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Bicyclic Substituted Hydroxyphenylmethanones as Novel Inhibitors of 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1) for the Treatment of Estrogen-Dependent Diseases†

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Estradiol (E2), the most important estrogen in humans, is involved in the initiation and progression of estrogen-dependent diseases such as breast cancer and endometriosis. Its local production in the target cell is regulated by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), which catalyzes E2-formation by reduction of the weak estrogen estrone (E1). Because the enzyme is expressed in the diseased tissues, inhibition of 17β-HSD1 is considered as a promising therapy for the treatment of estrogen-dependent diseases. For the development of novel inhibitors, a structure- and ligand-based design strategy was applied, resulting in bicyclic substituted hydroxyphenylmethanones. In vitro testing revealed high inhibitory potencies toward human placental 17β-HSD1. Compounds were further evaluated with regard to selectivity (17β-HSD2, estrogen receptors ERα and ERβ), intracellular activity (T47D cells), and metabolic stability. The most promising compounds, **14** and **15**, showed IC_{50} values in the low nanomolar range in the cell-free and cellular assays (8−27 nM), more than 30-fold selectivity toward 17β-HSD2 and no affinity toward the ERs. The data obtained make these inhibitors interesting candidates for further preclinical evaluation.

Introduction

Breast cancer and endometriosis are estrogen-dependent diseases, which show a strong global prevalence. Breast cancer is one of the two leading causes of cancer death in women. Although some therapeutic approaches are already available, the five-year survival rate for breast cancer patients only remains around 80% (www.cancer.org). The Endometriosis Foundation of America has pointed out that endometriosis is one of the top three causes of female infertility (www.endofound.org). However, until recently, there has been no oral medication available to cure this disease.

The local production of estradiol (E2[a]) plays a pivotal role for the initiation and development of both diseases.[1,2] Thus, the reduction of tissue levels of active estrogens by inhibiting their synthetic pathway moves steadily to the forefront. Two therapeutic approaches have mainly been investigated within the past years. Aromatase inhibitors[3−8] (to reduce the transformation of androgens into estrogens) and sulfatase inhibitors[9] (to reduce the conversion of estrone sulfate (E1S) into estrone (E1)) intervene in the penultimate step of E2 biosynthesis. Both therapeutic approaches show disadvantages by their rather radical reduction of systemic estrogen concentration. A softer therapy seems to be the inhibition of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1). This enzyme regulates the ratio of the weak active estrogen E1 and its potent metabolite E2 by catalyzing the reduction of E1 to E2 using NAD(P)H as a cofactor (Chart 1).

Interestingly, mRNA of 17β-HSD1, which is also used as a prognostic marker, is often overexpressed in breast cancer tissues[10,11] and endometriotic lesions.[12] Considering the fact that therapeutic approaches regulating intracellular hormone concentrations have already been used successfully in androgen dependent diseases such as benign prostatic hyperplasia and alopecia,[13−16] 17β-HSD1 appeared to be a promising target for the local reduction of E2 and accordingly for the treatment of breast cancer and endometriosis. In contrast to the applied therapies, a basal estrogenic activity will be maintained because the E1 level should not be affected. Thus, side effects caused by a total absence of estrogenic activity could be avoided. The tissue specific influence of 17β-HSD1 inhibitors seems especially suitable for the therapy of postmenopausal women, in which the local production (e.g., in breast or endometrium) represents the major source of E2.

As it has recently been suggested, 17β-HSD1 is also involved in the metabolism of retinoic acid,[17,18] a natural compound which is known to have antitumor activity.[19] Thus, inhibition of 17β-HSD1 should have an additional beneficial effect.

Efficacy of 17β-HSD1 inhibitors has been shown by several groups in different animal models. Husen et al.[20,21] and Day et al.[22] used xenograft models with MCF7 breast cancer cells stably expressing recombinant human 17β-HSD1 or the breast cancer cell line T47D, respectively, in order to demonstrate in vivo efficacy of their inhibitors by decreasing tumor growth. In

†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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[a] Abbreviations: 17β-HSD1, 17β-hydroxysteroid dehydrogenase type 1; 17β-HSD2, 17β-hydroxysteroid dehydrogenase type 2; E1, estrone; E2, 17β-estradiol; E1S, estrone sulfate; ER, estrogen receptor; NADP(H), nicotinamide adenine dinucleotide phosphate; NAD(H), nicotinamide adenine dinucleotide; IBX, 2-iodoxybenzoic acid; SAR, structure−activity relationship; RBA, relative binding affinity; SF, selectivity factor; HPLC, high performance liquid chromatography; CC, column chromatography; TLC, thin layer chromatography; IUPAC, international union of pure and applied chemistry.

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transgenic mice, it was proven that 17β-HSD1 inhibitors are able to reduce the conversion of E1 into E2.[23] With regards to endometriosis, two in vivo models have been established.[24,25] Using nude mice implanted with endometrial tissue from human donors, the expression of steroid hormone receptors and steroid converting enzymes, as well as the proliferation of the ectopic tissue, can be analyzed.[24] In a second model, endometriosis is induced in marmoset monkeys, either by endometrial reflux or invasively by laparotomy.[25] Despite encouraging in vivo data, no 17β-HSD1 inhibitor has entered clinical evaluation yet. Thus, the applied inhibitors do not seem to be appropriate candidates for clinical application.

The therapeutic concept requires selectivity of potent inhibitors toward 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2) because it catalyzes the reverse reaction, the oxidation of E2 into E1. Furthermore, to reduce the risk of intrinsic estrogen effects, affinity toward both estrogen receptors α and β (ERα and ERβ) should be avoided, even if the pathophysiological role of ERβ is not completely clarified.

The selective inhibition of 17β-HSD1 was the goal of several groups investigating inhibitors over the past years.[18,26−30] Their drug design efforts were facilitated by the existence of several X-ray structures of human 17β-HSD1 and will be further alleviated by the recently published first insights in 17β-HSD1 enzyme kinetics and ligand binding by dynamic motion investigations.[31] Although running the higher risk of unwanted side effects, most of these groups focused on the development of compounds bearing a steroidal scaffold. Regarding nonsteroidal inhibitors, aside from phytoestrogens[27,32] and thiophenepyrimidinones,[33,34] there are two additional compound classes showing inhibitory activity in the low nanomolar range, namely the (hydroxyphenyl)naphthols[35−39] and the bis(hydroxyphenyl)heterocycles.[40−45] Recently, we also reported on heterocyclic substituted biphenylols,[46] which demonstrate notable inhibitory activity. Molecular modeling studies indicated that linear and angulate molecules out of this class unfold their activity via interaction with the catalytic center and a rather lipophilic subpocket located below the catalytic site, respectively.[46]

In the following, we will report on the design, synthesis, and biological evaluation of a novel class of 17β-HSD1 inhibitors, namely bicyclic substituted hydroxyphenylmethanones.

