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Cinnamic acid esters as potent inhibitors of fungal 17β-hydroxysteroid dehydrogenase—a model enzyme of the short-chain dehydrogenase/reductase superfamily

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Abstract—We present the synthesis of a new family of nonsteroidal inhibitors of 17β-hydroxysteroid dehydrogenase, designed from flavones and chalcones. Their inhibitory potential was screened on 17β-hydroxysteroid dehydrogenase from the fungus *Cochliobolus lunatus* (17β-HSDcl), a model enzyme of the short-chain dehydrogenase/reductase superfamily. In a series of cinnamates and related coumarin-3-carboxylates, a number of compounds proved to be potent inhibitors of both the oxidative and reductive reactions catalyzed by 17β-HSDcl, with IC_{50} values in the low micromolar range.

1. Introduction

17β-hydroxysteroid dehydrogenases (17β-HSDs) are involved in the last step of the biosynthesis of sex hormones.[1] They convert inactive 17-keto-steroids into their active 17β-hydroxy-forms (such as estradiol, testosterone, and dihydrotestosterone) or vice versa, using NAD(P)H or $NAD(P)^+$ as cofactor, thus acting as molecular switches.[2,3] These enzymes play a key role in hormonal regulation and function in the human and constitute emerging therapeutic targets for the control of estrogeno- and androgeno-sensitive diseases like breast cancer, endometrial cancer, prostate cancer, benign prostatic hyperplasia, acne, hair loss, etc. 17β-HSDs are implicated also in the development of polycystic kidney disease, pseudohermaphroditism, Zellweger syndrome and Alzheimer's disease.[1,4] 11 different mammalian 17β-HSDs have been described that belong to protein superfamilies of short-chain dehydrogenases/reductases (SDR) or aldo–keto reductases (AKR). 17β-HSDs differ in their tissue distribution, catalytic preference, substrate specificity, subcellular localization, and mechanism of regulation.[1,5]

The design and synthesis of inhibitors of steroidogenesis is a fast-growing field in biomedical research. During the last decade numerous potent inhibitors of 17β-HSDs have been reported.[6] A serious drawback preventing the therapeutic use of many steroid biosynthesis inhibitors is the residual estrogenic or androgenic activity due to their steroid nucleus. The development of 17β-HSD inhibitors consisting of a nonsteroid core and devoid of residual steroidogenic activity is thus a very promising strategy for obtaining new drugs. Among nonsteroidal inhibitors of 17β-HSDs, attention has recently been focused on phytoestrogens, especially flavonoids like flavones and chalcones (Fig. 1). Flavonoids are small molecular substances having a remarkable variety of biological activities, including effects on estrogen receptors, sex hormone binding globulin, signaling via tyrosine kinase, and various enzyme systems.[7] They are formed in plants from aromatic amino acids and their biosynthesis proceeds via cinnamic acid (Fig. 1) or related phenolic acids like caffeic acid, ferulic acid, and chlorogenic acid.[8] Many flavonoids and analogous compounds were found to be potent inhibitors of type 1,[9,10] type 3[11,12], and type 5[13] 17β-HSD. Strong inhibitors of 17β-HSDs were also found among chalcones, which are unique in the flavonoid family in lacking a heterocyclic C ring, although their activity appears to be somewhat lower than that of their flavone analogues.[14,15]

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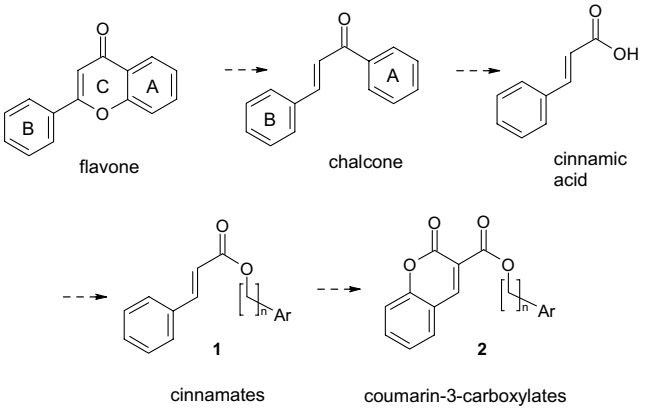


Figure 1. Design of new 17β-HSD inhibitors (Ar = aromatic, $n = 0$–2).

