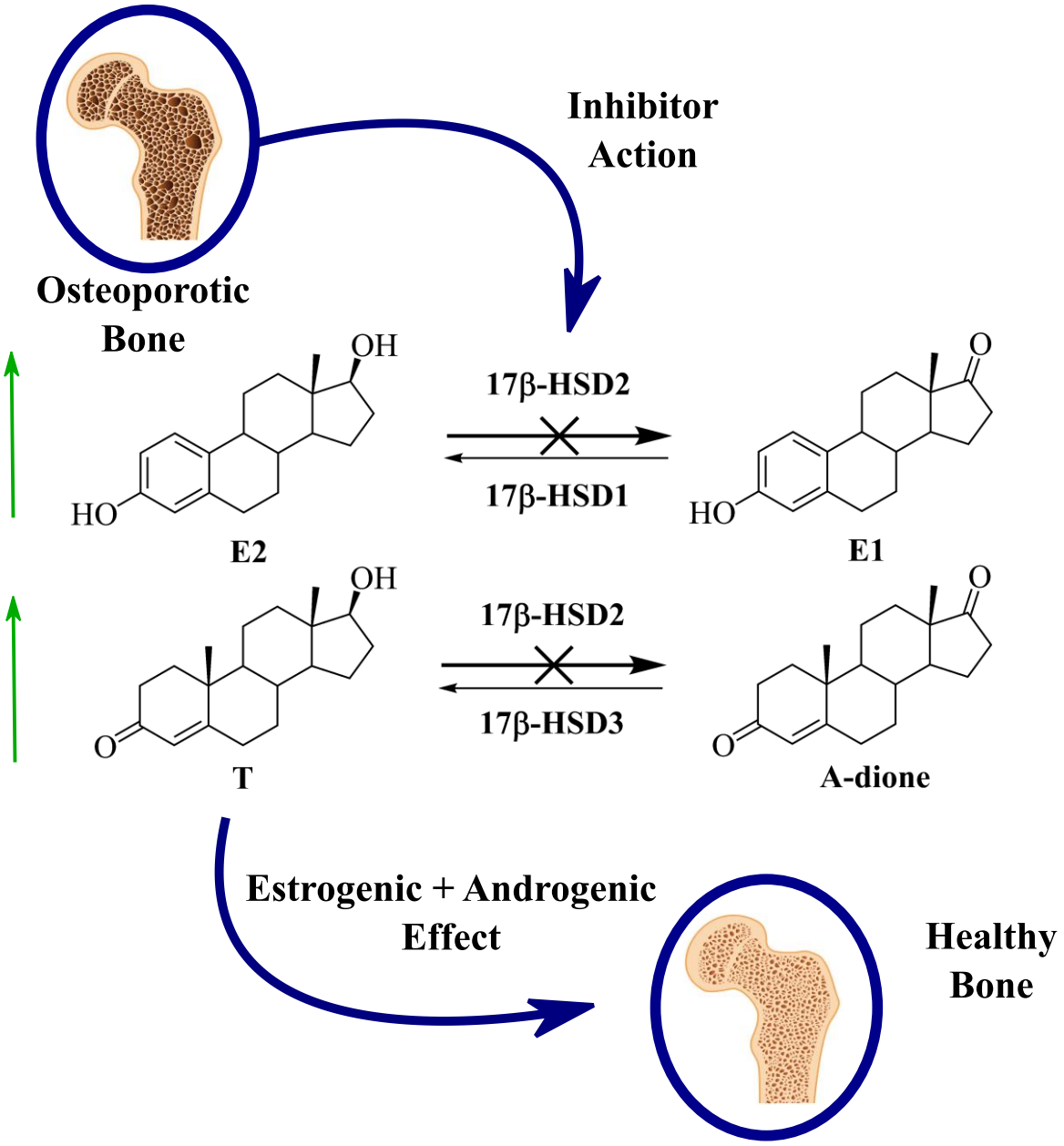


Figure 6.Expected effect of 17β-HSD2 on osteoporotic bone.

1.3.2 Estrogen-dependent diseases and 17β-HSD1

Estrogens are also implicated in the development and progression of numerous diseases (estrogen-dependent diseases), such as breast cancer and endometriosis (Deroo and Korach, 2006).

1.3.2.1 Breast cancer

Breast cancer is the most frequent cancer affecting women and, in North America, the second most important cause of death from cancer, after lung cancer (Jemal et al., 2008). This disease is most frequent in postmenopausal women and it is estradiol-dependent in 75% of the cases (Russo et al., 2003). However, the exact mechanism by which E2 contributes to the development of mammary cancer remains unclear. The most likely

hypothesis is that a higher rate of cell proliferation is induced by growth factors, whose synthesis is stimulated upon binding of E2 to ERα and β. Cell proliferation is oftenlinked to carcinogenesis. It implies DNA replication, which can lead to cancer when too many uncorrected errors in gene transcription take place,(Yue et al., 2005). To corroborate this hypothesis, there is the fact that 60% of postmenopausal breast cancer have measurable ERs (ER+), whereas in normal breast epithelial cells ERs are present in very low quantities (Cotterchio et al., 2003).

Besides surgery treatment, which remains the mainstay treatment for breast cancer, radiotherapy and chemotherapy, like the endocrine therapy, are also applied.

Gonadotropin-releasing hormone (GnRH) analoga(Emons et al., 2003), aromatase inhibitors (Herold and Blackwell, 2008)and sulfatase inhibitors (Aidoo-Gyamfi et al., 2009), all act by decreasing E2 levels.

1.3.2.2Endometriosis

Endometriosis is defined by the presence of endometrial cells and stroma outside of the uterine cavity. It is an estrogen-dependent disease.It mostly develops in women of reproductive age and regresses after menopause or ovariectomy. Its symptoms includepain, dysmenorrhea, deep dyspareunia and chronic pelvic pains as well as increased risk of infertility.

The pathogenesis of endometriosis remains unclear, but two main theories have been proposed: metastatic implantation after the reflux of endometrial cells through the fallopian tubes (Sampson, 1927), and metaplastic development such as coelomic metaplasia (Meyer, 1919).

The regulation of sex hormones producing enzymes plays a pivotal role in the onset and progression of the E2-dependent endometriosis.In endometriotic tissues an aberrant expression of aromatase, the over-expression of 17β-HSD1, and the deficiency in 17β-HSD2 is observed, which may lead to an accumulation of E2 (Tsai et al., 2001).

Endometriosis is mainly treated surgically, using laparoscopy, though ovariectomy is the most effective therapy(Kitawaki et al., 2002). Non-selective non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the synthesis of prostaglandins at both COX-1 and COX-2 enzymes , are the first line pharmacological treatment for this disease, although the severe associated adverse effects limit their use to patients with severe endometriosis under clearly defined conditions (Ebert et al., 2005). Further pharmacological treatments, aiming to produce a pseudo-pregnancy or pseudo-menopause, includeGnRHanaloga, oral contraceptives and androgens (Olive and Pritts, 2001). Aromatase inhibitors and SERMs showed so far promising results in the treatment of endometriosis, although they are not listed as official therapies, yet (Racine et al., 2010; Kulak et al., 2011).

1.3.2.3 Non-small cell lung cancer (NSCLC)

Lung cancer is divided into small cell lung cancer (SCLC) and non-small lung cancer (NSCLC), which accounts for about 80% of all lung cancers and is the most common type

in women (Muscat and Wynder, 1995). Despite the extensive research efforts fornew treatments, it is one of the most lethal human malignancies.
Several studies have demonstrated the relationship between E2 and NSCLC. E2 can promote the growth of both normal lung fibroblasts and lung cancer cells *in vitro* and *in vivo*(Weinberg et al., 2005; Stabile et al., 2002).E2 can also increase the secretion of growth factors such as the potent mitogen epidermal growth factor (EGF) in normal lung fibroblast and vascular-endothelial growth factor (VEGF) in lung cancer cell *in vitro*(Stabile et al., 2002; Pietras et al., 2005). A significant decrease in the proliferation of NIH-H23 lung cancer cells has also been observed *in vitro* for siRNA-mediated knockdown of the ERs (Márquez-Garbán et al., 2007). Further, the majority of NSCLC tumors result positive for the ERs, with ER β being the predominant subtype (Omoto et al., 2001).
Given the pivotal role of E2 in the progression of NSCLC, the possibility to interfere with the intratumoral synthesis of this sex hormone, has been regarded as a possible approach for the treatment.
Preclinical studies with the aromatase inhibitor exemestane have been conducted with a positive outcome (Márquez-Garbánet al., 2009).
Lately evidence has emerged that both 17βHSD1 and 17βHSD2 are expressed in NSCLC contributingto the tumor progression: the first by activating the available intratumoral E1 and the second by catalyzing the reverse reaction, thus playing a protective role against an excess of E2 (Drzewiecka and Jagodzinski, 2012; Drzewiecka et al., 2015; Verma et al., 2013).
In recent years 17βHSD1 has come up as new drug target for the treatment of NSCLC.

1.3.2.4 Summary

As so far described, E2 plays a crucial role in the maintenance of physiological processes, such as bone metabolism, which is negatively affected by E2 deficiency and in the onset and the progression of a number of illnesses, which are referred to as estrogens-dependent diseases: breast cancer, endometriosis and NSCLC, to name the most important ones.
A fine tuning of the local levels of estrogens seems to be possible by selectively acting on the two enzymes, mainly involved in the final steps of estrogens biosynthesis: 17βHSD1 and 17βHSD2.
The inhibition of these two enzymes seems particularly promising for the treatment of the mentioned diseases, in which hormone therapies or pharmacological treatments interfering with the endocrine system, proved a good efficacy but was associated with several adverse effects.
Since 17βHSD1 and 17βHSD2 are at the endof the estrogens biosynthesis, they might be idel targets to interfere with, to achieve the desired estrogen modulation and at the same time to avoid systemic adverse effects, which emerge with the traditional endocrine therapies.

1.4 State of Knowledge

1.4.1 Inhibitors of 17β-HSD2

17β-HSD2 inhibitors, bearing a steroidal core, were first identified by the group of Poirier (Sam et al., 2000; Bydal et al., 2004). Compound **A**(Figure 7) was the most interesting among them, exhibiting an IC_{50} value of 6 nM, but it was no longer investigated for further development, because of its lack of selectivity. In addition the steroidal core of this class of inhibitors increases the risk of interaction between the molecule and the steroid hormone receptors which could result in adverse effects.

A novel non-steroidal chiral 4,5-disubtituted *cis*-pyrrolidinone was discovered by Wood *et al*. (Gunn et al., 2005; Wood et al.; 2006) (Compound **B**, Figure 7). It showed an IC_{50} value of 50 nM, and was evaluated in a monkey osteoporosis model to establish a *proof of concept*. Slight decrease in bone resorption and increase in bone formation were observed subsequently to its administration, although non satisfactory pharmacokinetic profile of **B** led to strong variability in the observed effects.

In our group several classes of non-steroidal potent and selective 17β-HSD2 inhibitors were published. Among them the most interesting, depicted in Figure 7, are: 1. the hydroxyphenylnaphth-1-ol **C**(Wetzel et al., 2011), which displays an IC_{50} of 19 nM and s.f. over 17β-HSD1 of 32. Further development of **C** was stopped because of its metabolic instability, as proved by testing in human liver S9 fraction ($t_{1/2}$= 22 min). 2. the 2,5-thiophene amide **D**(Marchais-Oberwinkler et al., 2013), with an IC_{50} value of 58 nM and s.f. over 17β-HSD1 of 116, also displayed a rather high metabolic instability ($t_{1/2}$= 17 min). 3. The retro-*N*-methylsulfonamide **E**(Perspicace et al., 2013)showed high inhibition of 17β-HSD2 and only moderate selectivity over 17β-HSD1 (s.f. of 10).

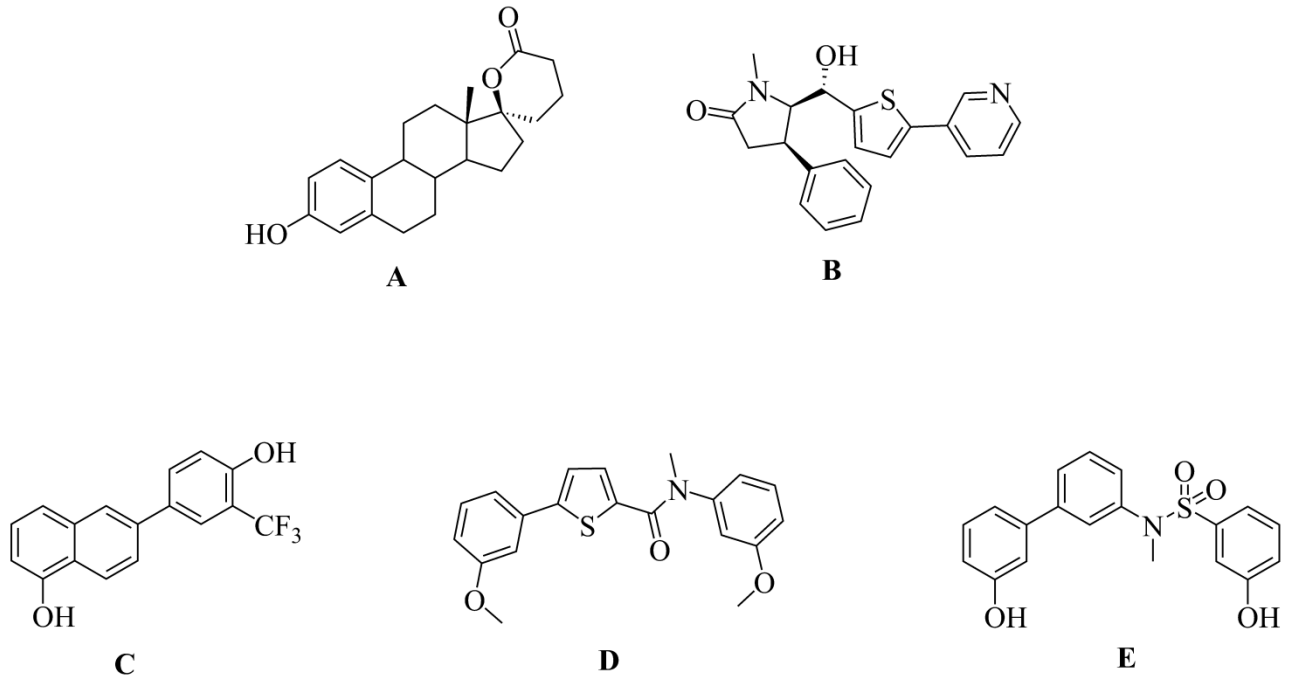


Figure 7. Selection of the most interesting 17β-HSD2 inhibitors.

1.4.2 Inhibitors of 17β-HSD1

Despite the many efforts dedicated over the past 30 years to the development of potent 17β-HSD1 inhibitors, it is only recently that lead candidates have been reported. Most of the 17β-HSD1 inhibitors reported are based on the steroid scaffold, including E1 and E2 derivatives (Poirier, 2003). As in the discussed case of 17β-HSD2 inhibitors, the presence of residual estrogenic activity is often associated with steroidal inhibitors, constituting a major drawback in the further progression of these compounds.

In order to overcome the risk of adverse effects caused by interaction with ERs or other steroid receptors, several classes of non-steroidal inhibitors have been developed: gossypol derivatives (Brown et al., 2003), phytoestrogens (Poirier, 2003), thiophenepyrimidinones (Messinger et al., 2006) and biphenyl ethanones(Allan et al., 2008).

Ourgroupstrongly contributed to the field of non-steroidal 17β-HSD1 inhibitors by developing several classes of selective 17β-HSD1 inhibitors for the treatment of estrogen-dependent diseases.

As an example some of the most interesting compounds are reported in Figure 8: 1. the heterocyclic substituted biphenylol **F**(Oster et al., 2010), with an IC_{50} value of 8 nM and a s.f. of 48, was one of the first potent compound reported by our group. 2. the bis(hydroxyphenyl) substituted arene **G** (IC_{50}= 8 nM, s.f.= 118) showed, in comparison to **F**, improved selectivity over 17β-HSD2 (Bey, et al., 2009). 3. the hydroxybenzothiazoles **H**(Spadaro et al., 2012)which showed an IC_{50} value of 13 nM and s.f. of 136. 4. The (hydroxyphenyl)naphthol **I**(Henn et al., 2012)displayed an IC_{50} value of 32 nM and s.f of 12. Compound **I** also inhibited 17β-HSD1 from *Callithrix jacchus*, 5. the most recent compound **J** (IC_{50}= 104 nM, s.f.= 2.4) is the most potent compound in a new class of inhibitors(Abdelsamie et al., 2014).

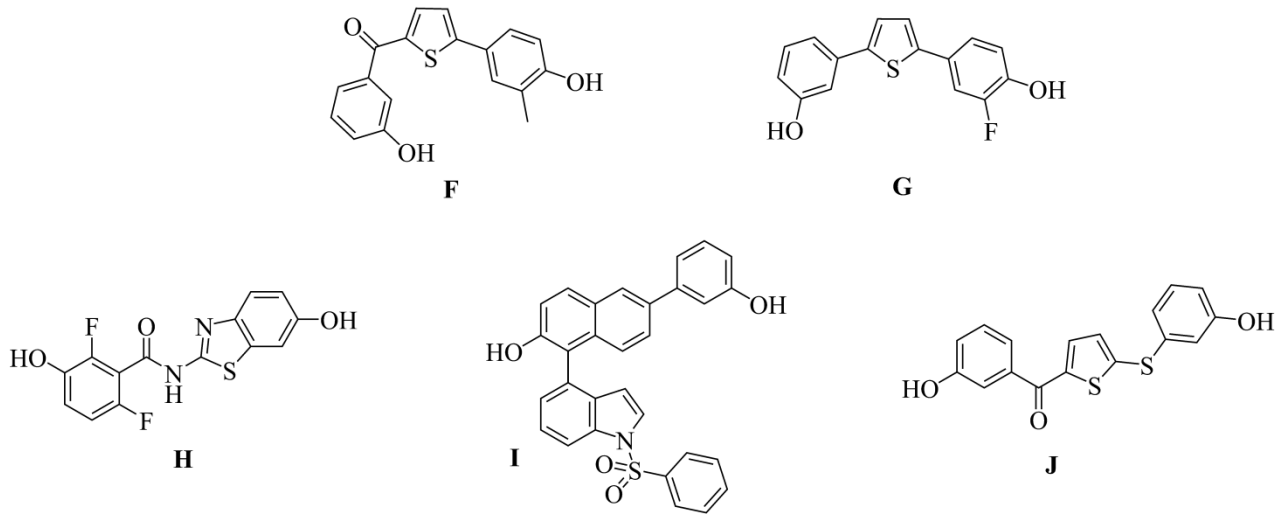


Figure 8. Selection of 17β-HSD1 inhibitors.

1.5 Aim of the Thesis

1.5.1 Scientific goal

Several diseases are associated with the endocrine system and the sex steroid hormones. Drop in E2 and T levels which occurs in post-menopause women and in elderly men constitutes the main cause of osteoporosis. 17β-HSD2 is responsible for the local inactivation of these hormones and is expressed in bone tissue. Thus inhibition of 17β-HSD2 might be a new promising approach for the treatment of this disease. Although the pyrrolidinone **B** has already been evaluated in a monkey osteoporosis model and its positive effects on bone health have been demonstrated, too strong variability in the results has also been reported, certainly due to non appropriate pharmacokinetic properties.

Therefore the goal of the first part of this thesis (Chapter 2.I-2.III) is the development of new inhibitors which might be suitable for an *in vivo* study in an animal model of the disease and at the same time present a pharmacological profile which makes them eligible for future human clinical studies. The mouse animal model was selected as the target model, because several murine model of osteoporosis are already established.

In order to achieve this goal, we designed a pharmacological flow chart for the development of new inhibitors or the optimization of in-house compounds (Figure 9).

In order to increase our chances of obtaining suitable compounds, different calculated parameters were determined prior to the synthesis of new molecules. cLogP, predicted solubility, polar surface area (PSA), pKa and tox liability were calculated *in silico*, in order to design drug-like inhibitors.

After testing on human and mouse 17β-HSD1 and 2 (percent of inhibition at 1 μM), the new molecular entities were evaluated for their metabolic stability (using human liver S9 fraction), plasma stability and plasma protein binding (PPB).

When the results were satisfactory the compounds were investigated with regard to their ability to inhibit hepatic CYP enzymes and to bind to the ERs. IC_{50} values were also determined at this point.

The following step includes the pharmacokinetic study of the compound in the mouse, together with an evaluation of toxicity in HEK 293 cells (using MTT assay) and induction of CYP enzymes.

A compound with positive results in all the above mentioned assays was considered suitable for an *in vivo* study and eventually for further clinical development.

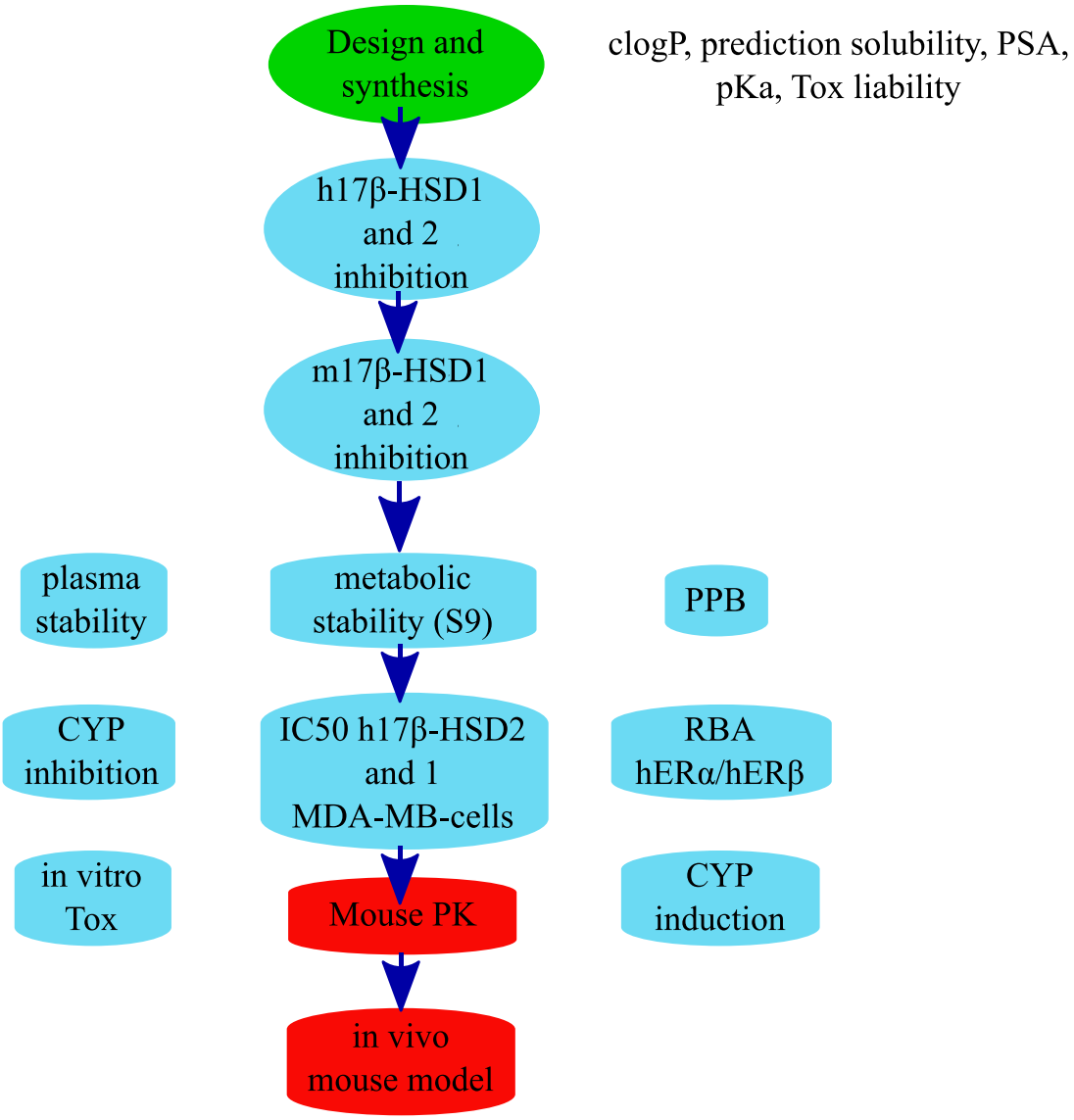


Figure 9. Pharmacological flowchart for the development of new 17β-HSD2 inhibitors

Whereas osteoporosis is caused by decreased levels of E2 and T, the so called estrogen dependent diseases depend on E2 presence. As previously listed, breast cancer, endometriosis and NSCLC are mainly estradiol-dependent. 17β-HSD1 and 17β-HSD2 are expressed in different amounts in estrogens target cells and play a pivotal role in the regulation of the growth of cancer tissues as well as endometrial tissue. Since 17β-HSD1 catalyses the last step in E2 biosynthesis, it is considered the key-target to block peripheral E2 production, with few or no adverse effects associated.

The *in vivo* efficacy of 17β-HSD1 inhibitors to reduce estrogen stimulated growth in breast cancer and endometriosis has already been proved, although up to date no 17β-HSD1 inhibitor has entered clinical trial yet.

Thereby the main goal of the second part of this thesis (Chapter 2.IV) is the development of new potent and selective 17β-HSD1 inhibitors, with a particular focus on their metabolic stability, in order to provide new chemical entities which might be successful in an animal and human *in vivo* study.

In recent years strong evidence of the pivotal role of 17β-HSD1 and 17β-HSD2 in the progression of NSCLC has emerged, although no experimental evidence of the effect of 17β-HSD1 inhibitors is available yet (Verma, et al., 2013). *In vitro* testing of the best new 17β-HSD1 inhibitor on a suitable NSCLC cancer cell line, to investigate the effect on cell growth should be performed.

1.5.2 Working strategy

1.5.2.1 Optimization of 17β-HSD2 inhibitors foran *in vivo* proof of concept

Metabolic stability optimization and metabolite identification of 17β-**HSD2 inhibitors** (Chapter 2.I): Although several classes of potent and selective 17β-HSD2 inhibitors were already synthesized, none of them presented an adequate metabolic stability. It was therefore decided to put the focus on this aspect first. A set of 11 2,5-thiophene amide compounds described by our group and carefully chosen as differing from one another by one structural change only, were tested for their metabolic stability using human liver S9 fraction, in order to gain information about the potential site of metabolism.
Taking into account the gathered information, a series of new compounds was designed and synthesized. Three strategies were followed (Figure 10): 1) exchange of the potential metabolic liability, according to the results of the starting experiment; 2) bioisosteric replacement of the central thiophene ring; 3) lowering of the cLogP.
Furthermore the metabolic pathway of three inhibitors, belonging to the 2,5-thiophene amide class, was investigated.The exact structure of the metabolites of one of these inhibitors was elucidated.

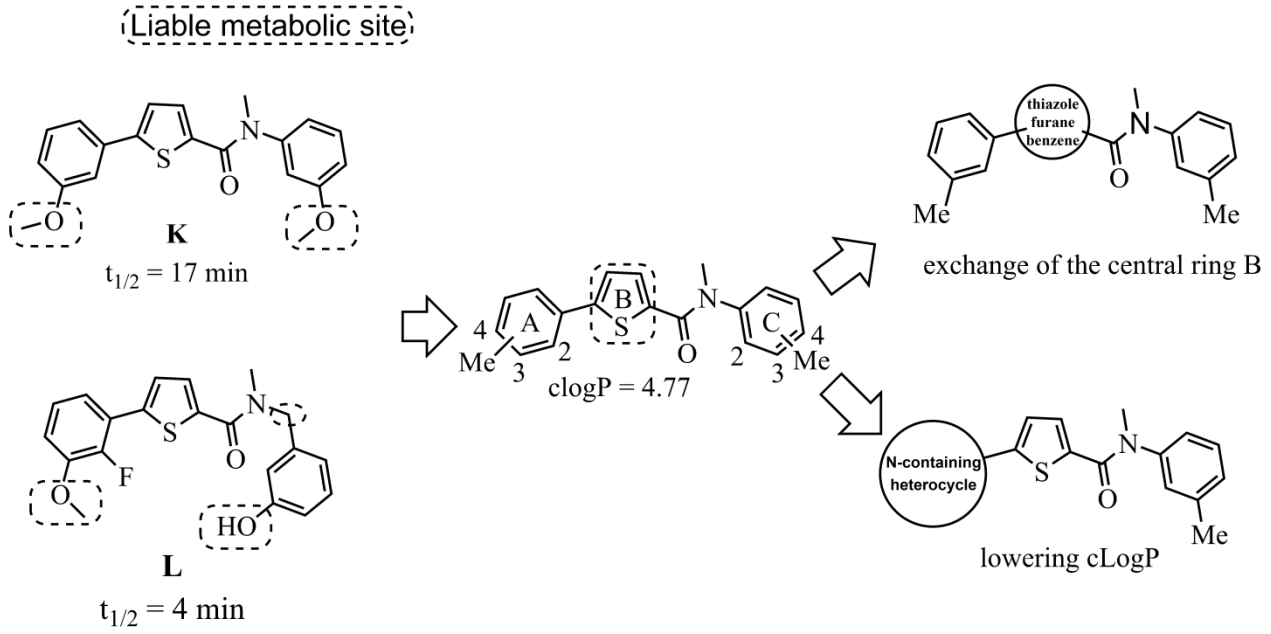
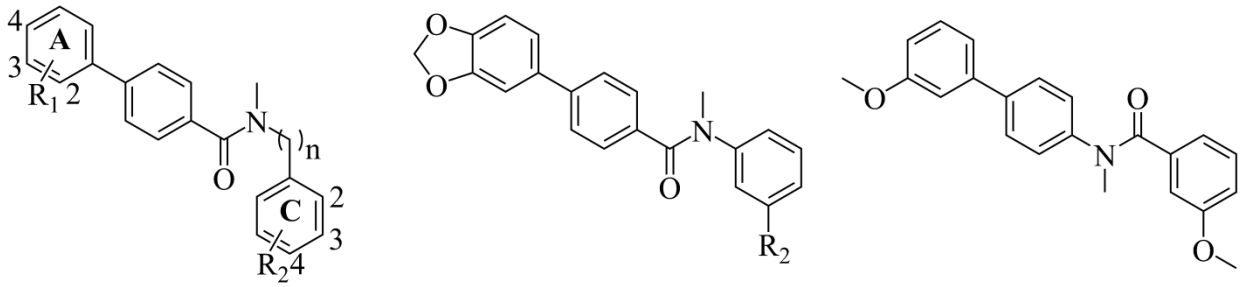


Figure 10. Structure overview of the metabolic stability optimization strategies. $t_{1/2}$= half life in human liver S9 fraction. Compounds **K** and **L** are displayed as examples of the 11 tested compounds.

Discovery of selective and metabolically stable compounds inhibiting both the Human and Mouse 17β-HSD2 (Chapter 2.II)**:** The aim of the second project was to obtain new compounds which could inhibit both the mouse and the human 17β-HSD2 and still retainthe metabolic stability of some of the inhibitors synthesized in the first project.
As starting point 25 previously described 17β-HSD2 inhibitors, belonging to the 2,5-thiophene amide, 1,3-phenyl amide and 1,4-phenyl amide class, were tested for m17β-HSD2 inhibition, in order to elaborate a comparative Structure Activity Relationship (SAR) and to develop an optimization strategy.
Combining the gathered information together with the knowledge obtainedabout metabolic stability presented in chapter 2.I, a small library of inhibitors, where the substitution pattern and the physicochemical nature of substituents on the A and C rings was varied, was designed and synthesized. The general structure of the compounds included in this chapter is presented in Figure 11.



R_1= -H, 3-Me, 2-OMe, 3-OMe
4-OMe, 3-OH, 3-F, 3-Cl, 3-$N(Me)_2$
R_2= 3-Me, 3OMe, 4-OMe, 3-OH

Figure 11.Structure overview of the compounds included in chapter 2.2.

Overcome of the cytotoxicity of the 1,4-biphenyl amide inhibitors (Chapter 2.III): In chapter 2.IIa lead candidate compound is discovered which potently and selectively inhibits the human and mouse 17β-HSD2 and which is metabolically stable. However this lead compound was found to be cytotoxic. In chapter 2.3 a preliminary study is presentedon a successful strategy to decrease cytotoxicity, without loss in 17β-HSD2 inhibitory activity. Decrease in cytotoxicity is achieved by disrupting the planarity of the biphenyl moiety (Figure 12).

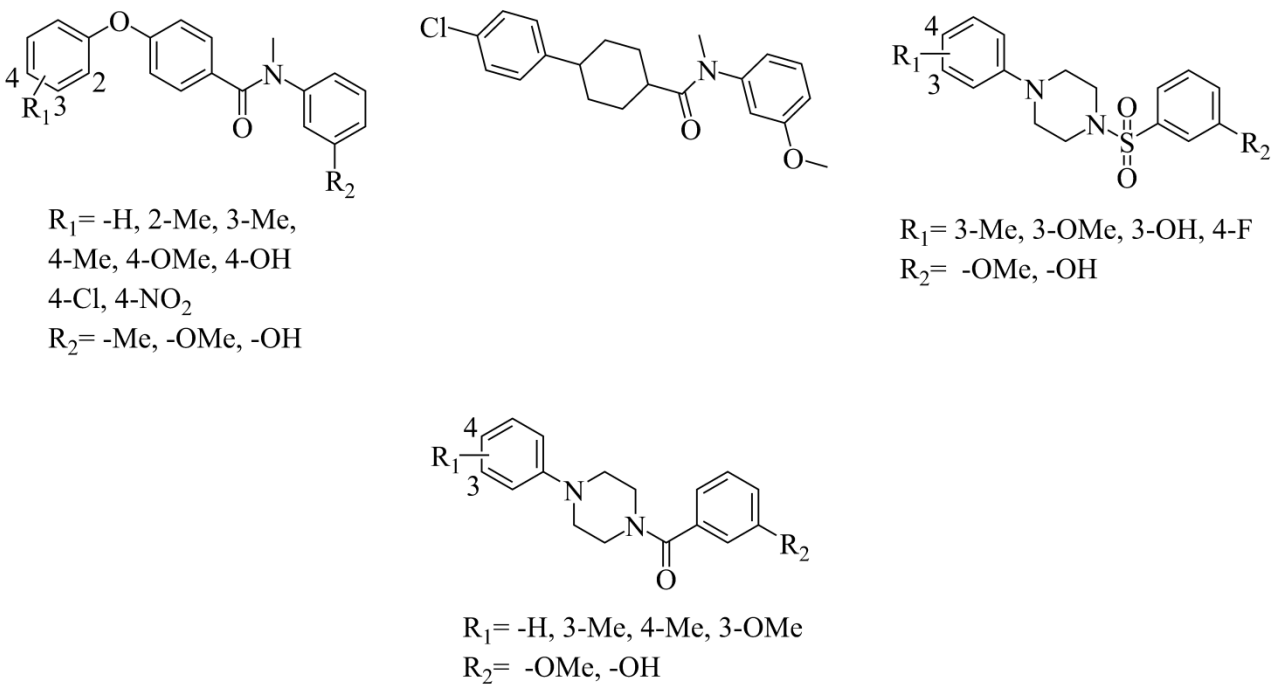


Figure 12. Structure overview of the compounds included in chapter 2.III.

1.5.2.2 Discovery of potent, selective and metabolically stable 17β-HSD1 inhibitors

Taking into account the information regarding 17β-HSD1/2 inhibitory activity and metabolic stability gathered in chapter 2.I, a series of new inhibitors was designed. Structural modification of the thiophene derivatives (exchange of the thiophene by furane ring, introduction of substituents on the phenyl residues of the molecules) has been studied to increase 17β-HSD1 inhibitory activity and reverse the selectivity in favour of 17β-HSD1. A structure overview of the compounds included in chapter 2.IV is presented in Figure 13.

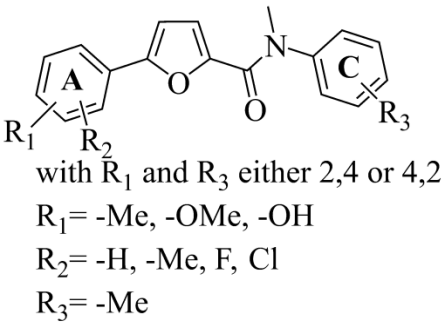


Figure 13. Structure overview of the compounds included in chapter 2.IV

2. Results

2.1 Metabolic stability optimization and metabolite identification of 2,5-thiophene amide 17β-hydroxysteroid dehydrogenase type 2 inhibitors

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Paper I

Abstract

17*β*-HSD2 is a promising new target for the treatment of osteoporosis. In this paper, a rational strategy to overcome the metabolic liability in the 2,5-thiophene amide class of 17*β*-HSD2 inhibitors is described, and the biological activity of the new inhibitors. Applying different strategies, as lowering the cLogP or modifying the structures of the molecules, compounds **27**, **31** and **35** with strongly improved metabolic stability were obtained. For understanding biotransformation in the 2,5-thiophene amide class the main metabolic pathways of three properly selected compounds were elucidated.

1. INTRODUCTION

Osteoporosis[1] is a systemic disease in which an unmatched activity of osteoclasts and osteoblasts leads to a decline in bone density and quality. It is also correlated with an increased risk of bone fracture. Osteoporosis is observed in 40% of postmenopausal women[2]. Considering the progressive ageing of the population this number is expected to constantly increase in the near future[3] and osteoporosis is therefore considered as a serious public health concern.

The available therapies today (bisphosphonates and selective estrogen receptor modulators) lack of a satisfying profile in terms of safety and efficacy. Bisphosphonates, with alendronate as the principal drug used in the treatment of osteoporosis, are effective in both postmenopausal women[4] and men[5, 6], though they are able to reduce the risk of fracture by only 50% and are associated with adverse effects such as osteonecrosis of the jaw. Raloxifene is, among the selective estrogen receptor modulators (SERMs), one of the most often used compounds for the treatment of osteoporosis[7]. SERMs are efficient too, but, as bisphosphonates, endowed with several adverse effects, such as an increased risk of

venous thromboembolism. As a consequence, the finding of new, safer and more effective treatments for osteoporosis is of particular importance for public health.

It has been proven that an important player in the maintenance of bone health is the physiological, pre-menopausal concentration of the estrogen estradiol (E2). That induces bone formation and represses bone resorption by acting on the osteoblasts[8]. Estrogen replacement therapy (ERT) was used in the past in postmenopausal osteoporosis patients[9, 10], successfully reducing the risk of fractures but increasing the incidence of cardiovascular diseases and breast cancer[10, 11], which was the reason for the cessation of this therapy. It has also been shown that testosterone (T) has beneficial effects on bone formation[12, 13].

Hence, an optimal strategy for the treatment of osteoporosis should be that to inducing a local increase inE2 concentration in bone tissue, without affecting the systemic levels of E2. Such an effect might be achieved by inhibiting 17*β*-hydroxysteroid dehydrogenase type 2[14] (17*β*-HSD2), an enzyme that catalyzes the conversion of the highly active 17*β*-hydroxysteroids into the low inactive 17-ketosteroids, that is the estrogen E2, and the androgen testosterone (T) into their inactive oxidatedforms estrone (E1) and Δ^4-androstene-3,17-dione (A-dione), respectively (Chart 1). The biological counterparts, 17*β*-hydroxysteroid dehydrogenase type 1 (17*β*-HSD1) and 17*β*-hydroxysteroid dehydrogenase type 3 (17*β*-HSD3) catalyse the reverse reactions. Inhibitors of 17*β*-HSD1are potential drugs for the treatment of breast cancer and endometriosis and have recently been described in a series of papers by us[15, 18].

As 17*β*-HSD2 is expressed in osteoblasticcells[19-21], its inhibition should lead to the desired local increase of E2 and T levels in bone tissue.

Ideally, the new 17*β*-HSD2 inhibitors should, in addition to their activity on the target enzyme, be selective over 17*β*-HSD1 and should have no affinity for the estrogen receptors (ER) α and β in order to avoid E2 related adverse effects.

The discovery of potent and selective nonsteroidal inhibitors of 17*β*-HSD2 has been already reported by our research group[22-30].

As drugs encounter formidable challenges to their stability *in vivo*, it is not sufficient to only focus on potency in the development process of a drug candidate. It is also important to profile the metabolic stability of a lead series and improve it when this constitutes a potential liability. As 17*β*-HSD2 should be inhibited with constancy to locally increase the E2 levels and to maintain them high over the time needed to re-establish bone metabolism, the development of very metabolically stable inhibitors is particularly important. The enhancement of the metabolic stability of a class of inhibitors is a very difficult task for the medicinal chemist but could strongly favor the emergence of potential preclinical candidates[31].

As a consequence, we decided to test three of our most promising, previously reported17*β*-HSD2 inhibitors (compounds **1-3**) for their metabolic stability in human liver microsomes S9 fraction (Chart 2), a powerful tool to explore both phase I and phase II major metabolic reactions[32]. **1**, **2** and **3** represent very good compounds in terms of

enzymatic inhibitory potency and selectivityover 17*β*-HSD1, however, they do not exhibit a satisfying stability displaying short half-lifes between 4 and 38 min.

In this report, we describe the analysis of the metabolic profile of some representative in house inhibitors, the design of new, more stable inhibitors based on the results from these studies, as well as their synthesis and biological evaluation. In addition three inhibitors were investigated for their specific metabolic fate in order to understand the particular biotransformation of this class of molecules.

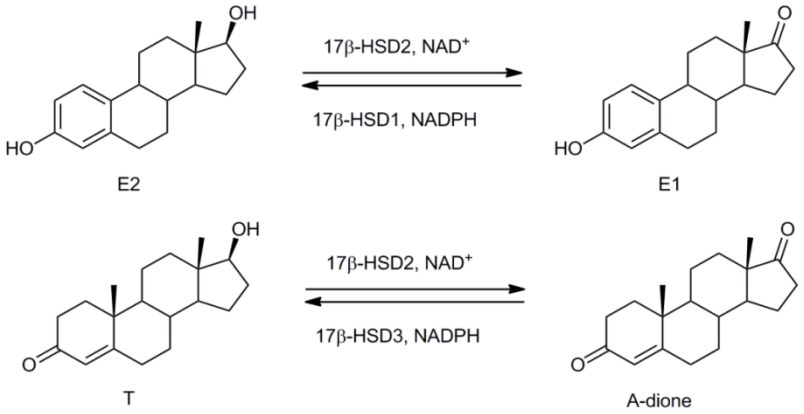


Chart 1. Interconversion of E2 to E1 by 17β-HSD2 and 17β-HSD1, and T to A-dione by 17β-HSD2 and 17β-HSD3.

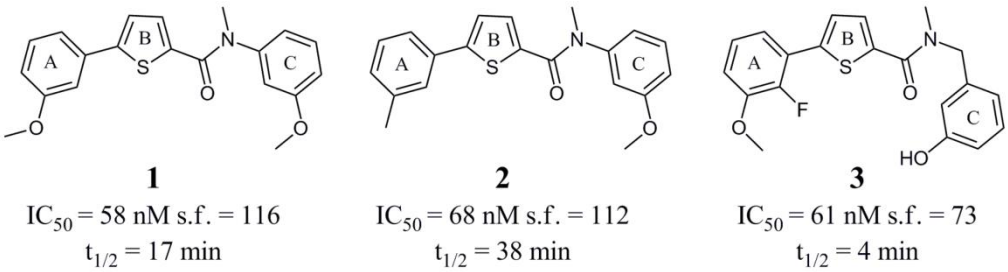


Chart 2. Previously described 17β-HSD2 inhibitors.

2. INHIBITOR DESIGN

2.1 Stability towards human S9 fraction

Different hepatic enzymes can be responsible for the metabolic transformation of a compound and it frequently happens that small structural changes on the molecule lead to a switch of the responsible metabolic enzyme[33], a phenomenon known as “metabolic switching” [33]. As a consequence introduction of several structural changes in the scaffold of a compound is not an appropriate method to evaluate liabilities of single moieties as changes in the core structure could have switched the main site of metabolism.

Therefore a set of 11 selected compounds described by our group[24, 25] and carefully chosen as differing from one another by one structural change only, were tested for their

phase I and II metabolic stability using human liver S9 fraction (Table 1), in order to find out the potential sites of metabolism.

From the comparison of compound **1** with **2** ($t_{1/2}$ = 17 min and 38 min, respectively) it appears that the methoxy group on the A ring in *meta* position is unfavorable for metabolic stability compared to the methyl group, whereas the presence of a *meta* dimethyl amino group (compound **4**, $t_{1/2}$ = 29 min) provides a similar stability as the methylated derivative**2** ($t_{1/2}$ = 38 min).

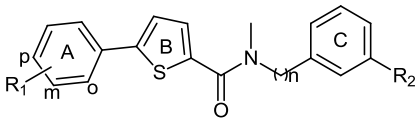
The exchange of the methoxy group (**5**) for a methyl (**6**) on the C ring results also in a slight increase in metabolic stability ($t_{1/2}$ = 12 min for **5**vs 20 min for **6**)

Compound **5** bears an additional fluorine in *ortho* position on the A ring in comparison to compound **1**. However introduction of this fluorine atom does not improve the stability of compound **5** (**5**, $t_{1/2}$ = 12 min *vs* 17 min for **1**).

Comparison of the unsubstituted inhibitor **7** ($t_{1/2}$ = 38 min), with **8** ($t_{1/2}$ = 1 min) bearing anhydroxy group on the A ring and with **9** ($t_{1/2}$ = 4 min) having an OH on the C ring shows that the phenolic OH group enhances the metabolic degradation of this class of compounds. This result was also observed in presence of a methylene linker in compound **3**, bearing an OH on the C ring and having a half-life of 4 min. On the other hand, the nature of substituents on the A ring (in the presence of the OH group on the C ring) plays an important role in the stabilization, as addition of the methyl group alone (**10**, $t_{1/2}$ = 17 min) or a hydroxy group (**11**, $t_{1/2}$ = 20 min) slightly increases the metabolic stability. Compounds **3**, **10** and **11**, in comparison with compound **1** have in their core two additional potential metabolic sites: the methylene linker and the phenolic group (liable for phase II metabolic attack). Despite the increase in liable metabolic sites their half-lives ($t_{1/2}$ = 17 min and 21 min, respectively) are still in the range of compound **1**.

In summary none of the tested inhibitors showed a satisfying metabolic stability profile (Table 1). However, from these results it is obvious that 1. the methyl group on the A ring is less prone to metabolic degradation than the methoxygroup (compounds **1** and **2**). 2. thephenolic hydroxy substituent on both the A ring or the C ring is subject to fast metabolism (compounds**8** and **9**), which can be slightly slowed down by introduction of substituents on the other ring (compounds **10** and **11**).

Table 1. Half-life in Human Liver Microsomes S9 fraction.



1 - 11

compd	R_1	R_2	n	Inhibitor $t_{1/2}$ (min)
1	*m*-OMe	-OMe	0	17[a]
2	*m*-Me	-OMe	0	38[a]
3	*o*-F, *m*-OMe	-OH	1	4[b,c]
4	*m*-$N(Me)_2$	-OMe	0	29[a]
5	*o*-F, *m*-OMe	-OMe	0	12[b,c]
6	*o*-F, *m*-OMe	-Me	0	20[a]
7	-H	-H	0	38[b,c]
8	*m*-OH	-H	0	1[b,c]
9	-H	-OH	0	4[b,c]
10	*m*-Me	-OH	1	17[a]
11	*o*-OH	-OH	1	21[a]

[a] Inhibitor tested at a final concentration of 3 µM, 1mg/ml, pooled human liver S9 fraction (IVT, Xenotech or TCS Cellworks), 1 mM NADPH regenerating system, 0.75 mM UDPGA, 0.05 mM PAPS, incubated at 37°C for 0, 5, 15, 30 and 45 minutes

[b]Inhibitor tested at a final concentration of 1 µM, 1 mg/ml pooled mammalian liver S9 fraction (BD Gentest), 2 mM NADPH regenerating system, 1 mM UDPGA and 0.1 mM PAPS, incubated at 37°C for 0, 5, 15 and 60 minutes.

[c] Mean of at least two determinations, standard deviation less than 25%.

2.2 Design of inhibitors

In the inhibitor design we took into account the information we had gained from the metabolic stability results. In addition we tried to modify some other moieties that have the potential of being metabolically labile. Doing this we kept in mind the structure-activity and structure-selectivity relationshipsobtained in our previous studies[24, 25].

As the methyl group resulted to be more stable than the methoxy group on the A and C ring , we decided to exchange each methoxy group with a methyl function and then to explore the effect of this substitution pattern on both activity and metabolic stability. It was also observed that most of the compounds with a hydroxy group in the presence of the methylene linker, known to be a critical point for metabolism, scored the worst results in

terms of metabolic stability. However since a hydroxy group is needed for activity[25] in the series of compounds having the methylene linker, we decided to stop further structural modifications on this series. Therefore compounds without linker with the general structure **A**, depicted in Chart 3 were synthesized.

The thiophene is also described as a potential site of metabolism[34]. Therefore, we tried to modify it by bioisosteric replacements using benzene, furane and thiazole (general structure **B**).

Moreover, it has been reported[35] that thelipophilicity parameters likecLogP couldplay an important role for the metabolic stability. Very often the simple strategy to lower the cLogP was appropriate to improve the metabolic stability of a compound. Therefore some inhibitors, modified at the A ring with a moiety reducingcLogP were designed (general structure **C**, Chart 3).

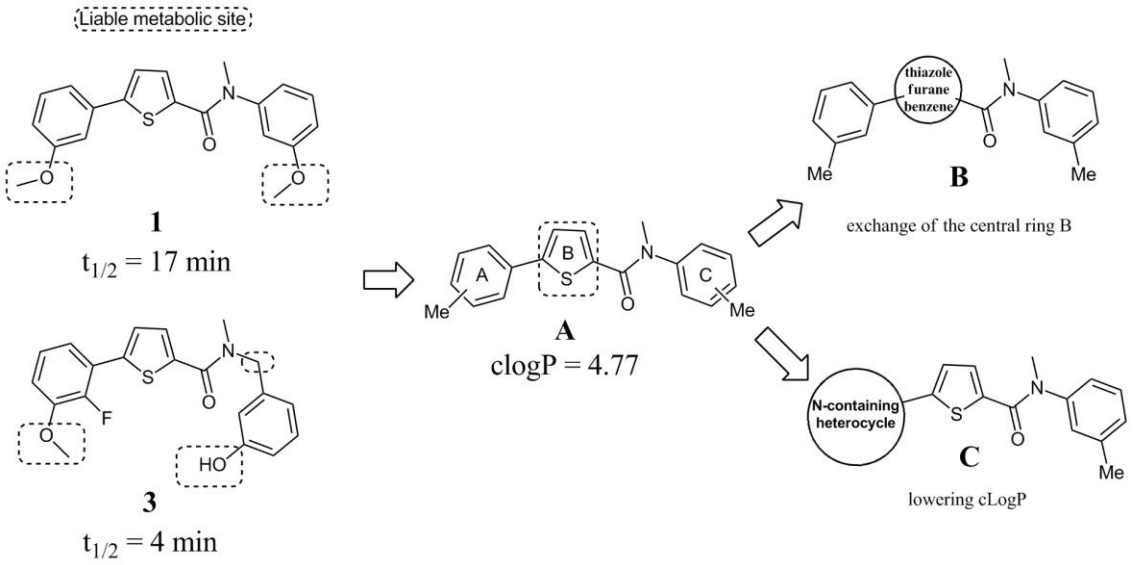


Chart 3. Designed inhibitors.

Furthermore, we profiled the metabolic pathway of the newly synthesized dimethylphenylthiophene**12** (Chart 4) looking for the major metabolites resulting from the mono-oxidation of the phenylthiophene part (Chart 4).

In order to identify the main metabolites, especially the one coming from mono-oxidation of the phenylthiophene fragment, we synthesized different hydroxylated derivatives of **12** bearing one additional oxygen in the positions that are likely to be attacked by the metabolizing enzyme (represented by the general structures **D** and **E,** Chart 4).

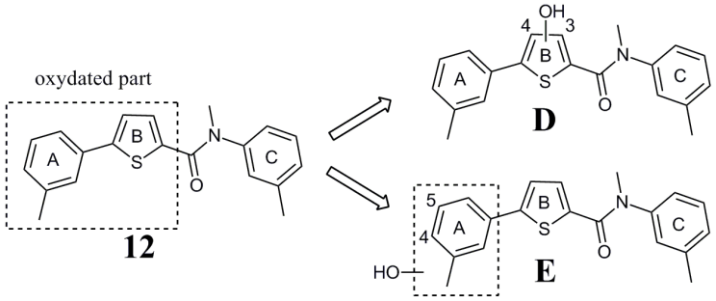
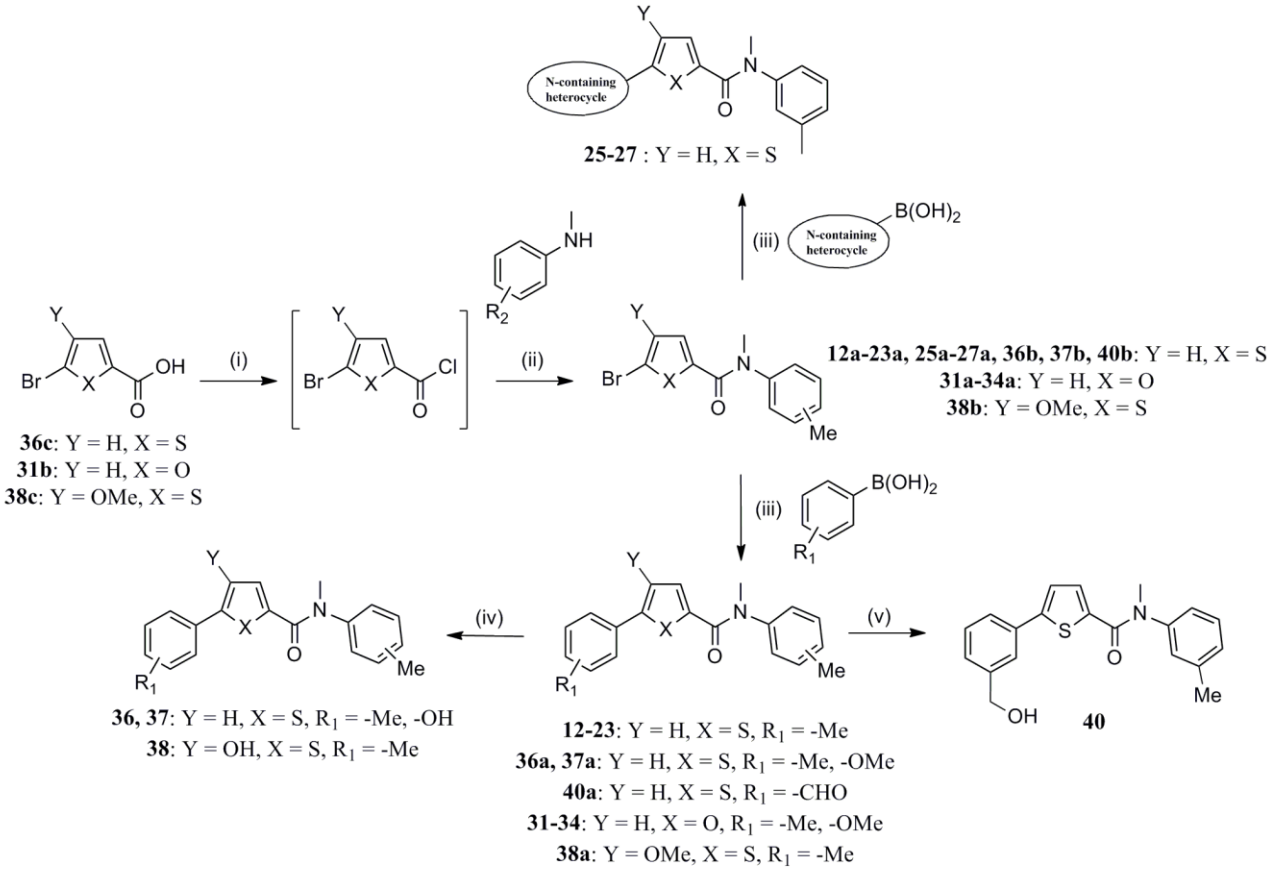


Chart 4. Structures of the most probable metabolites of **12**.