Design

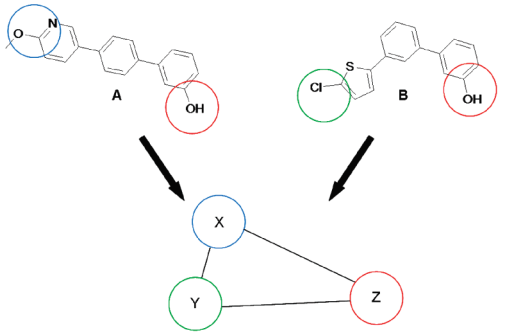
Molecular docking studies of the most potent heterocyclic substituted biphenylol inhibitors **A** and **B** (Chart 2) using the crystal structure 1EQU (PDB-ID) indicated that their heterocyclic part interacts with different areas of the active site.[46] Designed using E1 as a template, compound **A** shows the same interaction pattern as the natural substrate. In detail: the *meta*-OH-phenyl ring imitates the steroidal A-ring and the methoxypyridine interacts as hydrogen-bond acceptor with Ser142 and Tyr155, which together with Asn114 and Lys159 constitute the catalytic tetrade. While the OH-phenyl ring of **B** also mimics the steroidal A-ring, its chlorothiophene part points in the direction of a mainly apolar subpocket consisting of Leu95, Leu96, Asn152, Tyr155 and Phe192.

Our strategy focuses on the design of inhibitors, which fit into a three-point-pharmacophore model derived from the different interactions found for **A** and **B** (Chart 3). A closer analysis of the amino acids in these regions by computational docking studies provides ideas for substituents as appropriate interacting partners as well as for the structural architecture of the compounds.

To mimic the interactions established by the steroidal A-ring of the substrate with the amino acids His221 and Glu282, a hydroxy-substituted phenyl moiety will be one important component of our inhibitors. The second part should interact with the catalytic tetrade. Inhibitors with two H-bond acceptor groups located in this region are described to inhibit 17β-HSD1.[46] One functional group with two hydrogen bond acceptor properties could eventually be more appropriate to target the corresponding amino acids. A keto-group, as in the natural substrate E1, should be the functionality of choice. Moreover, it disrupts the linearity present in **A**, thus allowing for binding with a third interaction region, which is constituted by a mainly apolar subpocket located beneath the catalytic center. In this subpocket, Asn152 is the only hydrophilic amino acid. This amino acid was already exploited for active compound design: recently, Mazumdar et al.[47] reported on the crystal structure of the steroidal inhibitor **E2B** (Chart 2), whose *meta*-amidobenzyl substituent was found to interact with Asn152. Consequently, a second OH-phenyl moiety was

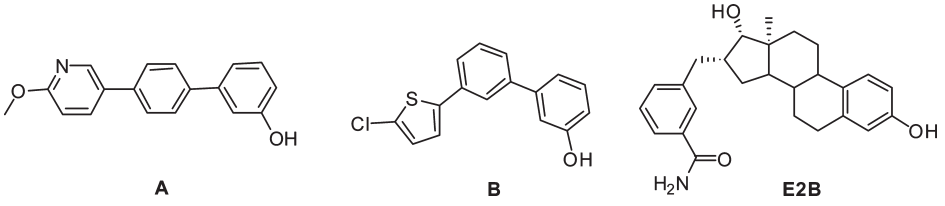
Chart 1. Interconversion of Estrone (E1) and Estradiol (E2)

Chart 3. Design Concept and Pharmacophore Model



X = pure hydrogen bond acceptor (ketone): interactions in catalytic center with Tyr155 + Ser142
Y = H-bond donor + lipohilic moiety (phenol): interactions in lipophilic subpocket with Asn152
Z = H-bond donor and/or H-bond acceptor (OH): interactions in C-terminal region with His221 + Glu282

Chart 2. Most Potent 17β-HSD1 Inhibitors of Heterocyclic Substituted Biphenylol Compound Class (Compounds **A** and **B**) and **E2B**



introduced as a further component of the inhibitor structure in order to fit in this apolar subpocket and interact with Asn152 (Chart 4).

The design concept resulted in compounds **1**−**5**, which were selected as the starting point for this study. Thiophene was chosen as the connecting group between the hydroxyphenyl and the keto-group in order to fit in the rather lipophilic substrate binding site (known as hydrophobic tunnel) and to fit the distance between the various interacting partners in the protein. After identifying **1** and **4** as most promising, small substituents were introduced into these compounds (**6**−**20**). Finally, the substitution pattern of the thiophene was changed (**21**) and it was replaced by thiazole (**22**−**24**).

Chemistry

The synthesis of the thiophene derivatives **1**−**20** is depicted in Scheme 1. The first step in the preparation of compounds **1**−**3** and **6**−**10** was the coupling of 2-bromothiophene with 3-methoxy- and 4-methoxybenzeneboronic acid, respectively, thus leading to intermediates **1b** and **6b**. A Friedel−Crafts acylation (method A) was then performed with the appropriate benzoyl chlorides, resulting in compounds **1a**−**3a** and **6a**−**10a**. Subsequently, treatment with boron tribromide led to the corresponding dihydroxylated final compounds **1**−**3** and **6**−**10**.

The brominated key intermediate **4b** was synthesized via Friedel−Crafts acylation of 2-bromothiophene with 3-methoxybenzoyl chloride. Using **4b**, Suzuki cross coupling reactions[48] with the appropriate commercially available boronic acids were carried out via two different methods (B for compounds **14a**−**16a** and C[41] for compounds **4a**, **5a**, **11a**−**13a**, and **18a**, Scheme 1). The methoxy functions were cleaved with BBr_3[49] yielding compounds **4**, **5**, **11**−**16**, and **18**.

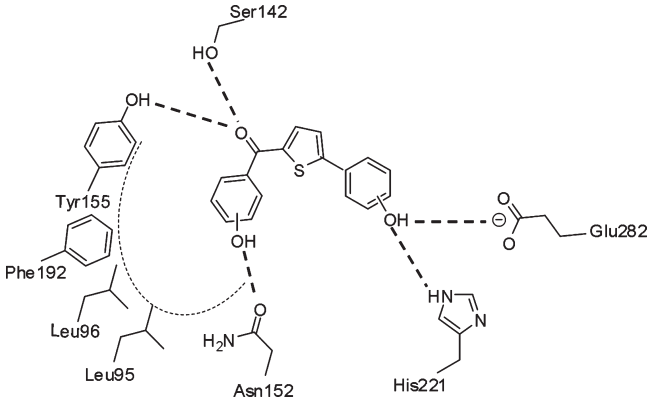
Ether cleavage of the key intermediate **4b** gave **17a** in quantitative yield. Cross coupling of **17a** with the appropriate hydroxylated benzene boronic acids resulted in the dihydroxylated compounds **17** and **19**.

3-Ethyl-4-methoxybenzeneboronic acid was synthesized using the method of Allan et al.[50] and was used for Suzuki reaction in order to yield the monomethoxy derivative **20a**. The latter was heated with pyridinium hydrochloride[51] to yield compound **20**.

