Synthesis, Reactivity and Biological Activity of 17β-HSD1 Inhibitors Based on a Thieno[2,3-d]pyrimidin-4(3H)-one Core

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ACADEMIC DISSERTATION

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ABSTRACT

Breast cancer is the most common cancer in women in Western countries. In the early stages of development most breast cancers are hormone-dependent, and estrogens, especially estradiol, have a pivotal role in their development and progression. One approach to the treatment of hormone-dependent breast cancers is to block the formation of the active estrogens by inhibiting the action of the steroid metabolising enzymes.

17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) is a key enzyme in the biosynthesis of estradiol, the most potent female sex hormone. The 17β-HSD1 enzyme catalyses the final step and converts estrone into the biologically active estradiol. Blocking 17β-HSD1 activity with a specific enzyme inhibitor could provide a means to reduce circulating and tumour estradiol levels and thus promote tumour regression. In recent years 17β-HSD1 has been recognised as an important drug target. Some inhibitors of 17β-HSD1 have been reported, however, there are no inhibitors on the market nor have clinical trials been announced. The majority of known 17β-HSD1 inhibitors are based on steroidal structures, while relatively little has been reported on non-steroidal inhibitors. As compared with 17β-HSD1 inhibitors based on steroidal structures, non-steroidal compounds could have advantages of synthetic accessibility, drug-likeness, selectivity and non-estrogenicity.

This study describes the synthesis of large group of novel 17β-HSD1 inhibitors based on a non-steroidal thieno[2,3-d]pyrimidin-4(3H)-one core. An efficient synthesis route was developed for the lead compound and subsequently employed in the synthesis of thieno[2,3-d]pyrimidin-4(3H)-one based molecule library. The biological activities and binding of these inhibitors to 17β-HSD1 and, finally, the quantitative structure–activity relationship (QSAR) model are also reported. In this study, several potent and selective 17β-HSD1 inhibitors without estrogenic activity were identified. This establishment of a novel class of inhibitors is a progressive achievement in 17β-HSD1 inhibitor development. Furthermore, the 3D-QSAR model, constructed on the basis of this study, offers a powerful tool for future 17β-HSD1 inhibitor development.

As part of the fundamental science underpinning this research, the chemical reactivity of fused (di)cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones with electrophilic reagents, i.e. Vilsmeier reagent and dimethylformamide dimethylacetal, was investigated. These findings resulted in a revision of the reaction mechanism of Vilsmeier haloformylation and further contributed to understanding the chemical reactivity of this compound class. This study revealed that the reactivity is dependent upon a stereoelectronic effect arising from different ring conformations.
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Helsinki, September 2007

Annamaria Lilienkampf
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.


### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Bn</td>
<td>Benzyl</td>
</tr>
<tr>
<td>Bu</td>
<td>Butyl</td>
</tr>
<tr>
<td>CoMFA</td>
<td>Comparative molecular field analysis</td>
</tr>
<tr>
<td>DDQ</td>
<td>2,3-Dichloro-5,6-dicyano-1,4-benzoquinone</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMFDMA</td>
<td>Dimethylformamide dimethylacetal</td>
</tr>
<tr>
<td>E1</td>
<td>Estrone</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>E1-S</td>
<td>Estrone-O-3-sulfate</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>Et</td>
<td>Ethyl</td>
</tr>
<tr>
<td>HSD</td>
<td>Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>17β-HSD1</td>
<td>17β-Hydroxysteroid dehydrogenase type 1 enzyme</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% Inhibition concentration value</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>Kₘ</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LDA</td>
<td>Lithium diisopropylamide</td>
</tr>
<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>Nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhouser effect spectroscopy</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PES</td>
<td>Potential energy surface</td>
</tr>
<tr>
<td>PPA</td>
<td>Polyphosphoric acid</td>
</tr>
<tr>
<td>SDR</td>
<td>Short-chain dehydrogenase reductase</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure–activity relationship</td>
</tr>
<tr>
<td>ROESY</td>
<td>Rotating frame Overhouser effect spectroscopy</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
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</table>
INTRODUCTION

Breast cancer is the most common cancer in women in Western Countries. It has been estimated that in the United States 178,480 new cases will be diagnosed and 40,460 women will die from breast cancer in 2007.\textsuperscript{1} Similarly in Finland, breast cancer is by far the most diagnosed cancer in women, with over 3900 new cases reported in the year 2004.\textsuperscript{2} The majority of breast cancers are initially hormone-dependent (estrogen receptor positive), and estrogens have a crucial role in their development and progression.\textsuperscript{3,4} One approach to the treatment of hormone-dependent cancers is to block the formation of the active estrogens by inhibiting the action of the steroid metabolising enzymes.\textsuperscript{5}

The biosynthesis of estrogens, the female sex steroids, starts from squalene and proceeds through cholesterol and androgens.\textsuperscript{6} 17\(\beta\)-Hydroxysteroid dehydrogenase type 1 (17\(\beta\)-HSD1) is a key enzyme in this biosynthetic pathway catalysing the final step (Figure 1) and converting estrone (E1) into the biologically active estradiol (E2). Blocking 17\(\beta\)-HSD1 activity with a specific enzyme inhibitor could provide a means to reduce both circulating and tumour E2 levels and thus promote tumour regression. Enzyme inhibitors are molecules that bind to an enzyme and prevent a substrate from entering the enzyme's active site or otherwise hinder the enzyme from catalysing its reaction.

This thesis describes the synthesis of potent non-steroidal 17\(\beta\)-HSD1 inhibitors based on a cycloalkeno thieno[2,3-d]pyrimidin-4(3\(H\))-one core. The biological activities and binding of these inhibitors to 17\(\beta\)-HSD1 and, finally, a quantitative structure–activity relationship (QSAR) model are reported. As a part of the study, investigation was made of the chemical reactivity of fused (di)cycloalkeno thieno[2,3-d]pyrimidin-4(3\(H\))-ones with electrophilic reagents, \textit{i.e.} Vilsmeier reagent and dimethylformamide dimethylacetal (DMFDMA). The findings resulted in a revision of the reaction mechanism of Vilsmeier haloformylation.

The literature review, which follows below, will focus on known inhibitors of 17\(\beta\)-HSD1, with a concise discussion of the structure and biological role of the enzyme.
1. **ENZYME 17β-HSD1**

17β-Hydroxysteroid dehydrogenases (17β-HSD) are a group of enzymes responsible for the NAD(P)(H) dependent reduction and oxidation reactions of C17 ketosteroids and C17 hydroxysteroids. As part of the prereceptor control mechanism, they activate and inactivate steroid hormones (Figure 1). To date 14 mammalian isoforms have been identified, of which at least 11 exist in humans. The different isoforms of 17β-HSD have relatively low sequence identity (25-30%) and they differ in catalytic direction, substrate and cofactor specificity, subcellular localisation and tissue distribution. The nomenclature of 17β-HSD enzymes follows the chronological order of description (17β-HSD types 1-14). 17β-HSDs are members of the short-chain dehydrogenase reductase (SDR) superfamily, except for 17β-HSD5 which is an aldo-keto reductase (AKR).

17β-HSD from human placenta (17β-HSD1, EC.1.1.1.62) was first described in 1958 by Engel & Langer who carried out extensive structure–activity experiments with a large and diverse set of natural and synthetic steroids. 17β-HSD1 is a key enzyme in the biosynthesis of female sex steroids, catalysing the final step which converts the less potent E1 into biologically active E2 (Figure 1). The reverse inactivating oxidative enzymatic reaction is catalysed by 17β-HSD2. In physiological environment, 17β-HSD1 is mainly a unidirectional reductive enzyme utilising NADPH as a cofactor, although in vitro the purified enzyme or cell homogenates can be driven to catalyse the oxidative direction. 17β-HSD1 is also capable of reducing some androgens, such as androstenedione, into testosterone, but only to a minor extent. 17β-HSD1 is a widespread enzyme expressed most abundantly in female ovaries, placenta and breast tissue but also found in other peripheral tissues.

### 1.1 Structure of 17β-HSD1

Human 17β-HSD1 is active as a soluble cytosolic homodimer. Both subunits of the homodimer have a molecular mass of 34.9 KDa and 327 amino acid residues. The 3D X-ray structure of native form 17β-HSD1 was first presented, at 2.2 Å resolution, in 1995. Since then, several crystal structures of 17β-HSD1 binary and ternary complexes with estrogenic or androgenic ligands have become available in the Protein Data Bank (PDB). 17β-HSD1 has also been crystallised with two known steroid based inhibitors. In addition, the binding of steroids and characteristics of the active site have been studied by molecular modelling. Site-directed mutagenesis experiments have been used to identify the critical residues for the enzyme activity and substrate binding. The inactivating isoform 17β-HSD2, which
Figure 1. The primary enzymes involved in the final steps of E2 biosynthesis.

catalyses the oxidation of E2 2 to E1 1 (Figure 1), is bound to the endoplasmic reticulum and its 3D structure has not been resolved.

The monomeric structure of 17β-HSD1 consists of seven parallel β-strands, forming a β-sheet, and eleven α-helices (Figure 2a). The β-sheet is surrounded on both sides by three parallel α-helices forming a typical "Rossmann fold", which is associated with NAD(P)H binding. The structure contains a flexible loop (Ala191 to Glu202) for which crystal structure determinations indicate several conformations (for the structures and abbreviations of amino acids see Appendix). The loop has been associated with NADP⁺ stabilisation and substrate entry and is suggested to adopt a closed conformation after substrate binding.

The ligand binding cavity of 17β-HSD1 is a narrow hydrophobic tunnel, hydrophobic atoms contributing 72% to the total surface area. As would be expected, the cavity is complementary to substrates resembling hormones both in structure and in volume. As a member of the SDR superfamily, 17β-HSD1 has the highly conserved and catalytically crucial Tyr-x-x-x-Lys sequence and generally conserved Ser in the active site (catalytic triad Tyr155, Lys159 and Ser142). E2, the reduced product of the natural substrate E1, binds to the active site via three to four putative hydrogen bond interactions, as well as several hydrophobic interactions involving the steroid core (Figure 2b). The hydroxy group at C17 in E2 forms a hydrogen bond to the conserved Ser142 and Tyr155 in the catalytic region. At the opposite end of the ligand binding cavity, His221 and C3 phenolic hydroxyl form a hydrogen bond, and also a hydrogen bond to Glu282 has been observed. It has been suggested that steroids are able to move inside
Figure 2. a) 3D structure of human 17β-HSD1 monomer crystallised with E2 (coloured magenta) and NADP⁺ (coloured blue) resolved to 2.2 Å (PDB code 1FDT). The flexible loop (Ala191 to Glu202) is coloured green. b) Orientation of E2 in the ligand binding cavity. The dashed lines indicate plausible hydrogen bonding.
the active site to some extent, and superimposition of the available crystal structures has revealed variable orientations of several active site residues, indicating some flexibility of the enzyme. C19 steroids, e.g. testosterone, can bind to 17β-HSD1 in a normal or reverse orientation, and the androgen discrimination is considered to result from the steric hindrance of the C19 methyl group with β-face Leu149.

1.2 17β-HSD1 and breast cancer

Initially most breast cancers are hormone-dependent, i.e. tumour cells expressing estrogen receptors (ER), and estrogens, especially E2, have a pivotal role in their development and progression. Breast carcinogenesis evolves over a period of time in a complex process from hormone-dependent status into hormone independence. The highest incidence of breast cancer occurs in postmenopausal women when ovarian estrogen production has ceased and E2 is formed locally in peripheral target tissues, as in the breast. Hormone-dependent breast cancer tissue can be considered an endocrine organ since it contains all the enzymes needed for the synthesis of E2 from its precursors.

Normal breast tissue expresses the reductive 17β-HSD1 and oxidative 17β-HSD2 isoforms, the type 2 activity being much higher. The situation is reversed in primary cultures of malignant hormone-dependent breast tumours, and 17β-HSD1 is expressed in much higher degree. Immunohistochemical studies have suggested that 17β-HSD1 plays an important role in the in situ regulation of breast tumour E2 production, and various breast cancer cell lines have been found to express 17HSD1, 17HSD2, or both isoforms. In addition, the expression of 17β-HSD1 has been shown to be an independent prognostic factor in breast cancer.

It is well established that sex steroid metabolising enzymes have an important role in the development and progression of breast tumours. One approach to the treatment of hormone-dependent breast cancer is to inhibit the action of these enzymes and prevent the formation of the sex steroids. ER positive breast cancer is usually treated with ER antagonists like tamoxifen, which by binding to ER prevent E2 induced cell proliferation. Inhibitors of aromatase (CYP19), the enzyme primary converting androstenedione into E1 (Figure 1), are already used as an adjuvant therapy in breast cancer treatment in postmenopausal women, although it has been shown that in malignant breast tumours E1 is formed predominantly via E1 sulfatase pathway (Figure 1). E1 sulfatase inhibitors are currently under development. Given its crucial role in E2 biosynthesis, 17β-HSD1 has become an attractive target for inhibitor development for the prevention and control of hormone-dependent breast tumour growth. Recently, in vivo efficacy of 17β-HSD1 inhibition on E2 dependent tumour growth was demonstrated in a mouse model.
expressing human 17β-HSD1.\textsuperscript{55,56} In addition to breast cancer, endometriosis is another hormone-dependent disorder that could benefit from 17β-HSD1 inhibition.\textsuperscript{57,58}

### 1.3 Methods of measuring 17β-HSD1 inhibition

Several methods are available for determining 17β-HSD1 inhibitory activity. An appropriate literature reference for each assay will be given where the inhibition studies are discussed in detail.

At present, many primary activity measurements are performed using a human recombinant enzyme in a protein based assay or using cell lines (MCF-7, HEK-293) transfected with 17β-HSD1 cDNA. Ovarian granulosa-luteal cells and wild-type ER positive human breast adenocarcinoma cell lines MCF-7 and T-47D have also been used. 17β-HSD1 is expressed to a much higher degree in T-47D cells than in MCF-7 cells.\textsuperscript{46,59} Purified 17β-HSD1 from human placenta and placental microsome or cytosol preparations were often used before techniques employing genetic engineering were readily available. Most of the methods rely on time-consuming TLC or HPLC separations. Radioactive substrates are commonly used since the conversion of labelled E1 into E2 is measured in the presence of potential inhibitors. In order to speed up the screening process for 17β-HSD1, homogeneous luminescence resonance energy transfer-based immunoassays have been developed utilising europium(III)-chelate-dyed nanoparticles.\textsuperscript{60,61} Recently, an animal model was reported for \textit{in vivo} evaluation of human 17β-HSD1 inhibitors.\textsuperscript{55,56}

The inhibitory potency of a compound is usually expressed as an IC\textsubscript{50} value or \(K_i\) value or as inhibition per cent. Frequently, the mechanism of inhibition is also reported, as reversible or irreversible inhibition. The IC\textsubscript{50} value is the inhibitor concentration at which half of the enzyme activity is inhibited. The \(K_i\) value (equilibrium dissociation constant) corresponds to the concentration of inhibitor at which the binding site on the enzyme is half occupied. The measured values are laboratory and assay dependent and thus comparison of inhibitory potencies is challenging. Similar assays can vary in molar proportions of inhibitors and enzyme, and amount of inactive enzyme.
2. INHIBITORS OF HUMAN 17β-HSD1: REVIEW OF THE LITERATURE

Recent years have seen an increase in the number of patents and publications concerning 17β-HSD1 inhibition as pharmaceutical companies have recognised the potential of 17β-HSD1 inhibitor development. Most of the known inhibitors of 17β-HSD1 are based on modifications of steroidal structures, while relatively little has been published on non-steroidal inhibitors.\textsuperscript{62,63} Currently there are no 17β-HSD1 inhibitors on the market.

