Synthesis and Characterization of Subtype-Selective Estrogen Receptor Ligands and their Application as Pharmacological Tools

Cross-Talk between Estrogen and NPY Y_1 Receptors in Human Breast Cancer Cells

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Martin Memminger

aus Heretsried (Landkreis Augsburg)

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Prüfungsausschuss:

Prof. Dr. F.-M. Matysik	(Vorsitzender)
Prof. Dr. G. Bernhardt	(Erstprüfer)
Prof. Dr. E. von Angerer	(Zweitprüfer)
Prof. Dr. A. Göpferich	(Drittprüfer)

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“New subtype-selective estrogen receptor antagonists as pharmacological tools for the investigation of estrogen receptor signalling pathways”

Contents

Abbreviations

abs.	absoluted
AC	adenylyl cyclase
AF	activation function
AMP	adenosine monophosphate
AP1	activator protein 1
aq	aqueous
ATP	adenosine triphosphate
B_{max}	maximum number of binding sites
BSA	bovine serum albumine
cAMP	cyclic adenosine monophosphate
CD	circular dichromism
cDNA	complementary DNA
CoA	coenzyme A (1), coactivator (2)
CoR	corepressor
ct-FCS	charcoal treated fetal calf serum
DNA	desoxyribonucleic acid
DCC	dextran coated charcoal
DCM	dichloromethane
DEA	diethylamine
dec.	decomposition
DEPC	diethylene pyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DPN	diarylpropionitrile, here: 2,3-bis(4-hydroxyphenyl)propionitrile
DTT	dithiotreitol
EC_{50}	agonist concentration which induces 50% of the maximum response
EDC	3-(3-dimethylaminopropyl)-1-ethyl carbodiimide
EDTA	ethylenedimaminetetraacetate
ee	enantiomeric excess
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
EGTA	ethyleneglycol tetraacetate
EMEM	Eagle’s minimum essential medium
ER	estrogen receptor
ERE	estrogen response element
Erk	extracellular-signal regulated kinase
EtOH	ethanol
FCS	fetal calf serum
GPCR	G-protein coupled receptor
h	hour(s)
HAP	hydroxyl apatite
HEL cells	human erythroleukemia cells
HEPES	2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid
HER	human epidermal growth factor receptor
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HR-MS	high resolution mass spectrometry
HSA	human serum albumine
IBMX	isobutylmethylxanthine
IC_{50}	antagonist/inhibitor concentration leading to 50% inhibition of an agonist induced effect or 50% displacement of a radiolihgand from the binding site
IGF-I	insuline like growth factor type I
kD	kilodalton
K_D	dissociation constant
LBD	ligand binding domain
MAPK	mitogen-activated protein kinase
MCPBA	meta-chloroperbenzoic acid
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NMR	nuclear magnetic resonance
NPY	neuropeptide Y
OD	optical density

p.a.	pro analysis
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate buffered saline
PE	petroleum ether
PI3K	phosphatidylinositol-3'-kinase
PKA	proteinkinase A
PKC	proteinkinase C
pNPY	porcine neuropeptide Y
PPT	"propylpyrazole triol"; 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole
PR	progesterone receptor
Ras	rat sarcoma
R_s	resolution
RBA	relative binding affinity
RLU	relative light units
RP	reversed phase
RT	retention time
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SERD	selective estrogen receptor down-regulator
SERM	selective estrogen receptor modulator
T/C	treated versus control
TFA	trifluoro acetate
THC	(R,R)-5,11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2-8-diol
THF	tetrahydrofuran
THIQ	1,2,3,4-tetrahydroisoquinoline
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet
Y_n R	neuropeptide Y receptor subtype, n = 1, 2, 4, 5

A General Introduction

1 Structure and Function of Estrogen Receptors (ERs) α and β

Estrogens are involved in a number of biological functions in many target organs such as mammary gland, uterus, bone, cardiovascular system and brain. Estrogen actions are predominantly mediated through two nuclear estrogen receptor (ER) subtypes namely α and β. The ER belongs to the superfamily of nuclear steroid hormone receptors that are ligand-activated transcription factors initiating gene transcription by interaction with specific hormone response elements of the DNA (Tsai and O'Malley, 1994).

An estrogen receptor protein was identified and isolated from the rat uterus in the 1960s (Toft and Gorski, 1966; Toft et al., 1967) and finally cloned from human tissue in the mid 1980s (Walter et al., 1985; Green et al., 1986; Greene et al., 1986). From the first discovery to the mid 1990s research was focused only on one ER protein. In 1996 a second ER protein was identified and cloned initially from rat (Kuiper et al., 1996), and shortly later also from man (Mosselman et al., 1996; Enmark et al., 1997) and mouse (Tremblay et al., 1997). Henceforward the originally known subtype was termed ERα and the 30 years later discovered isoform became known as ERβ. Several splice variants of the human ERβ subtype, designated ERβ long (Ogawa et al., 1998a), ERβ short (Mosselman et al., 1996) and ERβ cx (Ogawa et al., 1998b) have been identified.

Like other members of the superfamily of nuclear receptors, ERα and ERβ are made up of several distinct functional domains (Kumar and Thompson, 1999) (cf. Figure A1). The N-terminal A/B region is the one with the lowest sequence homology between ERα and ERβ. This domain harbors the transcriptional activation function AF-1 that was believed to be constitutively active and ligand independent (Kumar et al., 1987; Metzger et al., 1995). Ligand independent ER activation was shown to be induced by receptor phosphorylation via different cytoplasmic signaling pathways (cf. Paragraph 3.2).

The DNA binding domain (region C) is the most conserved region and shows approximately 95% sequence homology between ERα and ERβ. Eight highly conserved cysteine residues of this domain coordinate two zinc (II) cations to form two zinc finger

motifs that are involved in dimerization and DNA binding. The so-called P-box is a sequence of six amino acids located within the N-terminal zinc finger being responsible for the recognition of the ERE on the DNA. The second zinc finger harbors the so called D-box as a dimerization interface (Pettersson and Gustafsson, 2001). D-box mediated protein-protein interactions and P-box mediated DNA-protein binding are cooperative and stabilize the ER-DNA-complex in concert (Freedman, 1992).

The DNA binding domain is linked to the large ligand binding domain (LBD, region E) by a poorly characterized hinge region (D-domain). The LBD is a highly conserved region that mediates ligand induced transcriptional activation through the second ligand dependent activation function AF-2. The LBD is the key domain for the development of new ligands and therefore, a number of crystal structures of ERα and ERβ LBD in complex with various ligands have been reported (Brzozowski et al., 1997; Shiau et al., 1998; Pike et al., 1999; Pike et al., 2001; Shiau et al., 2002). The molecular mechanism of signal transduction after ligand binding will be explained in paragraph 3.1.

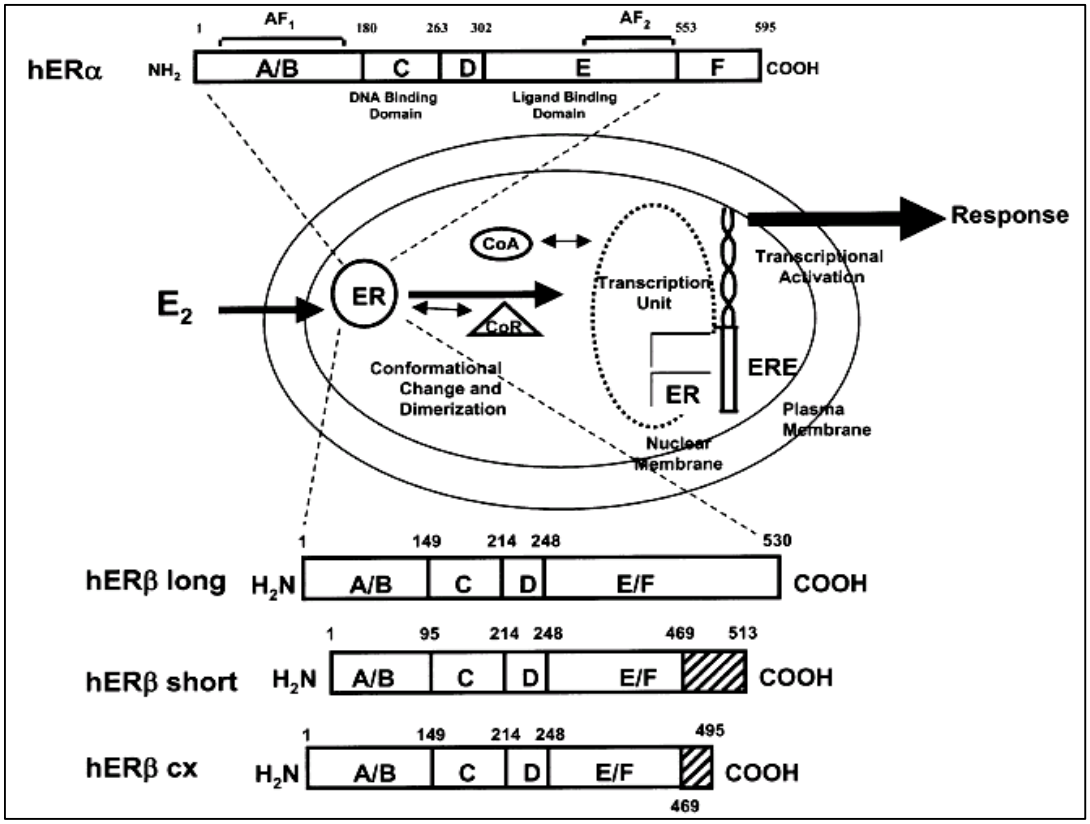


Figure A1: Schematic structure and signal transduction of human estrogen receptors α and β (from (Jordan, 2003a))

CoA: Coactivator, CoR: Corepressor, E2: 17β-estradiol

The ERα and ERβ proteins differ from each other in size and show a relatively low sequence homology of 47%. ERα is a 66 kD protein comprising 595 amino acids, whereas ERβ consists of 530 amino acids with a molecular weight of 59 kD. The ligand binding domain of ERα and ERβ show only 55% sequence homology, but the binding pockets of the two subtypes differ only in two amino acids: Leu 384 and Met 421 in ERα correspond to Met 336 and Ile 373 in ERβ (Pike et al., 1999). This similarity of the binding site explains the very similar affinity of the endogenous ligand 17β-estradiol to both estrogen receptors α and β (Kuiper et al., 1997).

The two estrogen receptor isoforms have different tissue distribution in the human body. ERα regulates the development and maintenance of both male and female reproductive organs and is the predominant subtype expressed in breast carcinoma. ERβ was reported to be the main isoform in normal breast tissue and was also found in a number of other tisssues such as the cardiovascular system, reproductive organs, brain and bone (Enmark and Gustafsson, 1998)

2 Ligands of the Estrogen Receptor

The increasing knowledge about the physiological functions of estrogens during the 20th century brought along the development of a large number of compounds active as agonists as well as partial- or full antagonists on ER. The class of antiestrogens and selective estrogen receptor modulators (SERMs) that are important in breast cancer therapy and ER subtype selective ligands developed in recent years will be reviewed in the following paragraphs.

2.1 Clinically Relevant ER Ligands: Antiestrogens and SERMs

Breast cancer is one of the most common forms of cancer in women. Approximately two third of all mammary carcinomas express estrogen- and progesterone receptors, which are well established prediction factors for the likelihood of response to hormonal therapy (Hopp and Fuqua, 2003). Besides other approaches focused on the blockade of estrogen biosynthesis, the functional blockade of the ER by antagonists (antiestrogens)

has become a leading strategy for the treatment of hormone sensitive mammary carcinoma. Tamoxifen (cf. Figure A2) was the first - and for a long time - the only available antiestrogen for breast cancer therapy on the market. The antiestrogen provides effective palliation in patients with advanced disease and reduces the risk of recurrence when applied as adjuvant therapy (Osborne, 1998). Contrary to its antagonistic activity in the breast, tamoxifen was found to be an agonist in other tissues such as bone, blood and endometrium. In the case of bone and blood the estrogen-like side effect was a benefit, as estrogen is important for preservation of bone mineral density (Turken et al., 1989; Love et al., 1992) and reduces blood lipids such as cholesterol as cardiovascular risk factors (Love et al., 1994). On the other hand, the estrogenic action in the endometrium brings along an increased risk for the development of endometrium carcinoma during tamoxifen treatment (Fisher et al., 1994). The tissue specific effects of tamoxifen were the basis for the development of selective estrogen receptor modulators (SERMs). Due to the estrogenic effect in bone SERMs were found to be a favorable form of treatment of osteoporosis, providing a lowered risk of breast cancer development as beneficial side effect. Osteoporosis is a common disease in aged women, which is caused by a reduced bone mineral density as a consequence of lowered estrogen production after menopause. The second generation SERM raloxifene (cf. Figure A2), was introduced for the treatment and prevention of osteoporosis and was also discussed as a preventive for breast cancer and coronary hart deseases (Jordan et al., 2001). A number of new SERMs based on the stilbene structure of tamoxifen, such as toremifene, droloxifene and idoxifen, or fixed ring analogues such as arzoxifene have been developed (Jordan, 2003b). Despite some improved pharmacokinetic and pharmacodynamic properties of most of the new compounds, clinical trials revealed no benefits of the new SERMs in terms of efficacy and tolerability in breast cancer therapy. Cross resistance to tamoxifen is another important problem on the search of usefull alternative SERMs (Jordan, 2003b). Up to date tamoxifen and raloxifene are the only SERMs available for the treatment of breast cancer and osteoporosis, respectively.

As a second line therapy of hormone sensitive breast cancer the “pure ER antagonist” fulvestrant (ICI 182.780, Faslodex^{TM}, cf. Figure A2) was introduced on the European market in 2004. Contrary to tamoxifen, the steroidal antiestrogen is completely devoid of any estrogenic activity and has been successfully applied in tamoxifen-resistant breast tumors in postmenopausal women (Robertson et al., 2001; Robertson et al., 2003).

Besides the effective blockade of the ER function, fulvestrant was shown to down-regulate the ER and PR expression (Robertson, 2001). This phenomenon suggests a mode of action different from SERMs such as tamoxifen and lead to the designation “selective estrogen receptor down-regulator (SERD)”.

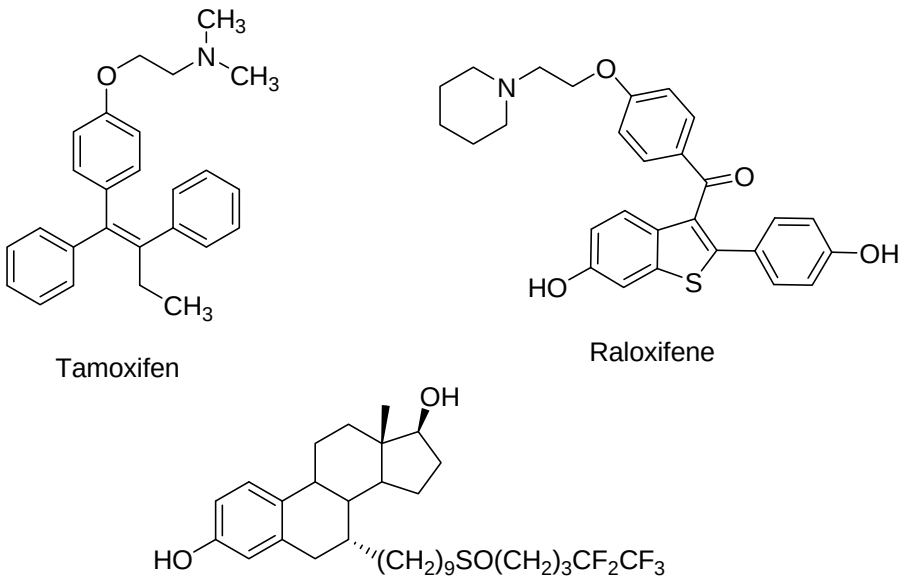


Figure A2: Estrogen receptor ligands with clinical relevance: The SERMs tamoxifen and raloxifene and the full ER antagonist fulvestrant

2.2 ER Subtype-Selective Ligands: Recent Advances

Induced by the discovery of ERβ as the second ER protein about ten years ago, an intensive search for selective ligands of both receptor subtypes began. As ER subtype specific physiological functions are unknown to a large extent, the initial goal of developing selective ligands has been their application as pharmacolocical tools.

In 2000 the synthesis and characterization of a library of pyrazole based compounds resulted in the 400-fold ERα selective highly potent agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (“propylpyrazole triol”, PPT, cf. Figure A3) (Stauffer et al., 2000). A shortly later developed class of ERα selective agonists was based on a triphenylfuran-scaffold (Mortensen et al., 2001). *In vivo* studies using PPT as a pharmacological tool

revealed that many classical estrogenic effects, such as uterotrophy, increase in bone mineral density and reduction of plasma cholesterol levels are mediated by ERα (Harris et al., 2002).

Besides full agonists, a number of potent SERMs with selectivity for ERα such as triarylpyrazoles (Stauffer et al., 2001) or tetrahydroisoquinolines (Renaud et al., 2003) have been reported. In the field of nonsteroidal "pure ER antagonists" first advances towards ERα selectivity have been achieved with the recent development of diphenylfuran based compounds in our work group (cf. Figure A3) (Zimmermann et al., 2005).

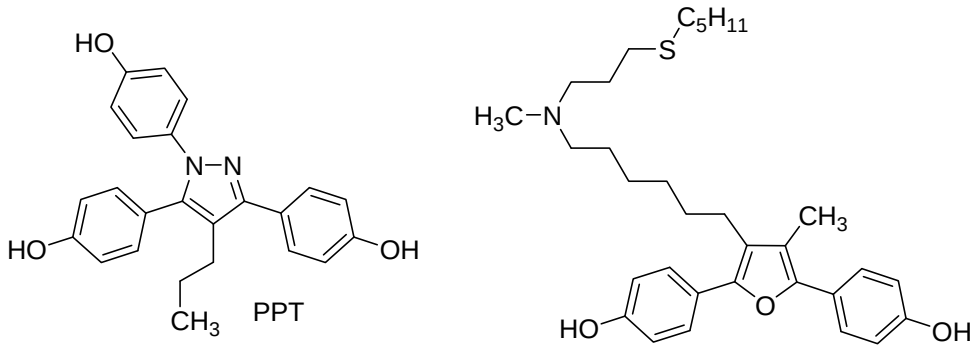


Figure A3: The ERα selective agonist PPT and a 2,5-diphenylfuran-based moderately ERα selective "pure antagonist"

Phytoestrogens were the first ER ligands to be characterized as moderately ERβ selective. The best known and most intensively studied isoflavone-type phytoestrogen genistein (cf. Figure A3) reveals an ERβ-selectivity of approximately 20-fold and was characterized as a full agonist via ERα but only a partial (50%) agonist via ERβ (Barkhem et al., 1998; Kuiper et al., 1998).

A first synthetic breakthrough in the field of selective ERβ agonists was achieved with DPN (cf. Figure A3). The compound shows increased ERβ selectivity referred to binding (70-fold over ERα) and agonist activity (78-fold over ERα) compared to genistein and is a full agonist via ERβ (Meyers et al., 2001). More recent studies of the Wyeth Research Institute resulted in potent ERβ agonists with highest selectivities known up to date. The compounds are based on a benzofuran or benzoxazole scaffold bearing small hydrophobic groups in position C7 (cf. Figure A4). The ERβ selectivities reach up to 100-fold in case of C7 substituted benzofurans and more than 200-fold in case of the

benzoxazole ERB-041, while their affinities for ERβ are comparable to 17β-estradiol. The C7-substituent was found to be an essential structural element to obtain the observed selectivities (Collini et al., 2004; Malamas et al., 2004). The molecular basis for this high selectivity affected by the small, relatively hydrophobic groups in position C7 was investigated in X-ray crystallography studies of benzofurans and benzoxazoles bound to the ERβ LBD. The 7-substituents extend into the relatively narrow groove formed by Ile 373, Ile367 and Phe 377. A substitution of the ERβ Ile 373 by a methionine mimicking ERα Met 421 is hypothesized to lead to a dropping binding affinity due to a combination of steric and electrostatic repulsion. The compounds named above were characterized as full agonists via ERβ in transcription assays using transfected cells overexpressing the recombinant ERβ. Furthermore the ERβ selective agonists were used as pharmacological tools for *in vivo* experiments to probe the physiological role of ERβ. For example, by using ERB-041 as ERβ selective agonist a role of ERβ in certain inflammatory diseases was demonstrated, while classical estrogenic effects were missing (Harris et al., 2003). This finding opened new field of potential clinical relevance of ERβ selective agonists

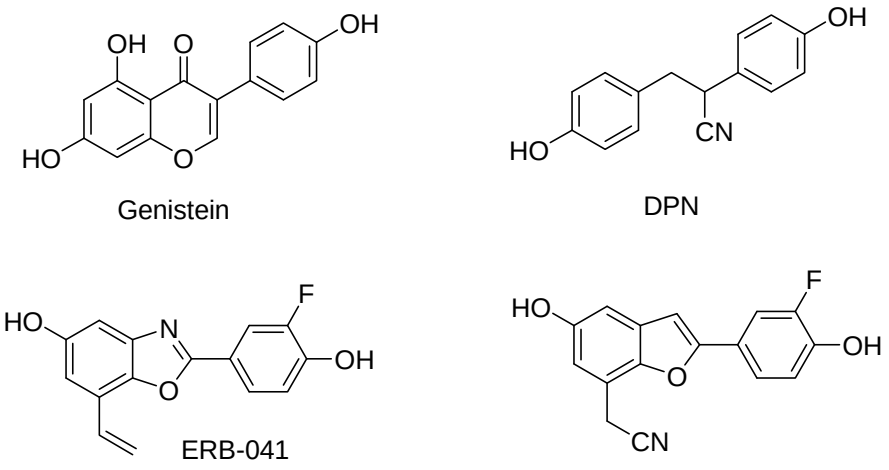


Figure A4: Top: The partial ERβ agonist genistein and the full agonist DPN; bottom: Highly potent benzoxazole- (ERB-041) and benzofuran-based ERβ selective agonists.

ERβ selective antagonists with selectivities comparable to agonists have not been described in literature. THC (cf. Figure A5) is a “pure antagonist” of ERβ that acts as an agonist via ERα. As a typical “antiestrogenic” side chain is lacking in the THC molecule, a “passive antagonism” has been proposed for its mode of action. In a high throughput

screening by the company Glaxo-Smith-Kline triazines were identified as SERMs with modest selectivity for ERβ. Structure optimization lead to a maximum selectivitiy of 26-fold in the case of the compound depicted in Figure A5 (Henke et al., 2002). Structure and substitution pattern of the triazines are not conform to common nonsteroidal ER ligands such as stilbenes or corresponding ring closed analogues.

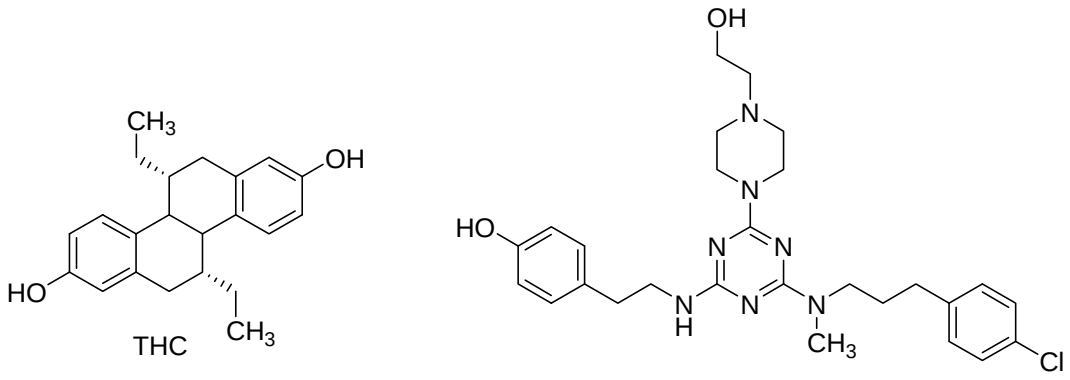


Figure A5: The "passive" ERβ antagonist THC and a triazine based SERM with modest ERβ selectivity

3 Molecular Mechanisms for Estrogen Action

3.1 The Classical Pathway to Transcription Activation: Ligand Binding

Estrogens exert their transcriptional activity by high affinity binding to a hydrophobic cleft localized in the LBD of ERα and ERβ. After binding of an estrogen, the ER dissociates from chaperone proteins, such as the heat-shock proteins Hsp90 and Hsp70, stabilizing the inactive receptor in a favorable conformation (Pratt and Toft, 1997). The activated estrogen receptor dimerizes, interacts with the ERE in the promoter region of estrogen responsive genes and activates transcription through activation of AF-1 and AF-2. The ERE of ERα is a specific DNA region compromising two inverted palindromic half-sites separated by three non-defined nucleotides (5'-AGGTCAnnnTGACCT-3') (Schwabe et al., 1993). The two activation functions AF-1 and AF-2 of ERα can mediate gene transcription independently, but in most cases they act synergistically in a cell-type and promoter context specific manner (Tzukerman et al., 1994). ERβ activates transcription similar to ERα and is capable of forming

functional heterodimers with the latter (Pace et al., 1997). Gene expression is not under direct control of the ERs. A number of coregulatory proteins (coactivators and corepressors) is involved as mediators in the transcription process that is not fully understood yet (Horwitz et al., 1996). Besides the classical ERE, several non-classical promoter sites, such as AP-1 have been identified. ERα stimulates gene expression through the AP-1 site indirectly by interacting with the DNA-bound transcription factors jun and fos. Contrary to ERα, the estradiol-ERβ complex was shown to inhibit transcription via the AP-1 promoter (Paech et al., 1997).

Crystal structures of the ERα and ERβ LBDs bound to various ER ligands have been available since 1997. They enabled a detailed insight into the molecular basis for agonistic and antagonistic ER action. Location and affinity of ligands is determined by a combination of hydrophobic interactions and specific hydrogen bonds.

The first crystal structure of ERα in complex with estradiol and raloxifene was published by Brzozowski and collegues (Brzozowski et al., 1997) (cf. Figure A6).