3. RESULTS

3.1. Chemistry

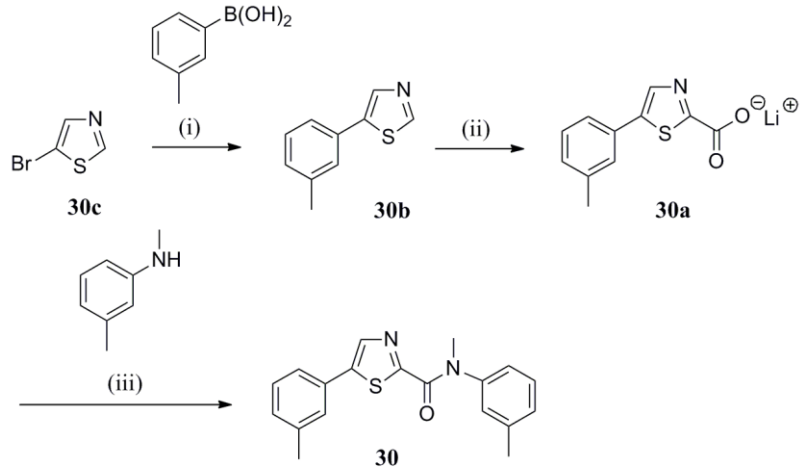
The synthesis of the 2,5-thiophene derivatives (compounds **12-23**,**25-27**,**36-38** and**40**) and the 2,5-furane derivatives (compounds **31-34**) is depicted in Scheme 1. The 5-bromothiophene-2-carbonic acid chloride and the 5-bromofuran-2-carbonic acid chloride were obtained from the corresponding carboxylic acids **36c** or **31b** by reaction with $SOCl_2$ and subsequently reacted with different anilines (Method A), providing the intermediates **12a**-**23a**,**25a**-**27a**,**36b, 37b, 40b** and **31a**-**34a** in good yields. Subsequently, Suzuki coupling (Method B) using tetrakis(triphenylphosphine)palladium and cesium carbonate in a mixture DME/EtOH/H_2O (1:1:1, 3 mL) as solvent and microwave irradiation (150°C, 150 W for 20 minutes), afforded the desired 2,5-thiophene derivatives **12-23**,**25-27**,**36a**, **37a**, **40a** and the 2,5-furane derivatives **31-34**. Methoxy compounds **36a** and **37a** were submitted to ether cleavage using boron trifluoride-dimethyl sulfide complex yielding the hydroxy compounds **36** and **37**. The aldehyde function of compound **40a** was reduced to primary alcohol using $NaBH_4$ to provide compound **40**. The synthesis of the 4-hydroxy-thiophene (compound **38**) is also depicted in Scheme 1 and followed the same synthetic pathway starting from the 5-bromo-4-methoxythiophene-2-carboxylic-acid **38c**. The methoxy group in position 4 of the thiophene (compound **38a**) was cleaved using boron trifluoride-dimethyl sulfide complex and yielded the hydroxy compound **38**.



[a]Reagents and conditions: (i) $SOCl_2$, DMF cat., toluene, reflux 4 h; (ii) Et_3N, CH_2Cl_2, room temperature, overnight; (iii) DME/EtOH/H_2O (1:1:1), Cs_2CO_3, $Pd(PPh_3)_4$, microwave irradiation (150°C, 150W, 20 min); (iv) $BF_3{\cdot}SMe_2$, CH_2Cl_2, room temperature, overnight; (v) $NaBH_4$, MeOH/Dioxane (1:1), 0 °C, 2 h.

Scheme 1. Synthesis of 2,5-thiophene derivatives **12-23**, **25-27**, **36-38** and **40** and 2,5-furane derivatives **31-34**.[a]

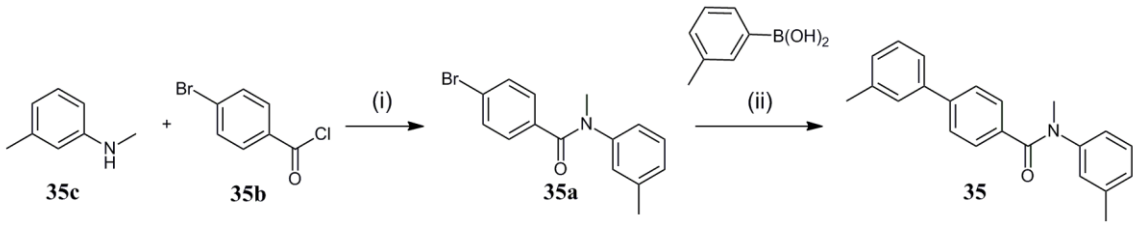
The synthesis of compound **30** was performed following the method depicted in Scheme 2. The intermediate **30b** was synthesized starting from 5-bromo-1,3-thiazole **30c** applying a Suzuki-Miyaura cross-coupling reaction with *m*-tolylboronic acid, using microwave irradiation. The acidic hydrogen in position 2 of the thiazole**30b** was easily removed using *n*-butyl lithium. The anion obtained reacted immediately with a dry flow of carbon dioxide leading to the corresponding carboxylate **30a** which was subsequentlyactivated using oxalyl chloride and reacted with 3-methyl-*N*-methylaniline to afford compound **30**.



[a]Reagents and conditions: (i) DME/EtOH/H_2O (1:1:1), Cs_2CO_3, $Pd(PPh_3)_4$, microwave (150°C, 150 W, 15 min); (ii) a) dry THF, *n*-BuLi, -78°C to 0°C, 4 h, b) dry CO_2 gas, -78°C, 5 h, room temperature, overnight; (iii) a) oxalyl chloride, DMF cat., CH_2Cl_2, 3 h, b) Et_3N, CH_2Cl_2, room temperature, overnight.

Scheme 2. Synthesis of compound **30**.[a]

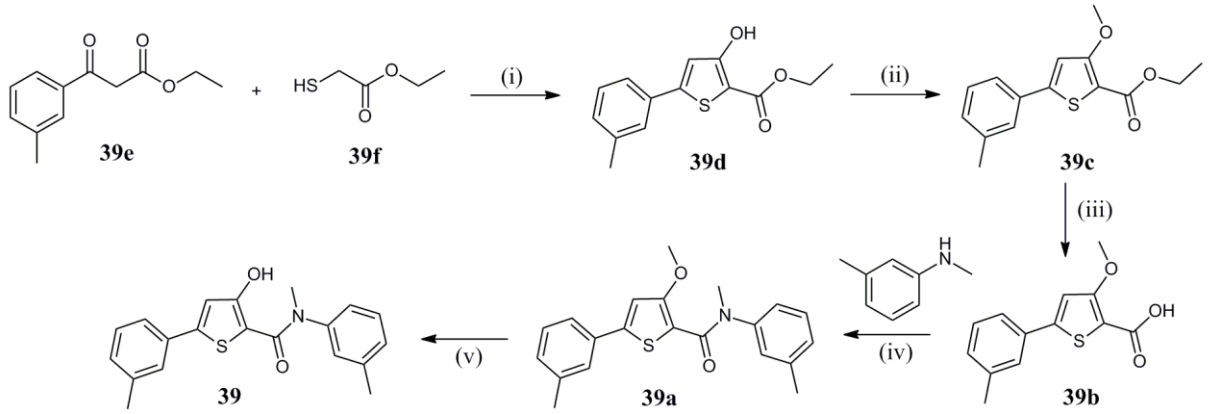
The 1,4-disubstituted phenyl derivative **35** was obtained following a two-step procedure (Scheme 3). First, amidation was carried out by reaction of 4-bromobenzoyl chloride **35b** with*N*,3-dimethylaniline **35c** (Method A) providing the brominated intermediate **35a**. Suzuki coupling (Method B) afforded the final product **35**.



[a]Reagents and conditions: (i) Et_3N, CH_2Cl_2, room temperature, overnight; (ii) DME/EtOH/H_2O (1:1:1), Cs_2CO_3, $Pd(PPh_3)_4$, microwave irradiation (150°C, 150W, 20 min).

Scheme 3. Synthesis of compound **35**.[a]

The synthesis of the 3-hydroxy-*N*-methyl-*N*,5-di-*m*-tolylthiophene-2-carboxamide (compound **39**) is depicted in Scheme 4. The ethyl 3-hydroxy-5-(*m*-tolyl)thiophene-2-carboxylate (compound **39d**) was obtained through the Fiesselmannthiophene synthesis[36] by reaction of the β-ketoester**39e** and the ethyl thioglycolate**39f**, according to the described procedure[36].The hydroxy group of compound **39d** was protected using MeI to afford compound **39c**in a moderate yield. The saponification of the ester function of **39c** was carried out using potassium hydroxide in water/THF (1:1) mixture, affording the corresponding carboxylic acid **39b**. Amidation was performed by reaction of **39b** with *N*,3-dimethylaniline, using the standard condition (method A), leading to the methoxy compound **39a**. The methoxy group of **39a** was cleaved, using boron trifluoride-dimethyl sulfide complex and yielded the hydroxyderivative**39**.



[a]Reagents and conditions: (i) a) HCl(g), room temperature, 5 h, b) NaOEt, room temperature, 2h; (ii) MeI, K_2CO_3, acetonitrile, reflux, 48 h; (iii) KOH, H_2O/THF (1:1), reflux, 4 h; (iv) a) $SOCl_2$, DMF cat., toluene, reflux 4 h, b) Et_3N, CH_2Cl_2, room temperature, overnight; (v) $BF_3 \cdot SMe_2$, CH_2Cl_2, room temperature, overnight.

Scheme 4. Synthesis of compound **39**.[a]

3.2.Biological

3.2.1. Inhibition of human 17β-HSD2 and selectivity over human 17β-HSD1 in cell-free assays.

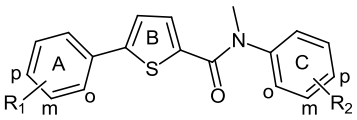
The synthesized compounds were tested for their ability to inhibit 17*β*-HSD2 and 17*β*-HSD1 using enzymes from placental source according to described methods[37-39]. Inhibitory activities of compounds **12-40** are shown in Tables 2-5, either as IC_{50} values or as percent of inhibition values determined at 1 µM. Compounds showing less than 10% inhibition at this concentration were considered to be inactive.

Influence of lipophilic substituents on rings A and C .

In the 2,5-thiophene class the methoxy groups in rings A and C can be replaced by methyl groups, without significantly affecting 17*β*-HSD2inhibitory potency and selectivity over 17*β*-HSD1, as it becomes apparent from comparing **1**, **2**and **12** (IC_{50} = 58 nM, s.f. = 116; 68 nM, s.f.= 112; 52 nM, s.f. = 83, respectively). This suggests that the methoxy groups on both rings do not function as H bond acceptors. Most likely the methyl substituents, being either part of a methoxy group or being directly connected to rings A and C, form important lipophilic interactions with the enzyme. This assumption is further supported by comparison of**12** with the corresponding compound without substituents on rings A and B, **24**[25], which is only moderately active (percent inhibition at 1 *µ*M, 48 %).

Encouraged by this result, nine compounds including all possible combinations of methyl substitutions on ring A and C were synthesized (compounds **12**-**20**), in order to investigate the influence of the methyl group position on the biological profile in this class of compounds. The substitution pattern indeed appears to be critical for both the 17*β*-HSD2inhibitory potency and the selectivity over 17*β*-HSD1 as indicated by the wide range of inhibitory activities displayed by compounds **12**-**20**(Table 2).

Table 2. Inhibition of human 17*β*-HSD2 and 17*β*-HSD1 by biphenyl-2,5-thiophene amide derivatives **12-24** with different substitution patterns on the A and C rings in cell-free systems.



1, 2, 12-24

compd	R_1	R_2	Inhibition IC_{50} (nM)[a] or % of inhibition at 1µM[a]		Selectivity factor[d]	cLogP[e]
			17*β*-HSD2[b]	17*β*-HSD1[c]		
1	*m*-OMe	*m*-OMe	58 nM	6728 nM	116	3.68
2	*m*-Me	*m*-OMe	68 nM	7616 nM	112	4.38
12	*m*-Me	*m*-Me	52 nM	4306 nM	83	4.77
13	*p*-Me	*m*-Me	58 nM	3825 nM	66	4.77
14	*o*-Me	*m*-Me	715 nM	4570 nM	7	4.77
15	*m*-Me	*o*-Me	380 nM	1177 nM	3	4.77
16	*p*-Me	*o*-Me	225 nM	698 nM	3	4.77
17	*o*-Me	*o*-Me	14%	ni	nd	4.77
18	*m*-Me	*p*-Me	892 nM	2687 nM	3	4.77
19	*p*-Me	*p*-Me	772 nM	1562 nM	2	4.77
20	*o*-Me	*p*-Me	27%	50%	nd	4.77
21	*m*-Me, *p*-Me	*m*-Me	33 nM	11576 nM	352	5.23
22	*m*-Me	*m*-Cl	327 nM	9412 nM	29	5.03
23	*m*-Cl	*m*-Me	474 nM	2144 nM	5	4.87
24	H	H	48%	14%	nd	nd

ni: no inhibition, nd: not determined

[a]Mean value of at least two determinations, standard deviation less than 25%.

[b]Human placental, microsomal fraction, substrate E2, 500 nM, cofactor NAD^+, 1500 µM.

[c]Human placental, cytosolic fraction, substrate E1, 500 nM, cofactor NADH, 500 µM.

[d]IC_{50}(17*β*-HSD1)/IC_{50}(17*β*-HSD2).

[e]Calculated data.

Results

The best 17*β*-HSD2 inhibitory potencyassociated with the best selectivity over 17*β*-HSD1 was achieved for compounds with the C ring bearing a methyl in *meta* position while on ring A the methyl can be in either in *meta* or *para* position (compounds **12** and **13**, IC_{50} = 52 nM and 58 nM; s.f. = 83 and 66, respectively). The selectivity factor is slightly decreased in comparison to the reference compounds **1** and **2**. Compound **14** with the methyl on the C ring in *meta* position and the methyl on the A ring in *ortho* position shows significantly decreased 17*β*-HSD2 inhibitory potency(IC_{50} = 715, s.f. = 7), indicating that the geometry of the A ring is critical for activity.

Substitution of the C ring with a methyl in *ortho* position, together with the A ring being either *para* or *meta*methyl substituted, leads to a slight decrease in 17*β*-HSD2 inhibitory potency and to an increase in 17*β*-HSD1 inhibition, providing compounds with a poor selectivity factor (compounds **15** and **16**, IC_{50} = 380 nM and 225 nM and s.f. = 3 and 3, respectively). If both A and C rings are *ortho* substituted, 17*β*-HSD2 and17*β*-HSD1 activity are strongly decreased (compound **17**, 14% inhibition and no inhibition for 17*β*-HSD2 and 17*β*-HSD1 at 1 µM, respectively).

Substitution of the C ring with a methyl group in *para* position in combination with a*meta* or *para* substituted A ring led to compounds with moderately decreased 17*β*-HSD2 inhibition and decreased selectivity over 17*β*-HSD1 (compounds **18** and **19**, IC_{50} = 892 nM and 772 nM, s.f. = 3 and 2, respectively). *Ortho* substitution of the A ring coupled with *para* substitution of the C ring, surprisingly leads to an inversion of selectivity (compound **20**, 26% and 50% inhibition of 17*β*-HSD2 and 17*β*-HSD1 at 1 µM, respectively).

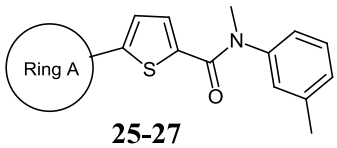
In the 2,5-thiophene class the highest inhibition is observed for acompound showing a*m*,*p*-dimethyl substitution on the A ring and a *meta* methyl substitution on the C ring (compound **21**, IC_{50} = 33 nM, s.f. = 352), indicating that the positive effect on the activity of the two methyl groups on the A ring is additive. Furthermore, the presence of the two methyl groups increases the selectivity over 17*β*-HSD1. Compound **21** turns out to be the most active and selective compound in this class.

The exchange of the methyl in *meta* position on either the Aor the C ring with a chlorine decreases both 17*β*-HSD2 inhibitory potency and selectivity over 17*β*-HSD1 (compounds **22** and **23**, IC_{50} = 327 nM and 474 nM, s.f. = 29 and 5, respectively). The importance of the molecular electrostatic potential (MEP) for the ability of 17*β*-HSD2 and 17*β*-HSD1 inhibitors to establish a π-stacking interaction with aromatic amino acids from the active site has been recentlydiscussed[40]. The decrease in the 17*β*-HSD2 inhibition of compounds **22** and **23** can be explained by the difference in the MEPs of the two aromatic rings, elicited by the exchange of the methyl group with a chlorine. This exchange on the A ring seems to be favorable for the 17*β*-HSD1 inhibitory potency (compound **23**, IC_{50} = 2144 nM for 17*β*-HSD1).

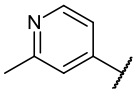
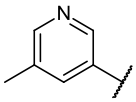
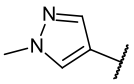
The introduction of lipophilic substituents on ring A and C increases cLogP values for compounds **12-23** slightlycompared to **1** and **2** (Table 2).However, the values are still below 5, which should be sufficient for an acceptable bioavailability[41]. Since lipophilic compounds are usually more susceptible to phase I metabolism[35], in a second step we

tried to lower cLogP of the 2,5-thiophene derivatives by exchanging the methylphenyl A ring for different heterocycles like 2-methylpyridinyl **25**, 3-methylpyridininyl **26** or 1-methyl-1*H*-pyrazolyl **27** (Table 3).
The calculated LogP values of compounds **25**, **26** and **27** are lower and therefore advantageous for their bioavailability compared to the one of compound **12** (3.38, 3.45 and 2.46 for **25**, **26** and **27**, respectively, 4.77 for **12**, Table 2 and 3).

Table 3. 2,5-thiophene amides *N*-containing heterocycle as A ring **25-27**. Inhibition of human 17*β*-HSD2 and 17*β*-HSD1.



25-27

compd	Ring A	% inhibition at 1 μM[a]		cLogP[d]
		17*β*-HSD2[b]	17*β*-HSD1[c]	
25		18%	n.i.	3.38
26		28%	n.i.	3.45
27		16%	n.i.	2.46

ni: no inhibition

[a]Mean value of at least two determinations, standard deviation less than 25%.

[b]Human placental, microsomal fraction, substrate E2, 500 nM, cofactor NAD^+, 1500 μM.

[c]Human placental, cytosolic fraction, substrate E1, 500 nM, cofactor NADH, 500 μM.

[d]Calculated data.

However the exchange of the A ring with these heterocycles led to a strong decrease in activity (compounds **25**, **26** and **27**: 18%, 28% and 16% inhibition at 1 μM, respectively). Once again the change in the MEP of the A ring might be responsible for this decreased activity.

As the methyl substituents on rings A and C in the 2,5-thiophene class were discovered to be very well tolerated by 17*β*-HSD2, especially in *meta* position, and to induce a good selectivity over 17*β*-HSD1 they will be maintainedfor the subsequent modifications.

Influence of the central core

In a previous study[42], the central 2,5-thiophene-carboxamide of **2** was replaced by a thiazole-2-carboxamide and by a thiazole-5-carboxamide (compounds **28** and **29**, IC_{50} values 296 nM and 621 nM, s.f. = 63 and 8, respectively, Table 4). Comparison of the potency of **28** and **29**,indicates that the thiazole-2-carboxamide is better in terms of 17*β*-

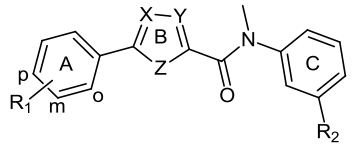
HSD2 inhibition and selectivity over 17*β*-HSD1 in presence of a methoxy group in position 3 on the C ring.As we had experienced that in the 2,5-thiophene class the methoxy group can be replaced by a methyl (compound **12**) we decided to synthesize the thiazole-2-carboxamide with a methyl group on the A and C ring (compound **30**, IC_{50} = 499 nM, s.f. = 51). This structure modification leads to a 2-fold decrease in activity compared to compound **28**, indicating that as observed in the 2,5-thiophene class, no crucial interaction is achieved by the oxygen atom of the methoxy group.

In addition the introduction of the methyl substituent in the thiazole-2-carboxamides did not sufficiently improve 17*β*-HSD2 inhibitory potency to make them superior to the 2,5-thiophene amides as already described by us[42].

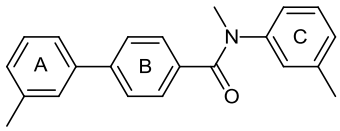
The replacement of the 2,5-thiophene for a 2,5-furane ring is detrimental for the 17*β*-HSD2 inhibitory potency as shown by compounds **31**-**34**. The strongest inhibition of 17*β*-HSD2 was achieved by compound **34** (38% inhibition at 1µM), demonstrating that the furane ring is not a suitablebioisostere for the thiophene in these compounds, in contrast to observations made in other compound classes[43]. The furane is less aromatic than the thiophene (electron rich system) because the electronegativity of the oxygen renders the electron pair on this atom less available for resonance. This makes the furane less suitable for π-stacking interactions. The electrostatic potential of the thiophene and furane derivatives are therefore different which might explain the decreasein the activity associated with the furanes.

Finally the replacement of the central ring by a 1,4-diphenyl substitution leads to compound **35**, with an IC_{50} value of 1126 nM and a s.f. of 10. The exchange of the thiophene with a benzene decreases 17*β*-HSD2 inhibition moderately. However, in comparison with the furane series, in the benzenes some selectivity over 17*β*-HSD1 is sustained (Table 4).

Table 4. Exchange of the central core B. Inhibition of human 17*β*-HSD2 and 17*β*-HSD1.



2, 12, 28-34



35

compd	X	Y	Z	R_1	R_2	Inhibition IC_{50} (nM)[a] or % of inhibition at 1µM[a]		Selectivity factor[d]	cLogP[e]
						17*β*-HSD2[b]	17*β*-HSD1[c]		
2	H	H	S	*m*-Me	-OMe	68 nM	7616 nM	112	3.68
12	H	H	S	*m*-Me	-Me	52 nM	4306 nM	83	4.77
28	H	N	S	*m*-Me	-OMe	296 nM	18663 nM	63	4.19
29	N	H	S	*m*-Me	-OMe	621 nM	4821 nM	8	3.91

30	H	N	S	*m*-Me	-Me	499 nM	25302 nM	51	4.58
31	H	H	O	*m*-Me	-Me	14%	31%	nd	3.86
32	H	H	O	*m*-OMe	-Me	ni	16%	nd	3.17
33	H	H	O	*o*-F, *m*-OMe	-Me	34%	26%	nd	3.62
34	H	H	O	*m*-Me, *p*-Me	-Me	38%	46%	nd	4.32
35	-	-	-	-	-	1126	11541	10	4.93

ni: no inhibition, nd: not determined

[a]Mean value of at least two determinations, standard deviation less than 25%.

[b]Human placental, microsomal fraction, substrate E2, 500 nM, cofactor NAD^+, 1500 µM.

[c]Human placental, cytosolic fraction, substrate E1, 500 nM, cofactor NADH, 500 µM.

[d]IC_{50}(17*β*-HSD1)/IC_{50}(17*β*-HSD2).

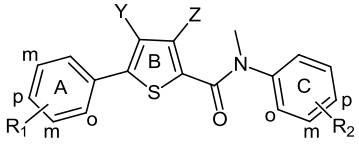
[e]Calculated data.

The exchange of the central thiophene ring of **12** (cLogP = 4.77) by thiazole (**30**, cLogP = 4.58) and by furane (**31**-**34**, cLogP = 3.86, 3.17, 3.62 and 4.32, respectively) lowers the cLogP value, whereas it is slightly increased in case of the benzene derivative**35**; (cLogP = 4.93, Table 4). However, all compounds show cLogP values in range, which is acceptable for a good bioavailability according to literature[41].

*3.2.2. Hydroxy derivatives of**12** and their precursors.*

Oxydation often occurs during metabolism and in some cases leads to highly active metabolites. Several oxidation products derived from **12** were synthesized and evaluated for their biological activity in vitro (Table 5).

Table 5. Inhibition of human 17*β*-HSD2 and 17*β*-HSD1 by the possible major metabolite of **12** and its precursors in cell-free systems.



12, 36a-40a, 36-40

compd	Y	Z	R_1	R_2	Inhibition IC_{50} (nM)[a] or % of inhibition at 1µM[a]		Selectivity factor[d]	cLogP[e]
					17*β*-HSD2[b]	17*β*-HSD1[c]		
12	-H	-H	*m*-Me	*m*-Me	52 nM	4306 nM	83	4.77
36a	-H	-H	*m*-Me, *p*-OMe	*m*-Me	66%	11%	nd	4.45

36	-H	-H	*m*-Me, *p*-OH	*m*-Me	62%	22%	nd	3.93
37a	-H	-H	*m*-Me, *m*- OMe	*m*-Me	56%	28%	nd	4.43
37	-H	-H	*m*-Me, *m*- OH	*m*-Me	53%	13%	nd	3.94
38a	-OMe	-H	*m*-Me	*m*-Me	59%	ni	nd	4.47
38	-OH	-H	*m*-Me	*m*-Me	64%	ni	nd	4.00
39a	-H	-OMe	*m*-Me	*m*-Me	127 nM	16732 nM	132	4.36
39	-H	-OH	*m*-Me	*m*-Me	67%	24%	nd	4.77
40a	-H	-H	*m*-CHO	*m*-Me	62%	ni	nd	3.84
40	-H	-H	*m*-CH_2OH	*m*-Me	55%	ni	nd	3.39

ni: no inhibition, nd: not determined

[a]Mean value of at least two determinations, standard deviation less than 25%.

[b]Human placental, microsomal fraction, substrate E2, 500 nM, cofactor NAD^+, 1500 µM.

[c]Human placental, cytosolic fraction, substrate E1, 500 nM, cofactor NADH, 500 µM.

[d]IC_{50}(17*β*-HSD1)/IC_{50}(17*β*-HSD2).

[e] Calculated data.

Introduction of an additional methoxy group in *para* position of compound **12** (*ortho* to methyl) leads to compound **36a** (Table 5) showing a significantly decreased activity (66% inhibition at 1 µM). A similar loss in activity is observed for the hydroxylated compound **36** (62% inhibition at 1 µM), indicating that in this position introductionof groups like a methoxy or hydroxy is not well tolerated.

This effect is also observed with the introduction of a second substituent in *meta* position of ring A in compound **12** (Table 5). In fact compound **37a**, bearing an additional methoxy group and compound **37**, with an additional hydroxy group in *meta* position, show a similar inhibitory potencyover 17*β*-HSD2 but lower than the one of the monomethyl**12** (56% and 53% inhibition at 1 µM, respectively) or the monomethoxy**1** (IC_{50} value 58 nM). This result indicates that no additive effect is obtained by addition of both methoxyand methyl group in *meta* position, perhaps due to a steric hindrance.

The introduction of a methoxy group or a hydroxy group in position 4 of the thiophene resulting in compounds **38a** and **38** (59% and 64% inhibition at 1 µM, respectively) is detrimental for activity. This might be due to conformational effects on the phenylA ring, steric hindrance in the binding site or repulsion of the polar groups caused by the lipophilic environment in the binding cavity.

On the other hand, a methoxy group in position 3 of the thiophene leading to compound **39a** (Table 5), decreases activity only slightly in comparison to **12** (IC_{50}= 127 nM and 52 nM, respectively) whereas selectivity is increased (s.f. = 132 and 83, respectively).

Exchanging this methoxy by a hydroxy group (compound **39**) leads to a decreased activity (67% inhibition at 1 µM, Table 5). This indicates that in position 3 of the thiophene a

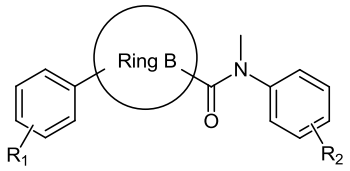
lipophilic group is tolerated whereas a hydrophilic substituent, such as a hydroxy, is detrimental for activity.

Exchange of the methyl group on ring A of compound **12** by an aldehyde (compound **40a**) or by a CH_2OH group (**40**) decreases inhibitory potency (62% and 55% inhibition at 1µM, respectively, Table 5).

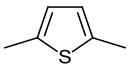
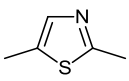
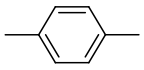
3.3. Affinity for the Estrogen Receptors

17*β*-HSD2 inhibitors should have no affinity for the estrogen receptors (ERs) α and β in order to avoid potential ER mediated adverse effects. Compounds **12**, **13**, **21**, **22**, **30** and **35** have been tested according to described methods[44] and the results have been expressed in terms of percent [^{3}H]-E2 displaced from the receptor by the inhibitor (Table 6). Shortly, ERα or ERβ were incubated with [^{3}H]-E2 and test compound. Subsequently the percent [^{3}H]-E2 displaced from the receptor by the inhibitors was determined, which were tested at concentrations 1000 times higher than the one of [^{3}H]-E2 (Table 6). All inhibitors tested were able to displace less than 50% of [^{3}H]-E2 from the corresponding receptor at this concentration. In terms of Relative Binding Affinity (RBA), their RBA values are <0.1% for both ER α and β, indicating a low binding affinity.

Table 6. Percentage of [^{3}H]E2 displaced from the ERα and β for selected compounds



12, 13, 21, 22, 30, 35

compd	Ring B	R_1	R_2	Percentage of [^{3}H]E2 displaced by the inhibitor[a,d]	
				Estrogen Receptor α[b]	Estrogen Receptor β[c]
12		*p*-Me	*o*-Me	12	21
13		*p*-Me	*m*-Me	11	34
21		*m*-Me, *p*-Me	*m*-Me	5	4
22		*m*-Me	*m*-Cl	16	11
30		*m*-Me	*m*-Me	9	0
35		-	-	8	11

[a]Mean value of at least two determinations, standard deviation less than 25%.

[b]Recombinant human receptor, ERα 1 nM, [^{3}H]E2 3 nM.

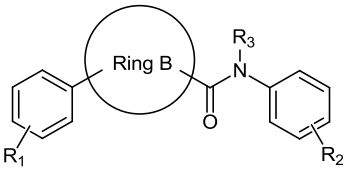
[c]Recombinant human receptor, ERβ 4 nM, [^{3}H]E2 10 nM.

[d]Inhibitor tested at 1000 fold the [^{3}H]E2 concentration, 3μM for ERα, 10 μM for ERβ.

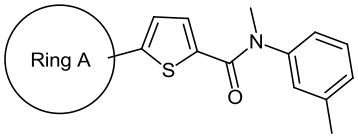
3.4. Metabolic stability in human S9 fraction.

We evaluated the most promising inhibitors for metabolic stability using human liver S9 fraction according to described methods[45-47]. Briefly, incubations were run with the S9 fraction, cofactors (for phase I and II reaction) and inhibitor. The concentration of the remaining test compound at the different time points was analyzed by LC-MS/MS and the results expressed as half-lifes ($t_{1/2}$) and reported in Table 7.

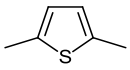
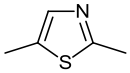
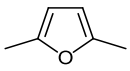
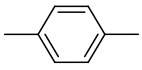
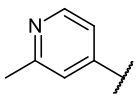
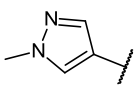
Table 7. Half-life of representative compounds measured in Human Liver Microsomes S9 fraction.



1, 12, 13, 16, 21, 22, 30, 31, 35, 41



25, 27

compd	Ring B	Ring A	R_1	R_2	R_3	Inhibitor[c] $t_{1/2}$(min)[a,b]
1		-	*m*-OMe	*m*-OMe	-Me	17
12		-	*m*-Me	*m*-Me	-Me	19
13		-	*p*-Me	*m*-Me	-Me	15
16		-	*p*-Me	*o*-Me	-Me	22
21		-	*m*-Me, *p*-Me	*m*-Me	-Me	20
22		-	*m*-Me	*m*-Cl	-Me	32
41		-	*o*-F, *m*-OMe	*m*-OMe	-H	117
30		-	*m*-Me	*m*-Me	-Me	5
31		-	*m*-Me	*m*-Me	-Me	92
35		-	-	-	-Me	>120
25	-		-	-	-Me	21
27			-	-	-Me	82

[a]Extrapolated value. Mean of at least two determinations, standard deviation less than 25%.

[b] 1 mg/ml pooled mammalian liver S9 fraction (BD Gentest), 2 mM NADPH regenerating system, 1 mM UDPGA and 0.1 mM PAPS at 37°C for 0, 5, 15 and 60 minutes.

[c]Inhibitor tested at a final concentration of 1 µM.

Exchange of the two methoxy of compound **1** ($t_{1/2}$ = 17 min) by two methyl groups provides compound **12** ($t_{1/2}$ = 19 min), which is also rather metabolically labile (Table 7). Changing the substitution pattern of the methyl functions does not influence metabolic stability, as it results from comparison of *meta-*,*meta-* **12**,*para-*,*meta-* **13** and*para-*,*ortho-* **16** ($t_{1/2}$ = 19 min, 15 min and 22 min, respectively).

Compound **21**($t_{1/2}$ = 20 min), bearing an additional methyl in *para-* position on ring A , in comparison to **12** does not show an improved stability (**21** $t_{1/2}$ = 20 min *vs.***12** $t_{1/2}$ = 19 min).

Compound **22**, with a chlorine on ring C, shows a slightly increased half-life ($t_{1/2}$ = 32 min) in comparison to **12** ($t_{1/2}$ = 19 min).

In addition compound **41**, previously described[25], and bearing a free carboxamide moiety was also tested for stability. In comparison to the others thiophene derivatives and more especially to the methylated analogue**5**, it is exceptionally stable ($t_{1/2}$ = 117 min). It is not clear whether the methyl group of the N-CH_3 amide is the metabolic reactive site or whether its absence stabilizes another site in the molecule.

Replacement of the thiophene by a furane (compound **31**, $t_{1/2}$ = 92 min) or by a benzene (compound **35**, $t_{1/2}\geq$120 min) results in strong improvement of metabolic stability. On the contrary, exchange of the thiophene by a thiazole leads to very labile compound (**30**, $t_{1/2}$ = 5 min). From these results, it is likely that the thiophene plays a major role in the metabolic fate in the 2,5-thiophene class of compounds.

The strategy of lowering the cLogP of compound **12** (cLogP = 4.77), in order to improve the metabolic stability, led to the pyrazole**27** (cLogP = 2.46) and the pyridine **25** (cLogP = 3.38). As expected the most hydrophilic compound **27** ($t_{1/2}$ = 82 min), is more stable than compounds**25** ($t_{1/2}$ = 21 min), and**12** ($t_{1/2}$ = 19 min).

3.5. Metabolites Identification.

Three compounds (**1**, **2** and **12**) were investigated regarding their metabolic fate and tested in the human S9 fraction using a similar procedure as already described for determination of the metabolic stability. Inhibitors were incubated at a concentration of 10 µM inhibitors without phase II cofactors for three hours (no phase II metabolism is expected).After extraction the samples were analyzed using a high resolution LC-MS (LTQ/Orbitrap). Potential metabolites were identified by searching for expected biotransformations(Table 8). The results are presented as area percentage relative to the remaining parent compound. In some cases two different peaks were found for one biotransformation, due to its occurrence at two different sites of the molecule.

Table 8. List of expected metabolites and their amounts, expressed as area percentage of the remaining parent compound.

Mass Difference	Formula	Description	Percentage of metabolite relative to parent compound[a]		
			1	**2**	**12**
0	-	Parent	100	100	100
+2	$-CH_2+O$	Demethylation + Hydroxylation	nf	4	6
+16	+O	Hydroxylation, N, S oxidation, epoxydation	12	14 5	20 4
+30	$+O_2-H_2$	CH_3 to CO_2H	nf	nf	5
+32	$+O_2$	Dihydroxylation	2	nf	7
-14	$-CH_2$	Demethylation	27 9	7	10
-28	$-C_2H_4$	Didemethylation	2	nf	nf

nf: not found

[a]Area percentage is calculated based on the assumption that each metabolite is equally responsive as parent.

For the most prominent metabolites, we propose a structure based on comparison of the fragmentation pattern of the parent compound and the ones of the metabolites (Figure 1). The spectrum of **1** (Fig. 1a) contains strong signals at *m/z* 136 and 164, representing the substituted aniline ring without or with the amide function, respectively. It also contains the signal *m/z* 217 representing the left part of the molecule after cleavage of the amide function. This fragmentation is observed in all the tested 2,5-thiophene amide inhibitors and their metabolites. The main metabolite of compound **1** is a demethylated product that accounts for 27 % of the remaining parent compound (Table 8). The spectrum of thisdemethylation product (Fig. 1b) still contains the signals *m/z* 136 and 164, indicating that no demethylation occurred either on the C ring or on the amide. A new signal at *m/z* 203, coming from the left part of the molecule, indicates that the cleavage of the methoxy occurred on the A ring. In addition a second demethylation product was also observed representing 9 % of the remaining parent compound as well as an+O product (12%, Table 8). Other biotransformations result in negligible amounts (Table 8). The exact structure of these metabolites was not identified.

In case of **2** (Fig. 1c and 1d) the metabolic demethylation (7 % of the remaining parent compound) was less intense compared to the methoxy compound **1**, whereas the +16 biotransformation (Table 8) results in two different products, accounting for 14 % and 5 % of the remaining parent compound. Comparing the fragmentation pattern of **2** (Fig. 1c) and its main oxidation product (Fig.1d) it can be concluded that the oxygen addition occurs on the thiophene phenyl A ring part of the molecule highlighted with the dotted line (Fig. 1d). The structure of the metabolite was not elucidated. Also in this case, the other biotransformationswere negligible.

For compound **12**, the main metabolic product derives from an oxidation (Table 8). Two different peaks for the +16 biotransformation can be observed (Table 8), one representing 20 % of the remaining parent compound and the other 4 %. Demethylation (10 %), and

oxidation of the -CH_3 into carboxylic acid (5 %) are also present. For **12**, the demethylation alone can only occur at the amide function. The transformation demethylation+ hydroxylation and the di-hydroxylation were also detected for this compound (Table 8).
Comparing the fragmentation pattern of **12** (Fig. 1e) and its main oxidation product (Fig. 1f), it can be concluded that the oxygen incorporation occurs on the thiophene phenyl part of the molecule highlighted with the dotted line. Comparing the fragmentation pattern of the -CH_3 to -CO_2H biotransformation in the same way (Fig. 1g), it becomes apparent that the carboxylic acid group is on the A ring.

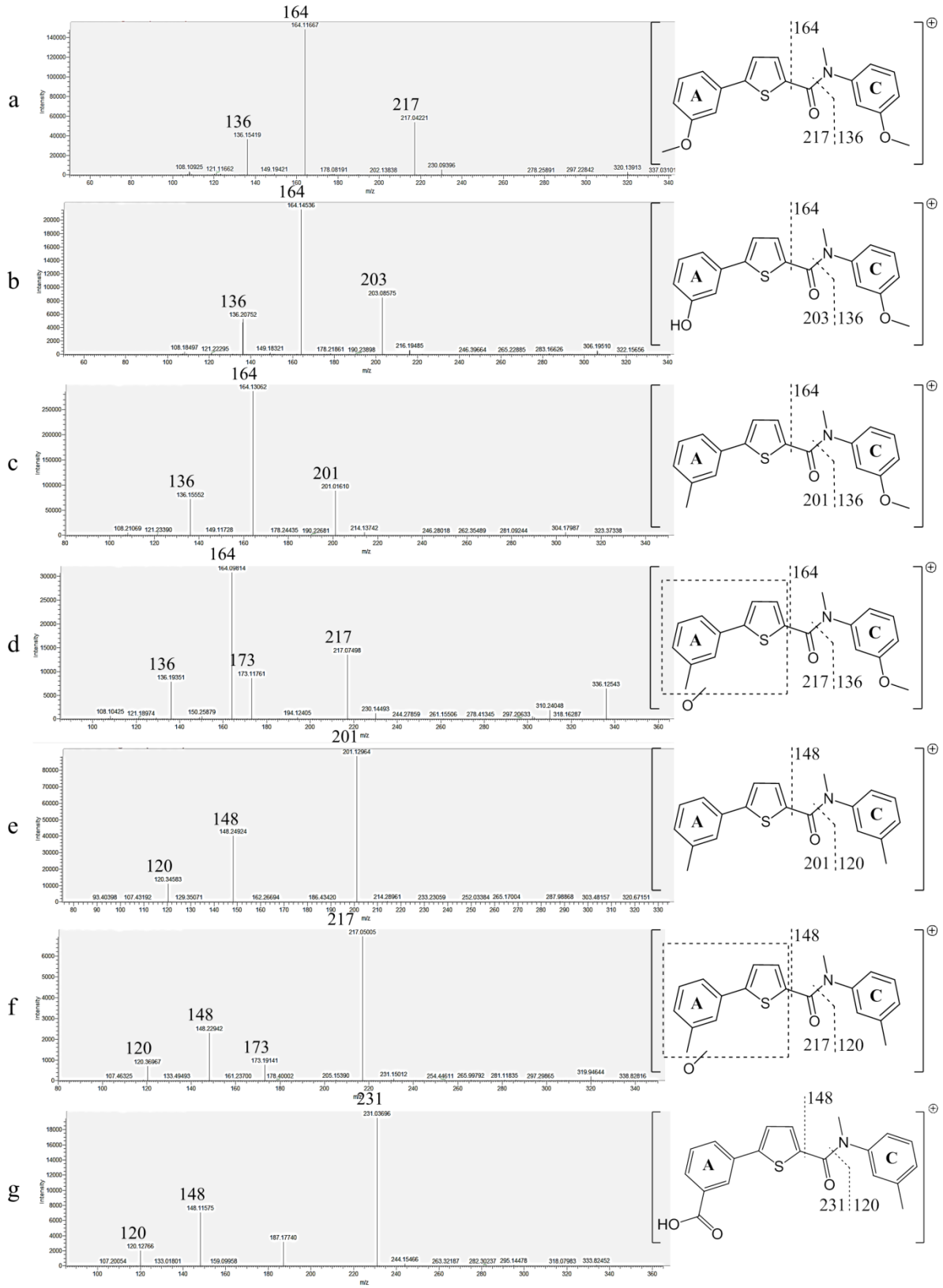


Fig. 1. MS2 spectra of a) **1**; b) **1** major demethylation product; c) **2**; d) **2** major oxidation product; e) **12**; d) **12** oxidation product; g) **12** CH_3 to CO_2H with proposed structures.

Since we were interested in the identification of the exact hydroxylation site for **12** and since the fragmentation pattern using only the MS/MS data was not conclusive, we synthesized the most probable metabolites. They are represented in Figure 2.

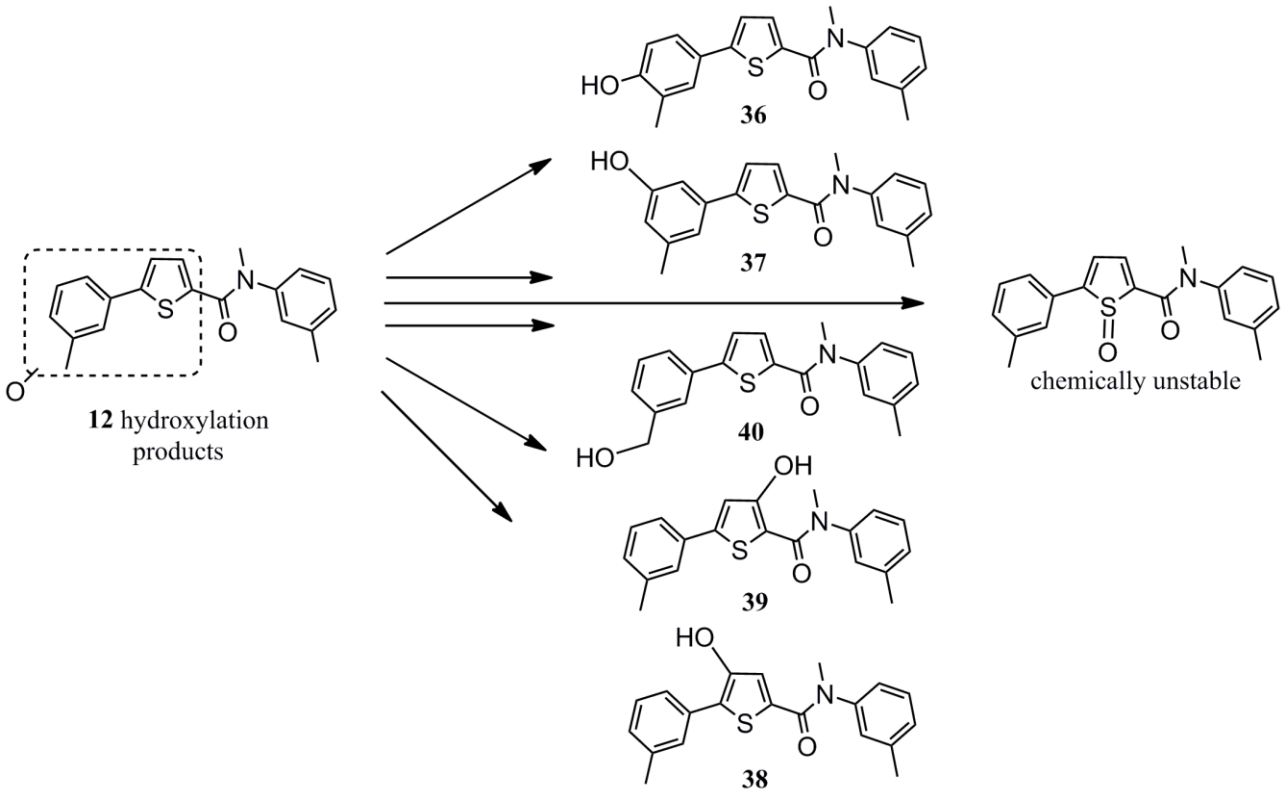


Fig. 2. Potential oxidized metabolites of compound **12**.

Compound **36**-**40**are derived from compound **12** with addition of one hydroxy group in different positions (Figure 2). The mass +16 (Table 8) observed in our experiment could in theory come from an S-oxidation of the thiophene. The S-oxide thiophene is known to be chemically highlyunstable[48] and after synthesis it was rapidly degraded (data not shown). In addition since the procedures for metabolitesynthesis and identification require the samples to be exposed to light at room temperature, able to even accelerate the S-oxide degradation[48], it was concluded that the biotransformation product observed is unlikely to be the thiophene S-oxide.

The other 5 potential metabolites, depicted in Figure 2, were synthesized and co-injected in the LC-MS/MS together with a solution of the metabolites of **12** (Figure 3).

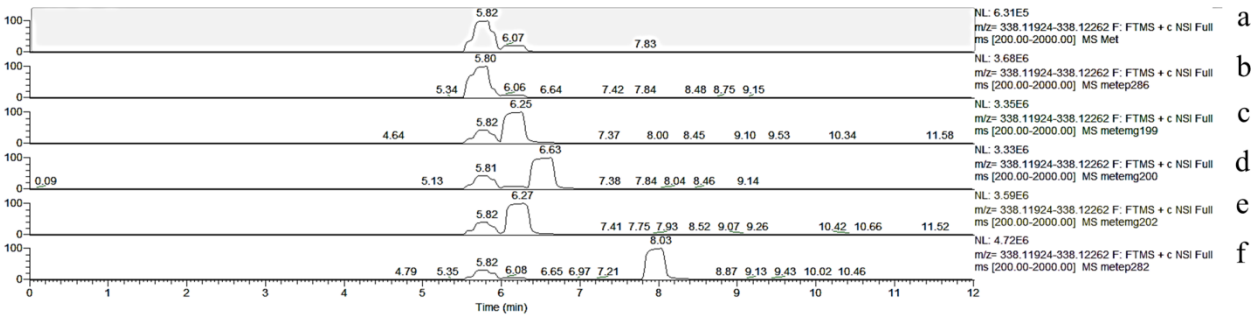


Fig. 3. Ion chromatogram of **12** major oxidation product and its co-injection with the synthesized potential metabolite. On the top of the peaks, the retention times are shown. From the top to the bottom are displayed the six chromatograms of the a) hydroxylation products after incubation with the S9 fraction; b) co-injected with **40**; c) co-injected with **36**; d) co-injected with **38**; e) co-injected with **37**; f) co-injected with **39**.

The chromatograms of the hydroxylated metabolites of **12** alone (Fig. 3a) and the one of the co-injection with compound **40** (Fig. 3b) are identical with a main peak observed with a retention time of 5.82 minutes and a minor peak at 6.07 minutes. The co-injection of any other potential metabolite synthesized (**36**-**39**) produces spectra with additional peaks having different retention time in comparison to the original spectrum: **36** (Fig. 3c), **38** (Fig. 3d), **37** (Fig. 3e) and **39** (Fig. 3f) produce new peaks at 6.25 min, 6.63 min, 6.27 min and 8.03 min, respectively (Fig. 3). This experiment demonstrates the identity of the hydroxylated metabolite of**12** with the hydroxy methylcompound**40**.

To confirm that **40** is the main metabolite of **12** we compared the fragmentation pattern of the metabolite and the synthesized compound (Figure 4).

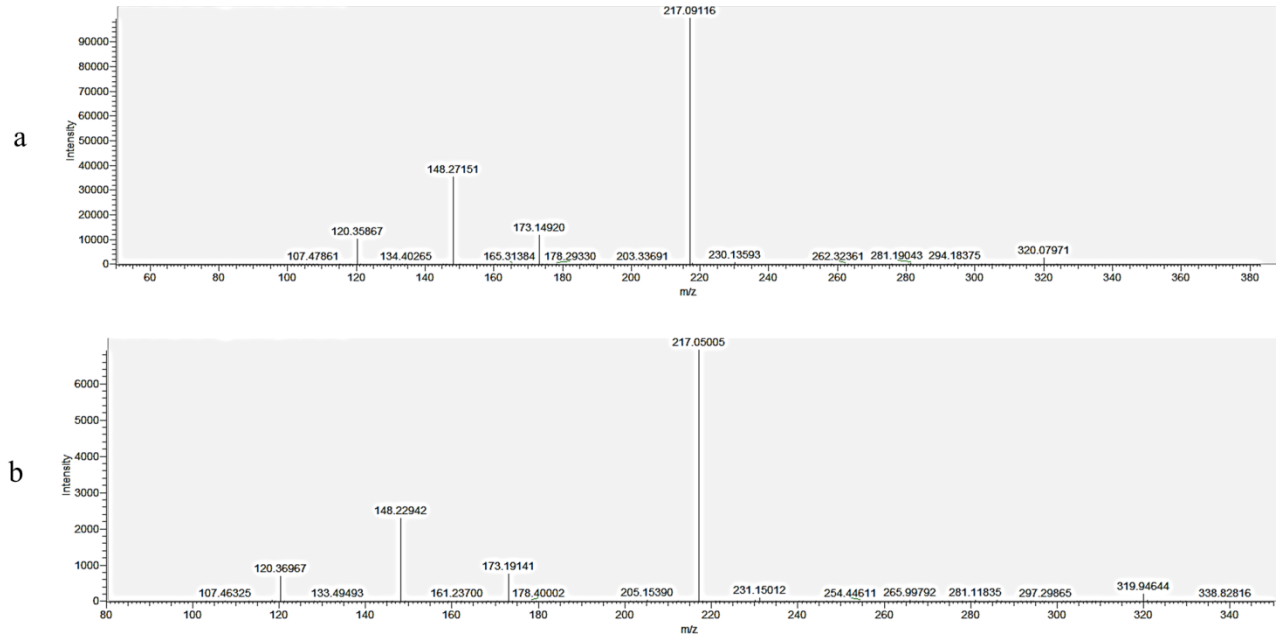


Fig. 4. a) Fragmentation pattern of the pure **40**; b) fragmentation pattern of **12** major hydroxylated product.

As displayed in Figure 4a and 4b, the two fragmentation pattern perfectly match. Prominent signals at *m/z* 120, 148, 173 and 217 are present in both spectra. Interestingly, the signal at *m/z* 173 is observed only in the MS2 spectra of **40** and in none of the fragmentation spectra produced for the other potential metabolites (data not shown). This signal is likely to represent the fragment depicted in Figure 5, derived from the loss of the hydroxy group on the methylene. Since the other potential metabolites all bear an OH directly linked to an aromatic ring (Fig. 2), they might lack the signal at *m/z* 173 because the fragmentation at an aromatic OH needs more energy to be produced. Since this same signal is present in the fragmentation spectrum of the hydroxylated metabolite of compound **2** (Fig. 1d) and given the high structural similarity with **12**, we could speculate that the hydroxylation reaction occurs at the methyl on the A ring of compound **2** as observed for**12**.

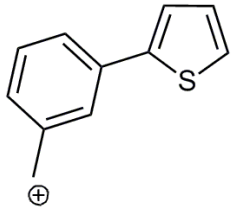


Fig. 5. Proposed structure for signal at m/z 173.

The main metabolic pathway for compound **12** is depicted in Figure 6. The different metabolites of **12** are expressed both as percentage of the total found metabolites and as percentage of the remaining parent compound in brackets. 39% of the metabolites derives from the hydroxylation of the methyl on the A ring (compound **40**) and its following transformation into a carboxylic acid group (compound**42**) accounts for 10% of the total biotransformations. The cleavage of the methyl on the amide function (compound **43**)represents 19% of all the metabolites and the combination of hydroxylation and demethylation(compound **44**) accounts for 12% of the detected biotransformation products.

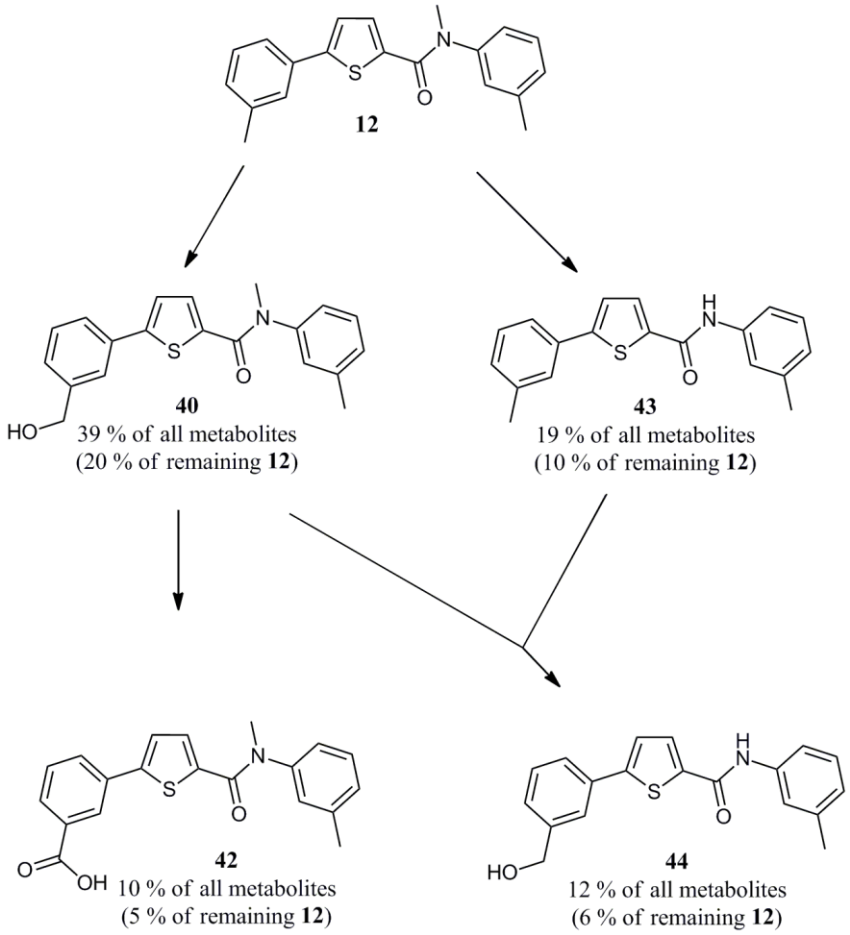


Fig. 6. Proposed pathway for the phase I metabolism of 12 in human S9 fraction.

4. DISCUSSION

The main goal of this study was the optimization of the metabolic stability of our in house 17*β*-HSD2 inhibitors, together with a satisfying understanding of their main routes of metabolism. The new 17*β*-HSD2 inhibitors were designed trying to keep potency and selectivity over 17*β*-HSD1 and the ERs in an acceptable range while improving their metabolic stability.

4.1. Potency and selectivity

In the design process we focused on compounds bearing methyl substituents on rings A and C. Compound **12** (IC_{50} = 52 nM) was obtained, which retains the activity of the dimethoxy analogue**1** (IC_{50} = 58 nM). This result indicates that a polar moiety is not necessary to achieve high potency as previously believed[24], therefore suggesting that the compounds are notsteroidomimetics and do not have the same binding mode as the natural substrate E2.

The effect of all the 9 possible substitution pattern of the methyl groups on rings A and C was studied. The substitution in *meta* or *ortho* position on ring A, while keeping a methyl in *meta* position on ring C (compounds **12** and **13**, IC_{50} = 52 and 58 nM) leads to a an increase

in activity of a factor of nearly 20, in comparison with the compound having the two unsubstituted phenyl rings (48% inhibition at 1 µM) [21]. Recently Leung *et al.* reported about the effects of the addition of a methyl group to a lead compound on its biological activity, revealing how, from analysis of more than 2000 examples described in the literature, an activity improvement of a factor of 10 or more is found with an 8% frequency only[49]. The authors pointed out that the highest activity increase is often the resultfrom the combination of good fitting of the methyl group into a hydrophobic region of the protein target together with a possible conformational gain, derived from an*ortho* methyl substitution at a phenyl ring. The increase in inhibitory potency observed in our class of inhibitors might come from the burial of the methyl group in *meta* position on ring A in a hydrophobic pocket of the enzyme, since any conformational restrain induced by*ortho* substitution at this ring (compounds**17** and**20**, 14% and 27% inhibition at 1 µM, respectively) is detrimental for activity.

By introducing two methyl groups in *para* and *meta* position on ring A, the most potent compound of this class (**21**, IC_{50} = 33 nM) was obtained, indicating additive effects of the two methyl groups. It also reveals the presence of a likely hydrophobic cavity around ring A.