An inhibitor of 17β-HSD1 should be selective over 17β-HSD2 and, most importantly, forego estrogenic effects. Antiestrogenic effects or inhibitory activity against aromatase or E1 sulfatase can be considered as an extra benefit. A good 17β-HSD1 inhibitor should exhibit certain drug-like properties in order to have sufficient absorption and permeation in biological systems. According to the rules of Lipinski,\textsuperscript{64} an orally active compound should not have more than 5 hydrogen bond donors or 10 hydrogen bond acceptors in its structure. In addition, the molecular weight should be under 500 gmol\textsuperscript{-1} and the partition coefficient (log P) less than 5. In general, depending on the biological assay, good 17β-HSD1 inhibition has been achieved with IC\textsubscript{50} values in (low) nM range or high inhibition% in nM or low μM test concentrations.

The review of the literature on inhibitors of human 17β-HSD1 is divided into three parts. First, the early work on 17β-HSD1 inhibition, at the time when the structure of the enzyme was not known, will be briefly discussed. Secondly, 17β-HSD1 inhibitors based on modifications of natural substrates or other steroidal structures will be covered. Finally, small non-steroidal molecules useful as 17β-HSD1 inhibitors will be reviewed. Figure 3 presents the prevailing labelling of the rings and numbering of the steroid skeleton, which will be employed throughout this thesis.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3}
\caption{Numbering and labelling of the steroid skeleton.}
\end{figure}
2.1 Early work on 17β-HSD1 inhibition: 1969-1990

Before the complete amino acid sequence\textsuperscript{21} or the first crystal structure\textsuperscript{22} of 17β-HSD1 was available, many of the inhibition studies were concentrated on affinity labelling of the enzyme’s active site or involved the determination of substrate specificities. Although the significance of these early inhibition studies can be debated, some of the results have guided later inhibitor development and are thus discussed here. Sections 2.1.1-2.1.3 will describe inhibition studies conducted between 1969 and 1990. During this period, 17β-HSD1 was considered a bidirectional enzyme, and thus most of the inhibition studies were performed under basic conditions measuring the oxidative direction of the enzyme. This should be taken into account in evaluating these early results.

2.1.1 The first inhibition studies

In 1969, ten years after the enzyme was first described, Jabarak & Sack\textsuperscript{65} discovered that the oxidative direction of purified soluble placental 17β-HSD was competitively inhibited by estrogen analogues as well as some non-steroidal compounds. A number of steroids were tested against 17β-HSD activity, and the most promising inhibitors were 1,3,5(10)-estratrien-3-ol 5 (17-desoxyestradiol), 17α-estradiol 6, 16-difluoroestrone 7 and equine estrogen equilin 8 (Figure 4). At pH 10.2, these compounds inhibited NAD\textsuperscript{+} dependent oxidation of E2 with IC\textsubscript{50} values of 0.12, 3.7, 2.8, 1.9 μM, respectively. \(K_i\) values ranged from 0.19 μM for 1,3,5(10)-estratrien-3-ol up to 4.5 μM for 17α-estradiol. In addition to the steroidal structures, mitotane (\(o,p\)-DDD) 9, antiestrogen nafoxidine 10, and diethylstilbestrol 11 (Figure 5) were potent inhibitors of 17β-HSD1, with IC\textsubscript{50} values of 0.16, 1.5 and 2.6 μM, respectively.

![Figure 4. Examples of the first steroidal 17β-HSD1 inhibitors.](image-url)
Equilin 8, which is used in present hormone replacement therapy, has been crystallised as 17β-HSD1–equilin–NADP⁺ complex (PDB code 1EQU), and this more contemporary study showed 8 at 1 μM concentration to inhibit the enzyme activity in transfected HEK-293 cells by 77%. Similar results were recently obtained in T-47D cells (IC₅₀ value 1.7 μM), but 8 was also shown to lack selectivity and to be an equally potent 17β-HSD2 inhibitor.

In addition to 1,3,5(10)-estratrien-3-ol 5, also other C18 and C19 steroids lacking the oxygen at C17 were potent inhibitors of the oxidative direction of soluble placental 17β-HSD. When E2 O-3 methyl ether was used as a substrate, 1,3,5(10)-estratrien-3-ol 5, 1,3,5(10),16-estratetraen-3-ol 12, 3β-hydroxy-5,16-andostanediene 13, and 5-androsten-3β-ol 14 were the most potent inhibitors, with $K_i$ values of 0.04, 0.17, 1.8, and 6.0 μM, respectively. For comparison, in this study $K_i$ value of 0.4 μM was measured for the natural ligand E1. In the same assay, non-steroidal alcohols were found to be poor inhibitors of 17β-HSD, whereas diethylstilbestrol 11 and the triphenylethylene derivative 15 had $K_i$ values of 0.8 and 2.5 μM, respectively. In phosphate buffer the oxidative direction of the purified enzyme was reversibly inhibited by 16-oxoestrone 16. The reductive direction was not inhibited, but instead 16 was found to be a substrate and was reduced to 16-oxoestradiol more efficiently than E1 to E2.

Unsaturated fatty acids were found to irreversibly inactivate purified placental 17β-HSD1 when the oxidative direction of 17β-HSD1 was investigated. Exposure to 200 μM of oleic acid or arachidonic acid led to complete inactivation in 30 minutes, whereas with linoleic or linolenic acid 42-66% of enzymatic activity remained. Saturated C₁₈ stearic acid, methyl and ethyl esters of oleic acid and with prostaglandins E₂ and F₂α were inactive. Pyridine nucleotides (NAD⁺, NADP⁺, NADH, NADPH), but not steroids like E1, had protective effects against the inactivation indicating that fatty acid micelles bind to or near the nucleotide binding sites.
2.1.2 Affinity labelling of the active site

Affinity labels are a class of irreversible inhibitors which covalently bind to the active site residues. These inhibitors resemble a particular substrate for the enzyme and are used to identify and locate residues in the enzyme’s active site. Electrophilic derivatives (17-20) of E1 and E2 with an iodine,\textsuperscript{71} bromoacetate,\textsuperscript{72,73,74,75} bromoacetamide\textsuperscript{76,77} or \textit{N-}(4-azido-2-nitrophenyl)-\textit{β}-alanine\textsuperscript{78} substituent were found to be 17\textit{β}-HSD1 affinity labels and were used to identify amino acid residues in the active site. Progesterone derivatives (21) bearing a bromoacetate moiety\textsuperscript{79,80,81} and 12\textit{β}-(bromoacetoxy)-estrene-4-ene-3,17-dione\textsuperscript{75} 22 were also used in the affinity labelling experiments (Figure 6). In addition to affinity labels resembling steroidal substrates, nucleotide analogues have been used to explore the cofactor binding site.\textsuperscript{82,83}

![Figure 6](image)

**Figure 6.** Steroid structures used in affinity labelling experiments. *Indicates alternative sites of the side chain attachment.

16-Oxoestrone 16 was found to be a 17\textit{β}-HSD1 affinity label in a borate buffer at pH 8.5.\textsuperscript{69} Unlike in phosphate buffer, 16 selectively reacted with arginine residues to form a diol intermediate, which subsequently reacted with the borate buffer to give an estratriene–arginine–borate complex. Also other dicarbonyl compounds (2,3-butanedione, phenylglyoxal), although not true affinity labelling agents, photochemically inactivated purified 17\textit{β}-HSD1 in borate buffer.\textsuperscript{84} The photooxidation with 2,3-butanedione destroyed all 17\textit{β}-HSD1 Met and Tyr residues, and partially His, Leu, Arg and Thr residues. In another study related to identifying active site residues, tetranitromethane was shown to modify Tyr residues in 17\textit{β}-HSD1.\textsuperscript{85}
2.1.3 Mechanism based 17β-HSD1 inactivation

The idea behind mechanism based enzyme inactivation is to create specific substrates that are inert until they are within the enzyme's active site. Enzymatic reaction on the substrate generates a reactive species which in turn will react with a nucleophilic residue in the active site and form a covalent bond yielding irreversible inhibition. Mechanism based inhibitors are often referred to as suicide inhibitors.

16-Methyleneestradiol 23 is a good substrate ($K_m$ value 8.0 μM, pH 9.2) as well as a mechanism based inhibitor of purified placental 17β-HSD1. 86 Enzymatic oxidation in the presence of NAD$^+$ produces 16-methyleneestrone 24 ($K_i$ value 261 μM, pH 7.0) which is a Michael acceptor and reacts in situ with an active site residue and inactivates the enzyme (Figure 7). As a downside, 23 enhances E2 potency in vivo. 87 17β-[1(R)-1-Hydroxy-2-propynyl]andros-4-en-3-one 25 is a rather poor substrate for 17β-HSD1, but the oxidised product 17β-(1-oxo-2-propynyl)androstan-4-en-3-one is a good substrate and a better affinity alkylator ($K_i$ value 190 μM) than 24. 88

[Diagram of enzyme activation and suicide inhibition]

Figure 7. The concept of mechanism based enzyme activation and the compounds used as 17β-HSD1 suicide inhibitors.

In view of the activities of 23 and 25, the E2 analogue 26, lacking the steroid ring D, was synthesised in order to create a mechanism based inhibitor with good affinity and reactivity. 89,90 Under basic conditions 26 had a $K_m$ value of 79 μM, and was oxidised approximately 400 times slower than the natural substrate E2. The oxidised product rapidly inactivated the enzyme resulting in the Cys and Lys Michael adducts, which were confirmed in solid state $^{13}$C NMR experiments of the lyophilised enzyme. 91 Unexpectedly the trifluoromethyl derivative 27 was not a mechanism based inactivator but instead an affinity label with $K_i$ values of 59 μM and 65 μM.
for the two diastereomers.\textsuperscript{92} It was suggested that the irreversible inhibition by 27 occurred by nucleophilic addition to one of the two acetylenic carbons.

2.2 Steroidal inhibitors of 17β-HSD1

Most of the known inhibitors of 17β-HSD1 are based on steroid structures, especially the natural ligands E1 and E2. The following sections introduce steroidal 17β-HSD1 inhibitors, which are divided into various subclasses mainly according to the modification site of the steroid core. Although the vast majority of these steroidal inhibitors are based on the estra-1,3,5(10)-trien-3-ol core, some progestins and E ring modified steroids are included as well.

2.2.1 Progestins

Synthetic progestins (Figure 8) have been found to inhibit 17β-HSD1 as well as E1 sulfatase activity in cell based assays. In intact T-47D cells the C\textsubscript{21} steroids nomegestrol acetate 28 and antiestrogen medrogestone (Prothil\textsuperscript{®}) 29 had IC\textsubscript{50} values of 0.8 µM and 0.45 µM, respectively, making them the best progestin based 17β-HSD1 inhibitors reported.\textsuperscript{93,94} Dydrogestone (Duphaston\textsuperscript{®}) 30, which has a 19α-methyl group, and its 20-dihydro metabolite 31 are less potent, the metabolite being a slightly better inhibitor with an IC\textsubscript{50} value of 9 µM.\textsuperscript{95} In MCF-7 cells, which have a much lower 17β-HSD1 expression,\textsuperscript{46} 28 and 29 had no or only moderate effect on the 17β-HSD1 activity.\textsuperscript{93,94}

19-Nortestosterone derivative tibolone 32, the active ingredient in the hormone replacement drug Livial\textsuperscript{®}, inhibited 17β-HSD1 activity in T-47D cells with an IC\textsubscript{50} value of 2.0 µM.\textsuperscript{96} Tibolone 3α-OH metabolite 33 was less than half as active as 32, whereas the 3β-OH metabolite 34 was a better inhibitor with an IC\textsubscript{50} value of 1.4 µM. The 4-Δ isomer 35 showed only weak inhibitory activity (IC\textsubscript{50} value 35.3 µM).Unlike 28 and 29, tibolone and its metabolites also showed inhibition against 17β-HSD1 in MCF-7 cells, with IC\textsubscript{50} values between 0.79 and 22.8 µM.
The 3D structure of 17β-HSD1 complexed with 20α-hydroxyprogesterone (20-hydroxy-(20S)-pregn-4-en-3-one) has been resolved, although the coordinates for the complex have not been published. 20α-Hydroxyprogesterone is reported to have a similar binding mode to E2. The crystallisation study along with the inhibition studies noted above show that C21 steroids are capable of binding to 17β-HSD1 and inhibit the enzyme activity.

Although the selectivity of these progestins over 17β-HSD2 is not known, 20α-hydroxyprogesterone inhibits the oxidation of testosterone 4 by microsomal 17β-HSD. On the other hand, the inhibitory activity also against E1 sulfatase can be considered beneficial because in breast cancer the sulfatase pathway is prominent for E1 formation. Evidently some progestins induce 17β-HSD1 activity, which could be one reason why these steroids have not been employed as lead compounds for 17β-HSD1 inhibition.

2.2.2 Derivatives of E1 and E2 substituted at C7 or C6

The estrogenic effects of E2 can be prevented by introducing a 7α-undecanamide group to the steroid core. The E2 derivative 36 (ICI 164384) is a known antiestrogen, which has also been shown to weakly inhibit 17β-HSD1 and E1 sulfatase activity in rat mammary and human breast tumour tissue (Figure 9). In a human tumour homogenate, 36 inhibited 17β-HSD1 activity with a very moderate IC50 value of 0.89 mM. In the same assay, 4-hydroxytamoxifen and
desmethyltamoxifen, which are metabolites of the commercial antiestrogen tamoxifen, had a minor inhibitory effect against 17β-HSD1 with IC₅₀ values up to 2.2 mM. In human placental cytosol preparations, 36 at 1 μM concentration inhibited 33% of 17β-HSD1 activity. In a mouse uterus assay, derivatives of 36, with an unsaturated ring D or a halogen group at C16, acted as potent antiestrogens and 17β-HSD1 inhibitors in vivo. Whether these derivatives also inhibit human 17β-HSD1 is not known.

![Figure 9. Derivatives of E2 and E1 substituted at C6 or C7.](image)

The inhibitory activity was improved by moving the N-butyl-N-methyl alkyl amide side chain to the C6 position of E2 (Figure 9). The 6β-thiaheptanamide derivative 37 had an optimised side chain length of five methylenes and was able to reversibly inhibit 17β-HSD1 in human placental cytosol preparations with an IC₅₀ value of 0.17 μM. Compound 37 showed almost identical activity in 17β-HSD1 transfected HEK-293 cell homogenates. The C6 β-configuration, C3 hydroxy group and the side chain amide moiety (-CONBuMe) were shown to be important structural features, as the removal of each significantly reduced the activity. Replacement of the 6β-thioether bond with a chemically more stable ether or carbon–carbon bond also reduced the activity, as was shown with compounds 38 (IC₅₀ value of 0.8 μM) and 39. In intact HEK-293 cells the inhibitory activity of 37 and 39 was reduced to approximately half, whereas in intact T-47D cells, which better represent physiological conditions, the activity was much higher, likely due to lower 17β-HSD1 expression. In the T-47D cells, 37 and 39 were equally potent, with 82% 17β-HSD1 inhibition at 1 μM. The C2 methoxy and E1 derivatives 40 and 41 were less potent, with 32% and 57% inhibition at 1 μM concentration, respectively.

Compounds 37 and 39-41 are reported to show some selectivity over the reductive isoforms 17β-HSD7 and 17β-HSD12, and 37 was also selective over 17β-HSD2 and 17β-HSD3.
Although structurally related to the antiestrogen 36, at 0.01 μM concentration 37 exhibited strong estrogenic effect in T-47D and MCF-7 cell lines. For 38 the proliferative effect in T-47D cells was only seen at higher concentrations (0.1 μM and 1.0 μM), and also 39 was less estrogenic than the 6β-thiaheptanamide derivative 36. As postulated, the C2 methoxy derivative 40 was not estrogenic at 1 μM concentration but had a proliferative effect at lower concentrations.