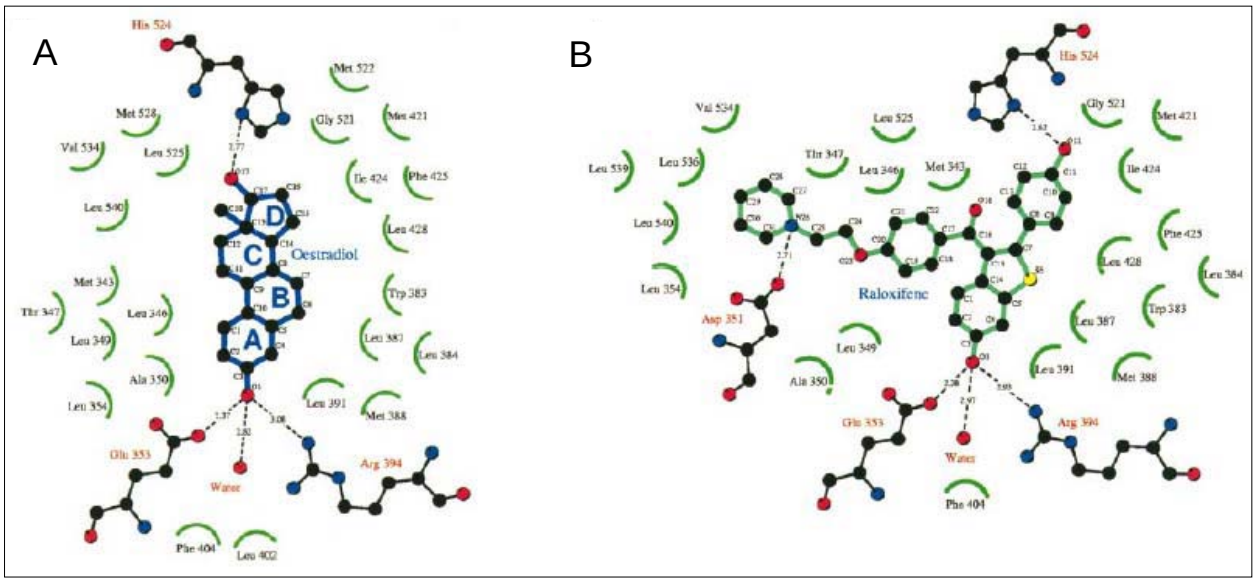


Figure A6: Arrangement of estradiol (A) and raloxifen (B) in the binding pocket of ERα, adapted from (Brzozowski et al., 1997)

The crystal structure of estradiol bound to the ERα LBD revealed two hydrogen bonds of the two hydroxy groups at both ends of the molecule. The phenolic hydroxy group localized at the A-ring of estradiol forms a multiple hydrogen bond to the carboxylate of Glu 353, the guanidinium group of Arg394 and an additional water molecule. Another

hydrogen bond is formed between the D-ring-hydroxy function and the His524 residue of the ERα binding pocket (cf. Figure A6 A).

With its two phenolic hydroxy functions, the SERM raloxifen forms the same hydrogen bonds as estradiol, whereas the OH-group positioned at the heterocyclic phenol ring corresponds to the A-ring of estradiol. As a characteristic structural element of SERMs, the amine sidechain of raloxifen is additionally anchored to the receptor by a hydrogen bond between the carboxylate of Asp351 and the protonated piperidine nitrogen (cf. Figure A6 B).

To be active as a full agonist of ERα, a ligand must be capable of being enveloped in the hydrophobic pocket that is closed by helix 12. The proper positioning of helix 12 over the hydrophobic pocket is crucial for the recruitment of coactivators and subsequent initiation of RNA polymerase activity. By the bulky side chains of SERMs the repositioning of helix 12 is prevented, leading to a conformational arrest in the inactive state of the receptor. The crystal structure of raloxifene in the rERβ LBD suggests an analogous mechanism as demonstrated for ERα (Pike et al., 1999). The position of helix H12 was accepted as a general key mechanism for estrogenic and antiestrogenic activity.

“Pure antagonists” generally have longer side chains than SERMs with additional functionalities at the outer extension. The crystal structure of ICI 164,384, bound to the ERβ LBD, suggests a “double blocking” effect of the extended side chain to the position of helix H12. The helix is prevented from sealing the ligand binding pocket and additionally from an alternative positioning along the coactivator binding site observed in the ER-SERM complex (Pike et al., 2001). Favored corepressor recruitment and neutralizing effects on AF-1 caused by the alternative H12 orientation were discussed as possible explanations for the full antagonism of fulvestrant and related compounds contrary to the partial antagonism of SERMs.

The binding mode of the partial agonist genistein was analyzed in X-ray crystallographic studies in complex with ERβ. The crystal structure revealed hydrogen bonds between the 4’-hydroxy group of genistein and the Glu305-Arg346-water triad and between the phenolic hydroxy group in position C7 and His 457 respectively. In the ERβ-genistein complex, helix 12 was found to seal the binding pocket not totally in the manner as it is the case for full agonists (Pike et al., 1999). This different conformation of H12 was suggested as an explanation for the poor agonist activity of the phytoestrogen via ERβ.

A Similar conformation of H12 was also found for the “passive” ERβ antagonist THC bound to the ERβ LBD (Shiau et al., 2002).

3.2 Cross-Talk Signaling Pathways: Estrogen Receptor Phosphorylation

In several studies it has been shown that activation of the ER by estrogens is associated with increases in overall receptor phosphprylation. Five different phosphorylation sites have been mapped within the ERα protein, which are predominantly serine residues and in rare cases also tyrosine residues. A detailed review on phosphorylation sites and mechanisms is given by Lannigan (Lannigan, 2003).

ER activation was reported to be induced by several compounds that are not ligands of the ER, such as cyclic adenosine monophosphate (cAMP), dopamine, epidermal growth factor (EGF) and insuline like growth factor type 1 (IGF-I). As such agents are not able to stimulate the ER by the classical way of ligand binding, the denotations “non-classical” or “ligand-independent” activation were formed. The growth factors and related compounds cross-talk with the ER in a complex system of cytoplasmic signaling pathways that involves cytoplasmic proteins or protein kinases leading to an ER phosphorylation (reviewed in (Driggers and Segars, 2002)).

cAMP activated protein kinase A (PKA) has been shown to activate the ERα via phosphorylation in a ligand independent manner (Aronica and Katzenellenbogen, 1993). Furthermore, an increased cAMP level triggered by the G-protein activators IBMX and cholera toxin enhanced the 17β-estradiol induced transcriptional activity in ER positive MCF-7 breast cancer cells (Cho and Katzenellenbogen, 1993). In similar experiments, a cAMP induced cross-talk activation of ERβ has been shown in HeLa cells transfected with the ERβ gene, whereas a distinct mechanism in ERα and ERβ phosphorylation was demonstrated (Coleman et al., 2003). As PKA induced activation was reported to be involved in the development of tamoxifen resistance, it is of potential clinical relevance (Fujimoto and Katzenellenbogen, 1994; Michalides et al., 2004). Contrary to tamoxifen, the “pure ER antagonist” fulvestrant inhibits cAMP induced ER activation (Michalides et al., 2004).

Another important cross talk activation of the ER is induced by growth factors such as EGF and IGF-1. EGF stimulates receptors of the EGFR family consisting of four distinct

members (EGFR/ErbB1/HER1, ErbB2/HER2/c-neu, ErbB3/HER3 and ErbB4/HER4) upon binding to the extracellular domain. The activated receptor undergoes dimerization, autophosphorylation at specific tyrosine residues and subsequently acquires the potential to activate a number of intracellular enzymatic activities. EGF can induce Erk1/2 / MAPK or phosphatidylinositol 3'-kinase (PI3K)/AKT pathways leading to ER phosphorylation and subsequent ligand independent activation (Smith, 1998). EGFRs - particularly HER2 - were found to be expressed in many tumors of the breast making them an important target for diagnosis and treatment of breast cancer. Furthermore the cross-talk between EGFRs and the ER was suggested as a possible molecular mechanism for the development of antiestrogen resistance (Osborne et al., 2005). Similarly, IGF-I was shown to activate the ER mediated luciferase activity in MCF-7 breast cancer cells transfected with a luciferase reporter plasmid. The IGF-I induced cross-talk activation was blocked by the full ER antagonist fulvestrant, demonstrating the ER specificity (Hafner et al., 1996).

The role of G-Protein coupled receptor (GPCR) mediated protein kinase C (PKC) signaling pathways in ER activation are less well understood than the PKA and growth factor pathways. GPCRs gained increasing interest in endocrine related cancer research in recent years. For example the neuropeptide Y (NPY) Y_1 receptor, a peptidergic GPCR was found to be expressed in many human cancers. Cell type specific regulative effects of NPY on cancer cell proliferation have been reported (Körner and Reubi, 2007). NPY was recently shown to induce Erk1/2 phosphorylation in prostate cancer cells that was cell line specifically blocked by a PKC inhibitor (Ruscica et al., 2006). Cross-talk effects between the ER and the NPY Y_1R in breast cancer cells remain to be investigated (cf. chapter D of this thesis). Furthermore, GPCRs can mediate the transactivation of growth factor receptors via tyrosin phosphorylation (Daub et al., 1997). Recently, it was shown that GPCR agonists such as thrombin or phospholipids can trigger an EGFR mediated signaling cascade in ER positive MCF-7- and ER negative MDA-MB-231 breast cancer cells (Hart et al., 2005).

A simplified overview of important cross-talk activation pathways is given in Figure A7.

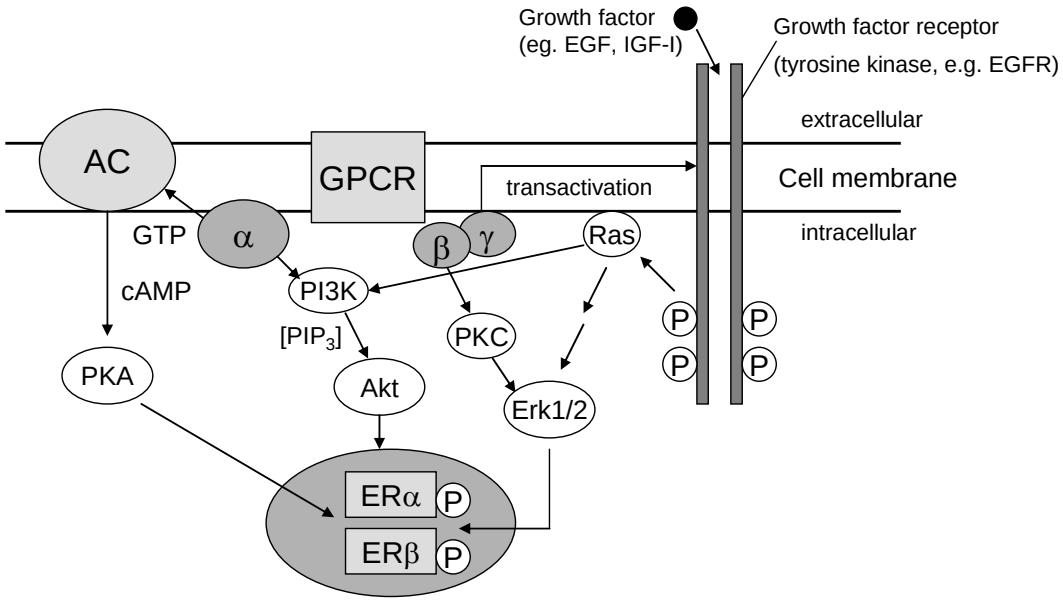


Figure A7: Schematic overview of important cross-talk ER activation pathways; due to clearness, not all, but only some key signaling molecules and enzymes involved in cross talk-pathways are depicted. For abbreviations see glossary.

4 Non Genomic Estrogen Action via Membrane Bound ER

Some estrogen actions cannot be explained by the classical functions of nuclear ERα and ERβ as transcription factors. For example, estrogen was reported to rapidly rise cAMP levels by stimulation of the adenylyl cyclase (AC) (Aronica et al., 1994) and to trigger the mobilization of intracellular calcium (Morley et al., 1992) and inosityltriphosphate (IP_3) (Le Mellay et al., 1997). As such effects are typical for membrane bound receptors, in particular GPCRs, a membrane ER (mER) was postulated in the 1990s. Evinger and Levin suggested a co-existent population of the nuclear ERα protein localized in the membrane (Evinger and Levin, 2005). Other studies demonstrated that estrogen induced Erk1/2 activation can occur independently of nuclear ERs as it also was observed in ER negative SKBR3 cells. The involvement of GPR30, so far known as an orphan GPCR, was suggested (Filardo et al., 2000). In the same study mechanistic investigations revealed an estrogen induced, GPR30 mediated transactivation of EGFR via activation of matrix metalloproteinase (MMP) and heparin bound (HB-) EGF release leading to Erk1/2 phosphorylation. GPR30 was originally cloned by Carmeci and coworkers within the scope of a differential screening study,

aiming on the identification of genes overexpressed in ER positive MCF-7 breast cancer cells but not in ER negative MDA-MB-231 cells (Carmeci et al., 1997). Besides in MCF-7 and SKBR3 breast cancer cells, GPR30 was later reported to be expressed in estrogen responsive tissues such as breast, heart, brain and leucocytes (Filardo, 2002). Thomas and co-workers finally demonstrated that GPR30 bears a single high affinity binding site for 17β-estradiol in ER-/GPR30+ SKBR-3 breast cancer cells and ER-/GPR30- human embryonic kidney (HEK) cells transfected with a GPR30 construct. Besides 17β-estradiol, the classical antiestrogens tamoxifen and fulvestrant were reported to induce an increased cAMP level in cells overexpressing GPR30, suggesting an agonist function towards GPR30 (Thomas et al., 2005). Another group confirmed estrogen binding to GPR30 and demonstrated its functional coupling to calcium- and IP_3 pathways (Revankar et al., 2005). In this study GPR30 was found to be localized in the endoplasmatic reticulum of cells overexpressing the receptor by confocal microscopy using a fluorescent estradiol analogue.

Recently, an agonist of GPR-30 named G-1 was identified by Bologna and co-workers. G-1 was reported to bind selectively to GPR30, but not to classical ERs and to trigger intracellular calcium mobilization selectively via GPR 30 (Bologa et al., 2006).

Taken together, the existence of GPR30 as functional estrogen binding GPCR appears to be evident. However there are still some open questions concerning the location within the cell (membrane or endoplasmatic reticulum) as well as its actual function and physiolocical role. The large number of recent reports on GPR30 mediated cellular functions suggests a key role in non-genomic estrogen action, but other mechanisms involving the classical ERs or other receptors might also contribute to this complex signaling. Compounds that are exclusively active via GPCR30 or the classical ERs respectively might open new doors for the understanding of nongenomic estrogen functions.

5 References

Aronica, S. M. and Katzenellenbogen, B. S., Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. *Mol Endocrinol* **1993,** 7, (6), 743-752.

Aronica, S. M.; Kraus, W. L. and Katzenellenbogen, B. S., Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci U S A* **1994,** 91, (18), 8517-8521.

Barkhem, T.; Carlsson, B.; Nilsson, Y.; Enmark, E.; Gustafsson, J. and Nilsson, S., Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol Pharmacol* **1998,** 54, (1), 105-112.

Bologa, C. G.; Revankar, C. M.; Young, S. M.; Edwards, B. S.; Arterburn, J. B.; Kiselyov, A. S.; Parker, M. A.; Tkachenko, S. E.; Savchuck, N. P.; Sklar, L. A.; Oprea, T. I. and Prossnitz, E. R., Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* **2006,** 2, (4), 207-212.

Brzozowski, A. M.; Pike, A. C.; Dauter, Z.; Hubbard, R. E.; Bonn, T.; Engstrom, O.; Ohman, L.; Greene, G. L.; Gustafsson, J. A. and Carlquist, M., Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **1997,** 389, (6652), 753-758.

Carmeci, C.; Thompson, D. A.; Ring, H. Z.; Francke, U. and Weigel, R. J., Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. *Genomics* **1997,** 45, (3), 607-617.

Cho, H. and Katzenellenbogen, B. S., Synergistic activation of estrogen receptor-mediated transcription by estradiol and protein kinase activators. *Mol Endocrinol* **1993,** 7, (3), 441-452.

Coleman, K. M.; Dutertre, M.; El-Gharbawy, A.; Rowan, B. G.; Weigel, N. L. and Smith, C. L., Mechanistic differences in the activation of estrogen receptor-alpha (ER alpha)- and ER beta-dependent gene expression by cAMP signaling pathway(s). *J Biol Chem* **2003,** 278, (15), 12834-12845.

Collini, M. D.; Kaufman, D. H.; Manas, E. S.; Harris, H. A.; Henderson, R. A.; Xu, Z. B.; Unwalla, R. J. and Miller, C. P., 7-Substituted 2-phenyl-benzofurans as ER beta selective ligands. *Bioorg Med Chem Lett* **2004,** 14, (19), 4925-4929.

Daub, H.; Wallasch, C.; Lankenau, A.; Herrlich, A. and Ullrich, A., Signal characteristics of G protein-transactivated EGF receptor. *Embo J* **1997,** 16, (23), 7032-7044.

Driggers, P. H. and Segars, J. H., Estrogen action and cytoplasmic signaling pathways. Part II: the role of growth factors and phosphorylation in estrogen signaling. *Trends Endocrinol Metab* **2002,** 13, (10), 422-427.

Enmark, E. and Gustafsson, J.-A., Estrogen receptor β - a novel receptor opens up new possibilities for cancer diagnosis and treatment. *Endocr Relat Cancer* **1998,** 5, 213-222.

Enmark, E.; Pelto-Huikko, M.; Grandien, K.; Lagercrantz, S.; Lagercrantz, J.; Fried, G.; Nordenskjold, M. and Gustafsson, J. A., Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* **1997,** 82, (12), 4258-4265.

Evinger, A. J., 3rd and Levin, E. R., Requirements for estrogen receptor alpha membrane localization and function. *Steroids* **2005,** 70, (5-7), 361-363.

Filardo, E. J., Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. *J Steroid Biochem Mol Biol* **2002,** 80, (2), 231-238.

Filardo, E. J.; Quinn, J. A.; Bland, K. I. and Frackelton, A. R., Jr., Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* **2000,** 14, (10), 1649-1660.

Fisher, B.; Costantino, J. P.; Redmond, C. K.; Fisher, E. R.; Wickerham, D. L. and Cronin, W. M., Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J Natl Cancer Inst* **1994,** 86, (7), 527-537.

Freedman, L. P., Anatomy of the steroid receptor zinc finger region. *Endocr Rev* **1992,** 13, (2), 129-145.

Fujimoto, N. and Katzenellenbogen, B. S., Alteration in the agonist/antagonist balance of antiestrogens by activation of protein kinase A signaling pathways in breast cancer cells: antiestrogen selectivity and promoter dependence. *Mol Endocrinol* **1994,** 8, (3), 296-304.

Green, S.; Walter, P.; Kumar, V.; Krust, A.; Bornert, J. M.; Argos, P. and Chambon, P., Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* **1986,** 320, (6058), 134-139.

Greene, G. L.; Gilna, P.; Waterfield, M.; Baker, A.; Hort, Y. and Shine, J., Sequence and expression of human estrogen receptor complementary DNA. *Science* **1986,** 231, (4742), 1150-1154.

Hafner, F.; Holler, E. and von Angerer, E., Effect of growth factors on estrogen receptor mediated gene expression. *J Steroid Biochem Mol Biol* **1996,** 58, (4), 385-393.

Harris, H. A.; Albert, L. M.; Leathurby, Y.; Malamas, M. S.; Mewshaw, R. E.; Miller, C. P.; Kharode, Y. P.; Marzolf, J.; Komm, B. S.; Winneker, R. C.; Frail, D. E.; Henderson, R. A.; Zhu, Y. and Keith, J. C., Jr., Evaluation of an estrogen receptor-beta agonist in animal models of human disease. *Endocrinology* **2003,** 144, (10), 4241-4249.

Harris, H. A.; Katzenellenbogen, J. A. and Katzenellenbogen, B. S., Characterization of the biological roles of the estrogen receptors, ERalpha and ERbeta, in estrogen target tissues in vivo through the use of an ERalpha-selective ligand. *Endocrinology* **2002,** 143, (11), 4172-4177.

Hart, S.; Fischer, O. M.; Prenzel, N.; Zwick-Wallasch, E.; Schneider, M.; Hennighausen, L. and Ullrich, A., GPCR-induced migration of breast carcinoma cells depends on both EGFR signal transactivation and EGFR-independent pathways. *Biol Chem* **2005,** 386, (9), 845-855.

Henke, B. R.; Consler, T. G.; Go, N.; Hale, R. L.; Hohman, D. R.; Jones, S. A.; Lu, A. T.; Moore, L. B.; Moore, J. T.; Orband-Miller, L. A.; Robinett, R. G.; Shearin, J.; Spearing, P. K.; Stewart, E. L.; Turnbull, P. S.; Weaver, S. L.; Williams, S. P.; Wisely, G. B. and Lambert, M. H., A new series of estrogen receptor modulators that display selectivity for estrogen receptor beta. *J Med Chem* **2002,** 45, (25), 5492-5505.

Hopp, T. A. and Fuqua, S. A. W., Estrogen and progesterone receptors in breast cancer. In: *Encyclopedia of hormones*. L., H.H. and Norman, A.W., eds. San Diego, Academic Press: 2003, Vol., 1, 573-577.

Horwitz, K. B.; Jackson, T. A.; Bain, D. L.; Richer, J. K.; Takimoto, G. S. and Tung, L., Nuclear receptor coactivators and corepressors. *Mol Endocrinol* **1996,** 10, (10), 1167-1177.

Jordan, V. C., Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. *J Med Chem* **2003a,** 46, (6), 883-908.

Jordan, V. C., Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J Med Chem* **2003b,** 46, (7), 1081-1111.

Jordan, V. C.; Gapstur, S. and Morrow, M., Selective estrogen receptor modulation and reduction in risk of breast cancer, osteoporosis, and coronary heart disease. *J Natl Cancer Inst* **2001,** 93, (19), 1449-1457.

Körner, M. and Reubi, J. C., NPY receptors in human cancer: a review of current knowledge. *Peptides* **2007,** 28, (2), 419-425.

Kuiper, G. G.; Carlsson, B.; Grandien, K.; Enmark, E.; Haggblad, J.; Nilsson, S. and Gustafsson, J. A., Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **1997,** 138, (3), 863-870.

Kuiper, G. G.; Enmark, E.; Pelto-Huikko, M.; Nilsson, S. and Gustafsson, J. A., Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* **1996,** 93, (12), 5925-5930.

Kuiper, G. G.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; van der Saag, P. T.; van der Burg, B. and Gustafsson, J. A., Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **1998,** 139, (10), 4252-4263.

Kumar, R. and Thompson, E. B., The structure of the nuclear hormone receptors. *Steroids* **1999,** 64, (5), 310-319.

Kumar, V.; Green, S.; Stack, G.; Berry, M.; Jin, J. R. and Chambon, P., Functional domains of the human estrogen receptor. *Cell* **1987,** 51, (6), 941-951.

Lannigan, D. A., Estrogen receptor phosphorylation. *Steroids* **2003,** 68, (1), 1-9.

Le Mellay, V.; Grosse, B. and Lieberherr, M., Phospholipase C beta and membrane action of calcitriol and estradiol. *J Biol Chem* **1997,** 272, (18), 11902-11907.

Love, R. R.; Mazess, R. B.; Barden, H. S.; Epstein, S.; Newcomb, P. A.; Jordan, V. C.; Carbone, P. P. and DeMets, D. L., Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* **1992,** 326, (13), 852-856.

Love, R. R.; Wiebe, D. A.; Feyzi, J. M.; Newcomb, P. A. and Chappell, R. J., Effects of tamoxifen on cardiovascular risk factors in postmenopausal women after 5 years of treatment. *J Natl Cancer Inst* **1994,** 86, (20), 1534-1539.

Malamas, M. S.; Manas, E. S.; McDevitt, R. E.; Gunawan, I.; Xu, Z. B.; Collini, M. D.; Miller, C. P.; Dinh, T.; Henderson, R. A.; Keith, J. C., Jr. and Harris, H. A., Design and synthesis of aryl diphenolic azoles as potent and selective estrogen receptor-beta ligands. *J Med Chem* **2004,** 47, (21), 5021-5040.

Metzger, D.; Ali, S.; Bornert, J. M. and Chambon, P., Characterization of the amino-terminal transcriptional activation function of the human estrogen receptor in animal and yeast cells. *J Biol Chem* **1995,** 270, (16), 9535-9542.

Meyers, M. J.; Sun, J.; Carlson, K. E.; Marriner, G. A.; Katzenellenbogen, B. S. and Katzenellenbogen, J. A., Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J Med Chem* **2001,** 44, (24), 4230-4251.

Michalides, R.; Griekspoor, A.; Balkenende, A.; Verwoerd, D.; Janssen, L.; Jalink, K.; Floore, A.; Velds, A.; van't Veer, L. and Neefjes, J., Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer. *Cancer Cell* **2004,** 5, (6), 597-605.

Morley, P.; Whitfield, J. F.; Vanderhyden, B. C.; Tsang, B. K. and Schwartz, J. L., A new, nongenomic estrogen action: the rapid release of intracellular calcium. *Endocrinology* **1992,** 131, (3), 1305-1312.

Mortensen, D. S.; Rodriguez, A. L.; Carlson, K. E.; Sun, J.; Katzenellenbogen, B. S. and Katzenellenbogen, J. A., Synthesis and biological evaluation of a novel series of furans: ligands selective for estrogen receptor alpha. *J Med Chem* **2001,** 44, (23), 3838-3848.

Mosselman, S.; Polman, J. and Dijkema, R., ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett* **1996,** 392, (1), 49-53.

Ogawa, S.; Inoue, S.; Watanabe, T.; Hiroi, H.; Orimo, A.; Hosoi, T.; Ouchi, Y. and Muramatsu, M., The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. *Biochem Biophys Res Commun* **1998a,** 243, (1), 122-126.

Ogawa, S.; Inoue, S.; Watanabe, T.; Orimo, A.; Hosoi, T.; Ouchi, Y. and Muramatsu, M., Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor ofestrogen action in human. *Nucleic Acids Res* **1998b,** 26, (15), 3505-3512.

Osborne, C. K., Tamoxifen in the treatment of breast cancer. *N Engl J Med* **1998,** 339, (22), 1609-1618.

Osborne, C. K.; Shou, J.; Massarweh, S. and Schiff, R., Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. *Clin Cancer Res* **2005,** 11, (2 Pt 2), 865s-870s.

Pace, P.; Taylor, J.; Suntharalingam, S.; Coombes, R. C. and Ali, S., Human estrogen receptor beta binds DNA in a manner similar to and dimerizes with estrogen receptor alpha. *J Biol Chem* **1997,** 272, (41), 25832-25838.

Paech, K.; Webb, P.; Kuiper, G. G.; Nilsson, S.; Gustafsson, J.; Kushner, P. J. and Scanlan, T. S., Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* **1997,** 277, (5331), 1508-1510.

Pettersson, K. and Gustafsson, J. A., Role of estrogen receptor beta in estrogen action. *Annu Rev Physiol* **2001,** 63, 165-192.

Pike, A. C.; Brzozowski, A. M.; Hubbard, R. E.; Bonn, T.; Thorsell, A. G.; Engstrom, O.; Ljunggren, J.; Gustafsson, J. A. and Carlquist, M., Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *Embo J* **1999,** 18, (17), 4608-4618.

Pike, A. C.; Brzozowski, A. M.; Walton, J.; Hubbard, R. E.; Thorsell, A. G.; Li, Y. L.; Gustafsson, J. A. and Carlquist, M., Structural insights into the mode of action of a pure antiestrogen. *Structure* **2001,** 9, (2), 145-153.

Pratt, W. B. and Toft, D. O., Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev* **1997,** 18, (3), 306-360.

Renaud, J.; Bischoff, S. F.; Buhl, T.; Floersheim, P.; Fournier, B.; Halleux, C.; Kallen, J.; Keller, H.; Schlaeppi, J.-M. and Stark, W., Estrogen Receptor Modulators: Identification and Structure-Activity Relationships of Potent ERα-Selective Tetrahydroisoquinoline Ligands. *J Med Chem* **2003,** 46, (14), 2945-2957.

Revankar, C. M.; Cimino, D. F.; Sklar, L. A.; Arterburn, J. B. and Prossnitz, E. R., A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **2005,** 307, (5715), 1625-1630.