Exchange of the A ring of compound **12** by different heterocycles like methyl pyridine leads to a significant decrease of activity. This might be due to an unfavorable electrostatic potential of the ring, compared to the phenyl (Table 3) or to the presence of the polar nitrogen, which does not fit well into the enzyme, confirming the presence of a lipophilic cavity. Compound **27**, bearing a 1-methyl-*1H*-pyrazole, is even less potent (Table 3) confirming that polar groups on the A ring are not compatible with a strong binding to the protein target.

The replacement of the central thiophene ring by a furane is also detrimental for activity (**31**, 14% of inhibition at 1 µM). A small study on the effects of different substituents the A ring resulted in compound **34** as best inhibitor of this series (38% of 17*β*-HSD2 inhibition at 1 µM and 46% of 17*β*-HSD1 inhibition at 1 µM), indicating the inadequateness of this central ring. The furane ring indeed seems to reverse the activity in favor of 17*β*-HSD1 inhibition, since **31** and **34** are slightly more active on 17*β*-HSD1 and much less active on 17*β*-HSD2, in comparison to compound **12** (table 4).

4.2. Metabolic stability

Compounds **1**, **2** and **3** taken as leads for the study of metabolic stability were found to be rather unstable ($t_{1/2}$ = 17 min, 38 min and 4 min, respectively). An important goal of this work was the improvement of the metabolic stability of this class of compounds. To achieve this goal two different techniques were applied: 1. modification of the potential reactive site of the inhibitors, 2. lowering theircLogP.

In order to understand the reason for the instability of **1**, **2** and **3** and in order to identify the metabolically reactive sites in these molecules, a small set of compounds was

synthesized and tested. As the methoxy groups seemed to be labile in presence of hepatic enzymes, they were exchanged by methyl groups, in principle slightly more stable. However, this strategy did not improve the stability of the inhibitors, as seen for compounds **12**,**13**, **22**, **21** and**22** with half-life in the same range as previous molecules ($t_{1/2}$ around 20 min). While replacement of the methoxygroup by methyl could preserve inhibitory potency, it did not influence metabolic stability. This can be explained looking at the primary metabolite of **1** and **12**: the methoxy group of **1** as well as the methyl group of **12** on ring Aare soon oxidized, thus leading to ether cleavage and CH_2OH formation, respectively. The rate of this reaction, which might be catalyzed by the same enzyme, appears to be in the same order of magnitude for both molecule and the metabolic stability is clearly not influenced by the nature of the substituents on the two compounds.

Given the high structural similarity of compounds **1** and **12** with others 2,5-thiophene amide inhibitors and considering the instability of the tested compounds of this class, it is very likely that the oxidation observed for **1** and **12** is the main metabolic route for the other compounds as well.

Compounds bearing hydroxy groups display half-lifes either similar or lower than inhibitors without hydroxy groups (Table 1) and are likely to be also affected by metabolic phase II reactions.

This metabolite identification study clearly indicates that the central thiophene of compound **12** does not constitute a metabolic reactive site and is not susceptible to any biotransformation. Therefore it is striking that the exchange of the thiophene either by a furaneor by benzene ring leads to more stable compounds like **31** and **35** ($t_{1/2}$ = 92 min and >120 min, respectively). This means that it is possible to modulate the metabolic rate of compound **12** by modification of the central ring, although it does not constitute a metabolic labile site.

The stability of compound **31** (cLogP = 3.86) might be explained by the decrease in cLogPaccomplished by the furane ring in comparison to the thiophene derivative**12** (cLogP = 4.77). This result is also confirmed by the 1-methyl-*1H*-pyrazole compound**27** (cLogP = 2.46) which shows a half-life in human liver S9 fraction of 82 minutes. Exchange of the thiophene by a benzene ring does not significantly modify the cLogP for **35** (cLogP = 4.93). The improvement in the $t_{1/2}$ of this compound might be causedby a decreased affinity for the metabolizing enzyme, due to a higher steric hindrance.

4.3. CONCLUSION

In the drug discovery process it is important to take into account the metabolic properties of lead compounds as early as possible. The design of metabolically stable molecules, which maintain the pharmacological activity, is a challenging task. In this work we identified the metabolic liability of a series of 2,5-thiophene amide inhibitors. We have also detailed the specific metabolic pathway of compound **12** and profiled the metabolic fate of **1** and **2**, leading to an overall comprehension of the metabolic liabilities of the 2,5-thiophene

amide class of inhibitors. Using two different strategies we discovered three new inhibitors with a highly improved metabolic stability: **27** ($t_{1/2}$ = 82 min), **31** ($t_{1/2}$ = 92 min) and **35** ($t_{1/2}$ = >120 min). The inhibitory potency and selectivity profiles of the synthesized compounds were also evaluated.

Among the three most stable inhibitors, **35** also retains a good activity and selectivity (IC_{50} = 1126 nM, *s.f.* = 10) and shows no affinity for the estrogen receptors. The relative structural simplicity of compound **35** still leaves room for an appropriate molecular optimization.

EXPERIMENTAL SECTION

5.1. Chemical Methods. Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Combi-Blocks, Enamine or Fluka and were used without purification.

Column chromatography was performed on silica gel (70-200 μm) and reaction progress was monitored by TLC on Alugram SIL G/UV254 (Macherey-Nagel). Visualization was accomplished with UV light.

^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM500 spectrometer (at 500 MHz and 125 MHz, respectively) at 300 K and on Bruker Fourier 300 (at 300 MHz and 75 MHz, respectively) at 300K. Chemical shifts are reported in δ (parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard: 2.05 ppm (1H NMR) and 29.8 and 206.3 ppm (^{13}C NMR) for CD_3COCD_3, 7.26 ppm (^{1}H NMR) and 77.0 ppm (^{13}C NMR) for $CDCl_3$. Signals are described as br (broad), s (singlet), d (doublet), t (triplet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplets) and m (multiplet). All coupling constants (J) are given in Hertz (Hz).

Melting points (mp) were measured in open capillaries on a Stuart Scientific SMP3 apparatus and are uncorrected.

Mass spectrometry was performed on a TSQ® Quantum (ThermoFisher, Dreieich, Germany). The triple quadrupole mass spectrometer was equipped with an electrospray interface (ESI). The purity of the compounds was assessed by LC/MS. The Surveyor®-LC-system consisted of a pump, an auto sampler, and a PDA detector. The system was operated by the standard software Xcalibur®. A RP C18 NUCLEODUR® 100-5 (3 mm) column (Macherey-Nagel GmbH, Dühren, Germany) was used as stationary phase. All solvents were HPLC grade. In a gradient run the percentage of acetonitrile (containing 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 needle voltage of 3000 V and a capillary temperature of 350 °C. Mass spectra were acquired in positive mode from 100 to 1000 m/z and UV spectra were recorded at the wave length of 254 nm and in some cases at 360 nm.

All microwave irradiation experiments were carried out in a 507 CEM-Discover microwave apparatus.

All tested compounds exhibited ≥ 95% chemical purity as measured by LC/MS, after dissolving them in methanol.

The following compounds were prepared according to previously described procedures: 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a**[25], lithium 5-(3-methylphenyl)-1,3-thiazole-2-carboxylate **30a**[42].

5.1.1. Method A, general procedure for amide formation:

A mixture of bromo-*N*-heteroarylcarboxylic acid (2 mmol), thionyl chloride (4 mmol) and DMF (5 drops) in toluene (10 mL) was refluxed at 110°C for 4 hours. The reaction mixture was cooled to room temperature; the solvent and the excess of thionyl chloride were removed under reduced pressure. The corresponding *N*-methylamine (2 mmol) and Et_3N (2 mmol) in CH_2Cl_2 (10 mL) was added at 0°C under N_2 atmosphere to the acyl chloride. After 30 minutes at 0°C, the ice bath was removed and the solution was warmed up and stirred at room temperature overnight. The reaction mixture was extracted twice with CH_2Cl_2 (2 × 15 mL); the organic layer was dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by silica gel column chromatography using *n*-hexane and EtOAc as eluent or by trituration in a mixture of diethyl ether / petroleum ether to afford the desired compound.

5.1.2. Method B, general procedure for Suzuki-Miyaura coupling:

In a sealed tube the previously prepared bromo-*N*-heteroarylcarboxamide derivative (1 eq.) was introduced followed by the corresponding boronic acid (1.5 eq.), cesium carbonate (3 eq.), tetrakis(triphenylphosphine)palladium (0.02 eq.) and a mixture of DME/EtOH/H_2O (1:1:1, v:v:v, 3 mL) as solvent. The reactor was flushed with N_2 and submitted to microwave irradiation (150°C, 150 W) for 20 minutes. After cooling to room temperature, a mixture of EtOAc/H2O (1:1, v:v, 2 mL) was added to stop the reaction. The aqueous layer was extracted with EtOAc (3 × 10 mL). The organic layer was washed once with brine and once with water, dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by column chromatography using *n*-hexane and EtOAc as eluent to afford the desired compound.

*5.1.3. Detailed Synthesis Procedures of the Most Interesting Compounds.*5.1.3.1.***N*-Methyl-*N*,5-bis(3-methylphenyl)thiophene-2-carboxamide (12).**The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (150 mg, 0.48 mmol), m-tolylboronic acid (86 mg, 0.63 mmol), cesium carbonate (469 mg, 1.44 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow solid (117 mg, 76 %). $C_{20}H_{19}NOS$; MW 321; mp: 98 – 101°C; MS (ESI) 322 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz) 2.36 (s, 3H), 2.39 (s, 3H), 3.47 (s, 3H), 6.68 (d, J = 4.1 Hz, 1H), 6.97 (d, J = 4.1 Hz, 1H), 7.09 – 7.12 (m, 3H), 7.21 – 7.25 (m, 2H), 7.28 – 7.29 (m, 1H), 7.31-7.34 (m, 2H); ^{13}C NMR

($CDCl_3$, 125 MHz)δ 21.3, 21.4, 39.4, 122.7, 123.2, 125.0, 126.7, 128.5, 128.9, 129.2, 129.7, 133.4, 133.5, 136.3, 138.6, 140.0, 143.8, 149.7, 163.1; IR (cm^{-1}) 3048, 2921, 1599, 1584.

5.1.3.2.***N*-Methyl-*N*-(3-methylphenyl)-5-(4-methylphenyl)thiophene-2-carboxamide (13).** The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (100 mg, 0.32 mmol), *p*-tolyl boronic acid (65 mg, 0.48 mmol), cesium carbonate (313 mg, 0.96 mmol) and tetrakis(triphenylphosphine) palladium (8 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow solid (63 mg, 61 %). $C_{20}H_{19}NOS$; MW 321; mp: 95 – 98°C; MS (ESI) 322 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz) 2.32 (s, 3H), 2.36 (s, 3H), 3.37 (s, 3H), 6.49 (d, *J* = 3.9 Hz, 1H), 7.07 (d, *J* = 3.9 Hz, 1H), 7.14 – 7.16 (m, 1H), 7.19 – 7.22 (m, 3H), 7.24 – 7.26 (m, 1H), 7.35 (t, *J* = 7.8 Hz, 1H), 7.46 (dt, *J* = 2.0, 8.3 Hz, 2H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 21.2, 21.3, 39.1, 123.4, 126.1, 126.6, 129.6, 129.7, 130.5, 130.6, 131.8, 133.3, 138.6, 139.3, 140.8, 145.5, 149.3, 162.5; IR (cm^{-1}) 3050, 2921, 1599, 1585.

5.1.3.3.**5-(3,4-Dimethylphenyl)-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide (21).**The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (150 mg, 0.48 mmol), 3,4-dimethylphenyl boronic acid (94.5 mg, 0.63 mmol), cesium carbonate (469 mg, 1.44 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as colorless solid (55 mg, 34 %). $C_{21}H_{21}NOS$; MW 335; mp: 138 – 140°C; MS (ESI) 336 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz) 2.26 (s, 3H), 2.27 (s, 3H), 2.38 (s, 3H), 3.45 (s, 3H), 6.57 (d, *J* = 4.0 Hz, 1H), 6.92 (d, *J* = 4.0 Hz, 1H), 7.08 – 7.11 (m, 3H), 7.20 – 7.21 (m, 1H), 7.23 (dd, *J* = 2.1, 7.8 Hz, 1H), 7.28 – 7.33 (m, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 19.5, 19.7, 21.3, 39.1, 122.2, 123.4, 125.0, 127.2, 128.5, 128.9, 129.6, 130.1, 131.2, 132.8, 136.6, 137.0, 137.1, 139.8, 144.1, 149.4, 162.6; IR (cm^{-1}) 3029, 2918, 1612, 1602.

5.1.3.4.***N*-Methyl-*N*-(3-methylphenyl)-5-(1-methyl-1H-pyrazol-4-yl)thiophene-2-carboxamide (27).** The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (150 mg, 0.48 mmol), 1-methyl-1H-pyrazole-4-boronic acid (96 mg, 0.63 mmol), cesium carbonate (626 mg, 1.92 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as yellow solid (70 mg, 53 %). $C_{17}H_{17}N_3OS$; MW 311; mp: 132 – 134°C; MS (ESI) 312 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)2.37 (s, 3H), 3.43 (s, 3H), 3.89 (s, 3H), 6.49 (d, *J* = 4.0 Hz, 1H), 6.70 (d, *J* = 4.0 Hz, 1H), 7.06 – 7.10 (m, 2H), 7.19 – 7.21 (m, 1H), 7.30 (t, *J* = 7.7 Hz, 1H), 7.47 (s, 1H), 7.57 (d, *J* = 1.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 21.6, 29.6, 39.0, 116.4, 122.0, 125.0, 127.4, 128.5, 128.9, 129.5, 132.7, 135.4, 137.0, 140.0, 140.2, 144.1, 162.4; IR (cm^{-1}) 3075, 2923, 2854, 1616, 1601, 1582.

5.1.3.5.***N*-Methyl-*N*,5-bis(3-methylphenyl)-1,3-thiazole-2-carboxamide (30).** To a solution of lithium 5-(3-methylphenyl)-1,3-thiazole-2-carboxylate **30a** (100 mg, 0.44 mmol) in CH_2Cl_2 (15 mL) was added drop wise oxalyl chloride (80 µL, 0.88 mmol) followed by few drops of DMF at 0°C under N_2 atmosphere. The reaction mixture was stirred at 0°C for 10 min and then at room temperature for 3 h. The solvent was removed under reduced pressure (bath temperature of rotavapor at 20°C). This residue was diluted in dry CH_2Cl_2 (10 mL) and *N*,3-dimethylaniline (0.06 mL, 0.44 mmol) was added followed by triethylamine (0.10 µL, 0.44 mmol). The solution was stirred overnight at room temperature under N_2 atmosphere. The solvent was removed under reduced pressure (bath temperature of rotavapor at 20°C). A mixture of Na_2CO_3 2N (15 mL) and EtOAc (15 mL) were added. The aqueous layer was extracted with EtOAc (15 mL). The organic layer was washed twice with Na_2CO_3 2N (15 mL), once with water (15 mL), dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as pale brown solid (51 mg, 36 %). $C_{19}H_{18}N_2OS$; MW 322; mp: 79 – 80°C; MS (ESI) 323 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz) 2.32 (s, 3H), 2.36 (s, 3H), 3.54 (br s, 3H), 7.07 – 7.09 (m, 1H), 7.10 – 7.12 (m, 1H), 7.15 (br s, 1H), 7.20 – 7.26 (m, 1H), 7.32 (t, J = 7.7 Hz, 1H), 7.44 – 7.46 (m, 2H), 7.49 (br s, 1H), 7.93 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) 21.3, 39.6, 124.9, 125.0, 128.3, 128.4, 128.6, 130.0, 130.1, 130.6, 131.5, 139.69, 139.70, 139.9, 144.2, 145.4, 161.2 . IR (cm^{-1}) 3096, 2922, 1624, 1606, 1585.

5.1.3.6.***N*-Methyl-*N*,5-bis(3-methylphenyl)furan-2-carboxamide (31).** The title compound was prepared by reaction of **31a** (150 mg, 0.51 mmol), m-tolylboronic acid (104 mg, 0.77 mmol), cesium carbonate (499 mg, 1.53 mmol) and tetrakis(triphenylphosphine) palladium (12 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as orange oil (100 mg, 64 %). $C_{20}H_{19}NO_2$; MW 305; MS (ESI) 306 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz) 2.30 (s, 3H), 2.38 (s, 3H), 3.37 (s, 3H), 6.67 (d, *J* = 3.7 Hz, 1H), 6.74 (d, *J* = 3.7 Hz, 1H), 7.00 – 7.01 (m, 1H), 7.07 – 7.12 (m, 2H), 7.16 – 7.22 (m, 3H), 7.24 – 7.27 (m, 1H), 7.35 (t, *J* = 7.8 Hz, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 21.3, 38.7, 107.1, 119.4, 122.3, 125.3, 125.4, 128.8, 129.0, 129.5, 129.9, 130.2, 130.7, 139.2, 140.3, 145.9, 148.2, 155.8, 159.1.

5.1.3.7.***N*,3'-Dimethyl-*N*-(m-tolyl)-[1,1'-biphenyl]-4-carboxamide (35).** The title compound was prepared by reaction of**35a** (120 mg, 0.39 mmol), *m*-tolylboronic acid(69 mg, 0.51 mmol), cesium carbonate (381 mg, 1.17 mmol) and tetrakis(triphenylphosphine) palladium (9 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow oil (105 mg, 85 %). $C_{22}H_{21}NO$; MW 315; MS (ESI) 316 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 300 MHz)□2.24 (s, 3H), 2.37 (s, 3H), 3.42 (s, 3H), 6.93 – 6.96 (d, *J* = 9Hz,

1H), 6.98 – 7.00 (d, *J* = 7Hz, 1H), 7.07 (s, 1H), 7.11 – 7.18 (m, 2H), 7.30 (t, *J* = 8 Hz, 1H), 7.37 – 7.42 (m, 4H), 7.47 – 7.50 (m, 2H); ^{13}C NMR (CD_3COCD_3, 75 MHz)□21.2, 21.4, 38.5, 124.8, 125.1, 126.8, 127.9, 128.4, 128.44, 129.3, 129.6, 129.7, 130.1, 136.5, 139.3, 139.9, 140.8, 142.7, 146.2, 170.2. IR (cm^{-1}) 3037, 2920, 2862, 1740, 1639, 1603.

5.1.3.8.**5-[3-(Hydroxymethyl)phenyl]-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide (40).** To a solution of 5-(3-formylphenyl)-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **40a** (76 mg, 0.23 mmol) in a mixture of dry MeOH (3 mL) and dry dioxane (3 mL) was added at 0°C sodium borohydride (87 mg, 0.46 mmol). After 2 hours at 0°C, water was added to quench the reaction. The aqueous layer was extracted three times with EtOAc (3 × 5 mL). The organic layer was washed once with saturated solution of $NaHCO_3$ and once with water, dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by preparative HPLC (using acetonitrile/water from 10% to 95% of acetonitrile) to afford the desired product as colorless oil (14 mg, 18 %). $C_{20}H_{19}NO_2S$; MW 337; MS (ESI) 338 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz)□ 2.36 (s, 3H), 3.43 (s, 3H), 4.70 (s, 2H), 6.57 (d, *J* = 4.0 Hz, 1H), 6.96 (d, *J* = 4.0 Hz, 1H), 7.04 – 7.06 (m, 1H), 7.06 – 7.10 (m, 1H), 7.17 – 7.22 (m, 1H), 7.27 – 7.36 (m, 3H), 7.40 (dt, *J* = 1.8, 7.2 Hz, 1H), 7.49 – 7.51 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz)□21.3, 39.1, 65.0, 122.9, 124.5, 125.0, 125.2, 126.8, 128.5, 129.0, 129.1, 129.6, 132.9, 133.9, 137.4, 139.9, 141.7, 144.1, 148.7, 162.6. IR (cm^{-1}) : 3397, 3027, 2921, 2865, 1598, 1582.

*5.3. logP Determination:*The logP values were calculated from ACD/Labs Percepta 2012 Release program. The logarithm of partition constant P (log P) was calculated using the "GALAS" method (Global Adjusted Locally According to Similarity). The program predicts clog P by comparing the molecule with structurally similar molecules where experimental data are known.

*5.4. Biological Methods***.** [2,4,6,7-^{3}H]-E2 and [2,4,6,7-^{3}H]-E1 were purchased from Perkin-Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt. Other chemicals were purchased from Sigma, Roth or Merck.

*5.4.1. 17β-HSD1 and 17β-HSD2 enzyme preparation.*Cytosolic (17*β*-HSD1) and microsomal (17*β*-HSD2) fractions were obtained from human placenta according to previously described procedures[38, 39, 50]. Fresh tissue was homogenized and the enzymes were separated by fractional centrifugation at 1000g, 10,000g and 150,000g. The pellet fraction containing the microsomal 17*β*-HSD2 was used for the determination of 17*β*-HSD2 inhibition, while 17*β*-HSD1 was obtained after precipitation with ammonium sulfate from the cytosolic fraction for use of testing of 17*β*-HSD1 inhibition. Aliquots containing 17*β*-HSD1 or 17*β*-HSD2 were stored frozen.

5.4.2 Inhibition of 17β-HSD2 in cell-free assay. Inhibitory activities were evaluated by an established method with minor modifications[37, 51, 52]. Briefly, the enzyme preparation was incubated with NAD^+ [1500 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 [^{3}H]-E2 (final concentration: 500 nM, 0.11 µCi). After 20 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with ether. After evaporation, the steroids were dissolved in acetonitrile/water (45:55). E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 RP chromatography column (Nucleodur C18, 3µm, 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 the following equation: %conversion = (%E1/(%E1+%E2))×100. Each value was calculated from at least three independent experiments.

*5.4.3. Inhibition of 17β-HSD1 in cell-free assay.*The 17*β*-HSD1 inhibition assay was performed similarly to the 17*β*-HSD2 test. The microsomal fraction was incubated with NADH [500 µM], test compound and a mixture of unlabelled- and [^{3}H]-E1 (final concentration: 500 nM, 0.15 µCi) for 10 min at 37°C. Further treatment of the samples and HPLC separation was carried out as mentioned above for 17*β*-HSD2.

*5.4.4. Estrogen receptor affinity in a Cellular Free Assay.*The binding affinity of selected compounds to ERα and ERβ was determined according to the recommendations of the US Environmental Protection Agency (EPA) by their Endocrine Disruptor Screening Program (EDSP) [44] using recombinant human proteins. Briefly, 1 nM of ERα and 4 nM of ERβ, respectively, were incubated with [^{3}H]-E2 (3 nM for ERα and 10 nM for ERβ) and test compound for 16-20 h at 4°C.

The potential inhibitors were dissolved in DMSO (5% final concentration). Evaluation of non-specific-binding was performed with unlabeled E2 at concentrations 100-fold of [^{3}H]-E2 (300 nM for ERα and 1000 nM for ERβ). After incubation, ligand-receptor complexes were selectively bound to hydroxyapatite (83.5 g/LinTE-buffer). The bound complex was washed three times and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (1450 LSC & Luminescence Counter, Perkin Elmer).

From these results the percentage of [^{3}H]-E2 displacement by the compounds was calculated. The plot of % displacement versus compound concentration resulted in sigmoidal binding curves. The compound concentrations to displace 50% of the receptor bound [^{3}H]-E2 were determined. Unlabeled E2 IC_{50} values were determined in each

experiment and used as reference. The E2 IC_{50} values accepted, were 3±20% nM for ERα and 10±20% nM for ERβ.

Relative Binding Affinity was determined by applying the following equation: RBA[%] = (IC_{50}(E2)/IC_{50}(compound)) · 100[44]. This results in an RBA value of 100% for E2.

After the assay was established and validated, a modification was made to increase throughput. Compounds were tested at concentrations of 1000 · IC_{50}(E2). Compounds with less than 50% displacement of [3H]-E2 at a concentration of 1000 ·IC_{50}(E2) were classified as RBA <0.1%.

5.4.5. Metabolic Stability in a Cell Free Assay. Compounds **3, 5, 7-9, 12, 13, 16, 21, 22, 25, 27, 30, 31, 35** and **41** were tested according to established method[45-47].For evaluation of phase I and II metabolic stability 1 µM compound was incubated with 1 mg/ml pooled mammalian liver S9 fraction (BD Gentest), 2 mM NADPH regenerating system, 1 mM UDPGA and 0.1 mM PAPS at 37°C for 0, 5, 15 and 60 minutes at a final volume of 100 µL. The incubation was stopped by precipitation of S9 enzymes with 2 volumes of cold acetonitrile containing internal standard. Concentration of the remaining test compound at the different time points was analyzed by LC-MS/MS and used to determine half-life ($t_{1/2}$).

Compounds **1, 2. 4, 6, 10** and **11** were tested following a similar procedure with the following changes: 3 µM compound was incubated with 1 mg/ml pooled mammalian liver S9 fraction (IVT, Xenotech or TCS cellworks), 1mM NADPH regenerating system, 0.75mM UDPGA and 0.05mM PAPS at 37°C for 0, 5, 15 and 45 minutes.

5.4.6. Metabolite identification in a Cell Free Assay. Compounds **1, 2** and **12** were incubated at a final concentration of 10 µM with 1 mg/ml pooled mammalian liver S9 fraction(BD Gentest) and 2 mM NADPH regenerating system for three hours(final volume 3 mL). The water phase was extracted with dichloromethane (3 × mL), which was evaporated to dryness at room temperature. The residue was resuspended in 500 µL methanol. Separation of 2 µL sample was accomplished on a Dionex Ultimate 3000 RSLC system using a BEH C18 50 ×2.1 mm, 1.7 µM d_p column (Waters, Germany). Separation was achieved by a linear gradient from (A) H_2O + 0.1% formic acid (FA) to (B) ACN + 0.1% FA at a flow rate of 600 µL/min and 45 °C. The gradient was initiated by a 0.33 min isocratic step at 5% B, followed by an increase to 95% B in 9 min to end up with a 1 min step at 95% B before reequilibration under the initial conditions. The flow entered the LTQ/Orbitrap (Thermo Scientific, Germany) using the NanomateAdvionNanospray ion source (NSI). The Mass Spectrometer was set with the following parameters: scan range 200-2000 m/z in positive ionization mode; 200 °C Capillary Temperature; 1,7 kV Voltage at NSI; Fourier Transform Mass Spectrometry (FTMS) Resolution of 30000; MS/MS analysis was data-dependent and triggered by the most abundant ions..

Data Acquisition was performed with Xcalibur version 1.0.2.65 (Thermo Electron, San Jose, Ca, USA). Five-decimal monoisopic masses of **1**, **2** and **15** and their predicted metabolites, calculated from CambidgeSoftChem& Bio Draw 11.0 using the ChemDrawPro

1.0 program, were used for the parent data and to filter data in Qual Browser (Thermo Electron, San Jose, Ca, USA) with a mass tolerance threshold of 5 ppm.

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2.1.1 Supporting Information

Table of contents

a) Chemical synthesis and spectroscopic data of all compounds
b) NMR spectra of representative compounds
c) References

a) Chemical Methods. Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Combi-Blocks, Enamine or Fluka and were used without purification.

Column chromatography was performed on silica gel (70-200 μm) and reaction progress was monitored by TLC on Alugram SIL G/UV254 (Macherey-Nagel). Visualization was accomplished with UV light.

^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM500 spectrometer (at 500 MHz and 125 MHz, respectively) at 300 K and on Bruker Fourier 300 (at 300 MHz and 75 MHz, respectively) at 300K. Chemical shifts are reported in δ (parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard: 2.05 ppm (^{1}H NMR) and 29.8 and 206.3 ppm (^{13}C NMR) for CD_3COCD_3, 7.26 ppm (^{1}H NMR) and 77.0 ppm (^{13}C NMR) for $CDCl_3$. Signals are described as br (broad), s (singlet), d (doublet), t (triplet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplets) and m (multiplet). All coupling constants (*J*) are given in Hertz (Hz).

Melting points (mp) were measured in open capillaries on a Stuart Scientific SMP3 apparatus and are uncorrected.

Mass spectrometry was performed on a TSQ® Quantum (ThermoFisher, Dreieich, Germany). The triple quadrupole mass spectrometer was equipped with an electrospray interface (ESI). The purity of the compounds was assessed by LC/MS. The Surveyor®-LC-system consisted of a pump, an auto sampler, and a PDA detector. The system was operated by the standard software Xcalibur®. A RP C18 NUCLEODUR® 100-5 (3 mm) column (Macherey-Nagel GmbH, Dühren, Germany) was used as stationary phase. All solvents were HPLC grade. In a gradient run the percentage of acetonitrile (containing 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 needle voltage of 3000 V and a capillary temperature of 350 °C. Mass spectra were acquired in positive mode from 100 to 1000 m/z and UV spectra were recorded at the wave length of 254 nm and in some cases at 360 nm.

All microwave irradiation experiments were carried out in a 507 CEM-Discover microwave apparatus.

Results

All tested compounds exhibited ≥ 95% chemical purity as measured by LC/MS , after dissolving them in methanol.

The following compounds were prepared according to previously described procedures:5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a**[1], lithium 5-(3-methylphenyl)-1,3-thiazole-2-carboxylate **30a**[2].

Method A, general procedure for amide formation:

A mixture of bromo-*N*-heteroarylcarboxylic acid (2 mmol), thionyl chloride (4 mmol) and DMF (5 drops) in toluene (10 mL) was refluxed at 110°C for 4 hours. The reaction mixture was cooled to room temperature; the solvent and the excess of thionyl chloride were removed under reduced pressure. The corresponding *N*-methylamine (2 mmol) and Et_3N (2 mmol) in CH_2Cl_2 (10 mL) was added at 0°C under N_2 atmosphere to the acyl chloride. After 30 minutes at 0°C, the ice bath was removed and the solution was warmed up and stirred at room temperature overnight. The reaction mixture was extracted twice with CH_2Cl_2 (2 × 15 mL); the organic layer was dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by silica gel column chromatography using *n*-hexane and EtOAc as eluent or by trituration in a mixture of diethyl ether / petroleum ether to afford the desired compound.

Method B, general procedure for Suzuki-Miyaura coupling:

In a sealed tube the previously prepared bromo-*N*-heteroarylcarboxamide derivative (1 eq.) was introduced followed by the corresponding boronic acid (1.5 eq.), cesium carbonate (3 eq.), tetrakis(triphenylphosphine)palladium (0.02 eq.) and a mixture of DME/EtOH/H_2O (1:1:1, v:v:v, 3 mL) as solvent. The reactor was flushed with N_2 and submitted to microwave irradiation (150°C, 150 W) for 20 minutes. After cooling to room temperature, a mixture of EtOAc/H_2O (1:1, v:v, 2 mL) was added to stop the reaction. The aqueous layer was extracted with EtOAc (3 × 10 mL). The organic layer was washed once with brine and once with water, dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by column chromatography using *n*-hexane and EtOAc as eluent to afford the desired compound.

5-Bromo-*N*-methyl-*N*-(2-methylphenyl)thiophene-2-carboxamide (15a)

The title compound was prepared by reaction of 5-bromo-thiophene-2-carboxylic acid**36c** (700 mg, 3.4 mmol), thionyl chloride (0.5 mL, 6.8 mmol) and *N*,2-dimethylaniline, HCl (536 mg, 3.4 mmol) according to method A. The residue was purified by silica gel column

chromatography (*n*-hexane/EtOAc 90:10) to afford the desired product as yellow solid (820 mg, 78 %).$C_{13}H_{12}BrNOS$; MW 310; mp: 59 – 62°C; MS (ESI) 310, 312$[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz)δ2.19 (s, 3H), 3.29 (s, 3H), 6.51 (d, *J* = 4.1 Hz, 1H), 6.93 (d, *J* = 4.1 Hz, 1H), 7.33 – 7.42 (m, 4H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 17.4, 37.7, 118. 6, 128.8, 130.0, 130.2, 131.1, 132.6, 133.1, 137.5, 140.8, 143.0, 161.1; IR (cm^{-1}) 3087, 2920, 1643, 1616, 1596.

5-Bromo-*N*-methyl-*N*-(4-methylphenyl)thiophene-2-carboxamide (18a)

The title compound was prepared by reaction of 5-bromo-thiophene-2-carboxylic acid**36c** (700 mg, 3.4 mmol), thionyl chloride (0.5 mL, 6.8 mmol) and *N*,4-dimethylaniline (412 mg, 3.4 mmol) according to method A. The residue was purified by crystallization in *n*-hexane, to afford the desired product as colorless solid (580 mg, 55 %).$C_{13}H_{12}BrNOS$; MW 310; mp: 87 – 88°C; MS (ESI) 310, 312 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ2.41 (s, 3H), 3.38 (s, 3H), 6.59 (d, *J* = 4.1 Hz, 1H), 6.76 (d, *J* = 4.1 Hz, 1H), 7.12 (dt, *J* = 2.4, 8.3 Hz, 2H), 7.22 – 7.23 (m, 2H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 21.4, 39.2, 118.9, 128.0, 129.8, 130.7, 132.6, 138.9, 139.4, 141.0, 161.6.IR (cm^{-1}) 3054, 3030, 2921, 1629.

5-Bromo-*N*-(3-chlorophenyl)-*N*-methylthiophene-2-carboxamide (22a)

The title compound was prepared by reaction of 5-bromo-furan-2-carboxylic acid **36c**(520 mg, 2.5 mmol), thionyl chloride (0. 36 mL, 5 mmol) and *N*-methyl-3-chloroaniline(354 mg, 2.5 mmol) according to method A. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc 90:10) to afford the desired product as brown solid (600 mg, 72 %). $C_{12}H_9BrClNOS$, MW 329; mp: 114– 116°C; MS (ESI) 330, 332, 333 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ3.40 (s, 3H), 6.61 (d, *J* = 4.0 Hz, 1H), 6.79 (d, *J* = 4.0 Hz, 1H), 7.13 (dt, *J* = 1.5 Hz, 7.5 Hz, 1H), 7.27 (t, *J* = 1.9 Hz, 1H), 7.35 (t, *J* = 8.1 Hz, 1H), 7.39 (dt, *J* = 1.5 Hz, 8.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ39.2, 119.2, 126.6, 128.5, 128.9, 130.0, 131.0, 133.0, 135.5, 138.9, 144.9, 161.5.

5-Bromo-*N*-methyl-*N*-(3-methylphenyl)furan-2-carboxamide (31a)

The title compound was prepared by reaction of 5-bromo-furan-2-carboxylic acid **31b**(1000 mg, 5.2 mmol), thionyl chloride (0.76 mL, 10.4 mmol) and *N*,3-dimethylaniline (634 mg, 5.2 mmol) according to method A. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc 80:20) to afford the desired product as yellow solid (1226 mg, 80 %).$C_{13}H_{12}BrNO_2$; MW 294; mp: 84 – 86°C; MS (ESI) 294, 296$[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz)δ2.35 (s, 3H), 3.33 (s, 3H), 5.87 (d, *J* = 3.5 Hz, 1H), 6.36 (d, *J* = 3.5 Hz, 1H), 7.08 – 7.10 (m, 1H), 7.15 – 7.16 (m, 1H), 7.21 – 7.24 (m, 1H), 7.33 (t, *J* =

7.8 Hz, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 21.2, 38.3, 113.8, 118.9, 125.0, 125.3, 128.8, 129.3, 130.2, 140.5, 144.9, 150.5, 158.1. IR (cm^{-1}) 3122, 2925, 1639, 1462.

4-Bromo-*N*-methyl-*N*-(*m*-tolyl)benzamide (35a)

To a solution of *N*,3-dimethylaniline**35c** (200 mg, 1.65 mmol) in CH_2Cl_2 were added the 4-bromobenzoyl chloride**35b** (724 mg, 330 mmol) and Et_3N (168 mg, 1.65 mmol) under N_2 atmosphere. The reaction was stirred at room temperature overnight. The reaction mixture was extracted twice with CH_2Cl_2 (2 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified on silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as a colorless solid (500 mg, 100 %). $C_{15}H_{14}BrNO$; MW 304; mp : 90 – 92 °C; MS (ESI) 304, 306 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 300 MHz)δ2.24 (s, 3H), 3.39 (s, 3H), 6.92 (d, *J* = 8Hz, 1H), 6.99 – 7.05 (m, 2H), 7.14 (t, *J* = 8Hz, 1H), 7.23 – 7.26 (m, 2H), 7.37 – 7.40 (m, 2H); ^{13}C NMR (CD_3COCD_3, 75 MHz)δ21.2, 38.4, 123.9, 125.2, 128.1, 128.5, 129.8, 131.4, 131.6, 137.0, 140.0, 145.8, 169.4; IR (cm^{-1}) 3086, 3058, 2923, 1638.

5-bromo-4-methoxy-*N*-methyl-*N*-(*m*-tolyl)thiophene-2-carboxamide (38b)

The title compound was prepared by reaction of 5-bromo-4-methoxythiophene-2-carboxylic acid**38c** (245 mg, 1.0 mmol), thionyl chloride (0.15 mL, 2.0 mmol) and *N*,3-dimethylaniline (125 mg, 1.0 mmol) according to method A. The residue was crystallized in water and ethanol, to afford the desired product as yellow solid (349 mg, 100%).$C_{14}H_{14}BrNO_2S$; MW 340; mp: 70 – 71°C; MS (ESI) 340, 342 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ2.38 (s, 3H), 3.39 (s, 3H), 3.66 (s, 3H), 6.61 (s, 1H), 7.05– 7.08 (m, 2H), 7.23– 7.25 (m, 1H), 7.33 (t, *J* = 7.6 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ21.4, 39.3, 58.9, 98.4, 121.2, 125.5, 129.1, 129.7, 129.9, 134.9, 140.4, 143.3, 154.3, 161.3. IR (cm^{-1}) 3047, 2934, 2848, 1623, 1604, 1587.

***N*-methyl-*N*,5-bis(2-methylphenyl)thiophene-2-carboxamide (17)**

The title compound was prepared by reaction of **15a** (100 mg, 0.32 mmol), *o*-tolylboronic acid (50 mg, 0.37 mmol), cesium carbonate (313 mg, 0.96 mmol) and tetrakis(triphenylphosphine) palladium (8 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as beige oil (40 mg, 39 %). $C_{20}H_{19}NOS$; MW 321; MS (ESI) 322 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz)δ 2.23 (s, 3H), 2.29 (s, 3H), 3.32 (s, 3H), 6.67 (d, *J* = 3.9 Hz, 1H), 6.86 (d, *J* = 3.9 Hz, 1H), 7.18 – 7.21 (m, 1H), 7.23 – 7.27 (m, 3H), 7.32 – 7.39 (m, 4H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 17.5, 21.1, 37.7, 126.9, 127.1, 128.6,

129.3, 129.8, 130.0, 130.7, 131.8, 132.4, 132.5, 134.1, 136.6, 137.4, 138.8, 143.7, 148.8, 162.2.IR (cm^{-1}) 3073, 3049, 2923, 1618.

N-Methyl-*N*-(2-methylphenyl)-5-(3-methylphenyl)thiophene-2-carboxamide (15)

The title compound was prepared by reaction of**15a** (100 mg, 0.32 mmol), *m*-tolylboronic acid (50 mg, 0.37 mmol), cesium carbonate (313 mg, 0.96 mmol) and tetrakis(triphenylphosphine) palladium (8 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as white solid (51 mg, 50%). $C_{20}H_{19}NOS$; MW 321; mp: 99 – 100°C; MS (ESI) 322 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ 2.24 (s, 3H), 2.34 (s, 3H), 3.38 (s, 3H), 6.63 (d, *J* = 4.0 Hz, 1H) 6.95 (d, *J* = 4.0 Hz, 1H), 7.08 – 7.10 (m, 1H), 7.19 – 7.37 (m, 7H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 17.6, 21.5, 37.9, 122.9, 123.2, 126.8, 127.8, 128.9, 129.0, 129.1, 129.2, 131.8, 132.8, 133.6, 136.6, 136.8, 138.7, 142.7, 149.6, 162.5. IR (cm^{-1}) 3089, 2921, 1615, 1595.

N-Methyl-*N*-(2-methylphenyl)-5-(4-methylphenyl)thiophene-2-carboxamide (16)

The title compound was prepared by reaction of**15a** (100 mg, 0.32 mmol), *p*-tolylboronic acid (50 mg, 0.37 mmol), cesium carbonate (313 mg, 0.96 mmol) and tetrakis(triphenylphosphine) palladium (8 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as white solid (60 mg, 58 %). $C_{20}H_{19}NOS$; MW 321; mp: 128 – 129°C; MS (ESI) 322 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ 2.24 (s, 3H), 2.33 (s, 3H), 3.38 (s, 3H), 6.63 (d, *J* = 4.0 Hz, 1H), 6.92 (d, *J* = 4.0 Hz, 1H), 7.12 – 7.14 (m, 2H), 7.23 (dd, *J* = 1.5, 7.6 Hz, 1H), 7.27 – 7.37 (m, 5H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 17.6, 21.3, 37.9, 122.4, 126.0, 127.8, 129.0, 129.1, 129.7, 131.0, 131.8, 132.9, 136.4, 136.6, 138.4, 142.7, 149.7, 162.6;IR (cm^{-1}) 3095, 2919, 1614, 1596.

N-Methyl-5-(2-methylphenyl)-*N*-(3-methylphenyl)thiophene-2-carboxamide (14)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (100 mg, 0.32 mmol), *o*-tolylboronic acid (65 mg, 0.48 mmol), cesium carbonate (313 mg, 0.96 mmol) and tetrakis(triphenylphosphine) palladium (8 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow solid (60 mg, 58 %). $C_{20}H_{19}NOS$; MW 321; mp: 88 – 91°C; MS (ESI) 322 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz)δ 2.30 (s, 3H), 2.36 (s, 3H), 3.38 (s, 3H), 6.66 (d, *J* = 3.9 Hz, 1H), 6.85 (d, *J* = 3.9 Hz, 1H), 7.16 – 7.18 (m, 1H), 7.19 – 7.29 (m, 6H), 7.36 (t, *J* = 7.7

Hz, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 21.1, 21.2, 39.0, 126.1, 126.96, 127.03, 129.26, 129.61, 129.65, 130.4, 130.8, 131.8, 132.6, 134.1, 136.6, 139.2, 140.7, 145.3, 148.5, 162.4;IR (cm^{-1}) 3050, 2921, 2855, 1614, 1586.

_N_-Methyl-_N_,5-bis(3-methylphenyl)thiophene-2-carboxamide (12)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a**(150 mg, 0.48 mmol), *m*-tolylboronic acid (86 mg, 0.63 mmol), cesium carbonate (469 mg, 1.44 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow solid (117 mg, 76 %). $C_{20}H_{19}NOS$; MW 321; mp: 98 – 101°C; MS (ESI) 322 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ 2.36 (s, 3H), 2.39 (s, 3H), 3.47 (s, 3H), 6.68 (d, *J* = 4.1 Hz, 1H), 6.97 (d, *J* = 4.1 Hz, 1H), 7.09 – 7.12 (m, 3H), 7.21 – 7.25 (m, 2H), 7.28 – 7.29 (m, 1H), 7.31-7.34 (m, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 21.3, 21.4, 39.4, 122.7, 123.2, 125.0, 126.7, 128.5, 128.9, 129.2, 129.7, 133.4, 133.5, 136.3, 138.6, 140.0, 143.8, 149.7, 163.1; IR (cm^{-1}) 3048, 2921, 1599, 1584.

_N_-Methyl-_N_-(3-methylphenyl)-5-(4-methylphenyl)thiophene-2-carboxamide (13)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (100 mg, 0.32 mmol), *p*-tolylboronic acid (65 mg, 0.48 mmol), cesium carbonate (313 mg, 0.96 mmol) and tetrakis(triphenylphosphine) palladium (8 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow solid (63 mg, 61 %). $C_{20}H_{19}NOS$; MW 321; mp: 95 – 98°C; MS (ESI) 322 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz)δ 2.32 (s, 3H), 2.36 (s, 3H), 3.37 (s, 3H), 6.49 (d, *J* = 3.9 Hz, 1H), 7.07 (d, *J* = 3.9 Hz, 1H), 7.14 – 7.16 (m, 1H), 7.19 – 7.22 (m, 3H), 7.24 – 7.26 (m, 1H), 7.35 (t, *J* = 7.8 Hz, 1H), 7.46 (dt, *J* = 2.0, 8.3 Hz, 2H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 21.2, 21.3, 39.1, 123.4, 126.1, 126.6, 129.6, 129.7, 130.5, 130.6, 131.8, 133.3, 138.6, 139.3, 140.8, 145.5, 149.3, 162.5;IR (cm^{-1}) 3050, 2921, 1599, 1585.

_N_-Methyl-5-(2-methylphenyl)-_N_-(4-methylphenyl)thiophene-2-carboxamide (20)

The title compound was prepared by reaction of **18a** (70 mg, 0.23 mmol), *o*-tolylboronic acid (37 mg, 0.27 mmol), cesium carbonate (221 mg, 0.68 mmol) and tetrakis(triphenylphosphine) palladium (5mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as white solid (40 mg, 54 %). $C_{20}H_{19}NOS$; MW 321; mp: 116 – 117°C;

MS (ESI) 322 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ 2.31 (s, 3H), 2.40 (s, 3H), 3.43 (s, 3H), 6.75 (d, *J* = 3.9 Hz, 1H), 6.76 (d, *J* = 3.9 Hz, 1H), 7.16 – 7.19 (m, 3H), 7.21 – 7.23 (m, 4H), 7.26 – 7.28 (m, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 21.2, 21.3, 39.3, 126.0, 126.2, 128.0, 128.4, 130.3, 130.5, 130.9, 132.4, 133.6, 136.2, 137.6, 138.4, 141.7, 148.5, 162.8;IR (cm^{-1}) 3081, 2922, 1604.

_N_-Methyl-5-(3-methylphenyl)-_N_-(4-methylphenyl)thiophene-2-carboxamide (18)

The title compound was prepared by reaction of**18a** (70 mg, 0.23 mmol), *m*-tolylboronic acid (37 mg, 0.27 mmol), cesium carbonate (221 mg, 0.68 mmol) and tetrakis(triphenylphosphine) palladium (5 mg, 0.02 eq) according to method B. The residue was purified by crystallization in *n*-hexane to afford the desired product as colorless needles (30 mg, 41 %). $C_{20}H_{19}NOS$; MW 321; mp: 111 – 112°C; MS (ESI) 322 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ 2.35 (s, 3H), 2.41 (s, 3H), 3.43 (s, 3H), 6.59 (d, *J* = 4.0 Hz, 1H), 6.95 (d, *J* = 4.0 Hz, 1H), 7.09 – 7.10 (m, 1H), 7.16 (dt, *J* = 2.0, 8.3 Hz, 2H), 7.21 – 7.24 (m, 3H), 7.28 – 7.31 (m, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 21.3 21.5, 39.3, 122.8, 123.3, 126.9, 127.9, 128.9, 129.2, 130.5, 132.9, 133.7, 137.3, 138.3, 138.7, 141.9, 149.3, 162.8;IR (cm^{-1}) 3081, 2919, 1602.

_N_-Methyl-_N_,5-bis(4-methylphenyl)thiophene-2-carboxamide (19)

The title compound was prepared by reaction of **18a** (70 mg, 0.23 mmol), *p*-tolylboronic acid (37 mg, 0.27 mmol), cesium carbonate (221 mg, 0.68 mmol) and tetrakis(triphenylphosphine) palladium (5 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as pale brown solid (43 mg, 58 %). $C_{20}H_{19}NOS$; MW 321; mp: 118 – 119°C; MS (ESI) 322 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ 2.34 (s, 3H), 2.41 (s, 3H), 3.43 (s, 3H), 6.59 (d, *J* = 4.0 Hz, 1H), 6.92 (d, *J* = 4.0 Hz, 1H), 7.13 – 7.17 (m, 4H), 7.21 – 7.23 (m, 2H), 7.38 (dt, *J* = 1.8, 8.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 21.3, 39.3, 122.4, 126.0, 127.9, 129.7, 130.5, 131.0, 133.0, 136.9, 138.3, 138.4, 141.8, 149.3, 162.8; IR (cm^{-1}) 3029, 2917, 1898, 1611.

5-(3,4-Dimethylphenyl)-_N_-methyl-_N_-(3-methylphenyl)thiophene-2-carboxamide (21)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (150 mg, 0.48 mmol), 3,4-dimethylphenyl boronic acid (94.5 mg, 0.63 mmol), cesium carbonate (469 mg, 1.44 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford

the desired product as colorless solid (55 mg, 34 %). $C_{21}H_{21}NOS$; MW 335; mp: 138 – 140°C; MS (ESI) 336 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ 2.26 (s, 3H), 2.27 (s, 3H), 2.38 (s, 3H), 3.45 (s, 3H), 6.57 (d, *J* = 4.0 Hz, 1H), 6.92 (d, *J* = 4.0 Hz, 1H), 7.08 – 7.11 (m, 3H), 7.20 – 7.21 (m, 1H), 7.23 (dd, *J* = 2.1, 7.8 Hz, 1H), 7.28 – 7.33 (m, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 19.5, 19.7, 21.3, 39.1, 122.2, 123.4, 125.0, 127.2, 128.5, 128.9, 129.6, 130.1, 131.2, 132.8, 136.6, 137.0, 137.1, 139.8, 144.1, 149.4, 162.6;IR (cm^{-1}) 3029, 2918, 1612, 1602.

5-(4-methoxy-3-methylphenyl)-*N*-methyl-*N*-(m-tolyl)thiophene-2-carboxamide (36a)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (150 mg, 0.48 mmol), (4-methoxy-3-methylphenyl)boronic acid (92 mg, 0.55 mmol), cesium carbonate (469 mg, 1.44 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as grey solid (70 mg, 42 %). $C_{21}H_{21}NO_2S$; MW 351; mp: 127 – 128°C; MS (ESI) 352 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ2.21 (s, 3H), 2.37 (s, 3H), 3.43 (s, 3H), 3.83 (s, 3H), 6.53 (d, *J* = 3.9 Hz, 1H), 6.78 (d, *J* = 8.4 Hz, 1H), 6.84 (d, *J* = 4 Hz, 1H), 7.07– 7.10 (m, 2H), 7.18– 7.20 (m, 1H), 7.27– 7.31 (m, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ16.4, 21.4, 39.3, 55.6, 110.3, 121.8, 124.8, 125.2, 126.1, 127.3, 128.6, 128.7, 129.0, 129.7, 133.0, 136.3, 140.0, 144.4, 149.5, 158.2, 162.8; IR (cm^{-1}) 3088, 2922, 2841, 1598, 1536.

5-(3-methoxy-5-methylphenyl)-*N*-methyl-*N*-(m-tolyl)thiophene-2-carboxamide(37a)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (200 mg, 0.64 mmol), (5-methoxy-3-methylphenyl)boronic acid (123 mg, 0.74 mmol), cesium carbonate (626 mg, 1.92 mmol) and tetrakis(triphenylphosphine) palladium (15 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow oil (184 mg, 82 %). $C_{21}H_{21}NO_2S$; MW 351; MS (ESI) 352 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ 2.32 (s, 3H), 2.37 (s, 3H), 3.44 (s, 3H), 3.79 (s, 3H), 6.54 (d, *J* = 4 Hz, 1H), 6.65 – 6.66 (m, 1H), 6.83 – 6.84 (m, 1H), 6.91 – 6.92 (m, 1H), 6.93 (d, *J* = 4 Hz, 1H), 7.06 – 7.10 (m, 2H), 7.18 – 7.20 (m, 1H), 7.30 (t, *J* = 8 Hz, 1H) ^{13}C NMR ($CDCl_3$, 125 MHz)δ121.3, 21.5, 39.1, 55.2, 108.7, 114.6, 119.4, 122.8, 124.9, 128.4, 128.9, 129.5, 132.6, 134.6, 137.2, 139.8, 140.0, 144.1, 148.9, 159.9, 162.5.

5-(3-Chlorophenyl)-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide (23)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (80 mg, 0.26 mmol), 3-chlorophenyl boronic acid (52.4 mg, 0.34 mmol), cesium carbonate (254 mg, 0.78 mmol) and tetrakis(triphenylphosphine) palladium (6 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as colorless solid (41 mg, 46 %). $C_{19}H_{16}ClNOS$; MW 342; mp: 143 – 145°C; MS (ESI) 343, 345 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ 2.39 (s, 3H), 3.45 (s, 3H), 6.60 (d, *J* = 4.1 Hz, 1H), 6.97 (d, *J* = 4.1 Hz, 1H), 7.07 – 7.10 (m, 2H), 7.21 – 7.23 (m, 1H), 7.24 – 7.29 (m, 2H), 7.32 (t, *J* = 7.8 Hz, 1H), 7.37 (dt, *J* = 1.9, 7.0 Hz, 1H), 7.47 – 7.48 (m, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 21.4, 39.3, 123.6, 124.3, 125.1, 126.0, 128.3, 128.6, 129.2, 129.8, 130.3, 133.0, 134.9, 135.5, 138.3, 140.1, 144.1, 147.2, 162.4; IR (cm^{-1})

_N_-Methyl-_N_-(3-methylphenyl)-5-(5-methylpyridin-3-yl)thiophene-2-carboxamide (26)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (80 mg, 0.26 mmol), 5-methyl-3-pyridine boronic acid (43 mg, 0.31 mmol), cesium carbonate (254 mg, 0.78 mmol) and tetrakis(triphenylphosphine) palladium (6 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as colorless solid (45 mg, 54 %). $C_{19}H_{18}N_2OS$; MW 322; mp: 167 – 170°C; MS (ESI) 323 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ 2.34 (s, 3H), 2.37 (s, 3H), 3.43 (s, 3H), 6.62 (d, *J* = 4.1 Hz, 1H), 6.99 (d, *J* = 4.1 Hz, 1H), 7.05 – 7.09 (m, 2H), 7.19 – 7.21 (m, 1H), 7.30 (t, *J* = 7.8 Hz, 1H), 7.56 (br s, 1H), 8.34 (br s, 1H), 8.54 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 18.5, 21.4, 39.2, 123.8, 125.1, 128.6, 129.2, 129.4, 129.8, 133.0, 133.4, 133.8, 138.6, 140.1, 144.0, 144.2, 145.1, 149.7, 162.3; IR (cm^{-1}) 3076, 2923, 1617, 1601.

_N_-Methyl-5-(2-methylpyridin-4-yl)-_N_-(_m_-tolyl)thiophene-2-carboxamide (25)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (80 mg, 0.26 mmol), (2-methylpyridin-4-yl)boronic acid (43 mg, 0.31 mmol), cesium carbonate (254 mg, 0.78 mmol) and tetrakis(triphenylphosphine) palladium (6 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as colorless solid (50 mg, 60 %). $C_{19}H_{18}N_2OS$; MW 322; mp: 131 – 133°C; MS (ESI) 323 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz)δ2.37 (s, 3H), 2.55 (s, 3H), 3.44 (s, 3H), 6.59 (d, *J* = 4.0 Hz, 1H), 7.05 – 7.08 (m, 2H), 7.11 (d, *J* = 4.0 Hz, 1H), 7.15 – 7.16 (m, 1H), 7.20 – 7.22 (m, 2H), 7.30 (t, *J* = 7.65 Hz, 1H), 8.43 (d, *J* = 5.1 Hz, 1H); ^{13}C NMR

($CDCl_3$, 125 MHz)δ21.4, 24.5, 39.3, 117.3, 119.6, 124.8, 125.1, 128.5, 129.3, 129.8, 132.8, 139.6, 140.2, 141.2, 144.0, 145.6, 149.7, 159.2, 162.2; IR (cm^{-1}) 3090, 2924, 1614, 1600.

N-Methyl-*N*-(3-methylphenyl)-5-(1-methyl-1*H*-pyrazol-4-yl)thiophene-2-carboxamide (27)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **12a** (150 mg, 0.48 mmol), 1-methyl-1*H*-pyrazole-4-boronic acid (96 mg, 0.63 mmol), cesium carbonate (626 mg, 1.92 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as yellow solid (70 mg, 53 %). $C_{17}H_{17}N_3OS$; MW 311; mp: 132 – 134°C; MS (ESI) 312 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ 2.37 (s, 3H), 3.43 (s, 3H), 3.89 (s, 3H), 6.49 (d, *J* = 4.0 Hz, 1H), 6.70 (d, *J* = 4.0 Hz, 1H), 7.06 – 7.10 (m, 2H), 7.19 – 7.21 (m, 1H), 7.30 (t, *J* = 7.7 Hz, 1H), 7.47 (s, 1H), 7.57 (d, *J* = 1.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 21.6, 29.6, 39.0, 116.4, 122.0, 125.0, 127.4, 128.5, 128.9, 129.5, 132.7, 135.4, 137.0, 140.0, 140.2, 144.1, 162.4; IR (cm^{-1}) 3075, 2923, 2854, 1616, 1601, 1582.

