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**Estrone C15 derivatives - a new class of 17 β -hydroxysteroid dehydrogenase type 1
inhibitors**

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Abstract

Lowering local estradiol concentration by inhibition of the estradiol-synthesizing enzyme 17 β -hydroxysteroid dehydrogenase type 1 (17 β -HSD1) has been proposed as a promising new therapeutic option to treat estrogen dependent diseases like endometriosis and breast cancer. Based on a molecular modelling approach we designed and synthesized novel C15-substituted estrone derivatives. Subsequent biological evaluation revealed that potent inhibitors of human 17 β -HSD1 can be identified in this compound class. The best, compound **21**, inhibited recombinant human 17 β -HSD1 with an IC₅₀ of 10 nM and had no effect on the activity of recombinant human 17 β -hydroxysteroid dehydrogenase type 2 (17 β -HSD2), the enzyme catalyzing estradiol inactivation. These properties were retained in a cell-based enzyme activity assays. In spite of the estrogen backbone compound **21** did not show estrogen receptor mediated effects in vitro or in vivo. In conclusion, estrone C15 derivative compound **21** can be regarded as a promising lead compound for further development as a 17 β -HSD1 inhibitor.

Keywords: 17 β -hydroxysteroid dehydrogenase, inhibitor, estrogen, estrone

1. Introduction

The oxidoreductase 17 β -hydroxysteroid dehydrogenase type 1 (17 β -HSD1, EC 1.1.1.62) catalyses the interconversion of the less potent estrogen estrone (E1) to the highly active estrogen estradiol (E2). In vivo, like in intact cultured cells, estradiol synthesis is prevailing (Luu-The et al., 1995; Miettinen et al., 1996; Husen et al. 2006a,b) meaning that this enzyme is able to control estradiol actions at the pre-receptor level (Penning 1996). Human 17 β -HSD1 is expressed only in a limited number of tissues, such as placenta, ovarian follicles, mammary gland and uterus (Martel et al., 1992) and is thought to be involved in diseases processes in these tissues due to local increase of estradiol levels. The expression of 17 β -HSD1 was shown to be elevated and to have prognostic significance in hormone-dependent breast cancer (Sasano et al, 1996; Gunnarsson et al., 2005; Gunnarsson et al, 2008), as well as in endometriosis and leiomyoma (Kasai et al., 2004; Tsuchiya et al. 2005; Smuc et al., 2007). Similar to selective estrogen receptor modulators, inhibitors targeting 17 β -HSD1 are emerging as a promising new option to treat estradiol-dependent diseases in a tissue-selective manner avoiding the unwanted side-effects of current therapies.

Several structural classes of reversible and irreversible 17 β -HSD1-inhibitors have been introduced, based on steroidal and non-steroidal core structures (reviewed by Penning, 1996; Poirier, 2003). Nonsteroidal, steroidomimetic pyrimidinones (Messinger et al., 2006), phytoestrogen derivatives and C7, C15, C16 - estradiol as well as C16 estrone derivatives (Pelletier et al., 1996; Allan et al., 2006a, b) have revealed therapeutic potential and have been pursued actively by different groups in recent years (reviewed by Brozic et al. 2008). So far, none of these inhibitors has entered clinical development.

We have focused on the synthesis of estrone derivatives based on the assumption that the natural substrate should have a stronger binding to the target than estradiol. First

investigations of C16 estrone oximethers (G. Schneider, personal communication) showed estrogenicity as an undesired side effect so we focused further on C15 estrone derivatives (Messinger et al., 2005) leading to promising drug candidates like compound **21** (Fig. 1).

2. Methods

2.1 Chemical Synthesis

The screening compounds can be synthesized in 8 to 12 steps. To introduce a substitution in C15 an activation of the position has to be carried out. Most suitable for a general path to the desired compounds is the introduction of a double bond in the D-ring in C15-C16 position to obtain an alpha/beta unsaturated carbonyl function. The first synthesis of this key intermediate (**6**) was already described 1932 by G. F. Marrian et al. (1932). The synthesis could be optimised further to be useful for kg synthesis (personal communication G. Schneider) as well. In the example outlined the benzyl ether was used (Fig. 2). A broad set of other ether (alkyl, benzyl, alkyl-aryl) can be synthesized according to the same principle.

The versatile intermediate **6** was used to introduce the C15 side chain. A 1,4 addition lead directly to a side chain in C15. Interestingly depending on the used reagents and condition the stereochemistry is exclusively alpha or beta. The chemical synthetic routes were already described in 1964 by E.W Cantrall et al. and have been explored later in more detail by the groups of Poirier (1991) and Künzer (Bojack and Künzer 1991). To obtain also longer side chains in C15 position a 1,2 addition is necessary followed by a Cope rearrangement to obtain the C15 alpha allyl derivative (Fig. 3), which can be nicely transformed further via metathesis (Kirschning et al., 2008).

The compounds **7 – 10** were useful for further derivatisation as depicted in schemes 3-5 to obtain the desired functional groups finally as ethers, amides, retroamides, carbamates,

urea, sulfonamides, sulfamates, sulfamides etc. in a library synthetic approach (Fig. 4, 5, 6). Compounds **11** – **20** finally have the desired functionality to be used as starting point for library synthesis.

In addition to the C3 benzyl ether also other ether derivatives have been prepared, with the methyl ether being most suitable for library synthesis even so it was known quite early that the activity on 17 β -HSD1 is significantly lower than the correspondent free phenols which were prepared from the benzyl ether by hydrogenation. With the methods outlined above more than 1000 screening compounds were prepared (see also Messinger et al., 2005).