2.2.3 C16 substituted E2 derivatives designed as irreversible inhibitors

Poirier and co-workers have developed a series of C16 substituted E2 derivatives designed to be irreversible inhibitors of 17β-HSD1. Affinity labelling experiments had already shown that inhibition of 17β-HSD1 could be achieved by an electrophilic substitution on E2 ring D. 16α-Propyl derivatives of E2 such as compounds 42a-42e, with a good leaving group at the end of the alkyl chain, were found to competitively and irreversibly inhibit partially purified placental 17β-HSD1 (Figure 10). 106 16α-(3'-Iodopropyl)estradiol 42d and 16α-(3'-bromopropyl)estradiol 42c were the most potent inhibitors, with IC50 values of 0.42 and 0.46 μM, respectively. Similar activity was obtained with the E1 derivative 16α-(3'-bromopropyl)estrone 43 (IC50 value 0.31 μM). 107 Introduction of the side chain to the 17α-position of E2, variation of the C16 configuration or variation of the side chain length reduced the activity. 108 In fact, the 16β-derivatives with a brominated ethyl or propyl chain were chemically unstable and readily underwent cyclisation. In addition to the partially purified enzyme, 16α-(3'-bromopropyl)estradiol 42c at 100 nM concentration inhibited approximately 88% of the 17β-HSD1 activity in transfected HEK-293 cells, exhibiting selectivity over 17β-HSD type 2, type 3 and type 5. 108 Unfortunately, the E2 derivatives, such as 42c are estrogenic and stimulate the growth of ZR-75-1 breast cancer cells.

In a subsequent study, a C16 bromobutyl side chain was attached to the known antiestrogen and 17β-HSD1 inhibitor ICI 164384 36 (see section 2.2.2) with a view to producing a dual action inhibitor with an additional antiestrogenic effect. This strategy led to decreased inhibitory potency compared with 36, although satisfactory antiestrogenic properties were retained. Better results were obtained when the N-methyl-N-butyl alkylamide moiety present in 36 was introduced to the C3’ position of 16α-(3'-bromopropyl)estradiol 42c to give compound 44a (Figure 10). For this dual biological action, the optimal length of the methylene linker between the C3’ and the amide moiety was determined to be five carbons (compound 44b).
When measured on partially purified 17β-HSD1, 44b had a moderate IC₅₀ value of 10.4 μM, but on the other hand it had no estrogenic activity up to 0.03 μM concentration, and at 1 μM concentration it also exhibited 74% antiestrogenic effect. Removal of the amide moiety from the side chain increased the inhibitory potency but restored the estrogenic activity. The enzyme activity was unexpectedly restored after incubation of 17β-HSD1 with NADH and 44, indicating reversible inhibition. Replacement of bromine at C3′ with a bromomethylene group gave the primary bromide 45 with enhanced inhibitory activity (IC₅₀ value 4.5 μM) and partial estrogen antagonism.

The described approach to create dual action 17β-HSD1 inhibitors failed to produce compounds with high affinity to the enzyme. Nevertheless these studies importantly showed that 17β-HSD1 inhibition can be achieved with steroids bearing relatively large substituents at the C16 position.

2.2.4 Derivatives of E1 and E2 substituted at C16

The investigation of C16 substituted steroids was continued by Potter and co-workers, who have developed a large and versatile library of C16 substituted E1 and E2 derivatives bearing additional substituents at C2, C6 or O-3 positions. Poirier and co-workers had already established that large substituents at C16 of E2 are tolerated, and molecular modelling studies suggested space for significant expansion from the C16 position of E1 resulting in possible interactions with the cofactor. The addition of substituents of various size to the C16 position was conducted in stepwise manner. First, a series of compounds 46, 47 and 48 with small hydrophobic alkenyl and alkyl moieties was synthesised and their biological activity evaluated (Table 1). The C16 alkenyl derivatives of E1 46a-46h can be synthesised from E1
Table 1. Selected examples of C16 alkenyl E1 and E2, and 16β-alkyl E2 derivatives, and their biological activity. Dashed line indicates the point of attachment.

<table>
<thead>
<tr>
<th>17β-HSD inhibition% at 10 μM</th>
<th>Purified 17β-HSD1 IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1a</td>
<td>Type 2b</td>
</tr>
<tr>
<td>a 82</td>
<td>31</td>
</tr>
<tr>
<td>b 17</td>
<td>&lt;10</td>
</tr>
<tr>
<td>c 34</td>
<td>25</td>
</tr>
<tr>
<td>d 23</td>
<td>16</td>
</tr>
<tr>
<td>e 44</td>
<td>10</td>
</tr>
<tr>
<td>f 72</td>
<td>13</td>
</tr>
<tr>
<td>g 38</td>
<td>3.8-4.0</td>
</tr>
<tr>
<td>h 34</td>
<td>0.26-0.33</td>
</tr>
<tr>
<td>i 32</td>
<td>0.72</td>
</tr>
<tr>
<td>j 14</td>
<td>0.72</td>
</tr>
</tbody>
</table>


via straightforward aldol condensation with an appropriate aldehyde. Subsequent NaBH₄ reduction of the C17 carbonyl moiety followed by hydrogenation of the double bond gives the E2 derivatives 47a-47f and 48a-48f. Some of the compounds 46-48 showed auspicious activity against 17β-HSD1 in T-47D cells and selectivity over 17β-HSD2 (Table 1). The generally improved activity with the 16β-alkyl derivatives 48a-48f indicated that flexibility of the C16 side chain allows more propitious orientation of substituents. The most potent inhibitors in this series were 6β-(ethoxymethyl)estrone 49 (IC₅₀ value 0.32 μM) and 16-dimethylestrone with 92% and 95% 17β-HSD1 inhibition at 10 μM concentration, respectively. Also compounds containing a
The pyridyl moiety in the side chain showed promising inhibition. In general, similar results to those obtained in the cell based assay were obtained by Poirier et al.,\textsuperscript{116} who evaluated compounds of type 46-48 against purified human 17\(\beta\)-HSD1 (Table 1). The best inhibitor of the purified enzyme was found to be the 16\(\beta\)-benzyl substituted E1 derivative 48b with an IC\textsubscript{50} value of 0.8–1.0 \(\mu\)M. Docking of 48b into the active site of 17\(\beta\)-HSD1–E2 complex (PDB code 1IOL) did not reveal any significant interactions between the benzyl moiety at C16 and the catalytic triad, but the E2 core was slightly shifted enabling the phenyl group to approach the hydrophobic region formed by Leu96 and Val196.\textsuperscript{116} Whether the 17\(\beta\)-HSD1 inhibition with compounds 46a-46i is reversible or irreversible has not been investigated. 16-Methyleneestrone 24, which Poirier et al. used as a lead and reference compound, nevertheless, is a known 17\(\beta\)-HSD1 alkylating agent.\textsuperscript{86} It is not likely that series 47 and 48 are irreversible inhibitors as they lack a suitable electrophilic moiety.

Potter and co-workers\textsuperscript{113} continued to explore the C16 substitution by incorporating carboxyl or methyl carboxyl side chains to E1 and E2 (compounds 50-52, Figure 11, Table 2). These compounds can be synthesised by alkylating O-3 protected E1, e.g. by using LDA and bromoethylacetate, which produces the ethyl estrone-16-acetate 51b. Compounds bearing amide sidechains were synthesised from the carboxylic acid derivatives 50a and 51b by standard amine coupling reactions or on a solid phase employing an oxime resin.

![Figure 11. Selected examples of C16 carboxyl and C16 methylene carboxyl derivatives of E1 and E2 tested against 17\(\beta\)-HSD1 as mixture of diastereomers.](image-url)
Table 2. Selected examples of C16 alkenyl E1 and E2, and 16β-alkyl E2 derivatives, and their biological activity. Dashed lines indicate the point of attachment.

<table>
<thead>
<tr>
<th>R</th>
<th>Inhibition% at 10 μM</th>
<th>Inhibition% at 1 μM</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17β-HSD1⁶</td>
<td>17β-HSD2⁷</td>
<td>17β-HSD1</td>
</tr>
<tr>
<td>a</td>
<td>OH</td>
<td>39</td>
<td>13</td>
</tr>
<tr>
<td>b</td>
<td>O</td>
<td>97</td>
<td>67</td>
</tr>
<tr>
<td>c</td>
<td>H</td>
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<tr>
<td>f</td>
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<td>82</td>
<td>91</td>
</tr>
<tr>
<td>g</td>
<td>N</td>
<td>87</td>
<td>85</td>
</tr>
<tr>
<td>h</td>
<td>N</td>
<td>90</td>
<td>83</td>
</tr>
<tr>
<td>i</td>
<td>N</td>
<td>85</td>
<td>45</td>
</tr>
<tr>
<td>j</td>
<td>N</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>k</td>
<td>N</td>
<td>91</td>
<td>95</td>
</tr>
<tr>
<td>l</td>
<td>N</td>
<td>82</td>
<td>&lt;10</td>
</tr>
<tr>
<td>m</td>
<td>N</td>
<td>58</td>
<td>14</td>
</tr>
</tbody>
</table>


In the C16 carboxyl series 50a-50k, the E2 derivatives bearing an amide moiety in the side chain showed promising inhibition and excellent selectivity to 17β-HSD1 over 17β-HSD2 (Table 2). In T-47D cells the 17β-HSD1 inhibition ranged between 70% and 91% when measured at 10 μM concentration as a mixture of diastereomers. The C16 methylene carboxyl derivatives 51a-51m of E1 were generally equally or more potent than the corresponding E2 carboxyl derivatives 50a-50k. The m-pyridyl derivative 51k, as a 2:1 mixture of diastereomers, was discovered to be a strong and selective inhibitor of 17β-HSD1 with a low IC₅₀ value of 37 nM.¹¹⁴ The major isomer 16β of 51k was isolated and found to have the same inhibitor potency.¹¹³ The corresponding E2
derivatives 52a-52f showed reduced activity indicating that the E1 core is preferred. However, only inhibitors with aliphatic amide side chains were reported.

With a view to reducing the possible estrogenic effects of the potent inhibitor 51k, synthesis of the corresponding C2 methoxy and C2 ethyl derivatives 55 and 53k (Figure 12) was undertaken. Estrogen derivatives bearing substituents at C2 position have reduced estrogenic activity, and in silico studies have suggested that substitution at C2 disrupts key hydrophobic interactions between E1 and the binding site of ERα. The 2-methoxy derivative 55 (mixture of diastereomers) showed one–eight the inhibitory activity of 51k, whereas the enantiopure 16β 2-ethyl derivative 53k exhibited enhanced activity with an excellent IC₅₀ value of 27 nM (Figure 12). The substitution at C2 also improved selectivity between the isoforms, and 53k did not show any inhibition of 17β-HSD2. When the ethyl moiety was introduced to the other C16 acetamide derivatives, an additive effect on biological activity was only observed with the o-pyridyl derivatives 53l (Table 2). Surprisingly, the C2 ethyl derivatives bearing an aliphatic amide side chain were inactive, possibly due to a change in the inhibitor binding conformation.

![Figure 12. The effect of substitution at C2 position on 17β-HSD1 inhibition.](image)

The addition of a 3-O-sulfamoyl to 53k significantly reduced the inhibitory activity, compound 54k having an IC₅₀ value of 5.1 μM. It was postulated that the sulfamate group could act as a larger hydrogen bond acceptor/donor moiety, or possibly as a prodrug for the free phenol at C3 position. In addition, the 3-O-sulfamate E1 based inhibitors (54) could reversibly bind to carbonic anhydrase II enzyme in red blood cells and in this way be transported to tumours. In general, the 3-O-sulfamate series 54 (Figure 11, Table 2) exhibited only weak or moderate activity suggesting loss of a hydrogen bond interaction, e.g. with His221 and Glu282, or loss of hydrophobic interactions to the ethyl group at C2. It should be noted that except for 53k, which was a pure 16β-isomer, the C2 ethyl libraries 53 and 54 were approximately a 3:1 mixture of α/β diastereomers. The phenol and sulfamate libraries 53 and 54 were synthesised from 2-ethylestrone in seven steps by a parallel solid-phase methodology utilising a 2-chlorotrityl chloride resin.
Docking of the best two inhibitors, 51k and 53k, into the active site of 17β-HSD1–E2–NADP⁺ complex (PDB code 1FDT) suggested a binding mode where the steroid core of the inhibitor was closely overlaid with E2. The side chain pyridyl m-nitrogen in 51k and 53k was close to the phosphate oxygen of NADP⁺. The proximity of the side chain C16 methylene with the cofactor pro(S) hydrogen could cause interference with the hydrogen transfer to the C17 carbonyl and prevent these inhibitors from acting as substrates. Compound 53k exhibited additional hydrophobic interaction involving the ethyl group at C2, which could have contributed to the slightly better inhibitory activity than for the desethyl analogue 51k. The inhibitory data for few selected compounds without a substituent at C2 or O-3 position was utilised to create a QSAR model with reasonable predictive power. This study is one of only two small QSAR studies with 17β-HSD1 reported so far.

These extensive studies by Potter and co-workers have confirmed that good inhibitory activity and selectivity can be obtained by introducing a large and flexible side chain to the C16 position of E1. The inhibition measurements were performed using T-47D breast cancer cells which represent the physiological situation and show that these types of inhibitors are able to pass through the cell membrane. Although the C2 substituted derivatives were synthesised with a view to reducing the possible estrogenicity of these inhibitors, actual data regarding estrogenic effects were not reported. 2-Ethyl-3-hydroxy-17-oxo-N-(3-pyridinylmethyl)-(16β)-estra-1,3,5(10)-triene-16-acetamide 53k (IC₅₀ value 27 nM) is one of the most potent 17β-HSD1 inhibitors reported to date and has the lowest IC₅₀ value obtained with a cell based assay.

### 2.2.5 Derivatives of E1 and 1,3,5(10)-estratrien-3-ol substituted at C15

Continuing to explore substitution on the D ring of E1, Solvay Pharmaceuticals has patented C15 substituted E1 and O-3 methyl E1 derivatives as 17β-HSD1 inhibitors. This group of inhibitors includes compounds with an amide, sulfonamide, urea, carbamate or hydroxyl side chain connected via an alkyl linker to the C15 position of E1 or 3-methoxyestrone. In a further patent, more inhibitors bearing C15 amide side chains were reported with additional substituents at C2 and modifications of the steroid core. In general, these C15 substituted E1 derivatives were synthesised in steps from the O-3 protected 15,16-unsaturated estrones. Michael addition of different nucleophiles bearing, for example, an ester or nitrile moiety, produced C15 side chains that could be further functionalised.

Representative inhibitor structures 56-65 and their rec17β-HSD1 inhibitory activity in a protein based assay are shown in Table 3. The most potent of these inhibitors was N-cyclooctyl-3-methoxy-17-oxo-estra-1,3,5(10)-triene-15β-propanamide 56a, producing 85% and 95% 17β-
Table 3. Selected examples of C15 substituted E1 and estrien derivatives patented by Solvay Pharmaceuticals, and their rec17β-HSD1 inhibitory activity.$^{119,120}$

<table>
<thead>
<tr>
<th></th>
<th>100 nM</th>
<th>1 µM</th>
<th>100 nM</th>
<th>1 µM</th>
<th>100 nM</th>
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<tr>
<td>56a</td>
<td>85</td>
<td>95</td>
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<td>57a</td>
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<td>57d</td>
<td>3</td>
<td>27</td>
<td>61</td>
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<td>76</td>
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</table>
HSD1 inhibition at 100 nM and 1 μM concentration, respectively. These results demonstrate that a hydroxy group at C3 of the steroid core is not essential for the binding of these inhibitors. Besides the amide side chain (e.g. in 56a and 57a), also the other side chain types, bearing alkyl, aromatic or heterocyclic moieties, were potent inhibitors, the differences in biological activity being more apparent at the lower, 100 nM test concentration. In general, 15β-configuration seemed to be favoured, with the exception of the sulfoamides (e.g. compound 62), where only the 15α substitution was reported. Compound 57a bearing a 4-(4-morpholinyl)-4-oxobutyl side chain at the C15 β-position inhibited rec17β-HSD1 activity 50% at 100 nM concentration. The efficacy of compound 57a was additionally demonstrated in vivo in a nude mouse model. Inoculation of the nude mice with MCF-7 tumour cells expressing human 17β-HSD1 led to the growth of hormone-dependent tumours. Four-week subcutaneous administration of 57a (5 μM/kg/day) resulted in 60% reduction in tumour size as compared with the non-treated controls.