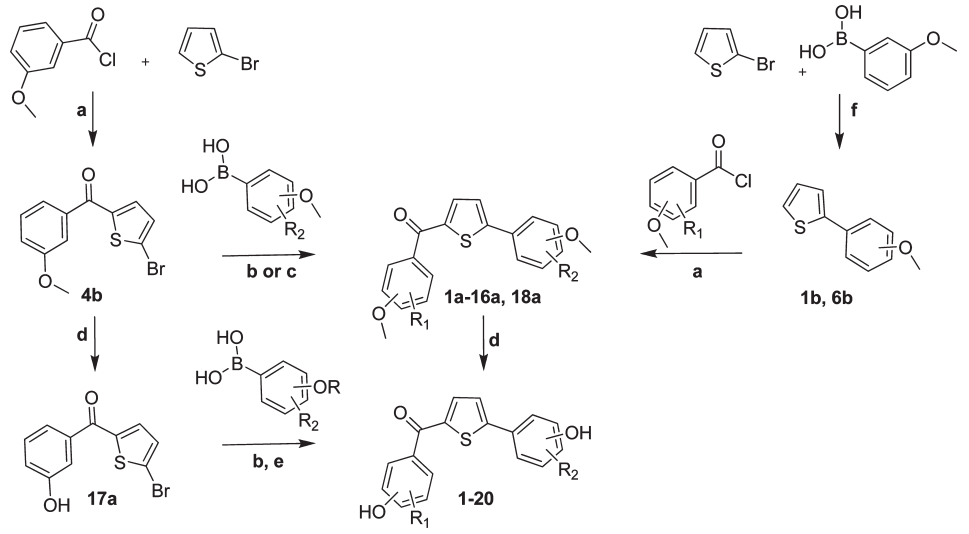
The preparation of compounds **21**−**24** (Scheme 2) started with a Grignard reaction using 3-methoxyphenylmagnesium bromide and the appropriate brominated aromatic carbaldehydes (method F). The OH-groups of compounds **21c**−**23c** were subsequently oxidized to the keto-functions with 2-iodoxybenzoic acid (IBX) (method G). Suzuki cross coupling reactions using the 4-methoxy-3-methylbenzeneboronic acid and the brominated methanones **21b**−**23b** led to compounds **21a**−**23a**. Demethylation with BBr_3 (method D) yielded the final compounds **21** and **23**, whereas pyridinium hydrochloride (method E) was used for the preparation of **22**.

For the preparation of **24**, Suzuki coupling was performed using the hydroxylated intermediate **24c** because its oxidation failed. Subsequently, **24b** was oxidized by means of IBX in anhydrous THF. Reaction of **24a** with pyridinium hydrochloride led to **24**.

Chart 4. Schematic Representation of the Inhibitor Template Binding to the Amino Acids of the Active Site (Possible Hydrogen Bond Interactions Are Depicted in Dashed Lines)



Scheme 1. Synthesis of Compounds **1**−**20**[a]

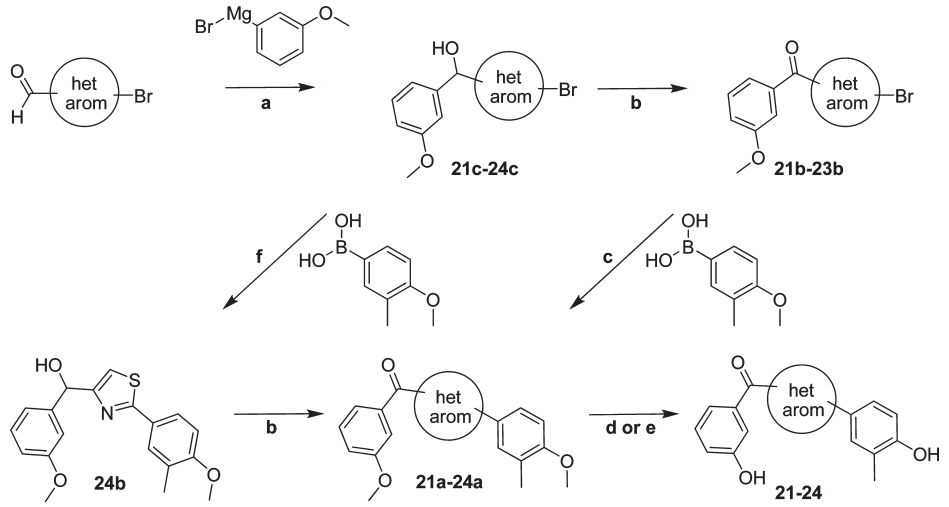


[a] Reagents and conditions: (a) method A, $AlCl_3$, anhydrous CH_2Cl_2, 0 °C, 0.5 h and then rt, 1 h, for compounds **1a**−**3a**, **4b**, **6a**−**10a**; (b) method B, Cs_2CO_3, $Pd(PPh_3)_4$, DME/water (1:1), reflux, 2 h, for compounds **14a**−**16a**, **17**, **19**, **20a**; (c) method C, Cs_2CO_3, $Pd(PPh_3)_4$, DME/EtOH/water (1:1:1), microwave conditions (150 W, 15 bar, 150 °C, 15 min), for compounds **4a**, **5a**, **11a**−**13a**, **18a**; (d) method D, BBr_3, CH_2Cl_2, −78 °C, 20 h, for compounds **1**−**16**, **17a**, **18**; (e) method E, pyridinium hydrochloride, 220 °C, 18 h, for compound **20**; (f) Na_2CO_3, $Pd(PPh_3)_4$, THF/water (1:1), reflux, 18 h, for compound **1b**.

Biological Results

Activity: Inhibition of Human 17β-HSD1. Placental enzyme was isolated following a described procedure[38] and

Scheme 2. Synthesis of Compounds **21**−**24**[a]



[a] Reagents and conditions: (a) method F, anhydrous THF, 80 °C, 3 h, for compounds **21c**−**24c**; (b) method G, 2-iodoxybenzoic acid, anhydrous THF, 0 °C, 10 min and 60 °C, 18 h, for compounds **21b**−**23b**, **24a**; (c) method B, Cs_2CO_3, $Pd(PPh_3)_4$, DME/water (1:1), reflux, 2 h, for compounds **21a**−**23a**; (d) method D, BBr_3, CH_2Cl_2, −78 °C, 20 h, for compounds **21**, **23**; (e) method E, pyridinium hydrochloride, 220 °C, 18 h, for compounds **22**, **24**; (f) Cs_2CO_3, $Pd(PPh_3)_4$, DME/water (1:1), reflux, 4 h, for compound **24b**

incubated with tritiated E1, cofactor, and inhibitor. After HPLC separation of substrate and product, the amount of labeled E2 formed was quantified. The inhibition values of compounds **1**−**24** are presented in Tables 1 and 2.