Robertson, J. F., ICI 182,780 (Fulvestrant)--the first oestrogen receptor down-regulator--current clinical data. *Br J Cancer* **2001,** 85 Suppl 2, 11-14.

Robertson, J. F.; Nicholson, R. I.; Bundred, N. J.; Anderson, E.; Rayter, Z.; Dowsett, M.; Fox, J. N.; Gee, J. M.; Webster, A.; Wakeling, A. E.; Morris, C. and Dixon, M., Comparison of the short-term biological effects of 7alpha-[9-(4,4,5,5,5-

pentafluoropentylsulfinyl)-nonyl]estra-1,3,5, (10)-triene-3,17beta-diol (Faslodex) versus tamoxifen in postmenopausal women with primary breast cancer. *Cancer Res* **2001,** 61, (18), 6739-6746.

Robertson, J. F.; Osborne, C. K.; Howell, A.; Jones, S. E.; Mauriac, L.; Ellis, M.; Kleeberg, U. R.; Come, S. E.; Vergote, I.; Gertler, S.; Buzdar, A.; Webster, A. and Morris, C., Fulvestrant versus anastrozole for the treatment of advanced breast carcinoma in postmenopausal women: a prospective combined analysis of two multicenter trials. *Cancer* **2003,** 98, (2), 229-238.

Ruscica, M.; Dozio, E.; Boghossian, S.; Bovo, G.; Martos Riano, V.; Motta, M. and Magni, P., Activation of the Y1 receptor by neuropeptide Y regulates the growth of prostate cancer cells. *Endocrinology* **2006,** 147, (3), 1466-1473.

Schwabe, J. W.; Chapman, L.; Finch, J. T. and Rhodes, D., The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. *Cell* **1993,** 75, (3), 567-578.

Shiau, A. K.; Barstad, D.; Loria, P. M.; Cheng, L.; Kushner, P. J.; Agard, D. A. and Greene, G. L., The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **1998,** 95, (7), 927-937.

Shiau, A. K.; Barstad, D.; Radek, J. T.; Meyers, M. J.; Nettles, K. W.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A.; Agard, D. A. and Greene, G. L., Structural characterization of a subtype-selective ligand reveals a novel mode of estrogen receptor antagonism. *Nat Struct Biol* **2002,** 9, (5), 359-364.

Smith, C. L., Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biol Reprod* **1998,** 58, (3), 627-632.

Stauffer, S. R.; Coletta, C. J.; Tedesco, R.; Nishiguchi, G.; Carlson, K.; Sun, J.; Katzenellenbogen, B. S. and Katzenellenbogen, J. A., Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *J Med Chem* **2000,** 43, (26), 4934-4947.

Stauffer, S. R.; Huang, Y. R.; Aron, Z. D.; Coletta, C. J.; Sun, J.; Katzenellenbogen, B. S. and Katzenellenbogen, J. A., Triarylpyrazoles with basic side chains: development of pyrazole-based estrogen receptor antagonists. *Bioorg Med Chem* **2001,** 9, (1), 151-161.

Thomas, P.; Pang, Y.; Filardo, E. J. and Dong, J., Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* **2005,** 146, (2), 624-632.

Toft, D. and Gorski, J., A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proc Natl Acad Sci U S A* **1966,** 55, (6), 1574-1581.

Toft, D.; Shyamala, G. and Gorski, J., A receptor molecule for estrogens: studies using a cell-free system. *Proc Natl Acad Sci U S A* **1967,** 57, (6), 1740-1743.

Tremblay, G. B.; Tremblay, A.; Copeland, N. G.; Gilbert, D. J.; Jenkins, N. A.; Labrie, F. and Giguere, V., Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol* **1997,** 11, (3), 353-365.

Tsai, M. J. and O'Malley, B. W., Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* **1994,** 63, 451-486.

Turken, S.; Siris, E.; Seldin, D.; Flaster, E.; Hyman, G. and Lindsay, R., Effects of tamoxifen on spinal bone density in women with breast cancer. *J Natl Cancer Inst* **1989,** 81, (14), 1086-1088.

Tzukerman, M. T.; Esty, A.; Santiso-Mere, D.; Danielian, P.; Parker, M. G.; Stein, R. B.; Pike, J. W. and McDonnell, D. P., Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by

two functionally distinct intramolecular regions. *Mol Endocrinol* **1994,** 8, (1), 21-30.

Walter, P.; Green, S.; Greene, G.; Krust, A.; Bornert, J. M.; Jeltsch, J. M.; Staub, A.; Jensen, E.; Scrace, G.; Waterfield, M. and et al., Cloning of the human estrogen receptor cDNA. *Proc Natl Acad Sci U S A* **1985,** 82, (23), 7889-7893.

Zimmermann, J.; Liebl, R. and von Angerer, E., 2,5-Diphenylfuran-based pure antiestrogens with selectivity for the estrogen receptor alpha. *J Steroid Biochem Mol Biol* **2005,** 94, (1-3), 57-66.

B Scope and Objectives

1 Scope and Objectives of the Thesis

The discovery of an estrogen binding protein in the 1960s and the increasing knowledge about its key role in the growth and development of the majority of breast cancers opened the doors for antiestrogens in breast cancer therapy. For many years tamoxifen has been the drug of choice in adjuvant therapy and the treatment of advanced and metastasized estrogen receptor (ER) positive breast cancer. Ten years ago, the discovery of a second ER protein designated ERβ complicated endocrine research, because until then endocrinologists focused on the existence of only one receptor protein that was believed to mediate all estrogenic effects. The physiological roles of the two distinct ER subtypes, in particular in the tumorigenesis and the growth of breast cancer, are only understood to some extent.

While highly selective agonists and moderately selective SERMs for both ER subtypes α and β are available, there is still a gap in the field of subtype selective “pure antagonists”. Compounds based on a diphenylfuran scaffold, that were recently developed in our group provide a first approach towards ERα selective “pure antagonists”, although the compounds with the highest selectivities are only weak antiestrogens (Zimmermann et al., 2005). Potent subtype selective ER antagonists are required as pharmacological tools to investigate, if cellular effects such as cross-talk signaling are subtype specific. Furthermore, ERα selective “pure antagonists” might be attractive candidates in the therapy of ER positive breast cancer, as ERα has been reported to be the main subtype involved in tumor growth.

Therefore, one aim of this thesis was the development of new non-steroidal subtype selective pure antagonists of the estrogen receptor. 2-Phenylbenzofurans are known as ERβ selective agonists from literature and from our own studies performed during the past few years (Collini et al., 2004; Zimmermann, 2005). It was shown that the introduction of long functionalized side chains in position C3 of the benzofuran core leads to antagonists with high potency, but lack of subtype selectivity. Within the scope of this thesis side chains characteristic for pure ER antagonists have to be linked to

position C7 of the benzofuran molecule, as C7-substituents were recently shown to be crucial for ERβ selectivity of benzofurans and benzoxazoles. A straight forward Sonogashira coupling route should afford a 7-formyl-2(4-methoxypenyl) building block, which has to be linked to aliphatic Grignard-nucleophiles or Wittig-ylides via the benzaldehyde function.

In the field of ERα selective ligands, tetrahydroisoquinolines (THIQs) were recently reported as ERα selective SERMs (Renaud et al., 2003; Renaud et al., 2005). In the present study, the replacement of the side chains in position C1 of the THIQ based SERMs by a long functionalized aliphatic side chain should give rise to full antagonists. The consequences of these structural modifications with respect to receptor binding and subtype selectivity had to be investigated. A published Bischler-Napiralski synthetic route, and an alternative route via a dihydroisoquinolone building block were evaluated regarding their applicability in an effective parallel synthesis in view of a library of THIQ based target compounds.

Binding affinities and selectivities of all synthesized compounds were to be determined in a radiometric binding assay with recombinant ERα and ERβ proteins and compared to known reference compounds. For compounds revealing sufficiently high receptor binding, further *in vitro* characterization, namely regarding (ant)agonistic activity in a gene reporter transcription assay and antiproliferative activity in a cytotoxicity assay with human breast cancer cells, had to be carried out.

The side chains of the devised THIQs are anchored via the chiral C1 atom to the THIQ core, yielding pairs of enantiomers, so that an appropriate method for enaniomeric separation had to be worked out. In order to identify eutomers and distomers, after successful resolution of the enantiomeric mixtures, the individual enantiomers had to be characterized with respect to ERα and ERβ binding .

In recent years membrane bound receptors such as GPCRs and tyrosine kinases (e.g EGFRs) gained increasing interest in breast cancer research. Cytoplasmic signaling pathways mediated by membrane receptors were shown to activate the unoccupied nuclear ER via phosphorylation at specific serine residues. The NPY Y_1 receptor (Y_1R), a peptidergic GPCR was reported to be expressed in many human primary tumors with particularly high incidence (85%) and receptor densities in breast cancers (Körner and Reubi, 2007). In MCF-7 breast cancer cells the Y_1R mRNA was recently shown to be up-regulated by estrogen (Amlal et al., 2006).

Within this project different breast cancer cell lines were selected to be analyzed with respect to Y_1R expression on the protein level, using the recently developed Y_1R selective radioligand [^{3}H]-UR-MK114 (Keller et al., 2008). In different subclones of MCF-7 breast cancer cells ER and Y_1R expression had to be quantified using tritiated 17β-estradiol or [^{3}H]-UR-MK114, respectively. ERα and β subtype expression in the investigated MCF-7 sublines had to be analyzed by Western-Blots using specific antibodies on the protein level on one hand, and on the mRNA level by reverse transcription - polymerase chain reaction (RT-PCR) on the other hand. Furthermore, the effect of (anti)estrogens on Y_1R protein expression in ER positive breast cancer cell lines had to be characterized. Available or newly synthesized (ant)agonists, selective for either ERα or ERβ, were considered to provide information on subtype specificity of estrogen induced Y_1R expression.

As reports on the function of Y_1Rs in breast cancer cells are scarce and contradictory in part, NPY induced inhibition of adenyl cyclase activity and its effect on the mobilization of intracellular calcium were investigated. Moreover, efforts were made to explore, if ER-mediated transcriptional activity and cell proliferation depend on Y_1R activation.

2 References

Amlal, H.; Faroqui, S.; Balasubramaniam, A. and Sheriff, S., Estrogen up-regulates neuropeptide Y Y1 receptor expression in a human breast cancer cell line. *Cancer Res* **2006,** 66, (7), 3706-3714.

Collini, M. D.; Kaufman, D. H.; Manas, E. S.; Harris, H. A.; Henderson, R. A.; Xu, Z. B.; Unwalla, R. J. and Miller, C. P., 7-Substituted 2-phenyl-benzofurans as ER beta selective ligands. *Bioorg Med Chem Lett* **2004,** 14, (19), 4925-4929.

Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G. and Buschauer, A., Guanidine-Acylguanidine Bioisosteric Approach in the Design of Radioligands: Synthesis of a Tritium-Labeled N(G)-Propionylargininamide ([(3)H]-UR-MK114) as a Highly Potent and Selective Neuropeptide Y Y(1) Receptor Antagonist. *J Med Chem* **2008,** 51, (24), 8168-8172.

Körner, M. and Reubi, J. C., NPY receptors in human cancer: a review of current knowledge. *Peptides* **2007,** 28, (2), 419-425.

Renaud, J.; Bischoff, S. F.; Buhl, T.; Floersheim, P.; Fournier, B.; Geiser, M.; Halleux, C.; Kallen, J.; Keller, H. and Ramage, P., Selective Estrogen Receptor Modulators with Conformationally Restricted Side Chains. Synthesis and Structure-Activity Relationship of ERα-Selective Tetrahydroisoquinoline Ligands. *J Med Chem* **2005,** 48, (2), 364-379.

Renaud, J.; Bischoff, S. F.; Buhl, T.; Floersheim, P.; Fournier, B.; Halleux, C.; Kallen, J.; Keller, H.; Schlaeppi, J.-M. and Stark, W., Estrogen Receptor Modulators: Identification and Structure-Activity Relationships of Potent ERα-Selective Tetrahydroisoquinoline Ligands. *J Med Chem* **2003,** 46, (14), 2945-2957.

Zimmermann, J., Furan- and pyran-based heterocycles as subtype-selective ligands of the estrogen receptor. Synthesis and biological characterisation. Doctoral thesis, Universität Regensburg, Regensburg, **2005**

Zimmermann, J.; Liebl, R. and von Angerer, E., 2,5-Diphenylfuran-based pure antiestrogens with selectivity for the estrogen receptor alpha. *J Steroid Biochem Mol Biol* **2005,** 94, (1-3), 57-66.

C Synthesis and Biological Characterization of New Estrogen Receptor Ligands

1 Pharmacological Test System

1.1 Radiometric Binding Assay

Radiometric binding assays are standard procedures in many academic and industrial research institutes. Target-specific binding is one of the most important criteria in the search for new drugs and pharmacological tools.

In most cases, the concentration dependant displacement of a well characterized, target selective radioligand with high binding affinity by a tested compound is analyzed. The radioligand is applied at a constant concentration, while the concentrations of the test compounds vary within a certain range. In the case of the estrogen receptor, the tritiated endogenous ligand 17β-estradiol is commonly used as radioligand. In our group, a cytosol prepared from calf uteri was used for many years as estrogen receptor source. Since ERβ as the second ER subtype besides ERα was discovered about ten years ago and recombinant full length human ERα and ERβ proteins have become available, these proteins are used for the determination of receptor affinity by most researchers. A new binding assay using commercial human ERα and ERβ proteins was established in our group by Dr. Zimmermann (Zimmermann, 2005). Within the scope of this thesis, all compounds were characterized by their binding profiles using both recombinant ER subtypes.

17β-Estradiol reveals similar affinities to both ER subtypes with K_D values of approximately 0.35 for ERα and 0.2 for ERβ (given by Invitrogen / pan Vera as the manufacturer of the recombinant proteins). In all experiments [^{3}H] estradiol was present at a concentration of 0.5 nM, guaranteeing the occupation of almost all binding sites. In each assay, the maximum number of occupied binding sites and the number of unspecific binding sites were determined by applying the radioligand alone and in presence of a 500-fold excess of unlabeled (“cold”) estradiol respectively. All new compounds and reference compounds including 17β-estradiol, 4-hydroxytamoxifen and

fulvestrant (ICI.182.780) were tested at six different concentrations each, covering a concentration range of two decades. According to this procedure, only the linear part of the displacement curve (20-80 % specific binding) was recorded. IC_{50} values (concentration of test-compound to inhibit specific radioligand binding by 50%) were determined after logit-log-transformation of the experimental data, where

logit = log [displacement (%) / (total specific binding (100%) – displacement (%))]

RBA (relative binding affinity) was calculated as the ratio of IC_{50} of estradiol and IC_{50} of test compound multiplied by 100. By definition the RBA value of the endogenous ligand 17β-estradiol is 100.

1.2 Luciferase assay

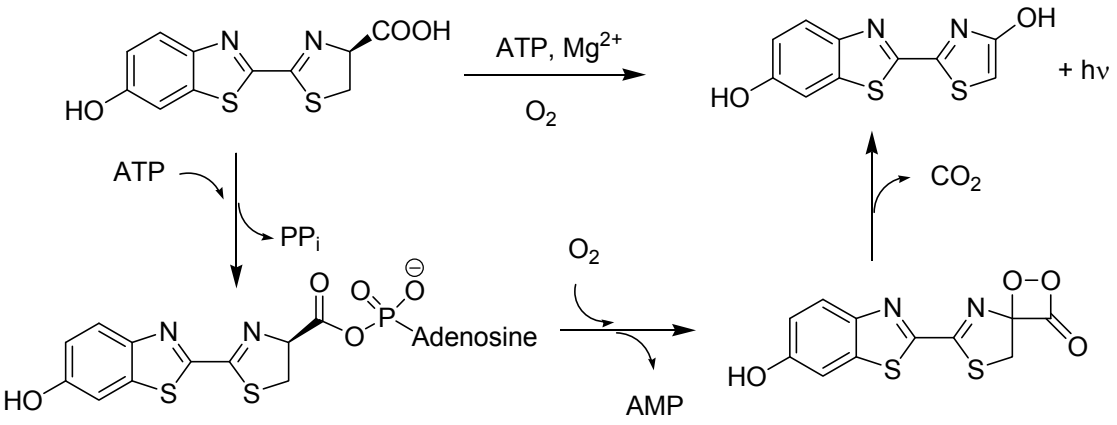
1.2.1 Principles of the Gene Reporter Assay

The luciferase assay is a very sensitive method for the determination of hormonal activity *in vitro*. In our research group, ER positive MCF-7 breast cancer cells were stably transfected with the plasmid 'EREwtc luc' harboring the ERE-controlled luciferase reporter gene (Meyer et al., 1994), isolated from the North American firefly *Photinus pyralis* (de Wet et al., 1987). The resulting subline has been termed MCF-7/2a and is routinely used in the screening of new compounds for estrogenic and antiestrogenic activity. Contrary to cell proliferation, gene expression is a very early event (proximal) in the estrogen signaling cascade and therefore exclusively mediated by the activation of the estrogen receptor.

When the assay is run in the agonist mode, potential estrogens are incubated with the cells for 48-50 h, until the luciferase expression reaches its maximum level. The estrogenic effect is generally expressed as the percentage of the luminescence evoked by 17β-estradiol at a concentration of 1nM. In the antagonist mode, potential antiestrogens are incubated with the cells in the presence of 17β-estradiol as stimulating agent.

The luciferase catalyses a reaction sequence known as firefly luminescence. The bioluminescence reaction requires the substrate D-luciferin and the cosubstrates ATP and Mg^{2+}. During a two-step-reaction sequence, the substrate undergoes oxidative

decarboxylation, which results in the production of oxyluciferin and visible light (cf. Scheme C1). The mechanism of this enzymatic reaction has been reported elsewhere (White et al., 1969).



Scheme C1: The luciferase catalyzed reaction

1.2.2 Optimization of the Luciferase Assay

In previous studies of our research group, the assay was performed using a commercially available luciferase assay kit (Promega). This procedure is rapid and convenient, but the screening of compound libraries is very expensive.

To perform the assay, two buffers, the substrate D-luciferin and the co-substrates ATP and Mg^{2+}, which are all included in the luciferase assay kit, are required. In initial experiments the feasibility of an economical method, using a self-made buffer system containig the substrate D-luciferin, purchased from a cheaper commercial source was explored, and the results were compared to those obtained with the luciferase assay kit.

The buffers were based on tricine (N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine), adjusted to pH 7.8. Among several common buffers, tricine buffer was reported to least affect luciferase activity (Webster et al., 1980). Additives and concentrations were adopted from a published protocol (Brasier, 1990). The lysis buffer for the preparation of cell extracts was supplemented with the detergent Triton^{TM} X-100, DTT and Mg^{2+}. The second buffer, termed “luciferase assay buffer”, contained the co-substrates ATP and Mg^{2+}. The selective calcium chelator EGTA (ethyleneglycoltetraacetic acid) was added

to both buffers. D-lucifrin was injected as a solution in luciferase assay buffer to the cell lysates.

Luminescence is known to have a relatively short half-life in the range of seconds. Therefore, a possible enhancing or stabilizing effect of coenzyme A (CoA) on the luminescence signal was investigated in an exploratory experiment. Such effects of CoA to the luciferase signal have been described by several groups (Airth et al., 1958; Ford et al., 1995; Fraga et al., 2005). The concentration range for CoA was 50-100 µM according to a literature report (Fraga et al., 2005). It should be mentioned that the previously reported stabilizing effect of CoA on the luciferase signal was obtained with the purified enzyme, rather than in a cellular assays.

Figure C2 shows the comparison of the signals measured with the new assay system in the presence and the absence of CoA with those obtained with the commercial luciferase assay kit.

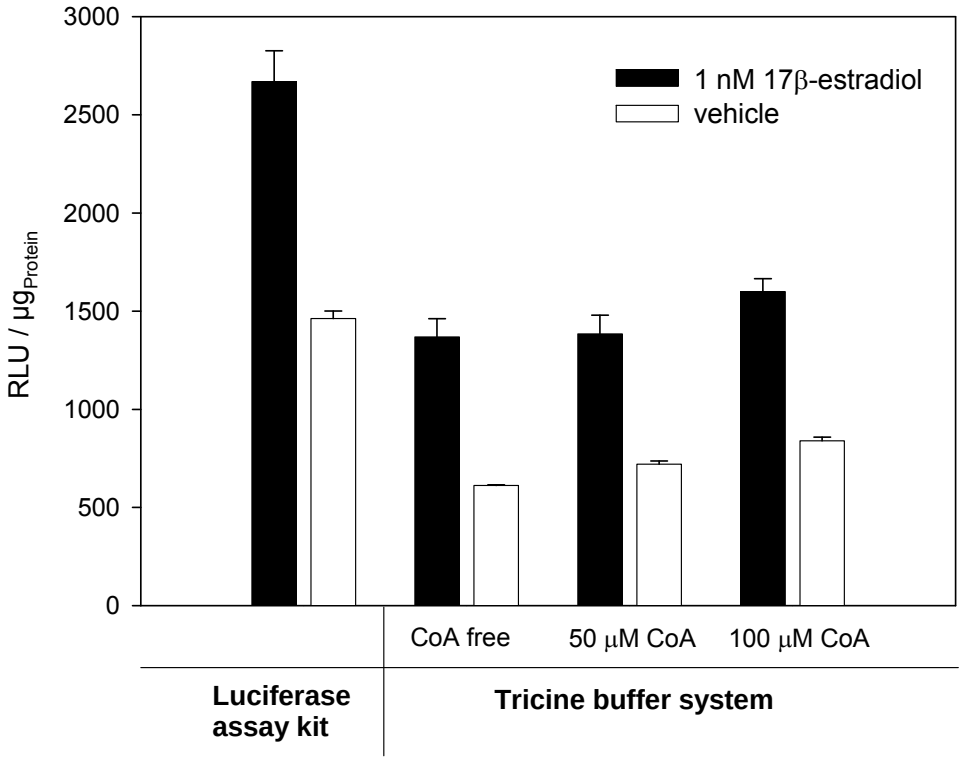


Figure C1: Comparison of luciferase signals in different assay systems
Values represent means of triplicates ± SEM

CoA had no significant effect to the luciferase signal up to a concentration of 100 µM. A possible explanation might be a stabilizing / enhancing effect of the detergent Triton™ X-100, a component of the lysis buffer on the luminescence signal, which cannot be

further enhanced by additives such as CoA. Effects of Triton™ X-100 on luciferase activity have been reported by Kricka and de Luca (Kricka and De Luca, 1982).
When the assay was performed in the self-made tricine buffer system, the absolute signal was decreased by approximately a factor of 2 compared to the signal obtained with the luciferase assay kit. However, for the determination of (anti)estrogenic effects not the absolute signal, but the signal to noise ratio is decisive. Irrespective of the height of the signals, the basal luciferase activity was approximately 50% of the 17β-estradiol stimulated control in all assay systems tested. This basal luciferase expression is assumed to mainly result from hormones and growth factors present in FCS (fetal calf serum), a culture medium supplement, at low concentrations and from ligand independent ER activation. Attempts to lower basal estrogen activity by depriving FCS from small molecular components using the dextran-coated charcoal (DCC) method failed, as the proliferation of MCF-7/2a cells ceased, when FCS was replaced with charcoal treated fetal calf serum (ct-FCS).
In a number of experiments the home-made tricine buffer system prooved to be appropriate for the reproducible determination of estrogenic and antiestrogenic activity. Thus, as alternative to the luciferase assay kit, an inexpensive and robust assay system was established for compound screening.

1.3 Proliferation Assay Using Human Mammary Carcinoma Cell Lines

Compounds revealing sufficient binding affinity and antiestrogenic potency in the luciferase assay were submitted to a microculture chemosensitivity assay described by Bernhardt et al. (Bernhardt et al., 1992). The assay relies on the quantification of cell mass by staining cells with crystal violet.
Two different breast cancer cell lines were used for the characterization of the new compounds: MCF-7 cells are endowed with a relatively high estrogen receptor content, and their growth is estrogen dependent. This cell line was used to demonstrate an estrogen receptor mediated antiproliferative effect of new antiestrogens. As the level of estrogen receptor expression in MCF-7 cells is not stable during long culture periodes (Bernhardt et al., 1992; Leichtl, 1994), the sensitivity of the cells against aniestrogen treatment must be routinely monitored using potent reference antiestrogens such as fulvestrant (ICI 182.780) or 4-hydroxytamoxifen. Detailed information on ER expression

in different MCF-7 subclones is given in section D of this thesis. As the growth of MDA-MB-231 cells is estrogen independent, these breast cancer cells are used to exclude unspecific toxic effects of (anti)estrogens.

In each assay identically treated cells were fixed at different time points within a period of eight to ten days of incubation. Analyses of growth curves provide information on the type of drug action: In case of a cytotoxic effect cell proliferation is initially inhibited, but with progressing incubation, the cell density of the treated population approximates that of the corresponding vehicle control. In the case of a cytostatic effect, the optical density remains either on the level of the initial value or is slightly increased and stays at a certain plateau. Cytocidal effects are characterized by a decrease in cell density below the initial level, as the main part of the cell population dies.

Corrected T/C (test/control) values ($T/C_{corr.}$) are calculated as the ratio of the optical densities of treated to non treated cells. Each density is corrected by subtraction of the initial cell density T_0.

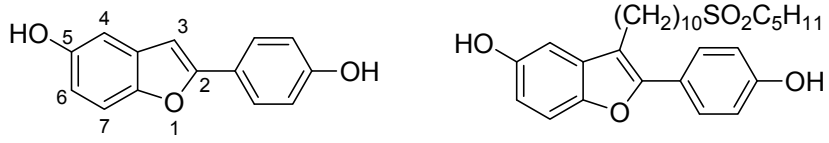
$$T/C_{corr.} = (T-T_0)/(C-T_0) \cdot 100\%$$

The corrected T/C values obtained after maximal incubation periods (end points) were plotted as a function of the concentration. From these graphs the IC_{50} values were determined as molar concentration, which is required to inhibit cell growth by 50 %.

2 2-Arylbenzo[b]furans

Compounds based on the 2-(4-hydroxyphenyl)benzo[b]furan scaffold have been extensively investigated in our research group (Erber, 1989; Leichtl, 1994; Zimmermann, 2005). In these studies, the benzo[b]furan partial structure has been substituted with a hydroxy function in position C5 or C6 and a wide variety of different side chains in position C3 (cf. Figure C2). The side chains of the earlier studies reached from simple alkyl moieties to monofunctionalised long chains. Dr. Zimmermann synthesized a library of 6-hydroxy-2(4-hydroxyphenyl)benzofurans equipped with aforementioned substituents as well as a newly developed bifunctional side chain (Walter, 2002) in position C3 of the benzofuran. The design of the side chains will be explained in the next paragraph.

The knowledge about the two subtypes of the ER, ERα and ERβ and a newly established binding assay using recombinant receptors enabled our group to screen the previously synthesized as well as the recent benzofuran libraries for subtype selectivity. In his studies, Dr. Zimmermann found, that C3-ususbstituted 5- and 6-hydroxy benzofurans bind selectively to ERβ (~20 fold over ERα). Introduction of small 3-alkyl substituents lowered or abrogated the selectivitiy as in case of long thioether, sulfone, and bifunctional chains (Zimmermann, 2005).



