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Novel estrone mimetics with high 17β-HSD1 inhibitory activity ☆

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ABSTRACT

17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) catalyzes the reduction of estrone into estradiol, which is the most potent estrogen in humans. Lowering intracellular estradiol concentration by inhibition of this enzyme is a promising new option for the treatment of estrogen-dependent diseases like breast cancer and endometriosis. Combination of ligand- and structure-based design resulted in heterocyclic substituted biphenylols and their aza-analogs as new 17β-HSD1 inhibitors. The design was based on mimicking estrone, especially focusing on the imitation of the D-ring keto group with (substituted) heterocycles. Molecular docking provided insights into plausible protein–ligand interactions for this class of compounds. The most promising compound **12** showed an inhibitory activity in the high nanomolar range and very low affinity for the estrogen receptors α and β. Thus, compound **12** is a novel tool for the elucidation of the pharmacological relevance of 17β-HSD1 and might be a lead for the treatment of estrogen-dependent diseases.

1. Introduction

Estrogens, especially the most active one estradiol (E2), are well known to be responsible for the development of estrogen-dependent diseases like breast cancer[1] and endometriosis.[2] Current endocrine therapies for breast cancer are either focused on blocking the estrogen action at the receptor level by selective estrogen receptor modulators (SERMs) and pure antiestrogens[3] or on decreasing the formation of estrogens by application of GnRH analogs and aromatase inhibitors.[4,5] In case of the latter compounds intensive efforts in the last two decades[6,7] resulted in therapeutics which—according to the FDA guidelines—are first-line therapeutics for the treatment of breast cancer.[7] However, all of these therapeutic approaches show disadvantages by causing side effects due to their rather radical reduction of systemic estrogen concentration.

A more sophisticated approach is focused on the hormone concentration in the target cell (intracrine approach). This strategy has been pursued for androgen-dependent diseases for quite some time. Steroidal[8] and later non-steroidal[8–12] 5α-reductase inhibitors have been developed and two of the former are used clinically today.[8] In case of estrogen-dependent diseases a promising target came up, 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1). This enzyme catalyzes the reduction of the weakly active estrone (E1) to yield the highly active E2 using NAD(P)H as cofactor, a reaction which represents the last step in E2 biosynthesis (Chart 1). Although the subtypes 17β-HSD7 and 12 are also able to catalyze this conversion, their main physiological roles are supposed to be restricted to cholesterol biosynthesis[13] and regulation of lipid biosynthesis,[14] respectively.

17β-HSD1 mRNA is often overexpressed in breast cancer tissues[15] and endometriotic lesions.[16] Therefore, the local reduction of estrogen action by inhibiting this enzyme appears to be a promising therapy with less side effects than existing therapies. Proof of concept for the treatment of breast cancer was recently reported in different mouse-models by applying steroidal compounds *intraperitoneally* or *subcutaneously*.[17,18] However, these compounds do not seem to be appropriate for clinical application.

The type 2 enzyme in the 17β-hydroxysteroid dehydrogenase family is responsible for the oxidation of E2 to E1 using NAD^+ as cofactor and acts as a biological counterpart of 17β-HSD1. Regarding the therapeutic concept, potential inhibitors of 17β-HSD1 should not affect 17β-HSD2. Furthermore, intrinsic estrogenic effects should be avoided, that is, potential inhibitors should have no or only little affinity to the estrogen receptors α and β (ERα and ERβ).

Abbreviations: 17β-HSD1, 17β-hydroxysteroid dehydrogenase type 1; 17β-HSD2, 17β-hydroxysteroid dehydrogenase type 2; E1, estrone; E2, 17β-estradiol; ER, estrogen receptor; SERM, selective estrogen receptor modulator; GnRH, gonadotropin-releasing hormone; NADP(H), nicotinamide adenine dinucleotide phosphate; NAD(H), nicotinamide adenine dinucleotide; RBA, relative binding affinity; SAR, structure–activity relationship; PDB, protein databank; HPLC, high performance liquid chromatography; CC, column chromatography; TLC, thin layer chromatography; FDA, food and drug administration.

☆ Remark: For the sake of clarity, IUPAC nomenclature is not strictly followed except for the experimental part where the correct IUPAC names are given.

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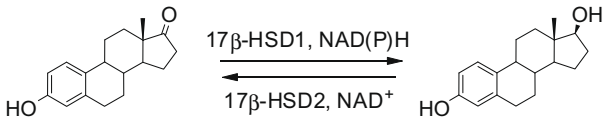


Chart 1. Interconversion of estrone (E1) and estradiol (E2).

Over the last decade, several groups have been working on the development of 17β-HSD1 inhibitors. Most of the latter are based on the steroidal scaffold with expansions at the 6, 15, 16 and 17 positions.[19,20] Only a few non-steroidal inhibitors have been published so far. In 2006, Messinger et al.[21] described thiophenepyrimidinones as the first non-steroidal inhibitors, which selectively inhibit 17β-HSD1. Mimicking the E1 skeleton, Allan et al.[22] investigated biphenyl ethanones containing side chains known from steroidal inhibitors. Recently, we reported on the development of two highly potent and selective, non-steroidal classes of 17β-HSD1 inhibitors, (hydroxyphenyl)naphthols[23–25] (**A**, Chart 2) and bis(hydroxyphenyl)heterocycles[26–31] (**B**, Chart 2). In these investigations molecular modelling studies with X-ray protein structures were performed revealing that all the mentioned inhibitors are likely to bind in the substrate binding site, except the bis(hydroxyphenyl)heterocycles. The latter most probably bind between the cofactor and substrate binding site showing interactions with NADPH.[29]

In both compound classes recently described by us (**A** and **B**), the most active inhibitors exhibit two OH-groups in a distance of about 11 Å.[23–29] Regarding pharmacokinetics, an exchange of one hydroxyphenyl moiety seems to be desirable because of the susceptibility of phenols to phase II metabolism.[32]

Therefore herein we report on the design of new steroidomimetics bearing only one OH-group. The compounds were designed using a combination of ligand- and structure-based approach. In the following, synthesis and biological evaluation of new non-steroidal 17β-HSD1 inhibitors of the heterocyclic substituted biphenylol type and their aza-analogs are described. Molecular modelling studies were performed in order to elucidate protein–ligand interactions in the active site.

2. Design

2.1. Ligand-based

In order to reduce the risk of undesired side effects, novel compounds should not have affinity to steroid receptors. Accordingly, we focused on the design of non-steroidal inhibitors. In this field we previously designed highly active inhibitors of 17β-HSD1 bearing two OH-groups mimicking the two hydrophilic features of the substrate.[23,26] Interestingly, some inhibitors containing only one OH-group showed still a notable activity.[27] A prerequisite for their activity is a *meta* OH-phenyl moiety. This substitution pattern will play an important role in the design of the compounds presented in this study.

We herein investigate E1, the natural substrate of 17β-HSD1 as template for ligand-based design, with a *meta* OH-phenyl group mimicking the steroidal A-ring and the replacement of the D-ring keto-function by means of different heterocycles avoiding a second OH-group. The distance between the two hydrophilic features of E1 is approximately 11 Å and will be considered as a further component in the drug design concept. In order to keep the lipophilic character of the steroidal scaffold, the two hydrophilic anchor points will be linked by a non-hydrophilic benzene moiety.

A similar approach was applied by Allan et al.[22] who also investigated E1-mimetics. In their study phenylindanone **C** (Chart 2) bearing a carbonyl function was found to be the most potent inhibitor with an IC_{50}-value of 1.7 μM. They also published steroidal compounds bearing a pyrazole E-ring, which showed also remarkable inhibitory activity (compound **D**, IC_{50} = 0.18 μM),[33] indicating that the heterocycle is well tolerated by the enzyme.

In this study, compound **1** (Chart 3) was chosen as starting point for the development of E1-mimetics as potential 17β-HSD1 inhibitors, with furane replacing the steroidal D-ring. Besides furane, 2-pyridone, morpholine and thiophene seemed to be appropriate moieties to investigate the suitability of further heteroatoms.

2.2. Structure-based

Among the numerous published crystal structures[34] there is no structure of the enzyme cocrystallized with E1 but with the closely related, well known 17β-HSD1 inhibitor equilin, an equine estrogen with a C7–C8 double bond and a carbonyl function in position 17. Therefore, the ternary complex of human 17β-HSD1 with equilin and $NADP^+$ (PDB-ID: 1EQU)[35] appeared to be suitable for studying the three-dimensional architecture of the enzyme. A substrate binding site, a cofactor binding pocket and a highly flexible substrate-entry loop (αG′βF) structure can be defined. The steroid binding site is almost exclusively hydrophobic except for two hydrophilic ends: the 17-keto oxygen of equilin accepts protons from Tyr155 and Ser142 at the catalytic end. At the other end, the 3-hydroxy group of equilin establishes bifurcated hydrogen bonds to His221 and Glu282. Closer analysis of the substrate binding pocket reveals three additional polar amino acids (Asn152, Tyr218 and Ser222) in the hydrophobic area, which are not involved in steroid binding. Introduction of heteroatoms in the benzene core of a potential inhibitor might be appropriate to explore supplementary interactions.

3. Chemistry

The synthesis of benzene derivatives **1**–**15** is depicted in Scheme 1. Compounds **1**–**3**, **4i**, **7**–**13** and **14i**–**15i** were synthesized via Suzuki reaction starting from the commercially available boronic acids and the brominated benzene derivatives. Microwave assisted Suzuki cross-coupling reactions were carried out as one-pot syntheses using method A[27] (Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bar, 15 min)). The ether functions of the methylated hydroxyphenyl derivatives **5**, **14** and **15** were cleaved according to method D[27] (BBr_3, CH_2Cl_2, −78 °C to rt, 18 h). The pyridin-2(1*H*)-one derivative **4** was prepared by demethylation of intermediate **4i** with borontrifluoride dimethyl sulfide complex by stirring the reaction mixture at rt for 20 h in anhydrous dichloromethane.