Compounds **1**−**5** with their keto and two OH functions were designed to interact with three distinct areas of the active site. Among these inhibitors, the *meta*−*meta* substituted **1** showed the best IC_{50} value (22 nM), demonstrating that in this class of compounds, the *meta*-OH substituted phenyl moiety is also very relevant for 17β-HSD1 inhibition as observed with the bis(hydroxyphenyl)heterocycles.[41] Displacement of the hydroxy group on the benzoyl ring led to a decrease in activity (**2** and **3**). The position of the OH group at the phenyl ring has a smaller impact on inhibitory activity: *para*-OH **4** (IC_{50} = 33 nM) was equipotent to *meta*-OH **1**, the potency of *ortho*-OH **5** was only slightly reduced (IC_{50} = 95 nM). The best substitution patterns identified in **1** (*meta*−*meta*) and **4** (*meta*−*para*) were sustained in the design of the further compounds. To examine the influence of additional small substituents in *para*-position of the benzoyl group, compounds **6**−**10** were synthesized. Introduction of a methyl substituent decreased the inhibitory activity in both series strongly (**6** and **8**). An exchange of methyl by fluorine did not affect inhibition (**7** and **9**).

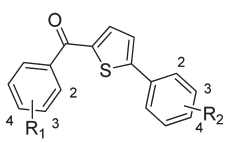
Introduction of methyl in *para*-position of the phenyl ring led to a 10-fold decrease in activity (**11**), whereas *meta*-CH_3, *para*-OH **14** turned out to be a very potent compound (IC_{50} = 8 nM). Fluorinated compounds with different substitution patterns showed a similarly high inhibition of 17β-HSD1 (IC_{50}s: **12**, 30 nM; **13**, 6 nM; **15**, 19 nM). It is remarkable that the substitution with fluorine, in contrast to methyl, does not decrease the high inhibitory potency of the parent compounds **1** and **4**, regardless of their position at the benzoyl or phenyl ring. To provide more insight into the role of different substituents, **14** and **15** were used as starting points for further structural modifications. To combine the characteristics of methyl and fluorine, a CF_3 group (**16**), as well as chlorine (**17**) as its bioisosteric equivalent, was introduced. Both inhibitors confirmed the expected high inhibitory activity with IC_{50} values of 14 and 5 nM, respectively. In contrast to this finding, a slight decrease in activity was caused by the replacement of the CH_3 for a hydroxy (**18**: IC_{50} = 86 nM) or a methoxy (**19**: IC_{50} = 108 nM). Interestingly, the ethyl derivative **20** once again revealed a high biological potency (IC_{50} = 20 nM), thus indicating that an oxygen in this position impinges on enzyme affinity.

Originally chosen as a suitable lipophilic component to fit the mainly apolar substrate binding site, we replaced the 2,5-disubstituted thiophene with 2,4-disubstituted thiophene and thiazole (**21**−**24**) in order to investigate the relevance of the middle ring on inhibitory activity. In the structurally related class of bis(hydroxyphenyl)heterocycles, a strong correlation between the nature of the heterocycle and the affinity toward 17β-HSD1 was already shown.[41] As **14** is one of the most active inhibitors in this series, its substitution pattern was maintained for this study. The biological results of compounds **21**−**24** are shown in Table 2.

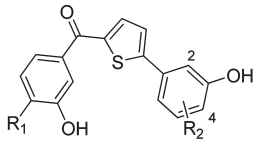
With regard to 2,4-disubstituted **21**, the decrease of activity (IC_{50} = 199 nM) underlines the importance of the sulfur position. Exchanging thiophene with thiazole led to compounds **22**−**24**. Compound **22**, which bears the sulfur of the thiazole in the same position as **14**, the thiophene-sulfur, still demonstrated a very good activity (IC_{50} = 35 nM). Interestingly, the potency of **23** and **24** strongly decreased, thereby confirming that the position of the sulfur plays a pivotal role for 17β-HSD1 inhibition.

Selectivity: Inhibition of Human 17β-HSD2 and Affinities to ERα and ERβ. Inhibition of 17β-HSD type 2 must be avoided, as it acts as a biological counterpart by catalyzing the reverse reaction: oxidation of E2 to E1. Compared with the type 1 enzyme, inhibition of 17β-HSD2 was determined in a similar assay using placental microsomes. These were incubated with tritiated E2 in the presence of NAD^+ and the inhibitor. The separation and quantification of the labeled product was performed by HPLC using radiodetection. IC_{50} values and the percentage of inhibition, respectively, are shown in Tables 1 and 2. In vitro selectivity toward 17β-HSD2 is also expressed as selectivity factor (SF) describing the ratio of the concentration required to inhibit the activity of the isoenzymes by 50% (IC_{50} 17β-HSD2/IC_{50} 17β-HSD1).

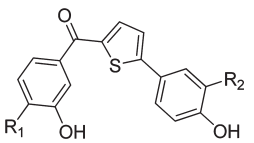
Table 1. Inhibition of Human 17β-HSD1 and 17β-HSD2 by Compounds **1**−**20**



1-5



8-13

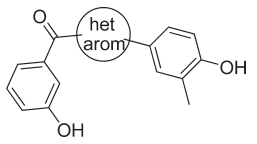


6, 7, 14-20

			IC$_{50}$ [nM][a]		
compd	R_1	R_2	17β-HSD1[b]	17β-HSD2[c]	SF[d]
1	3-OH	3-OH	22	109	5
2	4-OH	3-OH	368	376	1
3	2-OH	3-OH	945	567	0.6
4	3-OH	4-OH	33	478	14
5	3-OH	2-OH	95	18	0.2
1			22	109	5
8	CH_3		64%[e]	31%[e]	
9	F		18	49	3
10	NO_2		594	240	0.4
11		4-CH_3	207	465	2
12		4-F	30	57	2
13		2-F	6	19	3
4			33	478	14
6	CH_3		52%[e]	13%[e]	
7	F		21	69	3
14		CH_3	8	382	48
15		F	19	588	31
16		CF_3	16	95	6
17		Cl	5	246	49
18		OH	86	590	7
19		OCH_3	108	793	7
20		CH_2CH_3	20	786	39

[a] Mean value of three determinations, standard deviation less than 23%. [b] Human placenta, cytosolic fraction, substrate E1, 500 nM, cofactor NADH, 500 μM. [c] Human placenta, microsomal fraction, substrate E2, 500 nM, cofactor NAD^+, 1500 μM. [d] Selectivity factor: IC$_{50}$ (17β-HSD2)/IC$_{50}$ (17β-HSD1). [e] Inhibition at 1 μM (inhibitor concentration).

Table 2. Inhibition of Human 17β-HSD1 and 17β-HSD2 by Compounds **14**, **21**−**24**



cmpd	structure	IC$_{50}$ [nM][a]		SF[d]
		17β-HSD1[b]	17β-HSD2[c]	
14		8	382	48
21		199	719	4
22		35	485	14
23		32 %[e]	51 %[e]	
24		19 %[e]	57 %[e]	

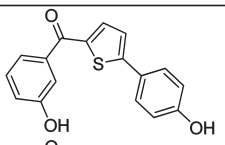
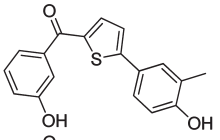
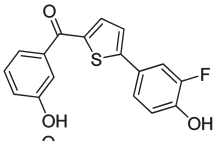
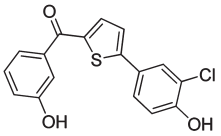
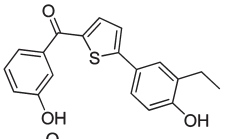
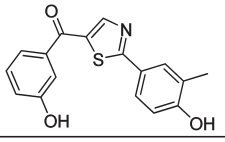
[a] Mean value of three determinations, standard deviation less than 14%. [b] Human placenta, cytosolic fraction, substrate E1, 500 nM, cofactor NADH, 500 μM. [c] Human placenta, microsomal fraction, substrate E2, 500 nM, cofactor NAD^+, 1500 μM. [d] selectivity factor: IC$_{50}$ (17β-HSD2)/IC$_{50}$ (17β-HSD1). [e] Inhibition at 1 μM (inhibitor concentration).