N-(3-Chlorophenyl)-*N*-methyl-5-(3-methylphenyl)thiophene-2-carboxamide (22)

The title compound was prepared by reaction of **22a** (80 mg, 0.24 mmol), *m*-tolylboronic acid (43 mg, 0.32 mmol), cesium carbonate (236 mg, 0.73 mmol) and tetrakis(triphenylphosphine) palladium (6 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow solid (49 mg, 59 %). $C_{19}H_{16}ClNOS$; MW 342; mp: 89 – 92°C; MS (ESI) 342, 344 $[M]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ 2.36 (s, 3H), 3.45 (s, 3H), 6.70 (d, *J* = 3.9 Hz, 1H), 6.99 (d, *J* = 3.9 Hz, 1H), 7.11 – 7.12 (m, 1H), 7.17 (dt, *J* = 2.0, 7.0 Hz, 1H), 7.24 (t, *J* = 7.6 Hz, 1H), 7.29 – 7.32 (m, 3H), 7.34 – 7.37 (m, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 21.5, 39.2, 122.9, 123.4, 126.5, 126.9, 128.3, 128.4, 129.0, 129.4, 130.9, 133.2, 133.5, 135.3, 136.4, 138.8, 145.7, 149.8, 162.8. IR (cm^{-1})

N-Methyl-*N*,5-bis(3-methylphenyl)-1,3-thiazole-2-carboxamide (30)

To a solution of lithium 5-(3-methylphenyl)-1,3-thiazole-2-carboxylate **30a** (100 mg, 0.44 mmol) in CH_2Cl_2 (15 mL) was added drop wise oxalyl chloride (80 µL, 0.88 mmol) followed by few drops of DMF at 0°C under N_2 atmosphere. The reaction mixture was stirred at 0°C for 10 min and then at room temperature for 3 h. The solvent was removed under reduced pressure (bath temperature of rotavapor at 20°C). This residue was diluted in dry CH_2Cl_2 (10 mL) and *N*,3-dimethylaniline (0.06 mL, 0.44 mmol) was added followed by

triethylamine (0.10 µL, 0.44 mmol). The solution was stirred overnight at room temperature under N_2 atmosphere. The solvent was removed under reduced pressure (bath temperature of rotavapor at 20°C). A mixture of Na_2CO_3 2N (15 mL) and EtOAc (15 mL) were added. The aqueous layer was extracted with EtOAc (15 mL). The organic layer was washed twice with Na_2CO_3 2N (15 mL), once with water (15 mL), dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as pale brown solid (51 mg, 36 %). $C_{19}H_{18}N_2OS$; MW 322; mp: 79 – 80°C; MS (ESI) 323 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ 2.32 (s, 3H), 2.36 (s, 3H), 3.54 (br s, 3H), 7.07 – 7.09 (m, 1H), 7.10 – 7.12 (m, 1H), 7.15 (br s, 1H), 7.20 – 7.26 (m, 1H), 7.32 (t, *J* = 7.7 Hz, 1H), 7.44 – 7.46 (m, 2H), 7.49 (br s, 1H), 7.93 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ 21.3, 39.6, 124.9, 125.0, 128.3, 128.4, 128.6, 130.0, 130.1, 130.6, 131.5, 139.69, 139.70, 139.9, 144.2, 145.4, 161.2. IR (cm^{-1}) 3096, 2922, 1624, 1606, 1585.

_N_-Methyl-_N_,5-bis(3-methylphenyl)furan-2-carboxamide (31)

The title compound was prepared by reaction of **31a** (150 mg, 0.51 mmol), *m*-tolylboronic acid (104 mg, 0.77 mmol), cesium carbonate (499 mg, 1.53 mmol) and tetrakis(triphenylphosphine) palladium (12 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as orange oil (100 mg, 64 %). $C_{20}H_{19}NO_2$; MW 305; MS (ESI) 306 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz)δ 2.30 (s, 3H), 2.38 (s, 3H), 3.37 (s, 3H), 6.67 (d, *J* = 3.7 Hz, 1H), 6.74 (d, *J* = 3.7 Hz, 1H), 7.00 – 7.01 (m, 1H), 7.07 – 7.12 (m, 2H), 7.16 – 7.22 (m, 3H), 7.24 – 7.27 (m, 1H), 7.35 (t, *J* = 7.8 Hz, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 21.3, 38.7, 107.1, 119.4, 122.3, 125.3, 125.4, 128.8, 129.0, 129.5, 129.9, 130.2, 130.7, 139.2, 140.3, 145.9, 148.2, 155.8, 159.1.

5-(3-Methoxyphenyl)-_N_-methyl-_N_-(3-methylphenyl)furan-2-carboxamide (32)

The title compound was prepared by reaction of **31a** (200 mg, 0.68 mmol), *m*-methoxyphenylboronic acid (155 mg, 1.02 mmol), cesium carbonate (665 mg, 2.04 mmol) and tetrakis(triphenylphosphine) palladium (16 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as colorless oil (200 mg, 94 %). $C_{20}H_{19}NO_3$; MW 321; MS (ESI) 322 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz)δ 2.37 (s, 3H), 3.37 (s, 3H), 3.80 (s, 3H), 6.61 (d, *J* = 3.7 Hz, 1H), 6.78 (d, *J* = 3.7 Hz, 1H), 6.83 – 6.86 (m, 2H), 6.93 – 6.95 (m, 1H), 7.09 – 7.12 (m, 1H), 7.20 – 7.26 (m, 3H), 7.33 (t, *J* = 7.8 Hz, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 21.2, 38.5, 55.7, 107.5, 110.2, 114.9, 117.5, 119.2, 125.1, 128.7, 129.0, 130.1, 130.6, 132.0, 140.3, 145.7, 148.3, 155.5, 159.1, 161.0.

5-(2-Fluoro-3-methoxyphenyl)-*N*-methyl-*N*-(3-methylphenyl)furan-2-carboxamide (33)

The title compound was prepared by reaction of **31a** (200 mg, 0.68 mmol), 2-fluoro-3-methoxyphenyl boronic acid (173 mg, 1.02 mmol), cesium carbonate (665 mg, 204 mmol) and tetrakis(triphenylphosphine) palladium (16 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as colorless solid (80 mg, 35 %). $C_{20}H_{18}FNO_3$; MW 339; mp: 99 – 101°C; MS (ESI) 340 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz)δ 2.37 (s, 3H), 3.38 (s, 3H), 3.88 (s, 3H), 6.60 – 6.64 (m, 2H), 6.74 (t, *J* = 3.8 Hz, 1H), 7.04 – 7.09 (m, 2H), 7.10 – 7.12 (m, 1H), 7.21 – 7.24 (m, 2H), 7.34 (t, *J* = 7.8 Hz, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 25.5, 42.9, 61.0, 116.2, 116.3, 118.1, 118.4, 122.3, 123.48, 123.53, 123.6, 129.41, 129.44, 129.5, 133.1, 133.3, 134.5, 144.7, 149.9, 152.46, 152.47, 153.0, 153.3, 153.4, 153.76, 153.78, 155.0, 163.2. IR (cm^{-1}) 3064, 2924, 1719, 1627, 1604, 1586.

5-(3,4-Dimethylphenyl)-*N*-methyl-*N*-(*m*-tolyl)furan-2-carboxamide (34)

The title compound was prepared by reaction of **31a** (150 mg, 0.51 mmol), (3,4-dimethylphenyl)boronic acid(115 mg, 0.77 mmol), cesium carbonate (499 mg, 1.53 mmol) and tetrakis(triphenylphosphine) palladium (12 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as a colorless oil (118 mg, 72 %). $C_{21}H_{21}NO_2$; MW 319; MS (ESI) 320 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz)δ2.22 (s, 3H), 2.23 (s, 3H), 2.38 (s, 3H), 3.44 (s, 3H), 6.44 (d, *J* = 3.6 Hz, 1H), 6.61 (d, *J* = 3.6 Hz, 1H), 6.96 (brs, 1H), 7.03 – 7.05 (m, 3H), 7.10 (s, 1H), 7.19 – 7.20 (m, 1H), 7.30 (t, *J* = 7.7 Hz, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ19.6, 19.7, 21.3, 38.7, 105.5, 119.3, 121.9, 124.4, 125.4, 127.5, 127.9, 128.3, 129.4, 129.8, 136.8, 137.1, 139.5, 144.6, 146.4, 155.7, 159.2.

***N*,3'-Dimethyl-*N*-(*m*-tolyl)-[1,1'-biphenyl]-4-carboxamide (35)**

The title compound was prepared by reaction of **35a** (120 mg, 0.39 mmol), *m*-tolylboronic acid(69 mg, 0.51 mmol), cesium carbonate (381 mg, 1.17 mmol) and tetrakis(triphenylphosphine) palladium (9 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow oil (105 mg, 85 %). $C_{22}H_{21}NO$; MW 315; MS (ESI) 316 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 300 MHz)δ2.24 (s, 3H), 2.37 (s, 3H), 3.42 (s, 3H), 6.95 (d, *J* = 9Hz, 1H), 6.99 (d, *J* = 7Hz, 1H), 7.07 (s, 1H), 7.11 – 7.18 (m, 2H), 7.30 (t,*J* = 8 Hz, 1H), 7.37 – 7.42 (m, 4H), 7.47 – 7.50 (m, 2H); ^{13}C NMR (CD_3COCD_3, 75 MHz)δ21.3,

21.5, 38.6, 124.8, 125.2, 126.9, 128.0, 128.4, 128.5, 129.4, 129.6, 129.7, 130.2, 136.5, 139.4, 140.0, 140.8, 142.7, 146.2, 170.2.IR (cm^{-1}) 3037, 2920, 2862, 1740, 1639, 1603.

4-methoxy-*N*-methyl-*N*,5-di-*m*-tolylthiophene-2-carboxamide (38a)

The title compound was prepared by reaction of 5-bromo-4-methoxy-*N*-methyl-*N*-(*m*-tolyl)thiophene-2-carboxamide **38b** (150 mg, 0.44 mmol), *m*-tolylboronic acid (72 mg, 0.53 mmol), cesium carbonate (430 mg, 1.32 mmol) and tetrakis(triphenylphosphine) palladium (10 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as a yellow oil (40 mg, 26 %). $C_{21}H_{21}NO_2S$; MW 351; MS (ESI) 352 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ2.34 (s, 3H), 2.39 (s, 3H), 3.47 (s, 3H), 3.65 (s, 3H), 6.73 (s, 1H), 7.04–7.06 (m, 1H), 7.11– 7.13 (m, 2H), 7.21 (t, *J* = 7.6 Hz, 1H), 7.24– 7.25 (m, 1H), 7.33– 7.40 (m, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ21.4, 21.6, 36.7, 58.5, 123.0, 124.6, 125.2, 127.8, 128.1, 128.4, 128.6, 128.7, 129.6, 129.9, 131.1, 132.3, 138.3, 140.3, 143.4, 152.6, 163.2; IR (cm^1) 2922, 2857, 1778, 1623, 1601, 1582.

4-hydroxy-*N*-methyl-*N*,5-di-*m*-tolylthiophene-2-carboxamide (38)

A solution of 4-methoxy-*N*-methyl-*N*,5-di-*m*-tolylthiophene-2-carboxamide **38a**(29 mg, 0.08mmol) and boron trifluoride methyl sulfide complex $BF_3.SMe_2$ (50 µL, 0.48mmol) in CH_2Cl_2 (3 mL) was stirred at room temperature overnight. Methanol (3 mL) was added to quench the reaction and the solution was stirred at room temperature for 30 minutes. The residual solvent was removed under reduced pressure at 25°C. The residue was triturated with cold water and stirred at room temperature for 2 hours. The precipitate formed was filtered off and washed once with water and twice with Et_2O to afford the desired product as colorless solid (27 mg, 100 %). $C_{20}H_{19}NO_2S$; mp: 156 – 157°C; MW 337; MS (ESI) 338 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ2.35 (s, 3H), 2.37 (s, 3H), 3.39 (s, 3H), 6.65 (s, 1H), 7.05 – 7.08 (m, 3H), 7.18 – 7.20 (m, 1H), 7.24 (t, *J* = 7.70 Hz, 1H), 7.29 (t, *J* = 7.7 Hz, 1H), 7.36 – 7.38 (m, 1H), 7.40 (brs, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz)δ21.3, 21.5, 39.3, 124.4, 125.0, 125.2, 126.5, 127.9, 128.1, 128.5, 128.7, 129.2, 129.6, 132.0, 132.3, 138.5, 139.9, 143.5, 148.4, 162.2. IR (cm^{-1}) : 3142, 3098, 2924, 1559.

5-(4-hydroxy-3-methylphenyl)-*N*-methyl-*N*-(*m*-tolyl)thiophene-2-carboxamide (36)

A solution of 5-(4-methoxy-3-methylphenyl)-*N*-methyl-*N*-(*m*-tolyl)thiophene-2-carboxamide **36a**(65 mg, 0.18 mmol) and boron trifluoride methyl sulfide complex $BF_3.SMe_2$ (120 µL, 1.11 mmol) in CH_2Cl_2 (3 mL) was stirred at room temperature overnight. Methanol (3 mL) was added to quench the reaction and the solution was stirred at

room temperature for 30 minutes. The residual solvent was removed under reduced pressure at 25°C. The residue was triturated with cold water and stirred at room temperature for 2 hours. The precipitate formed was filtered off and washed once with water and twice with Et_2O to afford the desired product as colorless solid (55 mg, 90 %).$C_{20}H_{19}NO_2S$; mp: 169 – 170°C; MW 337; MS (ESI) 338 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz)δ 2.21 (s, 3H), 2.36 (s, 3H), 3.36 (s, 3H), 6.43 (d, *J* = 4.1 Hz, 1H), 6.83 (d, *J* = 8.4 Hz, 1H), 6.95 (d, *J* = 3.8 Hz, 1H), 7.14-7.16 (m, 1H), 7.21 – 7.26 (m, 3H), 7.36 – 7.37 (m, 2H), 8.50 (s, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 16.1, 21.2, 39.1, 116.0, 122.2, 125.4, 125.92, 125.96, 126.0, 129.2, 129.4, 129.5, 130.4, 133.2, 137.4, 140.7, 145.6, 150.0, 156.9, 162.6. IR (cm^{-1}) : 3174, 2924, 1592, 1578.

5-(3-hydroxy-5-methylphenyl)-*N*-methyl-*N*-(*m*-tolyl)thiophene-2-carboxamide (37)

A solution of 5-(3-methoxy-5-methylphenyl)-*N*-methyl-*N*-(*m*-tolyl)thiophene-2-carboxamide**37a**(95 mg, 0.27 mmol) and boron trifluoride methyl sulfide complex $BF_3.SMe_2$ (170 µL, 1.62 mmol) in CH_2Cl_2 (3 mL) was stirred at room temperature overnight. Methanol (3 mL) was added to quench the reaction and the solution was stirred at room temperature for 30 minutes. The residual solvent was removed under reduced pressure at 25°C. The residue was triturated with cold water and stirred at room temperature for 2 hours. The precipitate formed was filtered off and washed once with water and twice with Et_2O to afford the desired product as colorless solid (87 mg, 96 %).$C_{20}H_{19}NO_2S$; mp: 173 – 174°C; MW 337; MS (ESI) 338 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz)□2.28 (s, 3H), 2.36 (s, 3H), 3.46 (s, 3H), 6.39 (d, *J* = 4 Hz, 1H), 6.67 (brs, 1H), 6.86 (brs, 1H), 6.91 (d, *J* = 4 Hz, 1H), 7.02 – 7.08 (m, 3H), 7.19 – 7.21 (m, 1H), 7.30 (t, *J* = 8 Hz, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ 21.3, 21.4, 39.5, 110.6, 116.5, 118.5, 123.0, 124.7, 128.2, 129.1, 129.6, 132.8, 134.5, 136.7. 140.0, 140.1, 144.0, 149.6, 156.9, 163.2.

Ethyl 3-hydroxy-5-(3-methylphenyl)thiophene-2-carboxylate (39d)

Through a solution of ethyl 3-(3-methylphenyl)-3-oxopropanoate**39e** (1 g, 4.9mmol) and ethyl thioglycolate**39f** (1.06 mL, 9.7 mmol) was bubbled dry HCl(g) at room temperature for 5 hours. Water was added to quench the reaction. The aqueous layer was extracted three times with Et_2O (3 × 15 mL). The organic layer was washed once with water, dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. To the residue was added drop wise a solution of sodium ethanolate (prepared from 900 mg of sodium metal). The reaction mixture was stirred for about 2 hours and then stopped with cold water. The basic pH was adjusted to neutral pH with a solution of HCl 1N. The aqueous layer was extracted three times with EtOAc (3 × 15 mL). The organic layer was washed once with water, dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure.

The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20 to 0:100) to afford the desired product as beige solid (267 mg, 21 %). $C_{14}H_{14}O_3S$; MW 262; mp: 49 – 51 °C; MS (ESI) 263 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz)δ1.36 (t, *J* = 7.2 Hz, 3H), 2.38 (s, 3H), 4.37 (q, *J* = 7.2 Hz, 2H), 7.15 (s, 1H), 7.23 – 7.26 (m, 1H), 7.33 (t, *J* = 7.7 Hz, 1H), 7.50 – 7.53 (m, 1H), 7.54 – 7.56 (m, 1H), 9.70 (br s, 1H); ^{13}C NMR (CD_3COCD_3, 125 MHz)δ14.6, 21.3, 61.8, 103.6, 116.0, 123.8, 127.2, 130.0, 131.1, 133.8, 139.8, 150.1, 165.4, 166.6.

Ethyl 3-methoxy-5-(3-methylphenyl)thiophene-2-carboxylate (39c)

A mixture of ethyl 3-hydroxy-5-(3-methylphenyl)thiophene-2-carboxylate**39d** (265 mg, 1.0mmol), potassium carbonate (207 mg, 1.5 mmol), methyl iodide (93 µL, 1.5 mmol) in dry acetonitrile (5 mL) was heated at reflux under N_2 atmosphere for 48 hours. The residual solvent was removed under reduced pressure and water was added to the residue. The organic layer was extracted three times with EtOAc (3 × 10 mL). The organic layer was dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as yellow oil (170 mg, 62 %). $C_{15}H_{16}O_3S$; MW 276; MS (ESI) 277 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 300 MHz)δ1.31 (t, *J* = 7.1 Hz, 3H), 2.38 (s, 3H), 4.01 (s, 3H), 4.26 (q, *J* = 7.1 Hz, 2H), 7.20 – 7.24 (m, 1H), 7.33 (t, *J* = 7.6 Hz, 1H), 7.44 (s, 1H), 7.50 – 7.55 (m, 1H), 7.56 – 7.58 (m, 1H); ^{13}C NMR (CD_3COCD_3, 75 MHz)δ14.3, 20.8, 58.8, 60.1, 108.5, 113.4, 123.0, 126.5, 129.4, 130.2, 133.6, 139.2, 147.9, 161.2, 162.5.

3-Methoxy-5-(3-methylphenyl)thiophene-2-carboxylic acid (39b)

A solution of ethyl 3-methoxy-5-(3-methylphenyl)thiophene-2-carboxylate **39c** (165 mg, 0.6 mmol) and potassium hydroxide (500 mg, 8.9 mmol) in a mixture of H_2O/THF (10 mL, 1:1, v:v) was heated at reflux for 4 hours. After cooling, the solution was extracted once with EtOAc (1 × 10 mL). The aqueous layer was acidified to pH = 2 at 0°C. The precipitate formed was filtered off and washed three times with water and two times with Et_2O to afford the desired product as pale brown solid (67 mg, 45 %). $C_{13}H_{12}O_3S$; MW 248; MS (ESI) 249 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 300 MHz)δ2.39 (s, 3H), 4.06 (s, 3H), 7.21 – 7.26 (m, 1H), 7.35 (t, *J* = 7.6 Hz, 1H), 7.49 (s, 1H), 7.52 – 7.57 (m, 1H), 7.58 – 7.60 (m, 1H), 10.55 (br s, 1H); ^{13}C NMR (CD_3COCD_3, 75 MHz)δ20.4, 58.6, 108.7, 113.0, 122.7, 126.1, 129.1, 129.9, 133.3, 138.9, 147.9, 161.4, 161.7.

3-Methoxy-*N*-methyl-*N*,5-bis(3-methylphenyl)thiophene-2-carboxamide (39a)

Results

The title compound was prepared by reaction of 3-methoxy-5-(3-methylphenyl)thiophene-2-carboxylic acid **39b** (67 mg, 0.27 mmol), thionyl chloride (78 µL, 1.1 mmol) and 3-methylaniline (34 µL, 0.27 mmol) according to method A. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc 70:30) to afford the desired product as yellow oil (84 mg, 88 %). $C_{21}H_{21}NO_2S$; MW 351; MS (ESI) 352 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 300 MHz)δ2.46 (s, 3H), 2.52 (s, 3H),3.53 (s, 3H), 3.62 (s, 3H), 7.12 – 6.14 (m, 1H), 7.17 – 7.19 (m, 1H), 7.06 – 7.09 (m, 1H), 7.24 (s, 1H), 7.30 – 7.35 (m, 2H), 7.45 (t, *J* = 7.7 Hz, 1H), 7.56 – 7.59 (m, 1H), 7.62 (s, 1H); ^{13}C NMR (CD_3COCD_3, 75 MHz)δ21.3, 21.4, 38.3, 58.6, 113.1, 123.2, 124.2, 126.7, 127.5, 127.9, 129.1, 129.9, 130.1, 134.6, 139.1, 139.7, 145.5, 146.2, 147.0, 156.3, 163.8.IR (cm^{-1}): 2925, 2856, 1622, 1602, 1586.

3-Hydroxy-*N*-methyl-*N*,5-bis(3-methylphenyl)thiophene-2-carboxamide (39)

A solution of 3-methoxy-*N*-methyl-*N*,5-bis(3-methylphenyl)thiophene-2-carboxamide **39a** (83 mg, 0.24 mmol) and boron trifluoride methyl sulfide complex $BF_3.SMe_2$ (150 µL, 1.42 mmol) in CH_2Cl_2 (3 mL) was stirred at room temperature overnight. Methanol (3 mL) was added to quench the reaction and the solution was stirred at room temperature for 30 minutes. The residual solvent was removed under reduced pressure at 25°C. The residue was triturated with cold water and stirred at room temperature for 2 hours. The precipitate formed was filtered off and washed once with water and twice with Et_2O to afford the desired product as beige solid (59 mg, 72 %). $C_{20}H_{19}NO_2S$; mp: 171 – 173°C; MW 337; MS (ESI) 338 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 300 MHz)δ2.33 (s, 3H), 2.47 (s, 3H), 3.61 (s, 3H), 7.04 (s, 1H), 7.23 – 7.31 (m, 3H), 7.33 – 7.36 (m, 1H), 7.48 – 7.53 (m, 2H),7.54 – 7.60 (m, 2H); ^{13}C NMR (CD_3COCD_3, 75 MHz)δ20.34, 20.35, 39.3, 115.18, 115.20, 123.0, 125.3, 126.4, 128.8, 129.1, 130.6, 130.9, 131.4, 132.3, 139.0, 139.8, 141.1, 155.7, 169.6.IR (cm^{-1}) : 3049, 2924, 2854, 1569, 1540, 1505.

5-(3-Formylphenyl)-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide (40a)

The title compound was prepared by reaction of 5-bromo-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide**12a**(620 mg, 2.0mmol), *m*-formylphenylboronic acid(450 mg, 3.0mmol), cesium carbonate (1.955 g, 6mmol) and tetrakis(triphenylphosphine) palladium (46 mg, 0.02 eq) according to method B. The residue was purified by recrystallization in EtOH/water to afford the desired product as pale brown solid (250 mg, 37 %). $C_{20}H_{17}NO_2S$; MW 335; MS (ESI) 336 $[M+H]^+$; ^1H NMR ($CDCl_3$, 500 MHz)δ2.38 (s, 3H), 3.45 (s, 3H), 6.60 (d, *J* = 4.1 Hz, 1H), 7.05 (d, *J* = 3.9 Hz, 1H), 7.06 – 7.11 (m, 2H), 7.20 – 7.23 (m, 1H), 7.31 (t, *J* = 7.7 Hz, 1H), 7.52 (t, *J* = 7.7 Hz, 1H), 7.74 (ddd, *J* = 1.2, 1.9, 7.9 Hz, 1H), 7.78 (dt, *J* = 1.8, 7.6 Hz, 1H), 7.98 (t, *J* = 1.6 Hz, 1H), 10.01 (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.3, 39.1, 123.7, 125.0, 126.7, 128.4, 129.1, 129.2,

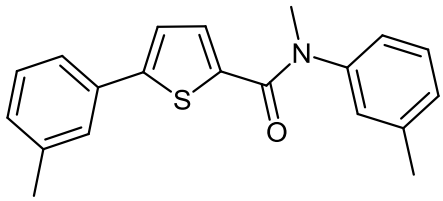
129.6, 129.7, 131.6, 132.8, 134.7, 136.9, 138.4, 140.0, 144.0, 146.9, 162.2, 191.8. IR (cm^{-1}) : 3083, 3055, 2922, 2840, 1694, 1601, 1584.

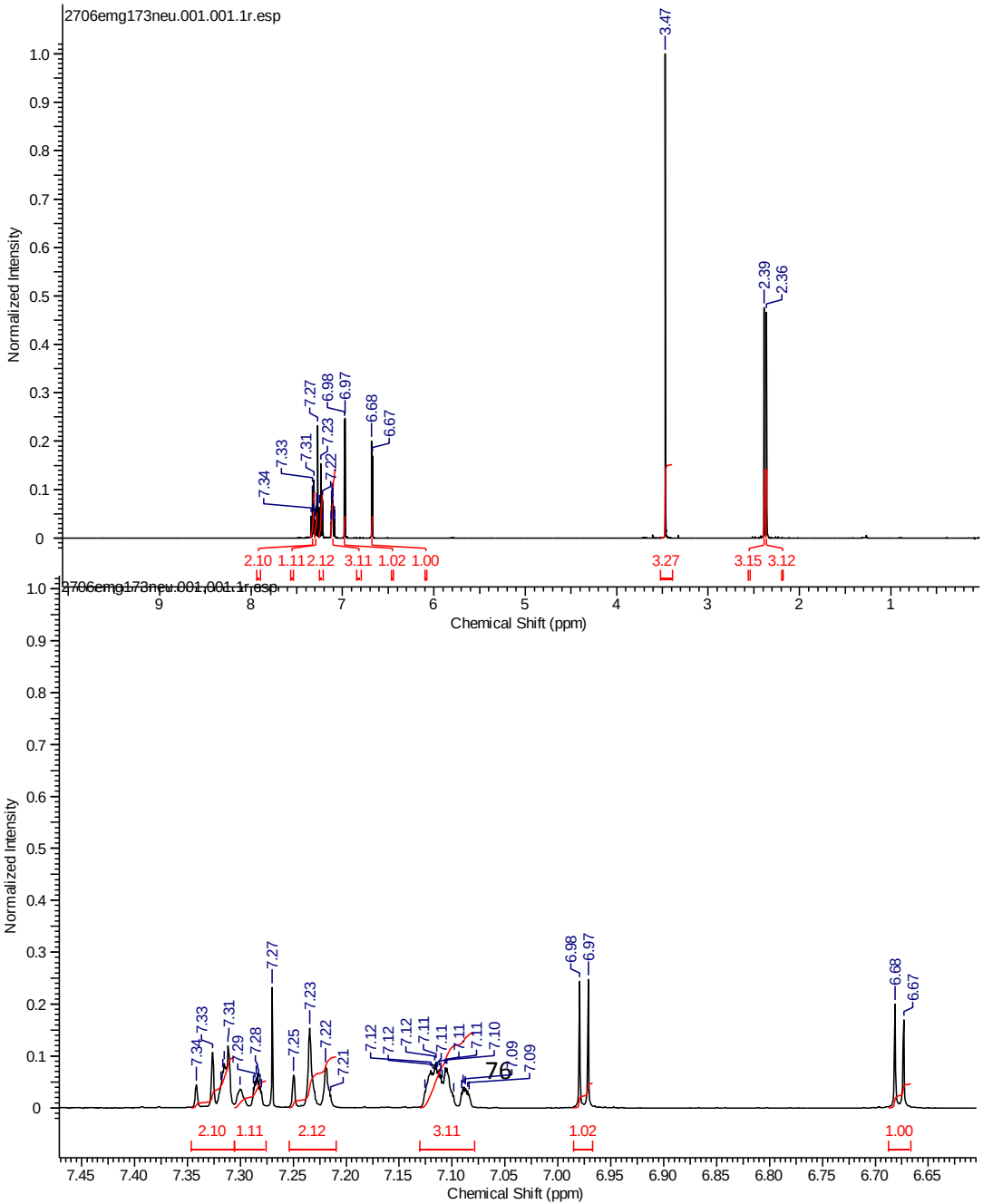
5-[3-(Hydroxymethyl)phenyl]-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide (40)

To a solution of 5-(3-formylphenyl)-*N*-methyl-*N*-(3-methylphenyl)thiophene-2-carboxamide **40a** (76 mg, 0.23 mmol) in a mixture of dry MeOH (3 mL) and dry dioxane (3 mL) was added at 0°C sodium borohydride (87 mg, 0.46 mmol). After 2 hours at 0°C, water was added to quench the reaction. The aqueous layer was extracted three times with EtOAc (3 × 5 mL). The organic layer was washed once with saturated solution of $NaHCO_3$ and once with water, dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by preparative HPLC (using acetonitrile/water from 10% to 95% of acetonitrile) to afford the desired product as colorless oil (14 mg, 18 %). $C_{20}H_{19}NO_2S$; MW 337; MS (ESI) 338 $[M+H]^+$; ^1H NMR ($CDCl_3$, 300 MHz)δ 2.38 (s, 3H), 3.44 (s, 3H), 4.71 (s, 2H), 6.58 (d, *J* = 4.0 Hz, 1H), 6.97 (d, *J* = 4.0 Hz, 1H), 7.06 – 7.09 (m, 2H), 7.19 – 7.22 (m, 1H), 7.28 – 7.36 (m, 3H), 7.41 (dt, *J* = 1.8, 7.2 Hz, 1H), 7.49 – 7.51 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz)δ21.3, 39.1, 65.0, 122.9, 124.5, 125.0, 125.2, 126.8, 128.5, 129.0, 129.1, 129.6, 132.9, 133.9, 137.4, 139.9, 141.7, 144.1, 148.7, 162.5.IR (cm^{-1}) : 3397, 3027, 2921, 2865, 1598, 1582.

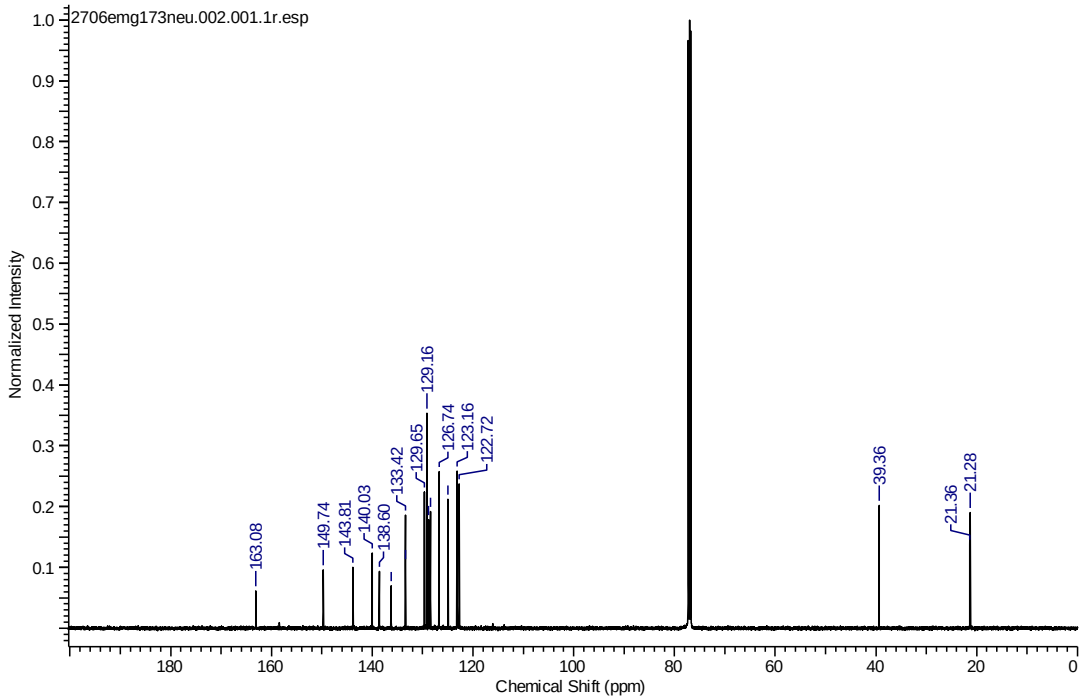
b) NMR spectra of representative compounds

Compound **12**:

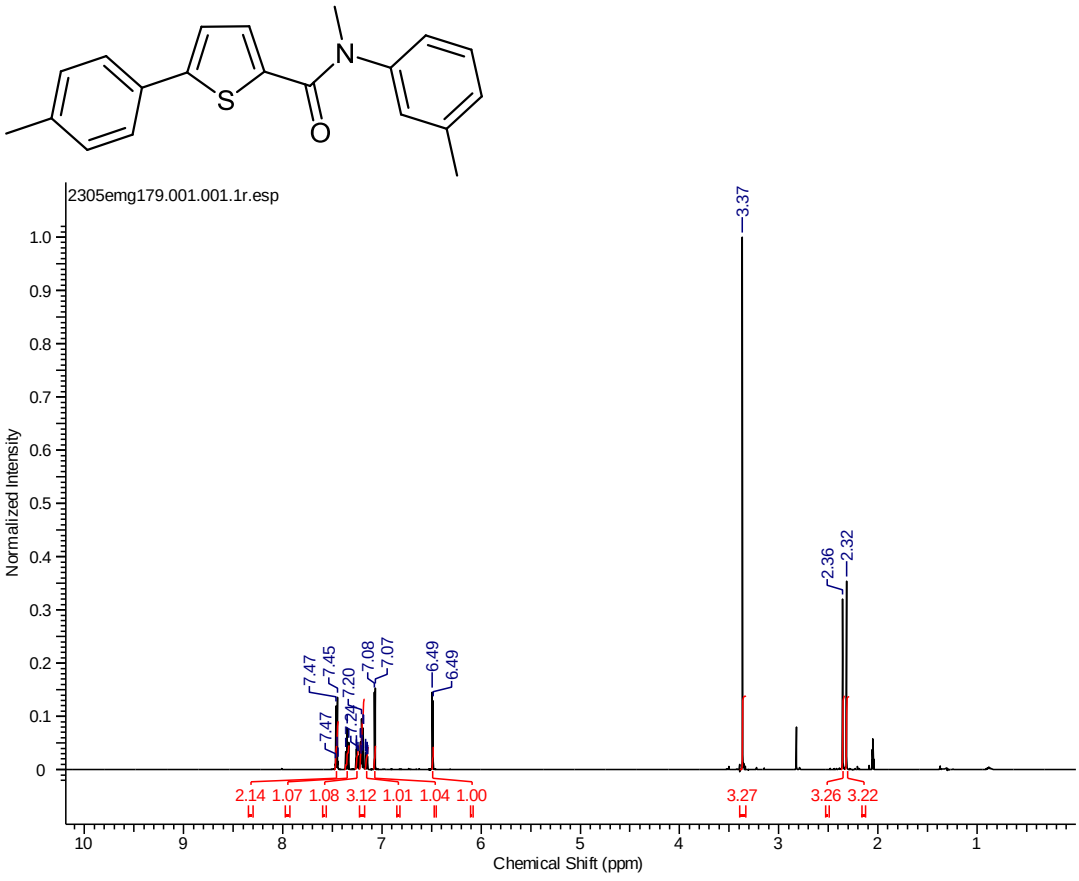




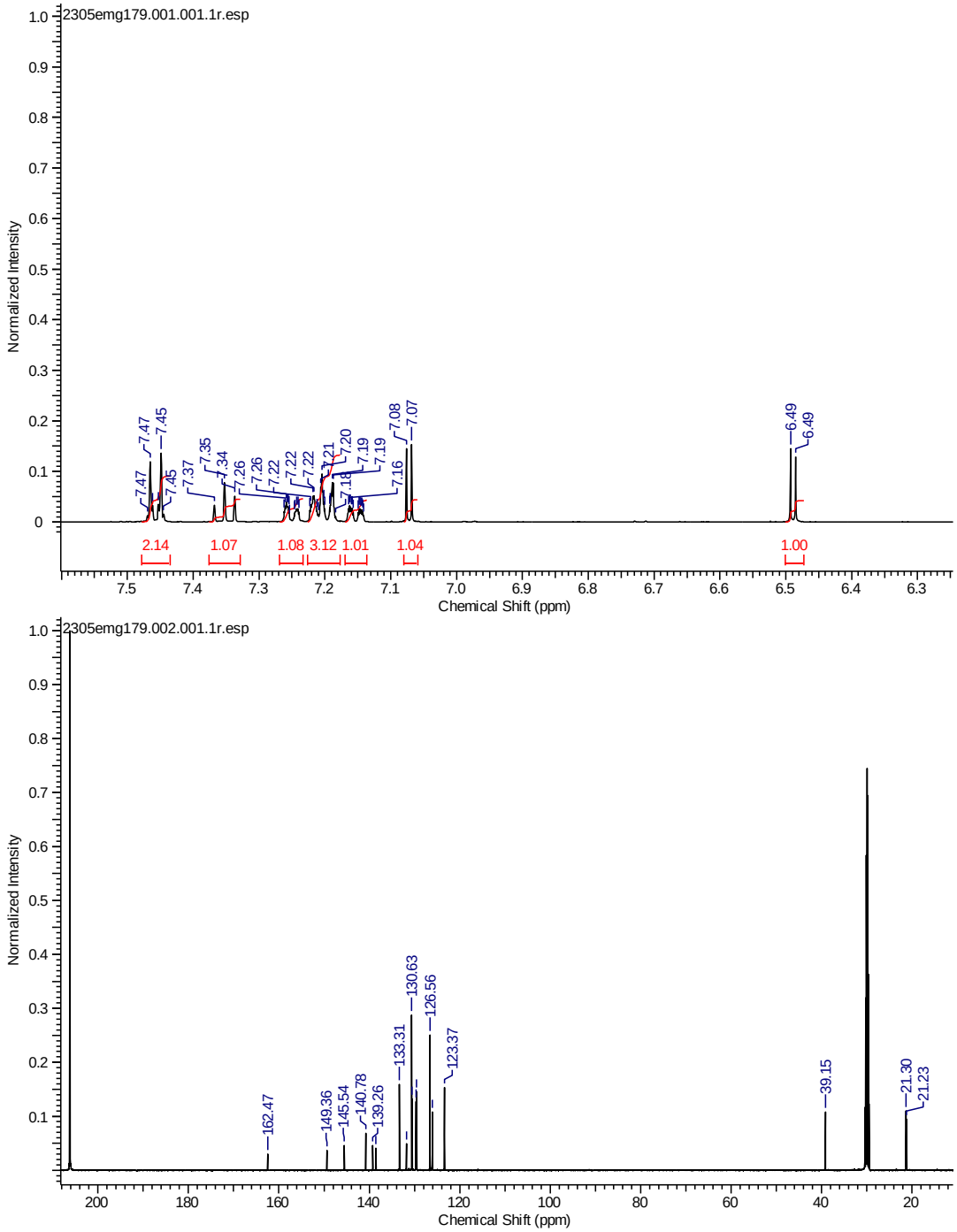
Results



Compound **13**:



Results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Normalized Intensity
Chemical Shift (ppm)
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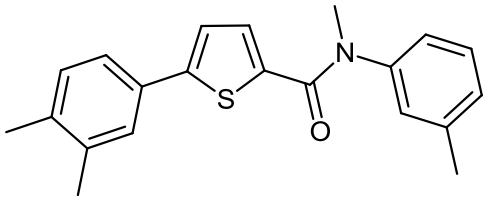
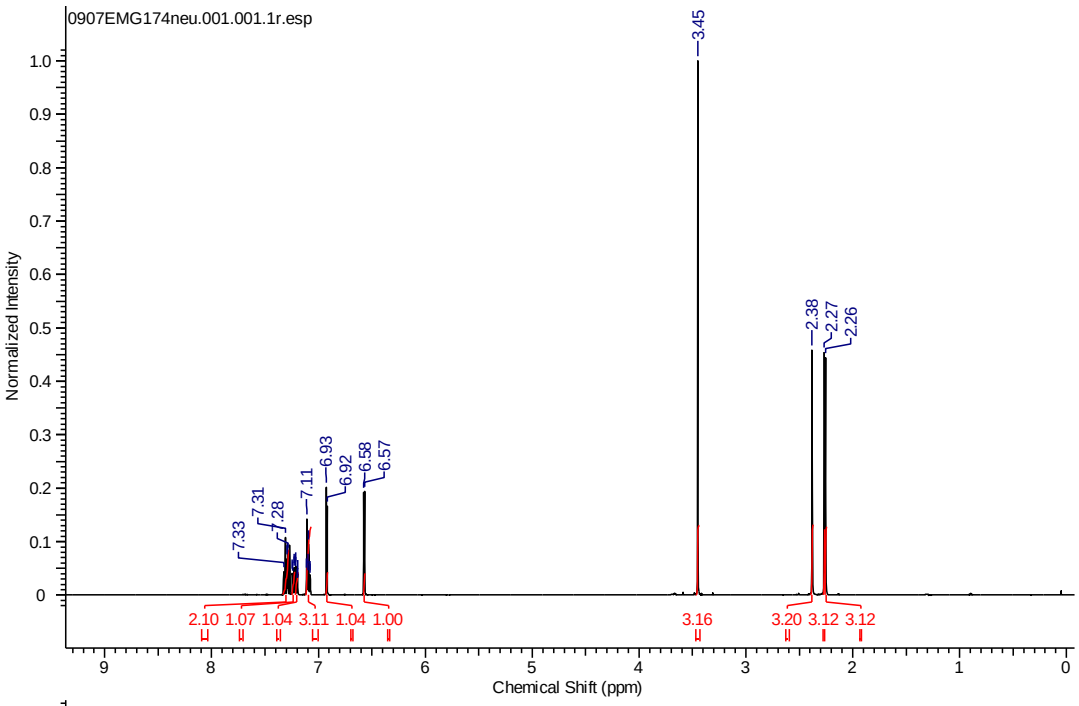
7.45
7.37
7.35
7.34
7.26
7.22
7.22
7.22
7.21
7.20
7.19
7.19
7.18
7.16
7.08
7.07
6.49
6.49
2.14
1.07
1.08
3.12
1.01
1.04
1.00
2305emg179.002.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
162.47
149.36
145.54
140.78
139.26
133.31
130.63
126.56
123.37
39.15
21.30
21.23

Compound **21**:


S
N
O


0907EMG174neu.001.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
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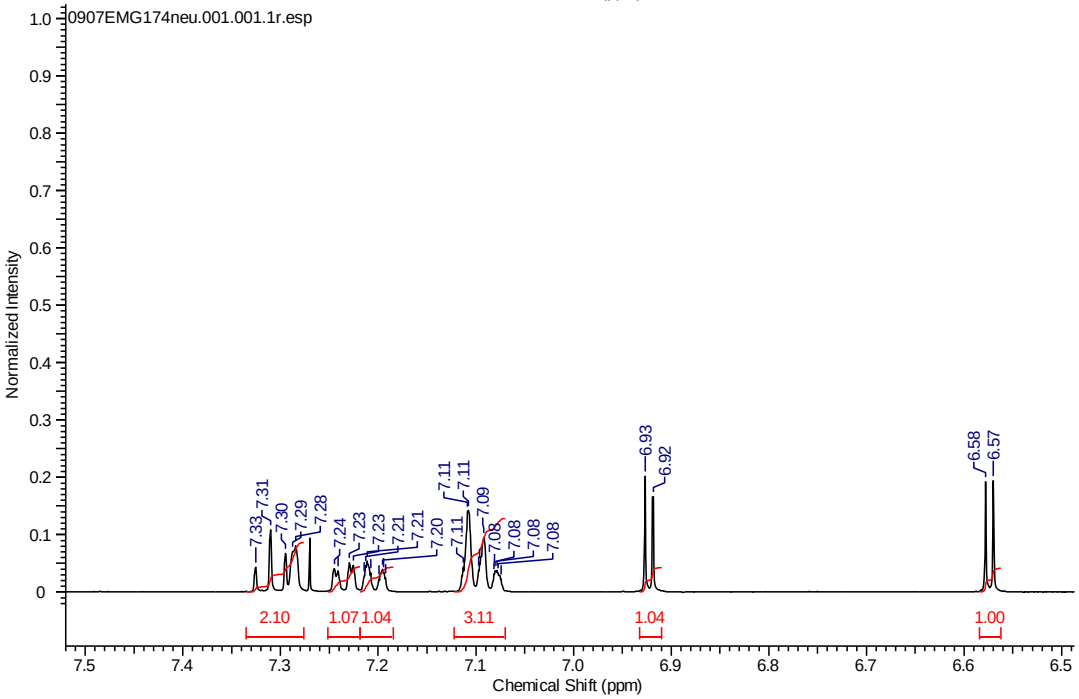
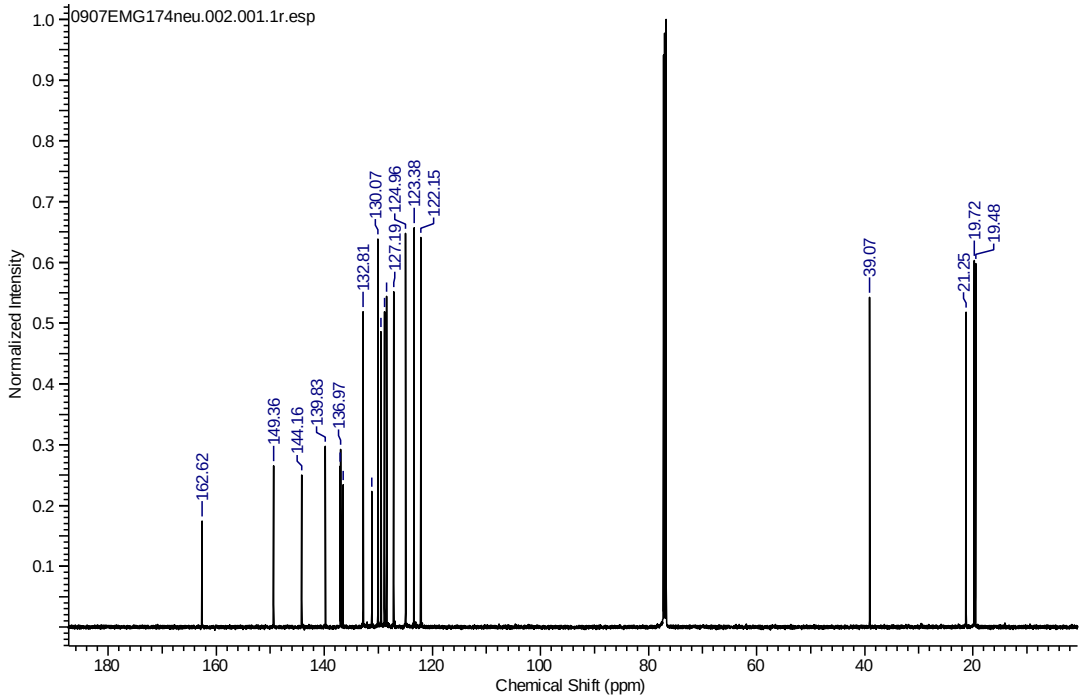
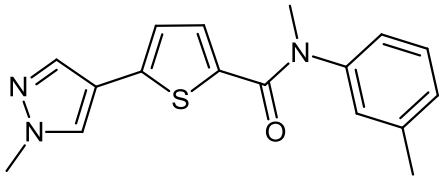
2.38
2.27
2.26
7.33
7.31
7.28
7.11
6.93
6.92
6.58
6.57
2.10 1.07 1.04 3.11 1.04 1.00
3.16
3.20 3.12 3.12

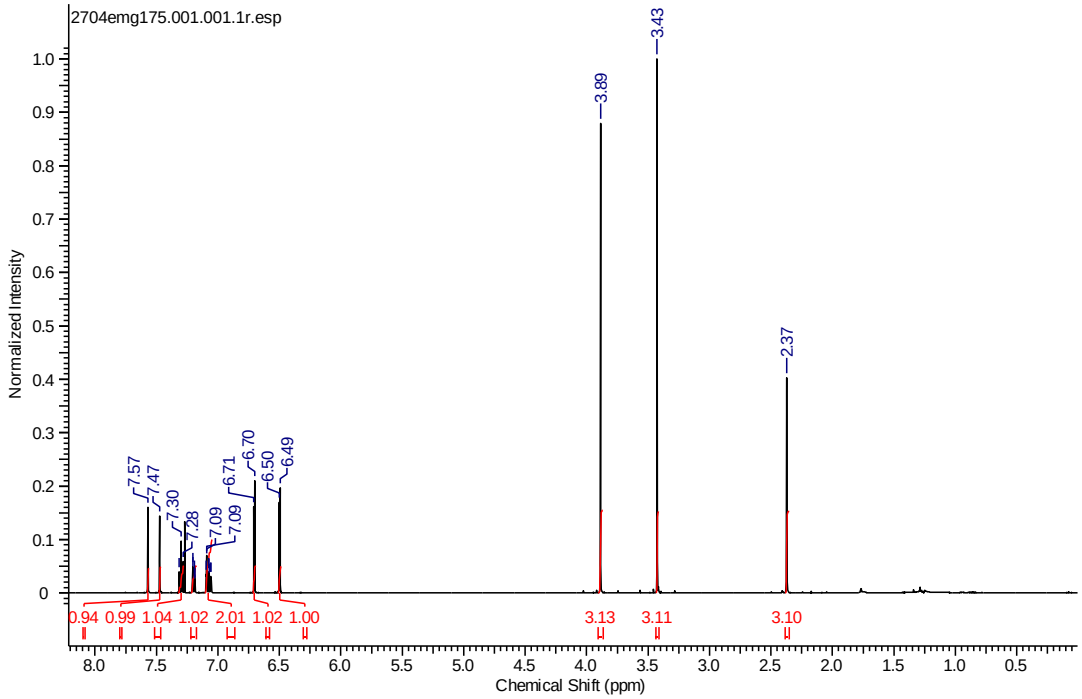

0907EMG174neu.001.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
7.33
7.31
7.30
7.29
7.28
7.24
7.23
7.23
7.21
7.21
7.20
7.11
7.11
7.11
7.09
7.08
7.08
7.08
7.08
6.93
6.92
6.58
6.57
2.10
1.07 1.04
3.11
1.04
1.00

Results

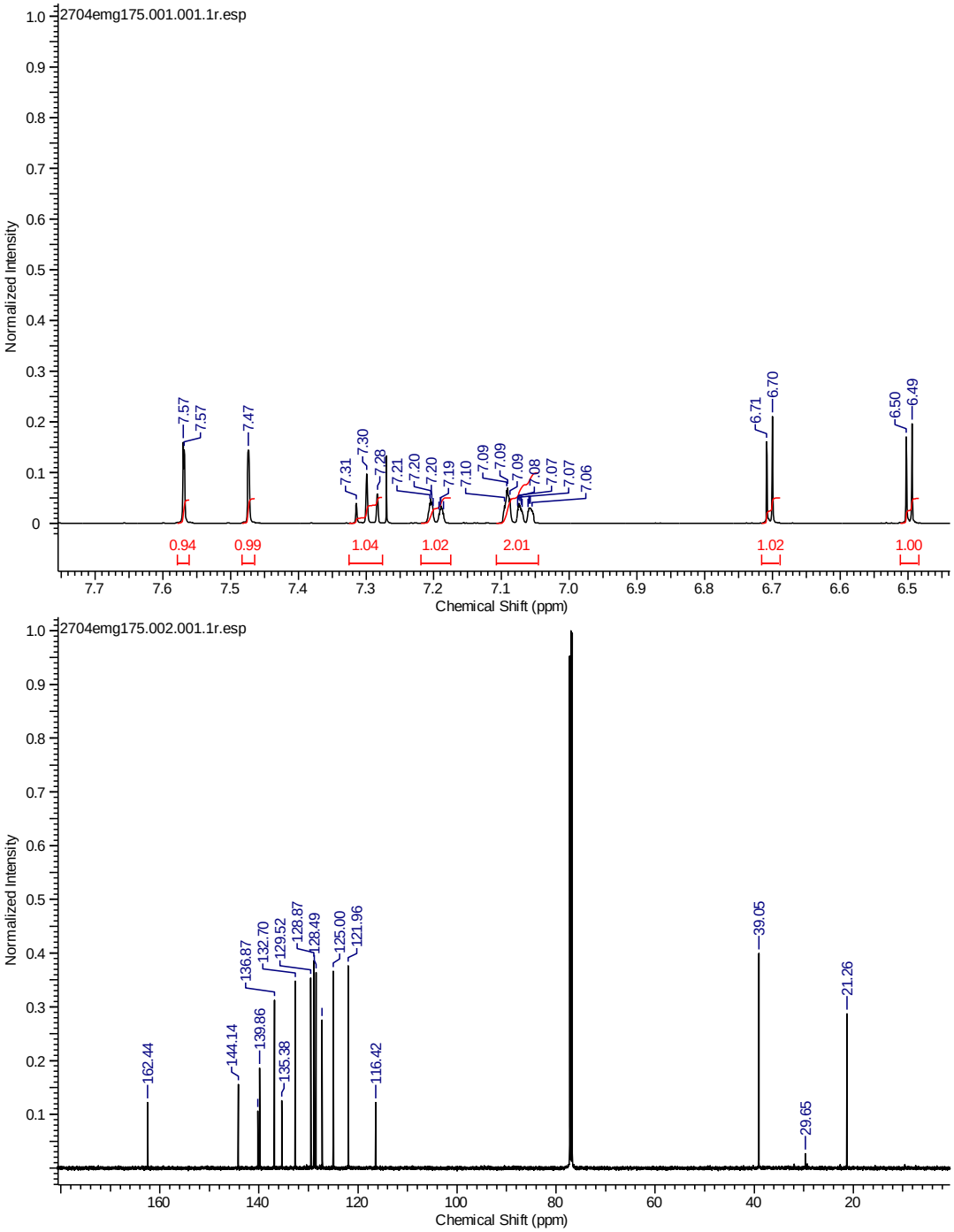


Compound **27**:

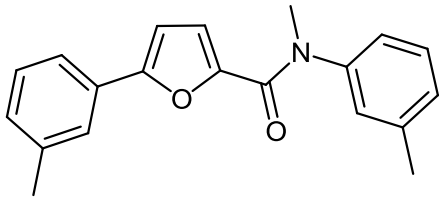


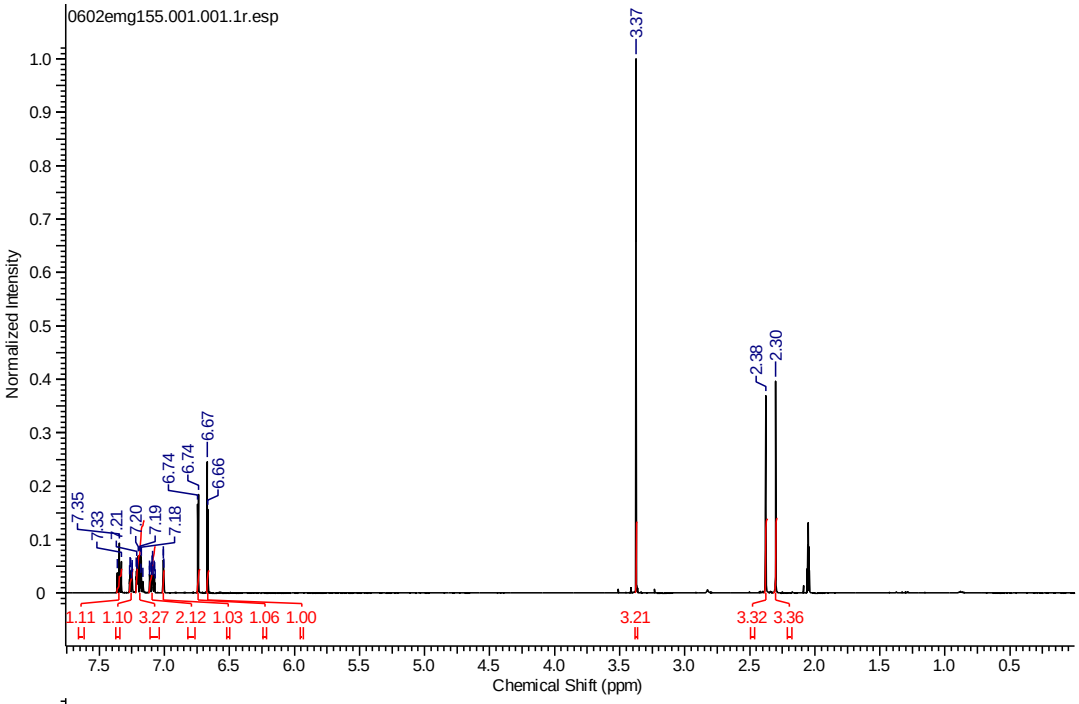


Results


2704emg175.001.001.1r.esp
Normalized Intensity
7.57
7.57
7.47
7.31
7.30
7.28
7.21
7.20
7.20
7.19
7.10
7.09
7.09
7.09
7.08
7.07
7.07
7.06
6.71
6.70
6.50
6.49
0.94
0.99
1.04
1.02
2.01
1.02
1.00
Chemical Shift (ppm)
2704emg175.002.001.1r.esp
Normalized Intensity
162.44
144.14
139.86
136.87
135.38
132.70
129.52
128.87
128.49
125.00
121.96
116.42
39.05
29.65
21.26
Chemical Shift (ppm)

Compound **31**:


