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17β-HSD2 inhibitors for the treatment of osteoporosis: Identification of a promising scaffold

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ABSTRACT

17β-Hydroxysteroid dehydrogenase type 2 (17β-HSD2) catalyses the conversion of active 17β-hydroxysteroids into the less active 17-ketosteroids thereby controlling the availability of biologically active estrogens (E2) and androgens (T) in the tissues. The skeletal disease osteoporosis occurs mainly in post-menopausal women and in elderly men when the levels of estrogens and androgens, respectively, decrease. Since 17β-HSD2 is present in osteoblasts, inhibition of this enzyme may provide a new and promising approach to prevent the onset of osteoporosis, keeping a certain level in estrogens and androgens in bone cells of ageing people. Hydroxynaphthyl, hydroxyphenyl and hydroxymethylphenyl-substituted moieties were synthesised as mimetics of the steroidal substrate. Compound **8** has been identified as promising scaffold for 17β-HSD2 inhibitors displaying high activity and good selectivity toward 17β-HSD1, ERα and ERβ.

1. Introduction

Osteoblasts (OB) and osteoclasts (OC), two bone cells, are responsible for bone formation and bone resorption, respectively. In bone physiology of healthy individuals, bone mass is maintained by a well regulated balance between the activity of OBs and OCs.

Osteoporosis[1] is a skeletal disease, characterised by a reduced bone mineral density (BMD), increased bone fragility and predisposition to fractures. The most common osteoporotic fractures occur at the hip, spine and wrist, leading to pain and amplifying mortality. In post-menopausal women and elderly men, there is an acceleration of bone loss, because of higher activity of the OCs compared to the one of OBs. As androgens and estrogens appear to be the most important sex steroids[2] involved in osteoclastic resorption and osteoblastic formation, a decrease in estrogens in post-menopausal women and in both androgens and estrogens in elderly men results in a disproportion between bone loss and bone formation and often leads to osteoporosis.

Antiresorptive agents, including bisphosphonates and selective estrogen receptor modulators (SERM) are often used to treat osteoporosis. Bisphosphonates (like alendronate)[3] are currently the most potent agents for the treatment of osteoporosis. However they lead to reduction of only 50% of fracture risks in post-menopausal women[3,4] and elderly men.[5] The SERM raloxifene,[6] is also efficient to treat osteoporosis but is associated with an increased risk of venous thromboembolism.[7]

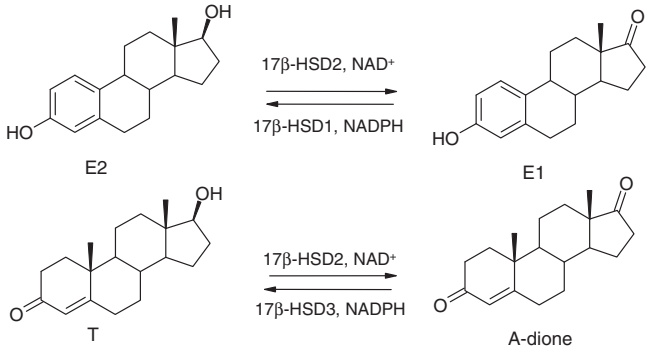

Chart 1. Interconversion of 17β-estradiol (E2) to estrone (E1) by 17β-HSD1 and 17β-HSD2 and of testosterone (T) to androstenedione (A-dione) by 17β-HSD2 and 17β-HSD3.

Abbreviations: 17β-HSD1, 17β-hydroxysteroid dehydrogenase type 1; 17β-HSD2, 17β-hydroxysteroid dehydrogenase type 2; E1, estrone; E2, 17β-estradiol; ER, estrogen receptor; RBA, relative binding affinity; SF, selectivity factor; T, testosterone; OPG, osteoprotegerin; NAD(P)H, nicotinamide adenine dinucleotide phosphate; A-dione, androstenedione; ALP, alkaline phosphatase; OB, osteoblast; OC, osteoclast; BMD, bone mineral density; SERM, selective estrogen receptor modulator.

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As all therapies used nowadays for the treatment of osteoporosis have limitations and none of them offers a complete cure, there is a need to develop other efficient drugs for this disease.

There is evidence that androgens and estrogens play a crucial role in the development of osteoporosis.[8] Although they belong to the antiresorptive agents, estrogens act on the OBs, regulating the ability of the OC precursors to differentiate to OCs (osteoclastogenesis). Estrogens can also enhance OB proliferation[9] as well as osteoprotegerin (OPG) gene expression[10] and alkaline phosphatase (ALP) activity.[10] While OB proliferation and ALP increase[11] are beneficial for bone formation, OPG via osteoclastogenesis inhibition reduces bone resorption. Therefore a drug, which could increase the estrogen levels in the OBs should result in a local increase of OB cell proliferation, OPG and ALP production, and should have beneficial effect on osteoporosis. While a decrease in estrogen and androgen levels can easily be obtained either systematically by the use of aromatase[12] and CYP17[13,14] inhibitors or locally by inhibitors of 17β-HSD1[15–19] or 5α-reductase,[20–22] an intracellular increase of both sex hormones should also be feasible.

High levels of E2 and T should be obtained in bone cells only in order to avoid development of breast and prostate cancer. This goal could be achieved by inhibiting 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2, Chart 1) in the target tissues.

17β-HSD2 catalyses the conversion of active 17β-hydroxysteroid estrogen E2 and androgen T into their less active 17-ketosteroids using the cofactor NAD^+. 17β-HSD2 is a trans-membrane protein[23] and its 3D-structure is unknown. It is mainly expressed in placenta, liver, small intestine, endometrium and osteoblastic cells.[24] As 17β-HSD2 is present in bone cells[24,25] and is responsible for the deactivation of active estrogens and androgens, inhibitors of 17β-HSD2 could maintain a certain level in active sex steroids in bone tissue, and therefore could protect against bone loss and structure deterioration. Therefore 17β-HSD2 is a new attractive target for the treatment of osteoporosis.

Only very few inhibitors of 17β-HSD2 (Chart 2) have been described until now.[26–29] The steroidal C-18 spirolactone (Chart 2) was the first 17β-HSD2 inhibitor identified.[26–28] Within the non-steroidal ones, some flavonoids like 3-hydroxyflavone have been described in the literature as 17β-HSD2 inhibitors.[30] However, regarding 17β-HSD1 they were not very selective. The pyrrolidinone **A** is until now the most potent inhibitor described in the literature. It was evaluated *in vivo* in a monkey osteoporosis model by Bagi et al.[31] In spite of the small effects observed at the highest dose and a strong variability when administrated orally, certainly

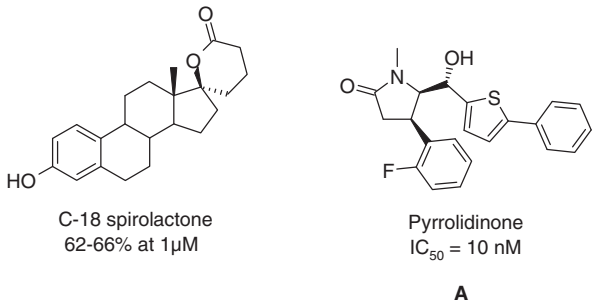
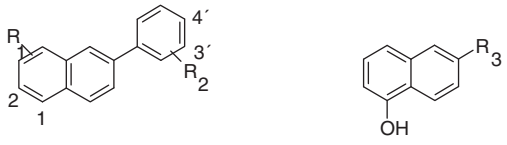


Chart 2. Inhibitors of 17β-HSD2.