Regarding compounds **1**−**5**, it became apparent that just the modification of the OH-substitution pattern influences the selectivity toward the type 2 enzyme. While compound **4** showed the best SF (14) out of these five, compounds **3** and **5**, which bear one of their OH functions in the *ortho* position, turned out to be potent inhibitors of 17β-HSD2 (SF = 0.6 and SF = 0.2, respectively). Introduction of further substituents on the benzoyl ring did not strongly influence the SF, with the exception of compound **10**, whose nitro group is also responsible for the reverse selectivity in favor of 17β-HSD2 (SF = 0.4). Concerning the different phenyl ring modified compounds, insertion of CH_3 (**14**, SF = 48) and F (**15**, SF = 31) in the *meta* position (*ortho* to OH) increased selectivity compared to the unsubstituted compound **4** (SF = 14). While other positions had less of a high impact (**11**−**13**), this *meta* position seems to be a key position for 17β-HSD1 activity and selectivity toward 17β-HSD2. Chlorine (**17**) and ethyl (**20**) showed comparable high selectivities (SFs = 49 for **17** and 39 for **20**). The fact that chlorine and trifluoromethyl substituents have comparable electronic influences on a phenyl ring implies that other parameters are responsible for the difference in selectivity between 17β-HSD1 and 2.

Regarding the influence of the different middle rings on selectivity toward 17β-HSD2, it is striking that when compared with **14**, the isomeric thiophene **21** showed a strong reduction of selectivity. Only thiazole **22** showed a comparatively high SF of 14. Surprisingly, thiazoles **23** and **24** turned out to be more suitable inhibitors for 17β-HSD2, thereby demonstrating that the nature of the middle ring has a major impact on activity and selectivity.

To keep the risk of unwanted side effects as low as possible, intrinsic estrogenic effects should be avoided. Therefore, it is a

Table 3. Inhibition of 17β-HSD1 in Cell-Free and Cellular Assay by Selected Compounds

cmpd	structure	IC_{50} [nM][a]	
		cell-free assay[b]	cellular assay[c]
4		33	30
14		8	27
15		19	17
17		5	126
20		20	367
22		35	107

[a] Mean values of three determinations, standard deviation less than 23%. [b] Human placental, cytosolic fraction, substrate E1, 500 nM, cofactor NADH, 500 μM. [c] T47D cells, substrate E1, 50 nM.

prerequisite for 17β-HSD1 inhibitors to show no or low affinity for the ERs. Relative binding affinities (RBA) of compounds, which showed a selectivity factor higher than 10, were determined using recombinant human protein in a competitive binding assay applying tritium labeled E2 and hydroxyapatite. Because RBA of E2 is equated with 100%, compounds showing a RBA value below 0.1% are considered to have neglectable receptor affinity. Within this study, none of the evaluated inhibitors crossed this value, neither for ERα nor ERβ.

Further Biological Evaluations. Besides the cell-free assay, T47D, a breast cancer cell line reflecting the conditions in many breast tumors by expressing subtypes 1 and 2 of 17β-HSD, was used to determine the intracellular potency of compounds having a SF higher than 10 (Table 3). While inhibitors **4**, **14**, and **15** confirmed their strong activity in T47D-cells with IC_{50} values in the low nanomolar range (between 17 and 30 nM), compounds **17** and **22** showed inhibitory activities in the 100 nM range. Compared to the cell-free assay, only chloro-(**17**) and ethyl-derivative (**20**) demonstrated a remarkable reduction of inhibitory activity in T47D cells.

Additionally, metabolic stability of compounds **14**, **17**, and **22** was evaluated using human liver microsomes. The corresponding data of the selected compounds and the reference compounds dextromethorphan and verapamil are presented in Table 4. All three inhibitors can be classified in the medium clearance category according to their half-life times around 100 min and thus are considered to have a reasonable metabolic stability. None of the small variations among these three inhibitors caused considerable differences in liability of the compounds to metabolic degradation.

Discussion and Conclusions

The results of molecular modeling studies in the class of heterocyclic substituted biphenylols[46] provided the basis for a successful design strategy using a new three-point-pharmacophore model. The determined biological results place emphasis on the validity of this structure- and ligand-based design approach, resulting in compound **1** as a new lead for further optimization. Interestingly, the position of a hydroxy function at the benzoyl ring has more influence on inhibitory activity than the one at the phenyl ring. This suggests that either the expected hydrogen-bond interactions of the latter with His221 and Glu282 are less important for 17β-HSD1 inhibition or that the protein is flexible enough, at least in this part of the enzyme, to allow equal interaction with each derivative **1**, **4**, and **5**. In contrast to previous studies,[43] an exchange of this OH-phenyl moiety might also lead to highly active 17β-HSD1 inhibitors.

The introduction of further substituents, such as methyl and fluorine in different positions on the benzoyl and phenyl ring, resulted in interesting modulations of biological activities. Compounds **6** and **8**, bearing the methyl substituent in the *para* position of the benzoyl ring, showed a strong decrease of inhibitory activity, once again emphasizing the sharp structure–activity relationships within the rather apolar subpocket. There is plausibly a sterical hindrance caused by the CH_3 group, thus leading to a shifted position of the molecule that is unable to establish suitable hydrogen bond interactions. Taking into account that the geometrical radius of fluorine is smaller than methyl, the fluorine substituted hydroxyphenyl-part of

Table 4. Metabolic Stability of Compounds **14**, **17**, and **22** and Reference Compounds Using Human Liver Microsomes

compd	CL_{int} [μL/min/mg protein]	$t_{1/2}$ [min]
dextrometorphan[a]	10.7	129
verapamil[a]	105	13.2
14[a]	13.8	100
17[b]	12.9	108
22[a]	17.0	81.4

[a] Test concentration = 3 μM. [b] Test concentration = 0.5 μM.

compounds **7** and **9** obviously still fits well into the subpocket, as can be seen by their high inhibitory activities.

Regarding the hydroxyphenyl, the introduction of methyl and fluorine in *ortho*- to the *para*-OH substituent (**14** and **15**) improved inhibitory activity and even selectivity toward 17β-HSD2. It is striking that derivatives with more polar substituents (OH in compound **18** and OCH_3 in compound **19**) in this position could not exert such a high inhibitory activity, and especially their selectivity factor decreased. The fact that the Cl and CH_2CH_3 compounds **17** and **20** have similar inhibitory properties comparable to those of **14** and **15**, led to the assumption that the amino acids surrounding this region of the 17β-HSD1 binding site are predominantly lipophilic. Only compound **16**, which bears a CF_3 group, demonstrated a low selectivity factor because of its strong inhibition of 17β-HSD2. The ethyl derivative **20**, which has a marginally lower SF than **14**, revealed a slightly reduced 17β-HSD2 inhibition, indicating that an enlargement of this lipophilic substituent might lead to lower inhibition of the type 2 enzyme.