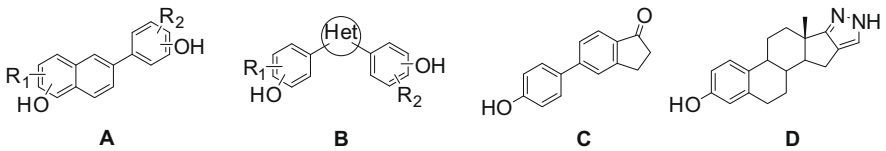
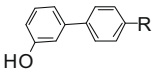
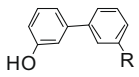


Chart 2. Recently published compound classes from our group (hydroxyphenyl)naphthols (**A**), bis(hydroxyphenyl)heterocycles (**B**) and two known inhibitors (**C**, **D**).



1-10



11-15

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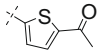
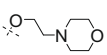
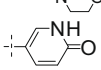
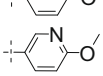
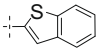
1		9	
2		10	
3		11	
4		12	
5		13	
6	H	14	
7		15	
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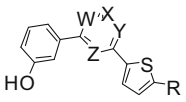


16-20

cmpd	R	W	X	Y	Z
16	H	CH	CH	N	CH
17	Cl	CH	CH	N	CH
18	Cl	CH	CH	CH	N
19	Cl	N	CH	CH	CH
20	Cl	CH	N	CH	CH

Chart 3. Title compounds.

The synthetic routes for the pyridine derivatives **16**–**20** are shown in Scheme 2. Starting from the commercially available dibrominated pyridines and boronic acid derivatives, compounds **17i** and **19ii** were obtained via Suzuki reaction following the conditions of method B (K_2CO_3, toluene/EtOH/water (1:1:1), $Pd(PPh_3)_4$, 100 °C, 18 h) and method C ($NaHCO_3$, toluene/water (1:1), $Pd(PPh_3)_4$, 100 °C, 18 h), respectively. Method A was used for the coupling of 3-hydroxybenzene boronic acid with compound **17i** to yield compound **17**. Compound **16** devoid of the chlorine substituent at the thiophene was isolated as a side product.

Cross-coupling of 5-chloro-2-thienyl boronic acid with brominated pyridine derivatives **18ii**–**20ii** according to method A (Cs_2CO_3, DME/EtOH/water (1:1:1), $Pd(PPh_3)_4$, MW (150 W, 150 °C, 15 bar, 15 min)) and method B (K_2CO_3, toluene/EtOH/water (1:1:1), $Pd(PPh_3)_4$, 100 °C, 18 h) led to compounds **19i** (method A), **18i** and **20i** (method B). Compounds **18**–**20** were prepared by cleaving the methoxy function with boron tribromide (method D: BBr_3, CH_2Cl_2, −78 °C to rt, 18 h).

4. Biological results

4.1. Activity: inhibition of human 17β-HSD1

Placental enzyme isolated following a described procedure,[31] was incubated with tritiated E1, cofactor and inhibitor. The separation of substrate and product was performed by HPLC. Compounds showing less than 10% inhibition at 1 μM were considered to be inactive. The percent inhibition values of compounds **1**–**20** are shown in Table 1. The IC_{50}-values of selected compounds are depicted in Table 2.

Compound **1**, bearing a furane moiety only showed marginal inhibitory activity of 17% at a concentration of 1 μM. To get more insight into the role of the heterocycles, we synthesized compounds **2**–**5**, **7** and **8** differing in the heteroatoms, the electronic and the geometric properties of the heterocycle. Compounds **2**–**3**, in which furane is replaced with non-aromatic heterocycles, showed no activity, indicating that a lack of aromaticity is counterproductive. The inactivity of pyridone **4** supports this hypothesis since its aromatic character is less pronounced compared to the methoxypyridine **5**.[36] Pyridine **5** and thiophenes **7**, **8** showing moderate activity (58%, 41% and 36% inhibition, respectively) seemed to be appropriate mimics of the steroidal keto-function. Regarding compound **5** (IC_{50} = 0.69 μM, Table 2), the methoxy-group and the pyridine–nitrogen offer hydrogen bond acceptor properties. This means that both the pyridine–nitrogen and the methoxy–oxygen are able to form hydrogen bonds with the catalytic amino acids. The slightly reduced activity of **7** and **8** might be explained by the lack of these hydrogen bond interactions. Introduction of an electron withdrawing (**9**) as well as an electron donating (**10**) substituent did not improve potency significantly.

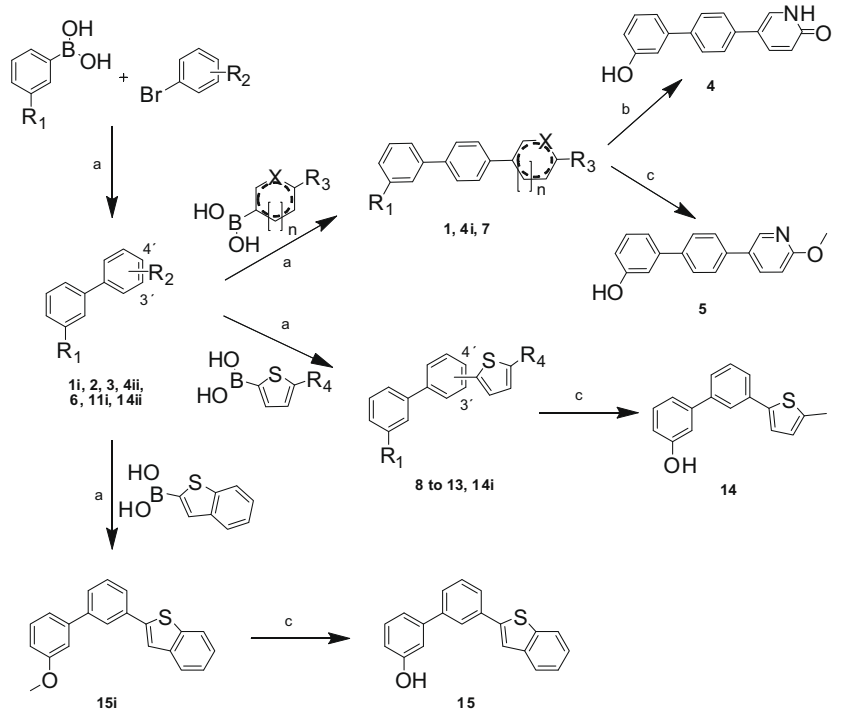
As the 1,4-disubstituted benzene derivatives **9** and **10** are larger than 11 Å, the 1,3-disubstituted **11** and **12** were synthesized in order to readjust the E1-derived requirements. While the activity of the acetylthiophene derivative **11** decreased, the chlorine substituted thiophene **12** is the most potent inhibitor of 17β-HSD1 identified in this study with an IC_{50} of 0.56 μM. Compounds **13** and **14** bearing H and CH_3 instead of Cl and compound **15** with benzothiophene substituent, were synthesized as tools to evaluate the impact of the chlorine atom on the activity and get insight into possible inhibitor–protein interactions. They all turned out to be less active than **12**, highlighting the positive effect of chlorine for enzyme affinity.

As discussed above, insertion of a heteroatom, capable of acting as hydrogen bond acceptor, in the central benzene core might lead to additional interactions with Tyr218 and Ser222. For the sake of clarifying this hypothesis, the benzene core was replaced by pyridine, leading to isomeric compounds **16**–**20**. None of the compounds in this series enhances 17β-HSD1 inhibition, indicating that the pyridine nitrogen is not able to target these hydrophilic amino acids. In case of compounds **17**, **18** and **20**, the insertion of a nitrogen even decreases inhibitory activity compared to **12**. Nevertheless, the isomer **19** with an IC_{50}-value of 0.85 μM showed nearly the same activity as compound **12**.

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

Since 17β-HSD2 catalyzes the oxidative transformation of E2 into E1, inhibitory activity toward this enzyme must be avoided. 17β-HSD2 inhibition was determined using an assay similar to the 17β-HSD1 test. Placental microsomes were incubated with tritiated E2 in the presence of NAD^+ and inhibitor. Quantification of labelled product formed was performed by HPLC and subsequent radio detection. Compounds showing less than 10% inhibition at 1 μM were considered to be inactive. Percentage of inhibition and IC_{50}-values are shown in Tables 1 and 2. All compounds, which did not inhibit 17β-HSD1 were also inactive on the type 2 enzyme.

Comparing the isomeric pyridines **17** and **19**, it is striking that the position of the nitrogen has a major impact on the selectivity. Compound **17**, bearing the pyridine nitrogen next to the thiophene, showed an IC_{50} of 0.47 μM for 17β-HSD2, which demonstrates a 5 fold higher inhibition than for the type 1 enzyme. Having the posi-



cmpd	R_1	R_2	R_3	X	n
1i	OH	4´-Br			
1	OH		H	O	0
2	OH	4´-morpholino			
3	OH	4´-(2-morpholinoethoxy)			
4ii	OCH_3	4´-Br			
4i	OCH_3		OCH_3	N	1
6	H	OH			
7	OH		H	S	0

cmpd	R_1	R_2	thienyl	R_4
8	OH		4´	H
9	OH		4´	CO-CH_3
10	OH		4´	Cl
11i	OH	3´-Br		
11	OH		3´	CO-CH_3
12	OH		3´	Cl
13	OH		3´	H
14ii	OCH_3	3´-Br		
14i	OCH_3		3´	CH_3

Scheme 1. Synthesis of compounds **1–15.** Reagents and conditions: (a) method A: Cs_2CO_3, $Pd(PPh_3)_4$, DME/EtOH/water (1:1:1), microwave conditions (150 W, 15 bar, 150 °C, 15 min); (b) $BF_3{\cdot}SMe_2$, CH_2Cl_2, rt, 20 h; (c) method D: BBr_3, CH_2Cl_2, −78 °C, 20 h.