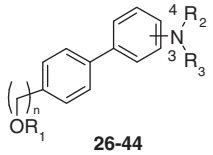
Table 1
Inhibition of human 17β-HSD2 and 17β-HSD1 by compounds **1**–**25**



Compd	R_1	R_2	R_3	Inhibition of 17β-HSD2[a] [%] at 1 μM	Inhibition of 17β-HSD1[b] [%] at 1 μM
B	2-OH	3′-OH	—	20	100
1	2-OH	2′-OH	—	n.i.	n.i.[c]
2	2-OH	4′-OH	—	n.d.	n.i.[c]
3	2-OH	H	—	17	44
4	H	3′-OH	—	12	61[c]
5	1-OH	H	—	20	n.i.
6	1-OH	3′-OH	—	32	23[c]
7	1-OH	4′-OCH_3	—	20	28
8	1-OH	4′-OH	—	74	20
9	1-OH	4′-CH_2OH	—	31	14
10	1-OH	4′-F	—	n.i.	n.i.
11	1-OH	4′-NO_2	—	13	15
12	1-OH	4′-CN	—	12	16
13	1-OH	4′-$COCH_3$	—	n.i.	16
14	1-OH	4′-COOH	—	16	n.i.
15	—	—	4-Pyridyl	26	n.i.
16	—	—	5-Pyrimidyl	n.i.	n.i.
17	—	—	4-OCH_3-3-pyridyl	n.i.	n.i.
18	—	—	3-Furanyl	13	n.i.
19	1-OH	4′-SO_2NHCH_3	—	57	12
20	1-OH	4′-$NHSO_2CH_3$	—	75	52
21	1-OH	3′-$NHSO_2CH_3$	—	22	19
22	1-OH	4′-$CONHCH_3$	—	13	n.i.
23	1-OH	3′-$CONHCH_3$	—	43	n.i.
24	1-OH	3′-$NHCOCH_3$	—	64	12
25	1-OH	3′-NHCOPh	—	n.i.	n.i.

[a] Human placenta, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], NAD^+ [1.5 mM], mean value of three determinations, relative standard deviation <10%.
[b] Human placenta, cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], NADH [0.5 mM], mean value of three determinations, relative standard deviation <10%.
[c] Recombinant human 17β-HSD1, substrate [^{3}H]-E1 + E1 [30 nM], cofactor NADPH [1 mM], mean value of two determinations, relative standard deviation <20%, [15]n.d.: not determined, n.i.: no inhibition (inhibition <10%).

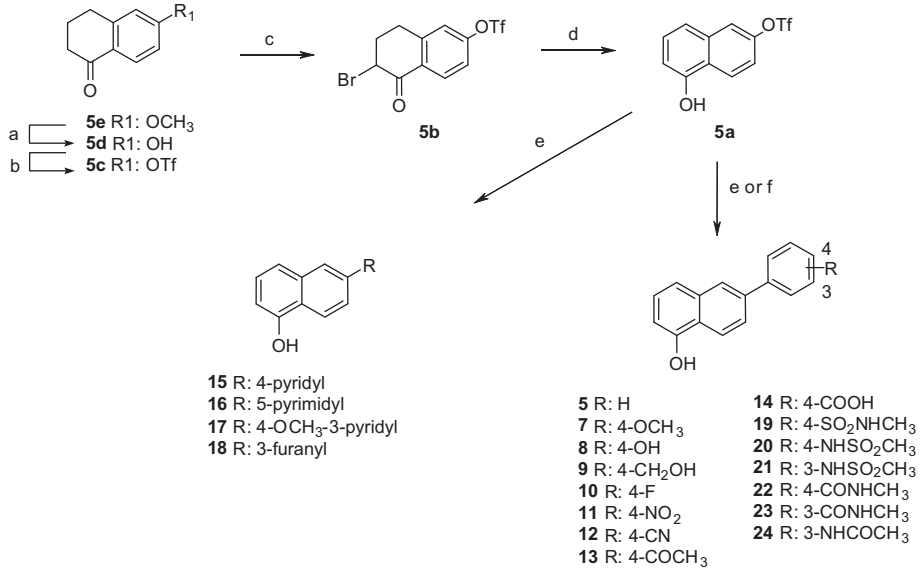
Table 2
Inhibition of human 17β-HSD2 and 17β-HSD1 by compounds **26**–**44**



26-44

Compd	*n*	R_1	Position substitution	R_2	R_3	Inhibition of 17β-HSD2[a] [%] at 1 μM	Inhibition of 17β-HSD1[b] [%] at 1 μM
26	1	H	3	H	$COCH_3$	n.i.	n.i.
27	1	H	3	H	COPh	n.i.	n.i.
28	1	H	3	CH_3	COPh	n.i.	n.i.
29	1	H	3	CH_3	COPh(3′-OMe)	15	n.i.
30	1	H	3	CH_3	COPh(4′-OMe)	14	n.i.
31	1	H	3	CH_3	$COCH_2$-Ph(3′-OMe)	12	n.i.
32	1	H	3	CH_3	$COCH_2$-Ph(4′-OMe)	n.i.	n.i.
33	1	H	4	H	SO_2CH_3	n.i.	n.i.
34	1	H	4	H	SO_2Ph(3′-CH_3)	12	n.i.
35	0	H	3	H	$COCH_3$	n.i.	n.i.
36	0	H	3	H	COPh	n.i.	n.i.
37	0	H	3	CH_3	COPh	46	n.i.
38	0	CH_3	3	CH_3	COPh(3′-OMe)	13	16
39	0	CH_3	3	CH_3	COPh(4′-OMe)	20	n.i.
40	0	CH_3	3	CH_3	$COCH_2$-Ph(3′-OMe)	n.i.	n.i.
41	0	H	3	CH_3	$COCH_2$-Ph(3′-OH)	39	n.i.
42	0	CH_3	3	CH_3	$COCH_2$-Ph(4′-OMe)	10	25
43	0	H	4	H	SO_2CH_3	n.i.	n.i.
44	0	H	4	H	SO_2Ph(3′-CH_3)	14	n.i.

[a] Human placenta, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], NAD^+ [1.5 mM], mean value of three determinations, relative standard deviation <10%.
[b] Human placenta, cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], NADH [0.5 mM], mean value of three determinations, relative standard deviation <10%, n.i.: no inhibition (inhibition <10%).