2.2 Molecular modelling

The available crystal structures of 17 β -HSD1 were superimposed using BODIL molecular modelling environment (Bermann et al., 2000; <http://www.abo.fi/fak/mmf/bkf/research/johnson/bodil/about.php>, Lehtonen et al., 2004). In general the x-ray structure 1A27 was used for comparison and to build the models on because it is co-crystallized with estradiol and NADP⁺ in a good resolution. This was especially helpful because we were focusing on a steroidal motive for lead optimisation.

2.3 Analysis of enzyme inhibition *in vitro*

Recombinant human 17 β -HSD1 and 17HSD2 were produced in Sf9-insect cells according to the method of Lu and coworkers (2002). The assay was performed in a final volume of 0.2 ml buffer (20 mM KH₂PO₄, 1 mM EDTA, pH 7.4) containing 0.1 μ g/ml protein, 1 mM cofactor (NADPH for 17 β -HSD1, NAD for 17HSD2), 30 nM substrate estrone or estradiol, 800 000 cpm/ml of [2,4,6,7-³H]-substrate estrone or estradiol and inhibitor concentrations in the range of 0.1-10 μ M. Duplicate samples were incubated for

25 min at room temperature. After incubation, the reaction was stopped by addition of 20 μ l 10% trichloroacetic acid per sample.

MCF-7 human breast cancer cells stably transfected with either human 17 β -HSD1 or human 17 β -HSD2 (Hirvelä et al., 2005) were used for the analysis of enzyme inhibition in intact cells. The cells were cultured in DMEM/ 10% FCS/ 2mM L-glutamine (Sigma Aldrich). The assay was performed in a final volume of 0.2 ml culture medium containing 2 nM substrate estrone or estradiol, 1.6×10^6 cpm/ml of [2,4,6,7- 3 H]-substrate estrone or estradiol and inhibitor concentrations in the range of 0.1-10 μ M. Triplicate samples were incubated for 1 h at 37 $^{\circ}$ C/ 5% CO₂. Afterwards, the reaction was stopped by addition of 22 μ l 25 % trichloroacetic acid per sample.

After incubation of recombinant enzyme or intact cells overexpressing the enzyme, the substrate [3 H]-E1 and the product of enzymatic conversion, [3 H]-E2, were separated and quantified by HPLC (Alliance 2790, Waters) connected to an online β -counter (Packard Flow Scintillation Analyzer). The ratio of [3 H]-E1 converted to [3 H]-E2 determines the conversion percentage of the samples. Inhibition efficiencies of the tested compounds were calculated by comparing the conversion percentages of the samples including tests compound with those of conversion controls (without compounds). An acetonitrile/water (48/52, v/v) solution is used as the mobile phase (flow rate 1 ml/min) in a Symmetry C₁₈ reverse-phase chromatography column (3.9 x 150mm) with a Symmetry C₁₈ guard column (Waters). Ecoscint A (National Diagnostics) is used as scintillation solution.

2.4 Receptor binding and reporter gene assay

Binding to estrogen receptor α was determined using a commercially available assay kit according to the manufacturer's instructions (PanVera LCC, Madison, WI). Estrogenic

agonism and antagonism was assessed in an estrogen receptor specific (ERE)-luciferase reporter gene assay (Burow et al., 2001).

2.5 Determination of estrogenicity in vivo

Lack of estrogenic activity in vivo was proven using the classical uterine growth test in immature rats (Lauson et al., 1939). Briefly, 18 days old immature, intact female rats were divided into experimental groups (3 animals/group). Inhibitors were administered subcutaneously at daily dose of 10 mg/kg for a period of 3 days. As a positive control 17 β -estradiol was administered in the same way at a dose of 50 μ g/kg s.c. A negative control group received vehicle only. The animals were killed by CO₂- asphyxiation 24 h after the last administration. At autopsy the uterus is carefully prepared free from the surrounding tissues and weighed. Relative weight is calculated according to the formula $100\,000 \times \text{uterus weight} / \text{body weight}$. The study protocol was approved by the local animal care committee.

3. Results and discussion

3.1 Molecular modelling

Estrone and estradiol were used as model compounds for in depth analysis of the substrate binding pocket of 17 β -HSD1 as well as of the receptor binding pocket of the estrogen receptors. The starting point for molecular modelling work was the analysis of the available high resolution X-ray structures (Bermann et al., 2000) of the estrogen receptor and the enzyme 17 β -HSD1. The available crystal structures of 17 β -HSD1 were superimposed (Lehtonen et al., 2004) to find out the more flexible and more static parts of the enzyme (Messinger et al., 2006). Analysis of the 17 β -HSD1 X-ray structure of Breton et al. 1996, co-crystallization of 17 β -HSD1 with estradiol, clearly shows a tight

grip of the enzyme around the steroidal backbone but also an opening towards the environment located in the proximity of the C15 carbon of the steroidal back bone. The hole is formed via the amino acids, e.g. Ser222, Leu219 and Met193 as well as Tyr218, Leu96 and Gly198. The position of the amino acids is highly flexible. A clear discrimination between hydrophilic and lipophilic areas can not be determined correctly. Due to the flexibility of the protein this region can also be entered by substitutions on C16 as shown by G.M. Allan et al. (2006a, b).