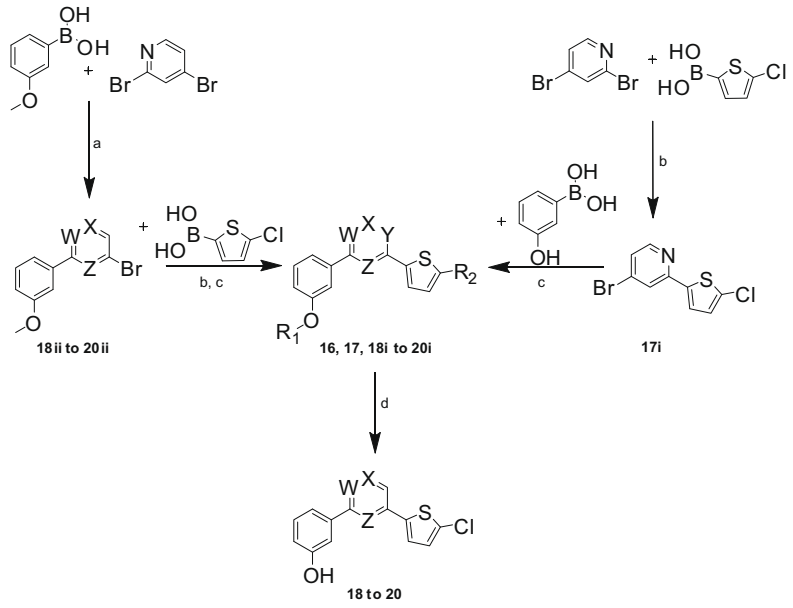
tion of the nitrogen next to the hydroxyphenyl ring (**19**), the selectivity changed completely (4-fold higher affinity toward 17β-HSD1). According to the position of the nitrogen, a 17β-HSD1 inhibitor can be transformed into a 17β-HSD2 inhibitor. Among the compounds revealing 17β-HSD1 activity, however, the chlorothiophene **10** showed the best selectivity (>10-fold) toward 17β-HSD2.

The therapeutic concept of 17β-HSD1 inhibition includes that potential inhibitors should have no or low affinity toward ERα and ERβ in order to avoid systemic effects. Binding affinities for ERα and ERβ were measured for selected compounds and are shown in Table 2. Using recombinant human protein, a competition assay applying tritium labelled E2 (RBA = 100%) was performed. All evaluated compounds present very low binding affinity to both estrogen receptors.

5. Molecular modelling

In order to gain deeper insight into plausible molecular interactions between the synthesized steroidomimetics and 17β-HSD1, docking experiments were performed using the monomer of the X-ray structure 1EQU (PDB-ID).

Selected compounds were docked into 17β-HSD1 by means of the docking software Autodock4.2[37] and GOLDv4.0.1.[38] In Figure 1A, a plausible binding pose for **5** is shown as an example. The compound is found in the steroidal binding mode. The *meta* OH-group establishes hydrogen bonds with His221 and Glu282 (d_{O-N} = 3.2 Å and d_{O-O} = 2.7 Å) in a bifurcated fashion, mimicking the OH-group of the steroidal A-ring. The methoxy substituted pyridine ring points toward the catalytic region of the protein. The pyridine nitrogen and the methoxy oxygen might be implicated



middle ring = pyridine
16, 17: Y = N
18ii, 18i, 18: Z = N
19ii, 19i, 19: W = N
20ii, 20i, 20: X = N

cmpd	R_1	R_2	method
16	H	H	c
17	H	Cl	c
18i	CH_3	Cl	b
19i	CH_3	Cl	c
20i	CH_3	Cl	b

Scheme 2. Synthesis of compounds **16**–**20**. Reagents and conditions: (a) method C: $NaHCO_3$, $Pd(PPh_3)_4$, toluene/water (1:1), 100 °C, 18 h; (b) method B: K_2CO_3, $Pd(PPh_3)_4$, toluene/EtOH/water (1:1:1), 100 °C, 18 h; (c) method A: Cs_2CO_3, $Pd(PPh_3)_4$, DME/EtOH/water (1:1:1), microwave conditions (150 W, 15 bar, 150 °C, 15 min); (d) method D: BBr_3, CH_2Cl_2, −78 °C, 20 h.

Table 1
Inhibition of human 17β-HSD1 and 17β-HSD2 by compounds **1**–**20**

Cmpd	% Inhibition[a]	
	17β-HSD1[b]	17β-HSD2[c]
1	17	n.i.
2	n.i.	n.i.
3	n.i.	n.i.
4	n.i.	n.i.
5	58	35
6	n.i.	n.i.
7	41	36
8	36	22
9	38	10
10	48	n.i.
11	22	23
12	71	31
13	48	40
14	32	36
15	49	39
16	33	62
17	39	63
18	40	21
19	61	25
20	40	46

n.i. = no inhibition (inhibition <10%).
[a] Mean value of three determinations, standard deviation less than 10%, compound concentration: 1 μM.
[b] Human placenta, cytosolic fraction, substrate [^{3}H]-E1, 500 nM, cofactor NADH, 500 μM.
[c] Human placenta, microsomal fraction, substrate [^{3}H]-E2, 500 nM, cofactor NAD^+, 1500 μM.

in hydrogen bonds with Ser142 (d_{O-N} = 4.0 Å) and Tyr155 (d_{O-O} = 3.8 Å), respectively. The docking studies also showed that the equipotent **10** binds in the same area as compound **5** (Fig. 1), comparable to equilin cocrystallized with 17β-HSD1 (1EQU). It is not clear if the interactions established by the methoxypyridine are relevant for the stabilization of **5** as the chlorothiophene moiety of **10** is located in the same area and is not able to establish the same H-bond interaction patterns.

Taking in account that compound **12** was designed to better fit the E1-distance requirements, the same interactions as with the linear molecules were expected for this compound.

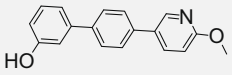
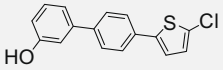
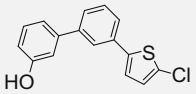
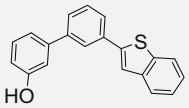
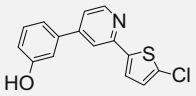
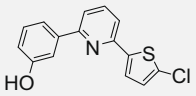
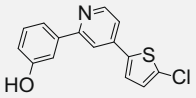
However, the results of the docking experiments indicate that the chlorothiophene moiety of **12** is not directed toward the catalytic centre (Fig. 1B). While the hydroxyphenyl group is still imitating the steroidal A-ring (d_{O-N} = 2.7 Å and d_{O-O} = 3.5 Å), the nonlinear scaffold enables the heterocycle to point to a rather lipophilic subpocket consisting of Leu95, Leu96, Asn152, Tyr155 and Phe192. Compounds **13**–**15** were found in the same binding mode as **12** (data not shown).

6. Discussion and conclusions

The biological results described herein provide evidence that mimicking the chemical features of E1 is an appropriate approach for the development of new 17β-HSD1 inhibitors. While in previous studies, the inhibitors bore two OH-groups, here only one OH-group was retained, namely the *meta* OH-phenyl group imitating the A-ring. The study was mainly focused on the investigation of (substituted) heterocycles as suitable D-ring mimics. It is shown that compounds **2** and **3**, with an aliphatic heterocycle replacing the D-ring, showed no inhibitory activity. This indicates that flatness and a delocalised π-system are prerequisites for inhibitory activity.

The interaction pattern of Ser142, Tyr155 and the 17β-OH group of E2 has been described to be a triangle-shaped hydrogen bond network.[39] Until now, it was not clarified whether the hydrogen bond donor function is a crucial component of this network. In this

Table 2
Inhibition of 17β-HSD1, 17β-HSD2 and binding affinities for the oestrogen receptors α and β by selected compounds

Cmpd	Structure	IC_{50}[a] (μM)		RBA[d] (%)	
		17β-HSD1[b]	17β-HSD2[c]	ERα	ERβ
5		0.69	2.97	0.001<RBA<0.01	<0.001
10		1.02	>10	0.01<RBA<0.1	0.001<RBA<0.01
12		0.56	2.37	<0.01	<0.001
15		1.37	1.94	<0.01	<0.001
17		2.38	0.47	0.001<RBA<0.01	<0.001
18		1.39	7.11	0.001<RBA<0.01	<0.001
19		0.85	3.64	<0.001	<0.001

[a] Mean value of three determinations, standard deviation less than 15%.
[b] Human placenta, cytosolic fraction, substrate [^{3}H]-E1, 500 nM, cofactor NADH, 500 μM.
[c] Human placenta, microsomal fraction, substrate [^{3}H]-E2, 500 nM, cofactor NAD^+, 1500 μM.
[d] RBA: relative binding affinity, E2: 100%, mean value of three determinations, standard deviation less than 10%.

study we were able to show that heterocycles like methoxypyridine and chlorothiophene are able to unfold high inhibitory activity.

The results of the docking study showed that the 1,3-benzene derivatives most likely do not exclusively cover the substrate binding area. Parts of them were found to reach into the rather lipophilic subpocket recently described by Mazumdar et al.[40] As the chlorothiophene moiety of compound **12** was found to bind into this subpocket, compounds **13**–**15** were designed to validate this finding. Since the benzothiophene moiety of **15** is too bulky to fit into the area of the catalytic centre as observed for compounds **5** and **10**, the only slightly reduced inhibitory activity of **15** compared to **12** supports our hypothesis of these ligands binding to the subpocket. The high to moderate inhibitory activity of compounds **12** and **15** can be explained by hydrophobic and van der Waals interactions within this subpocket. In order to investigate the relevance of the chloro substituent for the activity, compounds **13** and **14** were synthesized replacing Cl by H (**13**) and by CH_3 (**14**). Compound **13** is a weaker inhibitor of 17β-HSD1 compared to the parent compound (**12**), which might be due to its decreased lipophilicity. However, compound **14** shows less inhibition compared to **12**, although chloro and methyl exhibit very similar lipophilic properties. This indicates that not only lipophilicity is responsible for high affinity to this subpocket. In this context, the different electronic characteristics of the substituents as well as the possible formation of Cl–π interactions[41] might be involved in the increased potency of **12**.

With the aim of increasing activity and selectivity the middle benzene ring was exchanged by a pyridine in order to target the hydrophilic amino acids Tyr218 and Ser222 in the predominantly hydrophobic binding site. According to the expected binding mode, the distances between the pyridine nitrogens of compounds **16**–**20** and these residues should be in an appropriate range for hydrogen bonding. However, none of these aza-analogs enhanced 17β-HSD1 inhibitory activity compared to **12**. The nitrogens are obviously neither able to interact with these amino acids nor can any additional interaction of the nitrogens be observed. The biological data might be explained by differences in molecular electrostatic potential (MEP) distributions which were found to play an important role in the inhibition of 17β-HSD1 by other compounds, too.[27,29] For these it was shown that insertion of nitrogen and/or exchange of its position have a high impact on the MEP distributions.[27]

The selectivity of compounds **17**–**20** is influenced by the position of the nitrogen. As there is neither a crystal structure nor an appropriate homology model of 17β-HSD2 available, protein–ligand interactions cannot be interpreted. Comparing the aza-compounds it becomes apparent that only compound **17** showed a significantly higher inhibitory activity toward 17β-HSD2. In this compound, the nitrogen close to the chlorothiophene ring is responsible for a more negative electrostatic potential in this part of the structure (see MEP calculations in Supplementary data). This distribution might be beneficial for 17β-HSD2 inhibition whereas a negative MEP in the area of the *meta* hydroxyphenyl ring led to higher inhibitory activity toward 17β-HSD1.