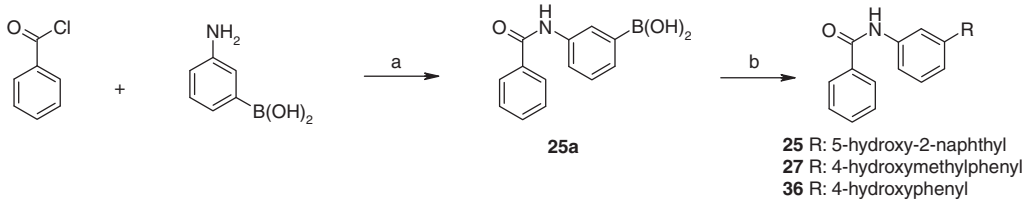
Scheme 1. Synthesis of compounds **5** and **7**–**24**. Reagents and conditions: (a) $AlCl_3$, toluene, reflux, 2 h; (b) $(CF_3CO)_2O$, pyridine, 0 °C, 30 min; (c) Br_2, CCl_4, diethylether, 0 °C, 4 h; (d) LiBr, Li_2CO_3, DMF, reflux, overnight; (e) R-boronic acid, $Pd(PPh_3)_4$, Na_2CO_3, DME/EtOH 1:1, 85 °C, 2 h for **5** and **7**–**18**, Method A; (f) R-boronic acid, $Pd(PPh_3)_4$, Na_2CO_3, DME/water 2:1, microwave irradiation (150 °C, 150 W, 25 min) for **19**–**24**, Method B.

due to an inappropriate pharmacokinetic profile, this study validates 17β-HSD2 as potential target for the treatment of osteoporosis. Therefore there is a need for new potent inhibitors with good pharmacokinetic properties for further in vivo experiments.

In this report, the design of novel and selective inhibitors derived from substituted 1-hydroxynaphthyl, 4-hydroxymethylphenyl and 4-hydroxyphenyl as well as their synthesis and biological evaluation regarding inhibition of 17β-HSD2 and 17β-HSD1 will be presented (Tables 1 and 2).

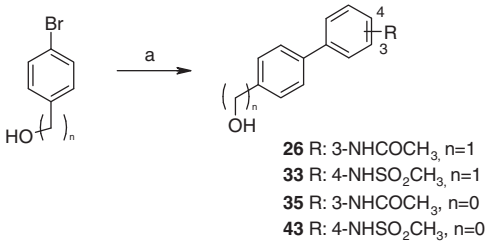
2. Design of inhibitors

As flavonoids are known to interact with several pharmacological targets, we refrained from using these structures as a starting point. A similar structure, 6-(3-hydroxyphenyl)-2-naphthol (compound **B**) was recently identified by Frotscher et al.[15] as a highly potent 17β-HSD1 inhibitor. In spite of the low sequence identity[32] and homology[32] (23% and 45%, respectively), the active site of 17β-HSD1 and 17β-HSD2 is considered to be very similar as the



Scheme 2. Synthesis of compounds **25**, **27** and **36**. Reagents and conditions: (a) Et_3N, CH_2Cl_2, room temperature, 1 h, Method C; (b) R-Br or R-OTf, $Pd(PPh_3)_4$, Na_2CO_3, DME/EtOH 1:1, 85 °C, 2 h, Method A.

substrate of one enzyme is the product of the catalytic reaction of the other enzyme (Chart 1). Taking advantage of our experience developing 17β-HSD1 inhibitors, the hydroxyphenylnaphthol scaffold of compound **B** was considered as promising starting point for the identification of new inhibitors of 17β-HSD2. To improve the activity of compound **B** in favour of 17β-HSD2 inhibition, the optimal position of the hydroxy groups was investigated first. In the second step, one hydroxy group was replaced by different substituents using the best scaffold. Subsequently, the naphthalene core was exchanged either by hydroxyphenyl or by hydroxymethylphenyl moiety.



Scheme 3. Synthesis of compounds **26**, **33**, **35** and **43**. Reagents and conditions: R-boronic acid, $Pd(PPh_3)_4$, Na_2CO_3, DME/water 2:1, microwave irradiation (150 °C, 150 W, 15 bar), 25 min, Method B.

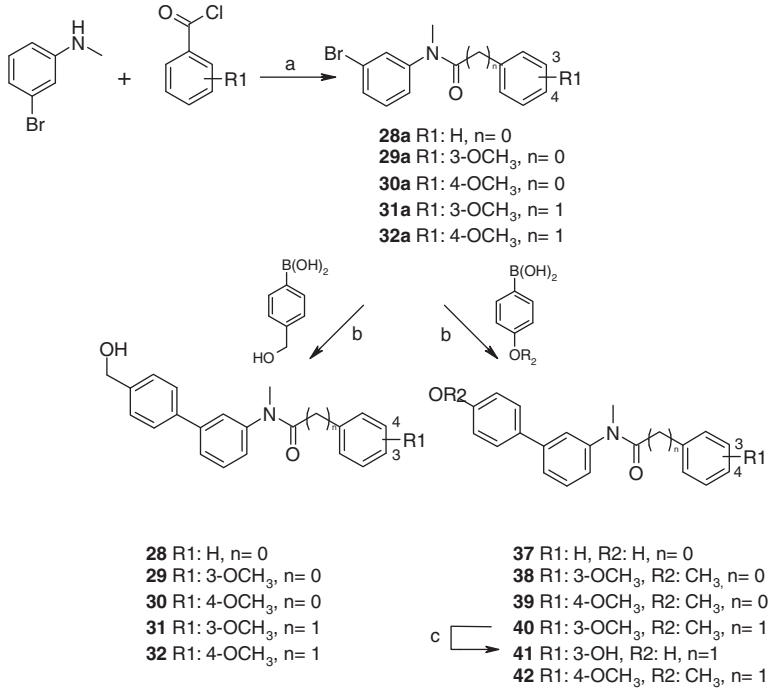
3. Chemistry

Compounds **1**–**4** and **6** were synthesised following the procedure described by Frotscher et al.[15] The synthesis of **5** started from the commercially available 6-methoxy-1-tetralone **5e**, which is converted to the hydroxy analogue **5d** by an ether cleavage using aluminium trichloride[33] and subsequently triflated[34] to **5c**. Bromination,[35] followed by dehydrobromination[35] gave the desired 1-naphthol **5a** in very good yield. Different substituted phenyl (**5**, **7**–**14**, **19**–**24**) or heteroaromatic rings (**15**–**18**) were introduced into the 6-position of the naphthalene core via Suzuki coupling[36] reaction (Scheme 1, Method A or B).

Synthesis of **25**, **27** and **36** occurred in a two steps reaction: amidation (Method C) between benzoyl chloride and 3-aminophenylboronic acid[37] afforded the intermediate **25a** and a subsequent Suzuki coupling (Scheme 2) with the appropriate boronic acid led to the target compounds.