In the search for new compounds with potential to inhibit 17β-HSDs, we became interested in the synthesis and biochemical evaluation of *trans*-cinnamic acid esters and related compounds. We postulated that cinnamic acid, esterified with phenols or alcohols having an aromatic/heteroaromatic ring attached to a hydroxyl group by a short alkyl spacer (compounds of general formula **1**, Fig. 1), could be potential inhibitors of 17β-HSDs, since their structure is similar to the structure of flavones and chalcones. In addition, as a replacement for cinnamic acid, a rigid structurally related analogue—coumarin-3-carboxylic acid—appeared interesting, giving potential inhibitors of general formula **2** (Fig. 1). To check the validity of our hypothesis we synthesized a series of compounds of general formula **1** and **2**, and screened their 17β-HSD inhibitory activities.

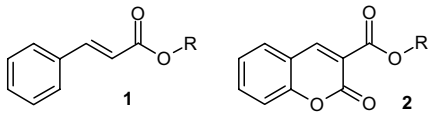
To evaluate their inhibitory potential toward human 17β-HSDs, we chose 17β-HSD from the fungus *Cochliobolus lunatus* (17β-HSDcl).[16] This readily available fungal enzyme is homologous to human 17β-HSD types 4 and 8, members of the SDR superfamily, for which it can, therefore, serve as a model enzyme.[16]

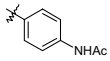
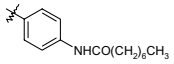
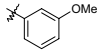
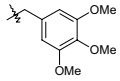
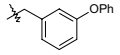
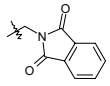
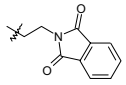
2. Results and discussion

Target compounds of general formula **1** and **2** (Table 1) are synthetically readily available from the corresponding carboxylic acids and alcohols or phenols. To form an ester bond we used standard methods (DCC[17] or BOP[18] mediated esterification).[19]

Compounds were tested for their inhibitory activity against recombinant 17β-HSDcl, which catalyzes the oxidation of 4-estrene-17β-ol-3-one to 4-estrene-3,17-dione in the presence of $NADP^+$, and the reduction of 4-estrene-3,17-dione to 4-estrene-17β-ol-3-one in the presence of coenzyme NADPH.[20] The reaction was followed spectrophotometrically by measuring the difference in NADPH absorbance ($\varepsilon_{\lambda 340} = 6270\ M^{-1}\,cm^{-1}$) in the absence and presence of inhibitor.[21,22] Assays were carried out in 0.6 mL 100 mM phosphate buffer (pH 8.0) containing 1% DMSO as cosolvent. Both the substrate and the coenzyme were 100 μM, the concentrations of inhibitors were from 0.01 to 100 μM and the enzyme was 0.5 μM. Initial velocities were calculated and IC_{50} determined (Table 1).

Table 1. Inhibitors of 17β-HSDcl



Compound	R	IC_{50} (μM)[a]	
		Oxidation	Reduction
1a		26	>200
1b		13	NI
1c		>200	>200
1d		>200	>200
1e		135	NI
1f		10	ND
1g		NI	NI
1h		0.7	7
2a		2.7	8
1i		14	NI
2b		NI	NI
1j		130	NI
1k		10	NI
2c		NI	NI
1l		5	90
2d		NI	NI

[a] NI = no inhibition observed; ND = not determined.