In summary, a new series of heterocyclic substituted biphenylols and their aza-analogs was designed as 17β-HSD1 inhibitors and synthesized by combining a ligand- and structure-based approach. Among these, a promising compound was discovered (**12**), which showed high activity, selectivity over 17β-HSD2 and very low affinity toward the ERs. The binding mode of the angulate 1,3-benzene derivatives differed from that observed for the linear compounds. The latter superimpose well to equilin which indicates that an OH-group pointing into the catalytic centre is not necessary for high inhibitory activity and can be taken over by other substit-

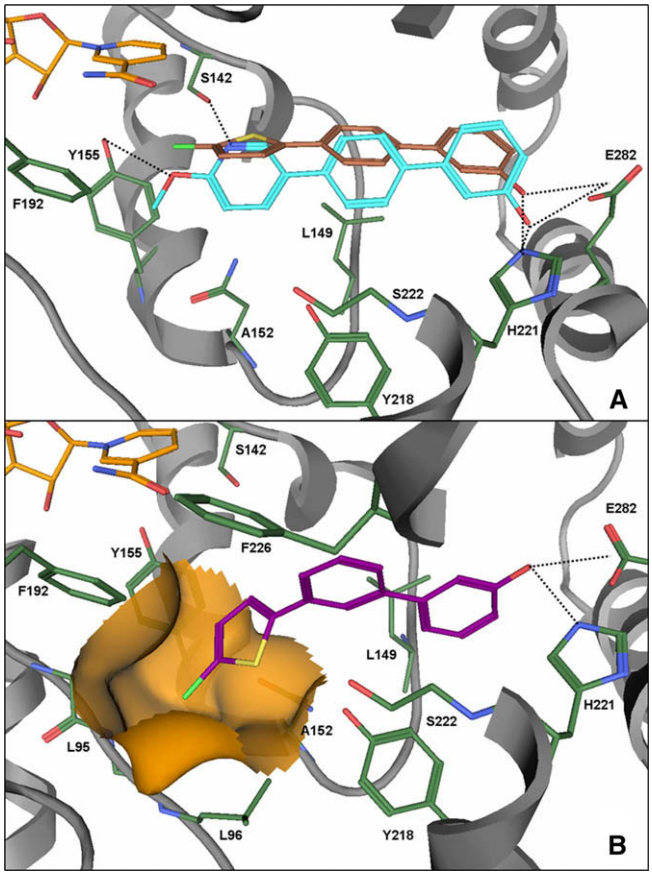


Figure 1. Docking complex between 17β-HSD1 (PDB-ID: 1EQU) and compounds **5** (cyan), **10** (brown) overlay depicted in A and **12** (violet) depicted in B. Cofactor NADPH (orange), interacting residues (green) and cartoon rendered tertiary structure (grey) of the active site are shown. Hydrogen bonds are drawn in black dashed lines. The surface of the subpocket is illustrated (light brown) in Figure 1B. Figures generated with MOE (Chemical Computing Group Inc., Montreal, Canada).

uents like methoxypyridine or chlorothiophene. This observation might be important for the design of other classes of 17β-HSD1 inhibitors. Furthermore it seems feasible that by combination of the two interaction patterns observed in this study (angulate-shaped structures interacting with the subpocket and linear structures with the catalytic centre) a further increase of activity and selectivity could be achieved leading to the development of clinical candidates.

7. Experimental section

7.1. Chemical methods

Chemical names follow IUPAC nomenclature. Starting materials were purchased from Aldrich, Acros, Lancaster, Roth, Combi Blocks, Merck or Fluka and were used without purification.

Column chromatography (CC) was performed on silica gel (70–200 μm) coated with silica, preparative thin layer chromatography (TLC) on 1 mm SIL G-100 UV_{254} glass plates (Macherey-Nagel) and reaction progress was monitored by TLC on Alugram SIL G UV_{254} (Macherey-Nagel).

IR spectra were recorded on a Bruker Vector 33 spectrometer (neat sample).

^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM500 spectrometer (500 MHz) at 300 K. Chemical shifts are reported in δ (parts per million: ppm), by reference to the hydrogenated residues of deuteriated solvent as internal standard ($CDCl_3$: δ = 7.24 ppm (^{1}H NMR) and δ = 77 ppm (^{13}C NMR), CD_3OD: δ = 3.35 ppm (^{1}H NMR) and δ = 49.3 ppm (^{13}C NMR), CD_3COCD_3: δ = 2.05 ppm (^{1}H NMR) and δ = 29.9 ppm (^{13}C NMR), CD_3SOCD_3 δ = 2.50 ppm (^{1}H NMR) and δ = 39.5 ppm (^{13}C NMR)). Signals are described as s, d, t, dd, ddd, m, dt, q for singlet, doublet, triplet, doublet of doublets, doublet of doublets of doublets, multiplet, doublet of triplets and quadruplet, respectively. All coupling constants (J) are given in hertz (Hz).

Mass spectra (ESI and APCI) were recorded on a TSQ Quantum (Thermo Finnigan) instrument.

Tested compounds are >95% chemical purity as measured by HPLC. The methods for HPLC analysis and a table of data for all tested compounds are provided in Supplementary data.

The following compounds were prepared according to previously described procedures: 4′-bromobiphenyl-3-ol (**1i**),[42] 4′-bromo-3-methoxybiphenyl (**4ii**),[43] biphenyl-3-ol (**6**),[44] 3′-bromobiphenyl-3-ol (**11i**),[45] 3-bromo-3′-methoxybiphenyl (**14ii**),[46] 2-bromo-6-(3-methoxyphenyl)pyridine (**18ii**),[47] 3-bromo-5-(3-methoxyphenyl)pyridine (**20ii**).[48]

7.1.1. General procedure for Suzuki coupling

7.1.1.1. Method A. A mixture of arylbromide (1 equiv), boronic acid derivative (1.2 equiv), caesium carbonate (4 equiv) and tetrakis(triphenylphosphine) palladium (0.03 equiv) was suspended in an oxygen-free DME/EtOH/water (1:1:1) mixture. The reaction mixture was exposed to microwave irradiation (15 min, 150 W, 150 °C, 15 bar). After cooling to rt, water was added and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, filtered and concentrated to dryness. The product was purified by CC, preparative TLC or preparative HPLC, respectively.

7.1.1.2. Method B. A mixture of arylbromide (1 equiv), boronic acid derivative (1.2 equiv), potassium carbonate (2 equiv) and tetrakis(triphenylphosphine) palladium (0.03 equiv) in an oxygen-free toluene/EtOH/water (1:1:1) mixture was stirred at 100 °C for 18 h under nitrogen atmosphere. The reaction mixture was cooled to rt. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, filtered and concentrated to dryness. The product was purified by CC.

7.1.1.3. Method C. A mixture of aryldibromide (1 equiv), methoxybenzene boronic acid (1 equiv), sodium hydrogencarbonate (2 equiv) and tetrakis(triphenylphosphine) palladium (0.03 equiv) in an oxygen-free toluene/water (1:1) mixture was stirred at 100 °C for 18 h under nitrogen atmosphere. The reaction mixture was cooled to rt. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered and concentrated in vacuo. The product was purified by CC.

7.1.2. General procedure for ether cleavage

7.1.2.1. Method D. To a solution of methoxybenzene derivative (1 equiv) in anhydrous dichloromethane at −78 °C (dry ice/acetone bath), boron tribromide in dichloromethane (1 M, 3 equiv) was added dropwise. The reaction mixture was stirred for 20 h to rt under nitrogen atmosphere. Water was added to quench the reaction, and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, evaporated to dryness under reduced pressure and purified by CC, preparative TLC or preparative HPLC, respectively.

7.1.3. General procedure for purification using preparative HPLC

All declared compounds were purified via an Agilent Technologies Series 1200-preparative HPLC using a linear gradient run (sol-

vents: acetonitrile, water) starting from 20% acetonitrile up to 100% in 36 min.

7.1.4. Detailed synthesis procedure for all compounds

7.1.4.1. 4′-(3-Furyl)biphenyl-3-ol (1). The title compound was prepared by reaction of 4′-bromobiphenyl-3-ol (**1i**) (150 mg, 0.60 mmol), furane-3-boronic acid (81 mg, 0.72 mmol), caesium carbonate (785 mg, 2.40 mmol) and tetrakis(triphenylphosphine) palladium (21 mg, 18 μmol) according to method A. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 3% (4 mg); MS (ESI): 237 $(M+H)^+$; ^{1}H NMR (CD_3COCD_3): 8.44 (s, 1H), 8.06 (dd, J = 0.9 Hz and J = 1.6 Hz, 1H), 7.69–7.67 (m, 2H), 7.65–7.63 (m, 3H), 7.28 (t, J = 8.1 Hz, 1H), 7.15–7.13 (m, 2H), 6.92 (dd, J = 0.9 Hz and J = 1.9 Hz, 1H), 6.84 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 162.50, 146.10, 144.00, 141.10, 133.50, 131.80, 129.10, 128.10, 128.00, 119.90, 118.30, 118.10, 116.30, 115.50, 110.50; IR: 3452, 3413, 3281, 1597, 1164, 835, 780 cm^{-1}.