Starting from 4-hydroxymethylbromophenyl or 4-bromophenol, compounds **26**, **33**, **35** and **43** were obtained via a cross-coupling reaction under microwave assisted conditions (Scheme 3, Method B).

The synthesis of compounds **28**–**32** and **37**–**42** started from the 3-bromo-*N*-methylaniline which was coupled to different benzoyl chlorides following method C (Scheme 4) to form the amides **28a**–**32a**. Subsequent Suzuki cross couplings with the appropriate



Scheme 4. Synthesis of compounds **28**–**32** and **37**–**42**. Reagents and conditions: (a) Et_3N, CH_2Cl_2, room temperature, 1 h, Method C; (b) $Pd(PPh_3)_4$, Na_2CO_3, DME/water 2:1, microwave irradiation (150 °C, 150 W, 15 bar, 25 min), Method B; (c) BBr_3, CH_2Cl_2, −78 °C to room temperature, overnight.

boronic acids afforded **28**–**32**, **37**–**40** and **42**. Compound **40** was further demethylated[38] with boron tribromide to give compound **41** in high yield.

The synthesis[37] of the sulfonamide derivative **34a** was carried out by reaction of 4-bromoaniline with 3-methylphenylsulfonylchloride (Scheme 5). Subsequent Suzuki coupling with the corresponding boronic acids afforded sulfonamides **34** and **44**.

4. Biological results

4.1. Inhibition of human 17β-HSD2 and selectivity toward 17β-HSD1, ERα and β

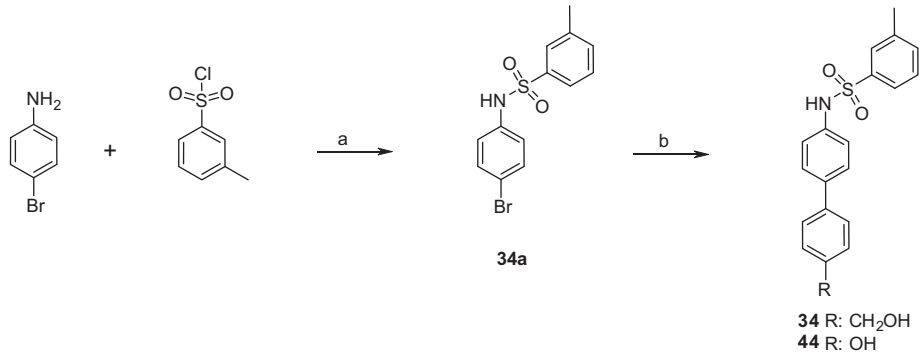
17β-HSD2 and 17β-HSD1 inhibitory activities of the synthesised compounds were evaluated in cell-free assays. As 17β-HSD1 catalyses the reduction of E1 to E2, it should not be affected by 17β-HSD2 inhibitors. Moreover, inhibitors of 17β-HSD2 should have no affinity for the estrogen receptors ERα and β, as it is expected that E2 effects are ER mediated.

Human placental enzymes were used as source of enzymes for both assays and were obtained according to described methods.[39–41] In the 17β-HSD2 assay, incubations were run with tritiated E2, cofactor and inhibitor.[41] The separation of substrate and product was accomplished by HPLC. The 17β-HSD1 assay was performed similarly using tritiated E1 as substrate. The percent inhibition values of compounds **1**–**44** are shown in Tables 1 and 2, and the IC_{50} values of selected compounds are reported in Table 3. Compounds showing less than 10% inhibition tested at a concentration of 1 μM were considered to be inactive. The 6-(3-hydroxyphenyl)-2-naphthol **B** identified in a previous work[15] was used as internal reference (20% 17β-HSD2 inhibition *vs* 100% 17β-HSD1 inhibition at 1 μM).

To identify the best scaffold for a high 17β-HSD2 inhibition, the questions were examined whether two hydroxy substituents are necessary for inhibition and which positions on the 6-phenylnaphthalene moiety were optimal. Compounds bearing only one hydroxy group, either on the naphthalene (compounds **3** and **5**) or on the phenyl (compound **4**) core turned out to have a weak 17β-HSD2 inhibitory activity (Table 1).

Compounds with two OH groups can be separated in two different groups depending on the position of the hydroxy moiety on the naphthalene ring: 2-naphthols (**B**, **1**–**2**) and 1-naphthols (**6**–**25**). 2-Naphthols are not appropriate as scaffold for 17β-HSD2 inhibitors as all compounds turned out to be inactive.

Concerning the 1-naphthols, compounds **6** and **7** substituted with a *meta*-hydroxy (**6**) or a *para*-methoxy (**7**) group on the



Scheme 5. Synthesis of compounds **34** and **44**. Reagents and conditions: (a) pyridine, room temperature, overnight; (b) R-phenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, DME/water 2:1, microwave irradiation 150 °C, 150 W, 15 bar, 25 min, Method B.

Table 3
IC_{50} values, selectivity factor and binding affinities for the estrogen receptors (ER) α and β for selected compounds

Compd	Cell-free assay			ERα RBA[d] (%)	ERβ RBA[d] (%)
	17β-HSD2 IC_{50}[a] [nM]	17β-HSD1 IC_{50}[b] [nM]	Selectivity factor[c]		
B	5641	116	0.02	0.2	0.8
8	302	2425	8	5	5
20	600	614	1	n.d.	n.d.
24	696	8516	12	n.d.	n.d.

[a] Human placenta, microsomal fraction, substrate [^{3}H]-E2 + E2 [500 nM], cofactor NAD^+ [1.5 mM], mean value of three determinations, relative standard deviation <10%.
[b] Human placenta, cytosolic fraction, substrate [^{3}H]-E1 + E1 [500 nM], cofactor NADH [0.5 mM], mean value of three determinations, relative standard deviation <20%.
[c] IC_{50} (17β-HSD1)/IC_{50} (17β-HSD2).
[d] RBA: relative binding affinity, E2: 100%; n.d.: not determined.

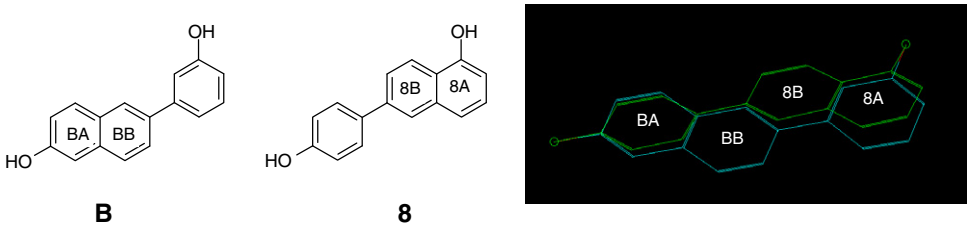


Chart 3. Superimposition of compounds **B** and **8**.

phenyl moiety displayed only very low activity. However, a *para*-hydroxy substituent (**8**) improved activity (74% inhibition at 1 μM) indicating that (1) the *para*-position is more appropriate than the *meta*-position and (2) H-bond interactions are likely to occur with the enzyme.