Introduction of an additional n-butyl or a methoxy or an ethoxy group to the C2 position of these E1 derivatives appeared to reduce the inhibitory activity slightly. Replacement of the C3 hydroxy or methoxy group in 57c with a sulfate or sulfamate moiety was not tolerated and the inhibitory activity was lost (compounds 57d and 57e). On the other hand, increased activity was obtained with compounds 63, 64a and 65 in which the E2 core was changed to 2H-estra-1,3,5(10)-trieno[17,16-c]pyrazol-3-ol 66 or to 17,17-difluoro-estra-1,3,5(10)-trien-3-ol 78. These compounds are known to be potent 17β-HSD1 inhibitors (see below).

Compounds of type 56-65 were patented by Solvay as selective and non-estrogenic or antiestrogenic 17β-HSD1 inhibitors. However, actual experimental data regarding the selectivity or estrogenic effects of these compounds has not been reported, except for compound 57a which did not show estrogenic activity in a rat uterus weight test.

2.2.6 Ring E modified steroids: 1,3,5(10)-estratrien-[17,16-c]-pyrazoles

Sweet et al. discovered that heterocyclic analogues of E1 with a pyrazole or an isoxazole fused to 16,17-position on ring D were competitive inhibitors of purified placental 17β-HSD1. Estra-1,3,5(10)-trieno[17,16-c]pyrazol-3-ol 66 (Figure 13) was the best inhibitor, with a Ki value of 4.08 μM when the oxidation of E2 was measured. The corresponding isoxazole derivative was only one-seventeenth as potent. Methylation of the hydroxy group at C3 position caused a 3- to 6-fold decrease in the affinity of the compounds, including E1.
Figure 13. 16-(Hydroxymethylene)estrones as bioisosteres of fused ring E.

Potter and co-workers utilised the pyrazole 66 and 16-(hydroxymethylene)estrone 67 as lead compounds to develop a series of N1’, N2’ or 5C’ substituted estra-1,3,5(10)-trieno[17,16-c]pyrazoles as 17β-HSD1 inhibitors (Figure 13, Table 4). In T-47D cells 16-(hydroxymethylene)estrone 67 (IC\(_{50}\) value 0.11 μM) and its alkylated derivatives 68 and 69 showed good selectivity and promising inhibition of 17β-HSD1. In view of the plausible intramolecular hydrogen bonding, they were postulated to be bioisosteres of a fused steroidal ring E (Figure 13). Similarly, 16-oximeoestrone was a good and selective inhibitor with an IC\(_{50}\) value of 1.1 μM. In this cell based assay the pyrazole 66, as a mixture of 1’-H and 2’-H tautomers, had an IC\(_{50}\) value of 0.18 μM and at 10 μM concentration was able to inhibit 97% of the enzymatic activity. The corresponding [17,16-c]-pyrazolone\(^{113}\) and [17,16-c]-pyrimidine\(^{117}\) analogues were slightly less potent, with 86% and 78% inhibition, respectively. Reduced activity was also observed with an addition of C6 carbonyl to 66 and 67.\(^{113}\)

The N1’ alkylated estra-1,3,5(10)-trieno[17,16-c]pyrazol-3-ols 70a-70e at 10 μM concentration proved to be good inhibitors of 17β-HSD1 in T-47D cells, the inhibition ranging from 77% to 95%.\(^{117}\) In general these compounds exhibited only moderate selectivity over 17β-HSD2 (Table 4). For example, the N-methoxyethyl derivative 70d had an IC\(_{50}\) value of 0.53 μM but lacked sufficient selectivity over 17β-HSD2. The corresponding N2’ alkylated derivatives 71a-71d were slightly less potent 17β-HSD1 inhibitors. C5’ alkylation of estra-1,3,5(10)-trieno[17,16-c]pyrazol-3-ols produced the ethyl ester 72a and hydroxymethyl 72b derivatives with IC\(_{50}\) values of 1.85 μM and 0.95 μM, respectively.\(^{117}\) Unlike the N-alkylated derivatives, 72a and 72b displayed excellent selectivity over 17β-HSD2. In a luciferase reporter gene assay, 66 was shown to be estrogentic already at 100 pM concentration, whereas the N-methoxyethyl derivative 70d did not show any estrogentic activity up to 100 nM concentration, and in silico evaluation of 70d indicated that it is unlikely to bind to ERα.\(^{117}\) The pyrazole derivatives 70-71 can be synthesised as a separable mixture by alkylation of O-3-Bn protected 66 with NaH and an alkylhalide, whereas 66 and 72a-72d are synthesised starting from the corresponding 16-(hydroxymethylene)estrone in a cyclisation reaction with NH\(_2\)NH\(_2\).\(^{117}\)
Table 4. N1’, N2’ and C5’ alkylated estr-1,3,5(10)-trieno[17,16-c]pyrazol-3-ols and their biological activity.\textsuperscript{117}

![Chemical structures](image)

<table>
<thead>
<tr>
<th>R</th>
<th>Inhibition % at 10 (\mu)M</th>
<th>IC\textsubscript{50} ((\mu)M)</th>
<th>Inhibition % at 10 (\mu)M</th>
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<tr>
<td></td>
<td>17(\beta)-HSD Type 1\textsuperscript{a}</td>
<td>17(\beta)-HSD Type 1</td>
<td>17(\beta)-HSD Type 2</td>
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<tr>
<td>H</td>
<td>66\textsuperscript{c}</td>
<td>97</td>
<td>32</td>
</tr>
<tr>
<td>CH\textsubscript{3}</td>
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<td>94</td>
<td>24</td>
</tr>
<tr>
<td>CH\textsubscript{2}CH(CH\textsubscript{3})\textsubscript{2}</td>
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<td>77</td>
<td>61</td>
</tr>
<tr>
<td>CH\textsubscript{2}CO\textsubscript{2}CH\textsubscript{3}</td>
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\textsuperscript{[a]} Measured in T-47D human breast cancer cell line. \textsuperscript{[b]} Measured in MDA-MB-231 human breast cancer cell line. \textsuperscript{[c]} A mixture of 1’-H and 2’-H tautomers.

Previous studies with C16 substituted E1 derivatives had indicated beneficial interactions between amide side chain and the cofactor (see section 2.2.4).\textsuperscript{113} This approach also proved successful in the C5’ substituted compounds 74a-74g (Table 5).\textsuperscript{117,122} 3-Hydroxy-N-[2-(3-pyridinyl)ethyl]-1’H-estra-1,3,5(10)-trieno-[17,16-c]-pyrazole-5’-carboxamide 74f was found to be highly selective and the most potent C5’ substituted inhibitor, with an IC\textsubscript{50} value of 0.30 \(\mu\)M. Also other compounds containing a pyridyl moiety in the C5’ side chain were found to be potent 17\(\beta\)-HSD1 inhibitors. However, primary aliphatic amides showed only moderate inhibitory activity. Addition of an ethyl group to C2 position of the steroid core, an approach which also previously has been propitious, led to decreased activity, as did the addition of O-3-sulfamoyl (Table 5, compounds 75-76).\textsuperscript{115}
Table 5. Selected examples of estra-1,3,5(10)-trieno[17,16-c]pyrazol-3-ols bearing a C5’ amide side chain, and their biological activity.\textsuperscript{115,117} Dashed line indicates the point of attachment.

| R | \begin{tabular}{c|c|c|c|c|c|c} 
 & \text{Inhibition\% at 10 \textmu M} & \text{IC\textsubscript{50}} & \text{Inhibition\% at 1 \textmu M} & \text{IC\textsubscript{50}} \\
\hline
<table>
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</thead>
<tbody>
<tr>
<td>(17\beta)-HSD\textsuperscript{a}</td>
<td>(17\beta)-HSD\textsuperscript{b}</td>
<td>(17\beta)-HSD1</td>
<td>(17\beta)-HSD1</td>
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<td></td>
</tr>
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<td>\text{a}</td>
<td>(\text{N}^{1})</td>
<td>88</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\text{b}</td>
<td>(\text{N}^{1}\text{N}^{2})</td>
<td>89</td>
<td>-4</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>\text{c}</td>
<td>(\text{N}^{1}\text{N}^{2})</td>
<td>80</td>
<td>61</td>
<td></td>
<td>(\text{ni}^{c})</td>
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<td>(\text{N}^{1}\text{N}^{2})</td>
<td>92</td>
<td>28</td>
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<td>17</td>
</tr>
<tr>
<td>\text{e}</td>
<td>(\text{N}^{1}\text{N}^{2})</td>
<td>93</td>
<td>9</td>
<td>0.88</td>
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<td>(\text{N}^{1}\text{N}^{2})</td>
<td>99</td>
<td>3</td>
<td>0.30</td>
<td>0.7</td>
</tr>
<tr>
<td>\text{g}</td>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>68</td>
</tr>
</tbody>
</table>

\textsuperscript{[a]} Measured in T-47D human breast cancer cell line. \textsuperscript{[b]} Measured in MDA-MB-231 human breast cancer cell line. \textsuperscript{[c]} No inhibition.

The binding modes of the \(N1’\) methoxyethyl derivative 70\text{d}, the most potent C5’ amide 74\text{f} and its C2 ethyl analogue 76\text{f} have been investigated by docking the structures into the active site of \(17\beta\)-HSD1–E2–NADP\textsuperscript{+} ternary complex (PDB code 1FTD).\textsuperscript{115,117} The steroid cores of 70\text{d} and 74\text{f} in the ligand binding cavity were overlaid and they formed the same hydrophobic interactions as E2. For both compounds, a possible favourable electrostatic interaction was suggested between the hydrogen bond acceptor in the side chain (ether oxygen or amide carbonyl) and the primary amide in NADP\textsuperscript{+}. In 76\text{f} the ethyl group at C2 was in a hydrophobic region and the hydroxy group at C3 was hydrogen bonded with His221, as in E2. In the best binding mode for 76\text{f} the pyrazole ring nitrogens were 1.9-2.1 Å from the NADP\textsuperscript{+} pro(S) hydrogen, and the C5’ amide carbonyl was in close proximity (3 Å) to the amide moiety in NADP\textsuperscript{+} possibly forming \(\pi-\pi\) interactions. The \(m\)-pyridyl moiety extended to the phosphate
region of NADP⁺, which could further aid the binding. Compound 76f was also docked into the ligand binding cavity of ERα (PDB code 3ERD), where a poor fit was observed indicating that this compound is probably not strongly estrogenic. In addition to the docking studies, comparative molecular field analysis (CoMFA) was used for few C5’ substituted pyrazole derivatives to create a QSAR model with reasonable prediction of biological activity.117

2.2.7 Fluorinated derivatives of 1,3,5(10)-estratrien-3-ol and gona-1,3,5(10)-trien-3-ol

The drug discovery efforts described above were mainly concentrated on introducing rather large and flexible substituents to the estrie ring D or to the heterocyclic ring E. The early work with steroidal 17β-HSD1 inhibitors had already established that relatively good inhibitory activity can be achieved with subtle changes in the estrien core.65 Adamski and co-workers123 recognised that good 17β-HSD1 inhibition can be obtained with fluorine substituted estriens. In theory, the polar non-hydrolysable fluorine groups at C17 imitate the transition state of the enzymatic reaction; fluorine, as a hydrogen bond acceptor, potentially mimics the carbonyl or hydroxyl group.

Fluorinated 1,3,5(10)-estratrien-3-ols 77-80 and gona-1,3,5(10)-trien-3-ols 81-82 were shown to inhibit various isoforms of 17β-HSD.123 In this compound class (Figure 14), 7,17,17-trifluoro-(7α)-estra-1,3,5(10)-trien-3-ol 77 was the most potent inhibitor of human rec17β-HSD1 with 74% inhibition at 2 µM concentration. Compound 78, which lacks the 7α-fluorine, showed slightly reduced activity. An introduction of a C2 methoxy or C2 ethoxy substituent to 78 further decreased the activity, and addition of a 16α-hydroxy group led to complete loss of the activity. Most of these fluorinated steroids were unselective 17β-HSD1 and 17β-HSD2 inhibitors, 77 showing some selectivity towards the type 1 enzyme. The C18 methyl group in gona-1,3,5(10)-trien-3-ols 81-82 seemed to promote 17β-HSD2 inhibition.

![Figure 14](image)

**Figure 14.** Examples of the most potent fluorinated estrien derivatives and their 17β-HSD1 inhibition% at 2 µM. The inhibition% of the oxidative 17β-HSD2 is given in parenthesis.123
As postulated, docking of 77 to the 17β-HSD1–E2–NADP⁺ complex (PDB code 1FTD) suggested a hydrogen bond between the 17β-fluorine and the catalytic residues Ser142 and Tyr155. This docking study also suggested that the 7α-fluorine occupies an additional cavity formed by Pro187, Val225 and Phe226, which could explain the slightly better activity and selectivity of this compound as compared with 78.

Although most of these fluorinated derivatives showed promising inhibitory activity, the lack of selectivity between the isoforms can be considered as a major disadvantage. Also, the possible estrogenic effects of the derivatives are not clear. Nevertheless these structures are good lead compounds for further inhibitor development.

2.2.8 C2 substituted estra-1,3,5(10)-trien-3-ols and D-homo-estra-1,3,5(10)-trien-3-ols

Most of the investigations described so far have targeted the steroidal D ring as the modification site for producing 17β-HSD1 inhibition. Additional substituents were introduced to the C2 position of the estrien core to increase selectivity and reduce possible estrogenic effects. Just recently, very good 17β-HSD1 inhibition was obtained by introducing small substituents to the C2 position of E1 (Figure 15). The estrogen metabolite 2-methoxyestrone (IC₅₀ value 2.4 μM) and 2-ethylestrone 83 at 10 μM concentration inhibited 17β-HSD1 activity 91-96% in T-47D cells. The inhibition by 2-methoxyestrone was unselective, as 87% inhibition of 17β-HSD2 was also observed in MDA-MB-231 cells.

Adamski and co-workers together with Schering AG have patented a series of 2-substituted estra-1,3,5(10)-triens 83-89 and D-homo-estra-1,3,5(10)-triens 90-95 as selective 17β-HSD1 inhibitors (Figure 15). In a human rec17β-HSD1 based assay, 2-chloroestrone 84a showed promising activity with an IC₅₀ value of 140 nM. The activity of 84a was increased with an addition of C18 methyl or C16 fluorine group, and 2-chloro-16β-fluoroestrone 85 with an IC₅₀ value of 35 nM was the most potent inhibitor reported among the E2 derivatives. Compounds 83, 88 and 89 also showed good inhibitory activity, indicating that bulky substituents at C2 position are well tolerated. Replacing the E2 core with a D-homoestrone core resulted in increase of activity (compounds 90-95). The best inhibitors were 2-(2-phenylethyl)-17a-oxo-17α-homoestra-1,3,5(10)-trien-3-ol 95 and 2-allyl-17a-oxo-17α-homoestra-1,3,5(10)-trien-3-ol 94 with IC₅₀ values of 15 nM and 24 nM, respectively. This makes them the most potent steroidal 17β-HSD1 inhibitors reported to date. The D-homo-estra-1,3,5(10)-triens 90a-90c bearing a halogen in the C2 position were also very potent inhibitors. Unlike in the E2 series an introduction of 17α-fluorine, as in 92, slightly decreased the activity, and 17β-fluorine derivative 91 was even less potent. Also 2-methoxy-17a-oxo-17α-homoestra-1,3,5(10)-trien-3-ol 93 showed
Figure 15. Examples of C2 substituted estra-1,3,5(10)-trien-3-ols and D-homo-estra-1,3,5(10)-trien-3-ols and their biological activity in rec17β-HSD1 based assay.\textsuperscript{124,125}

good activity, with an IC\textsubscript{50} value of 85 nM. For comparison, an IC\textsubscript{50} value of 109 nM was measured for the substrate E1.