7.1.4.2. 4′-Morpholin-4-ylbiphenyl-3-ol (2). The title compound was prepared by reaction of 4-(4-bromophenyl)morpholine (150 mg, 0.62 mmol), 3-hydroxybenzene boronic acid (103 mg, 0.74 mmol), caesium carbonate (807 mg, 2.48 mmol) and tetrakis(triphenylphosphine) palladium (22 mg, 19 μmol) according to method A. The product was crystallized in ethyl acetate; yield: 27% (42 mg); MS (ESI): 256 $(M+H)^+$; ^{1}H NMR (CD_3SOCD_3): 7.01 (d, J = 8.8 Hz, 2H), 6.73 (t, J = 7.9 Hz, 1H), 6.55–6.53 (m, 3H), 6.50 (t, J = 2.2 Hz, 1H), 6.22 (ddd, J = 0.9 Hz and J = 2.2 Hz and J = 7.9 Hz, 1H), 3.29 (t, J = 4.7 Hz, 4H), 2.69 (t, J = 4.7 Hz, 4H); ^{13}C NMR (CD_3SOCD_3): 149.10, 132.70, 122.20, 121.10, 118.40, 108.00, 106.60, 104.80, 104.00, 57.40, 39.90; IR: 3245, 2957, 1585, 1207, 1110, 828, 785 cm^{-1}.

7.1.4.3. 4′-(2-Morpholin-4-ylethoxy)biphenyl-3-ol (3). The title compound was prepared by reaction of 4-[2-(4-bromophenoxy)ethyl]morpholine (150 mg, 0.52 mmol), 3-hydroxybenzene boronic acid (87 mg, 0.63 mmol), caesium carbonate (678 mg, 2.08 mmol) and tetrakis(triphenylphosphine) palladium (18 mg, 16 μmol) according to method A. The product was purified by CC (dichloromethane/methanol 96:4) followed by preparative HPLC; yield: 20% (32 mg); MS (ESI): 300 $(M+H)^+$; ^{1}H NMR (CD_3COCD_3): 8.19 (s, 1H), 7.54 (d, J = 8.8 Hz, 2H), 7.23 (t, J = 7.6 Hz, 1H), 7.07–7.05 (m, 2H), 7.00 (d, J = 8.8 Hz, 2H), 6.78 (ddd, J = 1.3 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H), 4.18 (t, J = 5.7 Hz, 2H), 3.64 (t, J = 4.7 Hz, 4H), 2.82 (t, J = 5.7 Hz, 2H), 2.60 (t, J = 4.7 Hz, 4H); ^{13}C NMR (CD_3COCD_3): 164.50, 160.40, 159.70, 144.00, 135.40, 131.70, 129.70, 119.50, 116.70, 115.60, 115.20, 68.20, 67.60, 59.20, 55.80; IR: 3405, 2868, 1607, 1477, 1116, 835, 783 cm^{-1}.

7.1.4.4. 2-Methoxy-5-(3′-methoxybiphenyl-4-yl)pyridine (4i). The title compound was prepared by reaction of 4′-bromo-3-methoxybiphenyl (**4ii**) (300 mg, 1.14 mmol), 2-methoxy-5-pyridineboronic acid (209 mg, 1.37 mmol), caesium carbonate (1486 mg, 4.56 mmol) and tetrakis(triphenylphosphine) palladium (40 mg, 34 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 84% (280 mg); ^{1}H NMR ($CDCl_3$): 8.45 (d, J = 2.2 Hz, 1H), 7.83 (dd, J = 2.5 Hz and J = 8.5 Hz, 1H), 7.67 (d, J = 8.5 Hz, 2H), 7.60 (d, J = 8.5 Hz, 2H), 7.38 (t, J = 7.9 Hz, 1H), 7.23–7.22 (m, 1H), 7.17 (t, J = 2.1 Hz, 1H), 6.93–6.91 (m, 1H), 6.84 (dd, J = 0.6 Hz and J = 8.5 Hz, 1H), 4.00 (s, 3H), 3.88 (s, 3H); ^{13}C NMR ($CDCl_3$): 163.70, 160.00, 144.90, 142.10, 140.10, 137.30, 137.00, 129.90, 129.60, 127.70, 127.00, 119.50, 112.80, 110.90, 55.30, 53.50.

7.1.4.5. 5-(3′-Hydroxybiphenyl-4-yl)pyridin-2(1*H*)-one (4). To a solution of 2-methoxy-5-(3′-methoxybiphenyl-4-yl)pyridine (**4i**) (150 mg, 0.52 mmol, 1 equiv) in dry dichloromethane, borontrifluoride dimethyl sulfide complex (39 mmol, 75 equiv) was added dropwise at rt. The reaction mixture was stirred for 20 h at rt. Water was added to quench the reaction and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, evaporated to dryness under reduced pressure and purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 12% (17 mg); MS (ESI): 264 $(M+H)^+$; ^{1}H NMR (CD_3SOCD_3): 11.82 (s, 1H), 9.51 (s, 1H), 7.87 (dd, J = 2.9 Hz and J = 9.5 Hz, 1H), 7.75 (d, J = 2.9 Hz, 1H), 7.62 (s, 4H), 7.25 (t, J = 7.9 Hz, 1H), 7.10–7.08 (m, 1H), 7.04 (t, J = 2.1 Hz, 1H), 7.76 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H), 6.45 (d, J = 9.5 Hz, 1H); ^{13}C NMR (CD_3SOCD_3): 161.70, 157.80, 140.90, 139.80, 138.40, 135.10, 132.60, 129.90, 127.20, 126.90, 125.50, 122.80, 120.00, 117.20, 114.40, 113.20; IR: 3134, 2921, 1657, 1584, 1205, 825, 779 cm^{-1}.

7.1.4.6. 4′-(6-Methoxypyridin-3-yl)biphenyl-3-ol (5). The title compound was prepared by reaction of 2-methoxy-5-(3′-methoxybiphenyl-4-yl)pyridine (**4i**) (30 mg, 0.10 mmol) and boron tribromide (0.30 mmol) according to method D. The product was purified by preparative TLC (hexane/ethyl acetate 1:1); yield: 55% (16 mg); MS (ESI): 292 $(M+H)^+$; ^{1}H NMR (CD_3COCD_3): 8.49–8.47 (m, 2H), 7.99 (dd, J = 2.9 Hz and J = 8.9 Hz, 1H), 7.71 (s, 4H), 7.29 (t, J = 8.1 Hz, 1H), 7.17–7.16 (m, 2H), 6.87–6.85 (m, 2H), 3.94 (s, 3H); ^{13}C NMR (CD_3COCD_3): 165.60, 159.90, 146.70, 143.80, 141.90, 139.20, 138.60, 131.90, 131.30, 129.30, 128.70, 119.90, 116.40, 115.50, 112.60, 54.70; IR: 3234, 3023, 2955, 1601, 1487, 1298, 822 cm^{-1}.

7.1.4.7. 4′-(3-Thienyl)biphenyl-3-ol (7). The title compound was prepared by reaction of 4′-bromobiphenyl-3-ol (**1i**) (150 mg, 0.60 mmol), thiophene-3-boronic acid (93 mg, 0.72 mmol), caesium carbonate (785 mg, 2.40 mmol) and tetrakis(triphenylphosphine) palladium (21 mg, 18 μmol) according to method A. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 19% (28 mg); MS (ESI): 253 $(M+H)^+$; ^{1}H NMR (CD_3COCD_3): 8.44 (s, 1H), 7.79–7.77 (m, 3H), 7.67 (d, J = 8.5 Hz, 2H), 7.57 (d, J = 2.2 Hz, 2H), 7.28 (t, J = 8.2 Hz, 1H), 7.17–7.15 (m, 2H), 6.85 (ddd, J = 1.3 Hz and J = 2.2 Hz and J = 7.9 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 192.70, 159.80, 143.90, 143.60, 141.60, 136.90, 131.90, 129.20, 128.60, 128.50, 128.00, 122.40, 119.90, 116.30, 115.50; IR: 3479, 3394, 3096, 1596, 1200, 835, 775 cm^{-1}.

7.1.4.8. 4′-(2-Thienyl)biphenyl-3-ol (8). The title compound was prepared by reaction of 4′-bromobiphenyl-3-ol (**1i**) (150 mg, 0.60 mmol), thiophene-2-boronic acid (93 mg, 0.72 mmol), caesium carbonate (785 mg, 2.40 mmol) and tetrakis(triphenylphosphine) palladium (21 mg, 18 μmol) according to method A. The product was purified by preparative TLC (hexane/ethyl acetate 7:3); yield: 22% (34 mg); MS (APCI): 251 $(M-H)^+$; ^{1}H NMR (CD_3COCD_3): 8.47 (s, 1H), 7.74 (d, J = 8.6 Hz, 2H), 7.66 (d, J = 8.6 Hz, 2H), 7.49 (d, J = 3.8 Hz, 1H), 7.45 (d, J = 3.8 Hz, 1H), 7.29 (t, J = 8.1 Hz, 1H), 7.16–7.12 (m, 3H), 6.87–6.85 (m, 1H); ^{13}C NMR (CD_3COCD_3): 159.90, 145.60, 143.60, 142.00, 135.40, 131.90, 130.20, 129.30, 127.90, 127.00, 125.30, 119.80, 116.40, 115.40; IR: 3504, 3392, 1596, 1447, 1166, 821 cm^{-1}.

7.1.4.9. 1-[5-(3′-Hydroxybiphenyl-4-yl)-2-thienyl]ethanone (9). The title compound was prepared by reaction of 4′-bromobiphenyl-3-ol (**1i**) (100 mg, 0.40 mmol), 5-acetylthiophene-2-boronic acid (82 mg, 0.48 mmol), caesium carbonate (521 mg, 1.60 mmol) and tetrakis(triphenylphosphine) palladium (14 mg, 12 μmol) according to method A. The product was purified by CC (dichloromethane/methanol 98:2) followed by preparative HPLC; yield: 5% (6 mg); MS (APCI): 295 $(M+H)^+$; ^{1}H NMR (CD_3COCD_3): 8.47 (s, 1H), 7.87 (d, J = 4.1 Hz, 1H), 7.84 (d, J = 8.5 Hz, 2H), 7.72 (d, J = 8.5 Hz, 2H), 7.60 (d, J = 4.1 Hz, 1H), 7.30 (t,

J = 8.1 Hz, 1H), 7.19–7.16 (m, 2H), 6.87 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 7.9 Hz, 1H), 2.55 (s, 3H); ^{13}C NMR (CD_3COCD_3): 191.70, 153.40, 145.30, 136.10, 132.00, 129.50, 128.40, 126.40, 119.90, 119.30, 115.50, 27.50; IR: 3331, 1634, 1599, 1451, 804 cm^{-1}.