Concerning selectivity toward ERα and ERβ, each inhibitor out of this new compound class showed neglectable affinity to the ERs, which confirms our strategy of developing nonsteroidal inhibitors. Despite the fact that the keto function and one hydroxy group of the bicyclic substituted hydroxyphenylmethanones have to be considered as mimicks of the oxygen functions of E1, obviously, the absence of the sterane core and the additional phenolic ring are the main responsible factors that we succeeded in obtaining compounds without ER affinity.

While the presence of the keto linker is the only structural difference between this new class of 17β-HSD1 inhibitors and the previously described bis(hydroxyphenyl)heterocycles,[40−43] several structure−activity relationships are in accordance. For example, the influence of the (hetero)aromatic middle ring is comparable. The exchange of the 2,5-disubstituted thiophene with 2,4-disubstituted thiophene **21** or different thiazoles **22**−**24** revealed that the position of the sulfur plays a critical role for enzyme inhibition. However, only compounds in which the sulfur is located between the two substituents show inhibition values in the low nanomolar range. Because it was shown that 2,4-thiophenes are also able to exhibit strong 17β-HSD1 inhibition,[41] the molecular electrostatic potential of the inhibitors can be discussed as a possible reason for the differences in inhibitory activity.[41] With regards to thiazoles, the corresponding compounds in the class of bis(hydroxyphenyl)-heterocycles also revealed similar results. In both studies, there is a rather radical loss of inhibitory activity for all thiazole derivatives with nitrogen located between the two substituents, thus pointing out that this substitution pattern of thiazoles is detrimental for 17β-HSD1 inhibition. Interestingly, while compound **22** resembles the best inhibitor **14** in activity and selectivity, compounds **23** and **24** inhibited significantly stronger 17β-HSD2 than the type 1 enzyme. As there is neither an X-ray structure nor an applicable homology model of 17β-HSD2 available, these results cannot be interpreted on a sound basis.

The most important advantage of this compound class is its high cellular inhibitory activity. While most of the described intracellularly active 17β-HSD1 inhibitors were tested using low substrate concentrations (2−30 nM),[33,50] the new compound class revealed IC_{50} values in the low nanomolar range in spite of using a much higher substrate concentration of 50 nM. Evaluated in our assay, the most potent inhibitors of the bis(hydroxyphenyl)heterocycles showed cellular inhibitory activities around 300 nM (IC_{50}).[43] Therefore, the keto function seems to have a positive influence on cell permeation, protein binding, and/or intracellular metabolism. Only compounds **17** and **20** showed intracellularly a reduction of inhibitory activity compared to the cell-free assay. Increased lipophilicity might be responsible for this finding.

Concerning the metabolic stability study, the tested inhibitors **14**, **17**, and **22** revealed a medium intrinsic clearance confirming a reasonable metabolic profile. The fact that all three inhibitors showed comparable profiles indicates that neither the exchange thiophene-thiazole nor a variation of the additional substituent next to the hydroxy function can give rise to some significant metabolic liability.

In this paper, we described the structure-based design approach, synthesis, and biological evaluations of bicyclic substituted hydroxyphenylmethanones as new highly potent and selective inhibitors of 17β-HSD1. The applied design concept is based on the newly built pharmacophore model, which resulted in compound **1** as the starting point of the study. The position of the hydroxy functions, the influence of further small substituents and an alternative central ring were investigated. Structural optimization of this lead led to the development of **14**, **15**, **17**, and **22**, all of which show inhibitory activities in the very low nanomolar range in cell-free and cellular assays, remarkable selectivity toward 17β-HSD2, no affinity on both ERs, and very good metabolic stability (proven in case of **14**, **17**, and **22**) tested on human liver microsomes. Hence, they represent promising 17β-HSD1 inhibitors, which fulfill all prerequisites for consideration as clinical candidates. Nevertheless, further optimizations appear possible, especially with regards to the phenyl ring and will therefore be investigated in a future study.

Experimental Section

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

Column chromatography (CC) was performed on silica gel (70−200 μm), 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).

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

^{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 = 5.84 ppm (^{1}H NMR) and δ = 49.3 ppm (^{13}C NMR); CD_3COCD_3, δ = 2.05 ppm (^{1}H NMR) and δ = 30.8 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, td, and q for singlet, doublet, triplet, doublet of doublets, doublet

of doublets of doublets, multiplet, doublet of triplets, triplet of doublets, and quadruplet, respectively. All coupling constants (*J*) are given in hertz (Hz).

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

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

The following compounds were prepared according to previously described procedures: 4-fluoro-3-methoxybenzoyl chloride,[52] 4-nitro-3-methoxybenzoyl chloride,[52] 3-ethyl-4-methoxybenzeneboronic acid (**20b**),[50] 2-(4-methoxyphenyl)thiophene (**6b**).[53]

General Procedure for Friedel−Crafts Acylation. Method A. A mixture of monosubstituted thiophene derivative (1 equiv), arylcarbonyl chloride (1 equiv), and aluminumtrichloride (1 equiv) in anhydrous dichloromethane was stirred at 0 °C for 0.5 h. The reaction mixture was warmed to room temperature and stirred for 1 h. 1 M HCl was used to quench the reaction. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated to dryness. The product was purified by CC.

General Procedures for Suzuki Coupling. Method B. A mixture of arylbromide (1 equiv), boronic acid derivative (1.2 equiv), cesium carbonate (4 equiv), and tetrakis(triphenylphosphine) palladium (0.01 equiv) in an oxygen free DME/water (1:1) solution was refluxed under nitrogen atmosphere for 2 h. The reaction mixture was cooled to room temperature. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated to dryness. The product was purified by CC (except for **17** (CC followed by preparative HPLC) and **19** (CC followed by preparative TLC)).

Method C. A mixture of arylbromide (1 equiv), boronic acid derivative (1.2 equiv), cesium carbonate (4 equiv), and tetrakis-(triphenylphosphine) palladium (0.01 equiv) was suspended in an oxygen free DME/EtOH/water (1:1:1) solution. The reaction mixture was exposed to microwave irradiation (15 min, 150 W, 150 °C, 15 bar). After reaching room temperature, water was added and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated to dryness. The product was purified by CC.

General Procedures for Ether Cleavage. 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 eq per methoxy function) was added dropwise. The reaction mixture was stirred for 20 h at room temperature under nitrogen atmosphere. Water was added to quench the reaction, and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated to dryness. The product was purified by CC, CC followed by preparative TLC, or CC followed by preparative HPLC, respectively.

Method E. A mixture of methoxybenzene derivative (1 equiv) and pyridinium hydrochloride (37 equiv per methoxy function) was heated to 220 °C for 18 h. After cooling to room temperature, water, 1 M HCl and ethyl acetate were added. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over sodium sulfate, filtered, and concentrated to dryness. The product was purified by CC followed by preparative TLC or preparative HPLC, respectively.