Using a steroidal backbone as scaffold for an inhibitor lead optimisation program inherent estrogenicity was always considered as issue to take care about. A comparison of estradiol binding in X-ray structures of 17beta-HSD1 and estrogen receptor α (Fig. 7, 8) soon showed that there is a room for small substitution on C15 estrone position also in estrogen receptor α but polarity in this direction was disliked. However, this was considered to be beneficial for 17beta-HSD1 inhibitors. Furthermore antiestrogenicity was partly shown also by investigation of Poirier et al. (1996) using C15 estradiol derivatives, which in the end convinced us to use C15 estrone instead of estradiol C15 derivatives for further optimisation.

Superimposition of selected crystal structures of 17beta-HSD1 showed that the protein is highly flexible in certain areas. In Fig. 9 the co-crystallized estradiol of 1A27 is used to show the binding pocket. With the help of the ligand the flexibility of the protein in the proximity of C15 of estradiol can be demonstrated. The flexibility of the protein led to an ambiguous picture with regard to chain length and position of the hydrogen bond donors or acceptors of a possible side chain of an estrone derivative. In the end, this led us to a change in optimization strategy from a more rational approach towards a more combinatorial approach which resulted in the synthesis of several hundred compounds.

3.3 Enzyme inhibition *in vitro*

As expected by the computer-aided drug design (CADD) analysis of the X-ray structures the results show for more or less all synthesized compound at least some activity (>30% inhibition) in the recombinant 17 β -HSD 1 assay at 1 μ mol concentration.

It was also no surprise to find high activity within all spacer unit amides as well as urea or sulfonic acid amide derivatives. An analysis of the assay data indicates a cluster of higher activities for chains with 2-4 CH₂ groups. But an optimisation of one chain length could not be transferred to another one or from one isomer to the other even within of one functional spacer unit proving in the end that a combinatorial approach was the right choice finding the best inhibitor.

An overview of some potent inhibitors is given in Table 1 showing rather polar spacer units like sulfonamide derivatives as well as rather unpolar methyl-cyclohexyl amides and short as well as long chain length derivatives. The activity on the recombinant enzyme is retained in the cell based assay indicating good cell membrane penetration of the compounds. Different ways of analysing the screening data did not reveal a full SAR, which of course makes sense with regard to the outcome of the CADD investigations. The protein seems to be so flexible that it can change its conformation depending on the side chains offered; otherwise it is not understandable that compounds like **43** and **31** have quiet similar activity.

On the other hand even very small changes can make a big difference. In case of changing the position of the methyl group on the thiazole ring from 4 to 5 the activity of compound **21** drops 10 fold (compound **27**). Nevertheless some basic SAR rules can be established. (1) Compounds with a chain length of 2-4 CH₂ units are generally preferred, as demonstrated for morpholino side chains in Table 2. (2) Compounds with short chain length show a more pronounced effect between the isomeric forms with α - or β -

conformation than compounds with longer chain length (Table 2). (3) Less polar side chains do have the tendency to reveal some estrogenic activities, for example compound **43** shows minor activity in the estrogen receptor binding and functional assay as well as in a uterine weight test but the more polar side chains did not reveal any kind of estrogenicity (data not shown).

It may be concluded that in spite of the availability of a number of different X-ray structures a combinatorial approach had to be used to find the most active compounds, due to the flexibility of the enzyme. According to our selection criteria, compound **21** was identified as most promising candidate for further in depth pharmacological investigations.

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17 beta HSD1: 1A27, 1BHS, 1DHT, 1EQU, 1FDS, 1FDS, 1FDT, 1FDU, 1FDV, 1FDW, 1I5R, 1IOL, 1QYV, 1QYW, 1QYX, 3DHE,

ER: '1A52', '1ERE', '1ERR', '1G50', '1GWQ', '1GWR', '1HJ1', '1L2J', '1INDE', '1PCG', '1QKM', '1QKN', '1QKT', '1QKU', '1R5K', '1S9P', '1S9Q', '1SJ0', '1U3Q', '1U3R', '1U3S', '1U9E', '1UOM', '1X76', '1X78', '1X7B', '1X7E', '1X7J', '1X7R', '1XP1', '1XP6', '1XP9', '1XPC', '1XQC', '1YIM', '1YIN', '1YY4', '1YYE', '1ZAF', '2AYR', '3ERD', '3ERT'

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Figure captions

Figure 1: Inhibitor compounds reported in table 1

Figure 2: Synthesis of key intermediate **6**: **a** K₂CO₃, benzyl bromide, tetrabutylammonium fluoride, acetone (water free); **b** ethylene glycol, toluene, para toluene sulfonic acid; **c** dimethoxyethane, ethylene glycol, pyridinium perbromide; **d** potassium tert.-butylate, DMSO; **e** dimethoxyethane, para toluene sulfonic acid, overall yield 56%

Figure 3: Principal synthetic routes to C15 estrone derivatives (α or β isomers)

Figure 4: Derivatisation of the C15 side chain, starting from compound **8** (a. formation of the triflate, b. introduction of azide c. hydrogenation d. Jones oxidation)

Figure 5: Derivatisation of the C15 side chain, starting from compounds **9** and **10**

Figure 6: Derivatisation of the C15 side chain, starting from compound **7**

Figure 7: X-ray structure 1A27 (Breton et al., 1996), view towards the active site and C15 (Lehtonen et al., 2004)