7.1.4.10. 4′-(5-Chloro-2-thienyl)biphenyl-3-ol (10). The title compound was prepared by reaction of 4′-bromobiphenyl-3-ol (**1i**) (100 mg, 0.40 mmol), 5-chlorothiophene-2-boronic acid (78 mg, 0.48 mmol), caesium carbonate (521 mg, 1.60 mmol) and tetrakis(triphenylphosphine) palladium (14 mg, 12 μmol) according to method A. The product was purified by CC (dichloromethane) followed by preparative HPLC; yield: 18% (21 mg); MS (ESI): 287 (M+H)$^+$; ^{1}H NMR (CD_3COCD_3): 8.46 (s, 1H), 7.67 (s, 4H), 7.35 (d, J = 4.1 Hz, 1H), 7.29 (t, J = 8.1 Hz, 1H), 7.15–7.14 (m, 2H), 7.06 (d, J = 3.8 Hz, 1H), 6.86 (ddd, J = 0.9 Hz and J = 2.2 Hz and J = 7.9 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 159.90, 144.60, 143.40, 142.50, 134.40, 131.90, 130.10, 129.80, 129.40, 127.60, 124.90, 119.90, 116.60, 115.50; IR: 3484, 3412, 1584, 1436, 1186, 779 cm^{-1}.

7.1.4.11. 1-[5-(3′-Hydroxybiphenyl-3-yl)-2-thienyl]ethanone (11). The title compound was prepared by reaction of 3′-bromobiphenyl-3-ol (**11i**) (150 mg, 0.60 mmol), 5-acetylthiophene-2-boronic acid (123 mg, 0.72 mmol), caesium carbonate (785 mg, 2.40 mmol) and tetrakis(triphenylphosphine) palladium (21 mg, 18 μmol) according to method A. The product was purified by CC (dichloromethane/methanol 98:2) followed by preparative HPLC; yield: 27% (48 mg); MS (ESI): 295 (M+H)$^+$; ^{1}H NMR (CD_3COCD_3): 8.49 (s, 1H), 7.95 (t, J = 1.9 Hz, 1H), 7.84 (d, J = 4.1 Hz, 1H), 7.71–7.70 (m, 1H), 7.64–7.62 (m, 2H), 7.52 (t, J = 7.6 Hz, 1H), 7.31 (t, J = 8.1 Hz, 1H), 7.19–7.17 (m, 2H), 6.89 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H), 2.54 (s, 3H); ^{13}C NMR (CD_3COCD_3): 191.80, 159.80, 153.60, 145.40, 144.00, 143.60, 136.00, 135.80, 131.90, 131.60, 129.40, 127.00, 126.70, 126.40, 120.20, 116.70, 115.80, 27.50; IR: 3365, 3088, 1599, 1443, 1277, 780 cm^{-1}.

7.1.4.12. 3′-(5-Chloro-2-thienyl)biphenyl-3-ol (12). The title compound was prepared by reaction of 3′-bromobiphenyl-3-ol (**11i**) (100 mg, 0.40 mmol), 5-chlorothiophene-2-boronic acid (78 mg, 0.48 mmol), caesium carbonate (521 mg, 1.60 mmol) and tetrakis(triphenylphosphine) palladium (14 mg, 12 μmol) according to method A. The product was purified by CC (dichloromethane) followed by preparative HPLC; yield: 9% (10 mg); MS (APCI): 287 (M+H)$^+$; ^{1}H NMR (CD_3COCD_3): 8.50 (s, 1H), 7.82–7.81 (m, 1H), 7.58–7.56 (m, 2H), 7.49 (t, J = 7.8 Hz, 1H), 7.42 (d, J = 3.8 Hz, 1H), 7.30 (t, J = 8.2 Hz, 1H), 7.17–7.15 (m, 2H), 7.07 (d, J = 3.8 Hz, 1H), 7.89–7.86 (m, 1H); ^{13}C NMR (CD_3COCD_3): 159.90, 144.80, 144.00, 143.80, 135.90, 131.90, 131.60, 130.30, 129.80, 128.50, 126.30, 126.20, 125.70, 125.30, 120.20, 116.60, 115.80; IR: 3535, 3403, 1590, 1188, 778 cm^{-1}.

7.1.4.13. 3′-(2-Thienyl)biphenyl-3-ol (13). The title compound was prepared by reaction of 3′-bromobiphenyl-3-ol (**11i**) (100 mg, 0.40 mmol), thiophene-2-boronic acid (62 mg, 0.48 mmol), caesium carbonate (521 mg, 1.60 mmol) and tetrakis(triphenylphosphine) palladium (14 mg, 12 μmol) according to method A. The product was purified by preparative TLC (hexane/ethyl acetate 7:3) followed by preparative HPLC; yield: 36% (36 mg); MS (ESI): 253 (M+H)$^+$; ^{1}H NMR (CD_3COCD_3): 8.49 (s, 1H), 7.88 (t, J = 1.9 Hz, 1H), 7.66–7.64 (m, 1H), 7.56–7.54 (m, 2H), 7.50–7.47 (m, 2H), 7.30 (t, J = 7.9 Hz, 1H), 7.18–7.17 (m, 2H), 7.16–7.14 (m, 1H), 6.88 (ddd, J = 0.9 Hz and J = 2.2 Hz and J = 7.9 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 159.90, 145.80, 144.00, 136.80, 131.90, 131.50, 130.20, 128.00, 127.10, 126.60, 126.00, 125.60, 120.10, 116.50, 115.80; IR: 3365, 1593, 1456, 1186, 777, 692 cm^{-1}.

7.1.4.14. 2-(3′-Methoxybiphenyl-3-yl)-5-methylthiophene (14i). The title compound was prepared by reaction of 3′-bromo-3-methoxybiphenyl (**14ii**) (200 mg, 0.76 mmol), 5-methyl-2-thienyl boronic acid (129 mg, 0.91 mmol), caesium carbonate (991 mg, 3.04 mmol) and tetrakis(triphenylphosphine) palladium (26 mg, 23 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 99:1); yield: 94% (200 mg); ^{1}H NMR (CD_3COCD_3): 7.84 (t, J = 1.9 Hz, 1H), 7.58 (ddd, J = 1.3 Hz and J = 1.9 Hz and J = 7.6 Hz, 1H), 7.55–7.53 (m, 1H), 7.46 (t, J = 7.6 Hz, 1H), 7.39 (t, J = 7.9 Hz, 1H), 7.34 (d, J = 3.5 Hz, 1H), 7.25 (ddd, J = 0.9 Hz and J = 1.6 Hz and J = 7.6 Hz, 1H), 7.23 (t, J = 2.1 Hz, 1H), 6.96 (ddd, J = 0.9 Hz and J = 2.6 Hz and J = 8.2 Hz, 1H), 6.82–6.80 (m, 1H), 3.88 (s, 3H), 2.50 (s, 3H); ^{13}C NMR (CD_3COCD_3): 162.30, 143.70, 143.40, 141.50, 131.80, 131.70, 131.40, 128.50, 127.70, 126.20, 125.70, 125.50, 121.20, 115.00, 114.80, 114.50, 56.60, 16.30.

7.1.4.15. 3′-(5-Methyl-2-thienyl)biphenyl-3-ol (14). The title compound was prepared by reaction of 2-(3′-methoxybiphenyl-3-yl)-5-methylthiophene (**14i**) (200 mg, 0.71 mmol) and boron tribromide (2.13 mmol) according to method D. The product was purified by CC (hexane/ethyl acetate 99:1); yield: 95% (180 mg); MS (ESI): 267 (M+H)$^+$; ^{1}H NMR (CD_3COCD_3): 8.42 (s, 1H), 7.80 (t, J = 1.6 Hz, 1H), 7.57–7.56 (m, 1H), 7.51–7.49 (m, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.33 (d, J = 3.5 Hz, 1H), 7.30 (t, J = 8.2 Hz, 1H), 7.16–7.15 (m, 2H), 6.86 (ddd, J = 0.9 Hz and J = 2.6 Hz and J = 8.2 Hz, 1H), 6.81–6.80 (m, 1H), 2.50 (s, 3H); ^{13}C NMR (CD_3COCD_3): 159.80, 144.10, 143.80, 143.50, 141.40, 137.10, 131.90, 131.40, 128.50, 127.60, 126.10, 125.50, 125.40, 120.10, 116.50, 115.80, 15.50; IR: 3486, 3375, 1575, 1184, 777, 692 cm^{-1}.

7.1.4.16. 2-(3′-Methoxybiphenyl-3-yl)-1-benzothiophene (15i). The title compound was prepared by reaction of 3′-bromo-3-methoxybiphenyl (**14ii**) (200 mg, 0.76 mmol), benzothiophene-2-boronic acid (162 mg, 0.91 mmol), caesium carbonate (991 mg, 3.04 mmol) and tetrakis(triphenylphosphine) palladium (26 mg, 23 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 47% (113 mg); ^{1}H NMR (CD_3COCD_3): 8.05 (t, J = 1.8 Hz, 1H), 7.95–7.94 (m, 1H), 7.89 (s, 1H), 7.86 (dd, J = 1.9 Hz and J = 7.2 Hz, 1H), 7.77 (ddd, J = 0.9 Hz and J = 1.9 Hz and J = 7.5 Hz, 1H), 7.66 (ddd, J = 1.3 Hz and J = 1.9 Hz and J = 7.9 Hz, 1H), 7.56 (t, J = 7.8 Hz, 1H), 7.43–7.35 (m, 3H), 7.31 (ddd, J = 0.9 Hz and J = 1.6 Hz and J = 7.5 Hz, 1H), 7.29 (t, J = 2.1 Hz, 1H), 6.98 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H), 3.89 (s, 3H); ^{13}C NMR (CD_3COCD_3): 162.30, 143.80, 142.90, 141.20, 136.70, 131.90, 131.60, 130.70, 129.00, 127.30, 126.70, 126.60, 126.50, 125.70, 124.20, 122.20, 121.30, 115.20, 114.60, 56.70.