General Procedure for Grignard Reaction. Method F. To a solution of carbaldehyde derivative (1 equiv) in anhydrous THF, 3-methoxyphenylmagnesium bromide (1.0 M in THF/toluene, 2.2 equiv) was added dropwise. The reaction mixture was stirred for 3 h at 80 °C under nitrogen atmosphere. Brine was added to quench the reaction, and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated to dryness. The product was purified by CC.

General Procedure for Oxidation. Method G. A mixture of aliphatic alcohol derivative (1 equiv) and 2-iodoxybenzoic acid (2 equiv) in anhydrous THF was stirred at 0 °C. After 10 min, the reaction mixture was stirred and heated to 60 °C for 18 h. After cooling to room temperature, saturated sodium thiosulfate solution was added to quench the reaction and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed succesively with 1 M NaOH and brine, dried over magnesium sulfate, filtered, and concentrated to dryness. The product was purified by CC.

General Procedure for Purification Using Preparative HPLC. All declared compounds were purified via an Agilent Technologies series 1200−preparative HPLC using a RP C18 Nucleodur 100−5 column (30 mm × 100 mm/50 μM from Macherey Nagel GmbH) as stationary phase with a linear gradient run (solvents: acetonitrile, water) starting from 20% acetonitrile up to 100% in 36 min.

(3-Hydroxyphenyl)[5-(3-hydroxyphenyl)-2-thienyl]methanone (1). The title compound was prepared by reaction of (3-methoxyphenyl)[5-(3-methoxyphenyl)-2-thienyl]methanone (**1a**) (138 mg, 0.43 mmol) and boron tribromide (2.58 mmol) according to method D. The product was purified by CC (hexane/ethyl acetate 8:2) followed by preparative HPLC; yield: 75% (95 mg). ^{1}H NMR (CD_3OD): 7.64 (d, J = 4.1 Hz, 1H), 7.42 (d, J = 4.1 Hz, 1H), 7.33 (t, J = 7.9 Hz, 1H), 7.27 (dt, J = 1.3 Hz and J = 7.6 Hz, 1H), 7.24−7.17 (m, 3H), 7.12 (t, J = 2.1 Hz, 1H), 7.03 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 7.9 Hz, 1H), 6.80 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 7.9 Hz, 1H). ^{13}C NMR (CD_3OD): 189.90, 159.40, 159.00, 155.05, 140.60, 139.10, 137.90, 131.45, 130.80, 125.40, 121.30, 120.70, 118.60, 117.50, 116.55, 114.00.

(3-Hydroxyphenyl)[5-(4-hydroxyphenyl)-2-thienyl]methanone (4). The title compound was prepared by reaction of (3-methoxyphenyl)[5-(4-methoxyphenyl)-2-thienyl]methanone (**4a**) (178 mg, 0.55 mmol) and boron tribromide (3.30 mmol) according to method D. The product was purified by CC (hexane/ethyl acetate 8:2) followed by preparative TLC (hexane/ethyl acetate 1:1); yield: 51% (83 mg). ^{1}H NMR (CD_3COCD_3): 8.79 (s, 1H), 8.72 (s, 1H), 7.67−7.64 (m, 3H), 7.42 (d, J = 4.1 Hz, 1H), 7.37 (t, J = 7.9 Hz, 1H), 7.35 (td, J = 1.3 Hz and J = 7.6 Hz, 1H), 7.33−7.32 (m, 1H), 7.11 (ddd, J = 0.9 Hz and J = 2.5 Hz and J = 7.9 Hz, 1H), 6.95 (d, J = 8.8 Hz, 1H). ^{13}C NMR (CD_3COCD_3): 188.60, 160.60, 159.35, 155.20, 142.80, 141.50, 138.05, 131.50, 129.60, 126.80, 124.70, 122.00, 121.05, 117.95, 117.25.

[5-(4-Hydroxy-3-methylphenyl)-2-thienyl](3-hydroxyphenyl)-methanone (14). The title compound was prepared by reaction of [5-(4-methoxy-3-methylphenyl)-2-thienyl](3-methoxyphenyl)-methanone (**14a**) (180 mg, 0.53 mmol) and boron tribromide (3.18 mmol) according to method D. The product was purified by CC (hexane/ethyl acetate 7:3); yield: 90% (148 mg). ^{1}H NMR (CD_3COCD_3): 8.68 (s, 1H), 8.66 (s, 1H), 7.65 (d, J = 4.1 Hz, 1H), 7.57−7.56 (m, 1H), 7.47 (dd, J = 2.2 Hz and J = 8.2 Hz, 1H), 7.41 (d, J = 4.1 Hz, 1H), 7.39 (t, J = 7.8 Hz, 1H), 7.35 (dt, J = 1.3 Hz and J = 7.6 Hz, 1H), 7.32−7.31 (m, 1H), 7.11 (ddd, J = 1.3 Hz and J = 2.5 Hz and J = 7.9 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 2.27 (s, 3H). ^{13}C NMR (CD_3COCD_3): 188.55, 159.35, 158.65, 155.50, 142.65, 141.60, 138.05, 131.50, 130.70, 127.25, 126.90, 126.75, 124.55, 122.00, 121.00, 117.25, 117.20, 17.10.

[5-(3-Fluoro-4-hydroxyphenyl)-2-thienyl](3-hydroxyphenyl)-methanone (15). The title compound was prepared by reaction of [5-(3-fluoro-4-methoxyphenyl)-2-thienyl](3-methoxyphenyl)-methanone (**15a**) (200 mg, 0.58 mmol) and boron tribromide (3.48 mmol) according to method D. The product was purified by CC (hexane/ethyl acetate 7:3) followed by preparative TLC (hexane/ethyl acetate 6:4); yield: 90% (164 mg). ^{1}H NMR (CD_3COCD_3): 9.09 (s, 1H), 8.74 (s, 1H), 7.66 (d, J = 4.1 Hz, 1H), 7.56 (dd, J = 2.2 Hz and J = 12.3 Hz, 1H), 7.47−7.44 (m, 2H), 7.39 (t, J = 7.8 Hz, 1H), 7.35 (dt, J = 1.5 Hz and J = 7.6 Hz, 1H), 7.34−7.32

(m, 1H), 7.13 (ddd, J = 1.3 Hz and J = 2.5 Hz and J = 7.8 Hz, 1H), 7.09 (t, J = 8.8 Hz, 1H). ^{13}C NMR (CD_3COCD_3): 188.65, 159.35, 154.45, 153.45, 147.80, 143.50, 141.30, 137.95, 131.55, 127.50, 125.65, 124.70, 122.05, 121.15, 120.35, 117.25, 115.60.