Looking at these results, it appears that two hydroxy groups are crucial for the activity and the distance between them must be around 11 Å as observed for E2 and **8**. The structure of inactive **B** is very similar to the active **8**: they both consist of a hydroxylated naphthol and a hydroxylated phenyl, and the distance between the two OH groups are identical. These compounds differ only in their hydroxy substitution pattern which seems to play a very important role for 17β-HSD2 or 17β-HSD1 inhibition. In order to better understand these results, **8** and **B** were superimposed using their hydroxy groups as fixed points and energetically minimized with Hyper Chem Pro 6.0. (Chart 3). It can be observed that the central aromatic rings of **B** and **8** occupy a different space in the enzyme. It is likely that the presence of at least one amino acid of the active site of 17β-HSD2 around ring BB is responsible for the selectivity observed for **8** in favour of 17β-HSD2.

The dihydroxylated compound **8** can be considered as a promising core structure. In order to improve its activity and to limit the number of hydroxy functions (often responsible for a fast metabolism as OH groups are substrates for phase II metabolism), either the hydroxy group of the phenyl moiety was replaced by different substituents (**9**–**14**, **19**–**25**) or the 4-hydroxyphenyl was exchanged by different heteroaromatic rings (**15**–**18**).

Introduction of a methylene linker between the phenyl and the hydroxy group (**9**) led to a strong decrease in activity, possibly due to the flexibility of the OH group and a too long distance between the two hydroxy groups.

Exchange of the hydroxy moiety by a withdrawing group like fluoro (**10**), nitro (**11**), cyano (**12**), acetyl (**13**) or carboxylic (**14**) led to inactive compounds, indicating that only electron donating groups are tolerated by the enzyme in this region or that a H-bond donor group is necessary for activity. A series of sulfonamides, reversed sulfonamides and amides in positions 3 or 4 were introduced as replacement of the hydroxy function. Amides were better tolerated in 3-position and sulfonamides in 4-position (64% 17β-HSD2 inhibition at 1 μM for amide **24**; 57% and 75% for sulfonamide **19** and reverse sulfonamide **20**). These results point out the importance of the H-bond donor group directly attached to the phenyl ring: good inhibition data were obtained in case of the amide (in 3-position **24**) or sulfonamide (in 4-position **20**) which are linked to the ring by the NH and therefore able to mimic the OH group of **8**. The inactivity of **25** is probably due to a steric clash between the large phenylamide group and amino acids from the active site.

Various heteroaromatic rings were also introduced in 6-position of the naphthalene (compounds **15**–**18**). None of them turned out to be active, proving that the hydroxy substituent on the phenyl of **8** can not be replaced by a pure H-bond acceptor group, a H-donor in this position is essential for the activity.

In order to simplify the rather lipophilic scaffold of the naphthalene, the 1-naphthol moiety was replaced either by 4-hydroxymethylphenyl or by 4-hydroxyphenyl groups. The exchange of the 1-naphthol for a 4-hydroxymethylphenyl was detrimental for the activity: the amide **26** (to be compared to the 1-naphthol **24** 64% inhibition at 1 μM) and sulfonamide **33** (to be compared to the 1-naphthol **20** 75% inhibition at 1 μM) turned out to be completely inactive (Table 2). In order to gain activity in this class of compounds, different substituted amides (**27**–**32**) and sulfonamide (**34**) were introduced but also turned out to be either weakly active or inactive. Elimination of the methylene linker (compounds **35**–**44**) leading to 4-hydroxyphenyl derivatives was not appropriate to regain activity: the amide **35** (to be compared to the 1-naphthol **24** 64% inhibition at 1 μM) and sulfonamide **43** (to be compared to the 1-naphthol **20** 75% inhibition at 1 μM) were also inactive. These results emphasise the importance of the aromatic ring 8A of the 1-naphthol, which certainly stabilise the molecule in the active site via π–π-interactions. However, introduction of a methyl on the nitrogen of the phenyl amide **37** or of the benzylamide **41** led to a regain of activity (46% and 39% inhibition at 1 μM for **37** and **41**, respectively). All methoxy derivatives (**38**–**40** and **42**) were devoid of activity.

In order to evaluate the selectivity profile of these compounds, all synthesised molecules were tested for their 17β-HSD1 inhibition (Tables 1 and 2). For the most potent inhibitors identified in this report, IC_{50} data for 17β-HSD2 and 17β-HSD1 were determined as well as their binding affinity to ERα and β (Table 3). The most interesting compound identified in this study is the 4-hydroxyphenyl-1-naphthol **8**, with an IC_{50} of 302 nM for 17β-HSD2 inhibition. A certain selectivity toward 17β-HSD1 has been achieved (selectivity factor: 8). Taking into account the structural similarity between E2 and compound **8**, it was important to evaluate the affinity of **8** to the ERs, which is expressed as relative binding affinity.[42,43] The RBA measured represents the ligand affinity to ER, relative to that of E2 which is arbitrarily set up at 100%. With a RBA value of 5% for ERα and β, compound **8**, however, has a similar affinity as E1 which is weakly estrogenic.[44]

5. Discussion and conclusion

The goal of this study was the identification of a new non-steroidal scaffold for 17β-HSD2 inhibition. This was a difficult task, considering the structural similarities between 17β-HSD2 and 17β-HSD1 in their active sites. Taking advantage of our experience developing 17β-HSD1 inhibitors in the class of hydroxyphenylnaphthols,[15,16] compound **8** was identified as potent and selective 17β-HSD2 inhibitor while compound **B** showed a high inhibitory activity for 17β-HSD1 only. Compounds **B** and **8** share several structural similarities, they differ only in their hydroxy substitution pattern: in **B**, one OH is in 2-position of the naphthalene and the other is in *meta* on the phenyl while in **8**, the naphthalene is hydroxylated in 1-position and the phenyl in *para* (Chart 3). Because of the different hydroxy substitution pattern not only activity for 17β-HSD2 could be gained but also selectivity toward 17β-HSD1 could be reached. A minor structural change in the ligand can completely reverse the activity and selectivity profile. This indicates that the small differences between the active sites of these two enzymes are very important and can lead to a radical ligand discrimination. Further studies about selectivity regarding other 17β-HSDs and SDR enzymes will be evaluated in the future. Selectivity toward the ER which is an important issue could be gained by introduction of large substituents into this scaffold.