7.1.4.17. 3′-(1-Benzothien-2-yl)biphenyl-3-ol (15). The title compound was prepared by reaction of 2-(3′-methoxybiphenyl-3-yl)-1-benzothiophene (**15i**) (113 mg, 0.36 mmol) and boron tribromide (1.08 mmol) according to method D. The product was purified by preparative TLC (hexane/ethyl acetate 8:2); yield: 77% (83 mg); MS (ESI): 303 (M+H)$^+$; ^{1}H NMR (CD_3COCD_3): 8.45 (s, 1H), 8.01 (t, J = 1.8 Hz, 1H), 7.95–7.94 (m, 1H), 7.89 (s, 1H), 7.87–7.85 (m, 1H), 7.77 (ddd, J = 1.3 Hz and J = 1.9 Hz and J = 7.9 Hz, 1H), 7.62 (ddd, J = 0.9 Hz and J = 1.6 Hz and J = 7.6 Hz, 1H), 7.55 (t, J = 7.8 Hz, 1H), 7.41–7.31 (m, 3H), 7.22–7.20 (m, 2H), 6.90–6.88 (m, 1H); ^{13}C NMR (CD_3COCD_3): 171.90, 159.90, 143.90, 142.90, 141.20, 136.60, 131.90, 131.60, 128.90, 127.20, 126.70, 126.60, 126.50, 125.70, 124.20, 122.20, 120.20, 116.60, 115.80; IR: 3464, 3387, 1574, 1179, 779, 692 cm^{-1}.

7.1.4.18. 3-[2-(2-Thienyl)pyridin-4-yl]phenol (16). Compound **16** was obtained as a side product of the reaction of compound **17**; yield: 22% (17 mg); MS (ESI): 254 (M+H)$^+$; ^{1}H NMR (CD_3OD): 8.42 (dd, J = 0.6 Hz and J = 5.4 Hz, 1H), 7.91 (dd, J = 0.6 Hz and J = 1.6 Hz, 1H), 7.71 (dd, J = 0.9 Hz and J = 3.8 Hz, 1H), 7.46 (dd, J = 1.3 Hz and J = 5.1 Hz, 1H), 7.40 (dd, J = 1.6 Hz and J = 5.4 Hz, 1H), 7.27 (t, J = 7.9 Hz, 1H), 7.17 (ddd, J = 0.9 Hz and J = 1.9 Hz and J = 7.9 Hz, 1H), 7.11 (t, J = 2.1 Hz, 1H), 7.09 (dd, J = 3.5 Hz and

J = 5.1 Hz, 1H), 6.84 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H); ^{13}C NMR (CD_3OD): 159.40, 154.40, 151.40, 150.70, 145.30, 140.50, 131.40, 129.30, 128.90, 126.70, 121.30, 119.30, 118.20, 117.50, 114.80; IR: 3075, 1599, 1202, 781, 722, 692 cm^{-1}.

7.1.4.19. 4-Bromo-2-(5-chloro-2-thienyl)pyridine (17i). The title compound was prepared by reaction of 2,4-dibromopyridine (300 mg, 1.27 mmol), 5-chloro-2-thienyl boronic acid (206 mg, 1.27 mmol), potassium carbonate (350 mg, 2.54 mmol) and tetrakis(triphenylphosphine) palladium (44 mg, 38 μmol) according to method B using 1 equiv boronic acid. The product was purified by CC (hexane/ethyl acetate 99:1); yield: 24% (83 mg); ^{1}H NMR (CD_3COCD_3): 8.36 (dd, J = 0.6 Hz and J = 5.4 Hz, 1H), 8.07 (dd, J = 0.6 Hz and J = 1.9 Hz, 1H), 7.70 (d, J = 4.1 Hz, 1H), 7.48 (dd, J = 1.9 Hz and J = 5.4 Hz, 1H), 7.08 (d, J = 4.1 Hz, 1H); ^{13}C NMR (CD_3COCD_3): 154.80, 152.40, 144.40, 134.90, 134.40, 129.80, 127.40, 127.30, 122.90.

7.1.4.20. 3-[2-(5-Chloro-2-thienyl)pyridin-4-yl]phenol (17). The title compound was prepared by reaction of 4-bromo-2-(5-chloro-2-thienyl)pyridine (**17i**) (83 mg, 0.30 mmol), 3-hydroxybenzene boronic acid (50 mg, 0.36 mmol), caesium carbonate (391 mg, 1.20 mmol) and tetrakis(triphenylphosphine) palladium (11 mg, 9 μmol) according to method A. The product was purified by preparative TLC (hexane/ethyl acetate 6:4) followed by preparative HPLC; yield: 49% (42 mg); MS (ESI): 288 $(M+H)^+$; ^{1}H NMR (CD_3OD): 8.43 (dd, J = 0.9 Hz and J = 5.4 Hz, 1H), 7.90 (dd, J = 0.9 Hz and J = 1.9 Hz, 1H), 7.56 (d, J = 4.1 Hz, 1H), 7.43 (dd, J = 1.9 Hz and J = 5.4 Hz, 1H), 7.28 (t, J = 7.9 Hz, 1H), 7.18 (ddd, J = 0.9 Hz and J = 1.9 Hz and J = 7.9 Hz, 1H), 7.11 (t, J = 1.9 Hz, 1H), 6.97 (d, J = 3.8 Hz, 1H), 6.85 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H); ^{13}C NMR (CD_3OD): 159.40, 151.40, 150.80, 144.60, 140.40, 131.40, 128.80, 125.80, 121.60, 119.30, 118.30, 117.80, 117.50, 117.30, 114.80; IR: 3077, 1584, 1471, 799, 622 cm^{-1}.

7.1.4.21. 2-(5-Chloro-2-thienyl)-6-(3-methoxyphenyl)pyridine (18i). The title compound was prepared by reaction of 2-bromo-6-(3-methoxyphenyl)pyridine (**18ii**) (250 mg, 0.95 mmol), 5-chloro-2-thienyl boronic acid (185 mg, 1.14 mmol), potassium carbonate (262 mg, 1.89 mmol) and tetrakis(triphenylphosphine) palladium (33 mg, 28 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 33% (95 mg); ^{1}H NMR (CD_3COCD_3): 7.97 (dd, J = 0.6 Hz and J = 7.6 Hz, 1H), 7.80 (t, J = 7.8 Hz, 1H), 7.66–7.64 (m, 2H), 7.54 (dd, J = 0.6 Hz and J = 7.9 Hz, 1H), 7.41 (t, J = 8.2 Hz, 1H), 7.10 (d, J = 3.8 Hz, 1H), 7.05 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H), 7.02 (d, J = 4.1 Hz, 1H), 3.88 (s, 3H); ^{13}C NMR (CD_3COCD_3): 162.20, 159.80, 143.50, 141.80, 136.80, 133.70, 130.30, 129.60, 129.40, 128.50, 125.80, 121.20, 120.90, 117.30, 114.00, 56.70.

7.1.4.22. 3-[6-(5-Chloro-2-thienyl)pyridin-2-yl]phenol (18). The title compound was prepared by reaction of 2-(5-chloro-2-thienyl)-6-(3-methoxyphenyl)pyridine (**18i**) (95 mg, 0.32 mmol) and boron tribromide (0.96 mmol) according to method D. The product was purified by CC (hexane/ethyl acetate 8:2) followed by preparative HPLC; yield: 29% (27 mg); MS (ESI): 288 $(M+H)^+$; ^{1}H NMR (CD_3OD): 7.83 (t, J = 7.9 Hz, 1H), 7.71 (dd, J = 0.6 Hz and J = 7.9 Hz, 1H), 7.68 (dd, J = 0.6 Hz and J = 7.9 Hz, 1H), 7.63–7.62 (m, 1H), 7.57–7.55 (m, 1H), 7.54 (d, J = 4.1 Hz, 1H), 7.33 (t, J = 7.9 Hz, 1H), 7.02 (d, J = 4.1 Hz, 1H), 6.90 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H); ^{13}C NMR (CD_3OD): 159.00, 157.90, 152.70, 141.40, 139.00, 133.30, 133.20, 130.80, 129.80, 128.60, 125.10, 119.80, 119.10, 117.40, 114.70; IR: 3384, 1566, 1452, 1218, 775 cm^{-1}.

7.1.4.23. 4-Bromo-2-(3-methoxyphenyl)pyridine (19ii). The title compound was prepared by reaction of 2,4-dibromopyridine (300 mg, 1.27 mmol), 3-methoxybenzene boronic acid (193 mg, 1.27 mmol), sodium hydrogencarbonate (213 mg, 2.54 mmol) and tetrakis(triphenylphosphine) palladium (44 mg, 38 μmol) according to method C. The product was purified by CC (hexane/ethyl acetate 96:4); yield: 44% (147 mg); ^{1}H NMR (CD_3COCD_3): 8.54 (dd, J = 0.6 Hz and J = 5.4 Hz, 1H), 8.15–8.14 (m, 1H), 7.72–7.71 (m, 1H), 7.70–7.69 (m, 1H), 7.56 (dd, J = 1.9 Hz and J = 5.4 Hz, 1H), 7.41 (t, J = 7.8 Hz, 1H), 7.03 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H), 3.88 (s, 3H); ^{13}C NMR (CD_3COCD_3): 162.20, 160.20, 152.50, 141.20, 134.90, 131.70, 127.40, 125.30, 121.10, 117.40, 114.00, 56.70.

7.1.4.24. 4-(5-Chloro-2-thienyl)-2-(3-methoxyphenyl)pyridine (19i). The title compound was prepared by reaction of 4-bromo-2-(3-methoxyphenyl)pyridine (**19ii**) (147 mg, 0.56 mmol), 5-chloro-2-thienyl boronic acid (109 mg, 0.67 mmol), caesium carbonate (730 mg, 2.24 mmol) and tetrakis(triphenylphosphine) palladium (20 mg, 17 μmol) according to method A. The product was purified by CC (hexane/ethyl acetate 9:1); yield: 49% (83 mg); ^{1}H NMR (CD_3COCD_3): 8.65 (dd, J = 0.6 Hz and J = 5.1 Hz, 1H), 7.80 (dd, J = 0.6 Hz and J = 1.6 Hz, 1H), 7.77–7.76 (m, 1H), 7.75–7.73 (m, 1H), 7.72 (d, J = 3.8 Hz, 1H), 7.49 (dd, J = 1.6 Hz and J = 5.1 Hz, 1H), 7.41 (t, J = 7.9 Hz, 1H), 7.16 (d, J = 4.1 Hz, 1H), 7.02 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H), 3.88 (s, 3H); ^{13}C NMR (CD_3COCD_3): 162.20, 152.30, 143.10, 142.30, 142.00, 132.80, 130.10, 127.80, 121.00, 120.40, 117.60, 116.80, 114.20, 56.60.