Neither estrogenicity nor selectivity data was reported for these E1 and D-homoestrone derivatives. The C2 substitution is likely to prevent or reduce estrogenic effects. However, 2-methoxyestrone has been shown to be an equally potent 17β-HSD2 inhibitor,\textsuperscript{66} and one may suspect that the other compounds bearing solely a small substituent at C2 are unlikely to be selective. Nevertheless this approach elegantly confirms that strong 17β-HSD1 inhibition can be achieved with only subtle changes in the steroid core.
2.2.9 Substrate–cofactor hybrid inhibitors

Poirier and co-workers\(^{126}\) were the first to propose substrate–cofactor hybrid compounds as 17β-HSD1 inhibitors, \textit{i.e.} inhibitors interacting with both cofactor and steroid binding sites. Their hypothesis was that an ideal hybrid inhibitor should possess a complementary hydrophobic moiety, such as E2, to interact with the ligand binding domain of 17β-HSD1, and an additional polar moiety to interact with the hydrophilic cofactor binding site (Figure 16). This approach was first explored with compounds of type 96 where E2 was linked to a natural amino acid via a 16β-aminopropyl spacer. Although these amino acid derivatives of E2 failed to inhibit 17β-HSD1 activity, affinities towards steroid receptors were not detected either. In a subsequent study,\(^{127}\) analogues of 96 bearing a R\(^2\) methyl group and R\(^1\) benzyl group, were shown to be moderate 17β-HSD1 inhibitors.

![Figure 16](image)

*Figure 16. "Hybrid" inhibitors of 17β-HSD1 and the general structure of the non-active E2-amino acid derivatives.\(^{126}\)*

Further elucidation of the concept of dual-site inhibition by means of molecular modelling suggested E2-adenosine hybrid compounds as potential inhibitors.\(^{31}\) These E2-adenosine hybrids\(^{128}\) were found to be strong reversible competitive inhibitors of 17β-HSD1 in transfected HEK-293 cells.\(^{129}\) EM-1745 97 had an IC\(_{50}\) value of 52 nM and a \(K\)\(_i\) value of 3.0 nM, and was found to have a methylene linker of optimal length (8 carbons) between E2 and the adenosine moiety (Figure 17). In addition, the 16β-configuration was shown to be preferred. Neither 5-nonaoyl-O-adenosine nor 16β-nonylestradiol was an inhibitor, demonstrating the importance of both the E2 and adenosine moieties in 97. Poor results were also obtained when adenosine was replaced, for example, with N-adenine, N-methyl-N-butyl carbamide or hydroxymethyl group.
The binding mode of the hybrid inhibitor 97 was confirmed by resolving the X-ray crystal structure of 17β-HSD1–EM-1745 complex to 1.6 Å.\textsuperscript{31} Up to 40 Van der Waals contacts and several plausible hydrogen bond interactions were found between 97 and the substrate and the cofactor binding sites (Figure 17). Superimposition of complexes 17β-HSD1–EM-1745 (PDB code 1I5R) and 17β-HSD1–E2–NADP\textsuperscript{+} (PDB code 1FTD) revealed identical positioning of the E2 core in the two enzyme complexes.

Figure 17. Hybrid inhibitor EM-1745 and the plausible hydrogen bond interactions found from the 17β-HSD1–EM-1745 complex, and the structure of simplified hydrid inhibitors bearing an adenosine mimic.\textsuperscript{31,130}

Since 97 is not a drug-like molecule, simplified hybrid inhibitors were subsequently synthesised to improve bioavailability and \textit{in vivo} stability. The adenosine moiety was replaced with substituted aniline linked to E2 via saturated alkyl chain to produce compounds 98a–98c (Figure 17).\textsuperscript{130} Compound 98b bearing a carboxylic acid moiety in the aniline ring showed 90% inhibition at 1 \textmu M concentration, thus exhibiting approximately the same potency as EM-1745 97. Compounds 98a and 98c were less potent, with only 32% and 16% inhibition, respectively, at 1 \textmu M concentration. Carboxylic acid is a bioisostere of the NADPH phosphate, which could explain the better inhibitory potency of 98b. Compound 98b was less active at lower (0.1 \textmu M) concentration, providing only 56% inhibition as compared with the 90% inhibition achieved with 97 at the same concentration.
2.3 Non-steroidal inhibitors of 17β-HSD1

Drug discovery efforts to date have mostly concentrated on modifications of steroidal structures; much less work has been published on non-steroidal inhibitors of 17β-HSD1. As compared with inhibitors based on steroidal structures, non-steroidal compounds could have advantages of synthetic accessibility, drug-likeness, selectivity and non-estrogenicity.

2.3.1 Organotin compounds and carotenoids

Although the investigations to be described below are not connected with drug discovery efforts, they demonstrate the versatility of studies related to 17β-HSD1 inhibition. Organotin compounds are environmental contaminants suspected of being endocrine disruptors. Triphenyltin (Ph$_3$SnH), the major organotin in human blood, exhibits dose-dependent inhibition of placental 17β-HSD1 with an IC$_{50}$ value of 10.5 μM. Other sex steroid metabolising enzymes are inhibited to greater extent, e.g. 17β-HSD3 and aromatase with IC$_{50}$ values of 4.2 μM and 1.5 μM, respectively. It was proposed that organotins interact with cysteine residues, and this was supported by the compensatory effect of dithioerythritol (2R,3S-1,4-dimercapto-2,3-butanediol) on the triphenyltin induced inhibition. Contrary to this, a more recent study showed 17β-HSD1 reductive activity and mRNA transcription in human choriocarcinoma JAr cells to be enhanced by Ph$_3$SnH and other organotins, and thus Ph$_3$SnH at 10-100 nM concentration to be a potential stimulator of E2 biosynthesis in human placenta.

Palm oil carotenoid concentrate, which mainly consists of α-carotene (41.9%) and β-carotene (51.7%), was found at 1 μM concentration to inhibit E1 to E2 conversion by approximately 67% in MCF-7 cell line. On the other hand, at 10 μM concentration the concentrate had a strong stimulatory effect on the reductive activity. The oxidative activity (E2 to E1) was not affected. Trans-retinoic acid showed no inhibitory activity against stimulated E2 formation. It should be noted that inhibition of 17β-HSD1 activity by carotenoids may occur through an anti-oxidant effect on the co-factors.

2.3.2 Phytoestrogens and related compounds

After steroid derivatives, phytoestrogens are the most studied 17β-HSD1 inhibitor class. Phytoestrogens are a group of natural phenolic compounds, including flavonoids, isoflavonoids, coumestans and lignans, which possess a wide variety of biological activities (Figure 18).
Phytoestrogens can be obtained from diet, e.g. from vegetables and berries, and are currently of interest as dietary supplements. A number of phytoestrogens have been demonstrated to inhibit various isoforms of 17β-HSD,\textsuperscript{135,136} as well as other steroid metabolising enzymes, in several biological assays. Some contradictory results have nevertheless been reported.

Mäkelä and co-workers\textsuperscript{137,138,139} have evaluated several phytoestrogens and related compounds as 17β-HSD1 inhibitors, using purified placental 17β-HSD1 and T-47D cell lines (Table 6). Coumestrol 99 at 1.2 μM and 0.12 μM concentration was the most promising the inhibitor of the purified enzyme with 82% and 51% 17β-HSD1 inhibition,\textsuperscript{*} respectively.\textsuperscript{138} Unfortunately coumestrol was also found to be highly estrogenic. In general, flavones (100) were better 17β-HSD1 inhibitors than flavanones (101), which in turn were better than the corresponding isoflavones (102). Of the 14 flavones tested at 1.2 μM concentration, kaempferide 100a, acacetin 100b and fisetin 100c showed best activity against 17β-HSD1 with 73-53% inhibition.\textsuperscript{139} At the same concentration the isoflavones genistein 102a and daidzein 102b showed moderate 32-37% inhibition. The inhibitory activities were similar, although less effective, in wild type T-47D and 17β-HSD1 cDNA transfected T-47D\textsubscript{21} cells. Hydroxy groups at C5 and C7, and a hydroxy or methoxy group at C4\textsuperscript{*} seemed to be important structural features for the biological activity (Table 6). Mycotoxin zearalenone 103, coumarin and diethylstilbestrol 11, all potent estrogens, did not show inhibition. Plant sterols β-sitosterol and β-sitostanol exhibited moderate activity against purified 17β-HSD1 with approximately 31% inhibition at 1.2 μM concentration.\textsuperscript{138}

\textsuperscript{*}Mäkelä and co-workers reported the biological activity as E1 to E2 conversion%, which here is converted to inhibition% (inhibition% = 100 – conversion%).
Table 6. The most potent phytoestrogens and their 17β-HSD1 inhibition. [138,139,141,143]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Substitution pattern$^a$</th>
<th>Inhibition%$^b$</th>
<th>IC$_{50}$$^c$</th>
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<td>Coumestran</td>
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<tr>
<td>100a</td>
<td>Flavone</td>
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<td>100b</td>
<td>Flavone</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>100h</td>
<td>Flavone</td>
<td>OH OH OH OH</td>
<td>-11 ± 5</td>
<td>0.6</td>
</tr>
<tr>
<td>101a</td>
<td>Flavanone</td>
<td>OH OH OH OH</td>
<td>48 ± 11</td>
<td>15</td>
</tr>
<tr>
<td>102a</td>
<td>Isoflavone</td>
<td>OH OH OH OMe</td>
<td>37 ± 30</td>
<td>1.0</td>
</tr>
<tr>
<td>102b</td>
<td>Isoflavone</td>
<td>OH OH OMe</td>
<td>32 ± 10</td>
<td>10</td>
</tr>
</tbody>
</table>

[a] For the numbering and structure of the flavonoid skeleton see Figure 18. [b] Measured on purified placental 17β-HSD1. Inhibition% = 100 – conversion%. [c] Measured on human microsomal 17β-HSD1.

Genistein 102a and coumestrol 99 showed some selectivity towards 17β-HSD1, whereas the flavones kaempferol 100f, kaempferide 100a and galangin 100d were not selective, also inhibiting 17β-HSD2 activity in PC-3 prostate cancer cells. [139]

Studies on phytoestrogens and 17β-HSD1 inhibition have also been performed with use of placental microsomes. Le Lain et al. [140] found flavonoids to be weak inhibitors (IC$_{50}$ values >18 μM) of microsomal 17β-HSD1 type activity. In more recent studies by Le Bail and coworkers, [141,142,143] flavonoids and coumestrol 99 showed relatively high inhibitory activity against microsomal 17β-HSD1 from human placenta (Table 6). Again coumestrol 99 was the most potent phytoestrogen with an IC$_{50}$ value of 0.2 μM; medicarpin 104 with a related structure had an IC$_{50}$ value of >50 μM. In the flavonoid series the hydroxy group at C7 was essential for the activity, as 7-methoxyflavone was inactive. Apigenin 100e was the most potent flavonoid with an IC$_{50}$ value of 0.3 μM. In addition, the inhibitory activities of 12 chalcones (105) lacking the heterocyclic ring C were measured. [142] 4-Hydroxychalcone was the most potent chalcone with a moderate IC$_{50}$ value of 16.0 μM. Placental microsomes can be considered an unusual assay for 17β-HSD1 since they are usually associated with 17β-HSD2 expression and activity; [144] on the other hand they enable simultaneous measurement of aromatase inhibition. [143]

In MCF-7 cells, which are known to have low 17β-HSD1 expression, [46,59] genistein 102a and mammalian lignan enterolactone 106, but not enterodiol, showed 59% and 84% 17β-HSD1
inhibition, respectively, at high 50 μM concentration. Also the commercial aromatase inhibitor 4-hydroxyandrostene-3,17-dione exhibited weak 17β-HSD1 inhibition. In another study, 24 h exposure of MCF-7 cells to 100 nM of genistein 102a did not affect the conversion of E1 to E2. However, 48 h exposure resulted in elevated 17β-HSD2 activity and expression. Genistein 102 and apigenin 100e also weakly inhibit 17β-HSD1 activity in primary cultures of human granulosa-luteal cells. Surprisingly, synthetic 7-hydroxyflavones with small substituents at C4’ (e.g. halogen, Me, -OME, -NH₂, -NO₂) as well as genistein 102b failed to show any 17β-HSD1 inhibitory activity in human placental cytosol preparations at high 100 μM concentration.

In conclusion, several studies have established that phytoestrogen structures, especially coumestrol 99 and some flavonoids, are capable to bind to the active site of 17β-HSD1 and inhibit the enzyme activity. Although the estrogenic nature and inadequate selectivity of these

It has recently been proposed that 17β-HSD from the fungus Cochliobolus lunatus (17β-HSDcl) could act as a model enzyme for the SDR superfamily, even though it possesses only 21.2% and 18.1% sequence identity with human 17β-HSD1 and 17β-HSD2, respectively. This readily available enzyme might be useful in preliminary screening of compounds in a fast and low-cost spectrophotometric assay not requiring radioactive ligands. The most potent inhibitors of the reductive pathway of 17β-HSDcl are reportedly coumestrol 99, kaempferol 100f and flavones hydroxylated at C3, C5, C7 and C4’ positions (IC₅₀ values 1.2–4.8 μM). This substitution pattern was also preferred in 17β-HSD1 inhibition, although molecular modelling studies for kaempferol 100f suggested a different binding orientation from that of human 17β-HSD1. Possible applications of 17β-HSDcl in identifying new 17β-HSD1 inhibitors remain to be clarified.

In silico approaches have been used to investigate the binding modes of phytoestrogens to 17β-HSD1. When kaempferol 100f was docked into 17β-HSD1–equilin complex (PDB code 1EQU) it occupied the steroid binding domain. The hydroxy group at C7 pointed towards the catalytic region (Tyr155 and Ser142), and the C4 carbonyl and C5 hydroxy groups were facing Tyr218 and Ser222. The hydroxy group at C4’ was in close proximity to Glu282 and His221, which was suggested to explain why 4’-hydroxyflavones like 100f are good inhibitors in the flavonoid series. Coumestrol 99, docked into the 17β-HSD1–E2–NADP⁺ complex (PDB code 1FDT), superimposed well with E2, and the peripheral phenolic hydroxyls in 99 were suggested to form the same hydrogen bond interactions as those in E2. This similar binding mode of coumestrol 99 with E2, with an additional interaction with Tyr218, could explain the good inhibitory activity of coumestrol. Genistein 102a and apigenin 100e were also showed to occupy the ligand binding domain. In addition to the docking study, the activity data of Mäkelä and co-workers was utilised to derive a pharmacophore for the active site of 17β-HSD1.

In conclusion, several studies have established that phytoestrogen structures, especially coumestrol 99 and some flavonoids, are capable to bind to the active site of 17β-HSD1 and inhibit the enzyme activity. Although the estrogenic nature and inadequate selectivity of these
compounds prohibits their use as 17β-HSD1 inhibitors as such, it is surprising that no true drug discovery efforts exploiting phytoestrogens as lead compounds have been reported. The approach has nevertheless proven successful in studies of aromatase inhibition.\textsuperscript{153,154}

2.3.3 Gossypol derivatives: inhibitors targeted to the Rossmann fold

Gossypol\textsuperscript{107}, a bioactive polyphenol found in cottonseed, and four of its synthetic derivatives have been tested against purified placental 17β-HSD1 (Figure 19).\textsuperscript{155} The compounds were targeted to the Rossmann fold, \textit{i.e.} the cofactor binding domain of the enzyme (see section 1.1), as gossypol and its derivatives have been shown to inhibit other enzymes binding dinucleotides.\textsuperscript{156} In nature, gossypol\textsuperscript{107} occurs as a mixture of enantiomers as a result of restricted rotation around the central C–C bond. At 25 \( \mu \text{M} \) concentration gossypol\textsuperscript{107} showed a minor (<10%) inhibitory response in the oxidative direction (E2 to E1, pH 9.2) of 17β-HSD1.\textsuperscript{155} The synthetic derivatives gossylic lactone\textsuperscript{108a} and gossylic iminolactone\textsuperscript{108b} showed enhanced activity with 80-90% inhibition and IC\textsubscript{50} values of 2.2 \( \mu \text{M} \) and 4.3 \( \mu \text{M} \), respectively, and demonstrated competitive inhibition in respect of NAD\textsuperscript{+}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{gossypolderivatives.png}
\caption{Gossypol and its synthetic derivatives.}
\end{figure}

Docking studies indicated that\textsuperscript{108a} occupies the cofactor-binding region via up to eight hydrogen bond interactions. The lactone\textsuperscript{108a} was utilised as a lead compound, and active site analysis suggested that replacing the C5 isopropyl with a butylene group linked to a substrate mimic (C and D rings of E2) would result in a probable dual-site inhibitor\textsuperscript{109}. This study\textsuperscript{155} showed that 17β-HSD1 can be inhibited by compounds targeting the cofactor binding domain. This approach is likely to suffer from lack of selectivity, however, as the Rossmann fold moiety is found in numerous other enzymes as well.
2.3.4 Phenyl ketones and derivatives of benzofuranone, benzopyranone and tetralone

Derivatives of benzofuranone 110a-110d, benzopyranone 111-112 and tetralone 113, which can be considered to structurally resemble flavonoids, have been patented as 17β-HSD inhibitors (Figure 20).157,158 These compounds inhibit NADPH dependent conversion of E1 to E2 in human placental microsomes. Inhibition of 17β-HSD1 up to 77% has been reported for compounds 110-113. The inhibitor concentration used in the activity measurements was not reported, and thus evaluation and comparison of the inhibitory potency of these compounds is not possible. In the benzofuranone series 110, R⁴ methoxy substituent seemed to be unfavoured, whereas acetylation of the phenolic hydroxy group seemed to slightly improve the activity. In general, the benzofuranones are more potent than the benzopyranone and tetralone derivatives.