B1 **B2**

Figure C2: 5-Hydroxy-2-(4-hydroxyphenyl)benzo[b]furans; **B2** was synthesized by Dr. Leichtl (Leichtl, 1994)

In recent studies the Wyeth Research Institute focused on the development of new ERβ selective agonists, also including 2-aryl-benzo[b]furans (Collini et al., 2004; Manas et al., 2004). A library of 2-arylbenzo[b]furans substituted with small relatively lipophilic groups in position C7 was synthesized and tested for ERα/ERβ binding selectivity. The majority of 7-substituted 2-(4-hydroxyphenyl)benzo[b]furans exhibited a higher selectivity for ERβ in comparison to the corresponding unsubstituted analogue **B1**. Co-

crystallization experiments of the 7-substituted benzofurans in complex with ERβ show, that the C7 substituents extend into a narrow pocket formed by Ile 373, Ile367 and Phe 377. ERβ Ile373 corresponds to ERα Met421, the residue of which is supposed to cause a higher steric repulsion with the benzofuran C7-substituent compared to the isoleucine residue, explaining the selectivity of the compounds in favor of ERβ.

2.1 Design of Potential New ERβ-Selective 2-Arylbenzo[b]furan-based Antiestrogens

As Dr. Zimmermann's approach, developing new ERβ-selective antiestrogens by introducing "antiestrogenic" side chains into the benzofuran C3 position failed, a new strategy was followed. Inspired by the work of the Wyeth Research Institute, the approach for this study was the synthesis and biological evaluation of 2-(4-hydroxy)-phenylbenzofurans with antiestrogenic side chains in position 7.

Two types of side chains that should afford full antagonism were adopted from antiestrogens that have demonstrated high antiestrogenic potencies in previous studies of our research group (cf. Figure C3). The first type, the "monofunctional" side chains possess either a sulfanyl or a sulfonyl group in a distance of 9 or 10 carbon atoms from the benzofuran core. The "bifunctional" side chain has in addition to the sulfanyl group a basic methylamine function in place of the methylene group in position C7 of the side chain. This bifunctional chain with an additional terminal fluorination was first used by scientists in the research laboratories of Schering AG in Berlin to modify the structure of fulvestrant (ICI 182.780). The resulting steroidal antiestrogen was ZK191.703 (cf. Figure C3), which has shown a better oral bioavailability in comparison to fulvestrant (Hoffmann and Sommer, 2005).

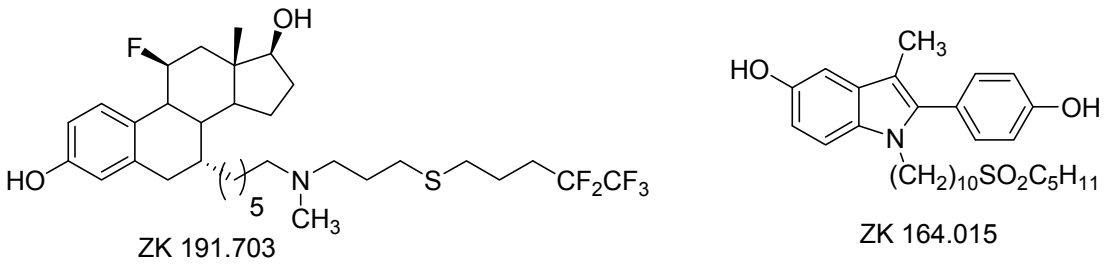


Figure C3: Potent steroidal and nonsteroidal antiestrogens

ERβ selective “pure antagonists” have not been described in literature. Such compounds would be useful tools for the investigation of ER subtype specific cellular functions, which is a topic in Chapter D of this thesis.

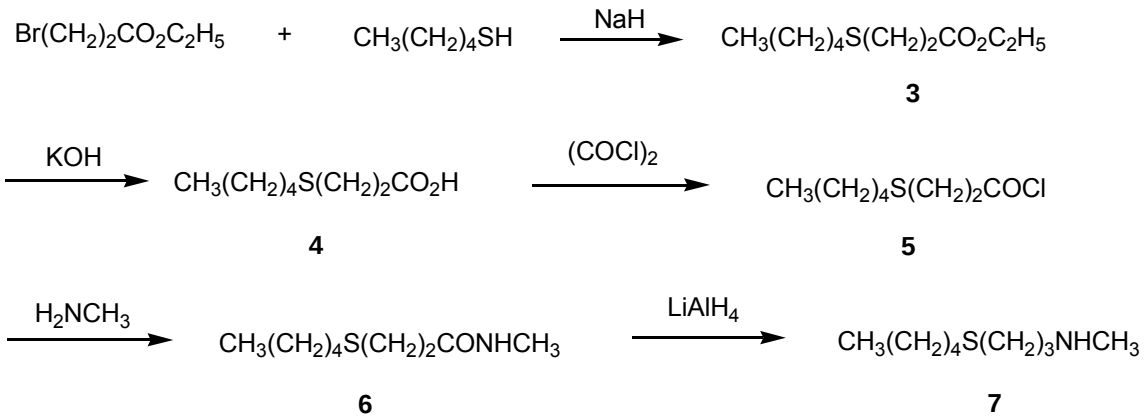
2.2 Chemistry

2.2.1 Synthesis of Side Chains

The monofunctional side chains were synthesized from pentanethiolate, generated with sodium hydride, and added to a 4-fold excess of 1,10-dibromodecane (Biberger, 1996). The desired monosubstituted product and the bisubstituted byproduct were separated by column chromatography (cf. Scheme C2).

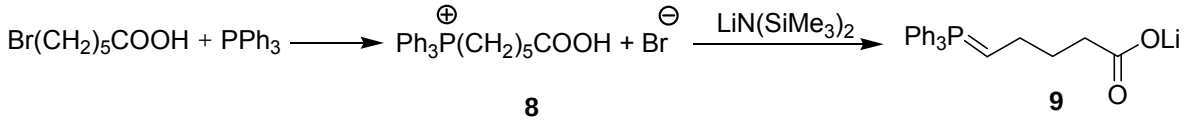
Scheme C2: Synthesis of thioether side chains

The synthesis of the amine precursor of the bifunctional side chain is depicted in Scheme C3. The multi-step reaction started from ethyl-3-bromopropionate, which was converted by nucleophilic substitution with pentanethiolate to the corresponding sulfide **3**. Subsequent ester hydrolysis to **4**, formation of the acid chloride **5** with oxalyl chloride and reaction with aqueous methylamine afforded the amide **6** that was reduced to the amine **7** by $LiAlH_4$ (Walter, 2002). In further reactions, **7** was alkylated or acylated by residues attached to the pharmacophore.



Scheme C3: Synthesis of the amine precursor for bifunctional side chains

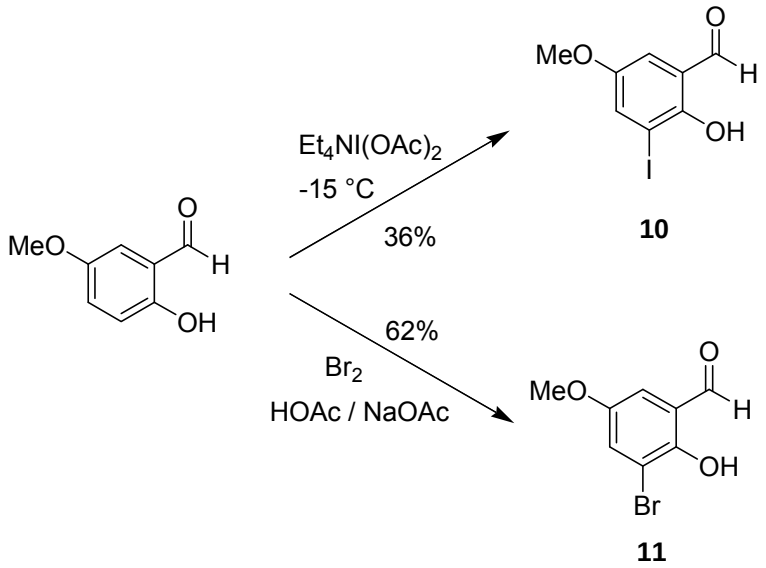
Phosphorous ylid **9** was required as Wittig reagent to build up the bifunctional side chain in the benzofuran C7 position. The synthesis of **9** started from 5-bromovaleric acid, which was converted by S_N reaction with triphenylphosphine to the triphenyl-phosphonium bromide **8** (Bhalerao et al., 1970). Deprotonation to the corresponding phosphorous ylid **9** was achieved by $LiN(SiMe_3)_2$ (Horillo-Martinez et al., 2007) (cf. Scheme C4).



Scheme C4: Synthesis of phosphonium ylid **9** from 5-bromovaleric acid

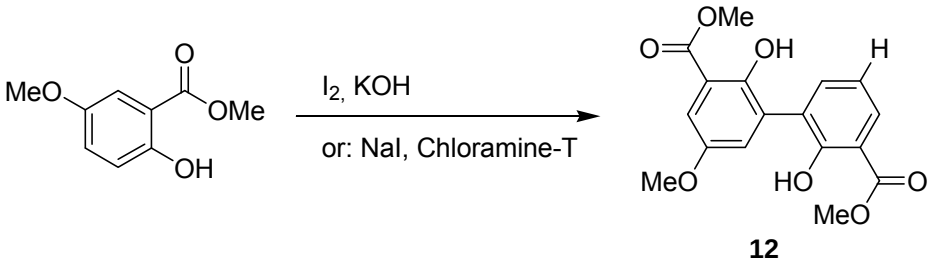
2.2.2 Synthesis of the 2-Aryl-7-formylbenzofuran Building Block

The 2-aryl-7-formyl benzofuran building block can be synthesized in a one-pot Sonogashira coupling and cyclisation. The palladium(0) / copper(I) catalyzed reaction requires a halogenated aryl component that is coupled to an acetylene moiety. Due to their high reactivity, aryliodides as starting materials are the first choice in this type of reaction. Starting from 2-hydroxy-5-methoxybenzaldehyde iodination in position 3 was achieved by the iodination reagent tetraethylammonium diacetoxyiodate (Doleschall and Toth, 1980; Hart and Mannino, 1996) in 36% yield (cf. Scheme C5).



Scheme C5: Iodination and bromination of 2-hydroxy-5-methoxybenzaldehyd

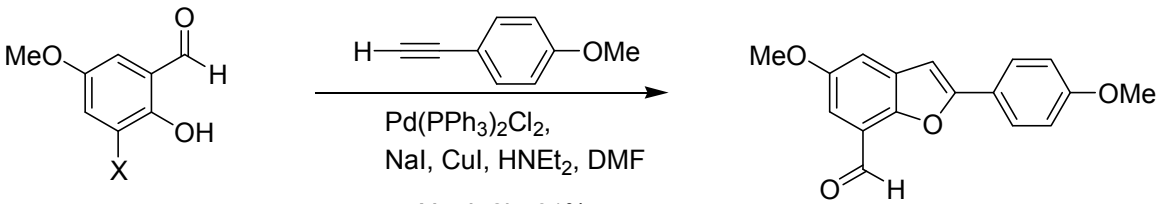
Conventional iodination methods were also tested using the model compound methyl-5-methoxy salicylate. The iodination of this educt by iodine and KOH has been described in a recent publication (Collini et al., 2004). As reported by other groups (Kometani et al., 1985; Hart and Mannino, 1996) the iodination by this method as well as by a mixture of chloramine T and sodium iodide lead almost exclusively to the formation of the biaryl product **12** (cf. Scheme C6).



12

Scheme C6: Formation of biaryl **12** by conventional iodination methods

For the preparation of the aryl iodides the only successful synthetic route required strictly controlled conditions and a long reaction time, while the yield was very moderate. Alternatively, the corresponding bromo analogue **11** was synthesized in a fast and simple reaction with bromine in acetic acid/sodium acetate (Evano et al., 2004) (Scheme C5). Amounting to 62%, the yield in this bromination reaction was acceptable. The aryliodides and arylbromides were then coupled to 4-ethinylanisol in a Sonogashira coupling reaction (Arcadi et al., 1986). The furan ring was closed *in situ* via the free hydroxy group and the product **13** was formed in a single step (Scheme C7). Due to the relatively mild coupling conditions, the aldehyde function was retained.



10 X = I
11 X = Br

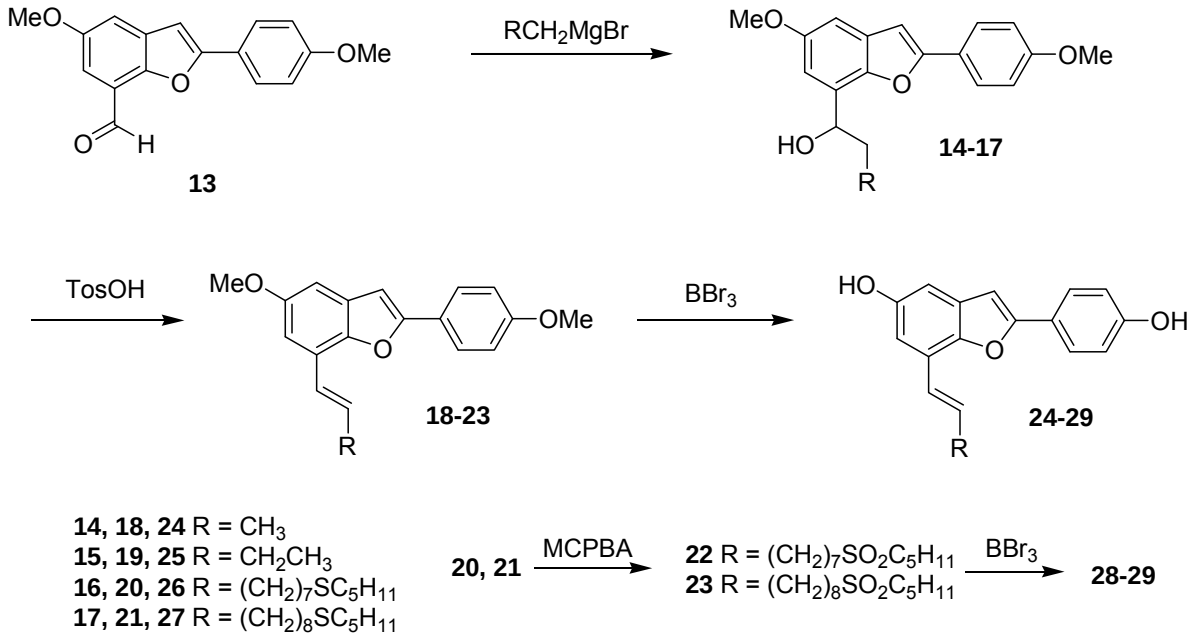
13

Scheme C7: Sonogashira coupling of iodo- and bromoaryl **10** and **11** with 4-ethinylanisol to the 2-aryl-7-formyl benzofuran building block **13**

Starting from aryliodide **10**, with 2 h and 61% the reaction time was shorter and the yield higher compared to 14 h and 46 % in case of arylbromide **11** as starting material. The overall yield of the two steps from 2-hydroxy-5-methoxybenzaldehyde to the building block **13** was 22 % via the iodide and 29 % via the bromide. Because of this slight difference in the overall yield and the fact, that the bromination was much faster and easier, the route via bromide **11** was preferred for up-scaling.

2.2.3 Introduction of Aliphatic Side Chains

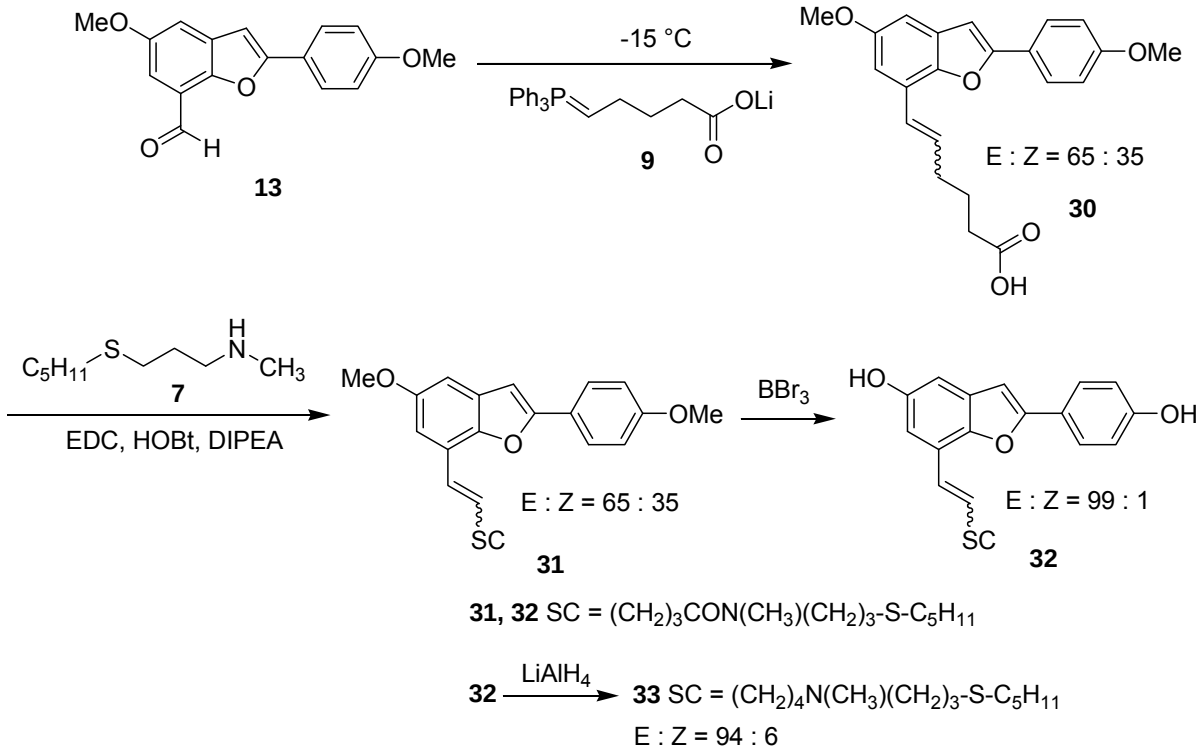
The alkyl groups were linked to the aldehyde function in the benzofuran C7 position by Grignard reaction. The commercially available or synthesized alkylbromides were converted into Grignard reagents by reaction with elementary magnesium. The Grignard nucleophiles attacked the aldehyde **13** to give the secondary alcohols **14-17**. Dehydratation under catalysis with p-toluenesulfonic acid led to the E-olefins **18-23**. In the case of compounds **20** and **21**, bearing thioether side chains, the sulfur was oxidized by meta-chloroperbenzoic acid to the corresponding sulfones **22** and **23**. The methoxy protecting groups were cleaved throughout by BBr_3 (Scheme C8). Mechanistic and experimental details to this ether cleavage will be given in the next paragraph.



Scheme C8: Synthetic route to 7-alkenyl benzofurans

Certain functional groups such as amines, amides, carboxylic acids and esters are not compatible with the Grignard reaction. Such groups are included in the bifunctional side chain itself or its synthetic precursors. Therefore the synthetic strategy, to attach a bifunctional side chain to the 7-aldehyde function of **13** was different from the one described for the other side chains.

The key reaction was a Wittig olefination of the benzaldehyde derivative **13** with the phosphourous ylide **9**, which was prepared as described in paragraph 2.2. Generally the E/Z stereoselectivity in the Wittig reaction is controlled by the properties of the phosphorous ylide. Compound **9** is a so-called labile ylide, as the electron donating alkyl chain effects a destabilization of the nucleophilic carbanion. Labile ylides commonly lead to Z selectivity. As an exception of this rule, the ω-carboxyl ylide **9** afforded the corresponding Wittig olefin **30** with a slight E-selectivity. Such directing effects of nucleophilic groups in appropriate distance to the ylide-phosphorous have been described in previous publications (Maryanoff et al., 1985; Ding et al., 1995). The stereoselectivity is of minor importance, as in the final deprotection step the the E-olefine is formed almost quantitatively.

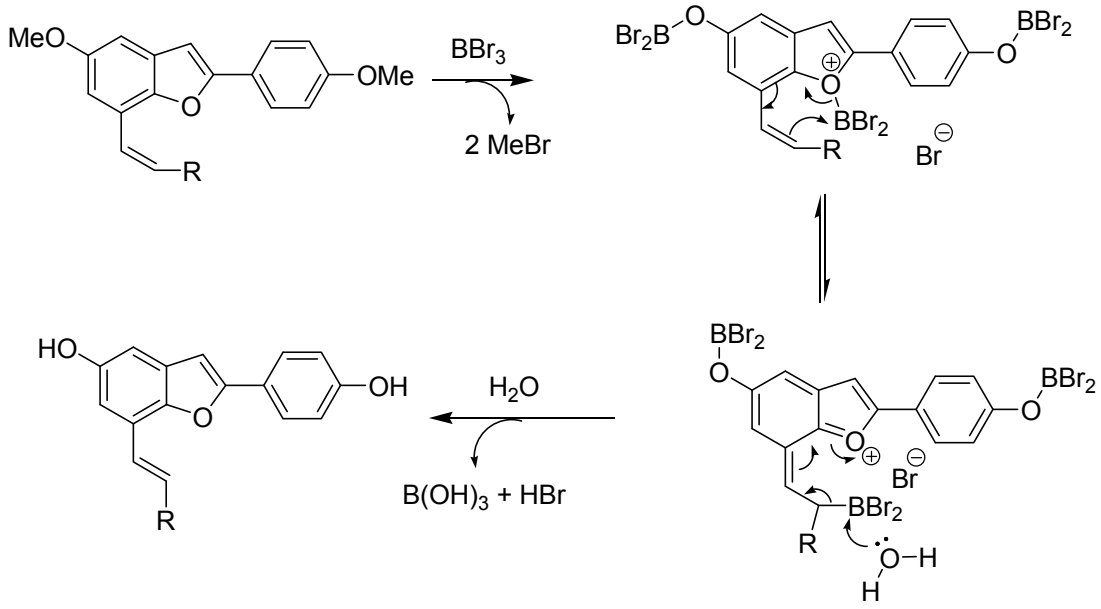


Scheme C9: Synthetic route to the 2-(4-hydroxyphenyl)benzofuran with a bifunctional sidechain in position C7

Amine **7** was attached to the carboxylic group of **30** by the coupling reagents EDC and HOBt to obtain amide **31**. The reaction sequence ended with the cleavage of the methoxy groups and the final reduction of amide **32** to the amine **33** (cf. sheme C9).

In all of the synthesized test compounds, the methyl ether cleavage with BBr_3 required a careful control of the reaction progress. In previous works of our group a reaction time of 7 h for the deprotection of 3-substituted benzofurans (Zimmermann, 2005) was reported. In the case of the 7-(alk-1-enyl)benzofurans, after 7 hours the compounds decomposed. Acceptable yields were obtained after reaction times of 1.5-2 h. This difference might possibly be due to a tautomeric stabilization (cf. Scheme C10) of the R_2O^+-BBr_2 intermediate at the ring oxygen by the C1-C2 doublebond in the sidechain. This effect possibly favors the opening of the heterocyclic benzofuran ring, which might be responsible for the observed decomposition of the compounds after long reaction periods.

As detected by proton NMR spectroscopy, deprotection of the E/Z mixture **31** with BBr_3 afforded **32** as an almost pure E olefin. A proposed mechanism for the doublebond inversion is depicted in Scheme C10.

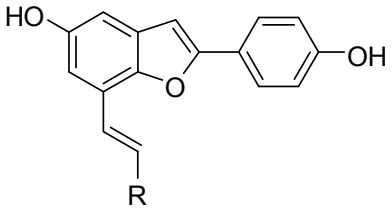


Scheme C10: Proposed mechanism for the BBr_3 induced doublebond inversion

A second isomeric form of the intermediate is possible, when the BBr_2^+ is shifted from the benzofuran ring-oxygen to the side chain position 2, while 3-electron-pairs are delocalized. The second isomer bears a single bond between C1 and C2 of the side

chain, which is able to rotate. This proposed mechanism explains, why only the thermodynamically favored E olefin was almost exclusively formed.
An overview of all synthesized test compounds based on a benzofuran scaffold is given in table C1.

Table C1: List of synthesized 2-phenylbenzofurans



Comp.	R
24	$-CH_3$
25	$-CH_2-CH_3$
26	$-(CH_2)_7-S-C_5H_{11}$
27	$-(CH_2)_8-S-C_5H_{11}$
28	$-(CH_2)_7-SO_2-C_5H_{11}$
29	$-(CH_2)_8-SO_2-C_5H_{11}$
32	$-(CH_2)_3-CO-N(CH_3)-(CH_2)_3-S-C_5H_{11}$
33	$-(CH_2)_4-N(CH_3)-(CH_2)_3-S-C_5H_{11}$

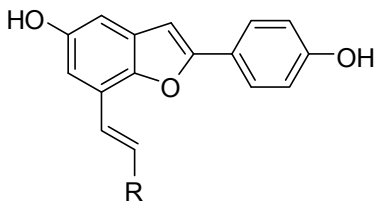
2.3 Biological Characterization of the 2-Phenylbenzofurans

2.3.1 Binding Affinities to Human Estrogen Receptors (ERα and ERβ)

Synthesized compounds and reference compounds were submitted to the competitive [^{3}H]-17β-estradiol binding assay. The relative binding affinities for both receptor subtypes are depicted in table C2.
IC_{50} values were determined by liner regression of the logits as a function of the logarithmic concentrations, comprising at least 4 data points. Generally the slopes of the linear regression functions were comparable to the slope of the logit-log-plot of 17β-estradiol, indicating a competitive binding mechanism.

The RBA values of all synthesized benzofurans and reference compounds are listed in table C2.

Table C2: Binding affinities of 2-phenylbenzofurans



Comp.	R	RBA[a] ERα	RBA[a] ERβ
24	Me	1.0 ± 0.4	34 ± 7
25	Et	0.57 ± 0.14	11 ± 2
26	$(CH_2)_7SC_5H_{11}$	<0.01	n.d.
27	$(CH_2)_8SC_5H_{11}$	<0.01	n.d.
28	$(CH_2)_7SO_2C_5H_{11}$	0.080 ± 0.021	0.65 ± 0.43
29	$(CH_2)_8SO_2C_5H_{11}$	0.066 ± 0.013	0.47 ± 0.29
32	$(CH_2)_3CON(CH_3)(CH_2)_3SC_5H_{11}$	< 0.02	< 0.02
33	$(CH_2)_4N(CH_3)(CH_2)_3SC_5H_{11}$	2.7 ± 0.5	0.77 ± 0.01
B1[b]		5.2	109
B2[b]		4.3	8.8
E2		100	100
Hexestrol		14 ± 5	10 ± 3

[a]RBA = IC_{50}(E2) / IC_{50}(test-compound) · 100; Values represent means of at least two independent determinations ± SEM; [b]determined by Dr. Zimmermann (Zimmermann, 2005)

Among the synthesized compounds, the 2-phenylbenzofurans with small alkenyl substituents in 7-position showed the highest affinities. Compound **24**, with a 7-propenyl moiety, revealed an RBA value for ERβ of 34 and a selectivity of >30 fold over ERα. For **25** the affinities for both subtypes decreased and the selectivity was about 20-fold. Compared to the unsubstituted benzofuran **B1** the total binding affinity of **24** was

reduced, but the selectivity was significantly increased. This result confirms a recent publication on 7-substiuated highly ERβ selective benzofurans (Collini et al., 2004).
The long thioether side chains in compounds **26** and **27** abolished binding affinity up to micromolar concentrations. Compounds **28** and **29** are moderate binders at ERβ with RBA values in the range of 0.5 with around 10 fold selectivities over ERα. Compared to compound **B2** with a sulfone side chain in position 3 the affinity is strongly decreased, when the side chain is moved to position 7. On the other hand the ERβ selectivity of **28** and **29** is higher, as it is only two fold for **B2**.
The binding profile of **33** with a bifunctional side chain is totally different from the other synthesized compounds. Surprisingly the compound binds preferably to ERα with a relatively high RBA of 2.7. From many examples the basic amine group in the side chain is known to form an essential hydrogen bond to the carboxylate of Asp351 in the ER binding pocket. This additional binding might effect, that the binding mode of compound **33** is different from the other benzofurans without an amine group in the side chain. Compound **32** bearing an amide side chain does not bind to any ER subtype up to micromolar concentrations. This underlines the theory of an additional binding effect of the basic amine group in the side chain of **33**.