As neither a 3D-structure nor a good homology model of 17β-HSD2 exists, the information on the active site architecture is limited and identification of amino acids which could be important for interaction with the potential inhibitors is not possible. Therefore in this study, drug design was focused on a ligand-based approach using the substrate E2 and other ligands like **B** (17β-HSD1 inhibitor). In case of 17β-HSD1, several X-ray structures of the enzyme with or without ligand are available.[45] In a previous work we mentioned that the hydroxyphenyl moiety of **B** certainly mimics the steroidal A-ring.[15] The superimposition between the OH moieties of **B** (17β-HSD1 inhibitor) and of **8** (17β-HSD2 inhibitor) (Chart 3) also indicates that the hydroxyphenyl moiety of **B** most likely does not overlap with the one of **8**, thus the OH-phenyl of **8** would mimic the steroidal D-ring, interacting with the catalytic triad. It can be expected that in this area of the enzyme, close to the catalytic triad, there is more free space available than close

to the A-ring for a large substituent. This results is indeed in accordance with the biological data obtained for **20** and **24**, where the hydroxy moiety (**8**, 74% inhibition at 1 μM) is replaced by the larger methylsulfonamide (**20**, 75% inhibition at 1 μM) or acetamide (**24**, 64% inhibition at 1 μM) without loss in activity. This finding will be further validated by substitution of OH-naphthalene or by introduction of substituent close to this hydroxy moiety in **8**.

Attempts to simplify the scaffold of compound **8** failed: one hydroxy group is not enough to stabilise the compound in the active site. The aromatic ring 8A of the naphthalene, mimicking the steroidal A-ring seems to be necessary for inhibitory activity, it might interact with the enzyme via π–π-interactions.

Exchange of the hydroxy group on the phenyl moiety by electron withdrawing groups or exchange of the hydroxyphenyl by heteroaromatic rings led to a complete loss in activity indicating the crucial role of the hydrogen bond donating properties of the OH-phenyl moiety to stabilise the compound in the active site.

The structure–activity relationship which can be deduced from the biological data gives very useful informations to map the unknown enzyme active site of 17β-HSD2.

In this report, a new scaffold for 17β-HSD2 inhibitors was identified: the 4-hydroxyphenyl-1-naphthol **8**, with a good inhibitory activity (IC_{50} = 302 nM), a selectivity factor of 8 toward 17β-HSD1 and only weak estrogen receptor binding affinity. As hydroxynaphthyl and hydroxyphenyl compounds have been associated with the formation of reactive intermediates and DNA-adducts, the safety of compound **8** will be further evaluated and attempts to improve the biological profile (activity, selectivity) will be performed.

6. Experimental section

6.1. Chemical methods

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX-500 instrument in $CDCl_3$, CD_3OD and acetone-d_6. Chemicals shifts are reported in δ values (ppm), the hydrogenated residues of deuterated solvent were used as internal standard ($CDCl_3$: δ = 7.26 ppm in ^{1}H NMR and δ = 77 ppm in ^{13}C NMR, CD_3OD: δ = 3.35 ppm in ^{1}H NMR and δ = 49.3 ppm in ^{13}C NMR, acetone-d_6: δ = 2.05 ppm in ^{1}H NMR and δ = 29.8 ppm and 206.3 ppm in ^{13}C NMR). Signals are described as br, s, d, t, dd, ddd, dt, qt and m for broad, singlet, doublet, triplet, doublet of doublets, doublet of doublet of doublets, doublet of triplets, quintuplet and multiplet, respectively. All coupling constants (J) are given in Hertz.

IR spectra were measured neat on a Bruker Vector 33FT-infrared spectrometer. Melting points were measured on Stuart Scientific apparatue SMP3.

Mass spectra (ESI) were measured on a TSQ Quantum (Thermo Electron Corporation) instrument.

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

Flash chromatography was performed using Silica Gel 40 (35/40–63/70 μM) with hexane/ethyl acetate mixtures as eluents. Reaction progress was monitored by thin-layer chromatography analyses on Alugram SIL G/UV254 (Macherey-Nagel). Visualisation was accomplished with UV light. Purifications with preparative HPLC were carried out on a Agilent 1200 series HPLC system from Agilent Technologies, using a RP C18 Nucleodur 100-5 column (30 × 100 mm/50 μm—from Macherey-Nagel GmbH) as stationary phase with acetonitrile/water as solvent in a gradient from 20:80 to 100:0.

Tested compounds show ⩾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 Supplementary data.

Chemical names follow IUPAC nomenclature. Starting materials were used as obtained from Aldrich, Acros and Combi-blocks without further purification. No attempts were made to optimise yields.

The following compounds were prepared according to previously described procedures: 6-(2-hydroxyphenyl)-2-naphthol **1**,[34] 6-(4-hydroxyphenyl)-2-naphthol **2**,[34] 6-phenyl-2-naphthol **3**,[15] 3-(2-naphthyl)phenol **4**,[46] 6-(3-hydroxyphenyl)-1-naphthol **6**,[34] 6-(4-hydroxyphenyl)-1-naphthol **8**,[34] 5-oxo-5,6,7,8-tetrahydro-2-naphthyl trifluoromethanesulfonate **5c**,[34] 6-hydroxy-1,2,3,4-tetrahydronapthalen-1-one **5d**,[33] 3-benzamidophenylboronic acid **25a**.[47]

6.1.1. General procedures for Suzuki coupling

6.1.1.1. Method A. To a mixture of arylbromide (1 equiv) and tetrakis(triphenylphosphine) palladium (0) (0.02 equiv) in DME was added a 2 M aqueous solution of sodium carbonate (2 equiv). The mixture was purged with N_2 and stirred at room temperature for 5 min. Subsequently a solution of boronic acid (1.2 equiv) in EtOH was added. The mixture was heated to 90 °C for 2 h. The reaction mixture was cooled to room temperature, quenched with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate and concentrated to dryness under reduced pressure. The product was purified by column chromatography.

6.1.1.2. Method B. A mixture of arylbromide (1 equiv), boronic acid (1.2 equiv), sodium carbonate (2 equiv), and tetrakis(triphenylphosphine) palladium (0) (0.02 equiv) was suspended in an oxygen-free DME/water (2:1) solution. The reaction mixture was exposed to microwave irradiation (25 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 sodium sulfate and concentrated to dryness under reduced pressure. The product was purified by column chromatography.

6.1.2. General procedure for amide bound formation

6.1.2.1. Method C. *N*-Methylaniline derivative (1 equiv) was mixed with Et_3N (2 equiv) in dichloromethane. The solution was cooled at 0 °C. The acyl chloride (1.2 equiv) was slowly added and the mixture stirred at room temperature for 1 h. The reaction mixture was extracted with ethyl acetate. The organic layer was washed with water, dried over sodium sulfate and concentrated to dryness under reduced pressure. The product was purified by column chromatography.