The first series of compounds synthesized and evaluated biochemically were phenyl cinnamates bearing different substituents on the phenol moiety (compounds **1a**–**e**). All were poor inhibitors of the reductive reaction catalyzed by 17β-HSDcl, however, phenyl cinnamate **1a** and 4-cyanophenyl cinnamate **1b** were good inhibitors in the oxidative direction (IC_{50} values 26 and 13 μM, respectively). Introduction of 4-acetylamino-, 4-(octanoylamino)-, and 3-methoxy-groups (compounds **1c**–**e**) significantly reduced inhibitory potency toward the oxidative reaction. This demonstrates that electron donating groups on the phenyl ring decrease the inhibitory activity relative to the parent phenyl cinnamate **1a**. If an additional phenyl ring was annulated to the phenolic moiety of phenyl cinnamate, the 1-naphthyl cinnamate product **1f** remained a good inhibitor of the

oxidative reaction, but did not inhibit the reductive reaction. To evaluate the importance of the aromatic ring in this series of compounds we prepared and tested cyclohexyl cinnamate **1g**, and found it to be devoid of oxidative and reductive inhibitory activity. Thus, for good inhibition of the 17β-HSD oxidative reaction, the alcohol component of cinnamate has to be aromatic.

Very interesting activities were observed among the benzyl cinnamates **1h–j**. The most active inhibitor of both oxidative and reductive reactions was benzyl cinnamate **1h** (oxidative $IC_{50} = 700\,nM$, reductive $IC_{50} = 7\,\mu M$). If the benzyl alcohol was replaced by 3,4,5-trimethoxybenzyl alcohol (compound **1i**), the oxidative inhibitory activity dropped by one order of magnitude ($IC_{50} = 14\,\mu M$) and reductive inhibitory activity was completely lost. Similarly, if 3-phenoxybenzyl alcohol was introduced (compound **1j**), oxidative inhibitory activity dropped by two orders of magnitude ($IC_{50} = 130\,\mu M$) with complete loss of reductive inhibition. Compounds **1k** and **1l**, in which cinnamic acid was esterified with *N*-(hydroxymethyl)phthalimide ($IC_{50} = 10\,\mu M$) and *N*-(2-hydroxyethyl)phthalimide ($IC_{50} = 5\,\mu M$), respectively, were also good inhibitors of the oxidative reaction. However, although these two compounds differ only in the length of the alkyl spacer, only compound **1l** inhibited also the reductive reaction ($IC_{50} = 90\,\mu M$).

In an attempt to improve the inhibitory properties of cinnamates we prepared some of their conformationally constrained analogues. Coumarin-3-carboxylates **2a–d** (Table 1) are in fact rigid cinnamates, in which position 2 of the cinnamic acid phenyl ring is attached to the α-carbon next to the carboxyl group by an oxycarbonyl moiety. Benzyl coumarin-3-carboxylate **2a** was a potent inhibitor of both oxidative and reductive reactions (IC_{50} values 2.7 and 8 μM, respectively). However, to our surprise, none of the remaining coumarin-3-carboxylates inhibited 17β-HSDcl, either in the oxidative or reductive directions, even though their alcohol components were the same as those used in some of the most active cinnamates (**1i**, **1k**, and **1l**).

3. Conclusion

We have designed and synthesized a series of lipophilic cinnamates and coumarin-3-carboxylates and shown them to be the first inhibitors of 17β-HSD from the fungus *Cochliobolus lunatus*—a model enzyme of the SDR superfamily. Some of the new 17β-HSD inhibitors are potential leads for the development of drugs that would block the action of reductive 17β-HSD isoforms and would thus prevent overproduction of active estrogens and androgens that can lead to development of hormone dependent forms of cancer. The other inhibitors that act preferentially in the oxidative direction may also be of interest. In Alzheimer's disease the oxidative 17β-HSD isoenzyme type 10 is highly expressed, resulting in neuronal estrogen deficiency, a potential risk factor. Specific inhibitors of oxidative 17β-HSD type 10 might, therefore, be valuable for treating this disease. Optimization of the reported inhibitors and in vitro determination of their inhibitory activity on human 17β-HSDs are in progress and will be reported in due course.