2.3.2 Determination of Estrogenic and Antiestrogenic Activity in the Luciferase Assay

The synthesized compounds were tested for estrogenic and antiestrogenic effects in the luciferase assay (cf. Figure C3 and C4). **24** and **25** were weak agonists in the micromolar range. At a concentration of 3 µM, both compounds induced a normalized lucifease activity of 80 % compared to 1nM 17β-estradiol.

In the antagonist mode, compounds **28** and **29** with sulfone side chains showed no inhibition of the 1 nM 17β-estradiol induced luciferase activity. Compound **33** with a bifunctional side chain proofed to be a poor antiestrogen. At a concentration of 3 µM the relative luciferase activity was still 40% of the 17β-estradiol control. For comparison, the “pure antagonist” fulvestrant reduced the E2 induced bioluminescence to 20% of the control (cf. Figure C4).

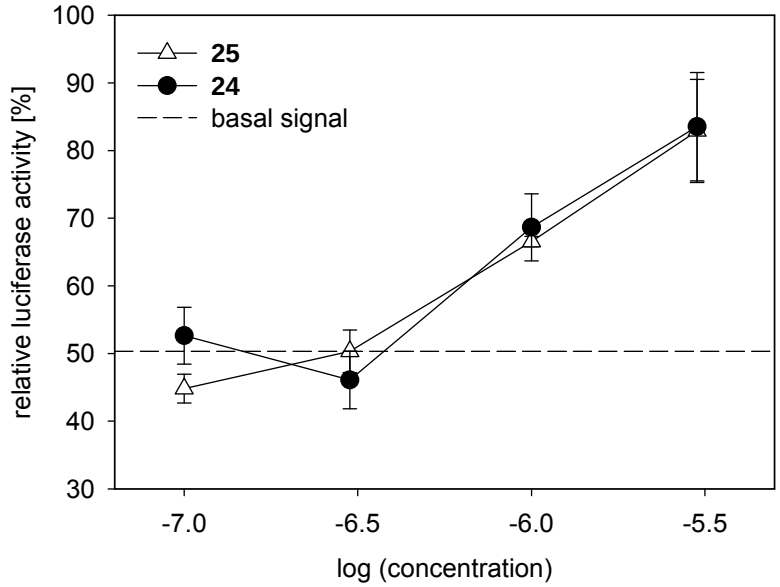


Figure C3: Estrogenic effects of benzofurans **24** and **25** in stably transfected MCF-7/2a cells
The luciferase activity induced by 1nM 17β-estradiol was set to 100%

Summarizing all the results from the luciferase assay, none of the compounds revealed a remarkable estrogenic or antiestrogenic effect. The strongest antiestrogenic effect was achieved by compound **33**, while binding experiments revealed a slight preference for ERα. All of the other benzofurans investigated in the luciferase assay showed some selectivity for ERβ, but with moderate binding affinities. This suggests ERα as the subtype which is mainly responsible for estrogenic activity in MCF-7/2a cells. Co-transfection assays with recombinant ERα or ERβ and an estrogen responsive gene reporter in an appropriate cell line might reveal subtype specific actions of synthesized compounds. Such assays have not been available for this study.

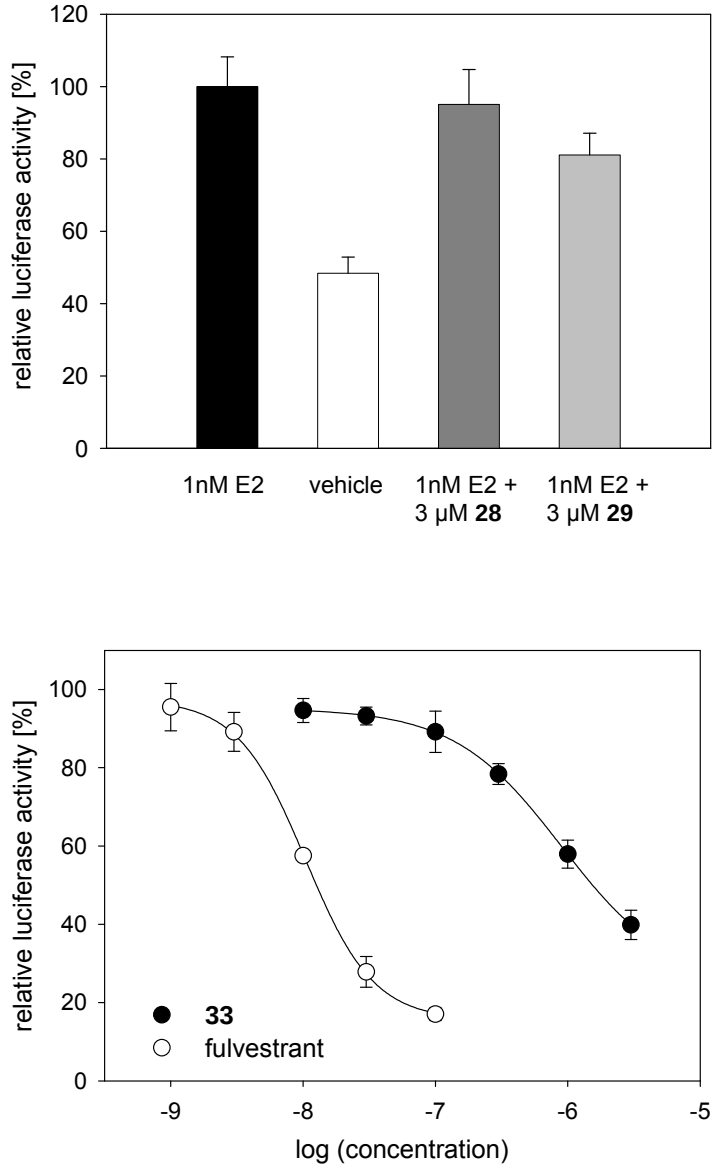


Figure C4: Effect of 2-phenylbenzofurans **28** and **29** with sulfone side chains (top) and compound **33** bearing a bifunctional side chain (bottom) on the 17β-estradiol-induced luciferase expression.

3 Estrogen Receptor Ligands Based on a Tetrahydroisoquinoline Scaffold

In terms of biological activity, 1,2,3,4-tetrahydroisoquinolines (THIQs) were first described in 1985 (Nagarajan et al., 1985). In this publication, the inhibitory activity on the attachment of the blastocyst to the epithelial lining of the uterus ("antiimplantation") by a library of 1,2-diaryl-THIQs was demonstrated. Almost 20 years later, this class of compounds was re-discovered by scientists at Novartis in a high throughput screening, aiming at the discovery of new SERMs (Renaud et al., 2003). To optimize this "hit", a series of new 1,2-diaryl-THIQs bearing side chains characteristic of SERMs at position 1 of the THIQ moiety was synthesized by varying of the substituents at the nitrogen. Most of the compounds exhibited high binding affinities and marked selectivities for ERα (Renaud et al., 2003; Renaud et al., 2005). A hydroxy group at the meta position of the aniline partial structure was shown to be favorable for preferential binding to ERα. A research group at Pfizer published other subtype-selective THIQs with various N-substituents such as benzyl, sulfonyl or trifluoroacetyl (Chesworth et al., 2004).

3.1 Design of Potential ERα Selective "Pure Antagonists"

The strategy of combining partial structures of known ERα-selective ligands with typical "antiestrogenic" side chains was partly successful in a recent study of our reaearch group (Zimmermann et al., 2005). Here the diphenylfuran core was adopted from ERα selective triphenylfurans published by others (Mortensen et al., 2001). A similar strategy was applied in this study, aiming at the synthesis of new ERα selective antagonists with a THIQ scaffold. Therefore the 1-aminoethoxyphenyl-group of the described THIQ-based SERMs was replaced by established "antiestrogenic" side chains (cf. paragraph 2.1).

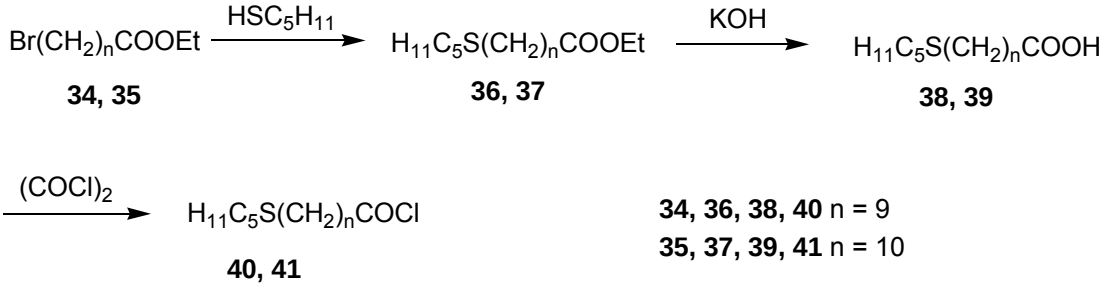
New potent ERα-selective full antagonists are useful as pharmacological tools to study subtype specific cellular effects. Additionally, such agents might be beneficial for breast cancer therapy, as ERα is claimed to be the main subtype involved in the proliferation of hormone sensitive breast cancer cells.

3.2 Chemistry

The tetrahydroisoquinolines were prepared via a previously published route involving a Bischler Napiralski cyclization (Nagarajan et al., 1985). The corresponding amide precursors were prepared by coupling of an appropriate phenylethylamine derivative to the aliphatic side chain activated as carboxylic acid chloride.

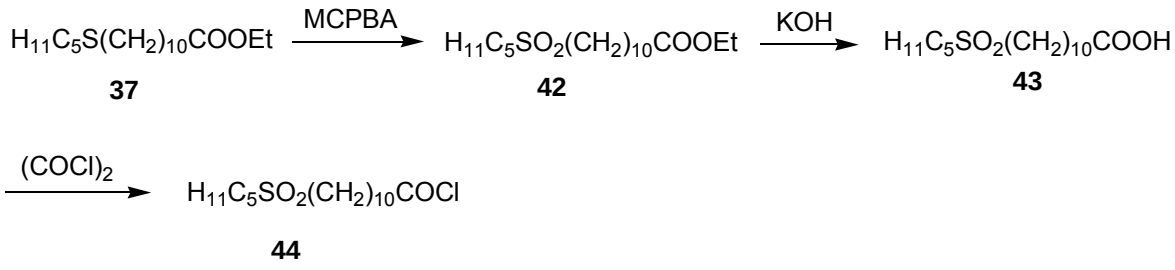
3.2.1 Synthesis of Side Chains

The thioether side chains were prepared from ω-bromo-carboxylic acid ethylesters **34** and **35** and pentanethiolate to obtain the thioethers **36** and **37**. Successive ester hydrolysis by KOH and chlorination with oxalyl chloride afforded the carboxylic acid chlorides **40** and **41** (cf. Scheme C11), which were ready to use for coupling to the phenyletylamine precursors



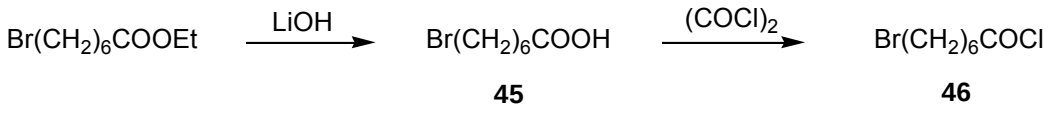
Scheme C11: Preparation of thioether side chains.

For the synthesis of the sulfone side chain, thioether **37** was oxidized by MCPBA. The following steps were the same as described for the thioether side chains to obtain the carboxylic acid chloride **44** (cf. Scheme C12).



Scheme C12: Preparation of the sulfone side chain.

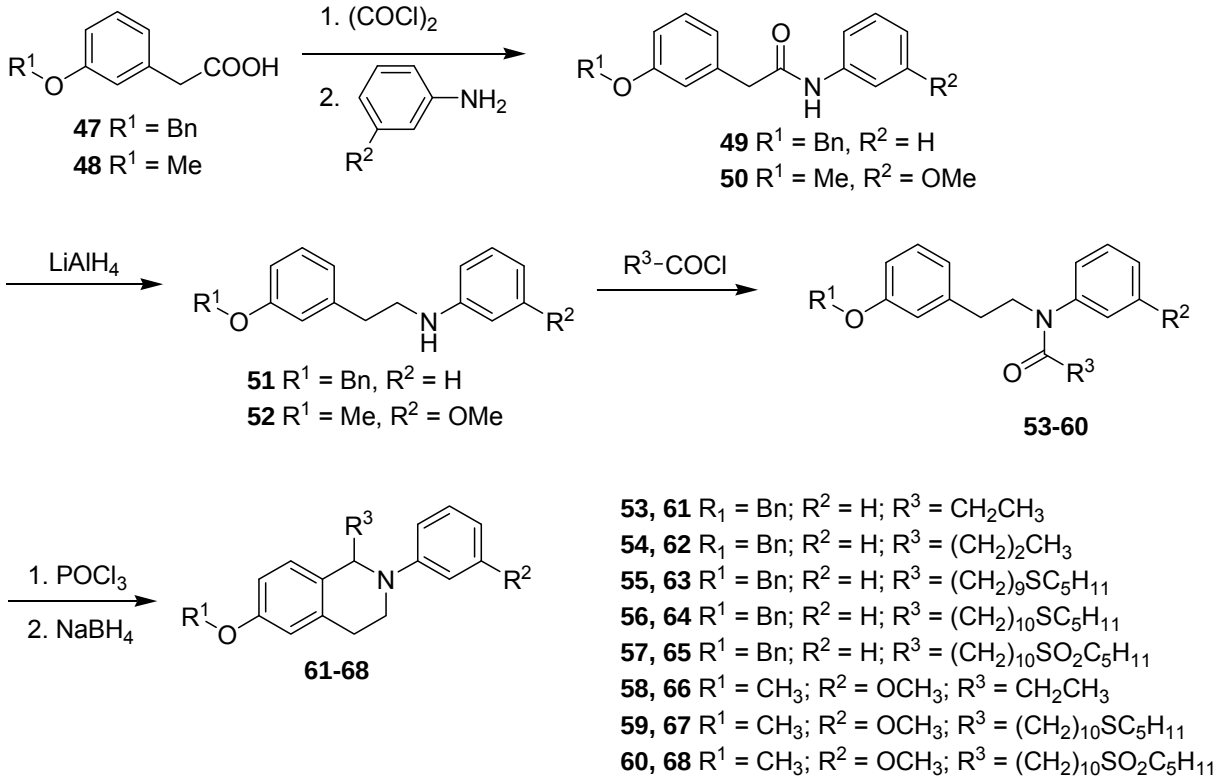
As precursor for the amine side chains 7-bromoheptanoic acid chloride was prepared from ethyl-7-bromoheptanoate by ester hydrolysis with LiOH and chlorination with oxalyl chloride (cf. Scheme C13)



Scheme C13: Preparation of 7-bromoheptanoic acid chloride

3.2.2 Synthesis of N-Aryltetrahydroisoquinolines

As depicted in Scheme C14, the synthesis of N-aryltetrahydroisoquinolines started from 2-phenyl-acetic acid derivatives **47** and **48**, which were chlorinated by oxalyl chloride to the corresponding acid chloride that was coupled to aniline or 3-methoxyaniline to yield the amides **49** and **50**.

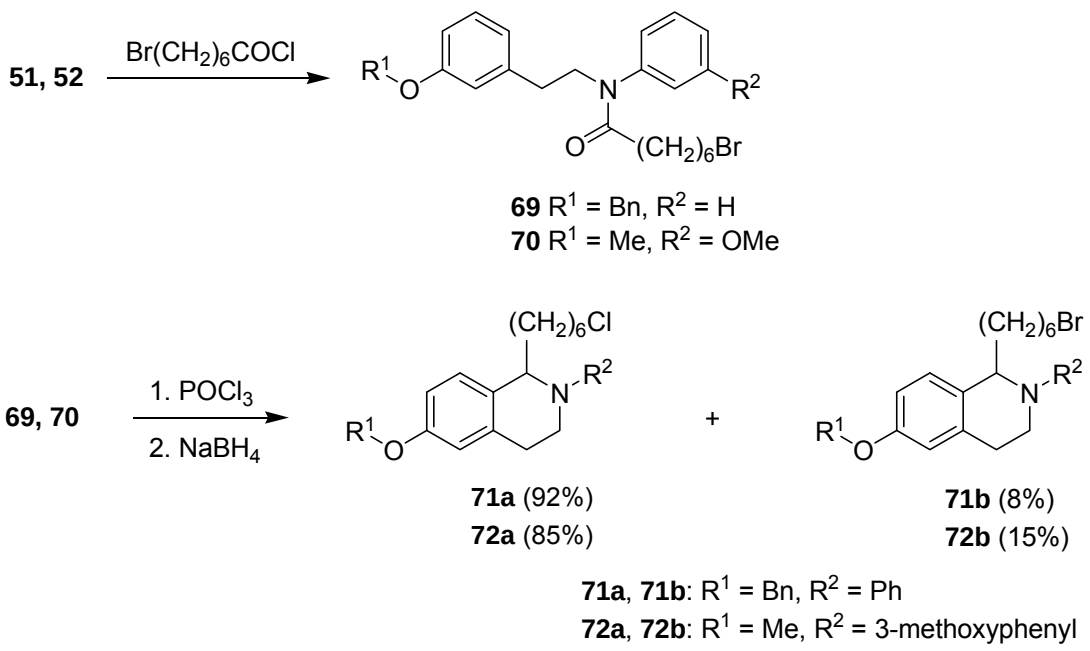


Scheme C14: Synthetic route to a library of THIQs.

After reduction with $LiAlH_4$ the phenylethylamine building blocks **51** and **52** were obtained. **51** and **52** were then linked to propionyl and buturyl chloride as well as the long-chaine carboxylic acid chlorides described in paragraph 3.2.1 to form the amides **53**-**60**.

Refluxing in $POCl_3$ afforded the dihydroisoquinolinium salts, which were directly reduced to give the tetrahydroisoquinolines **61**-**68**. Amide coupling and Bischler-Napiralski-cyclisation worked in good to excellent yields throughout.

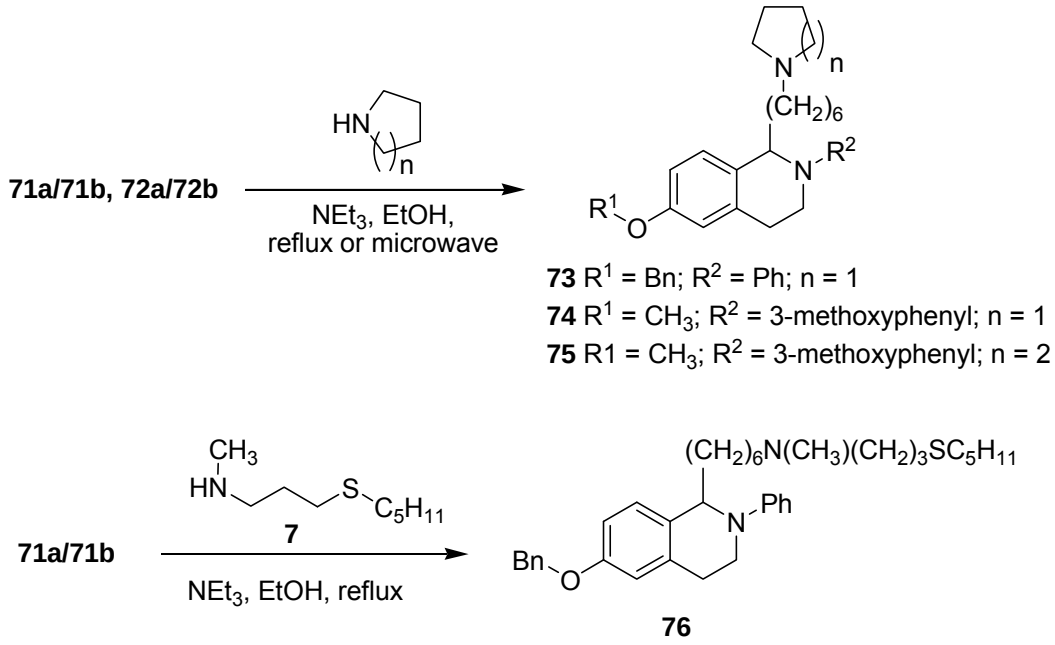
When ω-bromoheptanoyl amides **69** and **70** were converted into the tetrahydro-isoquinolines, the bromine was almost totally exchanged by chlorine (cf. Scheme C15). As the two products could not be separated chromatographically and both are synthetic equivalents for the alkylation of amines, the mixtures of chlorides **71a** and **72a** and the side products **71b** and **72b** were directly used for further transformations.



Scheme C15: Introduction of the alkylbromide chain and Bischler Napiralski cyclisation.

The chlorides **71a** and **72a** contaminated with their corresponding bromo analogues **71b** and **72b** were refluxed for three days with an excess of pyrrolidine in ethanol to obtain compounds **73** and **74**. As a synthetic alternative, for the introduction of the piperidine group, the mixture of **72a** and **72b** together with an excess of piperidine in ethanol was heated for 10 min to 180 °C under microwave-assistance. The chloride/bromide side chain of **71a**/**71b** was also converted into a bifunctional sidechain by reaction with amine **7** to afford **76** (cf. scheme C16). To complete this reaction, reflux

for 7 days was required. All amines were obtained in good yields (75-88%) with exception of compound **74** (49%).

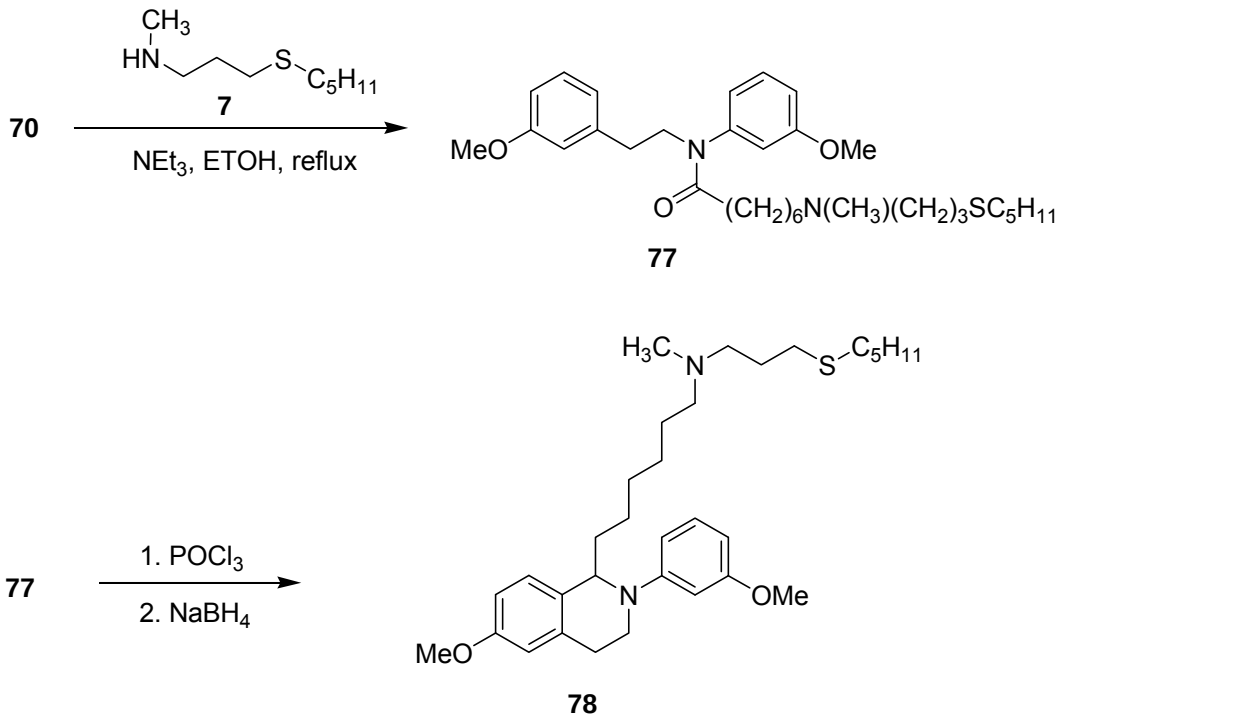


73 R^1 = Bn; R^2 = Ph; n = 1
74 R^1 = CH_3; R^2 = 3-methoxyphenyl; n = 1
75 R1 = CH_3; R^2 = 3-methoxyphenyl; n = 2

76

Scheme C16: Transformation of the alkyl chlorides/bromides **71a** / **71 b** and **72a** / **72b** to the amine side chains.