O
N
O


0602emg155.001.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
7.35
7.33
7.21
7.20
7.19
7.18
6.74
6.74
6.67
6.66
3.37
2.38
2.30
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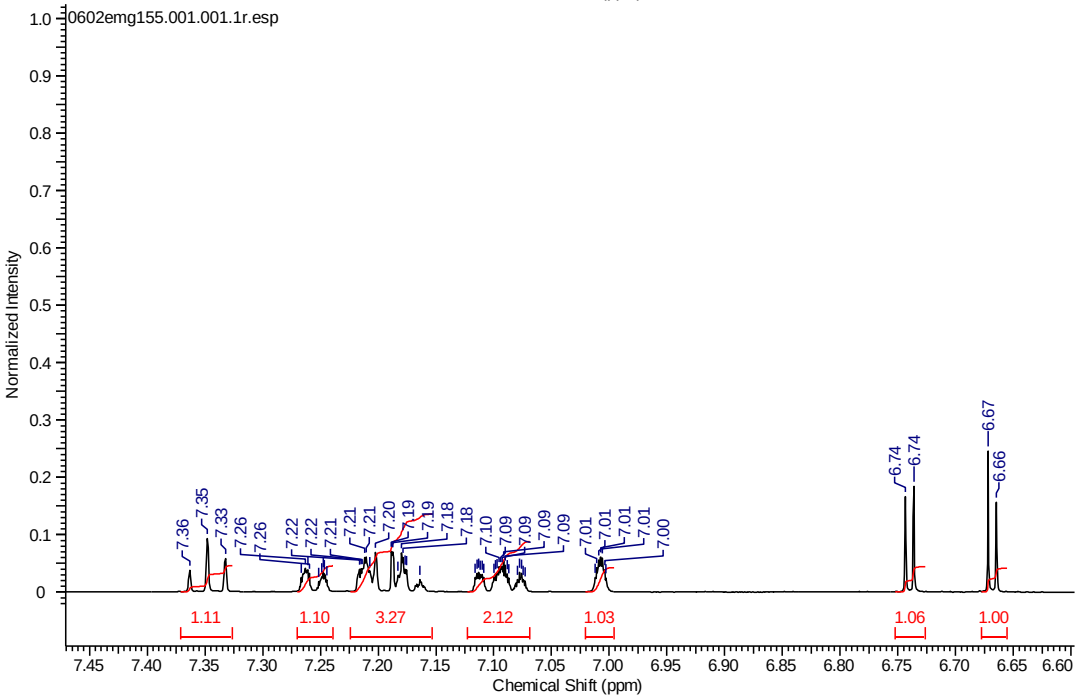
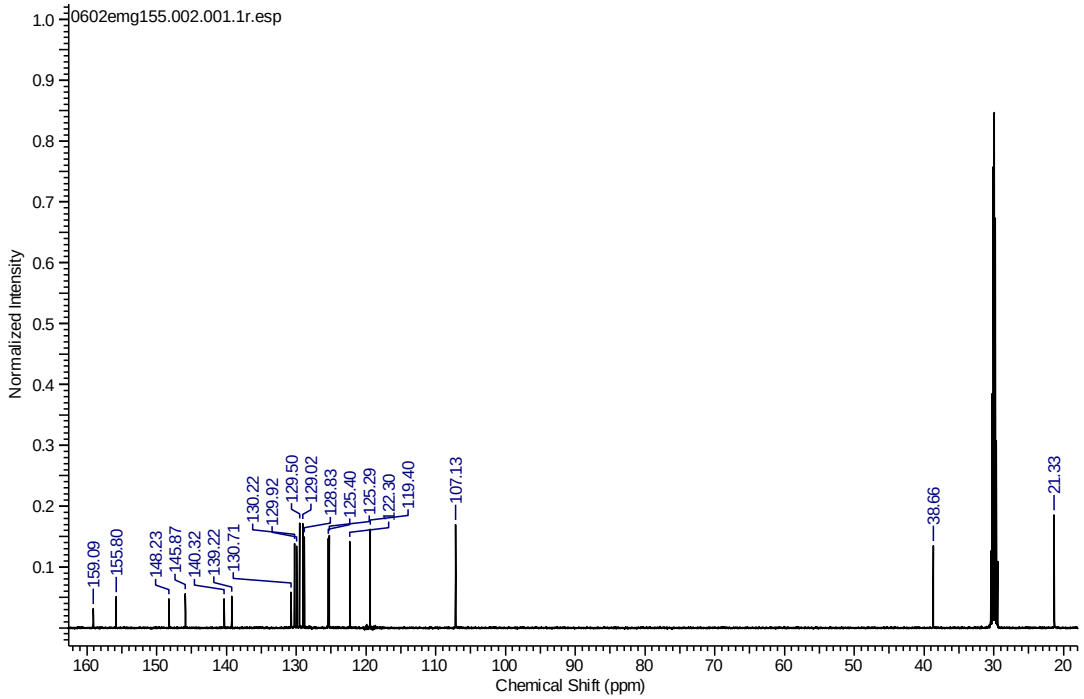
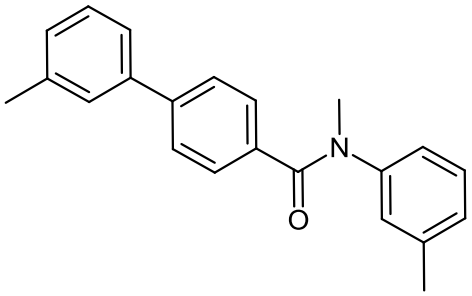
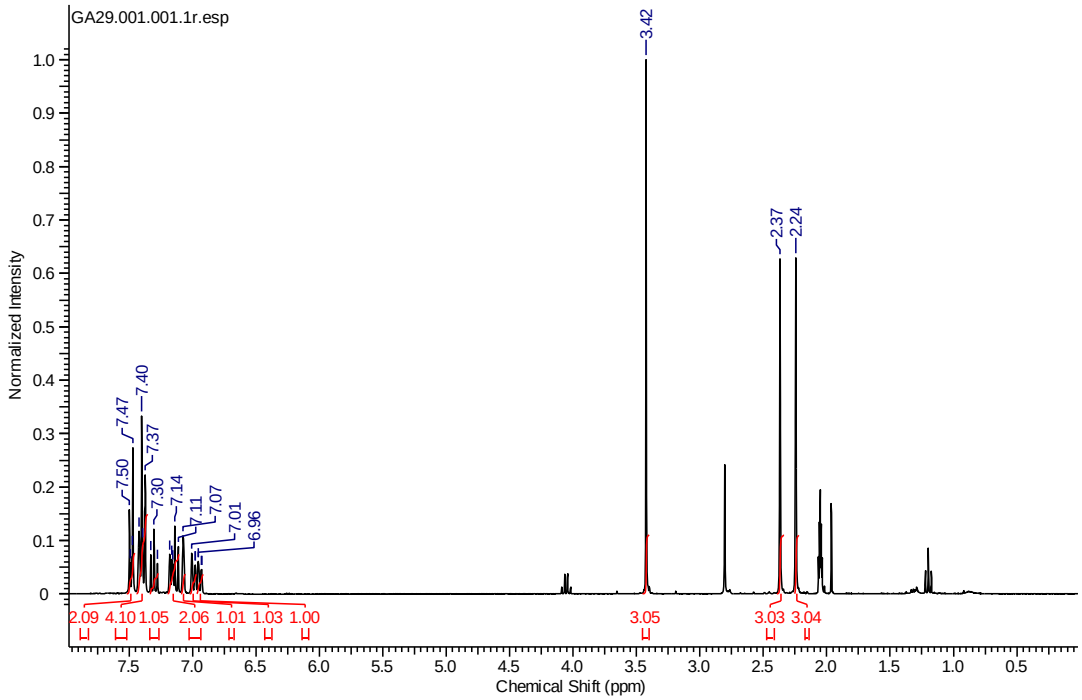
3.21
3.32 3.36

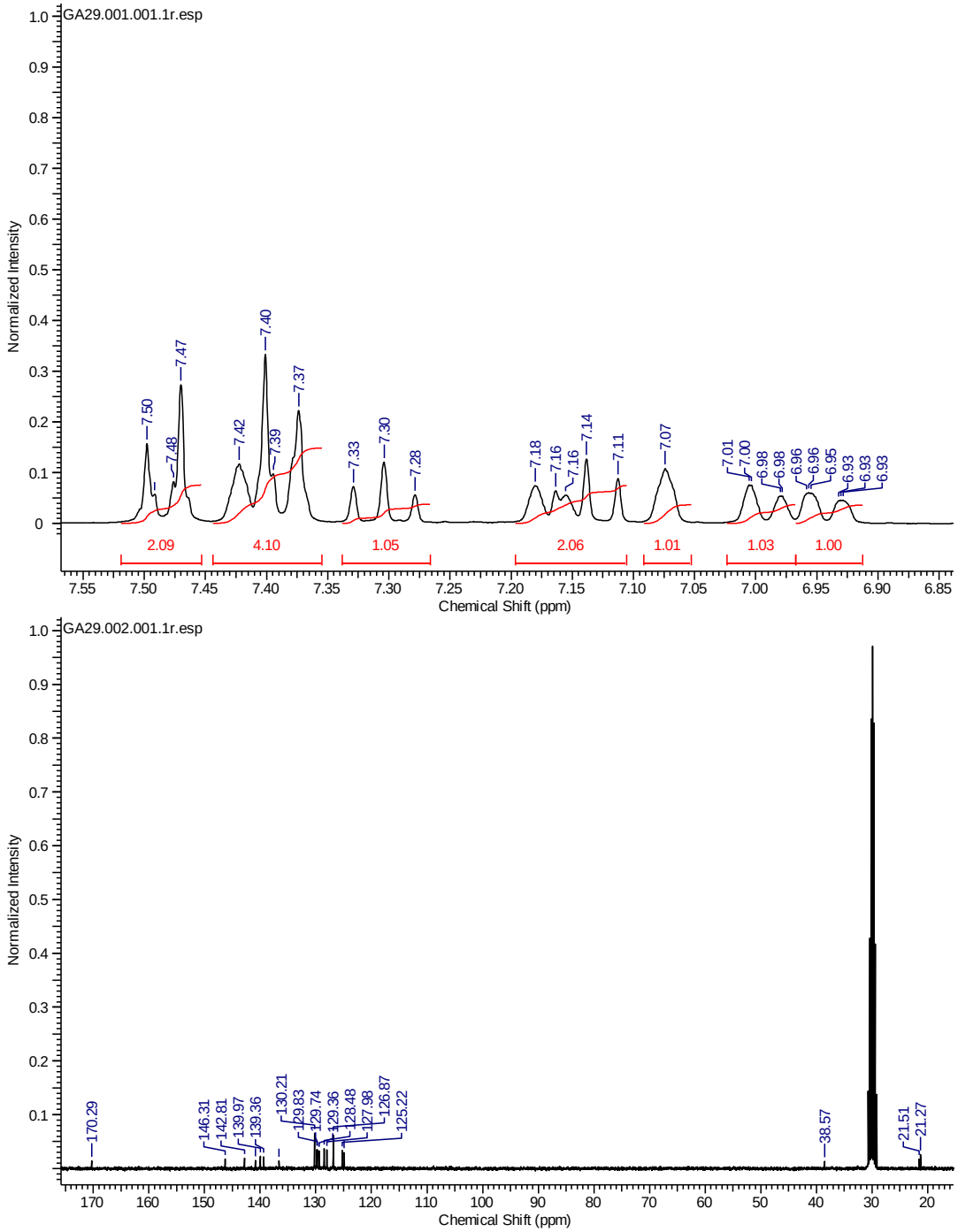

0602emg155.001.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
7.36
7.35
7.33
7.26
7.26
7.22
7.22
7.21
7.21
7.21
7.20
7.19
7.19
7.18
7.18
7.10
7.09
7.09
7.09
7.09
7.01
7.01
7.01
7.00
6.74
6.74
6.67
6.66
1.11
1.10
3.27
2.12
1.03
1.06
1.00


0602emg155.002.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
159.09
155.80
148.23
145.87
140.32
139.22
130.71
130.22
129.92
129.50
129.02
128.83
125.40
125.29
122.30
119.40
107.13
38.66
21.33

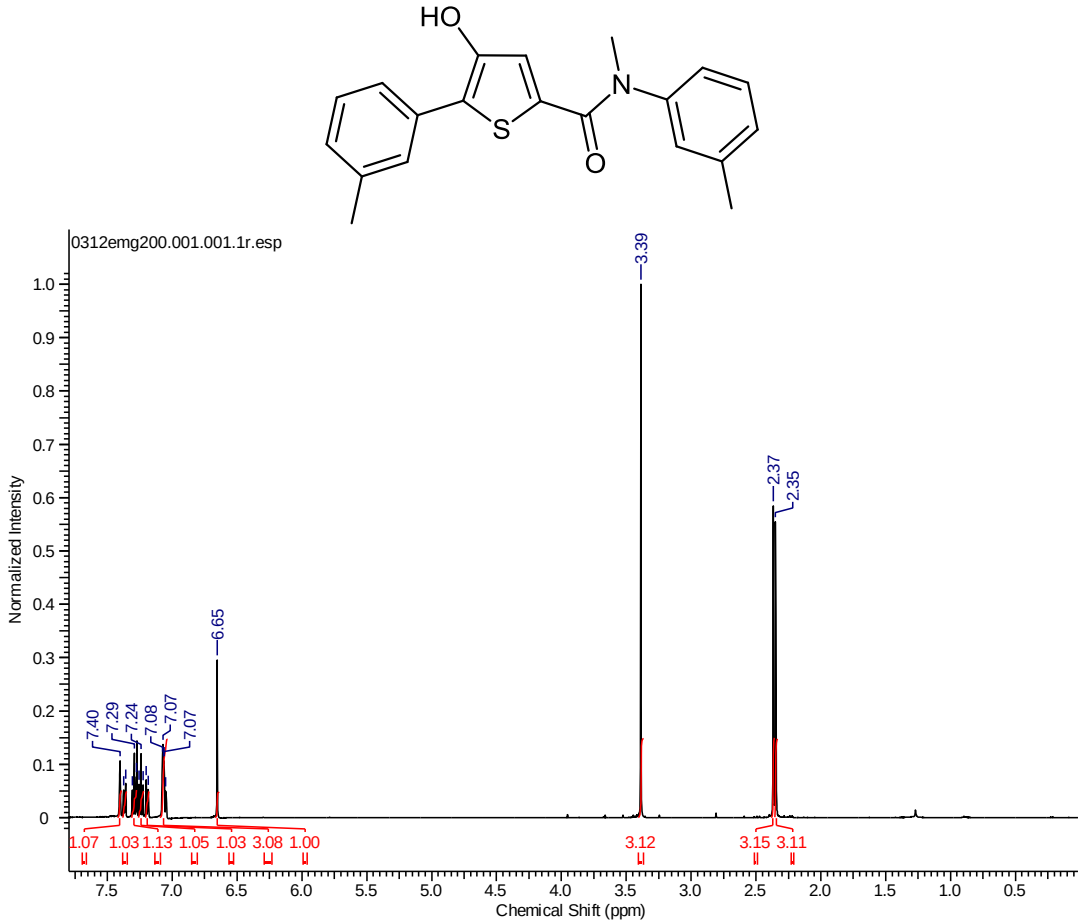
Compound **35**:

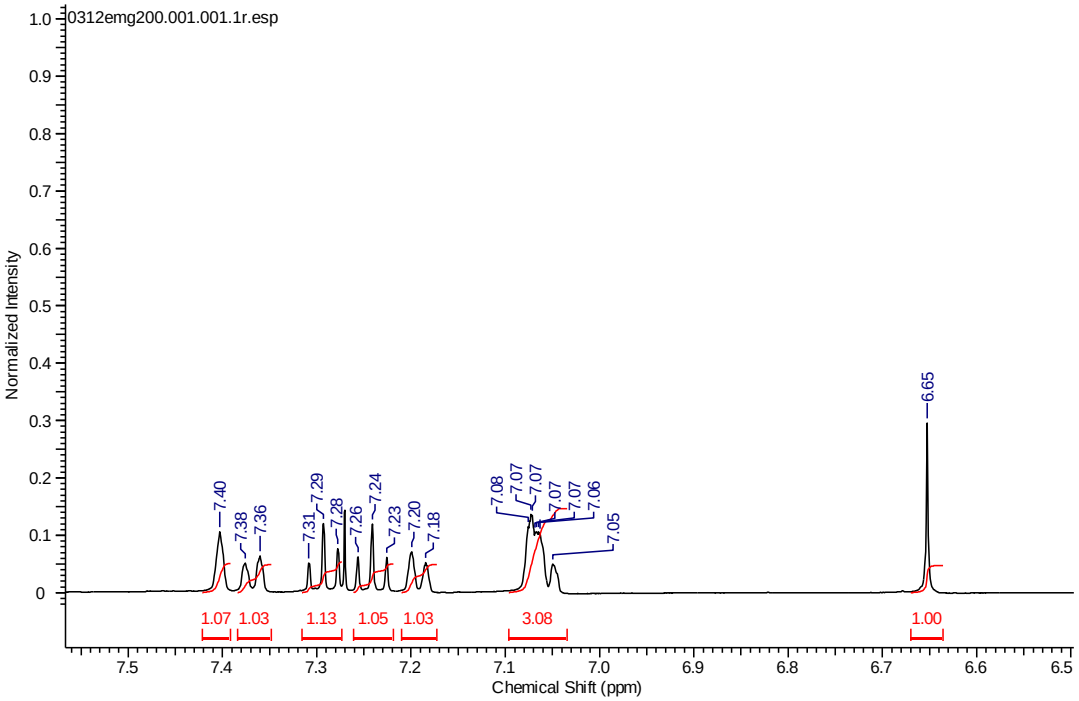

N
O


GA29.001.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
3.42
2.37
2.24
7.50
7.47
7.40
7.37
7.30
7.14
7.11
7.07
7.01
6.96
2.09
4.10
1.05
2.06
1.01
1.03
1.00
3.05
3.03
3.04

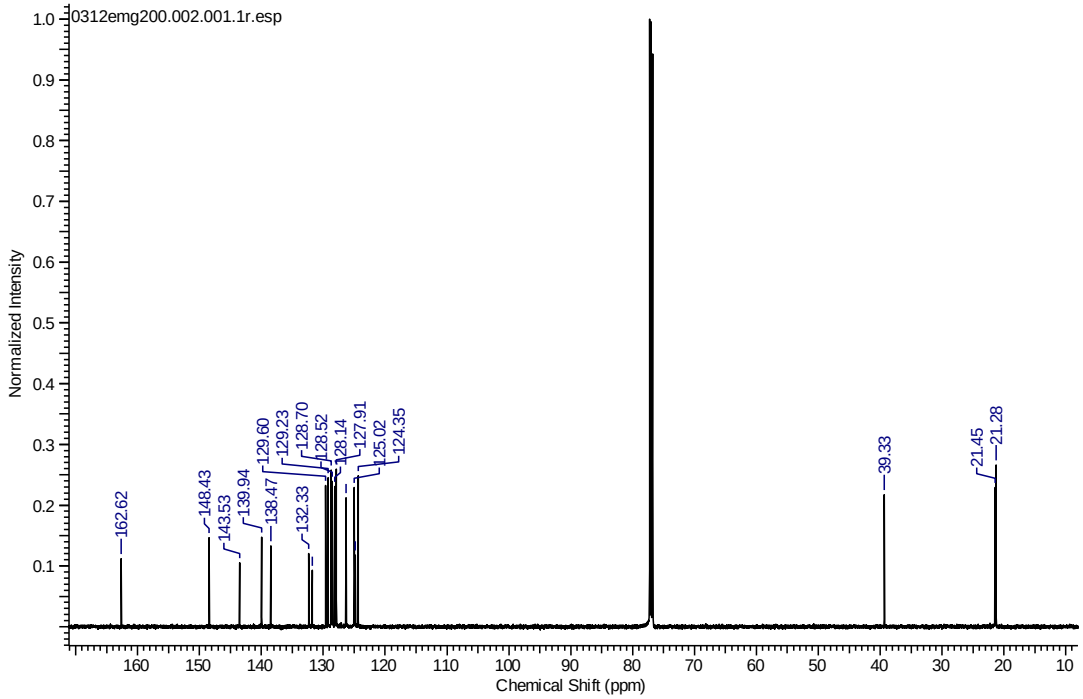

GA29.001.001.1r.esp
Normalized Intensity
7.50
7.48
7.47
7.42
7.40
7.39
7.37
7.33
7.30
7.28
7.18
7.16
7.16
7.14
7.11
7.07
7.01
7.00
6.98
6.98
6.96
6.96
6.95
6.93
6.93
6.93
2.09
4.10
1.05
2.06
1.01
1.03
1.00
Chemical Shift (ppm)
GA29.002.001.1r.esp
Normalized Intensity
170.29
146.31
142.81
139.97
139.36
130.21
129.83
129.74
129.36
128.48
127.98
126.87
125.22
38.57
21.51
21.27
Chemical Shift (ppm)

Compound **38**:

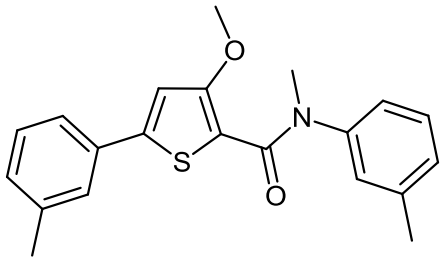

HO
S
N
O
0312emg200.001.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
7.40
7.29
7.24
7.08
7.07
7.07
6.65
3.39
2.37
2.35
1.07
1.03
1.13
1.05
1.03
3.08
1.00
3.12
3.15
3.11

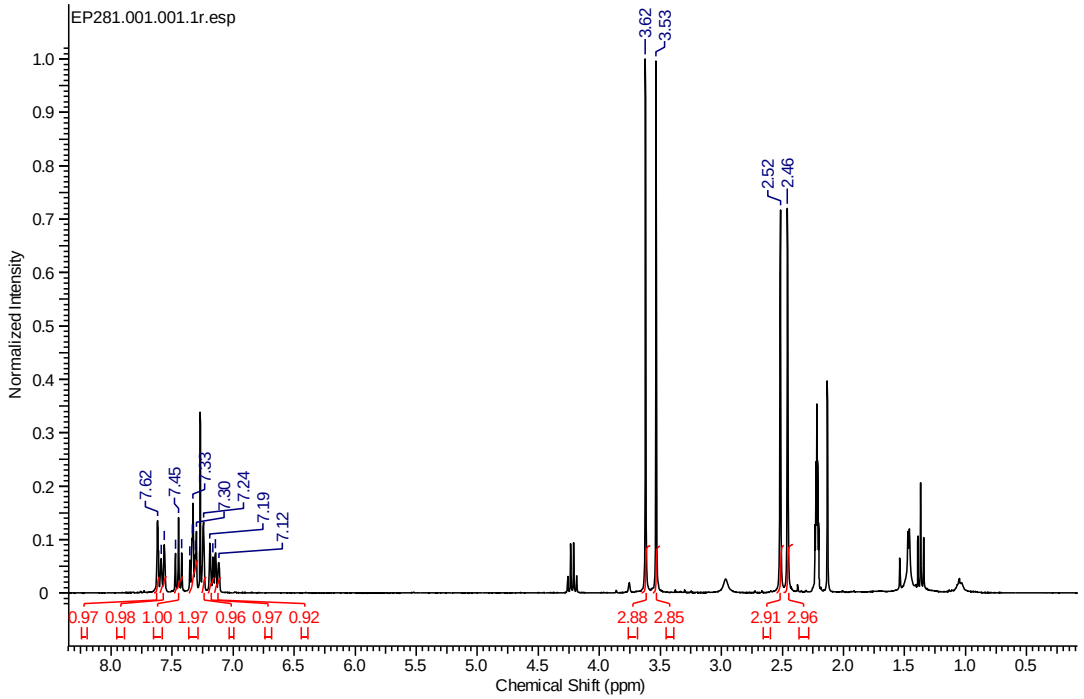

0312emg200.001.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
7.40
7.38
7.36
7.31
7.29
7.28
7.26
7.24
7.23
7.20
7.18
7.08
7.07
7.07
7.07
7.07
7.06
7.05
6.65
1.07
1.03
1.13
1.05
1.03
3.08
1.00

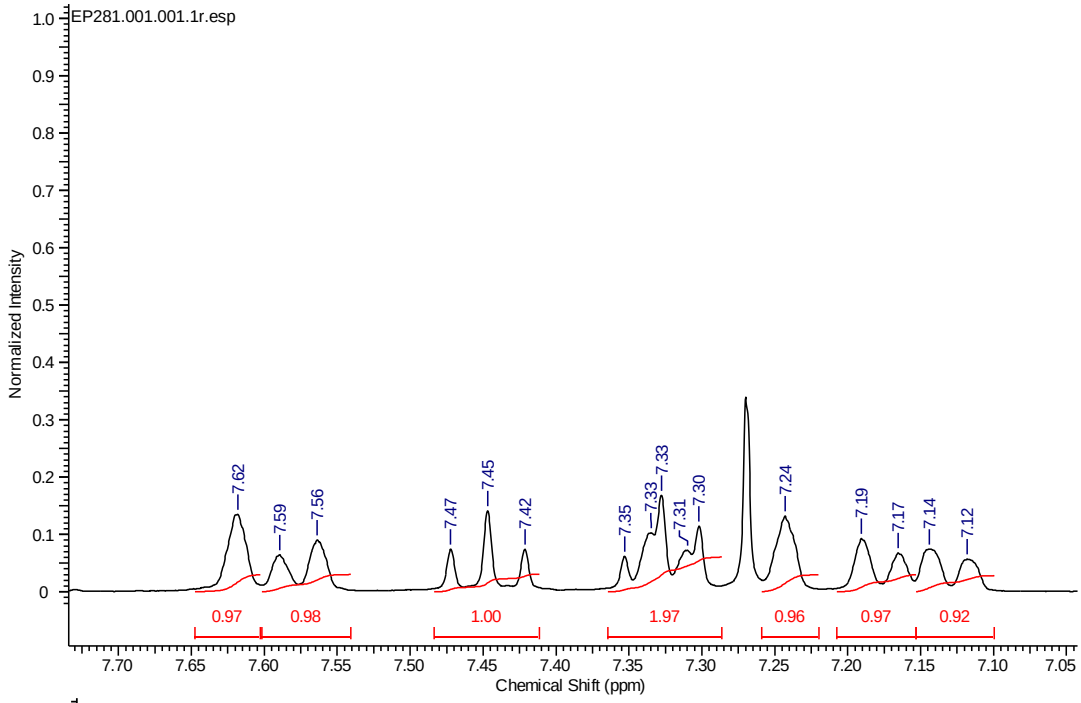
Results

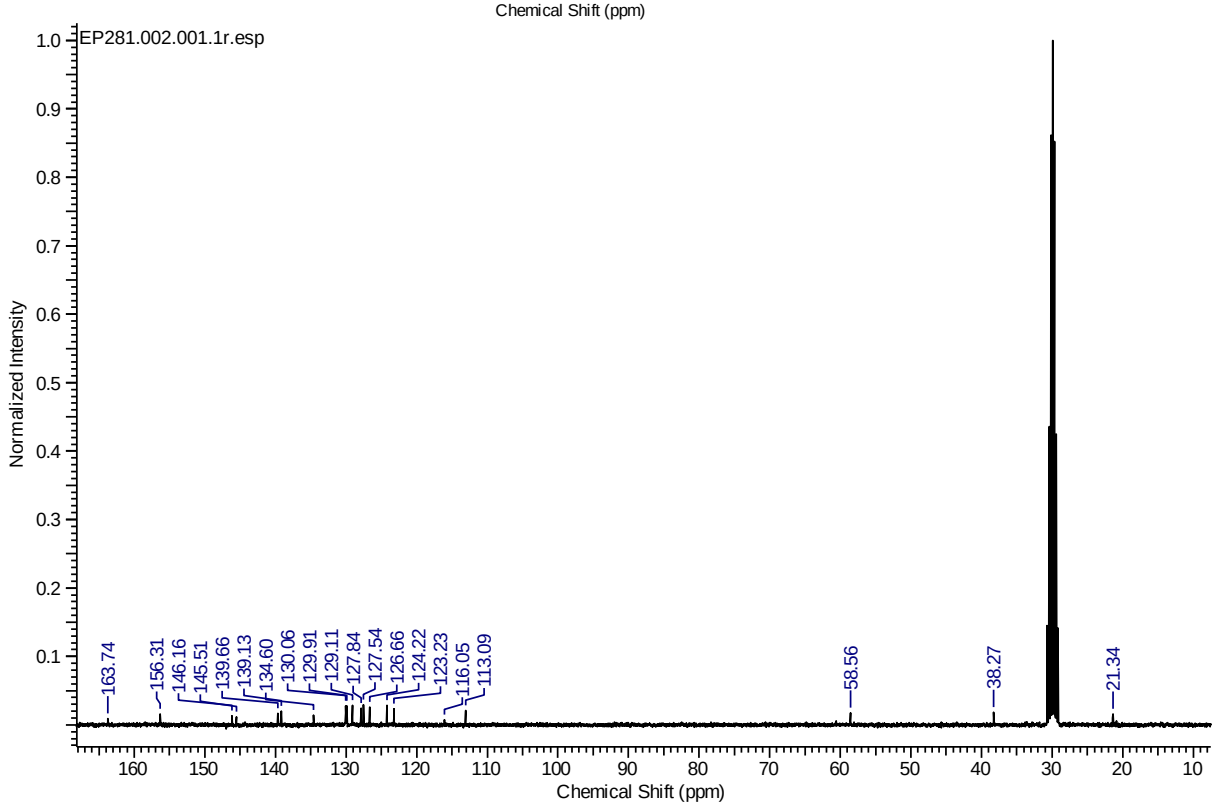


Compound **39a**:

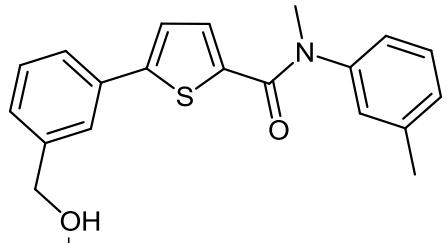


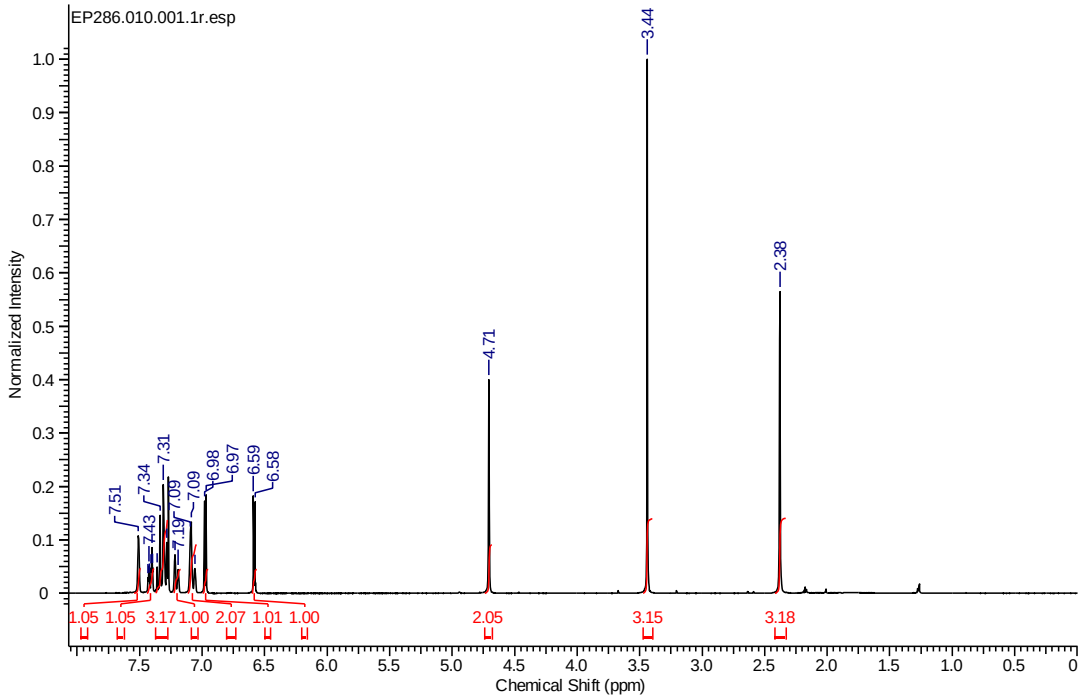



EP281.001.001.1r.esp
Normalized Intensity
7.62
7.59
7.56
7.47
7.45
7.42
7.35
7.33
7.33
7.31
7.30
7.24
7.19
7.17
7.14
7.12
0.97
0.98
1.00
1.97
0.96
0.97
0.92
Chemical Shift (ppm)


EP281.002.001.1r.esp
Normalized Intensity
163.74
156.31
146.16
145.51
139.66
139.13
134.60
130.06
129.91
129.11
127.84
127.54
126.66
124.22
123.23
116.05
113.09
58.56
38.27
21.34
Chemical Shift (ppm)

Compound **40**:


S
N
O
OH


EP286.010.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
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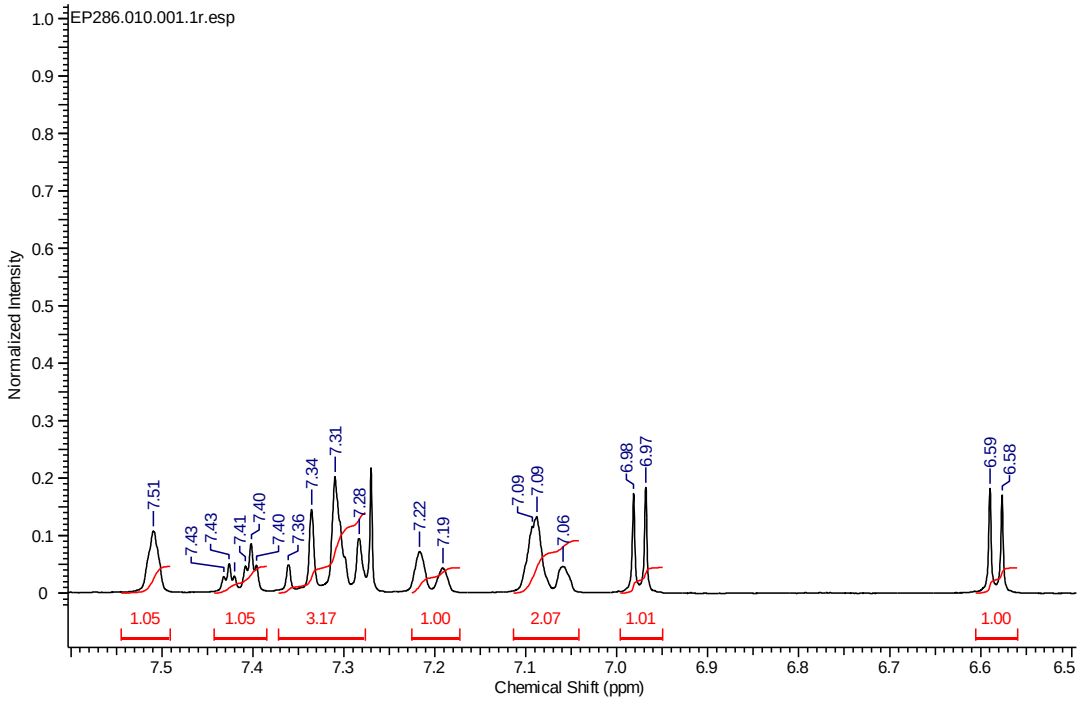
7.43
7.34
7.31
7.19
7.09
7.09
6.98
6.97
6.59
6.58
4.71
3.44
2.38
1.05 1.05 3.17 1.00 2.07 1.01 1.00
2.05
3.15
3.18


EP286.010.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
7.51
7.43
7.43
7.41
7.40
7.40
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7.31
7.28
7.22
7.19
7.09
7.09
7.06
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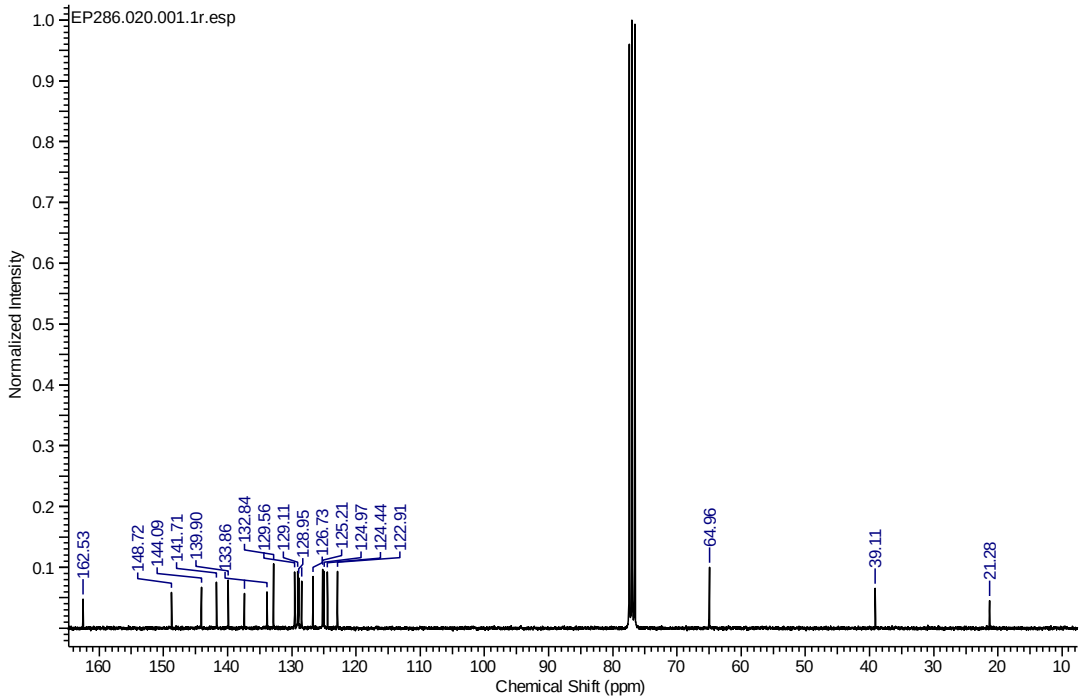
1.05
3.17
1.00
2.07
1.01
1.00



c) References

[1] S.Marchais-Oberwinkler, K.Xu, M. Wetzel, E.Perspicace, M.Negri, A. Meyer, A.Odermatt, G.Möller,J.Adamski, R. W. Hartmann, J. Med. Chem. 56 (2013) 167-181.

[2] E.Perspicace, L.Cozzoli, E. M.Gargano, N.Hanke, A.Carotti, R. W. Hartmann,Eur. J. Med. Chem. 83 (2014) 317-337.

2.2 17β-Hydroxysteroid Dehydrogenase Type 2 Inhibition: Discovery of Selective and Metabolically Stable Compounds Inhibiting Both the Human Enzyme and Its Murine Ortholog

Emanuele M. Gargano, Giuseppe Allegretta, Enrico Perspicace, Angelo Carotti, Chris Van Koppen, Martin Frotscher, Sandrine Marchais-Oberwinkler and Rolf W. Hartmann

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Paper II

Abstract

Design and synthesis of a new class of inhibitors for the treatment of osteoporosis and its comparative *h*17*β*-HSD2 and *m*17*β*-HSD2 SAR study are described. **17a** is the first compound to show strong inhibition of both *h*17*β*-HSD2 and *m*17*β*-HSD2, intracellular activity, metabolic stability, selectivity toward *h*17*β*-HSD1, *m*17*β*-HSD1 and estrogen receptors *α* and *β* as well as appropriate physicochemical properties for oral bioavailability. These properties make it eligible for pre-clinical animal studies, prior to human studies.

Introduction

Osteoporosis is a common, age-related disease, characterized by a systemic impairment of bone mass and microarchitecture, increasing bone fragility and risk of fractures[1]. It has been shown that the drop in 17β-estradiol (E2) and testosterone (T) levels, occurring with ageing, is the main factor driving the onset and progression of this disease[2]. 17β-Hydroxysteroid dehydrogenase type 2 (17*β*-HSD2) catalyzes the conversion of the highly active E2 and T into the weakly potent 17-ketosteroids estrone (E1) and Δ4-androstene-3,17-dione (Δ4-AD), respectively[3]. It is expressed in osteoblastic cells [4], therefore its inhibition can lead to the desired increase of E2 and T levels in the bone tissue and may thus be a novel approach for the treatment of osteoporosis.

Some steroidal[5-7] and non steroidal[8, 9] 17*β*-HSD2 inhibitors are already described. In our group we also developed and reported about several classes of non-steroidal 17*β*-HSD2 inhibitors[10-14], with a strong inhibition of human 17*β*-hydroxysteroid dehydrogenase type 2 (*h*17*β*-HSD2) and a good selectivity toward *h*17*β*-HSD1. Since *h*17*β*-HSD1 is the biological counterpart of *h*17*β*-HSD2, catalyzing the opposite conversion, selectivity toward this enzyme is an important feature to take into consideration. Potent and selective *h*17*β*-HSD1inhibitors have also been described for the treatment of estrogen-dependent diseases[15, 16].

Given that the most commonly used animal model for osteoporosis studies are established in rodents[17, 18], we aimed at the development of new inhibitors displaying a good inhibition of mouse 17*β*-hydroxysteroid dehydrogenase type 2 (*m*17*β*-HSD2) and a reasonable selectivity toward *m*17*β*-HSD1. In order to have a compound suitable for animal testing and following human studies, the designed inhibitors should also display *h*17*β*-HSD2 inhibitory activity and selectivity toward *h*17*β*-HSD1. Other characteristics to be implemented are preferably low affinity to the estrogen receptors (ERs) *α* and *β*in order tomaximize the E2 local effect and to minimize systemic adverse effects as well as metabolic stability.

Since the 3D-structure for both human and mouse 17*β*-HSD2 is up to date not available a ligand based approach was chosen for the design of new inhibitors. A set of *h*17*β*-HSD2 inhibitors was selected and tested for *m*17*β*-HSD2 inhibitory activity in order to get insight in the SAR and tracking the lead for the rational design of new inhibitors.

RESULTS AND DISCUSSION

*h*17*β*-HSD2, *h*17*β*-HSD1, *m*17*β*-HSD2 and *m*17*β*-HSD1 cell-free assays were performed similarly, by incubating enzyme, tritiated substrate, cofactor and inhibitor, according to described procedures[19-22].

As starting point 25 previously described *h*17*β*-HSD2 inhibitors (Fig. 1), belonging to the 2,5-thiophene amide, 1,3-phenyl amide and 1,4-phenyl amide class[12-14], were tested for *m*17*β*-HSD2 inhibition, in order to elaborate a comparative SAR and to develop an optimization strategy.

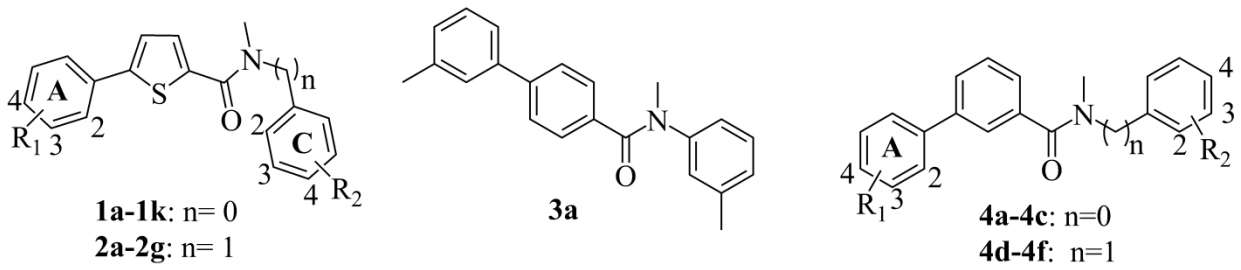

Fig 1. Previously described h17β-HSD2 inhibitors, tested form17β-HSD2 inhibition.

IC_{50} or percent of inhibition values for both *h*17*β*-HSD2 and *m*17*β*-HSD2 are given (Table 1, compounds **1a-2g**, **3a**, **4a-4f**) to facilitate comparison.

Table 1. Inhibition of Human and Mouse 17*β*-HSD2 by 2,5-Thiophene Amide, 1,3-Phenyl Amide and 1,4-Phenyl Amide Derivatives in a Cell-Free Assay.

			IC_{50} (nM)[a] or % inh. at 1 µM[a, d]	
Cmpd	R_1	R_2	*h*17*β*-HSD2[b]	*m*17*β*-HSD2[c]
1a	3-OMe	3-OMe	68	29%
1b	3-Me	3-Me	52	42%
1c	3-Me	3-OMe	58	30%
1d	2-OMe	3-OMe	490	19%
1e	3-OH	3-OH	33%	n.i.
1f	2-OH	3-OH	410	18%
1g	3-$N(Me)_2$	3-OMe	170	40%
1h	3-F	3-OMe	510	n.i.
1i	4-CN	3-OMe	48%	n.i.
1j	2-F, 3-OMe	3-OMe	62	26%
1k	2-F, 3-OMe	3-Me	62	45%
2a	2-F, 3-OMe	3-OH	61	65%
2b	3-OMe	3-OMe	370	16%
2c	3-OH	3-OH	390	26%
2d	4-OH	3-OH	330	35%
2e	3-Me	3-OH	160	45%
2f	3-F	3-OH	330	37%
2g	4-CN	3-OH	n.i.	n.i.
3a	3-Me	3-Me	1100	50%
4a	3-OMe	3-OMe	520	25%
4b	4-OMe	3-OMe	1200	11%
4c	3-OH	3-OH	35%	n.i.
4d	3-OMe	3-OMe	11%	n.i.
4e	3-OH	3-OH	640	29%
4f	4-OH	3-OH	480	22%

[a]Mean value of at least two determinations, standard deviation less than 20%. [b]Human placental microsomal fraction, substrate E2 [500 nM], cofactor NAD^+ [1500 µM]. [c]Mouse liver microsomal fraction, substrate E2 [500 nM], cofactor NAD^+ [1500 µM]. [d]n.i.: no inhibition, i.e., inhibition ≤10%.

Results

In the 2,5-thiophene amide class (compounds **1a-2g**), *h*17*β*-HSD2 inhibitors[13, 14], a broad range of inhibitory activities was detected, depending on the substitution pattern; the most active compounds show IC_{50} values around 60 nM (Table 1). Conversely, the inhibitory activity towards *m*17*β*-HSD2 was only marginally affected by these changes (inhibitory activity around 30% at 1 µM). Only compound **2a** shows a more pronounced *m*17*β*-HSD2 inhibition combined with good inhibition of the human enzyme (65% at 1 µM and IC_{50} = 61 nM). Unfortunately, compound **2a** turned out to be metabolically very unstable, with a half-life of only 4 minutes in the human liver S9 fraction[13]. In a further screening, all the tested 2,5-thiophene amide displayed a high metabolic instability [13].

It is striking that neither the nature of substituents on ring A and C or their substitution pattern does appear to exert an effect on *m*17*β*-HSD2 inhibition, whereas it is decisive for the *h*17*β*-HSD2 one. This result suggests that the inhibitors in this class are likely to have different binding modes in the two enzyme isoforms.

Exchange of the central thiophene by a 1,3-disubstituted phenyl led to compounds **4a-4c** for n = 0 and **4d-4f** for n = 1, with weak inhibitory activity towards both the human and the mouse enzyme (Table 1).

In contrast, compound **3a** (Table 1), bearing a 1,4-disubstituted phenyl moiety as central ring, shows moderate inhibition of both *h*17*β*-HSD2 and *m*17*β*-HSD2 and also revealed exceptional metabolic stability in the human liver S9 fraction, with a half-life time>120 minutes[13]. It was therefore taken as starting point for the design of a small library of inhibitors where the substitution pattern and the physicochemical nature of substituents on the A and C rings was varied (Fig. 2). Compounds **25a** and **25** were also synthesized to investigate the effect of the methylene linker between the amide function and the C ring.

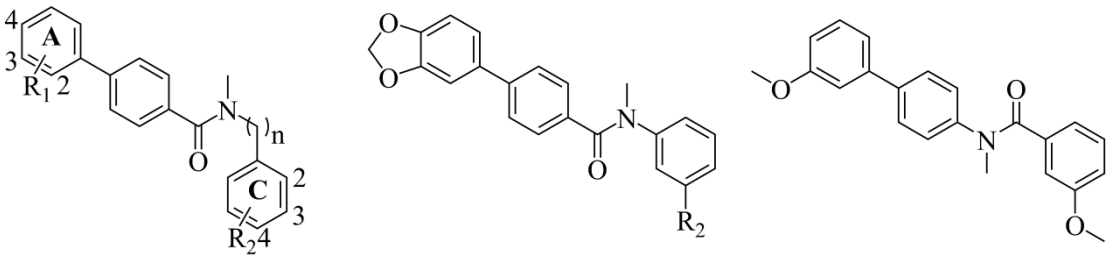

Fig. 2.Chemical Structures of the Designed Compounds

The synthesis of the 1,4-phenyl derivatives **5a-23a, 25a, 7**and **25**, depicted in Fig. 3, was accomplished following a two- or three-step reaction pathway.

First, amidation was carried out by reaction of the commercially available 4-bromobenzoyl chloride **5d** with substituted anilines **5c** or with the1-(3-methoxyphenyl)-*N*-methylmethanamine**25c**using standard conditions (method A: triethylamine, dichloromethane, from 0 °C to room temperature, overnight) affording the brominated

intermediates **5b, 6b** and **11b** in almost quantitative yields. Subsequently, Suzuki coupling (Method B) using tetrakis(triphenylphosphine)palladium and cesium carbonate in a mixture of DME/EtOH/H_2O (1:1:1,3 mL) as solvent and microwave irradiation (150°C, 150 W for 20 minutes), provided the biphenyl derivatives **5a-21a** and **25a**, and the 1,3-benzodioxole derivatives **22a** and **23a** in good yields. Compounds **7a** and **25a** were submitted to ether cleavage using boron trifluoride-dimethyl sulfide complex, yielding the hydroxylated molecules **7** and **25**.

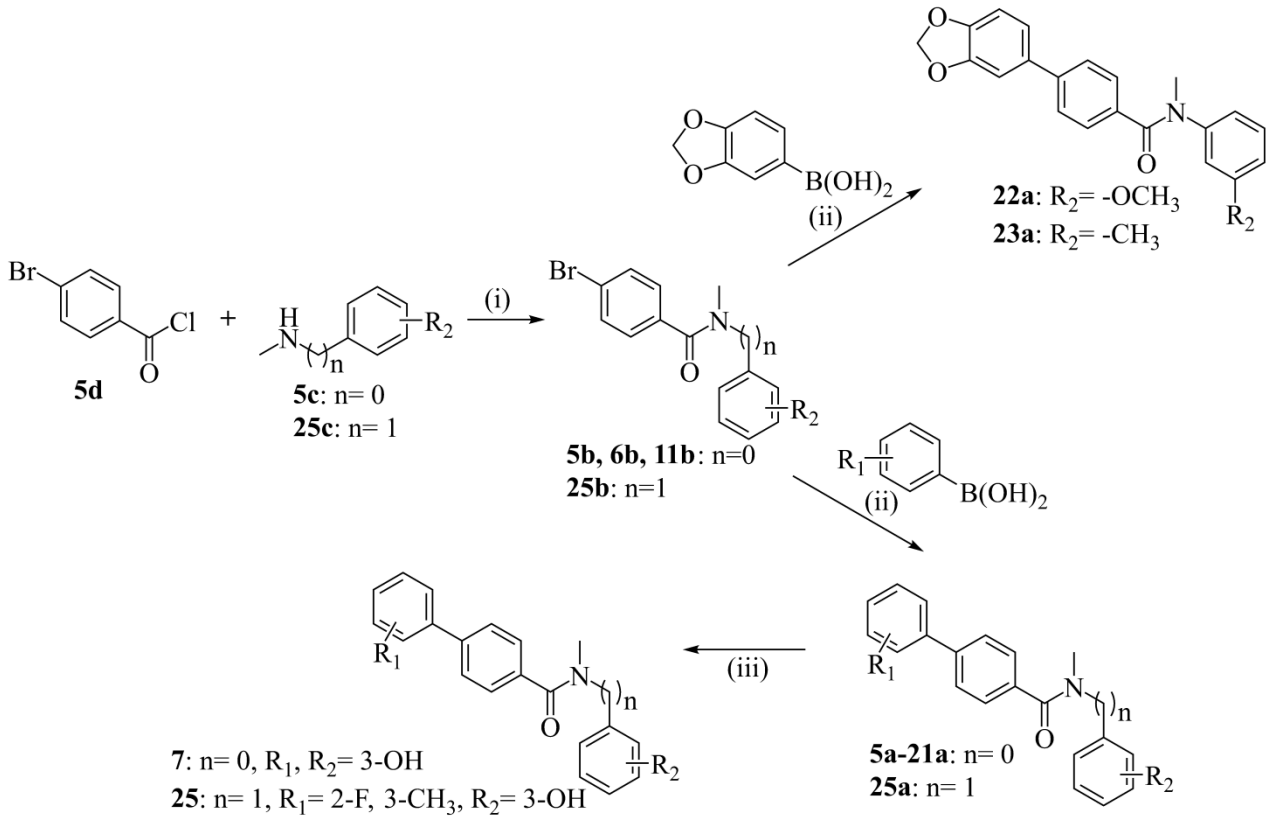

Fig. 3. Synthesis of 1,4-Phenyl Derivatives 5a-23a, 25a, 7, 25.
Reagents and conditions: (i) Et_3N, CH_2Cl_2, room temperature, overnight; (ii) DME/EtOH/H_2O (1:1:1), Cs_2CO_3, $Pd(PPh_3)_4$, microwave irradiation (150°C, 150W, 20 min); (iii) $BF_3{\cdot}SMe_2$, CH_2Cl_2, room temperature, overnight.

The synthesis of the retroamide**24a**, displayed in Fig. 4, follows a two-step procedure. First the commercially available 3-methoxybenzoyl chloride **24c** was reacted with 4-bromo-*N*-methyl aniline **24d** according to method A and afforded the brominated intermediate **24b** with 70% yield. Subsequently, Suzuki coupling following method B afforded compound **24a** in 68% yield.

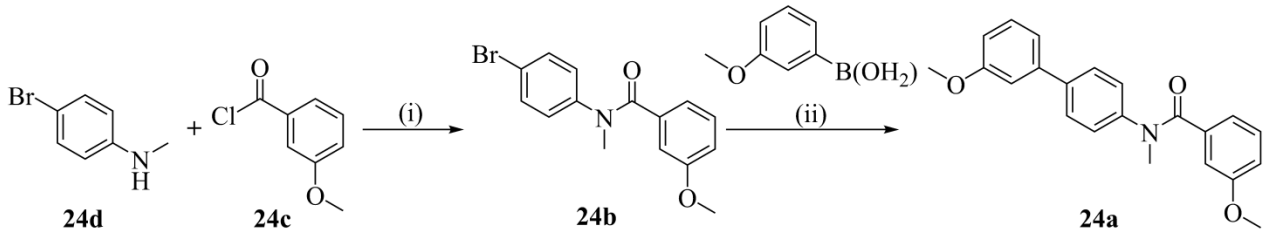

Fig. 4. Synthesis of 1,4-Phenyl Retroamide Derivative 24a.
Reagents and conditions: (i) Et_3N, CH_2Cl_2, room temperature, overnight; (ii) DME/EtOH/H_2O (1:1:1), Cs_2CO_3, $Pd(PPh_3)_4$, microwave irradiation (150°C, 150W, 20 min).

Compounds **5a-25a**, **7** and **25** were tested for *h*17*β*-HSD2, *m*17*β*-HSD2 and *h*17*β*-HSD1 inhibition (Table 2, results expressed as percentage of inhibition or IC_{50} value).

Compounds **18a** and **24a** were not tested for inhibition of *m*17*β*-HSD2, due to their low inhibitory activity towards the human enzyme. Among the different synthesized 1,4-biphenyl amides without methylene linker (n=0, Table 2, compounds **5a-21a**), the best *h*17*β*-HSD2 inhibitory activity and selectivity toward *h*17*β*-HSD1 was achieved for compounds **6a**, **7a** and **17a** (Table 2, IC_{50} values between 260 and 330nM, s.f. between 20 and 44), showing that a 3-OMe-group on ring C in combination with either a 3-OMe- or a 3-Me-group on ring A leads to a maximum in potency and selectivity in this series of compounds.

The presence of a methyl group in 4-position of ring A is tolerated by the *h*17*β*-HSD2 (compound **17a**, IC_{50} = 310nM) and increases the selectivity toward *h*17*β*-HSD1 (**17a**: s.f. 44; **6a**: s.f. 25). In contrast, compound **16a** bearing a 3,4-dimethoxy substituted A ring displays a slight decrease in *h*17*β*-HSD2 inhibitory activity if compared to the corresponding compound **7a** with only one methoxygroup on that ring. The rigidification of the two methoxy substituents by the synthesis of a 1,3-benzodioxole ring (compounds **22a** and **23a)** could not overcome the drop in potency.Compounds **6a**, **7a** and **17a** displayed the strongest *m*17*β*-HSD2 inhibitory activity (IC_{50} = 260, 290 and 140 nM, respectively).