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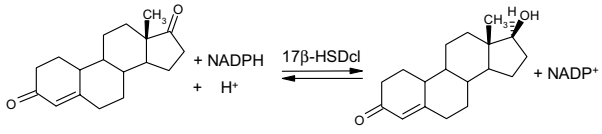
19. All compounds synthesized in this study gave satisfactory spectroscopic analytical results. Analytical data of some

selected compounds; **1i**: mp = 85–86 °C; FAB MS *m/z* 328 $[M+H]^+$, IR (KBr) ν_{max} 2936, 2834, 1710, 1638, 1593, 1508, 1423, 1332, 1245, 1124, 971, 858 cm^{-1}, ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 3.87 (s, 3H, OCH_3), 3.90 (s, 6H, 2×OCH_3), 5.20 (s, 2H, CH_2), 6.51 (d, 1H, $J = 16$ Hz, CH═C*H*–CO), 6.66 (br s, 2H, aromatic), 7.38–7.45 (m, 3H, aromatic), 7.50–7.75 (m, 2H, aromatic), 7.76 (d, 1H, $J = 16$ Hz, C*H*═CH–CO). Anal. Calcd for $C_{19}H_{20}O_5 \times 0.33H_2O$: C 68.25, H 6.23, found: C 68.35, H 6.21. **1j**: mp = 48–51 °C, FAB MS *m/z* 331 $[M+H]^+$, IR (KBr) ν_{max} 3038, 2942, 1713, 1637, 1583, 1484, 1378, 1312, 1250, 1218, 1165, 1074, 981, 803 cm^{-1}, ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 5.53 (s, 2H, CH_2), 6.52 (d, 1H, $J = 16$ Hz, CH═C*H*–CO), 6.96–7.21 (m, 6H, aromatic), 7.31–7.46 (m, 6H, aromatic), 7.50–7.60 (m, 2H, aromatic), 7.76 (d, 1H, $J = 16$ Hz, C*H*═CH–CO). Anal. Calcd for $C_{22}H_{18}O_3$: C 79.98, H 5.49, found: C 80.38, H 5.66. **1k**: mp = 146–147 °C, FAB MS *m/z* 308 $[M+H]^+$, IR (KBr) ν_{max} 2974, 1728, 1633, 1434, 1304, 1136, 976, 856 cm^{-1}, ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 5.89 (s, 2H, CH_2), 6.42 (d, 1H, $J = 16$ Hz, CH═C*H*–CO), 7.35–7.43 (m, 3H, aromatic), 7.50–7.56 (m, 2H, aromatic), 7.74 (d, 1H, $J = 16$ Hz, C*H*═CH–CO), 7.80–7.86 (m, 2H, phthalimido), 7.95–8.05 (m, 2H, phthalimido). Anal. Calcd for $C_{18}H_{13}O_4N$: C 70.36, H 4.23, N 4.56, found: C 70.03, H 4.23, N 4.52. **2b**: mp = 145–146 °C, FAB MS *m/z* 370 $[M+H]^+$, IR (KBr) ν_{max} 2936, 1741, 1709, 1612, 1566, 1509, 1457, 1372, 1307, 1128, 1008, 792 cm^{-1}, ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) 3.86 (s, 3H, OCH_3), 3.96 (s, 6H, 2×OCH_3), 5.36 (s, 2H, CH_2), 6.78 (br s, 2H, aromatic) 7.25–7.32 (m, 2H, aromatic), 7.60–7.66 (m, 2H, aromatic), 8.48 (s, 1H, CH═C). Anal. Calcd for $C_{20}H_{18}O_7$: C 64.86, H 4.86, found: C 64.75, H 4.91.

20. Oxido-reductive reaction monitored in 17β-HSDcl inhibitory assay:



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