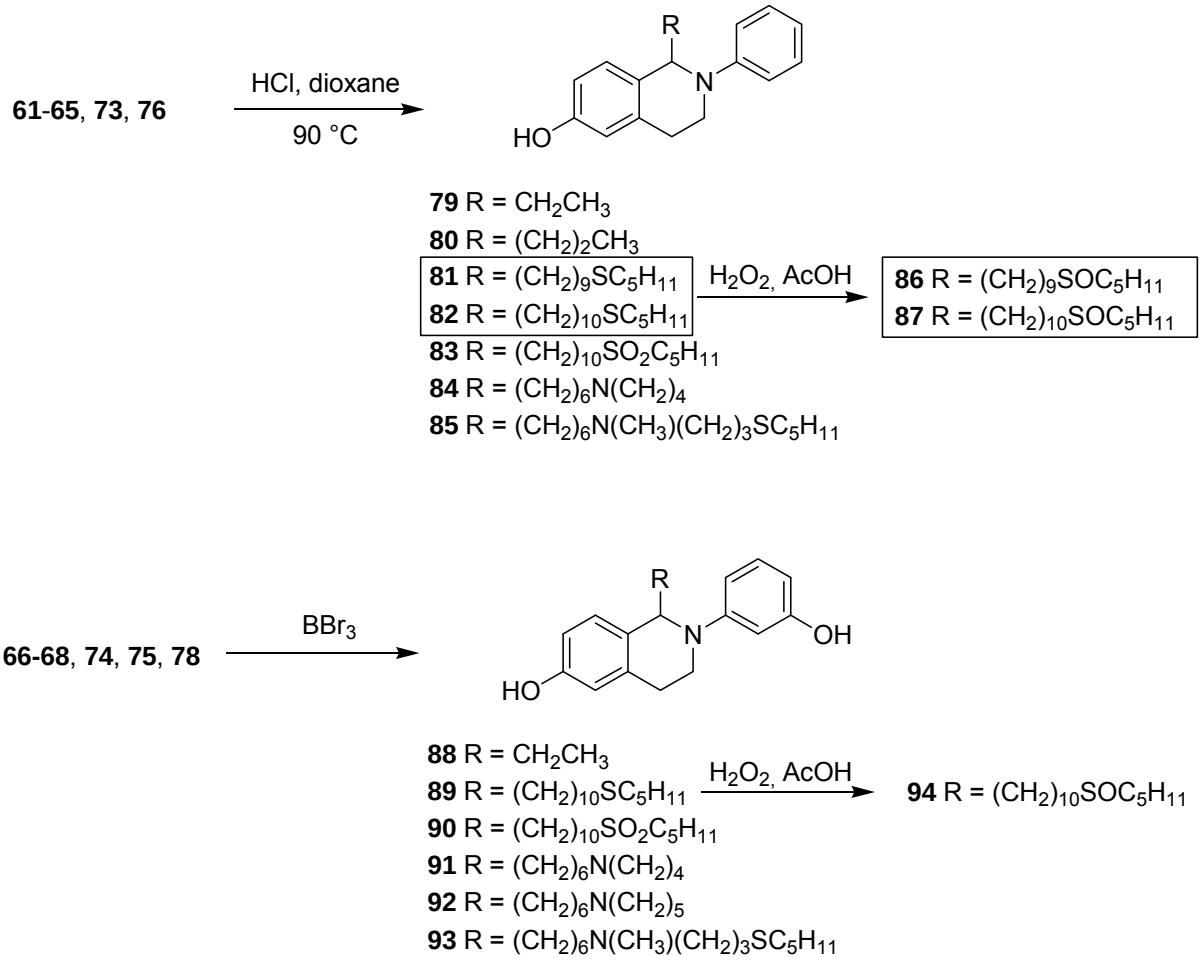
As bromides are much more reactive than chlorides in alkylation reactions, an alternative route to THIQs with amine side chains was performed. Amine **7** was fused to bromide **70** prior to Bischler Napiralski cyclisation (cf. Scheme C17). The reaction yielded THIQ **78** in an overall yield of 63%. This synthetic route was not successful in the case of pyrrolidine and piperidine, as the intermediate after the nucleophilic substitution could not be isolated.



Scheme C17: Alternative route to a THIQ with a bifunctional side chain: Alkylation prior to Bischler Napiralski cyclisation

The last step to the final tetrahydroisoquinlines was the cleavage of the benzyloxy and methoxy protecting groups. The benzyloxy groups were cleaved by heating in a mixture of HCl in dioxane. The two methoxy groups of the second series of compounds were removed by BBr_3 (cf. Scheme C18).

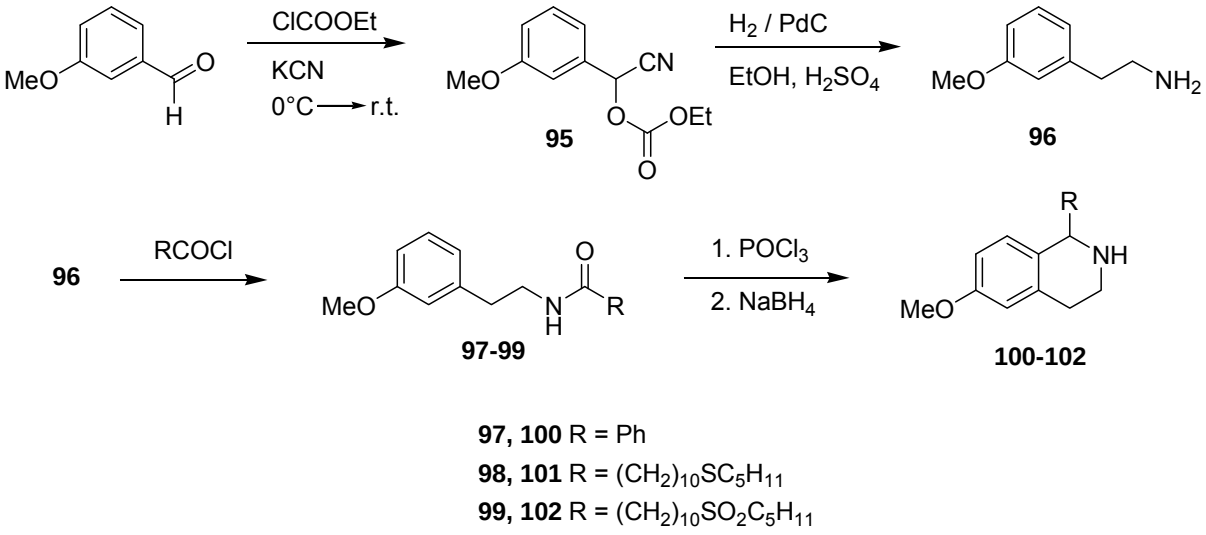
To obtain compounds containing sulfur atoms of different oxidation states in the side chain, initially a straight-forward strategy was followed to prepare the thioethers to be subsequently oxidized on a late step of the reaction sequence. In case of the THIQs the compounds were decomposed when the thioether was tried to be oxidized to the sulfone by MCPBA. With hydrogenperoxide as oxidation reagent the thioethers were successfully oxidized to sulfoxides as the very last step (cf. Scheme C18). The reaction at room temperature stopped after conversion to the sulfoxide. Heating should bring along further oxidation to the corresponding sulfone, but under these conditions the THIQs were unstable again. Thus, it was necessary to introduce the sulfone side chain from the beginning as described above (cf. Scheme C14), although this route was more time consuming.



Scheme C18: Deprotection and sulfur oxidation to the final THIQs

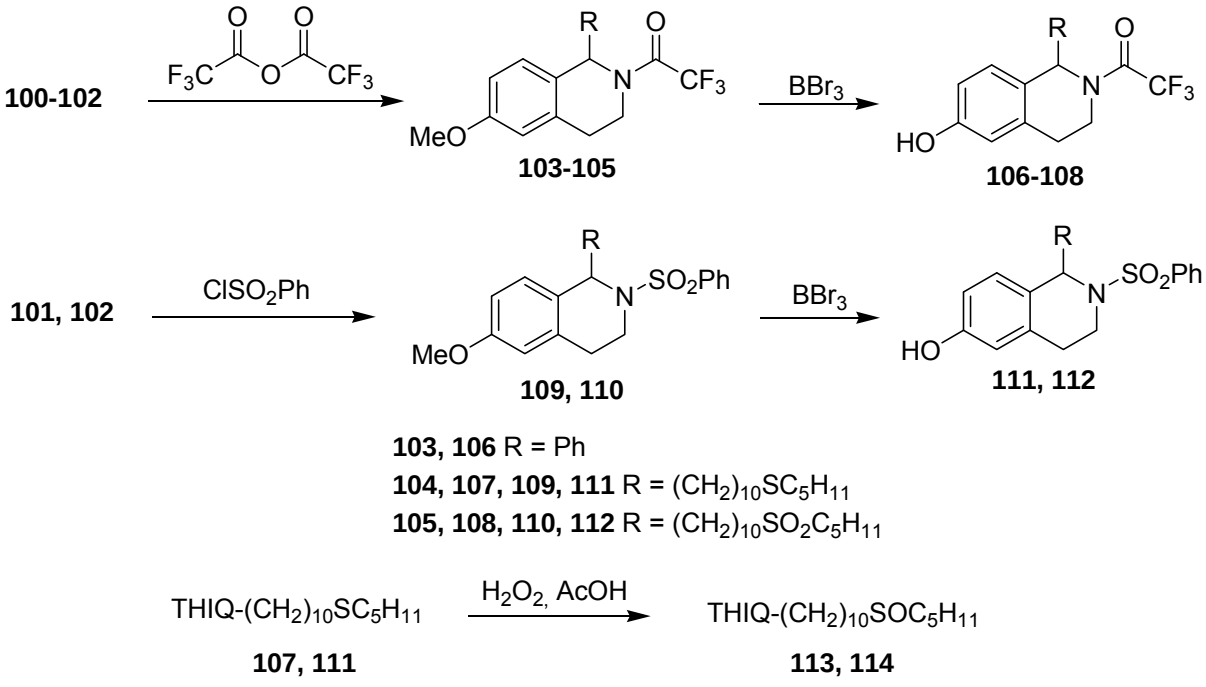
3.2.3 N-Trifluoroacetyl and N-Phenylsulfonyl Substituted Tetrahydroisoquinolines

Inspired by a recent publication (Chesworth et al., 2004), tetrahydroisoquinolines substituted with a 1-phenylsulfonyl- or a 1-trifluoroacetyl group instead of a 1-aryl substituent were synthesized. The N-unsubstituted phenylethylamine derivative **96** was synthesized starting from 3-methoxybenzaldehyde following a published procedure (Kashdan et al., 1982). A benzoyl substituent as well as a thioether- and sulfone side chain were fused to the amine by amide coupling with the corresponding acid chlorides described in paragraph 3.2.1. The amides were converted to the N-unsubstituted THIQs **100**-**102** by Bischler Napiralski cyclisation (cf. Scheme C19).



Scheme C19: Synthesis of 3-methoxyphenylethylamine and Bischler-Napiralski cyclisation to the N-unsubstituted THIQs.

The trifluoroacetyl group was coupled to the amine function of **100**-**102** by trifluoroacetic anhydride. Methoxy deprotection afforded the final trifluoracetates **106**-**108** containing a phenyl substituent as well as thioether and sulfone side chains in position 1. The coupling reactions of **101** and **102** with phenylsulfonylchloride followed by demethylation afforded **111** and **112**. Finally the thioether side chains were oxidized to the sulfoxides by hydrogenperoxide (cf. Scheme C20).



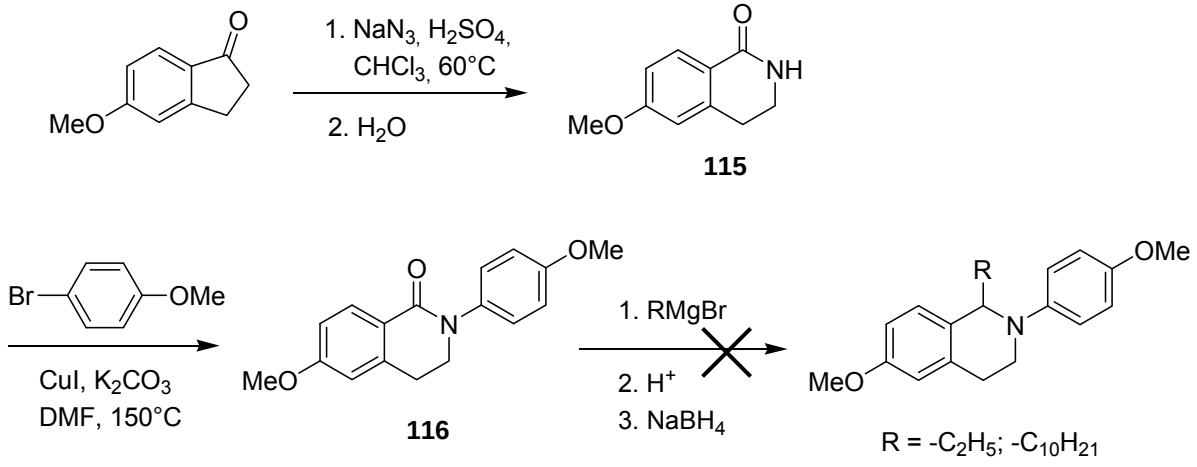
Scheme C20: Synthesis of N-trifluoroacetyl and N-phenylsulfonyl THIQs.

3.2.4 Unsuccessful Synthetic Approach to 1-Alkyl-2-aryltetrahydroisoquinolines

As shown in the last paragraph, the synthesis of 1-alkyl-2-aryl-THIQs required a time consuming multi step reaction sequence, as the cyclisation had to be performed for every single sidechain. An alternative, straight forward synthetic route to 1-2-diaryl-THIQs was recently described (Renaud et al., 2005). In this case, the key step of the reaction sequence is a nucleophilic attack of an aryl-lithium to a carbonyl function in position 1, followed by dehydratation and reduction of the dihydroisoquinolonium to the tetrahydroisoquinoline.

For this study aliphatic Grignard nucleophiles were applied as synthetic equivalents for lithiumorganic compounds described by others. This approach may open a short synthetic route to a library of THIQs containing aliphatic sidechains in position 1. The Grignard reagents should be prepared from the corresponding alkylbromides (cf. paragraph 2.2.1).

The N-aryl 3,4 dihydroisoquinolin-1-on building block was synthesized starting from 5-methoxy-2,3-dihydroinden-1-on that was transformed to the dihydroisoquinolin-1-on **115** in a Schmidt rearrangement (Tomita et al., 1969). The N-(4-methoxyphenyl) group was introduced by a Cu(I) catalyzed Ullmann-type condensation (Sugahara and Ukita, 1997) with the corresponding aryl bromide to afford compound **116**. The attempted Grignard reaction of **116** with ethyl-magnesiumbromide as well as with decylmagnesium bromide failed (cf. Scheme C21).

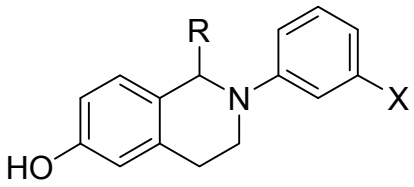


Scheme C21: Unsuccessful synthetic approach to 1-alkyl-2-aryl-THIQs.

3.2.5 Summary of Synthesized Test-Compounds with a Tetrahydroisoquinoline Scaffold

Tables C3 and C4 show the substitution profile of the two series of synthesized compounds. THIQs of series A are variably substituted in position 1 with simple alkyl groups as well as with long monofunctional and bifunctional chains. For each class of 1-substiuents the N-phenyl ring is either unsubstituted or carries a 3-hydroxy function.

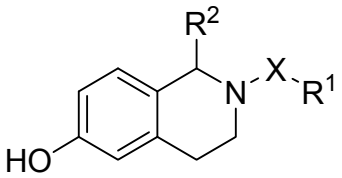
Table C3: Synthesized tetrahydroisoquinolines
Series A



Comp.	R	X
79	$-CH_2-CH_3$	H
80	$-(CH_2)_2-CH_3$	H
81	$-(CH_2)_9-S-C_5H_{11}$	H
83	$-(CH_2)_{10}-SO_2-C_5H_{11}$	H
84	$-(CH_2)_6-NC_4H_8$	H
85	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	H
86	$-(CH_2)_9-SO-C_5H_{11}$	H
87	$-(CH_2)_{10}-SO-C_5H_{11}$	H
88	$-CH_2-CH_3$	OH
90	$-(CH_2)_{10}-SO_2-C_5H_{11}$	OH
91	$-(CH_2)_6-NC_4H_8$	OH
92	$-(CH_2)_6-NC_5H_{10}$	OH
93	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	OH
94	$-(CH_2)_{10}-SO-C_5H_{11}$	OH

Synthesized tetrahydroisoquinolines of series B are substituted with a trifluoroacetate or a phenylsulfonyl group at the nitrogen. Compound **106** carrying a phenyl group in position 1 and an N-trifluoroacetate group was derscribed by others as ERβ selective agonist (Chesworth et al., 2004). **108**-**114** are substituted in position 1 with a sulfoxide or sulfone side chain.

Table C4: Synthesized tetrahydroisoquinolines
Series B



Comp.	X	R^1	R^2
106[a]	CO	CF_3	Ph
108	CO	CF_3	$-(CH_2)_{10}-SO_2-C_5H_{11}$
112	SO_2	Ph	$-(CH_2)_{10}-SO_2-C_5H_{11}$
113	CO	CF_3	$-(CH_2)_{10}-SO-C_5H_{11}$
114	SO_2	Ph	$-(CH_2)_{10}-SO-C_5H_{11}$

[a]Reference compound described in literature (Chesworth et al., 2004)

3.3 Pharmacological Characterization of the Tetrahydroisiquinolines

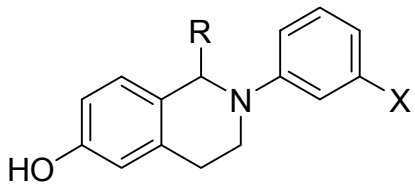
3.3.1 Binding to Human Estrogen Receptors (ERα and ERβ)

The two series of synthesized tetrahydroisoquinolines were submitted to the ER binding assay using the human recombinant ERα and ERβ.

Generally, in the range of the experimental precision, the slopes of the logit-log-plots of the test compounds were comparable to that of the 17β-estradiol, indicating a competitive displacement of the tritiated endogenous ligand from the ER binding pocket.

The binding data for series A of the investigated tetrahydroisoquinolines are summarized in table C5.

Table C5: Binding affinities of tetrahydroisoquinolines to human ERα and ERβ
Series A



Comp.	R	X	RBA[a] ERα	RBA[a] ERβ
79	$-CH_2-CH_3$	H	2.4 ± 0.2	2.6 ± 0.1
80	$-(CH_2)_2-CH_3$	H	3.1 ± 0.1	1.1[b]
88	$-CH_2-CH_3$	OH	5.5 ± 0.2	8.8 ± 3.2
81	$-(CH_2)_9-S-C_5H_{11}$	H	0.03[b]	0.02[b]
86	$-(CH_2)_9-SO-C_5H_{11}$	H	0.28 ± 0.05	0.13[b]
84	$-(CH_2)_6-NC_4H_8$	H	7.1 ± 1.3	8.2 ± 0.3
91	$-(CH_2)_6-NC_4H_8$	OH	11 ± 1	1.2 ± 0.2
92	$-(CH_2)_6-NC_5H_{10}$	OH	11.9 ± 0.2	1.08 ± 0.02
85	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	H	1.4 ± 0.2	1.43 ± 0.02
93	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	OH	14 ± 1	1.1 ± 0.1
87	$-(CH_2)_{10}-SO-C_5H_{11}$	H	0.37 ± 0.04	0.32 ± 0.15
94	$-(CH_2)_{10}-SO-C_5H_{11}$	OH	2.4 ± 0.1	0.14 ± 0.02
83	$-(CH_2)_{10}-SO_2-C_5H_{11}$	H	0.55 ± 0.25	1.3 ± 0.2
90	$-(CH_2)_{10}-SO_2-C_5H_{11}$	OH	2.9 ± 0.2	0.28 ± 0.04
E2			100	100
4-OH-Tam			11 ± 2	24[b]
ICI[c]			8.2 ± 0.3	12 ± 1

[a]RBA = IC_{50}(E2) / IC_{50}(test-comp.) · 100; values represent means of two independent determinations ± SEM; the concentration of the radioligand was 0.5 nM throughout; [b]single determination; [c]ICI = fulvestrant (ICI 182.780)

Compounds **79**, **80** and **88** substituted with simple alkl groups in position 1 bound to ERα with RBA values of 2.4 to 5 without any noteworthy selectivity over ERβ. For compound **88** harboring a 3'-hydroxy group, the affinity for both ER subtypes was increased by a factor 2 to 3 compared to the N-phenyl analogue **79**.

When these simple chains were replaced by side chains typical of aniestrogens, the binding affinity as well as the degree of selectivity became strongly dependent on the nature of the substituents included in the chain and on the presence of an additional hydroxy function in the 3'-position.

Compound **81** carrying a side chain with a sulfanyl group at a distance of 9 carbon atoms from the core was hardly able to compete with E2 for the ER binding site. The affinity for both receptor subtypes dropped by a factor of 100 compared to the ethyl- and propyl-substituted analogues. With an RBA value of 0.3, the corresponding sulfoxide derivative **86** revealed a 10-fold higher binding affinity, lacking any noteworthy subtype selectivity. The affinities for both ER-subtypes were approximately the same, when the chain was prolonged by one carbon atom in compounds **83** and **87** bearing a sulfone or a sulfoxide group, respectively.

If a second phenolic hydroxy group is present in position 3 of the N-phenyl (3'-position), the binding profiles of the tetrahydroisoquinolines carrying a sulfoxide- or sulfone side chain changed. The binding affinities for ERα were increased by a factor of 5 to 6 (RBA = 2.4-2.9) while binding to ERβ slightly decreased. Consequently, ERα selectivities reached from 10-fold in the case of sulfone **90** to 17-fold in the case of sulfoxide **94**.

Compounds **84**, **91** and **92** with pyrrolidine and piperidine side chains were found to bind with RBA values of 7-12 to ERα. The binding selectivity again strongly depended on an additional hydroxy group at the N-phenyl ring: compound **84** with only one phenolic hydroxy group already revealed a marked binding affinity with RBA values for both ER subtypes between 7 and 8. For **91** and **92** bearing two phenolic OH-groups the affinities to ERα were slightly increased (RBA = 11-12) compared to **84**. Both compounds bearing a 3'-hydroxy group bound with a selectivity of approximately 10-fold in favor of ERα. In this case the selectivity was mainly due to a dropping affinity at ERβ, when a 3'-hydroxy group was present.

The comparison of the binding profiles of compounds **85** and **93** bearing a bifunctional side chain demonstrates once more, that the second hydroxy function in 3' position of **93** is required for a high binding affinity (RBA = 14) and a 13-fold ERα selectivity.

Summarizing the binding data of series A of the investigated tetrahydroisoquinolines, two phenomena are noticeable: generally the highest binding affinities were found, if a basic nitrogen was present in the side chain. The relatively high binding affinities can be explained by a hydrogen bond between the basic amine group and the carboxylate of Asp351 in the ER binding pocket. The increase in binding affinity by amine groups in the side chain is well known, especially for agents belonging to the class of SERMs and for many antiestrogens developed in our group.

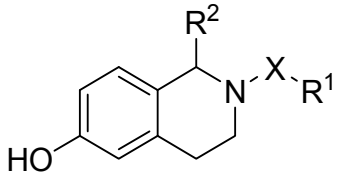
The second important observation was the fact that a meta-hydroxy group at the N-phenyl increases the selectivity in favor of ERα. This observation confirms the result of a recent study on tetrahydroisoquinoline based SERMs published by others (Renaud et al., 2005).

Among the synthesized compounds, the highest binding affinities (RBA = 10-14) in combination with selectivities of >10 fold for ERα were found for compounds **91**, **92** and **93**. These affinities for ERα are comparable to the highly potent antiestrogens fulvestrant (RBA = 8.2) and 4-hydroxytamoxifen (RBA = 11). For compound **94** the affinity was somewhat decreased (RBA = 2.4), but this compound was characterized by a 17-fold selectivity in favor of ERα.

In series B of the investigated tetrahydroisoquinolines, compound **106** revealed a high affinity (RBA = 14) and a 5-fold selectivity for ERβ. **106** was already investigated with a similar result by scientists at Pfitzer (Chesworth et al., 2004).

The combination of an N-phenylsulfonyl or N-trifluoroacetate with sulfoxide or sulfone side chains in compounds **108** and **112**-**114** led to unselective compounds with consistently low binding affinities. Binding data for the compounds of series B are listed in table C6.

Table C6: Binding affinities of tetrahydroisoquinolines to human ERα and ERβ
Series B



Comp.	X	R^1	R^2	RBA ERα	RBA ERβ
106	CO	CF_3	Ph	2.5 ± 0.3	14 ± 2
113	CO	CF_3	$-(CH_2)_{10}-SO-C_5H_{11}$	0.26[a]	0.4[a]
108	CO	CF_3	$-(CH_2)_{10}-SO_2-C_5H_{11}$	0.26 ± 0.07	0.22 ± 0.08
114	SO_2	Ph	$-(CH_2)_{10}-SO-C_5H_{11}$	0.19[a]	0.25[a]
112	SO_2	Ph	$-(CH_2)_{10}-SO_2-C_5H_{11}$	0.09 ± 0.02	0.11 ± 0.05

[a]single determination; for additional information see table C5

3.3.2 Functional Characterization of the THIQs in the Luciferase Assay

ER positive MCF-7 breast cancer cells, that have been stably transfected with a luciferase reporter gene under the control of an ERE, were used for the determination of antiestrogenic effects of synthesized tetrahydroisoquinolines.

The antiestrogenic activity was determined by simultaneous treatment of the cells with 1nM E2 and the respective tetrahydroisoquinoline or reference compound (fulvestrant or 4-hydroxytamoxifen) in various concentrations. Each compound was characterized by the IC_{50} value for the inhibition of the 1nM E2 induced luciferase expression that was determined by graphical regression of the respective concentration -response-plot. The graphs for inhibition of the luciferase activity in MCF-7/2a cells by the tested compounds are depicted in Figure C5.

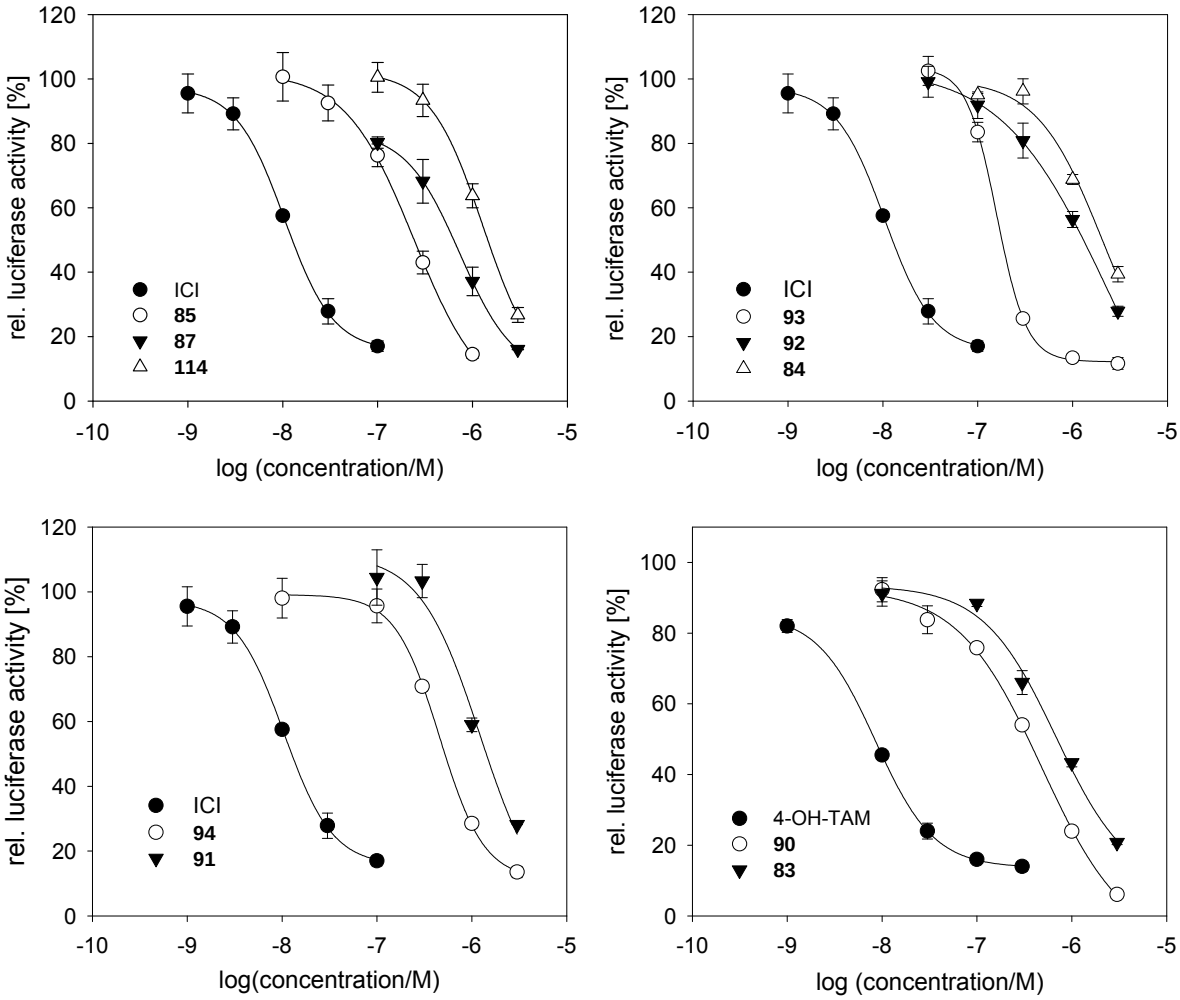
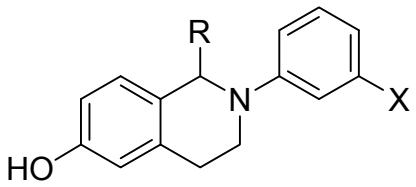


Figure C5: Inhibition of the E2-induced luciferase activity by various antiestrogens
The luciferase expression was mediated by 1nM E2 (control was set to 100 %); ICI = fulvestrant (ICI 182.780); 4-OH-Tam = 4-hydroxytamoxifen

All investigated compounds of series A equipped with an "antiestrogenic" side chain revealed an inhibiting effect on the 17β-estradiol stimulated luciferase expression in a concentration-dependent manner. Generally, the maximum inhibition of the luciferase activity by the test compounds was approximately 15-20% of the E2-control. The basal expression of nontreated cells was typically in the range of 40% to 50%. That means that the compounds were capable of inhibiting the luciferase activity far below the basal signal, which is a characteristic effect of potent ER antagonists. This level was also the limit of inhibition for the reference drugs fulvestrant and 4-hydroxytamoxifen.