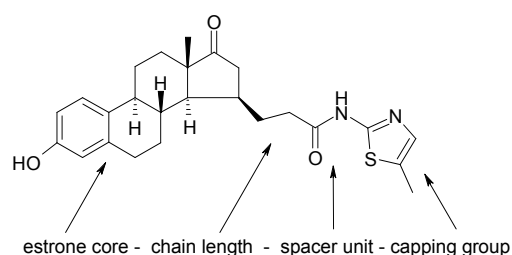
Figure 8: Estradiol in the binding pocket of estrogen receptor alpha (X-ray, 1A52, Tanenbaum et al., 1998), view into the pocket in front of C15

Figure 9: View on C15 in the binding pocket of human 17beta-HSD1, comparison of the different X-ray structures available, 1A27 bold

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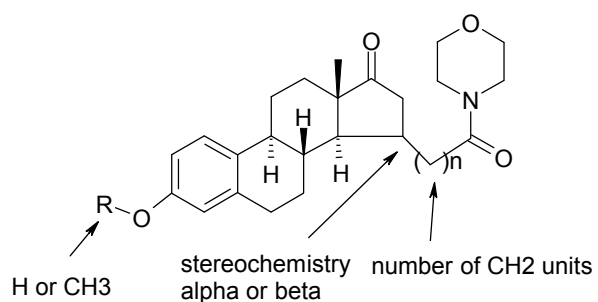
Tables

Table 1: Inhibitory potency of a selection of different C15-estrone derivatives versus 17beta-HSD1 and 17beta-HSD2



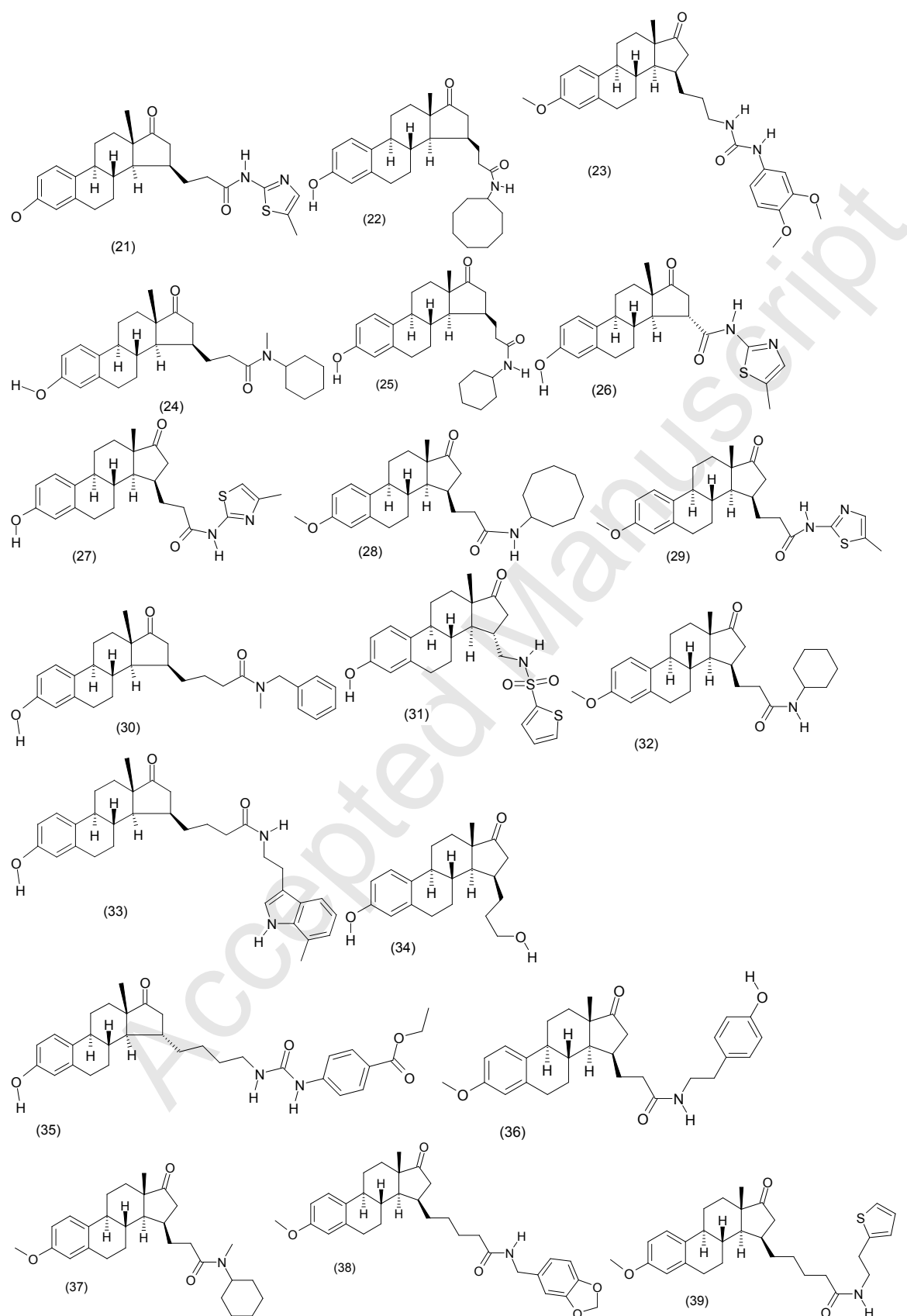
Compound	Recombinant human 17beta-HSD1			Recombinant human 17beta-HSD2		MCF-7-h17beta-HSD1 cells	
	%inhibition at 100nM	%inhibition at 1µM	IC50 [nM]	%inhibition at 100nM	%inhibition at 1µM	%inhibition at 1µM	%inhibition at 10µM
21	87	93	4	0	10	100	nt
22	89	93	14	7	9	100	nt
23	80	87	14	2	4	57	100
24	84	95	18	8	27	94	nt
25	75	87	23	3	6	93	nt
26	77	89	26	4	7	80	nt
27	68	84	39	4	13	70	nt
28	85	95	40	-2	8	72	100
29	79	96	43	-2	3	67	91
30	60	89	44	1	4	57	100
31	68	92	60	4	9	78	100
32	68	92	64	0	1	70	92
33	73	94	68	5	21	70	94
34	76	97	75	2	0	77	100
35	51	77	90	-2	10	24	65
36	58	91	96	-2	2	55	100
37	63	93	98	-2	3	51	94
38	59	90	98	1	7	45	89
39	58	92	109	0	3	50	87
40	51	87	115	7	10	70	91
41	42	82	129	3	4	68	100
42	44	89	130	0	5	57	97
43	49	93	138	2	0	50	97
44	47	87	145	8	13	43	88
45	49	92	198	2	7	50	100