7.1.4.25. 3-[4-(5-Chloro-2-thienyl)pyridin-2-yl]phenol (19). The title compound was prepared by reaction of 4-(5-chloro-2-thienyl)-2-(3-methoxyphenyl)pyridine (**19i**) (83 mg, 0.27 mmol) and boron tribromide (0.81 mmol) according to method D. The product was purified by CC (hexane/ethyl acetate 8:2) followed by preparative HPLC; yield: 47% (54 mg); MS (ESI): 288 $(M+H)^+$; ^{1}H NMR (CD_3SOCD_3): 8.63 (dd, J = 0.9 Hz and J = 5.4 Hz, 1H), 8.03 (dd, J = 0.9 Hz and J = 1.9 Hz, 1H), 7.85 (d, J = 4.1 Hz, 1H), 7.57–7.56 (m, 2H), 7.52 (dd, J = 1.9 Hz and J = 5.1 Hz, 1H), 7.30 (t, J = 8.1 Hz, 1H), 7.28 (d, J = 3.8 Hz, 1H), 6.86 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 8.2 Hz, 1H); ^{13}C NMR (CD_3SOCD_3): 157.70, 154.70, 140.60, 139.40, 129.60, 126.70, 118.00, 117.50, 116.30, 115.40, 113.50; IR: 3045, 1580, 1310, 780, 693 cm^{-1}.

7.1.4.26. 3-(5-Chloro-2-thienyl)-5-(3-methoxyphenyl)pyridine (20i). The title compound was prepared by reaction of 3-bromo-5-(3-methoxyphenyl)pyridine (**20ii**) (60 mg, 0.23 mmol), 5-chloro-2-thienyl boronic acid (44 mg, 0.27 mmol), potassium carbonate (49 mg, 0.46 mmol) and tetrakis(triphenylphosphine) palladium (8 mg, 7 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 8:2); yield: 68% (47 mg); ^{1}H NMR (CD_3COCD_3): 8.80 (t, J = 1.9 Hz, 2H), 8.18 (t, J = 2.2 Hz, 1H), 7.54 (d, J = 4.1 Hz, 1H), 7.43 (t, J = 8.2 Hz, 1H), 7.33–7.31 (m, 2H), 7.13 (d, J = 3.8 Hz, 1H), 7.03–7.01 (m, 1H), 3.89 (s, 3H); ^{13}C NMR (CD_3COCD_3): 169.00, 162.40, 149.30, 147.10, 140.50, 134.50, 133.00, 131.50, 131.30, 130.70, 129.90, 126.70, 121.30, 115.90, 114.60, 56.70.

7.1.4.27. 3-[5-(5-Chloro-2-thienyl)pyridin-3-yl]phenol (20). The title compound was prepared by reaction of 3-(5-chloro-2-thienyl)-5-(3-methoxyphenyl)pyridine (**20i**) (120 mg, 0.40 mmol) and boron tribromide (1.20 mmol) according to method D. The product was purified by CC (hexane/ethyl acetate 8:2) followed by preparative HPLC; yield: 37% (42 mg); MS (ESI): 288 $(M+H)^+$; ^{1}H NMR (CD_3OD): 8.79 (s, 1H), 8.75 (s, 1H), 8.27 (s, 1H), 7.51 (dd, J = 0.9 Hz and J = 3.8 Hz, 1H), 7.38 (t, J = 7.9 Hz, 1H), 7.20 (d, J = 7.9 Hz, 1H), 7.14 (s, 1H), 7.11 (dd, J = 0.9 Hz and J = 3.8 Hz, 1H), 6.94–6.92 (m, 1H); ^{13}C NMR (CD_3OD): 159.50, 132.30, 131.50, 129.20, 126.50, 119.50, 117.00, 115.50, 115.10; IR: 3188, 1589, 1329, 1014, 776, 693 cm^{-1}.

7.2. Biological methods

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

17β-HSD1 and 17β-HSD2 were obtained from human placenta according to previously described procedures.[31] Fresh human placenta was homogenized and cytosolic fraction and microsomes were separated by centrifugation. For the partial purification of 17β-HSD1, the cytosolic fraction was precipitated with ammonium sulfate. 17β-HSD2 was obtained from the microsomal fraction.

7.2.1. Inhibition of 17-HSD1

Inhibitory activities were evaluated by an established method with minor modifications.[31] Briefly, the enzyme preparation was incubated with NADH [500 μM] in the presence of potential inhibitors at 37 °C in a phosphate buffer (50 mM) supplemented with 20% of glycerol and EDTA (1 mM). Inhibitor stock solutions were prepared in DMSO. The final concentration of DMSO was adjusted to 1% in all samples. The enzymatic reaction was started by addition of a mixture of unlabelled- and [2, 4, 6, 7-^{3}H]-E1 (final concentration: 500 nM, 0.15 μCi). After 10 min, the incubation was stopped with $HgCl_2$ and the mixture was extracted with diethylether. After evaporation, the steroids were dissolved in acetonitrile. E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 reverse phase chromatography column (Nucleodur C18 Gravity, 3 μm, Macherey-Nagel, Düren) connected to a HPLC-system (Agilent 1100 Series, Agilent Technologies, Waldbronn). Detection and quantification of the steroids were performed using a radioflow detector (Berthold Technologies, Bad Wildbad). The conversion rate was calculated after analysis of the resulting chromatograms according to the following equation: $\%\text{conversion} = \frac{\%E2}{\%E2+\%E1} \times 100$. Each value was calculated from at least three independent experiments.

7.2.2. Inhibition of 17β-HSD2

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

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

7.2.3. ER affinity

The binding affinity of selected compounds to the ERα and ERβ was determined according to Zimmermann et al.[49] Briefly, 0.25 pmol of ERα or ERβ, respectively, were incubated with [2, 4, 6, 7-^{3}H]-E2 (10 nM) and test compound for 1 h at room temperature. The potential inhibitors were dissolved in DMSO (5% final concentration). Evaluation of non-specific-binding was performed with diethylstilbestrol (10 μM). After incubation, ligand–receptor complexes were selectively bound to hydroxyapatite (5 g/60 mL TE-buffer). The complex formed was separated, washed and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (Rack Beta Primo 1209, Wallac, Turku). For determination of the relative binding affinity (RBA), inhibitor and E2 concentrations required to displace 50% of the receptor bound labelled E2 were determined. RBA values were calculated according to the following equation: $RBA[\%] = \frac{IC_{50}(E2)}{IC_{50}(compound)} \times 100$. The RBA value for E2 was arbitrarily set at 100%.

7.3. Computational chemistry

7.3.1. Molecular modelling

All calculations and graphical manipulations were performed on Intel(R) Core(TM)2 Duo CPU 3.00 GHz running Linux CentOS 5.3 while using the software packages AutoDock4.2[37] as implemented through the graphical user interface AutoDockTools1.5.4[37], GOLDv4.0.1[38] and MOE2008.10 (Chemical Computing Group Inc., Montreal, Canada).

Atomic coordinates of 17β-HSD1 used during molecular modelling simulations were derived from the structure of the ternary complex between 17β-HSD1, $NADP^+$ and equilin (RCSB Protein Data Bank entry 1EQU).

To set the initial coordinates for the docking studies, chain A was isolated in the coordinate file and the cocrystallized equilin as well as all water molecules were removed. Missing protein atoms were added and correct atom types set. Ionization states and hydrogen positions were assigned using the Protonate 3D utility of MOE. Furthermore the crystal structure was energy minimized with MOE applying MMFF94× force field and generalized Born model, keeping the coordinates of protein backbone atoms fixed.

Inhibitors were energy minimized with MOE using the MMFF94× force field and the atomic partial charges for the inhibitors and the cofactor were calculated using the AM1-BCC approximation.

Two docking softwares, namely Autodock4.2 and GOLDv4.0.1, were used to evaluate the binding mode of the inhibitors.

7.3.2. Autodock4.2

The docking area has been defined by a box, centered on the steroid binding site. Grid points of 86 × 80 × 80 with 0.250 Å spacing were calculated around the docking area for all the ligand atom types using AutoGrid4.2. For each inhibitor, 100 separate docking calculations were performed. Each docking calculation consisted of 25×10^5 energy evaluations using the Lamarckian genetic algorithm local search (GALS) method. Each docking run was performed with a population size of 250. A mutation rate of 0.02 and a crossover rate of 0.8 were used to generate new docking trials for subsequent generations. The docking results from each of the 100 calculations were clustered on the basis of root-mean-square deviation (RMSD = 2.0 Å) between the Cartesian coordinates of the ligand atoms and were ranked on the basis of the free binding energy.

7.3.3. GOLDv4.0.1

Active site origin was set at the centre of the steroid binding site and a radius of 15 Å was chosen. The automatic active-site detection was switched on. Further, GOLDscore[38] fitness function was used and genetic algorithm default parameters were set as suggested by the GOLD authors. For each of the selected compounds 50 solutions were generated and compounds were ranked according to GOLDscore.

Both programs performed in a similar way, supporting the herein suggested binding modes. The quality of the docked poses was evaluated based mainly on visual inspection of the putative binding modes of the ligand, and secondly on the scoring functions.

7.3.4. MEP

For selected compounds ab initio geometry optimizations were performed for the gas phase at the B3LYP/6-311++G (d,p) level of density functional theory (DFT) by means of the Gaussian 03 software, and the molecular electrostatics potential map (MEP) was plotted using GaussView, version 3.0, the 3D molecular graphics package of Gaussian.[50] These electrostatic potential surfaces were generated by mapping 6-311++G electrostatic potentials onto sur-

faces of molecular electron density (isovalue of 0.004 e/Å). The MEP maps are colour-coded, where red stands for negative values (-1.0×10^{-2} Hartree) and blue for positive ones (2.5×10^{-2} Hartree).

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Supplementary data

Supplementary data (methods for analytical HPLC; purity data of all tested compounds, MEP calculations of compounds **17** and **19**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.065.

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