In general, the introduction of methyl- or methoxy-groups, especially in the 3-positions of rings A and B, had a positive impact on inhibitory activity, which is similar for both 17*β*-HSD2 isoforms (Table 2). Therefore, inhibitors belonging to the 1,4-phenyl class are likely to bind in a conserved area common to both enzymes, in contrast to the 2,5-thiophene amide class. As 17*β*-HSD2 belongs to the SDR superfamily, characterized by the conserved Rossmann fold and catalytic triad[23], it is possible that these inhibitors bind in or very close to this region. Furthermore, since compounds **6a** and **17a** lack the two oxygen functions to mimic the E2 interactions with the enzyme, they are likely to bind to the active site in an alternative mode, significantly influenced by the methyl substitution patterns.

Table 2. Inhibition of *h*17*β*-HSD2, *m*17*β*-HSD2 and *h*17*β*-HSD1 by Biphenyl Amide Derivatives with Different Substitution Patterns on the A and C Rings in Cell-Free System.

			IC_{50} (nM)[a] or % inh. at 1 μM[a, c]			IC_{50} (nM)[a] or % inh. at 1 μM[a, c]
Cmpd	R_1	R_2	*h*17*β*-HSD2[b]	*h*17*β*-HSD1[c]	s. f.[d,f]	*m*17*β*-HSD2[f,g]
3a	3-Me	3-Me	1100	11500	10	50%
5a	3-OMe	3-Me	44%	n.i.	n.d.	56%
6a	3-Me	3-OMe	260	6400	25	260
7a	3-OMe	3-OMe	330	6400	20	290
8a	-H	3-OMe	51%	22%	n.d.	48%
9a	2-OMe	3-OMe	50%	n.i.	n.d.	33%
10a	4-OMe	3-OMe	710	27000	38	66%
11a	3-OMe	4-OMe	27%	n.i.	n.d.	58%
12a	4-OMe	4-OMe	19%	n.i.	n.d.	62%
7	3-OH	3-OH	36%	15%	n.d.	42%
13a	3-F	3-OMe	1000	8000	8	57%
14a	3-Cl	3-OMe	950	12600	13	73%
15a	3-$N(Me)_2$	3-OMe	650	5000	8	67%
16a	3-OMe,4-OMe	3-OMe	520	85500	164	52%
17a	3-Me,4-Me	3-OMe	300	13300	44	140
18a	3-F,4-F	3-OMe	31%	n.i.	n.d.	n.d.
19a	2-F, 3-OMe	3-OMe	64%	35%	n.d.	67%
20a	2-F, 3-Me	3-OMe	460	11300	24	48%
21a	2-F, 3-Me	3-Me	330	3710	11	56%
22a	-	3-OMe	51%	n.i.	n.d.	56%
23a	-	3-Me	560	10900	20	70%
24a	-	-	11%	n.i.	n.d.	n.d.
25a	2-F, 3Me	3-OMe	310	87000	283	43%
25	2-F, 3-Me	3-OH	260	31000	118	190

[a]Mean value of at least two determinations, standard deviation less than 20% except for 11a (hHSD2): 26%, 7(hHSD1):25%,. [b]Human placental, microsomal fraction, substrate E2[500 nM], cofactor NAD^+[1500 μM]. [c]Human placental, cytosolic fraction, substrate E1[500 nM], cofactor NADH[500 μM]. [d]s.f. (selectivity factor)=IC_{50}(17*β*-HSD1)/IC_{50}(17*β*-HSD2). [e]n.i.: no inhibition (inhibition of <10%).[f]n.d.: not determined. [g]Mouse liver microsomal fraction, substrate E2 [500 nM], cofactor NAD^+ [1500 μM].

Compounds with a methylene linker **25a** and **25** (Table 2), showed *h*17*β*-HSD2 inhibitory activity in the same order of magnitude as the corresponding derivative **20a** lacking the methylene group, but displayed improved selectivities over *h*17*β*-HSD1. In contrast to what was observed for human 17*β*-HSD2, compounds **25a** and **25** showed a significant difference in terms of *m*17*β*-HSD2 inhibition, indicating that the hydroxy group of compound **25** might function as H-bond donor in the interaction with the enzyme.

The 1,4-phenyl amides are likely to be competitive inhibitors, as derivatives with a similar structure were found to inhibit the enzyme following this mode of action.

The most potent *m*17*β*-HSD2 inhibitors **6a-8a**, **10a**, **14a**, **15a**, **17a**, **19a**, **21a**, **23a** and **25** were also tested for their *m*17*β*-HSD1 inhibitory activity. None of the tested compounds showed any inhibition of *m*17*β*-HSD1 at a concentration of 1 μM, indicating a strong selectivity toward this enzyme.

The inhibitory activity of compounds **6a**, **17a** and **25** was also evaluated in the human mammary cell line MDA-MB-231 containing endogenous 17*β*-HSD2 (Table 3). The compounds were tested at 250 nM and 1250 nM, representing approximately the IC_{50}observed in the cell-free assay, and its 5-fold value. As displayed in Table 3, all three compounds showed an inhibition between 60% and 67%at the lower concentration and approximately 90% inhibition at the higher concentratio indicating that the inhibitors can permeate the membrane and are able to inhibit the enzyme in a concentration dependent manner.

Table 3.Human 17*β*-HSD2 Cellular Inhibition of Compounds 6a, 17a and 25.

Cmpd	% inh[b] HSD2[a] at 250 nM	% inh[b] HSD2[a] at 1250 nM
6a	60	87
17a	66	90
25	67	88

[a]MDA-MB-231 cell line, substrate E2[200 nM]. [b]Mean value of two determinations, standard deviation less than 15%.

Compounds **6a**, **7a**, **17a**, **21a**, **25a** and **25** were tested for their affinity toward the ERs α and β according to described methods[24] (assay details are available in the Supporting Information).Even when applied in a 1000-fold excess relative to E2, no inhibitor was able to displace more than 20% of the steroid from the corresponding receptor, indicating a very low binding affinity to the ERs.

The metabolic stabilities of the most active compounds**6a**,**17a** and **25** were evaluated using human liver microsomes (S9 fraction). In addition, compounds **5a**, **14a**, **20a** and **25a** were also tested in order to investigate whether structure modifications might exert an effect on metabolic stability (Table 4, assay details are available in the Supporting Information). All

compounds, except **25**, revealed a very high stability, which was not influenced by the nature of the substituents. Compound **25,** exhibiting a short half-life, bears a hydroxy group, potentially susceptible to phase II metabolism. Interestingly, all the tested inhibitors from the 1,4-phenyl amide class, for n= 0, demonstrated high metabolic stability, which seemingly constitutes a positive feature of the whole class. As species differences for the metabolism of drugs that are not or only partly metabolized are usually small [25], sufficient metabolical stability in species other than human can be anticipated.

Table 4. Half-life in Human Liver Microsomes S9 Fraction of Representative Compounds 3a, 5a, 6a, 14a, 17a, 20a, 25a and 25.

Cmpd	R_1	R_2	Inhibitor[c] $t_{1/2}$(min)[a,b]
3a	3-Me	3-Me	>120
5a	3-OMe	3-Me	116
6a	3-Me	3-OMe	106
14a	3-Cl	3-OMe	82
17a	3-Me,4-Me	3-OMe	107
20a	2-F, 3-Me	3-OMe	104
25a	2-F, 3-Me	3-OMe	103
25	2-F, 3-Me	3-OH	6

[a]Mean of at least two determinations, standard deviation less than 25%. [b]1 mg/ml pooled mammalian liver S9 fraction (BD Gentest), 2 mM NADPH regenerating system, 1 mM UDPGA and 0.1 mM PAPS at 37°C for 0, 5, 15 and 60 minutes. [c]Inhibitor tested at a final concentration of 1 µM.

CONCLUSION

The aim of this work was the design of a compound, which should be suitable for application in both an animal model of osteoporosis and in humans. We report here the discovery of compound **17a**, which is the first to show an appropriate profile for this purpose, with strong inhibition of both human and mouse 17*β*-HSD2 and selectivity toward the respective type 1 enzymes and the ERs. It also displayed good cellular inhibitory activity, high metabolic stability and good physicochemical parameters (MW= 345 and cLogP = 4.75)predictor for good oral bio-availability[26]. A comparative SAR study for *h*17*β*-HSD2 and *m*17*β*-HSD2 is also described for the 1,4-phenyl and the 2,5-thiophene classes of inhibitors, revealing that only compounds belonging to the first series similarly inhibit the two enzymes, probably through a similar binding mode. The species specific characterization of the thiophene and the phenyl derivatives pointed out the superiority of the latter class of inhibitors, which is able to equally inhibit the two isoenzymes and additionally displays a high metabolic stability. *In vivo* assays in a mouse osteoporosis

model will be carried out soon and the results reported in due course in a specialized journal dealing with bone diseases.

Materials and Methods

Chemical Methods

Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Combi-Blocks or Fluorochem and were used without purification.Column chromatography was performed on silica gel (70-200 μm) and reaction progress was monitored by TLC on Alugram SIL G/UV254 (Macherey-Nagel). Visualization was accomplished with UV light.^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM500 spectrometer (at 500 MHz and 125 MHz, respectively) at 300 K and on Bruker Fourier 300 (at 300 MHz and 75 MHz, respectively) at 300K. Chemical shifts are reported in δ(parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard: 2.05 ppm (^{1}H NMR) and 29.8 and 206.3 ppm (^{13}C NMR) for CD_3COCD_3, 7.26 ppm (^{1}H NMR) and 77.0 ppm (^{13}C NMR) for $CDCl_3$. Signals are described as br (broad), s (singlet), d (doublet), t (triplet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplets) and m (multiplet). All coupling constants (*J*) are given in Hertz (Hz).

Melting points (mp) were measured in open capillaries on a Stuart Scientific SMP3 apparatus and are uncorrected.

Mass spectrometry was performed on a TSQ Quantum (ThermoFisher, Dreieich, Germany). The triple quadrupole mass spectrometer was equipped with an electrospray interface (ESI). The Surveyor-LC-system consisted of a pump, an auto sampler, and a PDA detector. The system was operated by the standard software Xcalibur. A RP C18 NUCLEODUR 100-5 (3 mm) column (Macherey-Nagel GmbH, Dühren, Germany) was used as stationary phase. All solvents were HPLC grade. In a gradient run(acetonitrile/water) the percentage of acetonitrile (containing 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 needle voltage of 3000 V and a capillary temperature of 350 °C. Mass spectra were acquired in positive mode from 100 to 1000 m/z and UV spectra were recorded at the wave length of 254 nm and in some cases at 360 nm.

All microwave irradiation experiments were carried out in a 507 CEM-Discover microwave apparatus.

All tested compounds exhibited ≥ 95% chemical purity as measured by LC/MS.

The following compounds were prepared according to previously described procedures: 4-bromo-*N*-methyl-*N*-(*m*-tolyl)benzamide**7a**[13].

Method A, general procedure for amide formation

To a solution of bromobenzoylchloride (2 mmol) was added the corresponding *N*-methylaniline (2 mmol) followed by Et_3N (2 mmol) in CH_2Cl_2 (10 mL) at 0°C. After a few minutes, the ice bath was removed and the reaction mixture was warmed up to room temperature and stirred at room temperature overnight. The reaction mixture was extracted twice with CH_2Cl_2 (2 × 15 mL). The organic layer was dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by silica gel column chromatography using hexanes and EtOAc as eluent or by trituration in a mixture of diethyl ether / petroleum ether to afford the desired compound.

Method B, general procedure for Suzuki-Miyaura coupling

In a sealed tube the previously prepared bromo-*N*-heteroarylcarboxamide derivative (1 eq.) was introduced, followed by the corresponding boronic acid (1.5 eq.), cesium carbonate (3 eq.), tetrakis(triphenylphosphine)palladium (0.02 eq.) and a mixture of DME/EtOH/H_2O (1:1:1, v:v:v, 3 mL) as solvent. The reactor was flushed with N_2 and submitted to microwave irradiation (150°C, 150 W) for 20 minutes. After cooling to room temperature, a mixture of EtOAc/H_2O (1:1, v:v, 2 mL) was added to stop the reaction. The aqueous layer was extracted with EtOAc (3 × 10 mL). The organic layer was washed once with brine and once with water, dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by column chromatography using hexanes and EtOAc as eluent to afford the desired compound.

Detailed synthesis procedures of the compounds

4-Bromo-*N*-(4-methoxyphenyl)-*N*-methylbenzamide(11b)The title compound was prepared by reaction of 4-bromobenzoyl chloride(**5c**)(1272 mg, 5.8mmol) and 4-methoxy-*N*-methylaniline(**11d**) (400 mg, 2.9mmol) according to method A. The residue was purified by silica gel column chromatography (hexanes/EtOAc80:20) to afford the desired product as yellow solid (932 mg, 99%). $C_{15}H_{14}BrNO_2$; MW 320; mp: 61 – 62°C; MS (ESI) 320, 322 $[M]^+$; IR (cm^{-1}) 1635, 2840, 2850, 2917;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 3.36 (s, 3H), 3.74 (s, 3H), 6.82 (d, *J* = 9.0 Hz, 2H), 7.09 (d, *J* = 9.0 Hz, 2H), 7.23 (d, *J* = 8.0 Hz, 2H), 7.38 (d, *J* = 8.0 Hz, 2H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ (ppm) 38.5, 55.7, 115.2, 123.7, 129.3, 131.4, 131.6, 137.1, 138.6, 159.1, 169.5.

4-Bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide (**6b**)The title compound was prepared by reaction of 4-bromobenzoyl chloride(**5c**) (1272 mg, 5.8mmol) and 3-methoxy-*N*-methylaniline(**6d**) (400 mg, 2.9mmol) according to method A. The residue was purified by silica gel column chromatography (hexanes/EtOAc80:20) to afford the desired product as yellow solid (935 mg, quantitative). $C_{15}H_{14}BrNO_2$; MW 320; mp: 98 – 100°C; MS (ESI) 320, 322 $[M]^+$; IR (cm^{-1}) 1638, 2840, 2945, 3012;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 3.40 (s, 3H), 3.71 (s, 3H), 6.70 – 6.72 (m, 1H), 6.74 – 6.78 (m, 2), 7.17 (t,*J* = 8.0 Hz, 1H), 7.26 – 7.28 (m, 2H), 7.39 – 7.41 (m, 2H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ (ppm) 38.2, 55.7, 113.2, 113.8, 120.2, 123.9, 130.7, 131.3, 131.6, 137.0, 147.0, 161.2, 169.4.

4-Bromo-*N*-methyl-*N*-(*m*-tolyl)benzamide (**5b**)The title compound was prepared by reaction of 4-bromobenzoyl chloride(**5c**) (724 mg, 3.3mmol) and *N*-methyl-*m*-toluidine(**5d**) (200 mg, 1.6mmol) according to method A. The residue was purified by silica gel column chromatography (hexanes/EtOAc80:20) to afford the desired product as white solid (500 mg, 99%). $C_{15}H_{14}BrNO$; MW 304; mp: 90 – 92 °C; MS (ESI) 304, 306 $[M]^+$; IR (cm^{-1}) 1586, 1638, 2853, 2923, 3058;^{1}H NMR (C_2D_6CO, 300 MHz) δ (ppm) 2.24 (s, 3H), 3.39 (s, 3H), 6.90 – 6.93 (d, *J* = 8.0Hz, 1H), 6.99 – 7.05 (m, 2H), 7.14 (t,*J* = 8.0 Hz, 1H), 7.23 – 7.26 (m, 2H), 7.37 – 7.40 (m, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ (ppm) 21.2, 38.4, 123.9, 125.2, 128.1, 128.5, 129.8, 131.4, 131.6, 137.0, 140.0, 145.8, 169.4.

4-Bromo-*N*-(3-methoxybenzyl)-*N*-methylbenzamide (**25b**)The title compound was prepared by reaction of 4-bromobenzoyl chloride(**5c**) (579 mg, 2.6mmol) and 3-methoxy-*N*-methylbenzylamine(**25d**) (200 mg, 1.3mmol) according to method A. The residue was purified by silica gel column chromatography (hexanes/EtOAc80:20) to afford the desired product as yellow oil(430 mg, 97%).$C_{16}H_{16}BrNO_2$; MW 334; MS (ESI) 334, 336 $[M]^+$; IR (cm^{-1}) 1630, 2834, 2923;^{1}H NMR (C_2D_6CO, 500 MHz) δ(ppm) 2.91 (s, 3H), 3.79 (s, 3H), 4.52 – 4.70 (m, 2H), 6.76-6.80 (m, 1H), 6.86 (dd, *J* = 8.8 Hz, 5Hz, 1H), 6.95 (s, 1H), 7.28 (t, *J* = 8.0 Hz, 1H), 7.44 (d, *J* = 7.6 Hz, 2H), 7.62 (s, 2H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ(ppm) 37.2, 51.0, 55.5, 113.3, 113.6, 114.4, 119.7, 121.0, 123.9, 129.9, 130.5, 132.3, 137.0, 161.1.

***N*-(4-bromophenyl)-3-methoxy-*N*-methylbenzamide** (**24b**)The title compound was prepared by reaction of 3-methoxybenzoyl chloride(**24c**) (336 mg, 2.0mmol) and 4-bromo-*N*-methylaniline(**24d**) (367 mg, 2.0mmol) according to method A. The residue was purified by silica gel column chromatography (hexanes/EtOAc80:20) to afford the desired product as white solid (440 mg, 70%). $C_{15}H_{14}BrNO_2$; MW 320; mp: 97 – 100°C; MS (ESI) 320, 322 $[M]^+$; IR (cm^{-1}) 1634, 2834, 2929, 3009;^{1}H NMR (C_2D_6CO, 300 MHz) δ(ppm) 3.41 (s, 3H), 3.69 (s, 3H), 6.84 – 6.88 (m, 3H), 7.12 – 7.17 (m, 3H), 7.44 – 7.47 (m, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ(ppm) 38.3, 55.6, 114.8, 116.3, 120.1, 121.7, 129.8, 130.0, 133.1, 138.8, 145.7, 160.1, 170.3.

***N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide** (**8a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**)(80 mg, 0.25mmol), phenylboronic acid (40 mg, 0.33mmol), cesium carbonate (244 mg, 0.75mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) in DME/EtOH/H_2O (1/1/1/, 3 mL) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow solid (77 mg, 97%). $C_{21}H_{19}NO_2$; MW 317; mp: 102 – 104°C; MS (ESI) 318 $[M+H]^+$; IR (cm^{-1}) 1588, 1636, 2831, 2917, 2960, 3040;^{1}H NMR (C_2D_6CO, 300 MHz) δ (ppm) 3.44 (s, 3H), 3.70 (s, 3H), 6.72 – 6.77 (m, 2H), 6.80 (t,*J*= 2 Hz, 1H), 7.17 (t,*J* = 8 Hz, 1H), 7.33 – 3.37 (s, 1H), 7.41 – 7.46 (m, 4H), 7.51 – 7.54 (m, 2H), 7.60 – 7.63 (m, 2H); ^{13}C NMR (C_2D_6CO, 75

MHz) δ (ppm) 38.4, 55.7, 113.0, 113.8, 120.1, 126.9, 127.7, 128.6, 129.8, 130.1, 130.6, 136.7, 140.8, 142.6, 147.4, 161.2, 170.2.

2'-Methoxy-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**9a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (112 mg, 0.35mmol), 2-methoxyphenylboronic acid (70 mg, 0.46mmol), cesium carbonate (342 mg, 1.05mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 8 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as colorless oil (113 mg, 93%). $C_{22}H_{21}NO_3$; MW 347; MS (ESI) 348 $[M+H]^+$; IR (cm^{-1}) 1597, 1640, 2837, 2939;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 3.44 (s, 3H), 3.69 (s, 3H), 3.77 (s, 3H), 6.73 – 6.78 (m, 3H), 6.99 (dt, *J* = 1.0, 7.3 Hz, 1H), 7.07 (dd, *J* = 1.0, 8.3 Hz, 1H), 7.18 (dt, *J* = 1.0, 7.5 Hz, 1H), 7.24 (dd, *J* = 2.0, 7.5 Hz, 1H), 7.30 – 7.34 (m, 1H), 7.35 – 7.39 (m, 4H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ (ppm) 38.4, 55.6, 55.9, 112.5, 113.0, 113.7, 120.0, 121.7, 129.1, 129.5, 130.0, 130.4, 130.6, 131.3, 136.0, 140.7, 147.4, 157.5, 161.1, 170.4.

3'-Methoxy-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**7a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (81 mg, 0.25mmol), 3-methoxyphenylboronic acid (46 mg, 0.3mmol), cesium carbonate (247 mg, 0.75mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as white solid (74 mg, 85%). $C_{22}H_{21}NO_3$; MW 347; mp: 131– 134°C; MS (ESI) 348 $[M+H]^+$; IR (cm^{-1}) 1582, 1635, 2837, 2941, 2963;^{1}H NMR (C_2D_6CO, 500 MHz) δ(ppm) 3.43 (s, 3H), 3.70 (s, 3H), 3.84 (s, 3H), 6.73 – 6.76 (m, 2H), 6.80 – 6.81 (m, 1H), 6.92(ddd, *J* = 1.0, 2.0, 8.0 Hz, 1H), 7.14 – 7.19 (m, 3H), 7.32 – 7.35 (m, 1H), 7.41 – 7.42 (m, 2H), 7.51 – 7.53 (m, 2H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ(ppm) 38.4, 55.6, 55.7, 113.0, 113.1, 113.8, 114.3, 120.0, 120.1, 126.9, 130.0, 130.6, 130.8, 136.8, 142.2, 142.5, 147.37, 147.38, 161.2, 170.2.

3'-Hydroxy-*N*-(3-hydroxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**7**)To a solution of 3'-methoxy-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**7a**)(100 mg, 0.29mmol) in CH_2Cl_2 (10 mL) was added trifluorobromine dimethyl sulphide complex $BF_3.SMe_2$ (0.36 mL, 3.46 mM) at room temperature. The reaction mixture was stirred overnight. The reaction was quenched with MeOH (10 mL). The solvent was removed under reduced pressure at 25°C. Water was added to dissolve inorganic salts and the precipitate was filtered off and triturated with diethyl ether to afford the desired product as white solid (80 mg, 87%). $C_{20}H_{17}NO_3$; MW 319; mp: 168 – 171°C; MS (ESI) 320$[M+H]^+$; IR (cm^{-1}) 1589, 3191;^{1}H NMR (C_2D_6CO, 300 MHz) δ(ppm) 3.42 (s, 3H), 6.64 – 6.68 (m, 3H), 6.82 – 6.86 (m, 1H), 7.07 – 7.12 (m, 3H), 7.26 (t, *J* = 8.0 Hz, 1H), 7.39 – 7.44 (m, 2H), 7.47 – 7.49 (m, 2H), 8.42 (s, 1H), 8.48 (s, 1H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ(ppm) 38.4, 114.4, 114.6, 115.0, 115.6, 119.0, 119.1, 126.8, 130.1, 130.7, 130.8, 136.6, 142.3, 142.7, 147.4, 158.8, 158.9, 170.1.

4'-Methoxy-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**10a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (123 mg, 0.38mmol), 4-methoxyphenylboronic acid (75 mg, 0.49mmol), cesium carbonate (371 mg, 1.14mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 9 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc80:20) to afford the desired product as white solid (70 mg, 53%). $C_{22}H_{21}NO_3$; MW 347; mp: 128 – 130°C; MS (ESI) 348 $[M+H]^+$; IR (cm^{-1}) 1601, 1625, 2840, 2935, 2963;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 3.43 (s, 3H), 3.70 (s, 3H), 3.82(s, 3H), 6.72 – 6.75 (m, 2H), 6.78 – 6.79 (m, 1H), 6.98 (d, *J* = 2.3 Hz, 1H), 7.00 (d, *J* = 2.3 Hz, 1H), 7.15 – 7.18 (m, 1H), 7.37 – 7.40 (m, 2H), 7.46 – 7.48 (m, 2H), 7.55 – 7.58 (m,2H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ (ppm) 38.4, 55.6, 55.7, 112.9, 113.8, 115.2, 120.1, 126.3, 128.8, 130.1, 130.6, 130.1, 135.9, 142.4, 147.5, 160.7, 161.2, 170.3.

3'-Fluoro-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**13a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (100 mg,0.31mmol), 3-fluorophenylboronic acid (57 mg, 0.41mmol), cesium carbonate (303 mg, 0.93mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 7 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow solid (91 mg, 89%). $C_{21}H_{18}FNO_2$; MW 335; mp: 97 – 100°C; MS (ESI) 336 $[M+H]^+$; IR (cm^{-1}) 1587, 1637, 2834, 2923, 2960;^{1}H NMR (C_2D_6CO, 300 MHz) δ (ppm) 3.44 (s, 3H), 3.71 (s, 3H), 6.73 – 6.77 (m, 2H), 6.80 – 6.81 (m, 1H), 7.09 – 7.21 (m, 2H), 7.36 – 7.50 (m, 5H), 7.55 – 7.57 (m, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ (ppm) 38.4, 55.8, 113.1, 113.9, 114.3, 114.6, 115.2, 115.4, 120.2, 123.7, 127.1, 130.2, 130.8, 131.6, 131.8, 137.4, 141.2, 143.3, 143.4, 147.4, 161.3, 162.6, 165.8, 170.1.

3'-Chloro-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**14a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (80 mg, 0.25mmol), 3-chlorophenylboronic acid (52 mg, 0.33mmol), cesium carbonate (244 mg, 0.75mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as colorless oil (47 mg, 53 %). $C_{21}H_{18}ClNO_2$; MW 352; MS (ESI) 352, 354 $[M]^+$; IR (cm^{-1}) 1594, 1639, 2837, 2938;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 3.43 (s, 3H), 3.70 (s, 3H), 6.73 – 6.76 (m, 2H), 6.80 – 6.81(m, 1H), 7.17 (t,*J* = 8 Hz, 1H), 7.38 – 7.40 (m, 1H), 7.43 – 7.47 (m, 3H), 7.54 – 7.59 (m, 3H), 7.63 – 7.64 (m, 1H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ (ppm) 38.3, 55.7, 113.1, 113.8, 120.2, 126.3, 127.0, 127.6, 128.5, 130.1, 130.7, 131.4, 135.3, 137.4, 141.0, 142.9, 147.3, 161.2, 170.0.

***N*-(3-methoxyphenyl)-*N*,3'-dimethyl-[1,1'-biphenyl]-4-carboxamide** (**6a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (77 mg, 0.24mmol), 3-methylphenylboronic acid (42 mg, 0.31mmol),

cesium carbonate (235 mg, 0.72mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as colorless oil (56 mg, 71 %). $C_{22}H_{21}NO_2$; MW 331; MS (ESI) 332 $[M+H]^+$; IR (cm^{-1}) 1586, 1640, 2840, 2929;^{1}H NMR (C_2D_6CO, 300 MHz) δ(ppm) 3.38 (s, 3H), 3.44 (s, 3H), 3.70 (s, 3H), 6.74 – 6.76 (m, 2H), 6.79 – 6.80 (m, 1H), 7.15 – 7.20 (m, 2H), 7.31 (t,*J* = 8 Hz, 1H), 7.39 – 7.43 (m, 4H), 7.49 – 7.52 (m, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ(ppm) 21.5, 38.4, 55.7, 113.0, 113.9, 120.2, 124.9, 126.9, 128.5, 129.4, 129.8, 130.1, 130.7, 136.6, 137.4, 140.9, 142.9, 147.5, 161.3, 170.3.

3'-(Dimethylamino)-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**15a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**)(80 mg, 0.25mmol), 3-(dimethylamino)phenylboronic acid (54 mg, 0.33mmol), cesium carbonate (244 mg, 0.75mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow oil (49 mg, 54%). $C_{23}H_{24}N_2O_2$; MW 360; MS (ESI) 361 $[M+H]^+$; IR (cm^{-1}) 1597, 1639, 2797, 2834, 2939;^{1}H NMR (C_2D_6CO, 300 MHz) δ (ppm) 2.97 (s, 6H), 3.43 (s, 3H), 3.70 (s, 3H), 6.72 – 6.76 (m, 3H), 6.79 –-6.80 (m, 1H), 6.86 – 6.93 (m, 2H), 7.17 (t,*J* = 8.0 Hz, 1H), 7.23 (t, *J* = 8.0 Hz, 1H), 7.38 – 7.41 (m, 2H), 7.49 – 7.52 (m, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ (ppm) 38.5, 40.8, 55.7, 111.8, 113.0, 113.9, 116.0, 120.2, 127.0, 130.0, 130.4, 130.7, 136.5, 141.6, 143.9, 147.5, 152.2, 161.3, 170.4.

***N*-(3-methoxyphenyl)-*N*,3',4'-trimethyl-[1,1'-biphenyl]-4-carboxamide** (**17a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (100 mg, 0.31mmol), 3,4-dimethylphenylboronic acid (62 mg, 0.41mmol), cesium carbonate (303 mg, 0.93mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 7 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow oil (106 mg, 99%). $C_{23}H_{23}NO_2$; MW 345; MS (ESI) 346$[M+H]^+$; IR (cm^{-1}) 1599, 1640, 2840, 2914, 2942;^{1}H NMR (C_2D_6CO, 500 MHz) δ(ppm) 2.26 (s, 3H), 2.29 (s, 3H), 3.43 (s, 3H), 3.70 (s, 3H), 6.73 – 6.75 (m, 2H), 6.79 –-6.80 (m, 1H), 7.15 – 7.19 (m, 2H), 7.32 (dd, *J* = 2.0Hz, 8.0Hz, 1H), 7.38 – 7.40 (m, 3H), 7.47 – 7.49 (m, 2H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ(ppm) 19.4, 19.8, 38.4, 55.6, 112.9, 113.8, 120.1, 125.0, 126.5, 128.8, 130.0, 130.6, 131.0, 136.2, 137.0, 137.8, 138.3, 142.8, 147.4, 161.2, 170.3.

3',4'-Difluoro-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**18a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (100 mg, 0.31mmol), 3,4-difluorophenylboronic acid (65 mg, 0.41mmol), cesium carbonate (303 mg, 0.93mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 7 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow oil

(97 mg, 88%). $C_{21}H_{17}F_2NO_2$; MW 353; MS (ESI) 354$[M+H]^+$; IR (cm^{-1}) 1599, 1639, 2837, 2948;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 3.43 (s, 3H), 3.70 (s, 3H), 6.73 – 6.76 (m, 2H), 6.80 –-6.81 (m, 1H), 7.17 (t, *J* = 8.0 Hz, 1H), 7.35 – 7.41 (m, 1H), 7.42 – 7.44 (m, 2H), 7.45 – 7.48 (m, 1H), 7.52 – 7.54 (m, 2H), 7.56 (dd, *J* = 2.0 Hz, 8.0 Hz, 1H), 7.60 (dd, *J* = 2.0 Hz, 8.0 Hz, 1H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ (ppm) 38.3, 55.7, 113.0, 113.8, 116.6, 116.7, 118.5, 118.7, 120.2, 124.3, 124.4, 126.9, 130.1, 130.7, 137.3, 140.3, 147.3, 161.2, 170.0.

3',4'-Dimethoxy-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**16a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (100 mg, 0.31mmol), 3,4-dimethoxyphenylboronic acid (75 mg, 0.41mmol), cesium carbonate (303 mg, 0.93mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 7 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as white solid (83 mg, 71%). $C_{23}H_{23}NO_4$; MW 377; mp: 153 – 156°C; MS (ESI) 378$[M+H]^+$; IR (cm^{-1}) 1604, 1635, 2843, 2938, 2969, 3003, 3068;^{1}H NMR (C_2D_6CO, 300 MHz) δ(ppm) 3.51 (s, 3H), 3.69 (s, 3H), 3.90 (s, 3H), 3.92 (s, 3H), 6.62 – 6.72 (m, 3H), 6.89 – 6.91 (d, *J* = 8.0 Hz, 1H), 7.04 – 7.17 (m, 3H), 7.38 (s, 4H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ(ppm) 38.6, 55.6, 56.1, 110.4, 111.6, 112.2, 113.0, 119.4, 119.6, 126.1, 129.4, 130.0, 133.2, 134.4, 142.2, 146.3, 149.2, 149.3, 160.2, 170.5.

2'-Fluoro-3'-methoxy-*N*-(3-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**19a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (80 mg, 0.25mmol), 2-fluoro-3-methoxyphenylboronic acid (56 mg, 0.33mmol), cesium carbonate (244 mg, 0.75mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc 90:10) to afford the desired product as brown solid (43 mg, 47%). $C_{22}H_{20}FNO_3$; MW 365; mp: 125 – 127°C; MS (ESI) 366$[M+H]^+$; IR (cm^{-1}) 1592, 1644, 2837, 2948, 2975, 3009;^{1}H NMR (C_2D_6CO, 300 MHz) δ(ppm) 3.44 (s, 3H), 3.70 (s, 3H), 3.90 (s, 3H), 6.72 – 6.81 (m, 3H), 6.95 – 7.00 (m, 1H), 7.10 – 7.20 (m, 3H), 7.39 –-7.45 (m, 4H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ(ppm) 38.3, 55.7, 56.6, 113.1, 113.8, 113.9, 120.1, 122.4, 125.2, 125.3, 129.0, 129.1, 129.6, 130.6, 137.1, 137.5, 147.3, 161.2, 170.1.

2'-Fluoro-*N*-(3-methoxyphenyl)-*N*,3'-dimethyl-[1,1'-biphenyl]-4-carboxamide (**20a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (72 mg, 0.22mmol), 2-fluoro-3-methylphenylboronic acid (45 mg, 0.29mmol), cesium carbonate (215 mg, 0.66mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 5 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow solid (70 mg, 91%). $C_{22}H_{20}FNO_2$; MW 349; mp: 91 – 94°C; MS (ESI) 350 $[M+H]^+$; IR (cm^{-1}) 1599, 1635, 2840, 2920, 2963, 3055;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 2.29 (d, *J* = 2.5 Hz, 3H), 3.44 (s, 3H), 3.70 (s, 3H), 6.73 – 6.77 (m, 2H), 6.79-6.80 (m, 1H), 7.13 (t, *J* =

8.0 Hz, 1H), 7.18 (t, *J* = 8.0 Hz, 1H), 7.25 (t, *J* = 8.0 Hz, 1H), 7.39 – 7.44 (m, 4H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ (ppm) 14.6, 14.7, 38.3, 55.7, 113.1, 113.8, 120.1, 125.0, 125.1, 126.2, 126.3, 128.6, 128.7, 129.0, 129.07, 129.09, 129.6, 130.6, 131.9, 132.0, 137.0, 137.9, 147.3, 157.9, 159.9, 170.1.

4-(Benzo[d][1,3]dioxol-5-yl)-*N*-(3-methoxyphenyl)-*N*-methylbenzamide (**22a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxyphenyl)-*N*-methylbenzamide(**6b**) (80 mg, 0.25mmol), benzo[d][1,3]dioxol-5-ylboronic acid (55 mg, 0.33mmol), cesium carbonate (244 mg, 0.75mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow solid (68 mg, 76%). $C_{22}H_{19}NO_4$; MW 361; mp: 139 – 141°C; MS (ESI) 362 $[M+H]^+$; IR (cm^{-1}) 1592, 1644, 2837, 2948, 2975, 3009;^{1}H NMR (C_2D_6CO, 500 MHz) δ(ppm) 3.43 (s, 3H), 3.70 (s, 3H), 6.02 (s, 2H), 6.72 – 6.73 (m, 1H), 6.74 – 6.75 (m, 1H), 6.79 –-6.80 (m, 1H), 6.89 – 6.90 (m, 1H), 7.10 – 7.12 (m, 2H), 7.17 (t, *J* = 8.0 Hz, 1H), 7.37 – 7.39 (m, 2H), 7.44 – 7.46 (m, 2H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ(ppm) 38.4, 55.7, 102.3, 108.0, 109.4, 112.9, 113.8, 120.1, 121.4, 126.5, 130.0, 130.6, 135.0, 136.2, 142.4, 147.4, 148.5, 149.3 161.2, 170.2.

3'-Methoxy-*N*-(4-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**11a**)The title compound was prepared by reaction of 4-bromo-*N*-(4-methoxyphenyl)-*N*-methylbenzamide(**11b**) (100 mg, 0.31mmol), 3-methoxyphenylboronic acid (61 mg, 0.40mmol), cesium carbonate (303 mg, 0.93mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 7 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as colorlessoil (106 mg, 98%). $C_{22}H_{21}NO_3$; MW 347; MS (ESI) 348 $[M+H]^+$; IR (cm^{-1}) 1635, 2840, 2935;^{1}H NMR (C_2D_6CO, 300 MHz) δ (ppm) 3.40 (s, 3H), 3.75 (s, 3H), 3.85 (s, 3H), 6.82 – 6.86 (m, 2H), 6.92 (ddd, *J* = 1.0 Hz, 2.6 Hz, 8.3 Hz, 1H), 7.12 – 7.19 (m, 4H), 7.32 – 7.40 (m, 3H), 7.50 – 7.53 (m, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ (ppm) 38.7, 55.6, 55.7, 113.1, 114.3, 115.2, 120.0, 126.9, 129.2, 130.1, 130.8, 136.9, 139.0, 142.2, 142.3, 159.0, 161.2, 170.2.

4'-Methoxy-*N*-(4-methoxyphenyl)-*N*-methyl-[1,1'-biphenyl]-4-carboxamide (**12a**)The title compound was prepared by reaction of 4-bromo-*N*-(4-methoxyphenyl)-*N*-methylbenzamide(**11b**) (78 mg, 0.24mmol), 4-methoxyphenylboronic acid (48 mg, 0.32mmol), cesium carbonate (235 mg, 0.72mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc 90:10) to afford the desired product as yellow solid (73 mg, 88%). $C_{22}H_{21}NO_3$; MW 347; mp: 129 – 132°C; MS (ESI) 348 $[M+H]^+$; IR (cm^{-1}) 1635, 2840, 2932, 2957;^{1}H NMR (C_2D_6CO, 300 MHz) δ(ppm) 3.39 (s, 3H), 3.74 (s, 3H), 3.83 (s, 3H), 6.82 – 6.82 (m, 2H), 6.98 – 7.01 (m, 2H), 7.11 – 7.14 (m, 2H), 7.34 – 7.37 (m, 2H), 7.44 – 7.47 (m, 2H), 7.54 – 7.57 (m, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz)

δ(ppm) 38.7, 55.6, 55.7, 115.2, 126.2, 128.8, 129.2, 130.2, 133.0, 135.9, 139.1, 142.1, 159.0, 160.6, 170.3.

3'-Methoxy-*N*-methyl-*N*-(*m*-tolyl)-[1,1'-biphenyl]-4-carboxamide (**5a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methylphenyl)-*N*-methylbenzamide (**5b**) (123 mg, 0.40mmol), 3-methoxyphenylboronic acid (79 mg, 0.52mmol), cesium carbonate (393 mg, 1.20mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 9 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow oil (101 mg, 77%). $C_{22}H_{21}NO_2$; MW 331; MS (ESI) 332 $[M+H]^+$; IR (cm^{-1}) 1582, 1601, 1639, 2834, 2935, 3034;^{1}H NMR (C_2D_6CO, 300 MHz) δ(ppm) 2.24 (s, 3H), 3.42 (s, 3H), 3.84 (s, 3H), 6.90 – 7.00 (m, 3H), 7.08 (s, 1H), 7.11 – 7.18 (m, 3H), 7.33 (t, *J* = 8.0 Hz, 1H), 7.38 – 7.41 (m, 2H), 7.49 – 7.52 (m, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ(ppm) 21.2, 38.5, 55.6, 113.1, 114.3, 120.0, 125.1, 126.9, 127.9, 128.4, 129.8, 130.1, 130.8, 136.7, 139.9, 142.3, 142.5, 146.2, 161.2, 170.2.

2'-Fluoro-*N*,3'-dimethyl-*N*-(*m*-tolyl)-[1,1'-biphenyl]-4-carboxamide (**21a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methylphenyl)-*N*-methylbenzamide (**5b**) (93 mg, 0.31mmol), 2-fluoro-3-methylphenylboronic acid (62 mg, 0.40mmol), cesium carbonate (303 mg, 0.93mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 7 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as white solid (103 mg, quantitative). $C_{22}H_{20}FNO$; MW 333; mp: 73 – 76°C; MS (ESI) 334 $[M+H]^+$; IR (cm^{-1}) 1604, 1634, 2853, 2920;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 2.24 (s, 3H), 2.28 (d, *J* = 2.4 Hz, 3H), 3.43 (s, 3H), 6.94 – 6.97 (m, 1H), 6.98 – 7.01 (m, 1H), 7.07 (s, 1H), 7.11 – 7.16 (m, 2H), 7.23 –- 7.26 (m, 2H), 7.38 – 7.41 (m, 4H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ (ppm) 14.6, 14.7, 21.2, 38.5, 125.05, 125.09, 125.12, 126.2, 126.3, 128.5, 128.6, 128.7, 128.99, 129.01, 129.07, 129.09, 129.6, 129.8, 131.9, 132.0, 136.9, 137.8, 139.9, 146.1, 157.9, 159.9, 170.1.

4-(Benzo[d][1,3]dioxol-5-yl)-*N*-methyl-*N*-(*m*-tolyl)benzamide(**22a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methylphenyl)-*N*-methylbenzamide (**5b**) (79 mg, 0.26mmol), benzo[d][1,3]dioxol-5-ylboronic acid (56 mg, 0.34mmol), cesium carbonate (254 mg, 0.78mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow solid (44 mg, 49%). $C_{22}H_{19}NO_3$; MW 345; mp: 111 – 113°C; MS (ESI) 346$[M+H]^+$; IR (cm^{-1}) 1602, 1634, 2788, 2911, 2966;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 2.24 (s, 3H), 3.42 (s, 3H), 6.02 (s, 2H), 6.89 (d, *J* = 8.0 Hz, 1H), 6.94 (d, *J* = 8.0 Hz, 1H), 6.99 (d, *J* = 8.0 Hz, 1H), 7.07 – 7.11 (m, 3H), 7.14 (t, *J* = 8.0 Hz, 1H), 7.35 – 7.37 (m, 2H), 7.42 – 7.44 (m, 2H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ (ppm) 21.2, 38.5, 102.3, 107.9, 109.4, 121.4, 125.1, 126.5, 127.9, 128.4, 129.8, 130.2, 135.1, 136.2, 139.9, 142.3, 146.3, 148.5, 149.3, 170.2.

2'-Fluoro-*N*-(3-methoxybenzyl)-*N*,3'-dimethyl-[1,1'-biphenyl]-4-carboxamide (**25a**)The title compound was prepared by reaction of 4-bromo-*N*-(3-methoxybenzyl)-*N*-methylbenzamide (**25b**) (200 mg, 0.60mmol), 2-fluoro-3-methylphenylboronic acid (120 mg, 0.78mmol), cesium carbonate (587 mg, 1.80mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 14 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow oil (200 mg, 87%). $C_{23}H_{22}FNO_2$; MW 363; MS (ESI) 364 $[M+H]^+$; IR (cm^{-1}) 1630, 2834, 2923, 3034;^{1}H NMR (C_2D_6CO, 500 MHz) δ(ppm) 2.32 (s, 3H), 2.97 (s, 3H), 3.81 (s, 3H), 4.59 – 4.73 (m, 2H), 6.80 – 7.00 (m, 3H), 7.17 (t,*J* = 8 Hz, 1H), 7.27 – 7.32 (m, 2H), 7.33-7.36 (m, 1H), 7.57 – 7.59 (m, 2H), 7.62 (s, 2H); ^{13}C NMR (C_2D_6CO, 125 MHz) δ(ppm) 14.6, 14.7, 37.3, 51.0, 55.5, 113.6, 114.4, 121.0, 125.1, 125.2, 126.2, 126.3, 128.1, 128.7, 128.8, 129.1, 129.2, 129.7, 129.8, 130.5, 132.0, 132.1, 137.1, 138.0, 158.0, 159.9, 161.1.

2'-Fluoro-*N*-(3-hydroxybenzyl)-*N*,3'-dimethyl-[1,1'-biphenyl]-4-carboxamide (**25**)To a solution of 2'-fluoro-*N*-(3-methoxybenzyl)-*N*,3'-dimethyl-[1,1'-biphenyl]-4-carboxamide (**25a**) (100 mg, 0.28mmol) in CH_2Cl_2 (10 mL) was added trifluorobromine dimethyl sulphide complex $BF_3.SMe_2$ (0.18 mL, 1.68mM) at room temperature. The reaction mixture was stirred overnight. The reaction was quenched with MeOH (10 mL). The solvent was removed under reduced pressure at 25°C. Water was added to dissolve inorganic salts and the precipitated was filtered off and triturated with diethyl ether to afford the desired product as yellow solid (66 mg, 67%). $C_{22}H_{20}FNO_2$; MW 349; mp: 123 – 126°C; MS (ESI) 350 $[M+H]^+$; IR (cm^{-1}) 1600, 2917, 3181;^{1}H NMR (C_2D_6CO, 500 MHz) δ (ppm) 2.32 (s, 3H), 2.96 (s, 3H), 4.55 – 4.69 (m, 2H), 6.72 – 6.90 (m, 3H), 7.16 – 7.22 (m, 2H), 7.29 (t, *J* = 8.0 Hz, 1H), 7.33 –-7.36 (m, 1H), 7.57 – 7.58 (m, 2H), 7.62 (s, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ (ppm) 14.6, 14.7, 115.2, 125.1, 125.2, 126.2, 126.4, 128.0, 128.7, 128.9, 129.17, 129.21, 129.7, 129.8, 130.6, 132.0, 132.1, 137.0, 138.0, 157.3, 158.6, 160.6

3-Methoxy-*N*-(3'-methoxy-[1,1'-biphenyl]-4-yl)-*N*-methylbenzamide(**24a**)The title compound was prepared by reaction of *N*-(4-bromophenyl)-3-methoxy-*N*-methylbenzamide (**24b**) (80 mg,0.25mmol), 3-methoxyphenylboronic acid (50 mg, 0.33mmol), cesium carbonate (244 mg, 0.75mmol) and tetrakistriphenylphosphine palladium (0.02 eq., 6 mg) according to method B. The residue was purified by silica gel column chromatography (hexanes/EtOAc70:30) to afford the desired product as yellow oil (59 mg, 68%). $C_{22}H_{21}NO_3$; MW 347; MS (ESI) 348 $[M+H]^+$; IR (cm^{-1}) 1640, 2840, 2936;^{1}H NMR (C_2D_6CO, 300 MHz) δ(ppm) 3.45 (s, 3H), 3.66 (s, 3H), 3.85 (s, 3H), 6.80 –-6.84 (m, 1H), 6.90 – 6.94 (m, 3H), 7.09 – 7.19 (m, 3H), 7.24 – 7.27 (m, 2H), 7.34 (t, *J* = 8.0 Hz, 1H), 7.57 – 7.60 (m, 2H); ^{13}C NMR (C_2D_6CO, 75 MHz) δ(ppm) 38.4, 55.6, 55.7, 113.2, 114.0, 114.9, 116.4, 120.0, 121.9, 128.3, 128.4, 129.7, 130.9, 139.0, 139.6, 142.3, 145.7, 160.1, 161.3, 170.3.

Biological methods

[2,4,6,7-^{3}H]-E2 and [2,4,6,7-^{3}H]-E1 were purchased from Perkin-Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt. Other chemicals were purchased from Sigma, Roth or Merck.

*h*17*β*-HSD1 and *h*17*β*-HSD2 enzyme preparation

Cytosolic (*h*17*β*-HSD1) and microsomal (*h*17*β*-HSD2) fractions were obtained from human placenta according to previously described procedures[20, 27]. Fresh tissue was homogenized and the enzymes were separated from the mitochondria, cell membrane. Nucleus and other rests by fractional centrifugation at 1000 g, 10.000 g and 150.000 g. The pellet fraction containing the microsomal *h*17*β*-HSD2 was used for the determination of *h*17*β*-HSD2 inhibition, while *h*17*β*-HSD1 was obtained after precipitation with ammonium sulfate from the cytosolic fraction for use of testing of *h*17*β*-HSD1 inhibition. Aliquots containing *h*17*β*-HSD1 or *h*17*β*-HSD2 were stored frozen.

*m*17*β*-HSD2 enzyme preparation and inhibition

The microsomal fraction (*m*17*β*-HSD2) was obtained from mouse liver as described for *h*17*β*-HSD2. Inhibitory activities were evaluated by a method identical to the one described for the human enzyme.

*m*17*β*-HSD1 enzyme preparation

Recombinant *m*17*β*-HSD1 enzyme was produced by transfection of HEK 293 cells with a *m*17*β*-HSD1 expression plasmid (coding sequence of NM_010475 in pCMV6Entry vector, OriGene Technologies, Inc.) according to a described procedure[28]. 48 hours after transfection cells were homogenized by sonication (3 x 10 s) in a buffer containing saccharose (40 mMTris, 250 mMsaccharose, 5 mM EDTA, 7 mM DTT, 1 mM PMSF, pH 7,5). Cell lysate was centrifuged (1000 g, 15 min, 4°C) and 20% glycerol was added to the supernatant before aliquots were frozen and stored at -70°C.

Inhibition of *h*17*β*-HSD2 and *m*17*β*-HSD2 in cell-free assay

Inhibitory activities were evaluated following an established method with minor modifications[29-31]. Briefly, the enzyme preparation was incubated with NAD^{+} [1500 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 [^{3}H]-E2 (final concentration: 500 nM, 0.11 µCi). After 20 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with ether. After evaporation, the steroids were dissolved in acetonitrile/water (45:55). E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 RP chromatography column (Nucleodur C18, 3µm, 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 the following equation: %conversion = (%E1/(%E1+%E2))×100. Each value was calculated from at least two independent experiments.

Inhibition of h17β-HSD1 and m17β-HSD1 in cell-free assay

The 17*β*-HSD1 inhibition assay was performed similarly to the *h*17*β*-HSD2 test. The human cytosolic enzyme was incubated with NADH [500 µM] while the mouse recombinant enzyme was reacted with NADPH [500 µM].Test compound and a mixture of unlabelled- and [^{3}H]-E1 (final concentration: 500 nM, 0.15 µCi) were added and mixed for 10 min at 37°C. Further treatment of the samples and HPLC separation was carried out as mentioned above for *h*17*β*-HSD2.

Inhibition of *h*17*β*-HSD2 in a cellular assay

Cellular *h*17*β*-HSD2 inhibitory activity is measured using the breast cancer cell-line MDA-MB-231[32] (17*β*-HSD1 activity negligible). [^{3}H]-E2 (200 nM) is taken as substrate and is incubated with the inhibitor for 6 h at 37 °C. After ether extraction, substrate and product are separated by HPLC and detected with a radioflow detector. Potency is evaluated as percentage of inhibition (inhibitor concentrations used: 1250 nM and 250 nM).

Estrogen receptor affinity in a cell-free assay

The binding affinity of selected compounds to ER*α* and ER*β* was determined according to the recommendations of the US Environmental Protection Agency (EPA) by their Endocrine Disruptor Screening Program (EDSP)[24] using recombinant human proteins. Briefly, 1 nM of ER*α* and 4 nM of ER*β*, respectively, were incubated with [^{3}H]-E2 (3 nM for ER*α* and 10 nM for ER*β*) and test compound for 16-20 h at 4°C.

The potential inhibitors were dissolved in DMSO (5% final concentration). Evaluation of non-specific-binding was performed with unlabeled E2 at concentrations 100-fold of [^{3}H]-E2 (300 nM for ER*α* and 1000 nM for ER*β*). After incubation, ligand-receptor complexes were selectively bound to hydroxyapatite (83.5 g/LinTE-buffer). The bound complex was washed three times and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (1450 LSC & Luminescence Counter, Perkin Elmer).

From these results the percentage of [^{3}H]-E2 displacement by the compounds was calculated. The plot of % displacement versus compound concentration resulted in sigmoidal binding curves. The compound concentrations necessary to displace 50% of the receptor bound [^{3}H]-E2 were determined. Unlabeled E2 IC_{50} values were determined in each experiment and used as reference. The E2 IC_{50}determined were 3±20% nM for *ERα* and 10±20% nM for ER*β*.

Results

Relative Binding Affinity was determined by applying the following equation: RBA[%] = (IC_{50}(E2)/IC_{50}(compound)) · 100[24]. This results in a RBA value of 100% for E2.

After the assay was established and validated, a modification was made to increase throughput. Compounds were tested at concentrations of 1000 times the IC_{50}(E2). Compounds with less than 50% displacement of [^{3}H]-E2 at a concentration of 1000 timesIC_{50}(E2) were classified as RBA <0.1%.

Metabolic Stability in a cell-free assay

Compounds **7a, 8a, 16a, 19a, 22a, 27a** and **27** were tested according to established method[33-35]For evaluation of phase I and II metabolic stability 1 µM compound was incubated with 1 mg/ml pooled mammalian liver S9 fraction (BD Gentest), 2 mM NADPH regenerating system, 1 mM UDPGA and 0.1 mM PAPS at 37°C for 0, 5, 15 and 60 minutes at a final volume of 100 µL. The incubation was stopped by precipitation of S9 enzymes with 2 volumes of cold acetonitrile containing internal standard. Concentration of the remaining test compound at the different time points was analyzed by LC-MS/MS and used to determine half-life ($t_{1/2}$).

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2.3 Addressing cytotoxicity of 1,4-biphenyl amide derivatives: discovery of new potent and selective 17β-hydroxysteroid dehydrogenase type 2 inhibitors

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Paper III

Abstract

Four different classes of new 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) inhibitors were synthesized, in order to lower the cytotoxicity exhibited by the lead compound **A**, via disrupting the linearity and the aromaticity of the biphenyl moiety. Compounds **3**, **4**, **7a** and **8** displayed comparable or better inhibitory activity and selectivity, as well as a lower cytotoxic effect, compared to the reference compound **A**. The best compound **4** (IC_{50} = 160 nM, selectivity factor= 168, $LD_{50}\approx$ 25μM) turned out as new lead compound for inhibition of 17β-HSD2.

Introduction

Osteoporosis affects more than 75 million people in the United States, Europe and Japan, causing almost 9 million bonefractures annualy.[1] The current available therapies lack of sufficient safety and effectiveness,[2]and as consequence development of new treatments is needed.

17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) is responsible for the local reduction of the highly biologically active estradiol (E2) and testosterone (T)into the much less active estrone (E1) and androstenedione (A-dione), whereas 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), a target for the treatment of endometriosis,[3-5] type 3 (17β-HSD3) and type 5 (17β-HSD5) catalyze the opposite reaction (Fig. 1).[6]

The age-related decrease in the local levels of E2 and T is responsible for osteoporosis onset and progression.[7,8]Therefore the inhibition of 17β-HSD2, which is present in the bones,[9] should rebalance the steroid levels in this tissue and represents an appealing strategy for the treatment of this disease. Since 17β-HSD2 and 17β-HSD1 were shown to be both expressed in bone tissue,[9] 17β-HSD2 inhibitors should be selective over 17β-HSD1 (Fig. 1).

We previously reported on the discovery of compound **A** (Fig. 2)**,** which showed a good 17β-HSD2 inhibitory activity (IC_{50}= 300 nM) and good selectivity over 17β-HSD1 (IC_{50}= 13.3 μM, selectivity factor= 44) as well as an improved metabolic stability in human S9 fraction ($t_{1/2}$= 107 min) compared to the other 17β-HSD2 inhibitors described so far.[10] However, this lead compound **A** was found to exert some cytotoxicity.

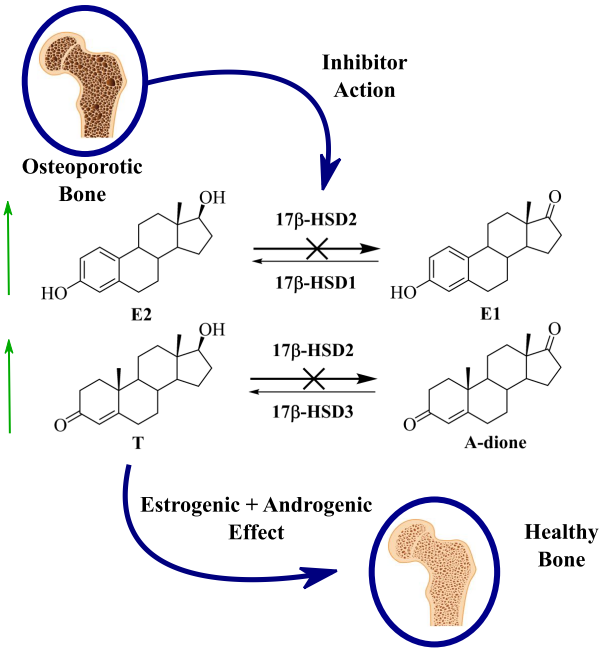


Figure 1. Blocking the reduction of estradiol and testosterone by using an inhibitor of 17β-HSD2.

Only 34% of the cellswere still alive were still alive after treatment with 6.25 µM of compound **A** in a MTT assay.[11]

The 1,1´-biphenyl moiety is known for its toxicity.[12] It might partly come from its planarity and the presence of two aromatic rings next to each other which might result in DNA intercalation by interaction with the nucleobases.[13]

Decrease in cytotoxicity should therefore be achieved by disrupting the planarity of the biphenyl moiety and/or avoiding the biphenyl moiety.