Table 2: Comparison of inhibitory potency of estrone C15 morpholino derivatives of different chain length



Compound	Recombinant 17beta-HSD1			Description of the estrone derivative		
	% inhibition at 100nM	% inhibition at 1μM	IC50 [nM]	Substitution in C3 position	number of CH2 groups	stereochemistry at C15 position
46	1	16		H	2	alpha
47	19	63		H	2	beta
48	25	75		CH3	2	beta
49	32	79	353	H	3	alpha
50	27	79	194	H	3	beta
51	12	40		CH3	3	beta
52	17	68		CH3	4	beta
53	29	80		CH3	5	beta
54	13	61		CH3	7	beta
55	1	41		CH3	8	beta

Figure 1



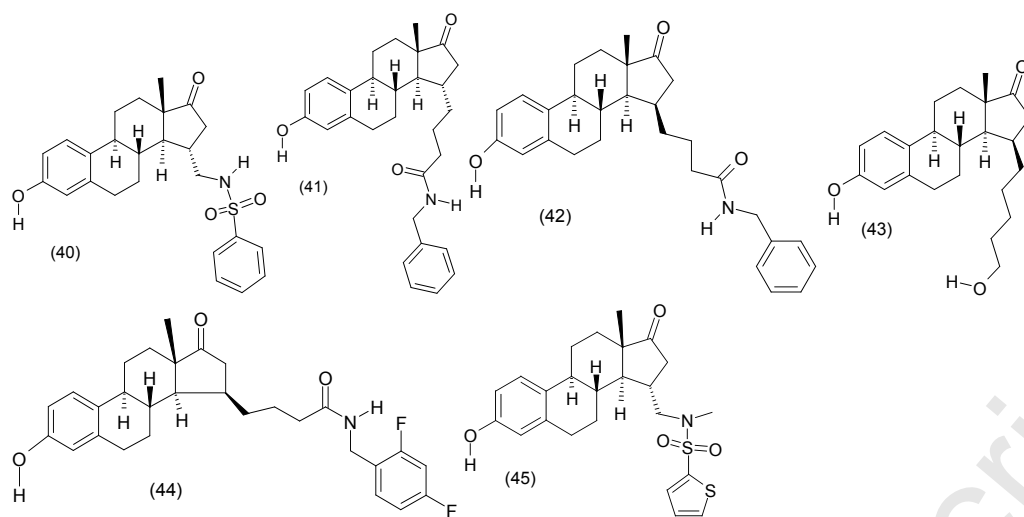


Figure 2

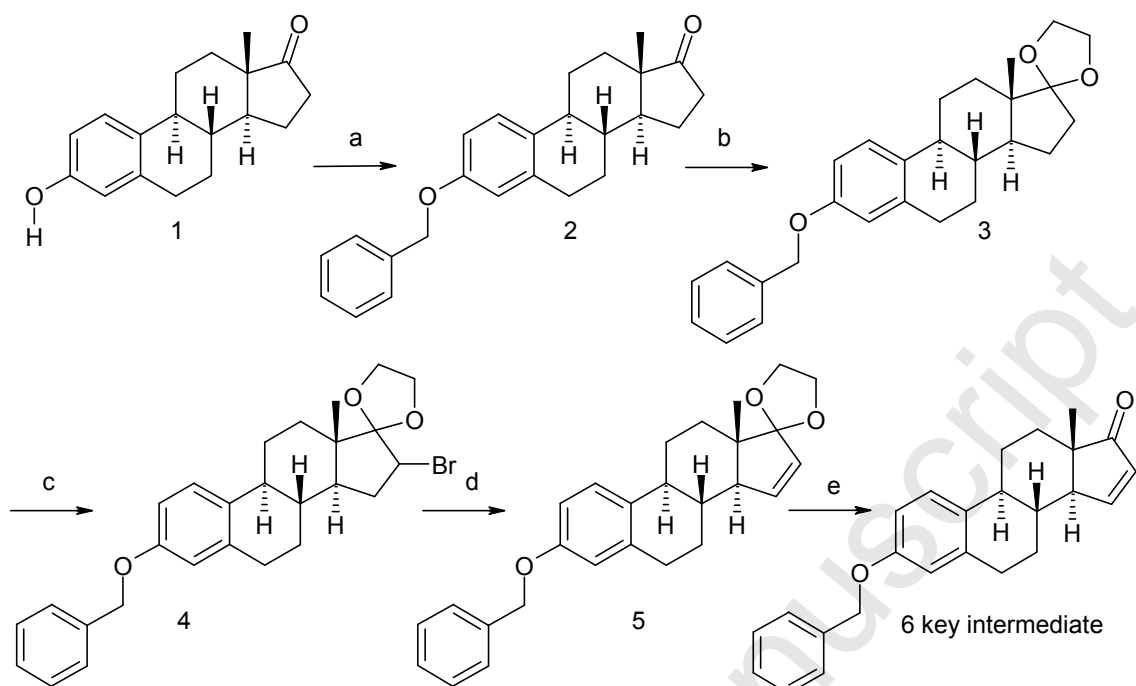


Figure 3

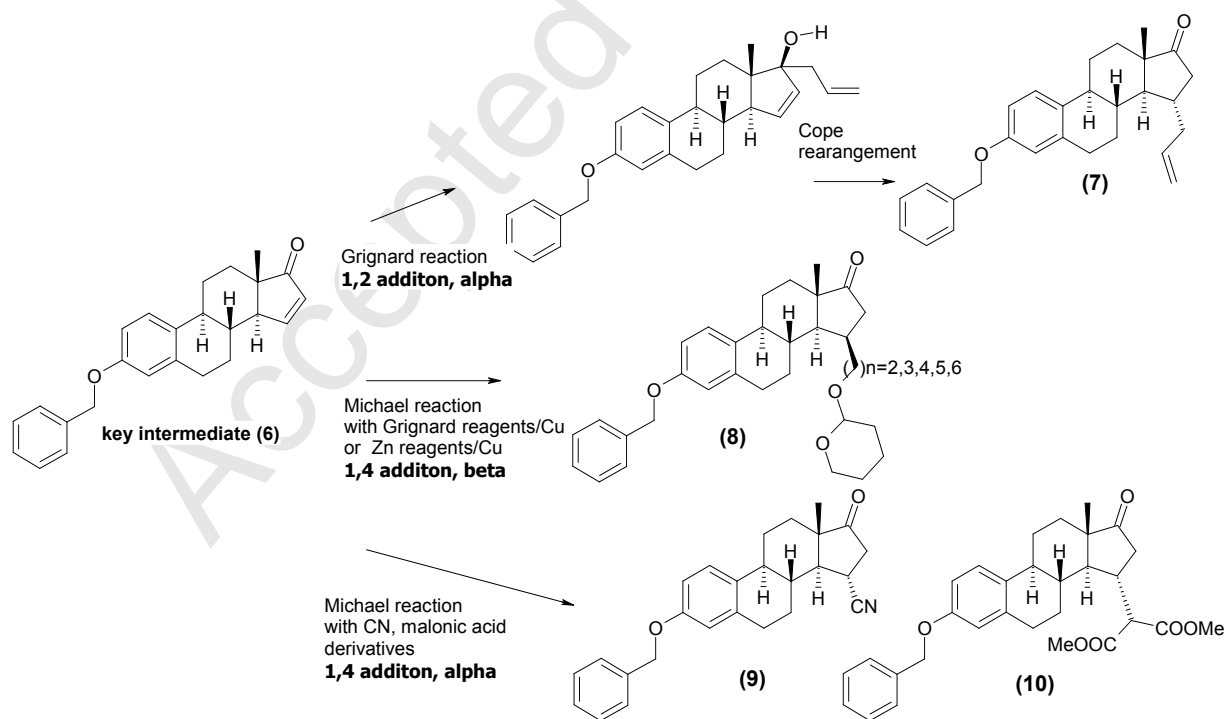


Figure 4

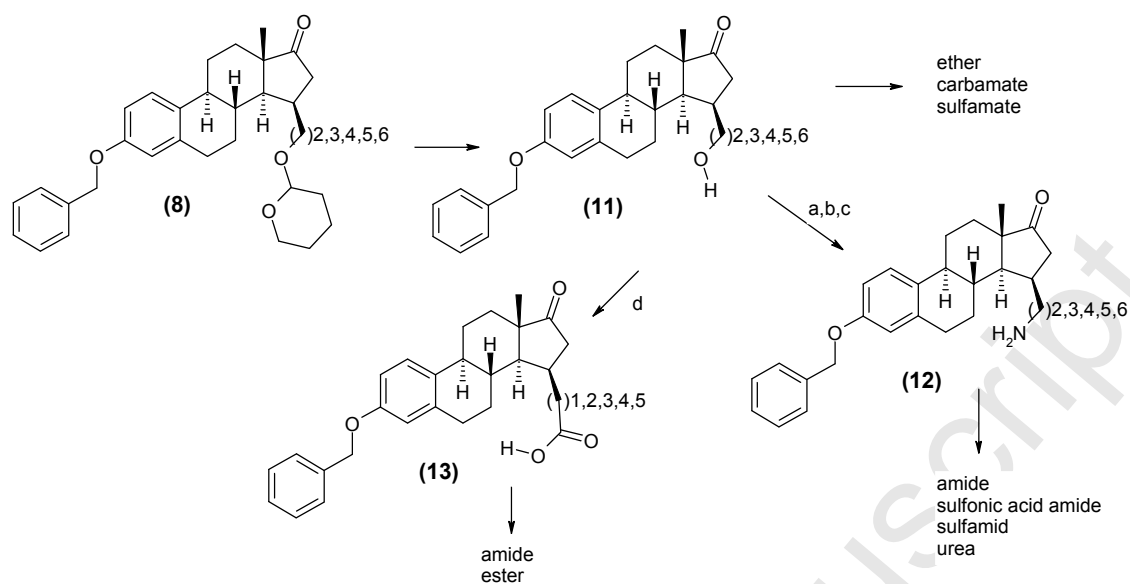