6.1.3. Detailed synthesis procedure for the most active compounds

6.1.3.1. 4-(5-Hydroxy-2-naphthyl)-*N*-methylbenzenesulfonamide (19). The title compound was prepared by reaction of 5-hydroxy-2-naphthyl trifluoromethanesulfonate **5a** (100 mg, 0.34 mmol, 1 equiv) with 4-[(methylamino)sulfonyl]phenylboronic acid (88 mg, 0.41 mmol, 1.2 equiv) according to method B. The product was purified by column chromatography (gradient hexane/ethyl acetate 1:0 to 3:1) to afford the analytically pure compound in 90% yield (84 mg) as a yellow powder. $C_{17}H_{15}NO_3S$; MW 313; mp: 204–207 °C; ^{1}H NMR (acetone-d_6): δ 9.10 (s, 1H), 8.36 (d, J = 8.6 Hz, 1H), 8.21 (d, J = 1.8 Hz, 1H), 8.04–8.01 (m, 2H), 7.99–7.95 (m, 2H), 7.85–7.84 (m, 1H), 7.51 (d, J = 8.4 Hz, 1H), 7.39–7.35 (m, 1H), 6.97 (dd, J = 1.0 Hz, J = 7.6 Hz, 1H), 6.36 (qt, J = 5.1 Hz, 1H), 2.64 (d, J = 5.1 Hz, 3H); ^{13}C NMR (acetone-d_6): δ 154.0, 145.7, 139.6, 137.7, 136.1, 133.2, 129.9, 128.6, 127.9, 127.8, 126.9, 125.4, 124.6, 124.1, 120.6, 109.7; IR: 3259, 2971, 1378 cm^{-1}; LC/MS m/z: 314 $(M+H)^+$.

6.1.3.2. *N*-[4-(5-Hydroxy-2-naphthyl)phenyl]methanesulfonamide (20). The title compound was prepared by reaction of

5-hydroxy-2-naphthyl trifluoromethanesulfonate **5a** (100 mg, 0.34 mmol, 1 equiv) with 4-[(methylsulfonyl)amino]phenylboronic acid (88 mg, 0.41 mmol, 1.2 equiv) according to method B. The product was purified by column chromatography (hexane/ethyl acetate 3:1) to give the analytically pure compound in 57% yield (61 mg) as an orange powder. $C_{17}H_{15}NO_3S$; MW 313; mp: 194–195 °C; ^{1}H NMR (acetone-d_6): δ 9.33 (s, 1H), 8.92 (s, 1H), 8.53 (d, J = 8.8 Hz, 1H), 8.30 (d, J = 1.6 Hz, 1H), 8.05–8.01 (m, 2H), 8.00 (dd, J = 1.6 Hz, J = 8.8 Hz, 1H), 7.72–7.69 (m, 2H), 7.67 (d, J = 8.8 Hz, 1H), 7.57–7.55 (m, 1H), 7.16–7.13 (m, 1H), 3.27 (s, 3H); ^{13}C NMR (acetone-d_6): δ 154.1, 138.9, 138.6, 137.8, 136.3, 131.6, 128.9, 127.7, 125.7, 125.0, 124.6, 123.8, 121.5, 120.9, 120.3, 109.1, 39.5; IR: 3384, 2934, 1692, 1519, 1322 cm^{-1}; LC/MS m/z: 314 (M+H)$^+$.

6.1.3.3. 3-(5-Hydroxy-2-naphthyl)-*N*-methylbenzamide (23).

The title compound was prepared by reaction of 5-hydroxy-2-naphthyl trifluoromethanesulfonate **5a** (100 mg, 0.34 mmol, 1 equiv) with 3-(*N*-methylaminocarbonyl)phenylboronic acid (74 mg, 0.41 mmol, 1.2 equiv) according to method B. The analytically pure product was obtained after column chromatography (gradient dichloromethane/methanol 1:0 to 20:1) in 54% yield (51 mg) as a brown oil. $C_{18}H_{15}NO_2$; MW 277; ^{1}H NMR (acetone-d_6): δ 9.14 (s, 1H), 8.34 (d, J = 8.6 Hz, 1H), 8.30 (t, J = 1.9 Hz, 1H), 8.14 (d, J = 1.9 Hz, 1H), 7.95–7.93 (m, 1H), 7.92–7.90 (m, 1H), 7.88 (s, 1H), 7.82 (dd, J = 1.9 Hz, J = 8.6 Hz, 1H), 7.60–7.57 (m, 1H), 7.48 (d, J = 8.2 Hz, 1H), 7.35 (t, J = 7.7 Hz, 1H), 6.95 (dd, J = 0.9 Hz, J = 7.6 Hz, 1H), 2.95 (d, J = 4.7 Hz, 3H); ^{13}C NMR (acetone-d_6): δ 154.2, 141.9, 132.8, 132.7, 130.5, 129.8, 129.5, 129.4, 127.8, 127.0, 126.6, 126.3, 124.7, 123.9, 120.3, 109.4, 26.7; IR: 3166, 1574, 1272 cm^{-1}; LC/MS m/z: 278 (M+H)$^+$.

6.1.3.4. *N*-[3-(5-Hydroxy-2-naphthyl)phenyl]acetamide (24).

The title compound was prepared by reaction of 5-hydroxy-2-naphthyl trifluoromethanesulfonate **5a** (100 mg, 0.34 mmol, 1 equiv) with 3-acetamidophenylboronic acid (74 mg, 0.41 mmol, 1.2 equiv) according to method B. The analytically pure product was obtained after column chromatography (gradient dichloromethane/methanol 1:0 to 20:1) in 42% yield (40 mg) as a yellow powder. $C_{18}H_{15}NO_2$; MW 277; mp: 105–106 °C; ^{1}H NMR (acetone-d_6): δ 9.24 (s, 1H), 9.04 (s, 1H), 8.32 (d, J = 8.6 Hz, 1H), 8.10–8.08 (m, 1H), 8.05 (d, J = 1.6 Hz, 1H), 7.75 (dd, J = 1.8 Hz, J = 8.8 Hz, 1H), 7.69 (d, J = 7.5 Hz, 1H), 7.49–7.45 (m, 2H), 7.42 (t, J = 7.9 Hz, 1H), 7.33 (t, J = 7.9 Hz, 1H), 6.93 (dd, J = 1.0 Hz, J = 7.4 Hz, 1H), 2.13 (s, 3H); ^{13}C NMR (acetone-d_6): δ 154.0, 147.6, 136.3, 130.1, 127.7, 126.0, 124.8, 123.7, 122.8, 120.3, 119.0, 118.7, 109.2, 24.3; IR: 3208, 3082, 2944, 1670 cm^{-1}; LC/MS m/z: 278 (M+H)$^+$.

6.1.3.5. *N*-(4′-Hydroxybiphenyl-3-yl)-*N*-methylbenzamide (37).