![Chemical structures of benzofuranone, benzopyranone, tetralone and phenylketone based 17β-HSD inhibitors](image)

Figure 20. Examples of benzofuranone, benzopyranone, tetralone and phenylketone based 17β-HSD inhibitors.157,158,159,160 The reported inhibition% (unspecified test concentration) and IC₅₀ values are given in parenthesis.

4'-Halogenated 2-(phenylmethyl)-6-hydroxy-3,4-dihydronaphthalen-1(2H)-ones 114a-114c showed only moderate inhibition of human placental soluble and microsomal 17β-HSD1 with IC₅₀ values of 14.6-21 μM.159 These compounds were better 17β-HSD3 inhibitors, with IC₅₀ values of 1.8-8.3 μM. Phenyl ketones 115 and biphenyl ketones 116 are reportedly very weak inhibitors of 17β-HSD1 in testicular micromes.160 The best inhibitors were 1-(4-hydroxyphenyl)-1-hexanone and 1-[1,1'-biphenyl]-4-yl-1-pentanone with approximately 45% inhibition at 100 μM concentration.
Although the structures described above represent attempts to create novel non-steroidal 17β-HSD1 inhibitors, the biological activity reported, at least for 114-116, can be considered modest at best.

2.3.5 Benzothienopyrimidones

Subsequent to our findings on non-steroidal 17β-HSD1 inhibitors based on a thieno[2,3-d]pyrimidin-4(3H)-one core,161 Solvay Pharmaceuticals reported a series of 8-hydroxy-benzothieno[2,3-d]pyrimidin-4(3H)-ones with various substituents at C2 and N3 positions as 17β-HSD1 inhibitors (Table 7).40,162 Compounds 117-118, at 100 nM concentration, were found to be potent inhibitors of rec17β-HSD1 with 82-94% inhibition in a protein based assay. On the basis of docking studies it was postulated that the pyrimidinone moiety and the phenolic hydroxy group at C8 are responsible for the 17β-HSD1 inhibitory activity, while the substituents at positions C2 and N3 of the pyrimidinone ring could influence the selectivity. With 117e, for example, the hydroxy group pointed towards the catalytic region and was within close proximity of Ser142 and Tyr155, while the benzyl ring was placed close to His221.

With compounds 117a-117f, which have a trimethoxyphenyl group at position C2, variation of the R1 substitution had little influence on the inhibitory activity against rec17β-HSD1. The selectivity over rec17β-HSD2 was slightly improved with alkyl substituents. A more dramatic effect was seen in MCF-7 cells expressing 17β-HSD1, where the R1 substitution seemed to affect the cell permeability (Table 7). Overall, with compounds 117a-117f the inhibitory activity was significantly reduced in a cell based assay. Here the benzyl derivative 117d was the most potent inhibitor with 52% inhibition at 1 μM concentration. With 117f (1 μM) the inhibition was reduced to just 14%, as compared with the 95% inhibition found in a protein based assay. Varying the R3 substitution in N-benzyl derivatives 118a-118c did not affect the activity against rec17β-HSD1, but it had an impact on the selectivity as well as cell permeability. Bulky C2 substituents like the trimethoxyphenyl group in 117b and 117d seemed to improve the selectivity towards 17β-HSD1. This substitution was also favoured in the cell based assay, indicating better cell membrane permeability of these compounds. Compound 119a exhibited only weak inhibition of rec17β-HSD1, and the addition of an n-butyl or benzyl group as R5 substituent in 119c and 119d resulted in loss of the activity, but n-butyl as R4 seemed to be tolerated. An IC50 value of 5 nM was reported for compounds 117d and 117e in the recombinant enzyme assay.40
Table 7. 8-Hydroxy-benzothieno[2,3-d]pyrimidin-4(3H)-ones with variable substituents at C2 and N3 position, and their 17β-HSD1 inhibition.\textsuperscript{30,162}

![Chemical Structures]

Inhibition % of 17β-HSD1 and 17β-HSD2

<table>
<thead>
<tr>
<th>Compd</th>
<th>Rec17β-HSD1 (100 \text{ nM}^b)</th>
<th>Rec17β-HSD1 (1 \text{ μM}^b)</th>
<th>Rec17β-HSD2 (1 \text{ μM}^b)</th>
<th>17β-HSD1\textsuperscript{40} (1 \text{ μM}^b)</th>
<th>17β-HSD2\textsuperscript{162} (1 \text{ μM}^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>117a</td>
<td>86</td>
<td>95</td>
<td>26</td>
<td>38</td>
<td>31</td>
</tr>
<tr>
<td>117b</td>
<td>91</td>
<td>94</td>
<td>43</td>
<td>47</td>
<td>24</td>
</tr>
<tr>
<td>117c</td>
<td>93</td>
<td>98</td>
<td>47</td>
<td>34/21\textsuperscript{c}</td>
<td>15</td>
</tr>
<tr>
<td>117d</td>
<td>94</td>
<td>99</td>
<td>64</td>
<td>52</td>
<td>22</td>
</tr>
<tr>
<td>117e</td>
<td>87</td>
<td>95</td>
<td>47</td>
<td>67/25\textsuperscript{c}</td>
<td>20</td>
</tr>
<tr>
<td>117f</td>
<td>90</td>
<td>95</td>
<td>58</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>118a</td>
<td>83</td>
<td>89</td>
<td>89</td>
<td>15</td>
<td>55</td>
</tr>
<tr>
<td>118b</td>
<td>82</td>
<td>97</td>
<td>87</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>118c</td>
<td>89</td>
<td>95</td>
<td>77</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>119a</td>
<td>33</td>
<td></td>
<td></td>
<td>78</td>
<td></td>
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<tr>
<td>119b</td>
<td>52</td>
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<td>81</td>
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</tr>
<tr>
<td>119c</td>
<td>9</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>119d</td>
<td>5</td>
<td></td>
<td></td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

[a] MCF-7 cell line stably expressing one of the respective 17β-HSD isoforms. [b] Inhibitor concentration. [c] Two different values reported.\textsuperscript{30,162}

Modifications of the benzothieno moiety, for example in the potent inhibitor 117d, did not yield better 17β-HSD1 inhibition in the cell based assay (Figure 21).\textsuperscript{162} Acetylation or methylation of the phenolic hydroxyl at C8 gave compounds 120 and 121 with 35% and 21% inhibition at 1 μM concentration, respectively, whereas the removal of the C8 phenolic hydroxyl from 117e resulted in complete loss of the activity. The 8,9,10,11-tetrahydrobenzothieno derivatives 122-124 also showed reduced activity.
Figure 21. Modifications of the benzothieno moiety.\textsuperscript{162}

The efficacy of compound 117e has been additionally proven \textit{in vivo} in a nude mouse model. Four-week subcutaneous administration of 117e (5 \(\mu\)M/kg/day) resulted in 54\% reduction in tumour (MCF-7 cells expressing human 17\(\beta\)-HSD1) size as compared with the non-treated controls.\textsuperscript{40} In addition, compound 117e did not show estrogenic activity in a rat uterus weight test. Although compound 117e was effective \textit{in vivo} it should be noted that all the compounds tested at 1 \(\mu\)M concentration in a cell based assay showed significantly reduced activity as compared with rec17\(\beta\)-HSD1 assay, indicating poor bioavailability of these compounds. Also in the cell based assay the compounds exhibited poor to moderate selectivity over 17\(\beta\)-HSD2.

\section*{2.4 Summary of 17\(\beta\)-HSD1 inhibition}

As described above, many potent 17\(\beta\)-HSD1 inhibitors have been identified in recent years. Table 8 lists representative 17\(\beta\)-HSD1 inhibitors with good affinity to the enzyme. To compare the inhibitory potencies of these compounds is difficult if not impossible, however, as various biological assays, with different substrate and enzyme concentrations, have been employed. The inhibitory potencies have been also expressed in various ways, though in general, IC\textsubscript{50} and \(K_I\) values are considered to be the most laboratory-independent. To allow reliable comparison, the inhibitors should be tested in parallel in the same assay. Moreover, a feasible clinical candidate should not only be a potent and selective 17\(\beta\)-HSD1 inhibitor but also possess suitable pharmacological properties. For example, the human recombinant 17\(\beta\)-HSD1 enzyme is an example of an assay that offers reliable information on direct interaction of the inhibitor with the enzyme but it does not give information about cell permeability or possible secondary metabolism of the compounds.
Table 8. Selected 17β-HSD1 inhibitors with good biological activity.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition%</th>
<th>IC₅₀</th>
<th>Assay</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>88% at 0.1 μM</td>
<td>52 nM</td>
<td>HEK-293 cells⁷</td>
<td>31</td>
</tr>
<tr>
<td>53k</td>
<td>94% at 1.0 μM</td>
<td>27 nM</td>
<td>T47-D cells</td>
<td>114</td>
</tr>
<tr>
<td>74f</td>
<td>99% at 10 μM</td>
<td>300 nM</td>
<td>T47-D cells</td>
<td>117</td>
</tr>
<tr>
<td>60</td>
<td>76% at 0.1 μM</td>
<td>-</td>
<td>rec17β-HSD1</td>
<td>119</td>
</tr>
<tr>
<td>56a</td>
<td>85% at 0.1 μM</td>
<td>-</td>
<td>rec17β-HSD1</td>
<td>119</td>
</tr>
<tr>
<td>85</td>
<td>-</td>
<td>35 nM</td>
<td>rec17β-HSD1</td>
<td>124</td>
</tr>
<tr>
<td>95</td>
<td>-</td>
<td>15 nM</td>
<td>rec17β-HSD1</td>
<td>125</td>
</tr>
<tr>
<td>100a</td>
<td>73% at 1.2 μM</td>
<td>-</td>
<td>purified 17β-HSD1</td>
<td>139</td>
</tr>
<tr>
<td>99</td>
<td>88% at 1.2 μM</td>
<td>-</td>
<td>purified 17β-HSD1</td>
<td>138</td>
</tr>
<tr>
<td>117d</td>
<td>94% at 0.1 μM</td>
<td>5 nM</td>
<td>rec17β-HSD1</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>52% at 1.0 μM</td>
<td>-</td>
<td>MCF-7 cells⁷</td>
<td></td>
</tr>
</tbody>
</table>

[a] Transfected with 17β-HSD1 cDNA
Currently there are no 17β-HSD1 inhibitors on the market, and although patent applications relevant to 17β-HSD1 inhibition have recently been published, clinical trials for potential inhibitors have not yet been reported. Clearly there is a demand for novel 17β-HSD1 inhibitors. Small non-steroidal molecules to be used as 17β-HSD1 inhibitors have so far received little attention, but with the aid of molecular modelling novel chemical entities could be found.
AIMS OF THE STUDY

The primary aim of the present study was to develop new non-steroidal 17β-HSD1 inhibitors for the treatment of breast cancer and other hormone-dependent disorders. A tetracyclic thieno[2,3-d]pyrimidin-4(3H)-one derivative that had been identified as a potent lead compound was used as a template for structural modification. The screening strategy comprised evaluation of the biological activity of the potential inhibitors against 17β-HSD1 in recombinant enzyme and cell based assays. The selectivity over isoform 17β-HSD2 was of interest. In connection with the drug discovery efforts, a further aim was to investigate the chemical properties of the fused (di)cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones.

More specifically, the aims of the study were

1. To develop an efficient synthetic route for the lead compound (I, II)

2. To synthesise a molecule library based on the lead compound with the aim of improving biological activity, and to study the QSAR of the 17β-HSD1 inhibitors (II, V)

3. To investigate the reactivity and structure of fused (di)cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones (IV)

4. To clarify the reaction mechanism of Vilsmeier haloformylation (III)
RESULTS AND DISCUSSION

At the start of our multidisciplinary research project, a commercial library of 1000 compounds was evaluated against 17β-HSD1. 1,2,7,8,9,10,11,13-Octahydro-13-oxo-4-(phenylthio)-[1]benzothieno-[2',3':4,5]pyrimido[1,2-a]azepine-3-carboxaldehyde* 125 (Figure 22) was identified as a potent inhibitor and selected as lead compound for 17β-HSD1 inhibitor development (Figure 23). Although compound 125 was commercially available in milligramme quantities, it was unknown in the chemical literature and development of a synthetic route was required.

![The Lead Compound](image)

Figure 22. The lead compound 1,2,7,8,9,10,11,13-octahydro-13-oxo-4-(phenylthio)-[1]benzothieno-[2',3':4,5]pyrimido[1,2-a]azepine-3-carboxaldehyde* 125 and the labelling of the rings.

Chapters 3 describes the synthesis and reactivity of 17β-HSD1 inhibitors based on a thieno[2,3-d]pyrimidin-4(3H)-one core. The mechanism of Vilsmeier haloformylation reaction was revised in connection to the reactivity studies and this is reported in section 3.4. The biological activity and the binding mode of selected thieno[2,3-d]pyrimidin-4(3H)-one based 17β-HSD1 inhibitors are discussed briefly in chapter 4.

* Chemical Abstracts Service (CAS) name
3. SYNTHESIS AND REACTIVITY OF CYCLOALKENO THIENO[2,3-d]PYRIMIDIN-4(3H)-ONES

3.1 Fused dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones

Thieno[2,3-d]pyrimidin-4(3H)-ones are a large group of organic heterocyclic compounds where a thiophene ring is fused at its 2,3 position to the 5,6 position (d bond) of a pyrimidin-4(3H)-one ring. More than 62,800 compounds have been registered to contain this fused heterocyclic moiety.\textsuperscript{164} Fused tetracyclic dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones (Figure 23) are a less studied subgroup.\textsuperscript{165,166,167,168,169,170}

![Figure 23. The general structure of fused dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones.](image)

Dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones, such as compound 126, are traditionally synthesised from ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate 127 in a condensation reaction with lactams\textsuperscript{165} or O-alkyl lactam ethers.\textsuperscript{167} The thiophene 127 and its analogues can be synthesised from cyclic ketones in a Gewald reaction (Scheme 1).\textsuperscript{171,172} Dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones have been reported to have weak antifungal,\textsuperscript{166} antibacterial\textsuperscript{167} (Staphylococcus aureus, Bacillus subtilis) and antiinflammatory\textsuperscript{168} activity.