[5-(3-Chloro-4-hydroxyphenyl)-2-thienyl](3-hydroxyphenyl)-methanone (17). The title compound was prepared by reaction of (5-bromo-2-thienyl)(3-hydroxyphenyl)methanone (**17a**) (150 mg, 0.53 mmol), 3-chloro-4-hydroxybenzene boronic acid (110 mg, 0.64 mmol), cesium carbonate (691 mg, 2.12 mmol), and tetrakis-(triphenylphosphine) palladium (6 mg, 5 μmol) according to method B. The product was purified by CC (hexane/ethyl acetate 8:2) followed by preparative HPLC; yield: 42% (73 mg). ^{1}H NMR (CD_3COCD_3): 7.79 (d, J = 2.2 Hz, 1H), 7.68 (d, J = 4.1 Hz, 1H), 7.60 (dd, J = 2.2 Hz and J = 8.5 Hz, 1H), 7.51 (d, J = 4.1 Hz, 1H), 7.39 (td, J = 0.6 Hz and J = 7.8 Hz, 1H), 7.35 (dt, J = 1.5 Hz and J = 7.8 Hz, 1H), 7.33−7.32 (m, 1H), 7.13−7.11 (m, 2H). ^{13}C NMR (CD_3COCD_3): 188.60, 159.40, 156.05, 153.20, 143.55, 141.35, 137.95, 131.55, 129.40, 128.10, 128.00, 125.65, 123.10, 122.00, 121.15, 119.30, 117.25.

[5-(3-Ethyl-4-hydroxyphenyl)-2-thienyl](3-hydroxyphenyl)-methanone (20). The title compound was prepared by reaction of [5-(3-ethyl-4-methoxyphenyl)-2-thienyl](3-hydroxyphenyl)methanone (**20a**) (116 mg, 0.34 mmol) and pyridinium hydrochloride (1.45 g, 12.58 mmol) according to method E. The product was purified by CC (hexane/ethyl acetate 6:4) followed by preparative TLC (hexane/ethyl acetate 6:4); yield: 47% (52 mg). ^{1}H NMR (CD_3COCD_3): 8.72 (s, 1H), 8.68 (s, 1H), 7.66 (d, J = 4.1 Hz, 1H), 7.57 (d, J = 2.5 Hz, 1H), 7.47 (d, J = 2.5 Hz and J = 8.2 Hz, 1H), 7.42 (d, J = 4.1 Hz, 1H), 7.39 (t, J = 7.9 Hz, 1H), 7.34 (dt, J = 1.5 Hz and J = 7.6 Hz, 1H), 7.32−7.31 (m, 1H), 7.11 (ddd, J = 1.3 Hz and J = 2.5 Hz and J = 7.9 Hz, 1H), 6.93 (d, J = 8.2 Hz, 1H), 2.70 (q, J = 7.6 Hz, 2H), 1.24 (t, J = 7.6 Hz, 3H). ^{13}C NMR (CD_3COCD_3): 187.60, 158.35, 157.30, 154.65, 149.55, 141.65, 140.60, 137.05, 132.35, 130.50, 128.25, 125.90, 123.60, 121.00, 120.00, 116.50, 116.25, 23.90, 14.50.

[2-(4-Hydroxy-3-methylphenyl)-1,3-thiazol-5-yl](3-hydroxyphenyl)methanone (22). The title compound was prepared by reaction of [2-(4-methoxy-3-methylphenyl)-1,3-thiazol-5-yl](3-methoxyphenyl)methanone (**22a**) (65 mg, 0.19 mmol) and pyridinium hydrochloride (1.63 g, 14.06 mmol) according to method E. The product was purified by CC (hexane/ethyl acetate 8:2) followed by preparative TLC (hexane/ethyl acetate 6:4); yield: 46% (27 mg). ^{1}H NMR (CD_3COCD_3): 8.26 (s, 1H), 7.87−7.86 (m, 1H), 7.78 (dd, J = 2.2 Hz and J = 8.5 Hz, 1H), 7.43−7.40 (m, 2H), 7.38−7.37 (m, 1H), 7.16−7.14 (m, 1H), 6.97 (d, J = 8.2 Hz, 1H), 2.29 (s, 3H). ^{13}C NMR (CD_3COCD_3): 188.25, 176.30, 161.20, 159.65, 151.65, 141.25, 131.75, 131.60, 128.20, 127.40, 126.35, 121.90, 121.65, 117.20, 17.15.

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.[38] 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.

1. Inhibition of 17β-HSD1. Inhibitory activities were evaluated by an established method with minor modifications.[38] Briefly, the enzyme preparation was incubated with NADH (500 μM) in the presence of potential inhibitors at 37 °C in a phosphate buffer pH 7.4 (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 unlabeled- 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.

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 unlabeled- 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:

$$\%\text{conversion} = \frac{\%E1}{\%E1 + \%E2} \times 100$$

3. ER Affinity. The binding affinity of selected compounds to the ERα and ERβ was determined according to Zimmermann et al.[54] 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 nonspecific-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, Finland). For determination of the relative binding affinity (RBA), inhibitor and E2 concentrations required to displace 50% of the receptor bound labeled E2 were determined. RBA values were calculated according to the following equation:

$$\text{RBA}[\%] = \frac{IC_{50}(E2)}{IC_{50}(\text{compound})} \times 100$$

The RBA value for E2 was arbitrarily set at 100%.

4. Inhibition of 17β-HSD1 in T47D Cells. A stock culture of T47D cells was grown in RPMI 1640 medium supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 μg/mL), insulin-zinc-salt (10 μg/mL), and sodium pyruvate (1 mM) at 37 °C under 5% CO_2 humidified atmosphere.

The cells were seeded into a 24-well plate at 1×10^6 cells/well in DMEM medium with FCS, and L-glutamine and the antibiotics added in the same concentrations as mentioned above. After 24 h, the medium was changed for fresh serum free DMEM and a solution of test compound in DMSO was added. Final concentration of DMSO was adjusted to 1% in all samples. After a preincubation of 30 min at 37 °C with 5% CO_2, the incubation was started by addition of a mixture of unlabeled- and [2,4,6,7-^{3}H]-E1 (final concentration: 50 nM, 0.15 μCi). After 0.5 h incubation, the enzymatic reaction was stopped by removing of the supernatant medium. The steroids were extracted with diethylether. Further treatment of the samples was carried out as mentioned for the 17β-HSD1 assay.

5. Metabolic Stability. Human liver microsomes (final protein concentration 0.5 mg/mL), 0.1 M phosphate buffer pH 7.4, and test compound (final substrate concentration = 3 μM; final

DMSO concentration = 0.25%) were preincubated at 37 °C prior to the addition of NADPH (final concentration = 1 mM) to initiate the reaction. Dextromethorphan and verapamil were used as references. All incubations were performed singularly for each test compound. Each compound was incubated for 0, 5, 15, 30, and 45 min (control: 45 min). The reactions were stopped by the addition of 50 μL of methanol containing internal standard at the appropriate time points. The incubation plates were centrifuged at 2000 rpm for 20 min at 4 °C to precipitate the protein. The sample supernatants were combined in cassettes of up to four compounds and analyzed using LC-MS/MS.

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Supporting Information Available: Experimental and spectroscopic data of all compounds as well as HPLC purity determination of all tested compounds. This material is available free of charge via the Internet at http://pubs.acs.org

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