The title compound was prepared by reaction of *N*-(3-bromophenyl)-*N*-methylbenzamide **28a** (100 mg, 0.34 mmol, 1 equiv) with 4-hydroxyphenylboronic acid (56 mg, 0.41 mmol, 1.2 equiv) according to method B. The analytically pure product was obtained after column chromatography (hexane/ethyl acetate 6:4) in 19% yield (20 mg) as a beige powder. $C_{20}H_{17}NO_2$; MW 303; mp: 175–176 °C; ^{1}H NMR (acetone-d_6): δ 8.51 (s, 1H), 7.34–7.31 (m, 3H), 7.30–7.29 (m, 2H), 7.28 (s, 1H), 7.26 (t, J = 8.0 Hz, 1H), 7.23–7.20 (m, 1H), 7.19–7.15 (m, 2H), 3.44 (s, 3H); ^{13}C NMR (acetone-d_6): δ 170.7, 158.4, 146.6, 142.7, 137.9, 132.1, 130.2, 130.1, 129.4, 128.8, 128.5, 126.1, 125.6, 124.9, 116.6, 38.2; IR: 3380, 3080, 1696, 1598, 1379, 1257 cm^{-1}; LC/MS m/z: 304 (M+H)$^+$.

6.1.3.6. *N*-(4′-Hydroxybiphenyl-3-yl)-2-(3-hydroxyphenyl)-*N*-methylacetamide (41).

To a solution of *N*-(4′-methoxybiphenyl-3-yl)-2-(3-methoxyphenyl)-*N*-methylacetamide **40** (30 mg, 0.083 mmol, 1 equiv) in anhydrous dichloromethane cooled at −78 °C under nitrogen was slowly added boron tribromide (0.83 mL, 0.83 mmol, 10 equiv). The reaction mixture was stirred at −78 °C for 1 h and allowed to warm up to room temperature overnight. The reaction was quenched by addition of water and extracted with ethyl acetate. The combined organic layers were washed with brine and dried over sodium sulfate evaporated to dryness under reduced pressure and purified by column chromatography (dichloromethane/methanol 20:1) to give the analytically pure compound in 72% yield (20 mg) as a yellow oil. $C_{21}H_{19}NO_3$; MW 333; ^{1}H NMR (acetone-d_6): δ 8.51 (s, 1H), 8.16 (s, 1H), 7.58 (d, J = 7.3 Hz, 1H), 7.49–7.45 (m, 3H), 7.42 (t, J = 1.8 Hz, 1H), 7.19 (ddd, J = 0.9 Hz, J = 2.0 Hz, J = 7.9 Hz, 1H), 7.05–7.00 (m, 1H), 6.94–6.90 (m, 2H), 6.68–6.64 (m, 2H), 6.50 (s, 1H), 3.43 (s, 2H), 3.27 (s, 3H); ^{13}C NMR (acetone-d_6): δ 158.4, 158.2, 150.9, 145.8, 130.8, 129.9, 129.0, 126.3, 121.0, 116.9, 116.6, 114.2, 41.4; LC/MS m/z: 334 (M+H)$^+$.

6.2. Biological assays

[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-HSD2 and 17β-HSD1 were obtained from human placenta according to previously described procedures.[39–41,48] Fresh human placenta was homogenised and centrifuged. The pellet fraction contains the microsomal 17β-HSD2, while 17β-HSD1 was obtained after precipitation with ammonium sulfate from the cytosolic fraction.

6.2.1. Inhibition of 17β-HSD2

Inhibitory activities were evaluated by a well established method with minor modifications.[39] Briefly, the enzyme preparation was incubated with NAD$^+$ [1500 μM] in the presence of potential inhibitors at 37 °C in a phosphate buffer (50 mM) supplemented with 20% of glycerol and EDTA 1 mM. Inhibitor stock solutions were prepared in DMSO. Final concentration of DMSO was adjusted to 1% in all samples. The enzymatic reaction was started by addition of a mixture of unlabelled- and [2,4,6,7-^{3}H]-E2 (final concentration: 500 nM, 0.11 μCi). After 20 min at 37 °C, the incubation was stopped with $HgCl_2$ and the mixture was extracted with ether. After evaporation, the steroids were dissolved in acetonitrile. E1 and E2 were separated using acetonitrile/water (45:55) as mobile phase in a C18 rp chromatography column (Nucleodur C18 Gravity, 3 μm, Macherey-Nagel, Düren) connected to a HPLC-system (Agilent 1100 Series, Agilent Technologies, Waldbronn). Detection and quantification of the steroids were performed using a radioflow detector (Berthold Technologies, Bad Wildbad). The conversion rate was calculated according to following equation: %conversion = (%E1/(%E1 + %E2) × 100). Each value was calculated from at least three independent experiments.

6.2.2. Inhibition of 17β-HSD1

The 17β-HSD1 inhibition assay was performed similarly to the 17β-HSD2 procedure. The cytosolic fraction was incubated with NADH [500 μM], test compound and a mixture of unlabelled- and [2,4,6,7-^{3}H]-E1 (final concentration: 500 nM, 0.15 μCi) for 10 min. Further treatment of the samples and HPLC separation was carried out as mentioned above.

6.2.3. ER affinity

The binding affinity of selected compounds to the ERα and ERβ was determined according to Zimmermann et al.[49] using recombinant human proteins. Briefly, 0.25 pmoles of ERα or ERβ, respectively, were incubated with [^{3}H]-E2 (10 nM) and test compound for 1 h at room temperature. The potential inhibitors were dissolved in DMSO (5% final concentration). Non-specific-binding

was performed with diethylstilbestrol (10 μM). After incubation, ligand–receptor complexes were selectively bound to hydroxyapatite (5 g/60 mL TE-buffer). The formed complex was separated, washed and resuspended in ethanol. For radiodetection, scintillator cocktail (Quickszint 212, Zinsser Analytic, Frankfurt) was added and samples were measured in a liquid scintillation counter (Rack Beta Primo 1209, Wallac, Turku). From these results the percentage of [^{3}H]-E2 displacement by the compounds was calculated. The plot of % displacement versus compound concentration resulted in sigmoidal binding curves. The compound concentration to displace 50% of the receptor bound [^{3}H]-E2 were determined. Unlabelled E2 was used as a reference. For determination of the relative binding affinity[42] the ratio was calculated according to the following equation: $\mathrm{RBA}\% = \frac{\mathrm{IC}_{50}(\mathrm{E2})}{\mathrm{IC}_{50}(\mathrm{compound})} \times 100$.[50] This results in an RBA value of 100% for E2. After the assay was established and validated a modification was made to increase throughput. Compounds were tested at concentrations of $1000 \times \mathrm{IC}_{50}$(E2) and $10{,}000 \times \mathrm{IC}_{50}$(E2). Results were reported as RBA ranges. Compounds with less than 50% displacement of [^{3}H]-E2 at a concentration of $10{,}000 \times \mathrm{IC}_{50}$(E2) were classified as RBA <0.01%, compounds that displace more than 50% at $10{,}000 \times \mathrm{IC}_{50}$(E2) but less than 50% at $1000 \times \mathrm{IC}_{50}$(E2) were classified as 0.01% < RBA < 0.1%.

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Supplementary data

Supplementary data (chemical synthesis and characterisation of all compounds as well as HPLC purity determination) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.12.013.

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