![Scheme 1. Example of a synthesis of a tetracyclic fused dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-one.\textsuperscript{167}](image)
3.2 Development of a synthetic route for the lead compound

The lead compound 125 was submitted to a retrosynthetic analysis, and the commercially available ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate 127 was found to be a logical starting material. A four-step synthetic route was designed (Scheme 2). Since the synthetic route was to be employed in the synthesis of derivatives of the lead compound, strong emphasis was put on the optimisation of the reaction conditions. First, POCl₃ catalysed cyclisation of 127 with ε-caprolactam was optimised to produce the tetracyclic dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-one core 126 in 90% yield. This cyclisation with POCl₃ as catalyst has earlier been reported to give 126 in 50% yield, but careful optimisation of the amount of the catalyst and the reaction conditions provided significantly improved yield. The published procedure for the oxidation of the thiophene moiety α-position in 126 with K₂Cr₂O₇ gave 128 in average 20% yield. In the second step, the yield of 128 was improved to 54% by using excess of PCC–Celite® as the oxidising reagent. The oxidation of 126 with the seldom used K₂S₂O₈–CuSO₄ in MeCN–H₂O, however, shortened the reaction time and improved the yield to 82%.

In step three, Vilsmeier haloformylation of the keto group was employed to produce the corresponding conjugated β-chlorovinylaldehyde 129. Compound 129 was considered to be a crucial intermediate in the synthetic route. Surprisingly, overnight stirring of ketone 128 with excess of DMF–POCl₃ (Vilsmeier reagent) led to the formation of the corresponding α-chloroalkene 130 and only a small amount of the desired haloformylation product 129. However, prolonged reaction time produced the β-chlorovinylaldehyde 129 in 78% yield. Monitoring of

Scheme 2. Synthetic route for the lead compound 125
the reaction by TLC and \(^1\)H NMR indicated that the reaction proceeds via the \(\alpha\)-chloroalkene \(130\). Haloalkene formation with Vilsmeier reagent has occasionally been reported in literature.\(^{176,177,178,179,180}\) In general, these chloroalkenes were considered to be abnormal sideproducts, but not intermediates, although Vilsmeier reagents are known to formylate activated double bonds.\(^{175,181}\) This observation of \(\alpha\)-chloroalkene formation led to the investigation and revision the reaction mechanism of Vilsmeier haloformylation (see section 3.4).\(^\text{III}\)

In the final step of the synthetic route for the lead compound \(125\), the aromatic thioether moiety was introduced into \(129\) in an addition–elimination reaction of thiophenol under basic conditions in 92% yield.\(^\text{II}\)

In conclusion, an efficient four-step synthetic route, starting from the commercially available ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate \(127\), was developed for the lead compound \(1,2,7,8,9,10,11,13\)-octahydro-13-oxo-4-(phenylthio)-[1]benzo[2',3':4,5]pyrimido[1,2-a]azepine-3-carboxaldehyde \(125\). Overall yield was 42%. This synthetic route was applied in the further development of inhibitors.

### 3.3 Structural modifications of the lead compound: synthesis of derivatives\(^{\text{I,II}}\)

After the development of a synthetic route for the lead compound \(125\), derivatives were synthesised with the aim of improving the biological activity and investigating the QSAR of the thieno[2,3-d]pyrimidin-4(3\(H\))-one based 17\(\beta\)-HSD1 inhibitors.\(^\text{I,II}\) Four sites were chosen for structural alterations of \(125\): rings A and D (homologation), the aldehyde functionality and the thioether moiety (Figure 24).

![Figure 24. Modification sites on the lead compound.](image)
3.3.1 Homologation of the cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-one core

Homologation of the fused thieno[2,3-d]pyrimidin-4(3H)-one skeleton was investigated in order to identify the optimal core for 17β-HSD1 inhibitors. The same synthetic route as for the lead compound was employed in the synthesis of homologues 131a-131c with a thiopropyl side chain in the ring A (Scheme 3). Five to eight membered lactams were fused with 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid ethyl ester 125 to give the ring D homologated derivatives 132a-132c in 76-90% yields. Similarly, cyclisation of ε-caprolactam with 2-amino-5,6,7,8-tetrahydro-4H-cyclohept[a]thiophene-3-carboxylic acid ethyl ester and with 2-amino-5,6-dihydro-4H-cyclopenta[b]thiophene-3-carboxylic acid ethyl ester gave the ring A homologated derivatives 132d and 132e, respectively. As with compound 126, K2S2O8-CuSO4 was the preferred oxidant for the homologated derivatives, producing the oxidised derivatives 133a-133e in 65-80% yields.

![Scheme 3. Homologation of the cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones.](image)

Unexpected difficulties emerged in the Vilsmeier haloformylation step when ketones 133a and 133b, having a five or six membered cycloalkeno ring fused to the pyrimidinone moiety, were treated with DMF-POCl3 (see Scheme 3). Complex mixtures of inseparable products, containing only small amounts of the desired β-chlorovinylaldehydes, were obtained under the same reaction conditions as produced compound 129 in good yield with only traces of side products. Various Vilsmeier reagents and reaction conditions were tested without significant improvement in the outcome of the reaction. For example, with 133a, after optimisation of the reaction...
conditions and a tedious chromatographic purification, a moderate 42% yield of the β-chlorovinylaldehyde 134a was obtained. However, 133c, which has an eight membered ring fused to the pyrimidinone moiety, produced the corresponding β-chlorovinylaldehyde 134c in 60% yield under the original reaction conditions. Vilsmeier haloformylation of ketones liberates HCl, which is considered to catalyse the keto–enol tautomerism required for the reaction.175 Treatment of 133a and 133b with HCl in CH₂Cl₂ was found rapidly give rise to insoluble imine salts. The salt formation was suspected to partly contribute to the complex reactivity of 133a and 133b with POCl₃–DMF. To test this hypothesis, the reaction was performed in an ionic liquid. Since the use of an ionic liquid as solvent in Vilsmeier haloformylation has not been reported in the literature, the applicability was first tested with a model compound. In [bmim][NTf₂] the model compound 2-butane reacted with POCl₃–DMF to give 3-chloro-2-methyl-2-butale as a mixture of diastereomers in good yield. Compounds 133a and 133b produced similar mixtures to those produced in organic solvents, however.

The different reactivities of the homologated compounds 133a-133b in the Vilsmeier haloformylation were also suspected to arise from conformational biases in the fused ring structures. This led us to investigate the structures of 133a-133e and 128, and their reactivity towards a similar electrophilic reagent, DMFDMA (see section 3.5). As has been shown, the amidine α-position is activated and a Vilsmeier reagent furnishes C9 enamines from 6,7,8,9-tetrahydro-4H-pyrido[1,2-alpyridine-4-ones.182 This cannot be the sole explanation of the different reactivities of 133a and 133b, however, since when non-oxidised 132a and 132b are exposed to the Vilsmeier haloformylation conditions, starting material is recovered.

In addition to the tetracyclic cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones 131a-131d, a tricyclic derivative 135 was synthesised in order to investigate the importance of hydrophobic ring D for the biological activity (Scheme 3).19 Again the same synthetic route (Scheme 2) was employed as for the lead compound, with N-methylacetamide supplying the starting material. For this tricyclic skeleton the Vilsmeier haloformylation step proceeded in high yield and purity. When an additional methyl group was introduced to the C2 position of the amidine ring, the Vilsmeier haloformylation again becomes unpropitious, indicating that the problems in the reaction do indeed arise from the amidine α-position.

The biological test performed on the homologated derivatives and with compound 136b (Figure 25),19 suggested that the original dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-one core, with a seven membered ring fused to the pyrimidone moiety, is ideal for the activity, and this was chosen for further structural modifications.
3.3.2 Modifications on rings A and D of the lead compound

To investigate the influence of the bulky thioether side chain in ring A of 125 on the biological activity, nine derivatives, 136a-136i, with an aliphatic thioether side chain and 12 derivatives, 137a-137l, with an aromatic thioether side chain were synthesised (Figure 25).\textsuperscript{1,II} The same synthetic route was employed as for the lead compound (Scheme 2). In addition, functional groups that could act as hydrogen bond acceptors or donors were introduced to the thioether side chain.\textsuperscript{II}

![Figure 25. Derivatives of the lead compound bearing a modified thioether moiety.\textsuperscript{1,II} The dashed lines indicate the point of attachment.](image)

Because it is highly reactive, an aldehyde is not an ideal functional group in a drug molecule and it was therefore replaced with a hydroxyl group. Reduction of the aldehyde moiety in 125 and 136b with NaBH\textsubscript{4} gave the corresponding alcohols 138 with retained biological activity. These allylic alcohols 138 were relatively unstable, however, and they were rejected (Figure 26). In order to produce chemically more stable compounds, derivatives with an aromatic ring A were synthesised. The aromatisation of the unsaturated ring A with DDQ produced the benzothieno[2,3-d]pyrimidin-4(3H)-one derivatives 139a and 139b in 84-91% yield (Scheme 4). Subsequent reduction of the aldehyde functionality with NaBH\textsubscript{4} in EtOH gave the desired benzylic alcohols 140a and 140b in good yield.

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For further studying the binding interactions of these thieno[2,3-d]pyrimidin-4(3H)-one based 17β-HSD1 inhibitors, the aldehyde functionality in 136b or 129 was converted to oxime, carboxylic acid or conjugated alkene in straightforward functional group interconversions (compounds 141-145, Figure 26). In addition to the ring A modification, compounds 146-148 bearing substituents at the C9 position of ring D were synthesised using C5-substituted caprolactams as a starting material.

### 3.4 Haloalkenes as intermediates in the Vilsmeier haloformylation of ketones

The unexpected reactivity and α-chloroalkene formation with cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones under Vilsmeier haloformylation conditions (see section 3.3.1) led to a reconsideration of the reaction mechanism. Investigation of cases of haloalkene formation reported in the literature revealed structural similarities. Except for one aberration involving a steroidal structure, in all cases reported the reactive carbonyl group was conjugated to a strong electron donating group. This conjugation was suspected to contribute to the haloalkene formation under Vilsmeier haloformylation conditions, and to confirm this, a study was performed with substituted aryl ketones as model compounds.
As postulated, 1 equiv. of Vilsmeier reagent (DMF–POCl₃) furnished 1-aryl-1-haloalkenes 150a-150i from the corresponding aryl ketones 149a-149i along with traces of β-halovinylaldehydes 151 as a side product (Scheme 5). The reaction requires a strong electron donating group, e.g., methoxy at the ortho- and/or para-position to the carbonyl group. The electron donating ability of the aromatic substituent determines the requisite reaction time and temperature as well as the conversion. As expected, unsubstituted and meta-substituted arylketones expectedly failed to give haloalkenes, and instead the corresponding β-halovinylaldehydes and starting material were obtained.

![Diagram](https://via.placeholder.com/150)

**Scheme 5. (Z)-1-Aryl-1-haloalkene formation.**

NOESY and 1D ROESY NMR experiments established the configuration of the double bond to be exclusively Z for the chloroalkenes 150a-150e. Small amounts (<10%) of the E-isomer were detected in the ¹H NMR spectrum when DMF–POBr₃ was used. If the formation of the Z-isomer is blocked, as in the case of 6-methoxytetralone 150f, the E-isomer is obtained. The bromoalkenes 150g-150i readily cleaved HBr to form the corresponding acetylenic compounds, and overall the haloalkenes 150a-150i were relatively unstable. Analytically pure samples of the (Z)-1-aryl-1-haloalkenes were obtained chromatographically with use of neutral Al₂O₃ as a stationary phase.

The (Z)-1-aryl-1-haloalkenes proved to be intermediates in the Vilsmeier haloformylation. β-Halovinylaldehydes 151a-151i were obtained from 149a-149i with excess of Vilsmeier reagent, and the haloalkene intermediates were detected by monitoring the reaction on TLC or by ¹H NMR. Similarly, when (Z)-1-aryl-1-haloalkenes 150a-150i were treated with Vilsmeier reagent, β-halovinylaldehydes were obtained as a mixture of isomers. This results offers solid evidence that haloalkenes are primary intermediates in the Vilsmeier haloformylation reaction of suitably substituted aryl ketones 149a-149i. The same reaction pathway is applicable to more complex molecules such as the oxidised cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones 133a-133d and
where there is a similar conjugation to the reactive carbonyl group. An alternative reaction mechanism is thus available in certain Vilsmeier haloformylations,\textsuperscript{111} in competition with the commonly accepted route by way of an enaminoketone.\textsuperscript{175}

The origin of the high stereoselectivity of the reaction was investigated with quantum-chemical density-functional calculations.\textsuperscript{111} The energy difference between the $Z$- and $E$-isomers of the products 150b, 150d, 150g and 150i was calculated (B3LYP/TZVP), and the relative energies between the isomers were found to correlate reasonably well with the selectivity of the isomer. However, the energy differences were not large enough to be the sole explanation for the selectivity. Utilising the final step of our postulated reaction mechanism (Scheme 6), we constructed a potential energy surface (PES) for species 150d$^*$ by scanning the torsion angle C1′–C1–C2–C3 and the bond length of the leaving proton (RI-BP/SVP). From the PES a clear pathway towards the $Z$-isomer can be discerned (Figure 27). From the start the $Z$-isomer is favoured, and the transition to the $E$-isomer becomes increasingly difficult as the double bond character between C1 and C2 in the intermediate 150d$^*$ increases.

Scheme 6. The reaction mechanism postulated for chloroalkene formation in the reaction of Vilsmeier reagents with aryl ketones.\textsuperscript{111} EDG = electron donating group; $X =$ Cl, Br

In conclusion, it was shown that the Vilsmeier haloformylation reaction can proceed via a haloalkene intermediate when suitable conjugation to an electron donating group is present. In addition, the haloalkene formation was shown to be highly stereoselective.
Figure 27. The potential energy surface for the final deprotonation step for species 150d. Energies relative to the lowest energy conformation for each proton bond length are shown. For the C1′–C1–C2–C3 torsion angle, 0° corresponds to the E-isomer, 180° to the Z-isomer. The fully relaxed geometry (B3LYP/TZVP) 150d* has a torsion angle of 109°.

3.5 Stereoelectronic effects in the reactivity of dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones: thermal condensation with DMFDMA

The unexpected results (see section 3.3.1) obtained in the reactions of 133a and 133b with DMF–POCl₃ (Vilsmeier reagent) raised the question of possible structural biases in the fused ring system contributing to the different reactivity. The reactivity of cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones towards electrophilic reagents was investigated with dimethylformamide dimethylacetal (DMFDMA). DMFDMA reacts via aza-oxo-stabilised carbenium ions and can thus be considered as a reagent related to DMF–POCl₃. Activated methylene groups, e.g. in carbonyl compounds and imines, react with DMF acetics in a condensation reaction. The thermal condensation of DMFDMA with carbonyl compounds produces enaminones, which are also intermediates in the direct Vilsmeier haloformylation of ketones. Treatment of enaminones with POCl₃ is known to produce the corresponding β-chlorovinylaldehydes, and it was postulated that this could have been an alternative two-step route to the β-chlorovinylaldehydes from 133a and 133b.

As it turned out, the condensation reaction of 133a and 133b with 4 equiv. of DMFDMA proceeded in high yield and regioselectivity, producing the corresponding conjugated (E)-enamines 152a and 152b (Scheme 7). The result indicated the amidine α-position to be more activated than the carbonyl α-position in ring A. Interestingly, 128 and 133e, with seven and
eight membered cycloalkeno rings fused to the pyrimidinone, did not give enamines, but instead the corresponding \((E)\)-enaminones \textbf{153a} and \textbf{153b}.

The regioselectivity of compounds \textbf{128}, \textbf{133a}, \textbf{133b} and \textbf{133e} was shown to originate from a stereoelectronic effect and to depend on the rate of the amidine–ketene–\(N,N\)-acetal tautomerism.\textsuperscript{IV} Deuterium exchange experiments showed this tautomerism to be prohibited with the larger ring systems (compounds \textbf{128} and \textbf{133e}). For the amidine–ketene–\(N,N\)-acetal tautomerism to occur, and thus the condensation and deuterium exchange reactions to proceed, the C-H bond at the \(\alpha\)-position must be able to orientate approximately parallel to the amidine double bond \(p\) orbitals. The crystal structures revealed that, in \textbf{133a} and \textbf{133b} the two amidine \(\alpha\)-protons (H\(_A\) and H\(_B\)) are positioned out of the plane of the ring with respect to the amidine double bond, with dihedral angles between 44° and 73° (Table 9, Figure 28), suggesting favourable \(p\) orbital interactions. In the crystal structures of \textbf{128} and \textbf{133e} with their larger fused cycloalkeno rings, the amidine \(\alpha\)-proton deviations from 90° dihedral are larger, leading to an unpropitious orientation of the \(\alpha\) C-H bonds that prohibits the amidine–ketene–\(N,N\)-acetal tautomerism. In compounds \textbf{128} and \textbf{133e}, the carbonyl \(\alpha\)-position is more activated than the amidine \(\alpha\)-position, and the enaminones \textbf{153a} and \textbf{153b} were the favoured products.