We already reported on compound **B** (Fig. 2) showing a 17β-HSD2 inhibitory activity (IC_{50}= 510 nM)slightly weakercompared to the one of **A**.[14] Whereas**B** and **A** share the biphenyl moiety, compound **B** bears,in place of a carboxamide, a sulfonamide linker. The O of the carbonamide**A** and of the sulfonamide **B** explores different regions of the protein and potentially achieves different H-bond interactions. Therefore the carboxamide of **A** and the sulfonamide of **B** were both taken as starting point for the design of the new inhibitors, in order to obtain a greater chemical diversity.

In order to develop new 17β-HSD2 inhibitors with a better toxicity profile and a good 17β-HSD2 inhibitory activity, we applied four strategies: (1) introduction of an ether bridge between the two phenyl rings, compounds **1-7**; (2) exchange of the phenyl central ring by a cyclohexane ring, compound **8**; (3) the exchange of the central ring by a piperazine ring linked to a sulfonyl group, compounds **9a-11a** and **9-11** and (4) the exchange of the sulfonyl function by an acyl function, compounds **12a-14a** and **12-15** (Fig. 2).

Results

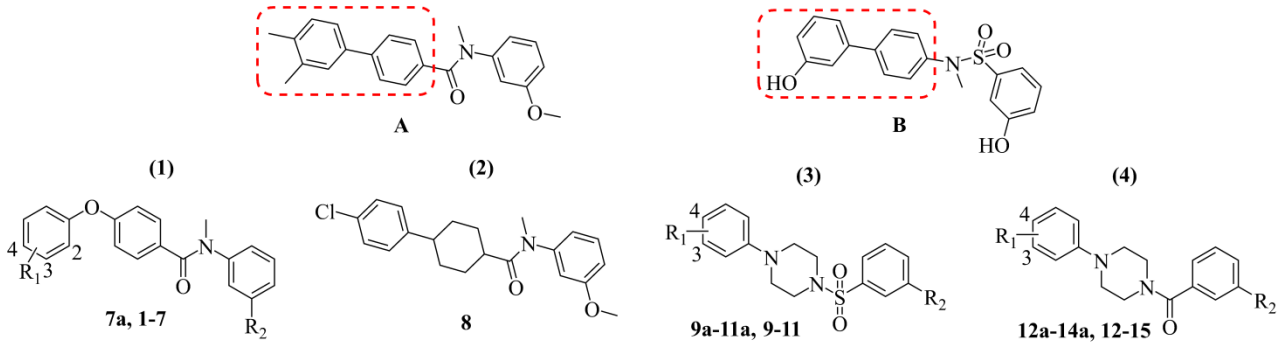


Fig. 2. Four different classes of inhibitors derived from the lead compounds **A** and **B**.

The reaction steps involved in the synthesis of the target compounds **1-8** are shown in Scheme 1. The 4-phenoxybenzoyl chlorides were obtained from the commercially available corresponding 4-phenoxybenzoic acids **1a-6a** and **7b** by reactionwith $SOCl_2$ and subsequently reacted with different anilines, according to the already described procedure,[15]providing compounds **1-6** and **7a**.

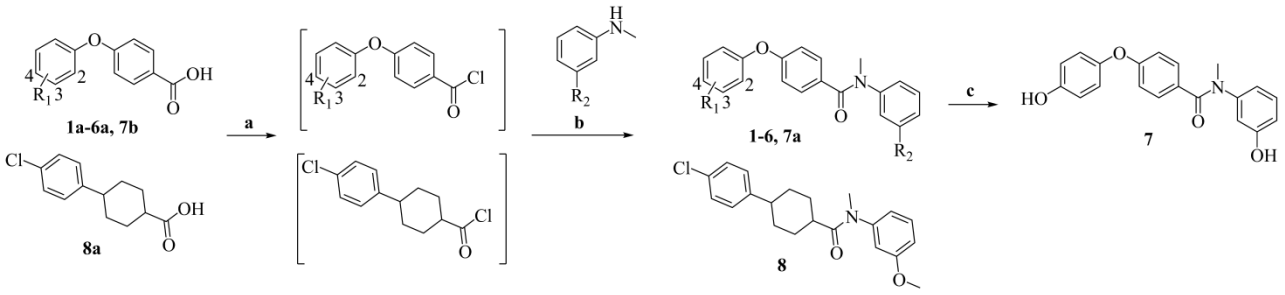
Compound **8** was synthesized using an identical method, starting from thecommercially available 4-(4-chlorophenyl)cyclohexane-1-carboxylic acid**8a** (Scheme 1).

The 3-(4-phenylpiperazin-1yl)sulfonyls **9a-11a** were prepared through the sulfonamide bond formation (Scheme 2), achieved by reaction of commercially available 1-phenylpiperazines **9b-11b** with 3-methoxybenzenesulfonyl chloride **9c**, according to a described procedure.[14] The following ether cleavage of **9a-11a**, using $BF_3 \cdot SMe_2$ in presence of triethylamine, as already described,[14] yielded the hydroxy compounds **9-11**.

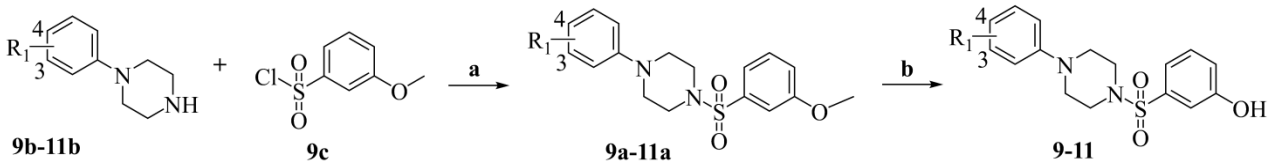
The synthesis of the phenylpiperazin-1-yl methanones**12-14** and **15a** are depicted in Scheme 3. The amide bond was formed, by reacting the commercially available 1-phenylpiperazines **9b**, **10b**, **12b** and **13b** and 3-methoxybenzoyl chloride**10c**, using triethylamine and dichloromethane as solvent. Compounds **12a-14a**were submitted to ether cleavage using boron trifluoride-dimethyl sulfide complex yielding the hydroxy compounds **12-14**. Conditions of both reactions were previously described.[7]

All final compounds as well as their intermediates were fully characterized (^{1}H NMR, ^{13}C NMR and LRMS) to confirm their chemical structure. The data of the representative compounds **4**, **8**, **9a** and **12** are presented as examples.[16-19]

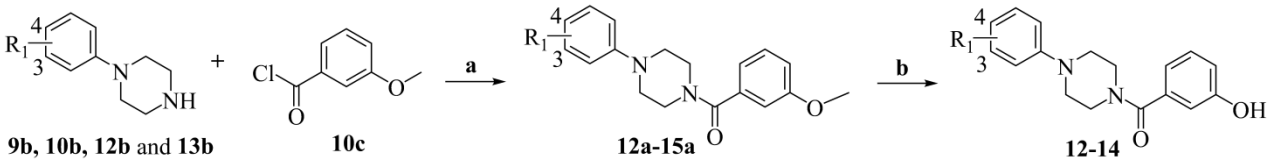
Scheme 1. Synthesis of 4-phenoxybenzamide derivatives **1-7** and **7a** and 4-(4-chlorophenyl)-*N*-methylcyclohexane-1-carboxamide **8**. Reagents and conditions: (a) $SOCl_2$, DMF cat, toluene, reflux, 4 h; (b) Et_3N, CH_2Cl_2, room temperature, overnight; (c) $BF_3{\cdot}SMe_2$, CH_2Cl_2, 0 °C to room temperature, overnight.



Scheme 2. Synthesis of 3-(4-phenylpiperazin-1yl)sulfonyls **9-11**. Reagents and conditions: (a) $(Bu_4)N{\cdot}HSO_4$, NaOH 50%, CH_2Cl_2, room temperature, 3h; (b) $BF_3{\cdot}SMe_2$, CH_2Cl_2, Et_3N, 0 °C to room temperature, overnight.



Scheme 3. Synthesis of phenylpiperazine-1-yl methanones **12-14** and **15a**. Reagents and conditions: (a) Et_3N, CH_2Cl_2, room temperature, overnight; (b) $BF_3{\cdot}SMe_2$, CH_2Cl_2, Et_3N, 0 °C to room temperature, overnight.



The inhibitory activities of compounds **7a**, **10a**, **11a**, **13a-15a** and **1-15** on 17β-HSD2 and 17β-HSD1 obtained from human placental source, were determined as previously described.[20]

The 4-phenoxybenzamides **7a** and **1-7**, as well as the phenylcyclohexanecarboxamide**8** (Table 1) displayed a good inhibition of 17β-HSD2. Compound **4**, with a bent shape and **8**, lacking the central aromatic ring, but conserving the overall linear shape, showed an inhibitory activity in the same order of magnitude as compound **A** and significantly improved selectivity against 17β-HSD1, thus demonstrating that neither the linearity of the biphenyl moiety nor the aromaticity of the central ring are essential for inhibitory activity.

Table 1
Inhibitory activities toward 17β-HSD2 and 17β-HSD1 of compounds **7a** and **1-8**.

			IC_{50} (nM)[a] or Inh. at 1 µM[a]		
Cmpd	R_1	R_2	17β-HSD2[b]	17β-HSD1[c]	s.f.[d]
A	-	-	75% (300nM)	n.i. (13300)	44
1	-H	-Me	59%	n.i.	n.d.
2	2-Me	-Me	55%	n.i.	n.d.
3	3-Me	-Me	300	16100	54
4	4-Me	-Me	160	26300	168
5	4-Cl	-OMe	51%	13%	n.d.
6	4-NO_2	-OMe	43%	n.i.	n.d.
7a	4-OMe	-OMe	310	9600	31
7	4-OH	-OH	43%	n.i.	n.d.
8	-	-	290	60100	209

[a]Mean value of at least two determinations, standard deviation less than 20%. [b]Human placental, microsomal fraction, substrate E2[500 nM], cofactor NAD^+[1500 µM]. [c]Human placental, cytosolic fraction, substrate E1[500 nM], cofactor NADH[500 µM]. [d]n.d.: not determined.

The phenylpiperazin-1-yl sulfonyls **9a-11a** and **9-11** (Table 2) displayed poor inhibition of the 17β-HSD2 enzyme,when compared to compounds**A**and **B**. Compound **10**, which can be directly compared to **B**, is a much weaker inhibitor of 17β-HSD2. Compound **11**is the best in the series. The improvement in activity between **10** and **11** comes from the introduction of the fluorine in *ortho* to the OH group. The Flikely positively influences the hydrogen bond on the OH group next to it.In comparison to compound **B**, the sulfonyl derivatives bear a much more hydrophilic central ring, which might explain the loss of activity. They also beara sulfonamide function condensed in the piperazine ring, whichrenders themolecules shorter than **B**. This feature might lead to a loss of important interactions with the enzyme, thus further explaining the lower inhibitory activity.

Table 2
Inhibitory activities toward 17β-HSD2 and 17β-HSD1 of compounds **9a-11a** and **9-11**.

			Inh. at 1 µM[a]	
Cmpd	R_1	R_2	17β-HSD2[b]	17β-HSD1[c]
B	-		66%	22%
9a	3-Me	-OMe	16%	n.i.
9	3-Me	-OH	27%	n.i.
10a	3-OMe	-OMe	n.i	n.i.
10	3-OH	-OH	25%	n.i.
11a	4-F, 3-OMe	-OMe	15%	n.i.
11	4-F, 3-OH	-OH	48%	n.i.

[a]Mean value of at least two determinations, standard deviation less than 20%. [b]Human placental, microsomal fraction, substrate E2[500 nM], cofactor NAD^+[1500 µM]. [c]Human placental, cytosolic fraction, substrate E1[500 nM], cofactor NADH[500 µM].

The phenylpiperazin-1-yl methanones **12a-15a** and **12-14** (Table 3) displayed no or very low 17β-HSD2 inhibitory activity, indicating that the acyl group does not bring any advantage in comparison with the sulfonyl group.

Table 3
Inhibitory activities toward 17β-HSD2 and 17β-HSD1 of compounds **12a-15a** and **12-14**.

			Inh. at 1 µM[a]	
Cmpd	R_1	R_2	17β-HSD2[b]	17β-HSD1[c]
12a	-H	-OMe	14%	n.i.
12	-H	-OH	27%	n.i.
13a	3-Me	-OMe	n.i	n.i.
13	3-Me	-OH	16%	n.i.
14a	4-Me	-OMe	16%	n.i.

14	4-Me	-OH	n.i.	n.i.
15a	3-OMe	-OMe	n.i.	n.i.

[a]Mean value of at least two determinations, standard deviation less than 20%. [b]Human placental, microsomal fraction, substrate E2[500 nM], cofactor NAD^+[1500 µM]. [c]Human placental, cytosolic fraction, substrate E1[500 nM], cofactor NADH[500 µM].

Cell viability in HEK293 cells was determined for the best compounds **3**, **4**, **7a** and **8**, using an MTT assay according to a described procedure.[11] The results are displayed in Fig. 3.

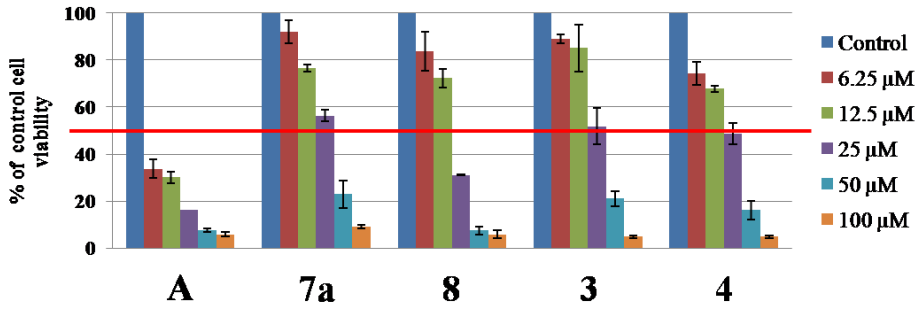


Fig. 3. Cytotoxicity of selected compounds is displayed in the order of increasing 17β-HSD2 inhibitory activity. Incubation was carried out at the indicated inhibitor concentrations for 66 hours at 37 °C. 100%-values were determined without inhibitor.

In comparison to compound **A** (LD_{50} less than 6.25 µM), all four compounds showed a better safety profile, with a LD_{50} around 25 µM for **3**, **4** and **7a** and above 12.5 µM for compound **8**. Compound **4** displays improved 17β-HSD2 inhibitory activity and much better selectivity against 17β-HSD1 as well as significantly decrease in cytotoxicity, when compared to compound **A**. These results confirm compound **4** as new lead compound for inhibition of 17β-HSD2.

Acknowledgments

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16. Data for *N*-methyl-*N*-(*m*-tolyl)-4-(*p*-tolyloxy)benzamide(compound **4**): yellow oil; $C_{22}H_{21}NO_2$;^{1}H-NMR–300 MHz (Acetone-d_6, δ, ppm) 2.25 (s, 3H), 2.30 (s, 3H), 3.39 (s, 3H), 6.71-6.76 (m, 2H), 6.84-6.92 (m, 3H), 6.98-7.01 (m, 2H), 7.12-7.20 (m, 3H), 7.27-7.32 (m, 2H);^{13}C NMR–75 MHz (Acetone-d_6, δ, ppm) 20.8, 21.3, 38.6, 117.5, 120.4, 125.1, 127.9, 128.5, 129.8, 131.4, 131.7, 132.0, 134.5, 139.9, 146.5, 154.9, 159.8, 169.9; LRMS (m/z) calcd. for $C_{22}H_{22}NO_2$ $[M+H]^+$ 332.16, found 332.18.

17. Data for 4-(4-chlorophenyl)-*N*-(3-methoxyphenyl)-*N*-methylcyclohexane-1-carboxamide(compound **8**): yellow solid; m.p.= 108-109 °C; $C_{21}H_{24}ClNO_2$; . ^{1}H-NMR–500 MHz (Acetone-d_6, δ, ppm) 1.18-1.25 (m, 2H), 1.62-1.71 (m, 2H), 1.77-

1.82(m, 4H), 2.38 (brs, 1H), 2.48-2.54 (m, 1H), 3.18 (s, 3H), 3.84 (s, 3H), 6.89-6.97 (m, 2H), 6.96-6.98 (m, 1H), 7.17 (d, *J*= 9 Hz, 2H), 7.23-7.25 (m, 2H), 7.39 (t, *J*= 8 Hz, 1H);^{13}C NMR–125 MHz (Acetone-d_6, δ, ppm) 30.5, 34.0, 37.4, 41.4, 43.9, 55.9, 114.0, 114.2, 120.4, 129.1, 129.4, 131.3, 131.9, 146.8, 147.1, 161.7, 175.5; LRMS (m/z) calcd. for $C_{21}H_{25}ClNO_2$ $[M+H]^+$ 358.16, found 358.21.

18. Data for 1-((3-methoxyphenyl)sulfonyl)-4-(m-tolyl)piperazine (compound **9a**): white solid; m.p.= 129-130 °C; $C_{18}H_{22}N_2O_3S$; ^{1}H-NMR–300 MHz (Acetone-d_6, δ, ppm) 2.24 (s, 3H), 3.11-3.14 (m, 4H), 3.22-3.25 (m, 4H), 3.91 (s, 3H), 6.63-6.66 (m, 1H), 6.70-6.76 (m, 2H), 7.08 (t, *J*= 8 Hz, 1H), 7.25-7.30 (m, 2H), 7.36-7.40 (m, 1H), 7.58 (t, *J*= 8 Hz, 1H);^{13}C NMR–75 MHz 21.7, 47.1, 49.7, 56.1, 113.7, 114.6, 118.2, 119.7, 120.7, 121.8, 129.7, 131.2, 137.9, 139.3, 151.9, 161.0; LRMS (m/z) calcd. for$C_{18}H_{23}N_2O_3S$ $[M+H]^+$ 346.14, found 346.89.

19. Data for (3-hydroxyphenyl)(4-phenylpiperazin-1-yl)methanone (compound **12**): white solid; m.p.= 174-175 °C;$C_{17}H_{18}N_2O_2$; ^{1}H-NMR–300 MHz (Acetone-d_6, δ, ppm) 4.00.4.14 (m, 8H), 6.94-6.97 (m, 1H), 6.95-7.02 (m, 3H), 7.30 (t, *J*= 8 Hz, 1H), 7.56-7.68 (m, 3H), 7.83-7.86 (m, 1H), 8.64 (brs, 1H); ^{13}C NMR–75 MHz (Acetone-d_6, δ, ppm) 56.5, 115.1, 118.0, 119.1, 122.0, 130.6, 131.0, 131.4, 137.1, 142.9, 158.4, 170.4;LRMS (m/z) calcd. for $C_{17}H_{19}N_2O_2$ $[M+H]^+$ 283.14, found 283.20.

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2.5 Monograph: Inhibition of 17β-Hydroxysteroid Dehydrogenase Type 1 for the Treatment of NSCLC: Discovery of Potent, Selective and Metabolically Stable Compounds for an *in vivo* Proof of Concept Study

The following people contributed to the experimental work of this monograph: Vilma Dollaku (synthesis of compound **5a** and **7b**); Arcangela Mazzini (analytical method development, estrogen receptor assay); Hanna Drzewiecka (xCELLigence experiments); Martina Jankowski (S9 fraction metabolic stability assay).

Abstract

Design, synthesis and biological evaluation of a new class of h17βHSD1 inhibitors for the treatment of NSCLC are described. Structural modifications turned selective h17βHSD2 inhibitors into potent and selective h17βHSD1 inhibitors. Compound **15** displays low nanomolar h17βHSD1 inhibition, high selectivity against 17βHSD2 and ERs, good in vitro and in vivo pharmacokinetics properties, r17βHSD1 inhibition and r17βHSD2. These properties make it eligible for an *in vivo* proof of concept study.

Introduction

Lung cancer is the leading cause of death from cancer worldwide.[1] Non-small-cell lung cancer (NSCLC) accounts for more than 85% of the cases, with a predicted 5-years survival rate for all stages of lung cancer of 15,9%, a figure which has only slightly improved during the past years.[2] Even if the disease is detected at an early stage and therefore still localized, the survival rate is only 49%, demonstrating that relapse and death from lung cancer are common. Thus, finding drugs with novel modes of action is an urgent necessity.
Over the last fifteen years, increasing evidence has demonstrated the pivotal role of the estrogen signalling pathway in lung tumorigenesis, in women and men.[3, 4] Both estrogen receptors (ERs) α and β are expressed in NSCLC cell lines, tumor tissues and normal lung-derived cells.Furthermoreestradiol (E2) can promote the growth of NSCLC cells *in vitro* and *in vivo*,[5, 6] and importantly a significant decrease in the proliferation of NIH-H23 lung cancer cells has been observed *in vitro* for siRNA-mediated knockdown of the ERs.[6]

Different strategies to target the estrogen signalling pathway have shown promising results.Fulvestrant, a down-regulator of ER function, displayed decreased tumor volume in an *in vivo* lung tumor xenograft model both alone and in combination with the EGFR tyrosine kinase inhibitor gefitinib.[7] Reduction of estrogen levels through aromatase inhibitor anastrozoleinhibited NSCLC growth *in vivo* in a lung tumor xenograft mouse model[8] and

promising results on the clinical efficacy of exemestane– another aromatase inhibitor - on breast cancer as well as NSCLC have been reported.[9]

The estradiol/estrone (E2/E1) ratio is locally regulated by the action of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) and type 2 (17β-HSD2) in the tissues where they are expressed. [10] Up to date evidence has been presented that both 17β-HSD1 and 17β-HSD1 are often expressed in NSCLC cells, contributing to tumor progression by regulating the E2/E1 ratioin a stimulatory fashion. Consequently theE2/E1 ratio is used as a prognostic factor. [3, 11, 12]

We have reported about the synthesis of different classes of 17β-HSD1[13, 14, 15] and 17β-HSD2 [16; 17; 18] inhibitors for the treatment of breast cancer and osteoporosis, respectively. In light of the fact that 17β-HSD2 mRNA expression is significantly higher in NSCLC cells than in breast carcinoma cells and that 17β-HSD2 positive cases are significantly associated with better overall survival when compared to 17β-HSD2 negative patients, thus suggesting a fundamental protective role of this enzyme in NSCLC,[(3)]we reasoned that a new 17β-HSD1 inhibitor should have an exceptionally high selectivity over 17β-HSD2, in order to show inhibitory effects in NSCLC cells.

This work provides the first evidence that it is possible inhibit NSCLC cell proliferation by using a highly selective17β-HSD1 inhibitor.

Results and discussion

In a previous study we described the optimization of 2,5-thiophene amides as selective inhibitors of the human 17βHSD2 (*h*17βHSD2), only bearing -Me groups on the two phenyl rings.[18] All the possible substitution patterns on the rings were evaluated and compound **1** turned out to be the best inhibitor (Fig. 1). In the same study it was pointed out that the exchange of the thiophene ring by a furane could improve the metabolic stability and significantly decrease the *h*17βHSD2 inhibition, without affecting the human 17βHSD1 (*h*17βHSD1) one, as it results by comparison of **1** with **2** (Fig. 1).

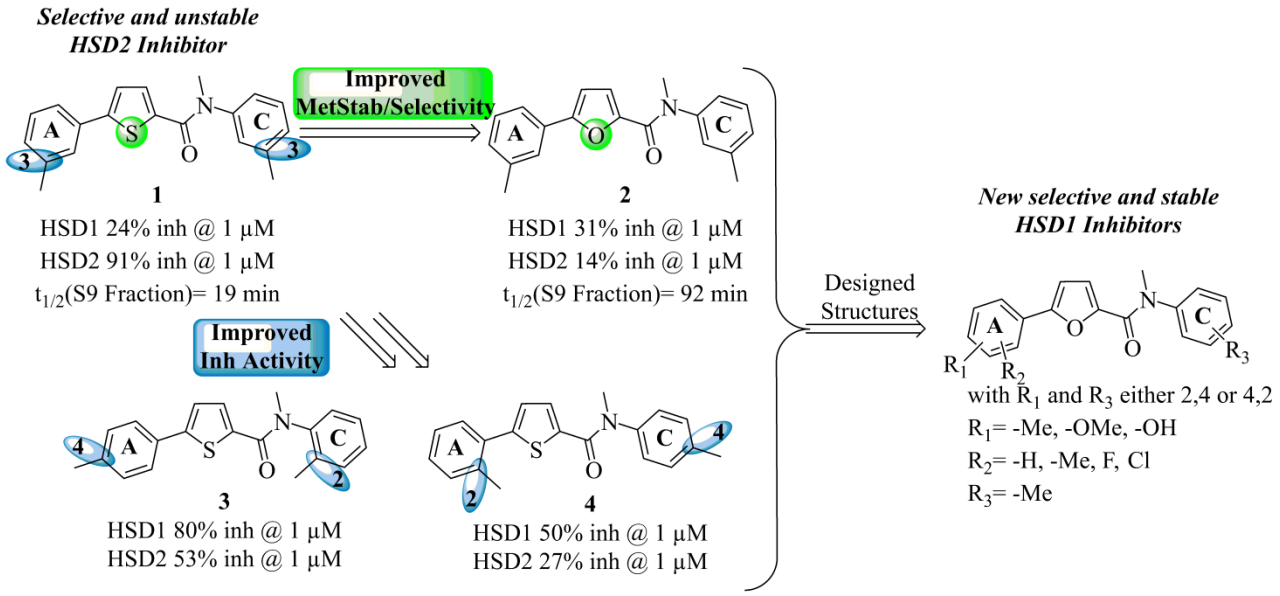


Figure 1. Design of the new 17βHSD1 inhibitors.

This effect is interesting for the purposes of the current study, whereas it was a brick wall for the development of *h*17βHSD2 inhibitors. It was also noted that the substitution pattern of the -Me groups had a major effect on *h*17βHSD1 and *h*17βHSD2 inhibition, with 4,2 and 2,4 substitution patterns (compound **3** and **4**, respectively) leading to the highest in *h*17βHSD1 inhibitory activity in the series (Fig. 1).[18]

We designed a series of 2,5-furane amides, with different substituents on ring A and C in position 2,4 or 4,2 (compounds **5-8**, **7a** and **8a**, Fig. 1) in order to investigate whether it was possible to further improve *h*17βHSD1 inhibition and selectivity against *h*17βHSD2, while retaining the good metabolic stability already displayed by compound **2**. Some more derivatives, also substituted in position 2 and/or 5 were designed and synthesized (compounds **9a-15a**, **9-17** Fig. 1).

The synthesis of the 2,5-furane derivatives (Compounds **7a-15a** and **5-17**) is depicted in Scheme 1. The 5-bromofuran-2-carbonic acid chloride was obtained from the corresponding carboxylic acid **5b** by reaction with $SOCl_2$ and subsequently reacted with different anilines (Method A), affording the intermediates **5a**, **6a** and **7b** in good yields. The subsequent Suzuki coupling (Method B), under microwave irradiation (150 °C, 150 W for 20 minutes), provided the desired 2,5-furane derivatives **5, 6, 7a-15a, 16** and **17**. The ether cleavage of methoxy compounds **7a-15a** was performed using boron trifluoride-dimethyl sulphide complex, yielding the hydroxy compounds **7-15**.

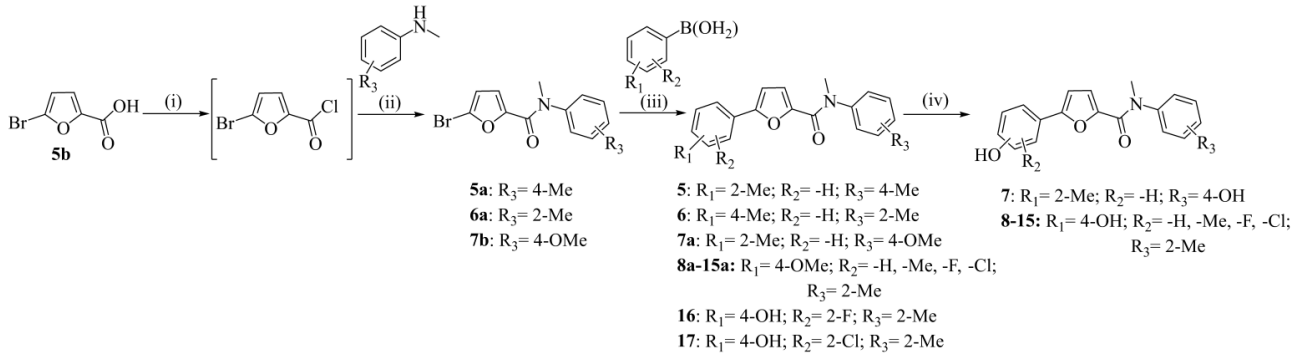
*h*17*β*HSD1 and *h*17*β*HSD2 and rat 17*β*HSD1 and 17*β*HSD2 (*r*17*β*HSD1 and *r*17*β*HSD2) cell-free assays were performed similarly, by incubating enzyme, tritiated substrate, cofactor and inhibitor, according to described procedures.[19-21] Assay details are available in the Supporting Information.

Compounds **5** and **6** in comparison with the corresponding 2,5-thiophene derivatives **3** display a decreased inhibition of *h*17*β*HSD2 and **4** and

comparable *h*17*β*HSD1 inhibition (Table 1), thus confirming the importance of the furane ring in enhancing the selectivity against the *h*17*β*HSD2. This effect might be explained by the different Molecular Electrostatic Potential (MEP) induced by the furane ring, which is tolerated by the *h*17*β*HSD1 enzyme, whereas decreases the affinity for the *h*17*β*HSD2. A similar effect has already been describe by E, Bey et al..[22]

A bulkier 4-OMe group on ring A does not affect the *h*17*β*HSD1 inhibitory activity, whereas a 2-OMe on ring A exerts a negative effect, as it results from comparison of compounds **7a** and **8a** with **5** and **6**, respectively (Table 1), suggesting that more space might be available around ring A and we therefore decided to exploit this feature for investigating the effect of different substituents on the ring in position 2 and 5 (Table 1, compounds **9a-15a**).

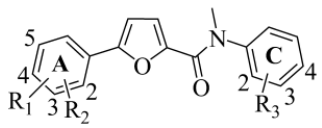
Scheme 1. Synthesis of 2,5-furane derivatives 7a-15a and 5-17.[a]



[a]Reagents and conditions:(i) $SOCl_2$, DMF cat., toluene, reflux 4 h**;** (ii) Et_3N, CH_2Cl_2, room temperature, overnight; (ii**i**) DME/EtOH/H_2O (1:1:1), Cs_2CO_3, $Pd(PPh_3)_4$, microwave irradiation (150°C, 150W, 20 min); (i**v**) $BF_3 \cdot SMe_2$, CH_2Cl_2, room temperature, overnight.

In all cases (**9a-15a**, Table 1), but compound **10a** the *h*17*β*HSD1 inhibitory activity is in the range of 50-70% inhibition at 1µM. Compounds **11a** and **12a** (Table 1) bearing small lipophilic substituents in position 3 (Table 1) showed the best inhibition in the set, with **12a** (*h*17*β*HSD1 70% and *h*17*β*HSD2 12% inh. at 1 µM) displaying similar *h*17*β*HSD1 and lower *h*17*β*HSD2 inhibition in comparison with the reference compounds **3** (*h*17*β*HSD1 80% and *h*17*β*HSD2 53% inh. at 1 µM).

Table 1. Inhibition of human 17βHSD1 and 17βHSD2 by biphenyl-2,5-furane without hydroxy group.



5, 6, 7a-15a

	Inh. at 1 µM[a,b]				
Cmpd	**R_1**	**R_2**	**R_3**	**h17β-HSD1[c]**	**h17β-HSD2[d]**
5	2-Me	-H	4-Me	44%	n.i.
6	4-Me	-H	2-Me	45%	n.i.
7a	2-Me	-H	4-OMe	24%	n.i.
8a	4-OMe	-H	2-Me	49%	18%
9a	4-OMe	2-Cl	2-Me	16%	13%
10a	4-OMe	2-Me	2-Me	24%	n.i.
11a	4-OMe	3-F	2-Me	61%	17%
12a	4-OMe	3-Me	2-Me	70%	12%
13a	4-OMe	3,5-F	2-Me	27%	n.i.
14a	4-OMe	3,5-Cl	2-Me	53%	30%
15a	4-OMe	3,5-Me	2-Me	57%	15%

[a]Mean value of at least two determinations, standard deviation less than 20%. [b]n.i.: no inhibition (inhibition of <10%). [c]Human placental, cytosolic fraction, substrate E1[500 nM], cofactor NADH[500 µM]. [d]Human placental, microsomal fraction, substrate E2[500 nM], cofactor NAD^+[1500 µM].

In a second step we decided to investigate the effect of an hydroxy group either on the A or the C ring, keeping the conformational constrain induced by a 2-Me group on one of the two rings (Table 2). An hydroxy group decreases the cLogP of a molecule, which is correlated to its oral bioavailability,[23] and might establish an hydrogen bond interaction with the enzyme, eventually boosting the inhibitory activity of the molecule.

The exchange of the 2-Me by 2-OH in compound **5** leads to **7** (Table 2), with worsen inhibition and selectivity and therefore it was not further investigated. On the contrary, the exchange of the 4-Me by a 4-OH group in compound **6** leads to **8** (Table 2), with a 10-folds increase of *h*17*β*HSD1 inhibitory activity and a selectivity factor of 52 against the *h*17*β*HSD2. The effect of the substitution with –Me, -F and –Cl in position 2, 3 and 5 was investigated in compounds **9**-**17** (Table 2).

The mono-substituted derivatives of **8**, all display an IC_{50} value below 40 nM (Table 2), with the 3-Cl (**17**, IC_{50} value of 8.9 nM) and 3-Me (**12**, IC_{50} value of 7.8 nM) substituted compounds showing the highest potency, thus pointing out a preference of the *h*17*β*HSD1 enzyme for lipophilic substituents in position 3 of ring A. Interestingly a 3-Me group also increases the selectivity against *h*17*β*HSD2 (Table 2, compound **12**), indicating that such substituent is not tolerated by this enzyme.

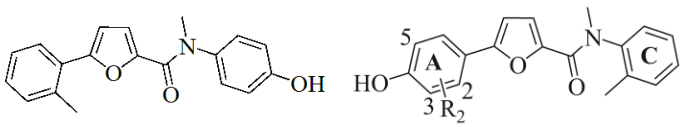
Results

Among the di-substituted derivatives of **8** (Table 2, **13-15**), the 3,5-Cl (**14**, IC_{50} value of 2.9 nM) and the 3,5-Me (**15**, IC_{50} value of 5.6 nM) display the highest *h*17*β*HSD1 inhibitory activity, indicating that the substitution with a second lipophilic group, exert an additive positive effect on the potency, if compared to **8**. The lipophilicity and electron-withdrawing properties of the two -Cl groups of compound **14**, results in the best combination for the inhibition of the *h*17*β*HSD1 enzyme. **14** is the most potent *h*17*β*HSD1 inhibitor so far described in the literature. By comparison of **12** and **15**, it appears that the substitution with a second -Me group further decreases the affinity for the *h*17*β*HSD2 enzyme, resulting in a selectivity factor of 563 (Table 2). Selectivity might be a pivotal issue given that both *h*17*β*-HSD1 and 2 are expressed in NSCLC cells, and therefore the inhibition of *h*17*β*-HSD2 might be counterproductive.

For compounds **8-17** the inhibitory activity against the rat *h*17*β*HSD1 and 2 was also determined and expressed as percent of inhibition at 1 µM (Table 2). For the most interesting compounds the IC_{50} values were determined (Table 2, compounds **12-15**).
Compounds **8-11**, **16** and **17**, all show other too low *r*17*β*-HSD1 inhibitory activity or lack in selectivity against *r*17*β*HSD2 (Table 2). Compound **13** and **14** also display a greater affinity for *r*17*β*HSD2 than *r*17*β*HSD1 (Table 2). On the contrary **12** (IC_{50} value of 176 nM) and **15** (IC_{50} value of 74 nM), which display the higher selectivity against the *h*17*β*-HSD2 also retain some selectivity against *r*17*β*HSD2 and show a good inhibition of the *r*17*β*HSD1, indicating that the -Me group play a role in the selectivity against both *h* and *r*17*β*-HSD2, probably due to its steric hindrance

The metabolic stability of compound **8** and compound **15** was evaluated using human liver microsomes (S9 fraction, assay details are available in the Supporting Information). They display an half life of 97 min and 51 min. Considering that the tested 2,5-thiophene derivatives (17 compounds tested) display a half life around 20 min[31] and that the tested 2,5-furane derivatives (**2**,**8**,**15**) display a half life between 51 and 97 min, it can be concluded that the furane ring plays a major role in increasing the stability of these compounds, probably decreasing the affinity for the metabolizing enzyme. Compound **15** was also tested for its affinity toward the ERα and ERβ according to described methods[24] (assay details are available in the Supporting Information), resulting in a Relative Binding Affinity (RBA) lower than 0.1% for both receptors. Since the RBA value of E1 itself is around 7%,[25] this value is sufficient to assume that the compound should not exert an activity through direct interaction with the ERs. The cytotoxicity of **15** was measured through Hek293 cell viability in the MTT assay (assay details are available in the Supporting Information). No toxic effect were detected up to 6.25 µM, which is more than 1000-folds the IC_{50} value and thus predicting a broad safety windows. In a preliminary *in vivo* PK study, the compound the compound was administered to a femal rat at a dose of 50 µM/Kg, subcutaneously (assay details are available in the Supporting Information). After 24h a concentration of 127 nM (almost 2-folds the IC50, Table 2) was still detectable, indicating that the compound has a sufficient PK profile for an *in vivo rat* application.

Table 2. Inhibition of human and rat 17β-HSD1 and 17β-HSD2 by biphenyl-2,5-furane without hydroxy group.



7 **8-11**

		IC$_{50}$ (nM)[a] or Inh. at 1 µM[a]			IC$_{50}$ (nM)[a] or Inh. at 1 µM[a]		
Cmpd	**R_2**	**h17β-HSD1[b]**	**h17β-HSD2[c]**	**s.f.[d]**	**r17β-HSD1[e]**	**r17β-HSD2[f]**	**s.f.[d]**
7	-	27%	30%	n.d.	n.d.	n.d.	n.d.
8	-H	80	4144	52	49%	37%	n.d.
9	2-Cl	28	301	11	71%	89%	n.d.
10	2-Me	31	1077	35	37%	50%	n.d.
11	3-F	15	447	30	69%	100%	n.d.
12	3-Me	7.8	1547	198	369	1200	3
13	3,5-F	18	56	3	291	16	0.05
14	3,5-Cl	2.9	71	25	176	40	0.2
15	3,5-Me	5.6	3155	563	74	1341	18
16	2-F	39	780	20	74%	100%	n.d.
17	3-Cl	8.9	265	30	89%	100%	n.d.

[a]Mean value of at least two determinations, standard deviation less than 20%. [b]Human placental, cytosolic fraction, substrate E1[500 nM], cofactor NADH[500 µM]. [c]Human placental, microsomal fraction, substrate E2[500 nM], cofactor NAD^+[1500 µM]. [d]n.d.: not determined. [e]Rat recombinant enzyme, expressed in HEK 293, substrate [^{3}H]-E1, 10 nM, cofactor NADH, 500 µM. [d]Rat liver, microsomal fraction, substrate [^{3}H]-E2, 10 nM, cofactor NAD^+, 1500 µM.

The efficacy of compound **15** was investigated in cellular experiments. For this purpose , two NSCLC cell lines Calu-1 and A549 were chosen.

E1 enhances the proliferation of Calu-1 cell line (Fig. 2, green line versus purple line). Compound **15** at the concentartion of 50 nM is able to decrease cell proliferation in the presence of E1 to the basal level (blue line) and this effect is stronger when the concentration of **15** is higher (red line).

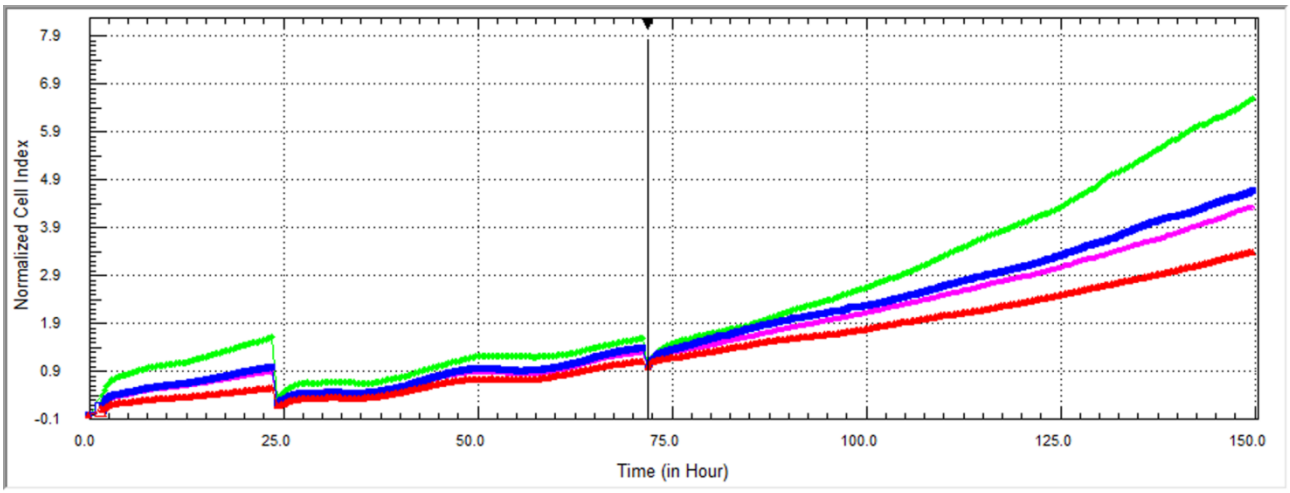


Figure 2. Control (DMSO) – purple curve; 5 uM E1 – green curve; 5 uM E1 + 50 nM of **15** – dark blue curve; 5 uM E1 + 500 nM of **15** – red curve.

The response of A549 cells is different, compared to the one of Calu-1 cells. E1 is able to enhance the proliferation of this cell line too Fig.3, but **15** did not inhibit cell proliferation as much as in Calu-1 cell line. At a concentration of 500 nM **15** is able to decrease A549 cells proliferation in presence of E1, although cells growth rate is significantly higher in comparison to control. This result might be explained by a different E1 metabolism pathway, which is able to bypass the 17βHSD1 inhibition.

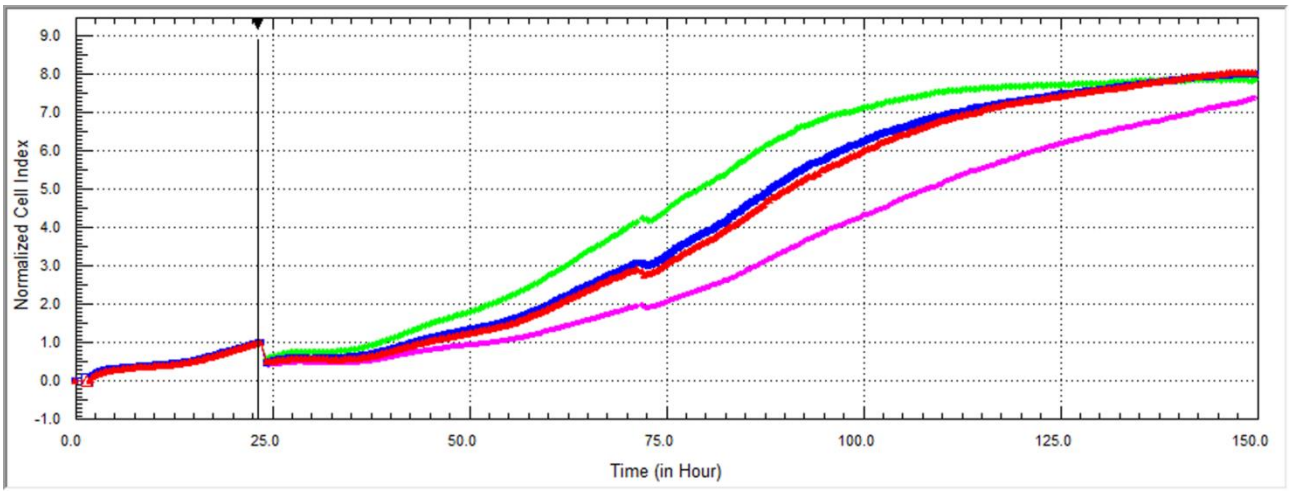


Figure 3. Control (DMSO) – purple curve; 5 uM E1 – green curve; 5 uM E1 + 50 nM of **15** – dark blue curve; 5 uM E1 + 500 nM of **15** – red curve.

CONCLUSION

The aim of this work was the discovery of a new class of selective 17βHSD1 inhibitors, which can be suitable for application in both a rat model of lung cancer and humans. We described how exchange of the central ring and substituents modulation can turn selective *h*17βHSD2 inhibitors into selective *h*17βHSD1. Compound **15** displays a high inhibitory

activity against *h*17*β*HSD1 and exceptional selectivity against *h*17*β*HSD2 and the ERs. It is also active and selective against the rat enzymes. Compound **15** provided the first convincing evidence that inhibition of *h*17*β*HSD1 can inhibit NSCLC cell proliferation *in vitro*, thus indicating a new theraupetic option for NSCLC treatment. Furthermore its low cytotoxicity and good pharmacokinetics properties *in vitro* and *in vivo*, makes **15** the ideal candidate for an *in vivo* proof of concept study.

EXPERIMENTAL SECTION

All reagents and solvents were obtained from commercial suppliers and used without further purification Column chromatography was performed on silica gel (70-200 μm) and reaction progress was monitored by TLC on Alugram SIL G/UV254 (Macherey-Nagel). ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM500 spectrometer (at 500 MHz and 125 MHz, respectively) at 300 K and on Bruker Fourier 300 (at 300 MHz and 75 MHz, respectively) at 300 K. Chemical shifts are reported in δ (ppm) and coupling constants (J) in Hertz (Hz). Melting points (mp) were measured in open capillaries on a Stuart Scientific SMP3 apparatus and are uncorrected.All microwave irradiation experiments were carried out in a 507 CEM-Discover microwave apparatus.All tested compounds exhibited ≥ 95% chemical purity as measured by LC/MS.

Method A, general procedure for amide formation:

A mixture of 5-bromofuran-2-carboxylic acid (1eq), thionyl chloride (2.5 eq) and DMF (5 drops) in toluene (10 mL) was refluxed at 110°C for 4 hours. The reaction mixture was cooled to room temperature; the solvent and the excess of thionyl chloride were removed under reduced pressure. The corresponding *N*-methylamine (1 eq) and Et_3N (1 eq) in CH_2Cl_2 (10 mL) was added at 0°C under N_2 atmosphere to the acyl chloride. After 30 minutes at 0°C, the ice bath was removed and the solution was warmed up and stirred at room temperature overnight. The reaction mixture was extracted twice with CH_2Cl_2 (2 × 15 mL); the organic layer was dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by silica gel column chromatography using hexanes and EtOAc as eluent or by trituration in a mixture of diethyl ether / petroleum ether to afford the desired compound.

Method B, general procedure for Suzuki-Miyaura coupling:

In a sealed tube the previously prepared 5-bromo-*N*-heteroaryl-furan-2-carboxamide derivative (1 eq.) was introduced followed by the corresponding boronic acid (1.5 eq.), cesium carbonate (3 eq.), tetrakis(triphenylphosphine)palladium (0.02 eq.) and a mixture of DME/EtOH/H_2O (1:1:1, v:v:v, 3 mL) as solvent. The reactor was flushed with N_2 and submitted to microwave irradiation (150°C, 150 W) for 20 minutes. After cooling to room temperature, a mixture of EtOAc/H_2O (1:1, v:v, 2 mL) was added to stop the reaction. The aqueous layer was extracted with EtOAc (3 × 10 mL). The organic layer was washed once with brine and once with water, dried over $MgSO_4$, filtered and the solution was

concentrated under reduced pressure. The residue was purified by column chromatography using hexanes and EtOAc as eluent to afford the desired compound.

Method C, general procedure fo ether cleavage:

To a solution of methoxyaryl compounds (1 eq.) in dry dichloromethane (5 mL/mmol of reactant), boron trifluoride-dimethyl sulfide complex (6 eq./methoxy function) was added dropwise at 0 °C and stirred for 6-14 h at room temperature. After the reaction was finished, the reaction mixture was diluted with dichloromethane and 5% aqueous $NaHCO_3$ was added until neutral pH was obtained. The aqueous layer wad extracted with dichloromethane. The combined organic layers were washes with brine, dried over sodium sulfate, evaporated to dryness under reduced pressure. The product was purified by column chromatography using EtOAc as eluent to afford the desired compound.

ACKNOWLEDGMENT

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2.4.1 Supporting Information

a) Chemical Methods. Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Combi-Blocks or Fluorochem and were used without purification.

Column chromatography was performed on silica gel (70-200 μm) and reaction progress was monitored by TLC on Alugram SIL G/UV254 (Macherey-Nagel). Visualization was accomplished with UV light.

^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM500 spectrometer (at 500 MHz and 125 MHz, respectively) at 300 K and on Bruker Fourier 300 (at 300 MHz and 75 MHz, respectively) at 300K. Chemical shifts are reported in δ (parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard: 2.05 ppm (^{1}H NMR) and 29.8 and 206.3 ppm (^{13}C NMR) for CD_3COCD_3, 7.26 ppm (^{1}H NMR) and 77.0 ppm (^{13}C NMR) for $CDCl_3$. Signals are described as br (broad), s (singlet), d (doublet), t (triplet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplets) and m (multiplet). All coupling constants (*J*) are given in Hertz (Hz).

Melting points (mp) were measured in open capillaries on a Stuart Scientific SMP3 apparatus and are uncorrected.

Mass spectrometry was performed on a TSQ Quantum (ThermoFisher, Dreieich, Germany). The triple quadrupole mass spectrometer was equipped with an electrospray interface (ESI). The purity of the compounds was assessed by LC/MS. The Surveyor-LC-system consisted of a pump, an auto sampler, and a PDA detector. The system was operated by the standard software Xcalibur. A RP C18 NUCLEODUR 100-5 (3 mm) column (Macherey-Nagel GmbH, Dühren, Germany) was used as stationary phase. All solvents were HPLC grade. In a gradient run the percentage of acetonitrile (containing 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 needle voltage of 3000 V and a capillary temperature of 350 °C. Mass spectra were acquired in positive mode from 100 to 1000 m/z and UV spectra were recorded at the wave length of 254 nm and in some cases at 360 nm.

All microwave irradiation experiments were carried out in a 507 CEM-Discover microwave apparatus.

All tested compounds exhibited ≥ 95% chemical purity as measured by LC/MS.

Method A, general procedure for amide formation:

A mixture of 5-bromofuran-2-carboxylic acid (1eq), thionyl chloride (2.5 eq) and DMF (5 drops) in toluene (10 mL) was refluxed at 110°C for 4 hours. The reaction mixture was cooled to room temperature; the solvent and the excess of thionyl chloride were removed

under reduced pressure. The corresponding *N*-methylamine (1 eq) and Et_3N (1 eq) in CH_2Cl_2 (10 mL) was added at 0°C under N_2 atmosphere to the acyl chloride. After 30 minutes at 0°C, the ice bath was removed and the solution was warmed up and stirred at room temperature overnight. The reaction mixture was extracted twice with CH_2Cl_2 (2 × 15 mL); the organic layer was dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by silica gel column chromatography using hexanes and EtOAc as eluent or by trituration in a mixture of diethyl ether / petroleum ether to afford the desired compound.

Method B, general procedure for Suzuki-Miyaura coupling:

In a sealed tube the previously prepared 5-bromo-*N*-heteroaryl-furan-2-carboxamide derivative (1 eq.) was introduced followed by the corresponding boronic acid (1.5 eq.), cesium carbonate (3 eq.), tetrakis(triphenylphosphine)palladium (0.02 eq.) and a mixture of DME/EtOH/H_2O (1:1:1, v:v:v, 3 mL) as solvent. The reactor was flushed with N_2 and submitted to microwave irradiation (150°C, 150 W) for 20 minutes. After cooling to room temperature, a mixture of EtOAc/H_2O (1:1, v:v, 2 mL) was added to stop the reaction. The aqueous layer was extracted with EtOAc (3 × 10 mL). The organic layer was washed once with brine and once with water, dried over $MgSO_4$, filtered and the solution was concentrated under reduced pressure. The residue was purified by column chromatography using hexanes and EtOAc as eluent to afford the desired compound.

Method C, general procedure fo ethe cleavage:

To a solution of methoxyaryl compounds (1 eq.) in dry dichloromethane (5 mL/mmol of reactant), boron trifluoride-dimethyl sulfide complex (6 eq./methoxy function) was added dropwise at 0 °C and stirred for 6-14 h at room temperature. After the reaction was finished, the reaction mixture was diluted with dichloromethane and 5% aqueous $NaHCO_3$ was added until neutral pH was obtained. The aqueous layer wad extracted with dichloromethane. The combined organic layers were washes with brine, dried over sodium sulfate, evaporated to dryness under reduced pressure. The product was purified by column chromatography using EtOAc as eluent to afford the desired compound.

5-bromo-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**6a**)

The title compound was prepared by reaction of 5-bromofuran-2-carboxylic acid (500 mg, 2.6 mmol), thionyl chloride (0.5 mL, 6.8 mmol) and *N*,2-dimethylaniline (315 mg, 2.6mmol) according to method A. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc70:30) to afford the desired product as grey solid (349 mg, 45 %).$C_{13}H_{12}BrNO_2$; MW 294; mp: 93 – 95°C; MS (ESI) 294, 296 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz) δ2.19 (s, 3H), 3.27 (s, 3H), 5.65 (d, *J* = 4 Hz, 1H), 6.33 (d, *J* = 4

Hz, 1H), 7.26 (d, *J* = 8 Hz, 1H), 7.29-7.33 (m, 1H), 7.36 (d, *J* = 4 Hz, 2H); ^{13}C NMR (CD_3COCD_3, 125 MHz) δ 21.2, 38.4, 113.9, 118.9, 125.1, 128.2, 131.1, 138.7, 142.5, 150.6, 158.3.

5-bromo-*N*-methyl-*N*-(*p*-tolyl)furan-2-carboxamide (**5a**)

The title compound was prepared by reaction of 5-bromofuran-2-carboxylic acid (500 mg, 2.6 mmol), thionyl chloride (0.5 mL, 6.8 mmol) and *N*,4-dimethylaniline (317 mg, 2.6 mmol) according to method A. The residue was purified by silica gel column chromatography (*n*-hexane/EtOAc 70:30) to afford the desired product as yellow solid (459 mg, 60 %).$C_{13}H_{12}BrNO_2$; MW 294; mp: 114 – 116°C; MS (ESI) 294, 296 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.38 (s, 3H), 3.32 (s, 3H), 5.83 (d, *J* = 4 Hz, 1H), 6.36 (d, *J* = 4 Hz, 1H), 7.26 (d, *J* = 8 Hz, 1H), 7.29-7.33 (m, 1H), 7.36 (d, *J* = 4 Hz, 2H); ^{13}C NMR (CD_3COCD_3, 125 MHz) δ 21.2, 38.4, 113.9, 118.9, 125.1, 128.4, 131.1, 138.74, 142.5, 150.6, 158.3.

N-methyl-5-(*o*-tolyl)-*N*-(*p*-tolyl)furan-2-carboxamide (**5**)

The title compound was prepared by reaction of **5a** (100 mg, 0.34mmol), *o*-tolylboronicacid(60 mg, 0.44mmol), cesium carbonate (332 mg, 1.02mmol) and tetrakis(triphenylphoshine) palladium (8 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as colorless solid (49 mg, 47%). $C_{20}H_{19}NO_2$; MW 305; mp: 94-97 °C; MS (ESI) 292 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.35 (s, 3H), 2.39 (s, 3H), 3.36 (s, 3H), 6.54 (d, *J* = 4 Hz, 1H), 6.58 (d, *J* = 4 Hz, 1H), 7.02 (d, *J* = 8 Hz, 1H), 7.11-7.15 (m, 1H), 7.17-7.22 (m, 4H), 7.26-7.29 (m, 2H); ^{13}C NMR (CD_3COCD_3, 125 MHz) δ 21.1, 21.9, 38.6, 110.8, 118.9, 126.7, 127.8, 128.0, 129.1, 129.9, 131.0, 132.0, 135.7, 138.1, 143.2, 147.8, 155.1, 159.3.

N-methyl-*N*-(*o*-tolyl)-5-(p-tolyl)furan-2-carboxamide (**6**)

The title compound was prepared by reaction of **6a** (100 mg, 0.34mmol), *p*-tolylboronicacid(60 mg, 0.44mmol), cesium carbonate (332 mg, 1.02mmol) and tetrakis(triphenylphoshine) palladium (8 mg, 0.02 eq) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow oil (30 mg, 29%). $C_{20}H_{19}NO_2$; MW 305; MS (ESI) 306 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ2.22 (s, 3H), 2.30 (s, 3H), 3.30 (s, 3H), 6.44 (d, *J*= 3Hz, 1H), 6.65 (d, *J*= 3Hz, 1H), 7.13 (d, *J*= 8 Hz, 2H), 7.22 (d, *J*= 8Hz, 2H), 7.27-7.40 (m, 4H); ^{13}C NMR (CD_3COCD_3, 125 MHz) δ 17.5, 21.2, 37.4, 106.6, 119.1, 125.0, 128.0, 128.3, 129.1, 130.1, 132.2, 136.7, 139.2, 144.2, 147.7, 156.2, 159.3.