As demonstrated in Figure C4 the inhibition curves for the tetrahydroisoquinolines were all shifted to higher concentrations compared to the potent reference antiestrogens fulvestrant and 4-hydroxytamoxifen. The IC_{50} values of all tested tetrahydroisoquinolines of series A are listed in table C7.

Table C7: Antiestrogenic activity of tetrahydroisoquinolines with functionalized side chains, Series A



Comp.	R	X	IC_{50} [µM]
84	$-(CH_2)_6-NC_4H_8$	H	2.0
91	$-(CH_2)_6-NC_4H_8$	OH	1.2
92	$-(CH_2)_6-NC_5H_{10}$	OH	2.3
85	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	H	0.24
93	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	OH	0.16
87	$-(CH_2)_{10}-SO-C_5H_{11}$	H	0.74
94	$-(CH_2)_{10}-SO-C_5H_{11}$	OH	0.47
83	$-(CH_2)_{10}-SO_2-C_5H_{11}$	H	0.67
90	$-(CH_2)_{10}-SO_2-C_5H_{11}$	OH	0.48
86	$-(CH_2)_9-SO-C_5H_{11}$	H	0.71
4-OH-Tam			0.0085
Fulvestrant			0.010

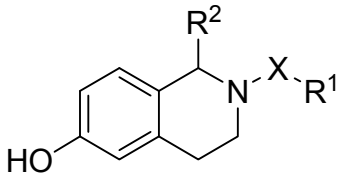
The lowest IC_{50} values of 160 nM and 240 nM respectively were obtained for tetrahydroisoquinolines **93** and **85** with the bifunctional side chain. The potencies of these compounds were by factor 15-25 lower than those of fulvestrant and 4-hydroxytamoxifen, both of which were in the range of 10 nM. The IC_{50} values of the second group of synthesized THIQs, bearing long sulfoxy and sulfone side chains were in a range of 0.5-0.7 µM. With approximately 0.5 µM, the antiestrogenic potencies of **90** and **94** carrying a 3-hydroxy group at the N-phenyl were slightly higher than those of the corresponding THIQs devoid of this hydroxy group.

Compounds **84**, **91** and **92**, having a terminal pyrrolidine or piperidine group at a C6-chain, inhibited gene transcription with IC_{50} values in the micromolar range. In this last class of THIQs, there is no correlation between the potencies in the luciferase assay and the binding affinities from the radiochemical assay. Pyrrolidines **84** and **91** and

piperidine **92** revealed throughout high affinities to the recombinant ERα, but in the cellular test system, they inhibited the E2- mediated gene transcription only weakly.
The compounds, which carry a relatively short side chain bear a basic amine group that is protonated at physiological pH. The lipophilicity of the salts is too low to readily penetrate accross cell membranes, so that the intracellular concentration at the site of action is much lower compared to the extracellular concentration. This may be an explanation for the relatively low antiestrogenic potency of THIQs **84**, **91** and **92**, in spite of their high binding affinities.
In the compounds with “bifunctional” chains, the lipophilic chain compensates the positive charge at the basic nitrogen to a certain degree, which possibly explains the relatively high potencies determined in the luciferase assay.

The tested THIQs of series B were all poor inhibitors of the estrogen mediated gene transcription with IC_{50} values in the micromolar range (> 3 µM for **112)** (cf. Table C8).

Table C8: Antiestrogenic activity of tetrahydroisoquinolines with functionalized side chains, Series B



Substanz	X	R^1	R^2	IC_{50} [µM]
113	CO	CF_3	$-(CH_2)_{10}-SO-C_5H_{11}$	1.3
114	SO_2	Ph	$-(CH_2)_{10}-SO-C_5H_{11}$	1.3
112	SO_2	Ph	$-(CH_2)_{10}-SO_2-C_5H_{11}$	> 3

3.3.3 Antiproliferative Activity

The antiproliferative potencies of the synthesized tetrahydroisoquinolines in ER positive MCF-7 breast cancer cells were determined as a second criterion for antiestrogenic efficacy. The assay was performed as described by Bernhardt et al (Bernhardt et al., 1992). The growth kinetics was determined by measuring the cell densities of treated and non-treated cell cultures after staining with crystal violet at five different time points. The characteristics of the growth curves reveal the type of growth inhibiting effect of a tested compound. The kinetics of growth inhibition for a number of tetrahydroisoquinolines and the reference fulvestrant is depicted in Figures C5 to C11.
The IC_{50} values for the antiproliferative effects of selected tetrahydroisoquinolines were determined by analyzing the concentration-response curves at the endpoint of each assay. IC_{50} values for the antiproliferative effects of some THIQs are listed in table C9.
The strongest inhibition of the cell proliferation was achieved with the tetrahydro-isoquinolines **87** and **94** bearing a long sulfoxide side chain The growth kinetics revealed a cytostatic effect over a concentration range from 0.1 to 1 µM, while the level of cell density decreased in a concentration dependent manner (cf. Figure 6). At the endpoint, IC_{50} values of 95 nM and 60 nM were determined for compounds **87** and **94,** respectively.
Tetrahydroisoquinoline **93,** bearing a bifunctional side chain was the most potent inhibitor of the 17β-estradiol-induced gene transcription in the lucifease assay (cf. paragraph 2.3.2). Surprisingly, the compound revealed a cytostatic effect only at a concentration of 1 µM. At 100 nM the curve is characteristic for a cytotoxic effect, as the cell density decreases only at the beginning, but with the progress of the incubation period the cell number of the cultures treated with 100 nM of **93** approximates the cell number of the corresponding control population. Consequently, the corrected T/C value shows a minimum at 140 h (cf. Figure C7). The IC_{50} value of **93** for the inhibition of the MCF-7 cell proliferation was 170 nM. A possible explanation for the lack of correlation between the results of the luciferase assay and the proliferation assay might be the difference in the incubation periods. While luciferase activity was determined after an incubation period of 48 hours, the time of incubation was more than 200 h in the crystal violet assay. The stability of the compounds is not guaranteed in an aqueous environment at 37 ° for such a long period of time. Compound **85** with “bifunctional” side

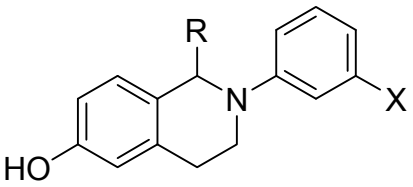
chain, but without a 3'-hydroxy group revealed a similar effect on MCF-7 cell proliferation than **93**. The IC_{50} value of **85** was 160 nM.

In contrast to compound **94,** bearing a solfoxide side chain, the corresponding sulfone analogue **90** revealed a weak cytotoxic effect at 100 nM and a cytostatic effect at a concentration of 1 µM (cf. Figure C8, top). Analysis of the concentration-response curve at the endpoint revealed an IC_{50} value of 240 nM.

Compound **91,** carrying a pyrrolidine side chain, exhibited no remarkable effect at 100 nM. At 1 µM a cytostatic effect was observed (cf. Figure C8, bottom). The relatively low antiproliferative potency of **91** was predictable from the gene reporter assay. The IC_{50} value of **91** with respect to growth inhibition was 180 nM.

As ER expression in MCF-7 cells is quite unstable and can strongly differ in different passages, the potent antiestrogens fulvestrant (cf. Figure C9) and 4-hydroxytamoxifen (not shown) were used as reference compounds. The fact that both antiestrogens inhibited growth at nanomolar concentrations (5 and 10 nM, respectively) totally, demonstrates the high responsiveness of the MCF-7 breast cancer cells to hormonal treatment.

Table C9: Antiproliferative activity of tetrahydroisoquinolines with functionalized side chains



Comp.	R	X	IC_{50} [µM]
91	$-(CH_2)_6-NC_4H_8$	OH	0.18[a]
85	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	H	0.16[a]
93	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	OH	0.17[a]
87	$-(CH_2)_{10}-SO-C_5H_{11}$	H	0.095[b]
94	$-(CH_2)_{10}-SO-C_5H_{11}$	OH	0.060[a]
90	$-(CH_2)_{10}-SO_2-C_5H_{11}$	OH	0.24[a]
ICI			< 0.005[b]

[a]MCF-7 passage 177; [b]MCF-7 passage 193

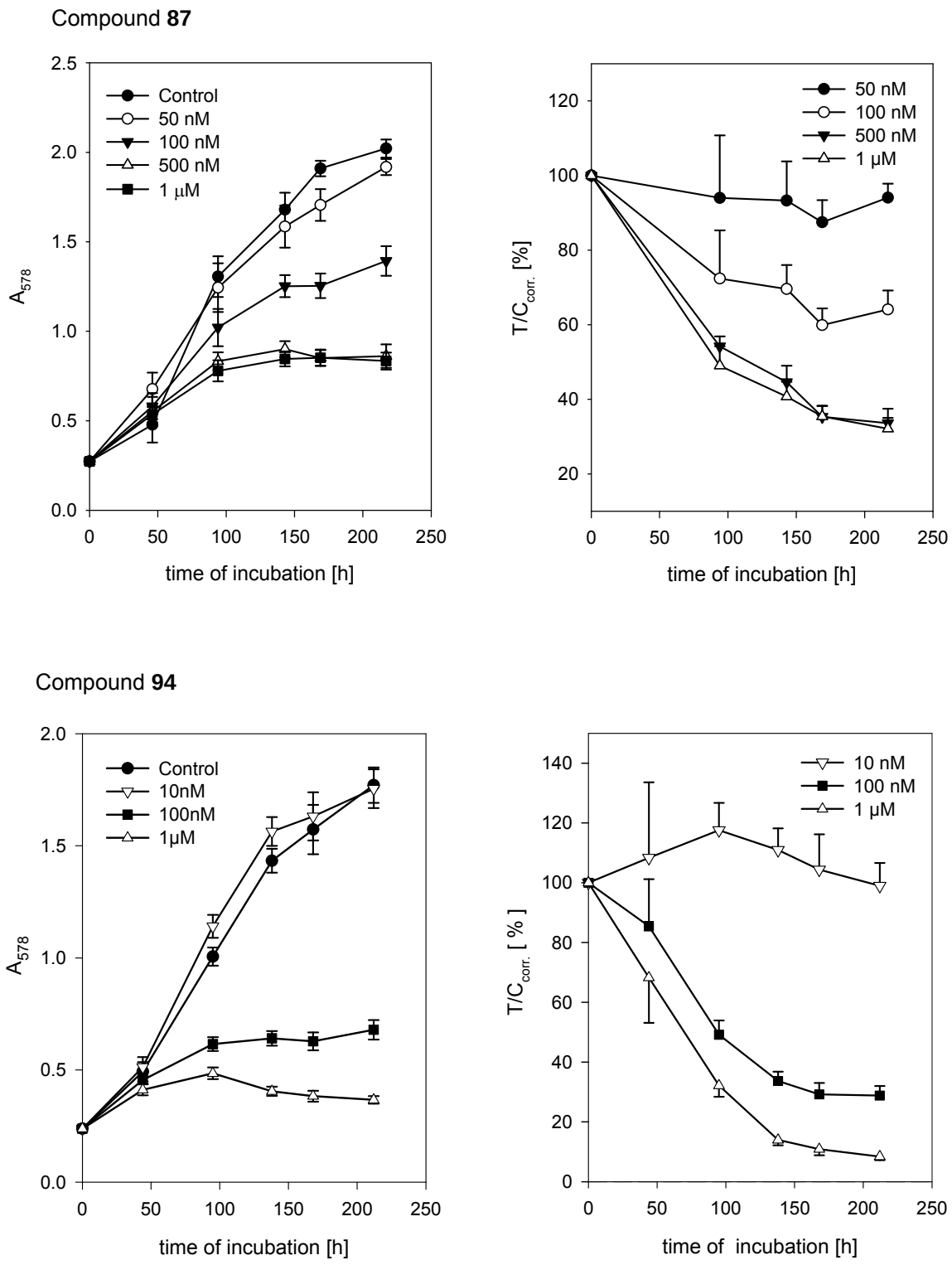


Figure C6: Proliferation kinetics (left) and growth inhibition of MCF-7 cells after treatment with compound **87** (top) and **94** (bottom) compared to the vehicle control.

Top: MCF-7 passage 177; bottom: MCF-7 passage 193; values represent means of at least 14 replicates ± SD; Errors of T/C were calculated according to the Gaussian law of error propargation.

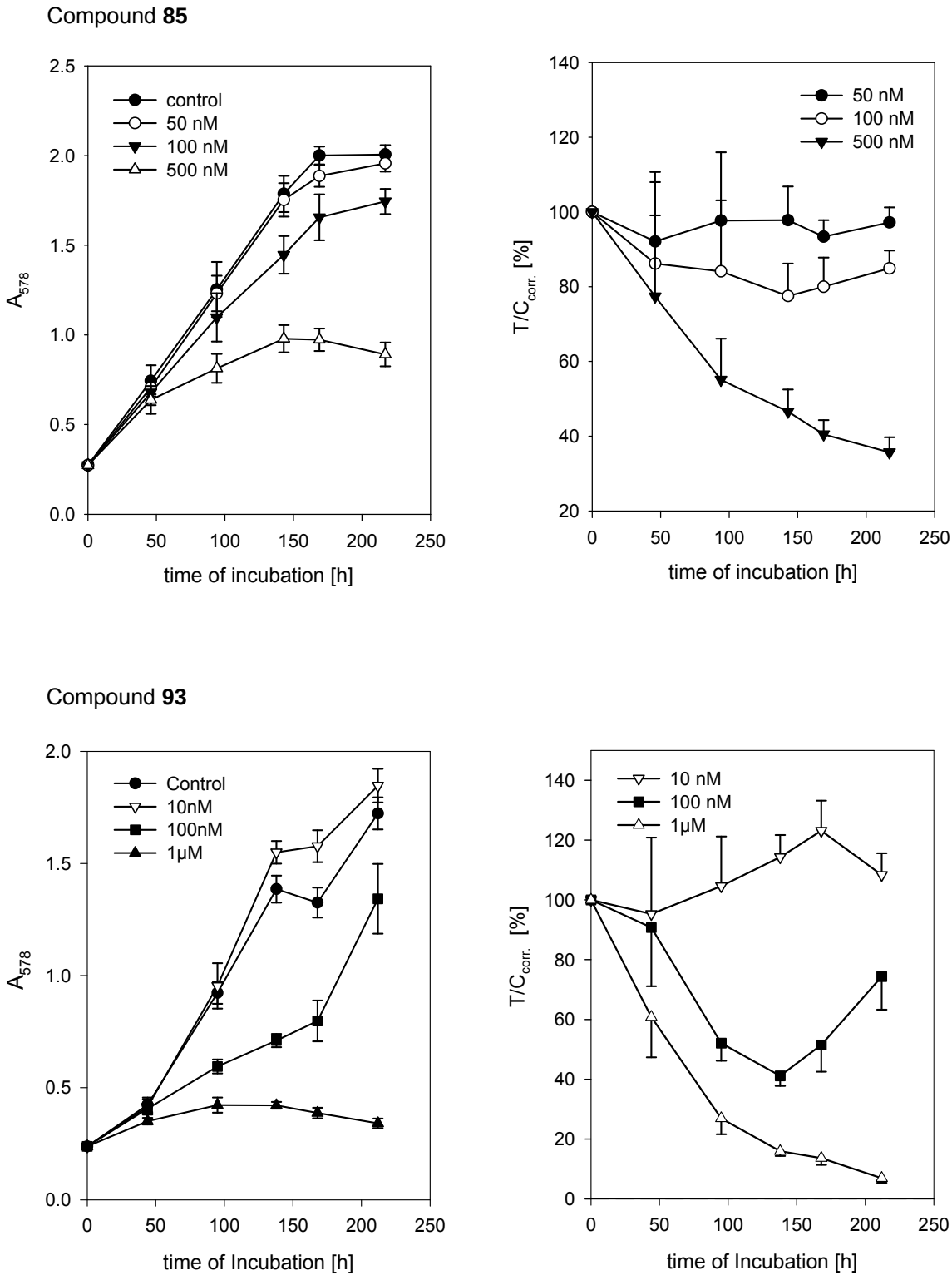


Figure C7: Proliferation kinetics (left) and growth inhibition of MCF-7 cells after treatment with **85** and **93** in various concentrations compared to the vehicle treated control. Top: *MCF-7 passage 193; bottom: MCF-7 passage 177; for additional information see Figure C6*

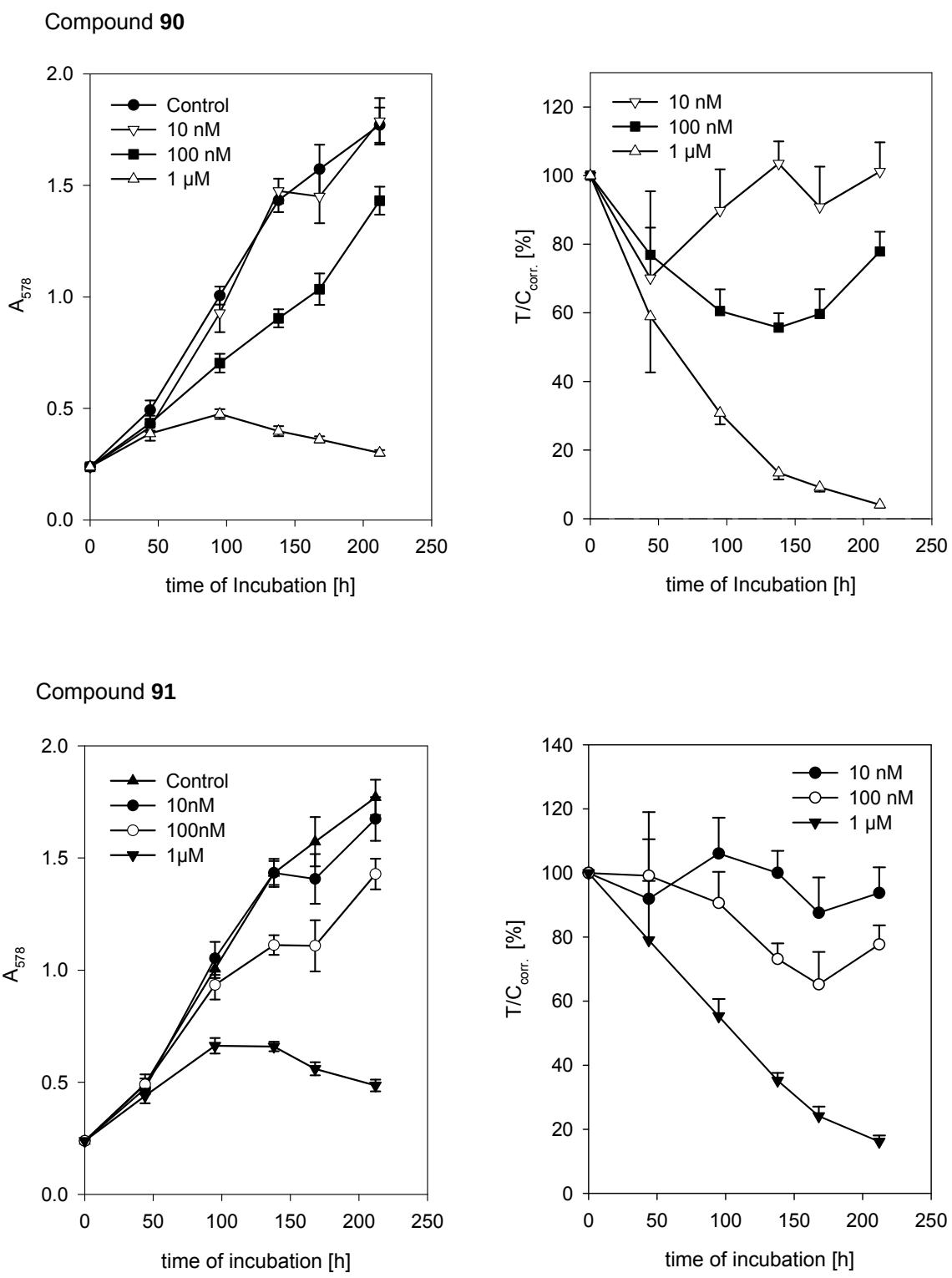


Figure C8: Proliferation kinetics (left) and growth inhibition of MCF-7 cells after treatment with compound **90** (top) and **91** (bottom) compared to the vehicle treated control.

MCF-7 passage 177; for additional information see Figure C6

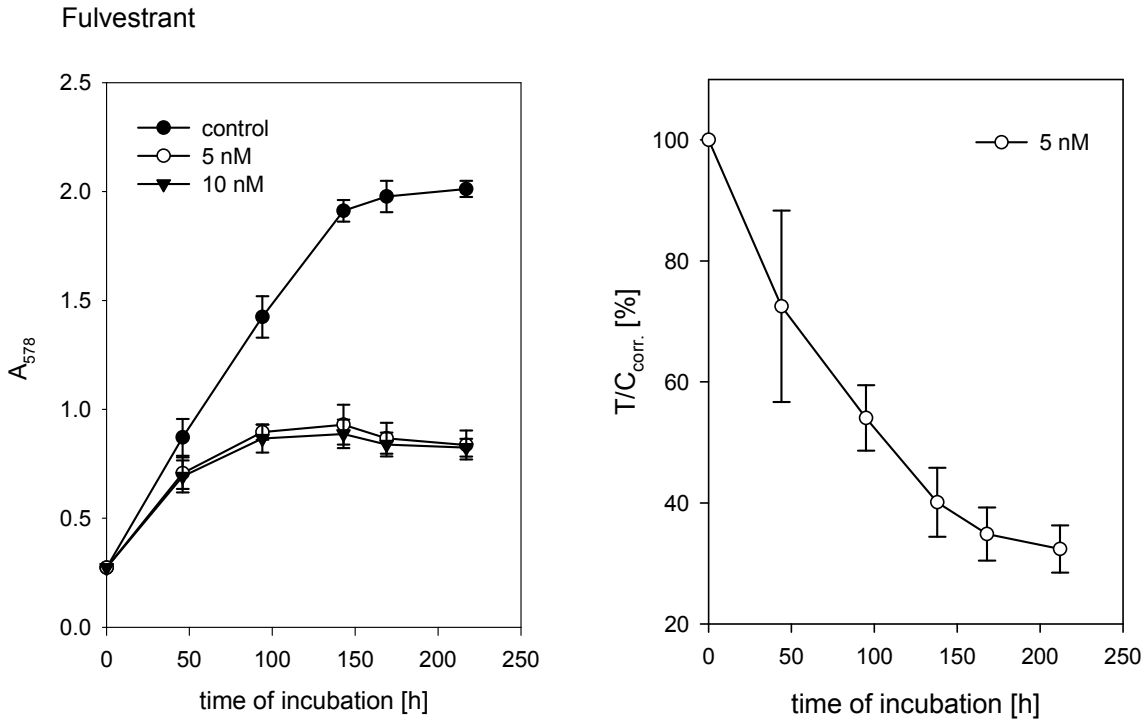


Figure C9: Proliferation kinetics (left) and growth inhibition of MCF-7 cells after treatment with 5 and 10 nM fulvestrant (ICI 182.780) compared to the vehicle treated control.

MCF-7 passage 193; for additional information see Figure C5

The observed antiproliferative effects are not necessarily due to antiestrogenic activities of the tested compounds, as cell proliferation is a relatively late event in the estrogen signaling cascade. In order to exclude any unspecific toxic effects, a additional crystal violet assay, using ER-negative MDA-MB-231 cells, was performed for each compound. The cells were incubated with the test compounds at the two highest concentrations applied to MCF-7 cells. As shown in Figure C10, sulfones **87** and **94** exhibited no antiproliferative activity on MDA-MB-231 cells at concentrations up to 1 µM. At these concentrations the compounds were efficient antiproliferative agents against MCF-7 cells. As demonstrated by the treatment with 10 nM vinblastin, the MDA-MB-231 cells used are sensitive to classical cytostatics. This result is representative for all the other compounds tested in this series. Thus, the observed antiproliferative activities of the investigated compounds in ER-positive MCF-7 cells are very likely due to their antiestrogenic potencies.