In the tricyclic derivative \textbf{154}, the amidine \(\alpha\)-methyl group is freely rotating and the required conformation for the tautomerism is readily obtained. According to \(^1\)H NMR, \textbf{155} has two rotamers at 27°C due to the restricted rotation of the \(–\text{NMe}_2\) moiety. An energy barrier (\(\Delta G^\ddagger\)) of 60 kJ between the rotamers was estimated by variable temperature NMR spectroscopy.\textsuperscript{IV}

\begin{center}
\includegraphics[width=\textwidth]{scheme7.png}
\end{center}

\textbf{Scheme 7.} Synthesis of isomeric enamine derivatives of fused cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones.\textsuperscript{IV}
Figure 28. The superimposed crystal structures of 133a (green) and 128 (orange) demonstrating the different conformational orientations of the protons at the amidine α-position. The superimposition (RMSD = 0.021 Å) was made by using the thiophene ring as a template. IV

Table 9. The dihedral (torsion) angles and the degree of H/D exchange at the imine α-position in compound 133a, 133b, 128 and 133e. IV

<table>
<thead>
<tr>
<th>Compound</th>
<th>N=C-C-H_A</th>
<th>N=C-C-H_B</th>
<th>D exchange_b</th>
<th>Product</th>
<th>Yield</th>
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</thead>
<tbody>
<tr>
<td>133a</td>
<td>58.1°</td>
<td>62.4°</td>
<td>68%</td>
<td>Enamine</td>
<td>152a</td>
</tr>
<tr>
<td>133b</td>
<td>72.8°</td>
<td>43.9°</td>
<td>75%</td>
<td>Enamine</td>
<td>152b</td>
</tr>
<tr>
<td>128</td>
<td>10.9°</td>
<td>127.5°</td>
<td>-</td>
<td>Enaminone</td>
<td>153a</td>
</tr>
<tr>
<td>133e</td>
<td>23.2°</td>
<td>139.8°</td>
<td>-</td>
<td>Enaminone</td>
<td>153b</td>
</tr>
</tbody>
</table>

[a] Dihedral angles measured from the crystal structures. [b] The H/D exchange% of the amidine α-protons after two days refluxing in MeOD with catalytic amount of NEt_3. IV

In conclusion, it was shown that regioselectivity in the reaction of DMF/DMA with fused cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones 128, 133a, 133b and 133e is controlled by the stereoelectronic effect at the amidine α-position in ring D. This observation contributes to the understanding of reactivity of fused cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones towards certain electrophilic reagents. The stereoelectronic effect is likely to contribute to the unexpected reactivity observed in the Vilsmeier haloformylation of 133a and 133b discussed in section 3.3.1.
4. BIOLOGICAL ACTIVITY AND BINDING MODE OF THIENO[2,3-d]PYRIMIDIN-4(3H)-ONE BASED 17β-HSD1 INHIBITORS

4.1 Inhibition of 17β-HSD1 by thieno[2,3-d]pyrimidin-4(3H)-one based compounds

The screening strategy for thieno[2,3-d]pyrimidin-4(3H)-one based inhibitors included evaluating their biological activity against 17β-HSD1 in a recombinant protein and for selected compounds also in a cell based assay. The compounds were also tested against 17β-HSD2 and 17β-HSD3 to evaluate their selectivity. In addition, the possible estrogenity of the compounds was investigated.

The lead compound 125 at 1 µM concentration showed promising activity in both rec17β-HSD1 and in cell based 17β-HSD1 assays, with 85% and 41% inhibition, respectively, and selectivity over 17β-HSD2 and 17β-HSD3. Homologation of ring A or ring D did not improve the inhibitory activity and thus the original tetracyclic skeleton with a seven membered cycloalkeno ring D was chosen for further structural modifications. I,II The aldehyde functionality and the thioether moiety were shown to be important structural features for the biological activity since the cyclised intermediates, e.g. compounds 126-130, failed to show good inhibitory activity against 17β-HSD1.

Most of the derivatives of the lead compound with a modified thioether moiety in ring A showed good activity against rec17β-HSD1 at 100 nM and 1 µM concentration. I,II Aromatisation of ring A and reduction of the aldehyde moiety seemed to slightly improve or retain the biological activity as compared with the non-aromatic counterparts. However, introduction of an amino or a carboxylic acid group to the C4’ position of thiophenyl side chain led to loss of activity (compounds 137g and 137j). The most potent inhibitor identified in this study was 4-(3-hydroxyphenylthio)-1,2,7,8,9,10,11,13-octahydro-13-oxo-[1]benzothieno[2',3':4,5]pyrimido[1,2-a]azepine-3-carboxaldehyde 137k, with 94% and 97% inhibition of rec17β-HSD1 at 0.1 µM and 1 µM concentration, respectively. I,II

Representative inhibitor structures and their 17β-HSD1, 17β-HSD2 and 17β-HSD3 inhibition data are presented in Table 10.
Table 10. Selected examples of thieno[2,3-d]pyrimidin-4(3H)-one based 17β-HSD1 inhibitors, and their biological activity.1,11

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>17β-HSD inhibition% in a protein and/or cell based assay (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rec17β-HSD1</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>1 μM</td>
</tr>
<tr>
<td>137k</td>
<td>94</td>
</tr>
<tr>
<td>137h</td>
<td>86</td>
</tr>
<tr>
<td>137c</td>
<td>89</td>
</tr>
<tr>
<td>137b</td>
<td>87</td>
</tr>
<tr>
<td>137f</td>
<td>79</td>
</tr>
<tr>
<td>136b</td>
<td>73</td>
</tr>
<tr>
<td>136e</td>
<td>50</td>
</tr>
<tr>
<td>139a</td>
<td>83</td>
</tr>
<tr>
<td>140b</td>
<td>81</td>
</tr>
</tbody>
</table>
Table 10. Continues

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>rec17β-HSD1</th>
<th>17β-HSD1</th>
<th>rec17β-HSD2</th>
<th>17β-HSD3</th>
</tr>
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<tbody>
<tr>
<td>140g</td>
<td>1</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>136i</td>
<td>0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>131a</td>
<td>17</td>
<td>67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>24</td>
<td>56</td>
<td>48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17</td>
</tr>
<tr>
<td>131d</td>
<td>63</td>
<td>75</td>
<td>57</td>
<td>37</td>
</tr>
<tr>
<td>141</td>
<td>0</td>
<td>3</td>
<td>35</td>
<td>41</td>
</tr>
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<td>147</td>
<td>18</td>
<td>66</td>
<td>47</td>
<td>3</td>
</tr>
<tr>
<td>129</td>
<td>32</td>
<td>79</td>
<td>78</td>
<td>2</td>
</tr>
<tr>
<td>133b</td>
<td>0</td>
<td>4</td>
<td>36</td>
<td>26</td>
</tr>
</tbody>
</table>

[a] Biological activity measurements were performed at Hormos Medical Ltd., Turku, Finland [b] Not fully soluble at 10 μM test concentration. [c] MCF-7 cell line stably expressing one of the respective 17β-HSD isoforms.
These inhibitors showed good to excellent selectivity over 17β-HSD2. The inhibitory activity was somewhat reduced in the cell based assay. In general, the inhibitors bearing an aliphatic thioether moiety in ring A (compounds 136a-136i) seemed to retain their biological activity better. Inhibitor 136b was shown to be a competitive 17β-HSD1 inhibitor and to lack cytotoxicity in MCF-7 cells.

Some of the potent 17β-HSD1 inhibitors, e.g. compounds 136b and 147, also showed good inhibition against 17β-HSD3 (Table 10), which is an enzyme converting androstenedione into testosterone (Figure 1). However, 17β-HSD3 is expressed almost exclusively in male testes and thus selectivity between 17β-HSD1 and 17β-HSD3 isoforms is not an essential feature for an inhibitor of 17β-HSD1. Nevertheless, these thieno[2,3-d]pyrimidin-4(3H)-one based compounds also offer good lead compounds for the development of 17β-HSD3 inhibitors, which could be used in the treatment of prostate cancer, for example.

In conclusion, several potent and selective 17β-HSD1 inhibitors based on a thieno[2,3-d]pyrimidin-4(3H)-one core were identified in this study. In addition, the compounds did not show estrogenic activity in ERα or ERβ receptor binding assay. The most active compounds showed high inhibitory potency (>80% inhibition) against the recombinant enzyme at 100 nM test concentration.

4.2 Binding mode of thieno[2,3-d]pyrimidin-4(3H)-one based 17β-HSD1 inhibitors

The binding mode of thieno[2,3-d]pyrimidin-4(3H)-one based 17β-HSD1 inhibitors was investigated by molecular modelling. Since previous studies had indicated alternative binding modes for substrates, as well as flexibility of the active site residues, molecular dynamics simulations of 17β-HSD1–NADP⁺–inhibitor complexes were performed, which could resolve the optimal binding mode of the inhibitors. Docking of inhibitors to the active site of a crystal structure of 17β-HSD1, while informative, does not reveal if an induced fit can be obtained. Unlike docking studies with crystal structure complexes, dynamic simulations can mimic the physiological conditions.

The lead compound 125 was docked to the active site of a relaxed 17β-HSD1–NADP⁺–E2 complex and molecular dynamic simulation was performed for the enzyme–cofactor–inhibitor complex with the aim of resolving the most plausible binding mode. Subsequently, a set of thieno[2,3-d]pyrimidin-4(3H)-one based inhibitors was docked to the representative active site of the 17β-HSD1–NADP⁺–125 complex simulation trajectory in order to investigate the structural
features contributing to the biological activity of these compounds. The most potent inhibitor, 137k, was found to be complementary in structure to the hydrophobic active site, the bulky ring D partly extending to the surface of the enzyme (Figure 29). Up to four hydrogen bond interactions were formed between 137k and the active site (Figure 30). In the ring A the aldehyde group forms hydrogen bonds to Asn152 and Tyr218, the thioether side chain occupies a hydrophobic pocket near the catalytic region, and the hydroxy group at the meta position forms a hydrogen bond to the backbone carbonyl of Gly144. The pyrimidone carbonyl in the inhibitor core forms a hydrogen bond to Arg254, and the thieno[2,3-d]pyrimidone core is suggested to have π-π interactions with Phe259. In the representative structure of the 17β-HSD1–NADP⁺–125 complex simulation, the inhibitor 125 (the lead compound), which differs from 137k in the lack of phenolic hydroxyl in the thioether side chain, forms two plausible hydrogen bond interactions: one between the ring A aldehyde and Tyr218 and another between the pyrimidone carbonyl and Arg258. The formation of the two additional hydrogen bonds with 137k is supported by the increase observed in the inhibitory activity.

Unexpectedly, docking studies to the relaxed 17β-HSD1–NADP⁺–E2 complex suggested a different binding mode for 136b. Here the cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-one core is flipped horizontally relative to 125 (Figure 31). The only difference between 125 and 136b is that 136b has an aliphatic thiopropyl side chain in ring A. During subsequent molecular dynamics simulation of the 17β-HSD1–NADP⁺–136b complex, 136b was found to turn and shift away from the cofactor resulting in longer equilibration time and thus a more unstable complex as compared with the 17β–HSD1–NADP⁺–125 complex. This, and the comparable biological activity, suggests that 136b could actually bind in similar mode to 125, although docking studies indicated otherwise. In the representative 17β-HSD1–NADP⁺–136b complex, only one plausible hydrogen bond is observed between the aldehyde group in 136b and Tyr218 (Figure 31). Figure 32 illustrates the orientation of E2 before and subsequent to the relaxation of the enzyme complex, and the orientation of inhibitors 136b and 137k in simulated 17β-HSD1-NADP⁺-inhibitor complexes.

Finally, with use of the docked conformations and the biological data of 28 selected inhibitors, a 3D-QSAR model was created to guide further inhibitor development. The model was validated with an external test set, and the correlation (r²) between the experimental and predicted biological activity for the training set was 0.994. The QSAR model shows good predictive power and offers a tool for rational design of new 17β-HSD1 inhibitors.
Figure 29. The surface of the active site around NADP⁺ (coloured blue) and inhibitor 137k (coloured orange).

Figure 30. The top ranked pose for the most potent inhibitor 137k docked into the active site of simulated 17β-HSD1–NADP⁺–125 complex.
Figure 31. Representative active site of 17β-HSD1–NADP⁺–136b complex simulation. The dashed line indicates plausible hydrogen bond interaction. NADP⁺ is coloured blue and 136b is coloured green.

Figure 32. The variable positions of the reduced substrate E2 and inhibitors in the active site of 17β-HSD1. E2 (coloured magenta) in the crystal structure of 17β-HSD1–NADP⁺–E2 complex (pdb code 1FDT) and E2 (coloured blue) after the relaxation of the complex. The most potent inhibitor 137k (coloured orange) docked into the active site of simulated 17β-HSD1–NADP⁺–125 complex and 136b (coloured green) from 17β-HSD1–NADP⁺–136b complex simulation. The corresponding enzyme structures are aligned on the basis on the enzyme backbone.
5. CONCLUSIONS

17β-HSD1 enzyme is an attractive biological target for the development of inhibitors useful for the treatment and prevention breast cancer and other hormone dependent disorders. Several potent and selective non-steroidal 17β-HSD1 inhibitors without estrogenic activity were identified in the study. An efficient synthetic route was developed for the lead compound and subsequently utilised in the synthesis of a cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-one based molecule library. Around 100 thieno[2,3-d]pyrimidin-4(3H)-one based compounds were synthesised, most of which were new chemical entities. 4-(3-Hydroxyphenylthio)-1,2,7,8,9,10,11,13-octahydro-13-oxo-[1]benzothieno[2′,3′:4,5]pyrimido[1,2-a]azepine-3-carboxaldehyde 137k is one the most potent 17β-HSD1 inhibitors reported to date, with 94% inhibition of the recombinant enzyme at 100 nM test concentration. Overall, the establishment of novel class of inhibitors is a progressive achievement in 17β-HSD1 inhibitor development.

The predictive 3D-QSAR model that was constructed on the basis of this study offers a powerful tool for future 17β-HSD1 inhibitor development. On the basis of the 3D-QSAR model we can now more efficiently utilise structure based drug design in the creation of dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-one based 17β-HSD1 inhibitors with good inhibitory potency. Future work should also take into consideration the pharmacological properties of thieno[2,3-d]pyrimidin-4(3H)-one based 17β-HSD1 inhibitors, to overcome among other things, the relatively poor solubility of some of these compounds. Clarification is also needed of the structural factors influencing cell membrane permeability.

The studies on the structure and reactivity of cycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones contribute to understanding the chemical reactivity of this compound class. The reactivity of dicycloalkeno thieno[2,3-d]pyrimidin-4(3H)-ones towards electrophilic reagents was shown to depend on a stereoelectronic effect arising from different ring conformations. In addition, the mechanism of the Vilsmeier haloformylation was reviewed and the reaction was shown to proceed via chloroalkene intermediate when conjugation to a suitable electron donating group is present.
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APPENDIX

Structure of the natural amino acids

Hydrophobic amino acids

- Alanine - Ala
- Valine - Val
- Leucine - Leu
- Isoleucine - Ile
- Methionine - Met
- Phenylalanine - Phe
- Tryptophan - Trp
- Proline - Pro

Polar amino acids

- Glysine - Gly
- Serine - Ser
- Threonine - Thr
- Cysteine - Cys
- Aspargine - Asn
- Glutamine - Gln
- Tyrosine - Tyr

Ionised amino acids

- Arginine - Arg
- Lysine - Lys
- Histidine - His
- Aspartic acid - Asp
- Glutamic acid - Glu