5-(4-methoxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**8a**)

Results

The title compound was prepared by reaction of **6a** (80 mg, 0.27mmol), (4-methoxyphenyl)boronic acid (54 mg, 0.35mmol), cesium carbonate (266 mg, 0.82mmol) and tetrakis(triphenylphoshine) palladium (6 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as yellow solid (52 mg, 29%). $C_{20}H_{19}NO_3$; MW 321; mp: 92-95 °C; MS (ESI) 322 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ2.22 (s, 3H), 3.29 (s, 3H), 3.80 (s, 3H), 6.43 (d, *J*= 3Hz, 1H), 6.59 (d, *J*= 3Hz, 1H), 6.89 (d, *J*= 8 Hz, 2H), 7.26-7.41 (m, 7H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ 17.5, 37.3, 55.7, 105.6, 114.9, 119.0, 123.5, 126.6, 128.2, 129.0, 129.2, 132.1, 136.7, 144.4, 147.6, 156.0, 159.1, 160.9.

5-(4-hydroxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**8**)

The title compound was prepared by reaction of **8a** (50 mg, 0.16 mmol) and $BF_3{\cdot}SMe_2$ (100 µL, 0.96 mmol) according to method C. The residue was purified silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as yellow solid (45 mg, 92%). $C_{19}H_{17}NO_3$; MW 307; mp: 172-175 °C; MS (ESI) 308 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ2.22 (s, 3H), 3.29 (s, 3H), 6.40 (d, *J*= 3Hz, 1H), 6.54 (d, *J*= 3Hz, 1H), 6.80 (d, *J*= 8 Hz, 2H), 7.19 (d, *J*= 8 Hz, 2H), 7.28 (dd, *J* = 2 Hz, 8 Hz, 1H), 7.31-7.41 (m, 3H), 8.60 (s, 1H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ 17.5, 37.3, 105.1, 116.4, 119.0, 122.6, 126.7, 128.2, 129.0, 129.1, 132.1, 136.7, 144.4, 147.3, 156.4, 158.8, 159.1.

N-(4-methoxyphenyl)-*N*-methyl-5-(*o*-tolyl)furan-2-carboxamide (**7a**)

The title compound was prepared by reaction of **7b** (150 mg, 0.48mmol), *o*-tolylboronic acid (85 mg, 0.62mmol), cesium carbonate (473 mg, 1.45mmol) and tetrakis(triphenylphoshine) palladium (11 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product as yellow solid (91 mg, 59%). $C_{20}H_{19}NO_3$; MW 321;mp : 103 – 106 °C; MS (ESI) 322 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.38 (s, 3H), 3.35 (s, 3H), 3.85 (s, 3H), 6.44 (d, *J* = 4 Hz, 1H), 6.59 (d, *J* = 4 Hz, 1H), 7.02 (dd, *J* = 2 Hz, 7 Hz, 2H), 7.14-7.15 (m, 2H), 7.19-7.22 (m, 2H), 7.27 (dd,*J* = 2 Hz, 7 Hz,2H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ 21.9, 38.7, 55.9, 110.8, 115.6, 118.8, 126.7, 127.9, 129.1, 129.4, 129.9, 132.0, 135.7, 138.4, 147.8, 155.1, 159.3, 160.0.

N-(4-hydroxyphenyl)-*N*-methyl-5-(*o*-tolyl)furan-2-carboxamide (**7**)

The title compound was prepared by reaction of **7a** (76 mg, 0.24 mmol) and $BF_3{\cdot}SMe_2$ (150 µL, 1.43 mmol) according to method C. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as white solid (30 mg, 41%). $C_{19}H_{17}NO_3$; MW 307; mp: 180-182 °C; MS (ESI) 308 $[M+H]^+$; ^{1}H NMR (DMSO-d6, 500 MHz) δ2.34 (s, 3H), 3.28 (s, 3H), 6.26 (br s, 1H), 6.64 (d, *J*= 4Hz, 1H), 6.82 (dd, *J*= 2Hz, 6 Hz, 2H), 7.14 (dd, *J*= 2Hz, 6 Hz, 2H), 7.18-7.25 (m,4H), 9.69 (s,

1H);^{13}C NMR (DMSO-d6, 125 MHz) δ21.3, 38.3, 110.2, 116.0, 117.9, 126.0, 126.7, 128.3, 128.40, 128.43, 131.2, 134.5, 135.2, 146.1, 153.5, 156.9, 158.2.

5-(2-fluoro-4-methoxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**16a**)

The title compound was prepared by reaction of **6a** (80 mg, 0.27mmol), (2-fluoro-4-methoxyphenyl)boronic acid (60 mg, 0.35mmol), cesium carbonate (266 mg, 0.82mmol) and tetrakis(triphenylphoshine) palladium (6 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product as yellow oil (60 mg, 67%). $C_{20}H_{18}FNO_3$; MW 339; MS (ESI) 340 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.22 (s, 3H), 3.30 (s, 3H), 3.84 (s, 3H), 6.49 (d, *J* = 4 Hz, 1H), 6.56 (t, *J* = 4 Hz, 1H), 6.74-6.59 (m,2H), 6.97 (t, *J*= 8 Hz, 1H), 7.29-7.42 (m, 4H); ^{13}C NMR (CD_3COCD_3, 125 MHz) δ17.6, 37.4, 56.3, 102.6, 102.8, 109.9, 110.0, 111.4, 111.48, 111.52, 111.54, 119.1, 128.0, 128.1, 128.4, 129.17, 129.23, 132.2, 136.8, 144.4, 145.3, 147.6, 150.2, 159.0, 159.7, 161.7, 162.1, 162.2.

5-(2-fluoro-4-hydroxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**16**)

The title compound was prepared by reaction of **16a** (57 mg, 0.17 mmol) and $BF_3{\cdot}SMe_2$ (110 µL, 1.01 mmol) according to method C. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as yellow oil (10 mg, 18%). $C_{19}H_{16}FNO_3$; MW 325; MS (ESI) 326 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.22 (s, 3H), 3.30 (s, 3H), 6.45 (d, *J* = 4 Hz, 1H), 6.50-6.52 (m, 1H), 6.62-6.66 (m, 2H), 6.92 (t, *J* = 8 Hz, 1H), 7.29-7.41 (m, 4H), 9.24 (br s, 1H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ 17.6, 37.5, 103.9, 104.0, 104.1, 109.5, 109.6, 110.5, 110.6, 112.7, 112.83, 112.85, 119.2, 128.3, 128.4, 129.18, 129.22, 132.6, 144.3, 147.2, 150.7, 159.2, 159.8, 160.1, 160.2, 161.8.

5-(3-chloro-4-hydroxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**17**)

The title compound was prepared by reaction of **6a** (80 mg, 0.27mmol), (3-chloro-4-hydroxyphenyl)boronic acid (61 mg, 0.35mmol), cesium carbonate (266 mg, 0.82mmol) and tetrakis(triphenylphoshine) palladium (6 mg, 0.02 eq.) according to method B. The residue was purified by crystallization in ethanol and water, to afford the desired product as white solid (36 mg, 39%). $C_{19}H_{16}ClNO_3$; MW 342;mp : 77 – 80 °C; MS (ESI) 342, 344 $[M+H]^+$; ^{1}H NMR (DMSO-d6, 500 MHz) δ2.15 (s, 3H), 3.24 (s, 3H), 6.50 (d, *J* = 4 Hz, 1H), 6.74 (d, *J* = 4 Hz, 1H), 6.91 (d, *J* = 8 Hz, 1H), 7.08 (d, *J* = 2 Hz, 1H), 7.14 (dd, *J* = 2 Hz, 8 Hz, 1H), 7.27-7.40 (m, 4H), 10.52 (s, 3H);^{13}C NMR (DMSO-d6, 125 MHz) δ 17.0, 36.9, 105.7, 116.8, 118.5, 120.2, 121.5, 124.0, 125.3, 127.3, 128.0, 128.3, 131.1, 135.2, 142.8, 146.0, 153.40, 153.42, 157.7.

5-(3-fluoro-4-methoxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**11a**)

The title compound was prepared by reaction of **6a** (100 mg, 0.34mmol), (3-fluoro-4-methoxyphenyl)boronic acid(75 mg, 0.44mmol), cesium carbonate (332 mg, 1.02 mmol) and tetrakis(triphenylphoshine) palladium (8 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30), to afford the desired product as ayellow oil (75 mg, 65%). $C_{20}H_{18}FNO_3$; MW 339; MS (ESI) 340 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.22 (s, 3H), 3.30 (s, 3H), 3.89 (s, 3H), 6.52 (d, *J* = 3 Hz, 1H), 6.69 (d, *J* = 3 Hz, 1H), 6.99-7.02 (m, 1H), 7.08-7.13 (m, 2H), 7.30 (dd, *J* = 2 Hz, 8 Hz, 1H), 7. 32-7.42 (m, 3H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ17.5, 37.4, 56.6, 106.7, 112.5, 112.7, 114.70, 114.71, 119.1, 121.46, 121.49, 123.87, 123.93, 128.3, 129.1, 132.1, 136.7, 144.3, 148.1, 148.76, 148.85, 152.1, 154.1, 154.7, 158.9.

5-(3-fluoro-4-hydroxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**11**)

The title compound was prepared by reaction of **11a** (60 mg, 0.18 mmol) and $BF_3{\cdot}SMe_2$ (110 µL, 1.06 mmol) according to method C. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as yellow solid (55 mg, 95%). $C_{19}H_{16}FNO_3$; MW 325; mp: 190-192 °C; MS (ESI) 326 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.22 (s, 3H), 3.30 (s, 3H), 6.49 (d, *J* = 4 Hz, 1H), 6.64 (d, *J* = 4 Hz, 1H), 6.93-7.00 (m, 2H), 7.04 (d, *J* = 8 Hz, 1H), 7.30 (dd, *J* = 2 Hz, 8 Hz, 1H), 7.32-7.42 (m, 3H), 8.98 (s, 1H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ17.5, 37.4, 106.3, 112.7, 112.9, 118.9, 119.0, 119.2, 121.72, 121.75, 123.17, 123.22, 128.3, 129.1, 132.1, 136.7, 144.3, 146.1, 146.2, 147.8, 151.4, 153.3, 155.03, 155.05, 159.0.

5-(4-methoxy-3,5-dimethylphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**15a**)

The title compound was prepared by reaction of **6a** (500 mg, 1.70mmol), (4-methoxy-3,5-dimethylphenyl)boronic acid(367 mg, 2.04mmol), cesium carbonate (1662 mg, 5.10 mmol) and tetrakis(triphenylphoshine) palladium (39 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product asyellow solid (75 mg, 65%). $C_{22}H_{23}NO_3$; MW 349; mp : 128-132 °C MS (ESI) 350 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.22 (s, 9H), 3.30 (s, 3H), 3.68 (s, 3H), 6.62 (d, *J* = 3 Hz, 1H), 6.64 (d, *J* = 3 Hz, 1H), 6.94 (brs, 2H), 7.29 (dd, *J* = 2, 8 Hz, 1H), 7.33-7.43 (m, 3H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ16.11, 16.16, 17.6, 37.5, 60.6, 106.4, 119.3, 125.6, 126.3, 128.3, 129.2, 132.1, 132.2, 136.8, 144.6, 148.1, 156.0, 158.4, 159.1;IR (cm^{-1}) 1626, 2856, 2924, 2981, 3049.

5-(3,5-difluoro-4-methoxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**13a**)

The title compound was prepared by reaction of **6a** (500 mg, 1.70mmol), (3,5-difluoro-4-methoxyphenyl)boronic acid(383 mg, 2.04mmol), cesium carbonate (1662 mg, 5.10 mmol) and tetrakis(triphenylphoshine) palladium (39 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 70:30) to afford the desired product asyellow oil (236 mg, 39%). $C_{20}H_{17}F_2NO_3$; MW 357; mp : 70-72

°C; MS (ESI) 357.9, 358.5, 359.2$[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.23 (s, 3H), 3.31 (s, 3H), 3.97 (s, 3H), 6.64 (d, *J* = 4 Hz, 1H), 6.84 (d, *J* = 4 Hz, 1H), 6.89 (d, *J* = 9 Hz, 2H), 7.30-7.43 (m, 4H);^{13}C NMR (CD_3COCD_3, 125 MHz)δ17.6, 37.5, 62.4, 108.6, 108.87, 108.92, 109.1, 119.3, 125.9, 126.0, 126.1, 128.4, 129.2, 129.3, 132.2, 136.8, 137.2, 144.3, 148.9, 153.2, 155.80, 155.85, 157.76, 157.81, 158.8;IR (cm^{-1}) 1625, 2847, 2921.

5-(3,5-dichloro-4-methoxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**14a**)

The title compound was prepared by reaction of **6a** (500 mg, 1.70mmol), (3,5-dichloro-4-methoxyphenyl)boronic acid (450 mg, 2.04mmol), cesium carbonate (1662 mg, 5.10 mmol) and tetrakis(triphenylphoshine) palladium (39 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 80:20) to afford the desired product asyellow solid(438 mg, 39%). $C_{20}H_{17}Cl_2NO_3$; MW 390; mp : 114-117 °C MS (ESI) 390, 392$[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 300 MHz) δ 2.25 (s, 3H), 3.33 (s, 3H), 3.89 (s, 3H), 6.78 (d, *J* = 4 Hz, 1H), 6.94 (d, *J* = 4 Hz, 1H), 7.24 (s, 2H), 7.31-7.46 (m, 4H);^{13}C NMR (CD_3COCD_3, 75 MHz) δ17.6, 37.5, 61.3, 109.0, 119.4, 125.2, 128.3, 128.4, 129.1, 129.5, 130.5, 132.2, 136.7, 144.3, 149.3, 152.5, 152.8, 158.7.

5-(4-methoxy-2-methylphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**10a**)

The title compound was prepared by reaction of **6a** (500 mg, 1.70mmol), (4-methoxy-2-methylphenyl)boronic acid(339 mg, 2.04mmol), cesium carbonate (1662 mg, 5.10 mmol) and tetrakis(triphenylphoshine) palladium (39 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 90:10) to afford the desired product aswhite solid (247 mg, 41%). $C_{21}H_{21}NO_3$; MW 335; mp : 90-93 °C MS (ESI) 336$[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ2.22 (s, 3H), 2.35 (s, 3H), 3.29 (s, 3H), 3.79 (s, 3H), 6.33 (d, *J* = 3 Hz, 1H), 6.42 (d, *J* = 3 Hz, 1H), 6.72 (dd, *J* = 2 Hz, 8 Hz, 1H), 6.78 (s, 1H), 6.99 (d, *J* = 7 Hz, 1H), 7.27-7.40 (m, 4H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ17.6, 22.1, 37.4, 55.6, 109.4, 112.4, 117.2, 118.6, 122.8, 128.4, 129.1, 129.2, 129.6, 132.3, 136.7, 137.6, 144.4, 147.2, 155.6, 159.3, 160.6;IR (cm^{-1}) 1639, 2835, 2859, 2936, 2960, 3052.

5-(4-methoxy-3-methylphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**12a**)

The title compound was prepared by reaction of **6a** (500 mg, 1.70mmol), (4-methoxy-3-methylphenyl)boronic acid(339 mg, 2.04mmol), cesium carbonate (1662 mg, 5.10 mmol) and tetrakis(triphenylphoshine) palladium (39 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 90:10) to afford the desired product ascolorless oil (213 mg, 37%). $C_{21}H_{21}NO_3$; MW 335; MS (ESI) 336 $[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ2.15 (s, 3H), 2.22 (s, 3H), 3.29 (s, 3H), 3.83 (s, 3H), 6.51 (d, *J* = 3 Hz, 1H), 6.57 (d, *J* = 3 Hz, 1H), 6.87 (d, *J* = 8 Hz, 1H), 7.02 (s, 1H), 7.18-7.19 (m, 1H), 7.28 (dd, *J* = 1 Hz, 9 Hz, 1H), 7.32-7.42 (m, 3H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ16.2, 17.6, 37.4, 55.9, 105.5, 111.1, 119.2, 123.1, 124.3, 127.3,

127.5, 128.3, 129.1, 129.2, 132.2, 136.8, 144.5, 147.6, 156.4, 159.1, 159.2;IR (cm^{-1}) 1627, 2838, 2933, 3052.

5-(2-chloro-4-methoxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**9a**)

The title compound was prepared by reaction of **6a** (500 mg, 1.70mmol), (2-chloro-4-methoxyphenyl)boronic acid(380 mg, 2.04mmol), cesium carbonate (1662 mg, 5.10 mmol) and tetrakis(triphenylphoshine) palladium (39 mg, 0.02 eq.) according to method B. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 90:10) to afford the desired product aswhite solid (150 mg, 25%). $C_{21}H_{18}ClNO_3$; MW 356; mp : 92-94 °C; MS (ESI) 356, 358$[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ2.22 (s, 3H), 3.30 (s, 3H), 3.85 (s, 3H), 6.51 (d, *J* = 3 Hz, 1H), 6.85-6.90 (m, 2H), 6.95 (d, *J* = 9 Hz, 1H), 7.00-7.01 (m, 1H), 7.28-7.41 (m, 4H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ17.6, 37.5, 56.2, 111.2, 114.4, 116.4, 118.7, 121.7, 128.4, 129.16, 129.22, 130.2, 131.8, 132.3, 136.8, 144.4, 147.7, 152.1, 159.0, 161.0;IR (cm^{-1}) 1619, 2844, 2915, 2944, 2996.

5-(4-hydroxy-3,5-dimethylphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**15**)

The title compound was prepared by reaction of **15a** (260 mg, 0.74 mmol) and $BF_3{\cdot}SMe_2$ (470 µL, 4.44 mmol) according to method C. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as yellow solid (74 mg, 30%). $C_{21}H_{21}NO_3$; MW 335; mp: 175-178 °C; MS (ESI) 336 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.22-2.23 (m, 9H), 3.37 (s, 3H), 6.32 (d, *J* = 3 Hz, 1H), 6.48 (d, *J* = 3 Hz, 1H), 6.88 (brs, 2H), 7.21-7.23 (m, 1H), 7.28-7.35 (m, 3H);^{13}C NMR ($CDCl_3$, 125 MHz) δ 16.51, 16.55, 17.6, 37.5, 105.0, 119.4, 122.4, 125.2, 125.5, 128.3, 129.1, 129.2, 132.2, 136.8, 144.6, 147.4, 154.9, 156.9, 159.3; IR (cm^{-1}) 1592, 1614, 2912, 2952, 3274.

5-(3,5-difluoro-4-hydroxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**13**)

The title compound was prepared by reaction of **13a** (180 mg, 0.50 mmol) and $BF_3{\cdot}SMe_2$ (320 µL, 3.00 mmol) according to method C. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as yellow solid (140 mg, 81%). $C_{19}H_{15}F_2NO_3$; MW 343; mp: 176-178 °C; MS (ESI) 344, 345$[M+H]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 2.22 (s, 3H), 3.31 (s, 3H), 6.59 (d, *J* = 3 Hz, 1H), 6.75 (d, *J* = 3Hz, 1H), 6.88 (d, *J* = 7 Hz, 2H), 7.30-7.42 (m, 4H), 9.25 (s, 1H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ17.6, 37.5, 107.5, 108.48, 108.54, 108.6, 108.7, 119.31, 121.97, 122.05, 122.13, 128.4, 129.2, 129.3, 132.2, 134.9, 135.0, 135.2, 136.8, 144.3, 148.4, 152.57, 152.63, 153.9, 154.50, 154.55, 158.9;IR (cm^{-1}) 1623, 2930, 3056.

5-(3,5-dichloro-4-hydroxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**14**)

The title compound was prepared by reaction of **14a** (240 mg, 0.61mmol) and $BF_3{\cdot}SMe_2$ (476 µL, 3.66mmol) according to method C. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as orange solid

(117 mg, 51%). $C_{19}H_{15}Cl_2NO_3$; MW 376; mp: 134-137 °C; MS (ESI) 376, 378 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz) δ2.23 (s, 3H), 3.31 (s, 3H), 6.71 (d, *J* = 3 Hz, 1H), 6.80 (d, *J* = 3 Hz, 1H), 7.19 (s, 2H), 7.30 (d, *J* = 8 Hz, 1H), 7.34-7.43 (m, 3H), 9.08 (s, 1H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ17.6, 37.5, 107.5, 119.4, 123.4, 124.1, 125.0, 128.3, 129.1, 129.5, 132.2, 136.7, 144.3, 148.6, 150.0, 153.2, 158.9;IR (cm^{-1}) 1620, 2853, 2923, 2957, 3129.

5-(4-hydroxy-2-methylphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**10**)

The title compound was prepared by reaction of **10a** (200 mg, 0.60mmol) and $BF_3{\cdot}SMe_2$ (468 µL, 3.60mmol) according to method C. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40) to afford the desired product as white solid (178 mg, 92%). $C_{20}H_{19}NO_3$; MW 321; mp: 219-221 °C; MS (ESI) 322 $[M+H]^+$; ^1H NMR (CD_3SOCD_3, 500 MHz) δ 2.15 (s, 3H), 2.23 (s, 3H), 3.23 (s, 3H), 6.20 (d, *J* = 3 Hz, 1H), 6.42 (d, *J* = 3 Hz, 1H), 6.55 (d, *J* = 7 Hz, 1H), 6.62 (s, 1H), 6.83 (d, *J* = 8 Hz, 1H), 7.26-7.38 (m, 4 HH), 9.64 (s, 1H);^{13}C NMR (CD_3SOCD_3, 125 MHz) δ 17.0, 21.3, 30.6, 36.9, 108.1, 113.0, 117.6, 117.8, 119.8, 127.4, 128.1, 128.4, 131.2, 135.2, 136.3, 142.8, 145.1, 154.6, 157.5, 158.0;IR (cm^{-1}) 1616, 2853, 2920, 2966, 3056, 3243.

5-(4-hydroxy-3-methylphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**12**)

The title compound was prepared by reaction of **12a** (224 mg, 0.67 mmol) and $BF_3{\cdot}SMe_2$ (420 µL, 4.02 mmol) according to method C. The residue was purified by crystallization in water and ethanol to afford the desired product as green solid (140 mg, 65%). $C_{20}H_{19}NO_3$; MW 321; mp: 183-186 °C; MS (ESI) 322 $[M+H]^+$; ^1H NMR (CD_3COCD_3, 500 MHz) δ2.16 (s, 3H), 2.22 (s, 3H), 3.29 (s, 3H), 6.47 (d, *J* = 3 Hz, 1H), 6.51 (d, *J* = 3 Hz, 1H), 6.79 (d, *J* = 8 Hz, 1H), 6.98 (s, 1H), 7.06 (d, *J* = 8 Hz, 1H), 7.28 (dd, *J* = 1 Hz, 8 Hz, 1H), 7.32-7.42 (m, 3H), 8.91 (s, 1H);^{13}C NMR (CD_3COCD_3, 125 MHz) δ16.1, 17.6, 37.4, 104.9, 115.8, 119.3, 122.2, 124.1, 125.6, 127.7, 128.3, 129.1, 129.2, 132.2, 136.7, 144.5, 147.2, 156.9, 157.2, 159.2;IR (cm^{-1}) 1573, 2907, 3132.

5-(2-chloro-4-hydroxyphenyl)-*N*-methyl-*N*-(*o*-tolyl)furan-2-carboxamide (**9**)

The title compound was prepared by reaction of **9a** (120 mg, 0.34 mmol) and $BF_3{\cdot}SMe_2$ (265 µL, 2.04 mmol) according to method C. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 60:40)to afford the desired product as pink solid (89 mg, 77%). $C_{19}H_{16}ClNO_3$; MW 342; mp: 198-201 °C; MS (ESI) 342, 344$[M+H]^+$; ^1H NMR (CD_3SOCD_3, 500 MHz) δ2.15 (s, 3H), 3.24 (s, 3H), 6.36 (d, *J* = 3 Hz, 1H), 6.70 (dd, *J* = 2 Hz, 9 Hz, 1H), 6.80-6.82 (m, 2H), 6.86 (d, *J* = 3 Hz, 1H), 7.28-7.39 (m, 4H), 10.2 (s, 1H);^{13}C NMR (CD_3SOCD_3, 125 MHz) δ16.9, 36.9, 109.7, 114.7, 116.9, 117.9, 118.5, 127.4, 128.1, 128.2, 129.2, 130.3, 131.2, 135.2, 142.7, 145.6, 151.1, 157.8, 158.3;IR (cm^{-1}) 1562, 2691, 2775, 2877, 2993, 3064.

b) Biological methods

[2,4,6,7-^{3}H]-E2 and [2,4,6,7-^{3}H]-E1 were purchased from Perkin-Elmer, Boston. Quickszint Flow 302 scintillator fluid was bought from Zinsser Analytic, Frankfurt. Other chemicals were purchased from Sigma, Roth or Merck.

***h*17*β*-HSD1 and *h*17*β*-HSD2 enzyme preparation.** Cytosolic (*h*17*β*-HSD1) and microsomal (*h*17*β*-HSD2) fractions were obtained from human placenta according to previously described procedures.[1-3] Fresh tissue was homogenized and the enzymes were separated from the mitochondria, cell membrane,nucleus and other rests by fractional centrifugation at 1000 g, 10.000 g and 150.000 g. The pellet fraction containing the microsomal *h*17*β*-HSD2 was used for the determination of *h*17*β*-HSD2 inhibition, while *h*17*β*-HSD1 was obtained after precipitation with ammonium sulfate from the cytosolic fraction for use of testing of *h*17*β*-HSD1 inhibition. Aliquots containing *h*17*β*-HSD1 or *h*17*β*-HSD2 were stored frozen.

***r*17*β*-HSD1 and *r*17*β*-HSD2 enzyme preparation.** Recombinant rat 17β-HSD1 enzyme was produced by transfection of HEK 293 cells with a rat 17β-HSD1 expression plasmid [coding sequences and NM_012851 in pCMV6Entry vector, OriGene Technologies, Inc.]. 48 hours after transfection cells were homogenized by sonication (3 x 10 s) in a buffer containing saccharose (40 mMTris, 250 mMsaccharose, 5 mM EDTA, 7 mM DTT, 1 mM PMSF, pH 7.5). Cell lysate was centrifuged (1000 g, 15 min, 4°C) and 20% glycerol was added to the supernatant before aliquots were frozen and stored at -70°C.[4] Microsomal 17β-HSD2 fractions were obtained from rat tissues.[5] Fresh tissue was homogenized and centrifuged. The pellet fraction contains the microsomal 17β-HSD2 and was used for the determination of E1 formation.

Inhibition of *h*17*β*-HSD2 and *r*17*β*-HSD2 in cell-free assay.Inhibitory activities were evaluated following an established method with minor modifications.[6, 7] Briefly, the enzyme preparation was incubated with NAD^+ [1500 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 [^{3}H]-E2 (final concentration: 500 nM, 0.11 µCi). After 20 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with ether. After evaporation, the steroids were dissolved in acetonitrile/water (45:55). E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 RP chromatography column (Nucleodur C18, 3µm, 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 the following equation: %conversion = (%E1/(%E1+%E2))×100. Each value was calculated from at least two independent experiments.

Results

Inhibition of *h*17*β*-HSD1 and *r*17*β*-HSD1 in cell-free assay.The 17*β*-HSD1 inhibition assay was performed similarly to the *h*17*β*-HSD2 test. The human cytosolic enzyme was incubated with NADH [500 µM] while the rat recombinant enzyme was reacted with NADPH [500 µM].Test compound and a mixture of unlabelled- and [^{3}H]-E1 (final concentration: 500 nM, 0.15 µCi) were added and mixed for 10 min at 37°C. Further treatment of the samples and HPLC separation was carried out as mentioned above for *h*17*β*-HSD2.

Estrogen receptor affinity in a cell-free assay. The binding affinity of compound **15** to ER*α* and ER*β* was determined according to the recommendations of the US Environmental Protection Agency (EPA) by their Endocrine Disruptor Screening Program (EDSP)[8] using recombinant human proteins. Briefly, 1 nM of ER*α* and 4 nM of ER*β*, respectively, were incubated with [^{3}H]-E2 (3 nM for ER*α* and 10 nM for ER*β*) and test compound for 16-20 h at 4°C.

The potential inhibitors were dissolved in DMSO (5% final concentration). Evaluation of non-specific-binding was performed with unlabeled E2 at concentrations 100-fold of [^{3}H]-E2 (300 nM for ER*α* and 1000 nM for ER*β*). After incubation, ligand-receptor complexes were selectively bound to hydroxyapatite (83.5 g/LinTE-buffer). The bound complex was washed three times and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (1450 LSC & Luminescence Counter, Perkin Elmer).

From these results the percentage of [^{3}H]-E2 displacement by the compounds was calculated. The plot of % displacement versus compound concentration resulted in sigmoidal binding curves. The compound concentrations necessary to displace 50% of the receptor bound [^{3}H]-E2 were determined. Unlabeled E2 IC_{50} values were determined in each experiment and used as reference. The E2 IC_{50}determined were 3±20% nM for *ERα* and 10±20% nM for ER*β*.Relative Binding Affinity was determined by applying the following equation: RBA[%] = (IC_{50}(E2)/IC_{50}(compound)) · 100.[10] This results in a RBA value of 100% for E2. After the assay was established and validated, a modification was made to increase throughput. Compounds were tested at concentrations of 1000 times the IC_{50}(E2). Compounds with less than 50% displacement of [^{3}H]-E2 at a concentration of 1000 timesIC_{50}(E2) were classified as RBA <0.1%.

Metabolic Stability in a cell-free assay. Compounds **15** were tested according to established method.[9-11]For evaluation of phase I and II metabolic stability 1 µM compound was incubated with 1 mg/ml pooled mammalian liver S9 fraction (BD Gentest), 2 mM NADPH regenerating system, 1 mM UDPGA and 0.1 mM PAPS at 37°C for 0, 5, 15 and 60 minutes at a final volume of 100 µL. The incubation was stopped by precipitation of S9 enzymes with 2 volumes of cold acetonitrile containing internal standard. Concentration of the remaining test compound at the different time points was analyzed by LC-MS/MS and used to determine half-life ($t_{1/2}$).

Results

MTT-Cytotoxicity assay. The number of living cells was evaluated measuringthe reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide(MTT). Experiments were performed in96-well cell culture plates in DMEM supplemented with 10% FCS. Cells were incubated for 66 h with 6.25, 12.50, 25, 50, and 100 µM of test compound at37 ˚C in a humidified atmosphere at 5% CO_2. For cleavage reactionMTT-solution (5mg/mL in PBS) was added and incubation wascontinued for another 66 h. Reaction stop and cell lysis were carriedout by addition of sodium dodecyl sulphate (SDS) in 0.01N HCl(10%). The produced blue formazan was quantifiedspectrophotometricallyat 590nm as described by Denizot and Lang[12]withminor modifications.

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3. Final Discussion

The present study aimed at the accomplishment of two main goals: 1) The optimization of the drug-like properties of*in house* 17β-HSD2 inhibitors for the development of a lead candidate which might be suitable for both an *in vivo* proof of concept in a mouse model of osteoporosis and for further development as a therapeutic (**Part One**). 2) Discovery of a new class of potent and selective human and rat 17β-HSD1 inhibitors, with good Phase I and II metabolic stability which might be used in a rat model of EDD (**Part Two**).

Part One.

In order to increase the chances of a compound to survive clinical trials, the medicinal chemist must optimize not only its pharmacological properties, but also its drug-like properties.
Drug-like properties is a term which refers to a large variety of characteristics of a molecule which, if carefully tuned, increase its chances of becoming a successful drug product and it includes: appropriate structural properties (i.e. hydrogen bonding, etc.), physicochemical properties (i.e. solubility, chemical stability, lipophilicity etc.), biochemical properties (i.e. metabolic stability, PPB) pharmacokinetics and toxicity (i.e. clearance, LD_{50}, etc.).
In the effort to efficiently optimize our *in house* 17β-HSD2 inhibitors, a pharmacological flowchart (Figure 9) was elaborated in order to obtain the desired drug-like properties. In addition the desired molecule must be suitable for an *in vivo* study in an animal model of osteoporosis.

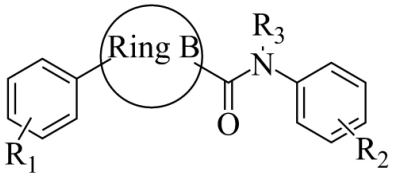
In chapter 2.I, the main focus was put on the metabolic stability of the inhibitors. In order to find out the potential sites of metabolism, eleven 2,5-thiophene amide inhibitors (**I.1-I.11**) were tested for their phase I and II metabolic stability using liver S9 fraction. In this study, none of the tested inhibitors showed a satisfying metabolic stability and it was concluded that the methyl group onthe A ring is less prone to metabolic degradation than the methoxy group and the phenolic hydroxy substituent on both the A and C rings is subject to fast metabolism. Combining this information with the knowledge that the thiophene is a potential site of metabolism and that the lipophilicity also play an important role in metabolic stability, several different compounds were designed and synthesized. Doing this we kept in mind the SAR data obtained from previous studies.

The exchange in compounds **I.1** (IC_{50}= 58 nM) of both methoxy groups on rings A and C by methyl moieties led to compound **I.12** (IC_{50}= 52 nM), with comparable inhibitory activity, thus confirming the previously observed SAR data about the equivalency of methoxy and methyl groups with regard to 17β-HSD2 inhibition. Despite the preliminary study on metabolic stability indicated that the methyl group on the A ring is less prone to metabolic degradation than the methoxy group, the parallel exchange of both methoxy, realised in

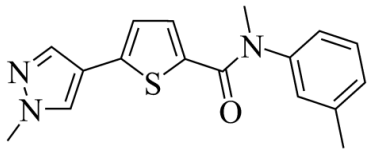
compound **I.12**, did not bring the desired improvement (Table 2), probably because of a switch of the responsible metabolizing enzyme.

On the other hand, replacement of the thiophene by a furane or by a benzene resulted in much more stable compounds **I.31** and **I.35**, respectively (Table 2), indicating that the thiophene plays an important role inthe metabolic fate of this class of compounds.
The strategy of lowering the cLogP of compound **I.12** (cLogP= 3.38) was also successful. In fact the pyrazole **I.27** (cLogP= 2.46) was, in comparison to **I.12**, much more stable (Table 2).
Compound **I.41** (Table 2), bearing a free carboxamide moiety, was also tested for metabolic stability, and was found to be exceptionally stable in comparison to the methylated analog **I.**5 (Table 2). It was not clear whether the methyl group on the N-Me amide is the metabolic reactive site or whether it only promotes metabolism atanother site of the molecule.

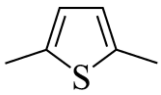
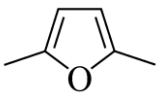
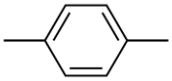
Table 2. Half-life of representative compounds measured in Human Liver Microsomes S9 fraction.



I.1, I.12, I.31, I.35, I.41



I.27

compd	Ring B	R_1	R_2	R_3	Inhibitor[c] $t_{1/2}$(min)[a,b]
I.1		3-OMe	3-OMe	-Me	17
I.12		3-Me	3-Me	-Me	19
I.41		2-F, 3-OMe	3-OMe	-H	117
I.31		3-Me	3-Me	-Me	92
I.35		-	-	-Me	>120
I.27		-	-	-	82

[a]Extrapolated value. Mean of at least two determinations, standard deviation less than 25%.

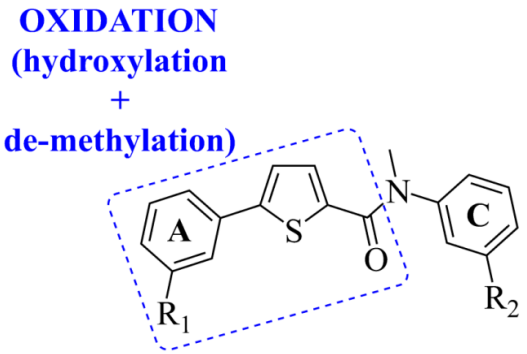
[b] 1 mg/ml pooled mammalian liver S9 fraction (BD Gentest), 2 mM NADPH regenerating system, 1 mM UDPGA and 0.1 mM PAPS at 37°C for 0, 5, 15 and 60 minutes.

[c]Inhibitor tested at a final concentration of 1 µM.

Among the metabolically stable compounds **I.27**, **I.31**, **I.35** and **I.41** described in Chapter 2.I, **I.35** was the only one retaining 17β-HSD2 inhibitory activity, with an IC_{50} value of 1126 nM.

The metabolic fate of compounds **I.1**, **I.2** and **I.12** was studied. The main metabolic pathway for all the compounds was found to be a oxidation of the left part of the molecule, resulting in either hydroxylation or de-methylation reactions (Figure 14).
In order to identify the exact oxidation site for **I.12**, all its potential metabolites were synthesized for comparison and a metabolic pathway for this compound in human S9 fraction was outlined. The principal metabolic reaction was revealed to be the hydroxylation of the methyl group of ring A.

Although this study clearly indicated that the central thiophene of compound **I.12** did not constitute a metabolic reactive site, it was also demonstrated that modulation of **I.12** metabolic rate was achievable by modification of the central ring.**I.35**, bearing a central 1,4-substituted phenyl ring, resulted in exceptional high metabolic stability and it was the starting point for following inhibitory activity optimizationstudy, as described in chapter 2.II.



I.1: R_1= -OMe, R_2= -OMe
I.2: R_1= -Me, R_2= -OMe
I.12: R_1= -Me, R_2= -Me

Figure 14. Metabolic fate of compounds **I.1**, **I.2** and **I.12**

The goal of the work described in chapter 2.II was the discovery of new potent and selective inhibitors of both h17β-HSD2 and m17β-HSD2 and therefore suitable for an *in vivo* study in a mouse model of osteoporosis.
In order to develop an optimization strategy, a comparative SAR was elaborated by testing 25 previously described h17β-HSD2 inhibitors, belonging to the 2,5-thiophene amide, 1,3-phenyl amide and 1,4 phenyl amide classes (Figure 15).

Final discussion

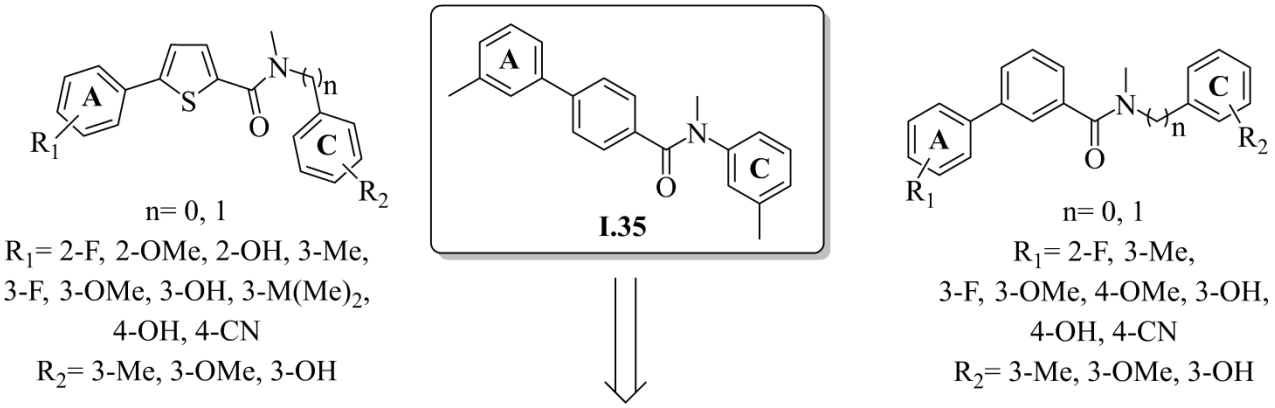
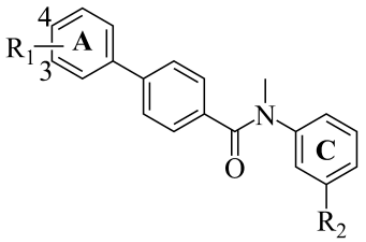


Figure 15. Previously described h17β-HSD2 inhibitors tested for m17β-HSD2 inhibition.

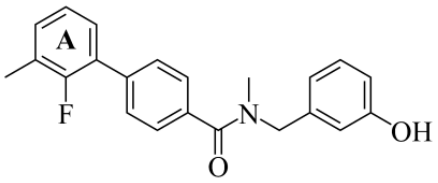
For all the tested 2,5-thiophene and the 1,3-phenyl amides both the nature of substituents on ring A and C and their substitution pattern influenced h17β-HSD2 inhibition, whereas they did not seem not exert an effect on the h17β-HSD2 enzyme, suggesting that inhibitors in these classes might have different binding modes in the two isozymes.
In contrast, compound **I.35**, bearing a 1,4-disubstituted phenyl moiety as central ring, showed moderate inhibition of both h17β-HSD2 and m17β-HSD2, as well as exceptional metabolic stability in the human liver S9 fraction, with a half life> 120 min. Therefore, it constituted the starting point for the design of a small library of inhibitors where the substitution pattern and the physicochemical nature of substituents on rings A and C were varied.

Inhibitors belonging to the 1,4-phenyl amide class similarly inhibited both h17β-HSD2 and h17β-HSD2. The different substituents introduced on rings A and B had comparable effects on the inhibitory activity in both 17β-HSD2 isoforms, in contrast with what was observed for 2,5-thiophene and 1,3-phenyl amides.
It is therefore likely that 1,4-phenyl amide derivatives bind in or very close to one of the conserved regions of the SDR superfamily members, such as the Rossmann fold or the catalytic triad.

Table 3. Human and mouse 17β-HSD2 and human h17β-HSD1 inhibition of representative 1,4-phenyl amides.



I.35, II.6a, II.7a and **II.17A**



II.25

Cmpd	R_1	R_2	Inhibition IC_{50} (nM)[a] or % inh. at 1μM[a,c]		s.f.[d,f]	Inhibition IC_{50} (nM)[a] or % inh. at 1μM[a,c]
			17β-HSD2[b]	17β-HSD1[c]		17β-HSD2[f,g]
I.35	3-Me	3-Me	1100 nM	11500 nM	10	50%
II.6a	3-Me	3-OMe	260 nM	6400 nM	25	260
II.7a	3-OMe	3-OMe	330 nM	6400 nM	20	290
II.17a	3-Me, 4-Me	3-OMe	300 nM	13300 nM	44	140
II.25	-	-	260 nM	31000	118	190

[a]Mean value of at least two determinations, standard deviation less than 20% except for 11a (hHSD2): 26%, 7(hHSD1):25%,. [b]Human placental, microsomal fraction, substrate E2[500 nM], cofactor NAD^+[1500 μM]. [c]Human placental, cytosolic fraction, substrate E1[500 nM], cofactor NADH[500 μM]. [d]s.f. (selectivity factor)=IC_{50}(17β-HSD1)/IC_{50}(17β-HSD2). [e]n.i.: no inhibition (inhibition of <10%).[f]n.d.: not determined. [g]Mouse liver microsomal fraction, substrate E2 [500 nM], cofactor NAD^+ [1500 μM].

Compounds **II.6a**, **II.7a**, **II.17a** and **II.25** displayed significantly improved h17β-HSD2 and m17β-HSD2 inhibitory activity and selectivity over h17β-HSD1 (Table 3). They all showed no inhibition of m17β-HSD1 at a concentration of 1 μM, suggesting a good selectivity over this enzyme, as well.

Seven of the newly synthesized 1,4-phenyl amides were tested for metabolic stability, using human liver S9 fraction. With the exception of compound **II.25**, they all revealed an exceptional metabolic stability, suggesting that this isa positive characteristic of the whole class. Since compound **II.25**was the only one bearing a hydroxyl group as well as the only tested compound which was metabolically unstable, it is likely to be subject to Phase II metabolism at this functional group.

Inhibitory activity assay, using the human mammary tumor cell line MDA-MB-231, for compounds **II.6a**, **II.17a** and **II.25** also revealed that these inhibitors can permeate the membrane and are able to inhibit the enzyme in a concentration dependent manner.

Compounds **II.6a**, **II.7a**, **II.17a** and **II.25** were also tested for their affinity toward the ERα and ERβ, resulting in a very low binding affinity (RBA< 0,1%).

In conclusion compound **II.17a** resulted in the best pharmacological profile, with strong inhibition of both h17β-HSD2 and m17β-HSD2 and selectivity toward the respective type 1

enzymes and the ERs. It also displayed good cellular inhibitory activity, high metabolic stability and good physicochemical parameters (MW= 345 and cLogP= 4.75) which are predictor for a good oral bio-availability.

Although compound **II.17a** seemed to meet most of the requirements of our pharmacological flowchart, it was later found to exert some cytotoxicity. In fact only 34% of the cells treated with 6.25 µM of **II.17a** were still alive in an MTT assay.

Therefore in chapter III is a successful strategy is described to address cytotoxicity and keep up potency and selectivity of the lead **II.17a**.

In order to achieve these goals, it was decided to disrupt the aromaticity and linearity of the biphenyl moiety. Four different classes of compounds were therefore explored (Figure 16): (1) the 4-phenoxybenzamides, compounds **III.7a** and **III.1-III.7**; (2) the phenylcyclohexene, compound **III.8**; (3) the phenylpiperazin-1-yl sulfonyls, compounds **III.9a-III.11a** and **III.9-III.11**; (4) the phenypiperazin-1-yl methanones, compounds **III.12a-III.15a** and **III.12-III.14**.

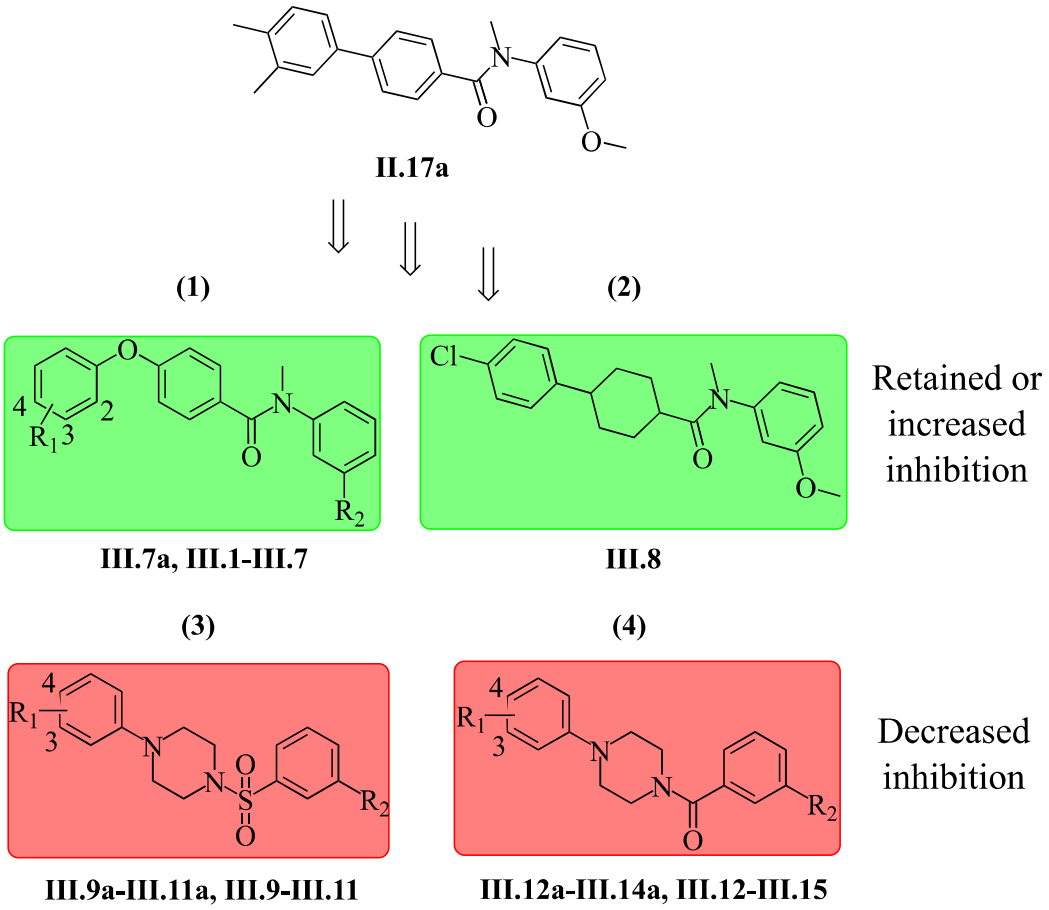


Figure 16. Structure overview of four different classes of inhibitors designed to address **II.17a** cytotoxicity.

In the class of 4-phenoxybenzamides, compounds **III.3**, **III.4** and **III.7a** displayed similar or better h17β-HSD2 inhibitory activity and selectivity, in comparison to **II.17a** (Table 4),

demonstrating that the linear shape of the 1,4-phenyl amide class is not necessary for inhibition.
Also the phenylcyclohexene **III.8** displayed similar inhibitory activity and a more than 4-fold higher selectivity over h17β-HSD1 in comparison to **II.17a** (Table 4), indicating that the aromaticity of the central ring is not needed for h17β-HSD2 inhibition.

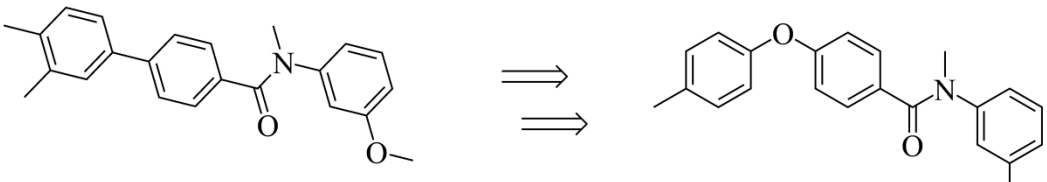
On the other hand, all the tested piperazine derivatives **III.9a-III.11a**, **III.9-III.11**, **III.12a-III.15a** and **III.12-III.14** showed weak or no inhibition of h17β-HSD2. Probably the introduction of the hydrophilic piperazine ring or the sulphonamide function condensed in the piperazine ring, which makes the molecule shorter, are responsible for the loss in inhibitory activity.

Table 4. Human 17β-HSD2 and 17β-HSD1 inhibition of **III.3**, **III.4III.7a** and **III.8**.

			IC_{50} (nM)[a] or Inh. at 1 μM[a]		
Cmpd	R_1	R_2	17β-HSD2[b]	17β-HSD1[c]	s.f.[d]
II.17a	-	-	75% (300nM)	n.i. (13300)	44
III.3	3-Me	-Me	300	16100	54
III.4	4-Me	-Me	160	26300	168
III.7a	4-OMe	-OMe	310	9600	31
III.8	-	-	290	60100	209

[a]Mean value of at least two determinations, standard deviation less than 20%. [b]Human placental, microsomal fraction, substrate E2[500 nM], cofactor NAD^+[1500 μM]. [c]Human placental, cytosolic fraction, substrate E1[500 nM], cofactor NADH[500 μM]. [d]n.d.: not determined.

The evaluation of cell viability in HEK293 cells for compounds **III.3**, **III.4**, **III.7a** (LD_{50}~ 25 μM) and **III.8** (LD_{50}> 12 μM), revealed a much improved safety profile for all the compounds, when compared to **II.17a**(LD_{50}< 6.25 μM).
Compound **III.4** also displayed improved h17β-HSD2 inhibitory activity and selectivity, confirming it as new lead compound (Figure 17).



II.17a
17β-HSD2 IC_{50}= 300
selectivity factor(HSD1)= 44
MTT test LD_{50}< 6.25 μM

III.4
17β-HSD2 IC_{50}= 160
selectivity factor(HSD1)= 168
MTT test LD_{50}~ 25 μM

Figure 17. New lead compound for h17β-HSD2 inhibition.

Part Two.

In chapters 2.I-2.III,the laborious process of obtaining potent 17β-HSD2 inhibitors, with significant selectivity over 17β-HSD1, together with well defined pharmacological properties, aimed to assure the "drug-likeness" of the new chemical entities is described.

In chapter 2.IV, a strategy to reverse the inhibitory activity of 17β-HSD2 inhibitors in favour of 17β-HSD1 is presented. The metabolic stability information, gained in the previous studies (chapter 2.I and 2.II) were also integrated in the inhibitor design process, in order to obtain metabolically stable compounds.

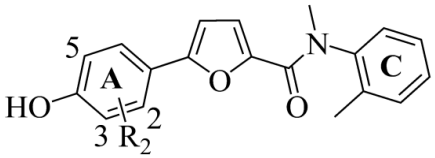
In the frame of the 17β-HSD2 project, the class of 2,5-thiophene inhibitors was extensively studied. Compound **I.12**, turned out to be a potent and selective h17β-HSD2 inhibitor, although it is metabolically unstable. Exchange of the thiophene by a furane ring, improved the metabolic stability strongly, as it results by comparison of **I.12** and **I.31** ($t_{1/2}$= 19 min and 92 min, respectively). In addition only the 2,4 and the 4,2 substitution on ring A and C were favourable for h17β-HSD1 inhibitory activity.

A small series of biphenyl-2,5-furane amides were synthesized, without hydroxy group on the phenyls. They inhibited h17β-HSD1 potently and selectively, with **IV.12a** (70% and 12% inhibition of h17β-HSD1 and h17β-HSD2 at 1 µM, respectively) being the best compound in the series. The design concept with regard to the inversion of inhibitory activity was therefore confirmed as correct.

Another small library of biphenyl-2,5-furane amides, with an hydroxy group on the A ring was also synthesized and tested (Table 5). All the compounds in this series showed exceptionally high h17β-HSD1 inhibitory activity, in comparison to the analogues not bearing the hydroxy function, thus suggesting that the hydroxy significantly contributes to the interaction with the enzyme. In particular **IV.12** and **IV.15** were also very selective over h17β-HSD2 (Table 5, selectivity factor= 198 and 563, respectively), indicating that the methyl groups in meta position are not tolerated by this enzyme.

The hydroxyl derivatives were also tested against the rat 17β-HSD1 (r17β-HSD1) and 17β-HSD2 (r17β-HSD2) enzymes (Table 5). Most of them were very potent in inhibiting r17β-HSD1, but only compound **IV.15** was also selective over r17β-HSD2.

Table 5. Inhibition of human and rat 17β-HSD1 and 17β-HSD2 by biphenyl-2,5-furane without hydroxy group.



IV.8-IV.17

		IC_{50} (nM)[a] or Inh. at 1 µM[a]			IC_{50} (nM)[a] or Inh. at 1 µM[a]		
Cmpd	**R_2**	**h17β-HSD1[b]**	**h17β-HSD2[c]**	**s.f.[d]**	**r17β-HSD1[e]**	**r17β-HSD2[f]**	**s.f.[d]**
IV.8	-H	80	4144	52	49%	37%	n.d.
IV.9	2-Cl	28	301	11	71%	89%	n.d.
IV.10	2-Me	31	1077	35	37%	50%	n.d.
IV.11	3-F	15	447	30	69%	100%	n.d.
IV.12	3-Me	7.8	1547	198	369	1200	3
IV.13	3,5-F	18	56	3	291	16	0.05

IV.14	3,5-Cl	2.9	71	25	176	40	0.2
IV.15	3,5-Me	5.6	3155	563	74	1341	18
IV.16	2-F	39	780	20	74%	100%	n.d.
IV.17	3-Cl	8.9	265	30	89%	100%	n.d.

[a]Mean value of at least two determinations, standard deviation less than 20%. [b]Human placental, cytosolic fraction, substrate E1[500 nM], cofactor NADH[500 µM]. [c]Human placental, microsomal fraction, substrate E2[500 nM], cofactor NAD^+[1500 µM]. [d]n.d.: not determined. [e]Rat recombinant enzyme, expressed in HEK 293, substrate [^{3}H]-E1, 10 nM, cofactor NADH, 500 µM. [d]Rat liver, microsomal fraction, substrate [^{3}H]-E2, 10 nM, cofactor NAD^+, 1500 µM.

Compounds **IV.8** and **IV.15** also displayed high metabolic stability ($t_{1/2}$= 97 min and 51 min, respectively). Considering that the seventeen 2,5-thiophene amides tested for metabolic stability displayed a mean half-life of 20 min and that the three tested 2,5-furane amides displayed an half-life between 51 and 97 min, it can be concluded, that the furane ring plays a major role in increasing the stability of these compounds, probably by decreasing the cLogP and therefore also the affinity for the metabolizing enzyme.

Compound **IV.15** was the most interesting compound in the series and was therefore tested for its affinity toward the ERs, and was found to display low affinity with an RBA value lower than 0.1%.

The effect of **IV.15** was then investigated in two NSCLC cell lines. The ability of compound **IV.15** of inhibiting the E1-induced growth of Calu-1 cells at low nanomolar concentration provided the first convincing evidence, that h17β-HSD1 is a suitable target for the treatment of NSCLC, thus opening new horizons for facing this lethal disease.

Also cytotoxicity was evaluated for this compound in the MTT assay. No toxic effect was detected up to 6.25 µM which is more than 1000-folds the IC_{50} value. A broad safety window *in vivo* can therefore be expected.

Data obtained in a preliminary PK study in the rat, indicated sufficient concentration of the compound after 24h. This observation, together with high h17β-HSD1 and r17β-HSD1 inhibitory activity and selectivity over h17β-HSD2, r17β-HSD2 and ERs, as well as with the low cytotoxicity, makes **IV.15** a good candidate for an *in vivo* proof of concept study in a rat model of non-small lung cell cancer.

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