Figure 5

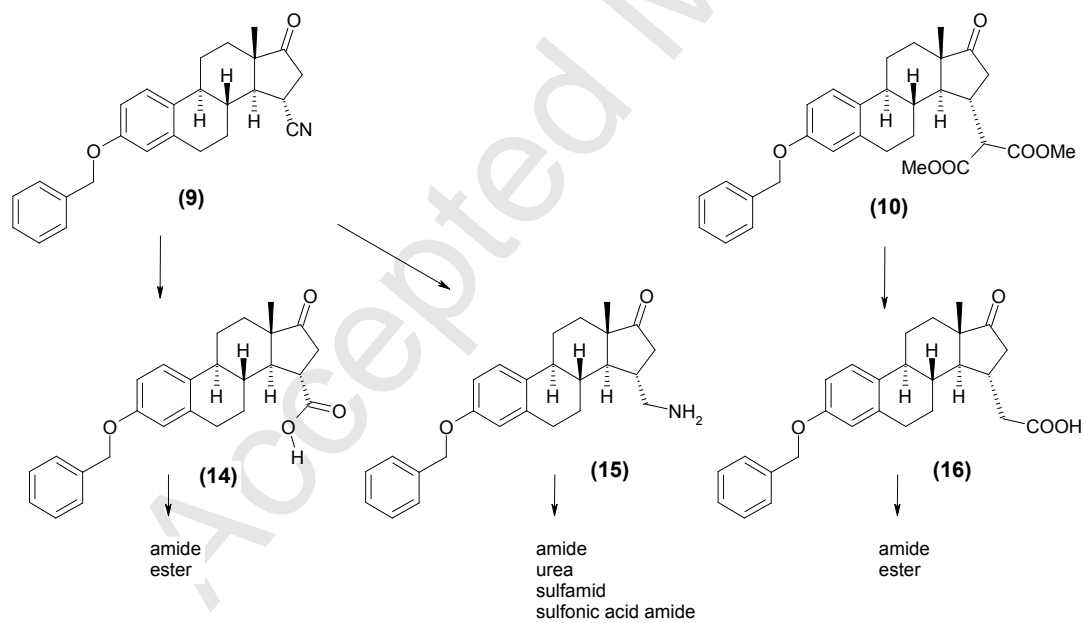


Figure 6

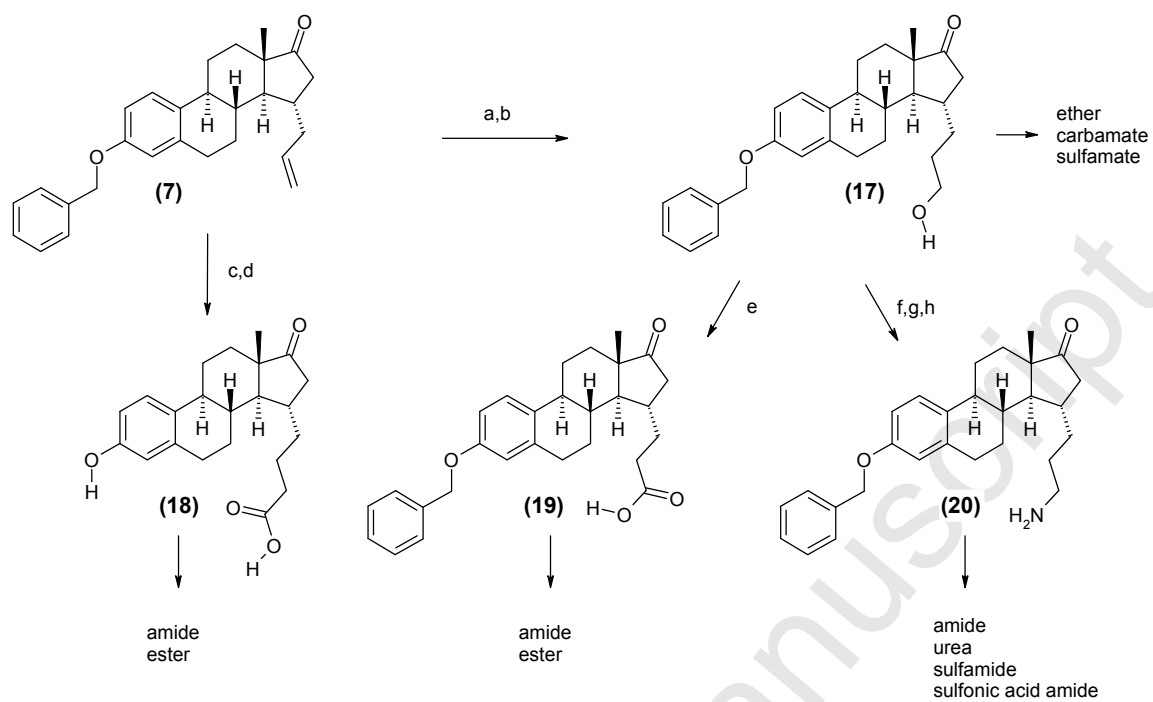


Figure 7

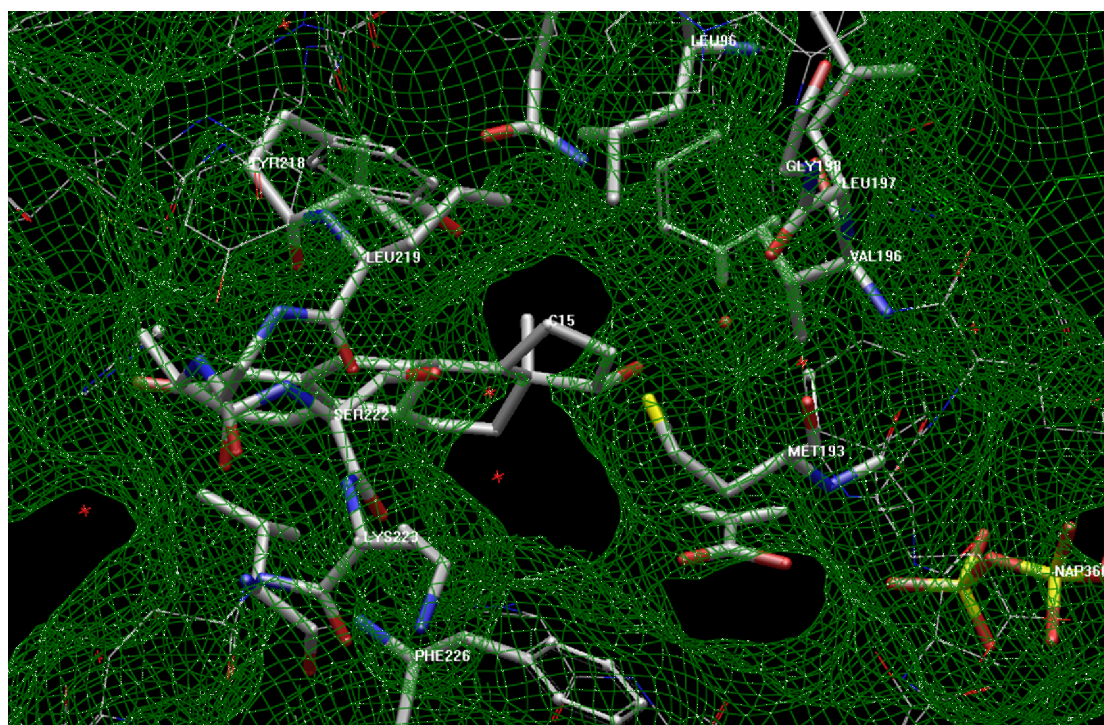


Figure 8

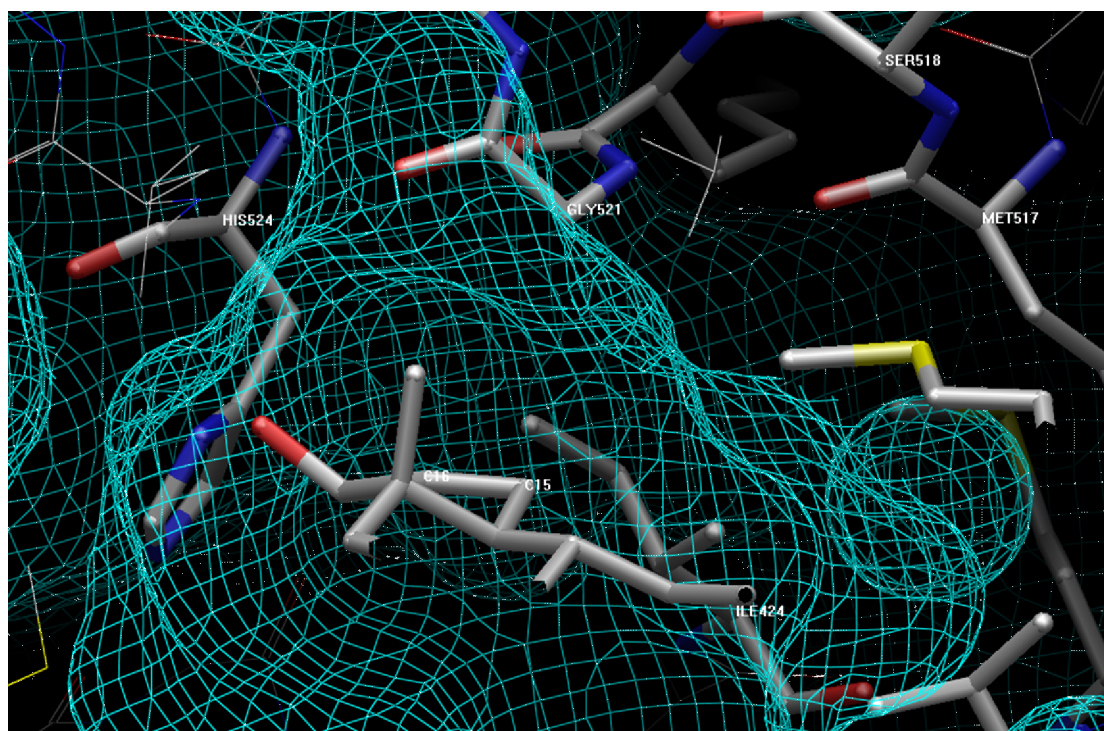


Figure 9