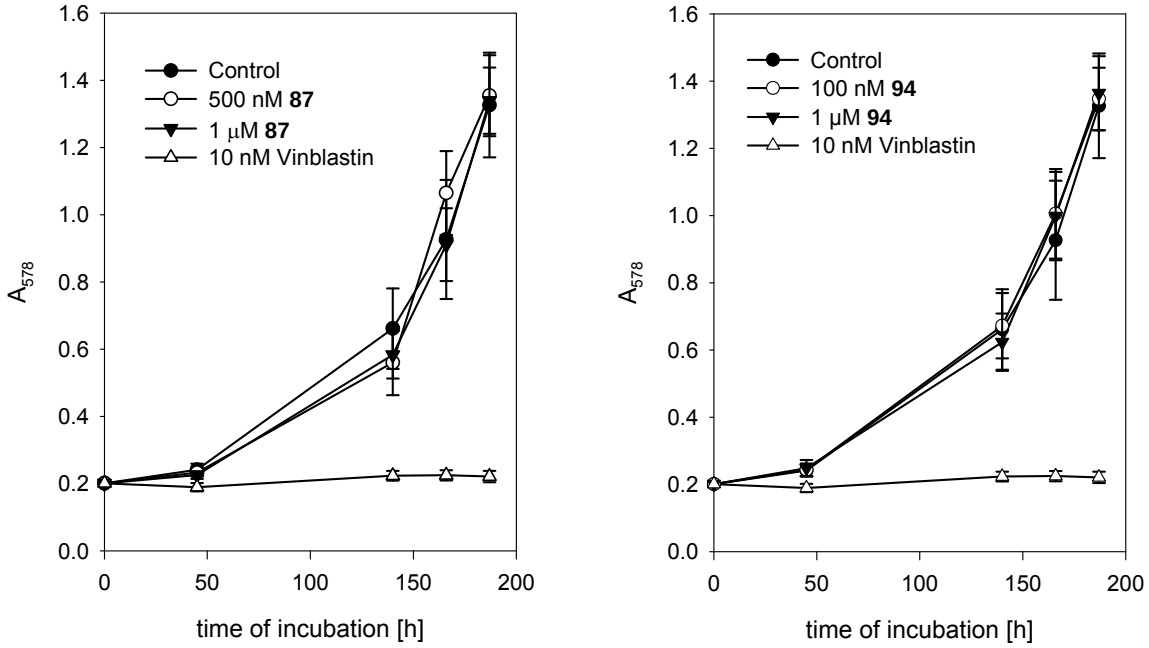


Figure C10: Growth curves of MDA MB-231 cells in presence of tetrahydroisoquinolines **87** and **94** compared to the vehicle control and 10 nM vinblastin.

3.4 Separation and Characterization of Enantiomeric Tetrahydroisoquinolines

All the synthesized tetrahydroisoquinolines are chiral due to an assymmetric sp^3 carbon atom in position 1. As the reductive hydration of the dihydroisoquinolines to the THIQs with $NaBH_4$ was not enantioselctive, the target compounds were obtained as racemates. As the biological assays revealed, the sidechains play a critical role for the biological activity of the corresponding THIQ, especially for binding affinity to the estrogen receptors (cf. paragraph 3.3.2).

Published crystal structures demonstrate that one stereoisomer, namely that with the *R*-configuration at C1, preferably binds to the ERα binding pocket, if the racemate of a THIQ-based SERM is co-crystallized with the ERα LBD (Renaud et al., 2005). Characteristic of these SERMs is the rigid conformation of the side chain.

By contrast, the synthesized ER antagonists are equipped with different aliphatic, non-rigid side chains. The study described in this paragraph reports on the separation of the enantiomers of some selected THIQs and their evaluation with respect to binding to the

human estrogen receptors ERα and ERβ. The investigation of a selection of three racemates aimed at the identification of individual eutomers and distomers.
As representative tetrahydroisoquinolines **92,** with a piperidine side chain, **93** with a bifunctional side chain and **94** with a sulfoxy side chain were chosen. All three compounds bear a hydroxy substituent in position 3 of the N-phenyl ring and were found to preferably bind to ERα.

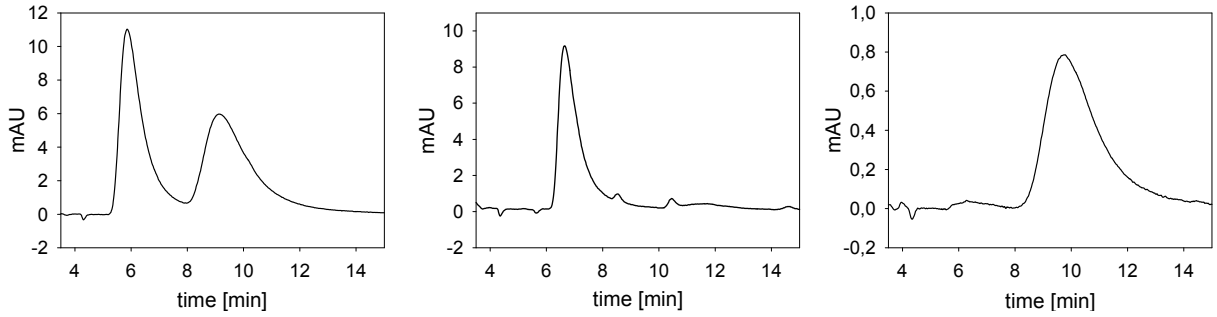
3.4.1 Separation of the Enantiomers

Two different methods for the preparative enantiomeric separation were investigated.
In the test compounds the tetrahydroisoquinoline-nitrogen is located in direct vicinity to the chiral center. A strong chiral acid such as *(R)*- or *(S)*-camphor-10-sulfonic acid should be capable of forming diastereomeric salts. All attempts to separate the enantiomers of the selected THIQs by crystallization as diastereomeric (R) or (S)-camphor-10-sulfonic acid salts failed. The compounds either did not crystallize at all from mixtures of ethanol and ether or, in the case compound **92**, the crystalline salt was a racemic mixture (analysed by chiral HPLC).
The second method concerning the separation of the racemates of **92**-**94** was chiral HPLC. The material Eurocel 01 form Knauer proved to be an appropriate chiral selector for the given class of compounds. According to the manufacturer's information, the chiral stationary phase consists of a silica based matrix coated with a derivatized polysaccharide. It is modified with the chiral selector 3,5-dimethyphenylcarbamate.
In all separations, HPLC was performed in the reversed phase mode, using mixtures of methanol and 0.05 % aqueous TFA. At identical coloumn loading, the resolution of the two enantiomers by HPLC was strongly different in compounds **92**-**94** (cf. table C10). **94** being devoid of a basic amine group showed two sharp, totally resolved peaks. The amine groups in compounds **92** and **93** affected a tailing that was particularly pronounced in the case of **92** bearing a piperidine group. At moderate column loading (injection of 50 µL of a 100 µM solution), the enantiomeric peaks of **92** were not totally resolved (R_s = 1.0) by the chiral selector.
As only an analytical column was available in our group, a semi-preparative separation was performed. Multiple injections (n = 8-10) were necessary for the accumulation of

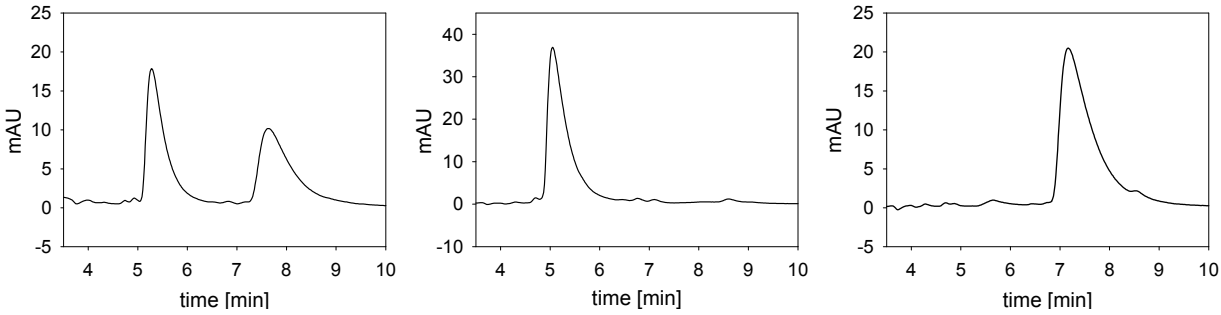
the substance amount required for proper biological assaying. As the ee value was below 80 % for enantiomer **92b** after the first separation, the separation procedure was repeated in this case.

Chromatograms of the chiral compounds **92**-**94** are depicted in Figure C11.

A) Compound **92**



B) Compound **93**



C) Compound **94**

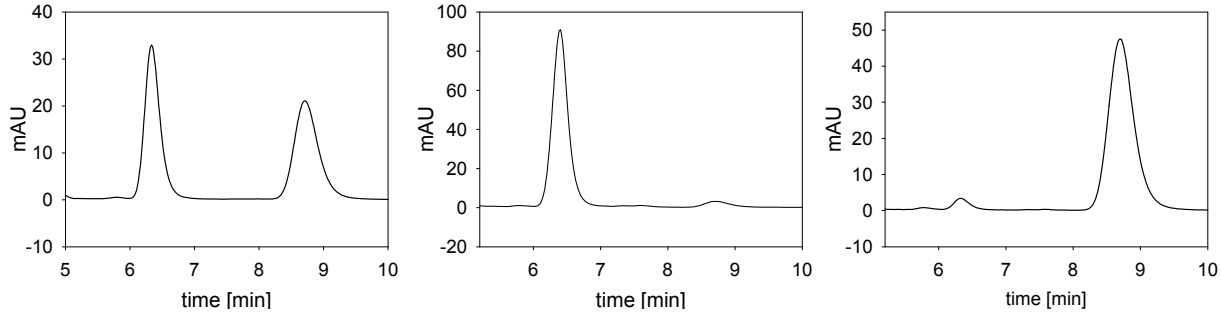


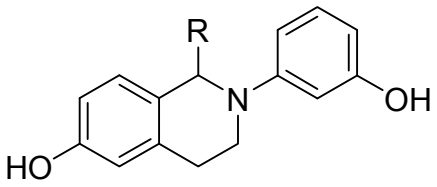
Figure C11: Zoom in on chromatogramms of selected chiral THIIQs: left: racemate, middle: first eluted enantiomer after separation, right: last eluted enantiomer after separation.

Stationary phase: Eurocel 01; A) mobile phase: MeOH/0.05 % TFA (aq) 50/50; B) mobile phase: MeOH/0.05 % TFA (aq) 70/30; C) mob. phase: MeOH/0.05 % TFA (aq) 90/10.

Before separation, all compounds were racemates. From the chromatograms after separation, the enantiomeric purity was determined by peak integration for each enantiomer. The circular dicromism (CD) of the individual enantiomers was determined at 245 nM using a CD detector directly coupled to the HPLC system. For all THIQs the first eluted enantiomer gave the positive CD signal. The absolute configuration was not accessible.

The enantiomeric purities of all isolated enantiomers are summarized in table C10.

Table C10: Enantiomeric purities of separated THIQ-enantiomers



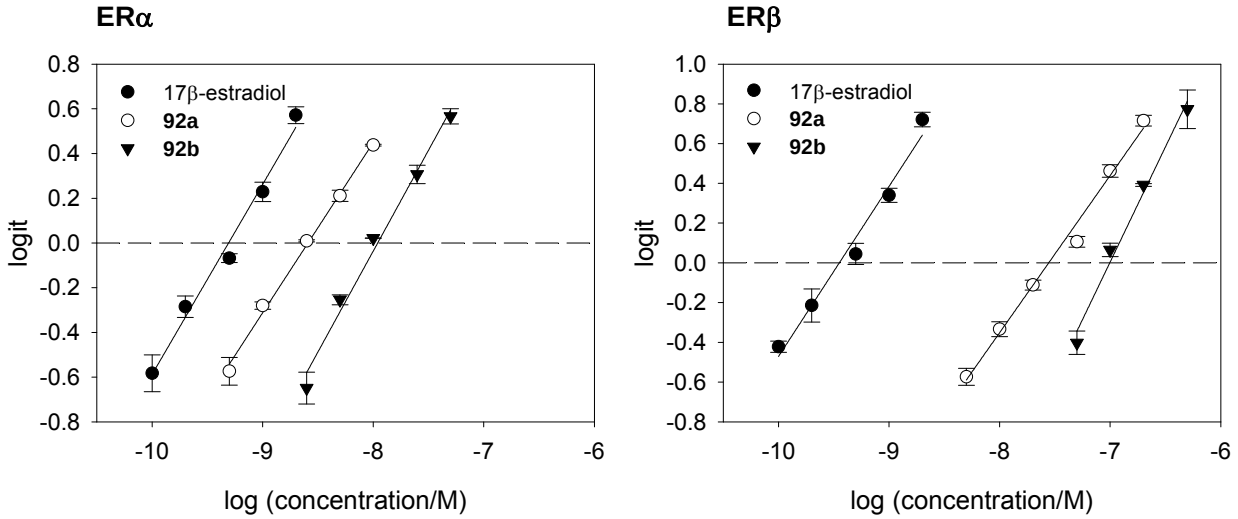
Comp.	R	R_s[a]	CD[b]	ee[c] [%]
92a	$-(CH_2)_6-NC_5H_{10}$	1.0	+	96.8
92b			-	94.9[d]
93a	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	1.6	+	98.4
93b			-	95.5
94a	$-(CH_2)_{10}-SO-C_5H_{11}$	3.1	+	91.2
94b			-	92.6

[a]R_s = resolution of HPLC-peaks; injection: 50 µL of a 100 µM solution; [b]circular dichromism at 245 nM; [c]enantiomeric excess; [d]after two successive separations

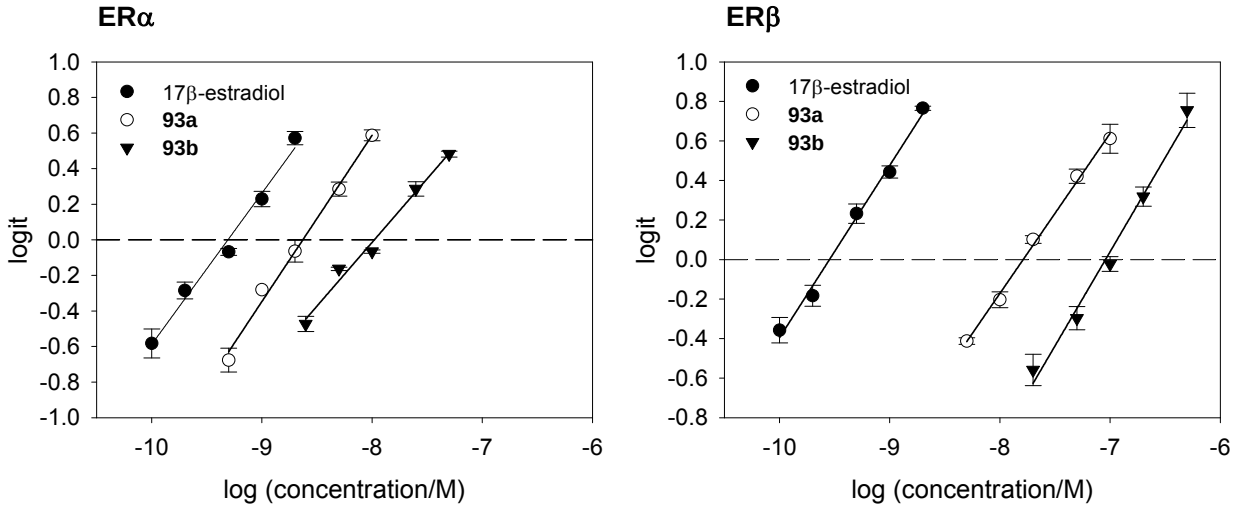
3.4.2 Binding Affinities of the Enantiomers to ERα and ERβ

The separated enantiomers were submitted to the ERα and ERβ binding assay described in paragraph 1.1. The logit-plots of the respective enantiomers of compounds **92**-**94** for both receptor subtypes are depicted in Figure C11. The slope of all regression curves is in the range of 1, indicating a competitive displacement of the radioligand binding.

A) Compound **92**



B) Compound **93**



C) Compound **94**

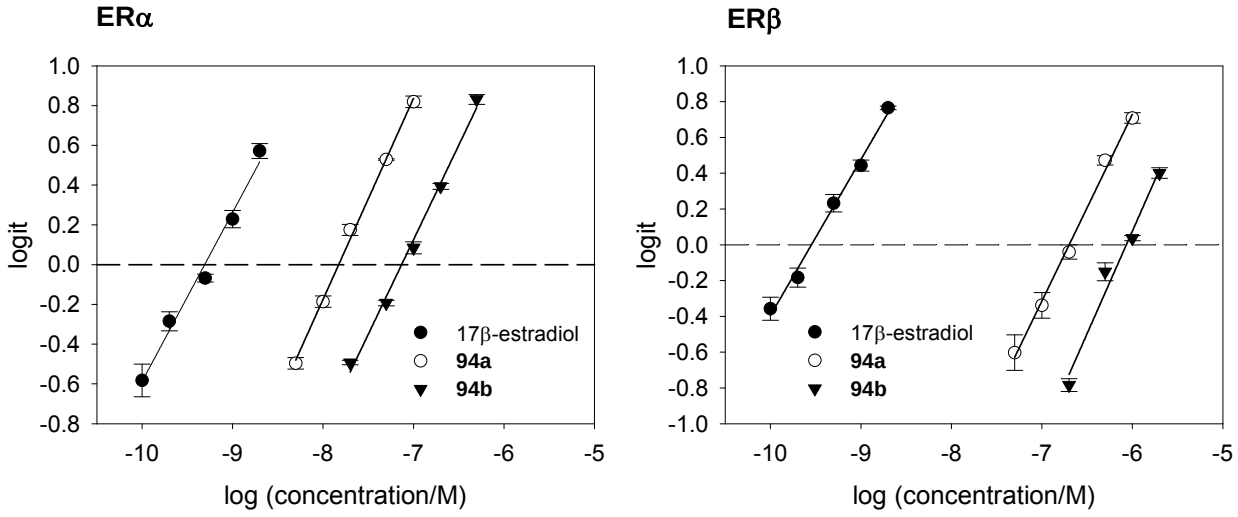
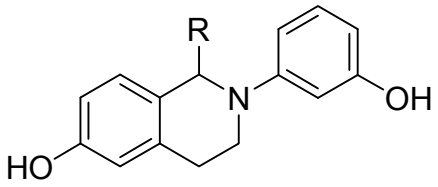


Figure C12: Logit linearization for binding of THIQ enantiomers to ERα and ERβ; *means of triplicates ±SEM*

The RBA values for the racemates and the respective single enantiomers are listed in table C11. For all compounds (**92**-**94**) the enantiomers giving the positive CD signal were identified as the eutomers concerning ER binding. They bound to both ER subtypes with a 3 to 6 fold higher RBA value than the corresponding distomers and with a slightly increased RBA value compared to the racemates.

In a published experiment, the distomer of an 1-aryl-tetrahydroisoquinoline was reported to lack binding affinity up to the micromolar range (Chesworth et al., 2004). A possible explanation for the difference between this example from literature and the compounds investigated within this project is the higher degree of rotation in the aliphatic side chains of THIQs **92**-**94** compared to a rigid phenyl ring. By conformational restriction, the ligand is fixed to the binding pocket in only one possible conformation. In the case of compounds **92**-**94**, the long aliphatic sidechains of the distomers seem to find their favorable orientation in the binding pocket anyhow by free rotation.

Table C11: Binding affinities of THIQ enantiomers



Comp.	R	RBA ERα	RBA ERβ
rac-92	$-(CH_2)_6-NC_5H_{10}$	11.9 ± 0.2	1.08 ± 0.02
92a		15.9 ± 0.4	1.2 ± 0.1
92b		3.4 ± 0.4	0.22 ± 0.01
rac-93	$-(CH_2)_6-N(CH_3)(CH_2)_3-S-C_5H_{11}$	14 ± 1	1.1 ± 0.1
93a		21± 1	1.7 ± 0.1
93b		7.0 ± 1.9	0.28 ± 0.1
rac-94	$-(CH_2)_{10}-SO-C_5H_{11}$	2.4 ± 0.1	0.14 ± 0.02
94a		3.0 ± 0.3	0.21 ± 0.04
94b		0.5 ± 0.1	0.065 ± 0.009

For additional information see table C5;

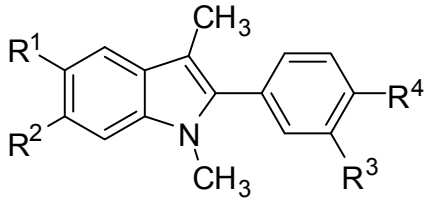
4 Binding Affinities of 2-Phenylindoles to ERα and ERβ

2-Phenylindoles as ER ligands were an important research area of our group mainly in the 1980s and 1990s. A large number of compounds has been synthesized and evaluated for biological activity aiming on new agents for the therapy of hormone receptor-positive breast cancer (von Angerer et al., 1984; von Angerer et al., 1990; von Angerer et al., 1994). ERβ as the second ER subtype was not yet discovered to that time, so that the binding profile of this class of compounds at both human estrogen receptors has not been investigated.

Within the scope of this project a small selection of isomeric 2-phenylindoles was investigated with regard to affinities and selectivities for ERα and ERβ. The differentiated evaluation of binding affinities to the currently known two human estrogen receptor subtypes might offer new insight into the binding characteristics of this class of compounds.

The isomeric indoles **In1**-**In4** of series 1 are all substituted with a methyl group in position 1 and 3. The only difference between these compounds are the positions of the hydroxy groups at the indol core and the 2-phenyl-ring. The RBA values of **In1**-**In4** for the human estrogen receptor subtypes and the calf ER are listed in table C12.

Table C12: Binding affinities of isomeric 2-phenylindoles (series 1) to ERα and ERβ



Compound	R^1	R^2	R^3	R^4	RBA calf-ER[a]	RBA ERα	RBA ERβ
In1	OH	H	OH	H	0.55	0.87	3.4
In2	H	OH	OH	H	0.6	1.3	6.6
In3	H	OH	H	OH	10	14	74
In4	OH	H	H	OH	4.6	18	95

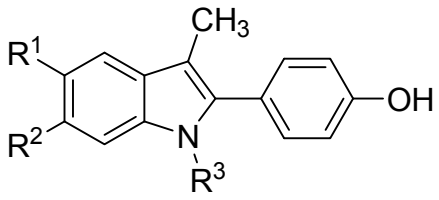
[a]from calf uterus cytosol (von Angerer et al., 1984); for additional information see table C5.

The RBA values of **In1** and **In2** for ERα were in the range of 1, which is in agreement with the RBA values determined for the calf uterus cytosol by the original publishers of the compounds. **In3** exhibited an approximately 10-fold higher affinity for ERα than **In1** and **In2**. This value also matches the corresponding value from calf ER. Exceptionally, the binding affinity of **In4** for ERα was approximately 4-fold higher than the affinity at the calf ER. Generally, the RBA values of compounds **In1** and **In2** bearing a hydroxy group in the 3' position are decreased by approximately one order of magnitude compared to the corresponding 4'-OH analogues. This trend became obvious for both human ER subtypes as well as for the calf ER.

Characteristic of all investigated indoles of this series was the selectivity in favor ERβ by a factor of 4-5. For indoles **In3** and **In4** already exhibiting high affinities for ERα, the RBA values for ERβ were 75 and 95, respectively, which is very close to the endogenous estrogen E2. The binding affinities of **In3** and **In4** for ERβ are in the same range as the binding affinity of benzofuran **B1** (cf. paragraph 2.3.1 table C2), but the selectivity of the indoles is by a factor of 4 lower compared to the benzofuran.

A second series of 2-phenylindole-based compounds which were differently substituted at the indole-nitrogen, was also submitted to ERα and ERβ binding. The compounds were mainly synthesized and biologically characterized by Dr. T. Golob as a former member of our group (Golob et al., 2002). Compounds ZK119.010 (von Angerer et al., 1990), **In5** (von Angerer et al., 1984) and **In8** (von Angerer et al., 1994) included in this second series of 2-phenylindoles have been described in earlier work of our research group. RBA values of the indoles of series 2 are listed in table C13.

Table C13: Binding affinities of various 2-phenylindoles (series 2) to ERα and ERβ



Comp.	R^1	R^2	R^3	RBA ERα	RBA ERβ	RBA Calf-ER[a]
In5	OH	H	Et	25	54	14
In6	H	H	Et	18	7.8	3.4
In7	H	OH	$-(CH_2)_5-CO-NC_4H_8$	4.2	3.6	12.2
In8	OH	H	$-(CH_2)_5-CO-NC_4H_8$	21	4.6	19
In9	H	OH	$-(CH_2)_6-NC_4H_8$	8.3	11	8.4
ZK119.010	OH	H	$-(CH_2)_6-NC_4H_8$	25	6.0	33

[a]from calf uterus cytosol (Golob et al., 2002); for additional information see table C5.

Within this second indol-series, compounds **In5** and **In6**, substituted with an N-ethyl group are different by one hydroxy group: **In5** carries a 5-OH group at the indol scaffold, while **In6** is unsubstituted at this ring. **In5** and **In6** are highly affinic to ERα with similar RBA values of 25 and 18, respectively. **In5**, bearing a 5-hydroxy group, revealed a 2 fold selectivity for ERβ, while **In6** lacking a hydroxy group at the indole core preferably bound to ERα. **In7** and **In8** are isomers with a carbonyl side chain at the indole nitrogen varying in the position of a hydroxy group at the indole partial structure. The RBA values of **In7** were in the range of 4 for both receptor subtypes. **In8** revealed a 5-fold selectivity for ERα (RBA = 21) over ERβ (RBA = 4.6).

In9 and ZK 19.010 are the amine analogues to the amides **In7** and **In8**. For ZK 119.010 the RBA value (25) for ERα was again close to the value determined with the calf uterus cytosol as receptor source (RBA = 33). ZK119.010 revealed a 4-fold selectivity for ERα, which is a notable parallel to the corresponding amide analogue **In8**. **In9** exhibits similar affinities for both receptor subtypes with RBA values in the range of 10.

5 Conclusion

Two types of potential estrogen receptor ligands were synthesized and evaluated for binding affinity and selectivity at ERα and ERβ and for (anti)estrogenic potency.
The first class of synthesized compounds were 2-(4-hydroxyphenyl)benzofurans with simple alkyl moieties as well as long functionalized side chains in position 7. Taking account of its binding and activity profile, the >30 fold ERβ-selective agonist (E)-2-(4-hydroxyphenyl)-7-(prop-1-enyl)benzofuran **24** can be used as a potential tool to investigate subtype specific cellular effects (cf. section D). By the introduction of long sulfone side chains into the benzofuran 7-position, the binding affinities were decreased, while the ERβ-selectivity was maintained to some degree (10-fold). The sulfones were inactive in the luciferase assay. Compound **33** with a bifunctional side chain revealed an increased binding affinity compared to the benzofurans with a sulfone side chain. It was preferentially bound to ERα, but exhibited a weak antagonistic effect in the luciferase assay. The present results demonstrate again, that the development of potent, ERβ-selective antagonists still remains a challenge.

2-Aryl-1,2,3,4-tetrahydroisoquinolines as a second class of synthesized compounds were known from literature as ERα-selective ligands. Within this project, the introduction of established side chains known to be favorable for antiestrogenic activity in position 1 aimed on ERα selective “pure ER antagonists”. The binding affinity of the THIQs depended from the nature of the side chain, while a hydroxy group in position 3 of the N-phenyl ring was necessary for ERα-selectivity. The highest RBA values were in the range of 10, which is comparable to the potent antiestrogens fulvestrant and 4-hydroxytamoxifen. In the case of three representative compounds the enantiomer which were eluted first and gave a positive CD were identified as the eutomers (eudysmic ratio 3-6) after separation of the racemates by chiral HPLC. Replacement of the N-aryl moiety by a trifluoroacetyl or a phenylsulfonyl group lead to decreased binding affinity and a lack of selectivity. The synthesized THIQs with long aliphatic chains were active as antagonists in a gene reporter (luciferase) assay. **93** harboring a bifunctional side chain and a 3’-hydroxy group revealed the highest potency among the compounds tested with an IC_{50} value of 160 nM. In agreement with our previous experience, the compounds with pyrrolidine and piperidine side chains were the least active, in spite of their high binding affinities. Selected compounds were tested for their

antiproliferative effect on hormone-sensitive MCF-7 breast cancer cells. Interestingly, compounds **87** and **94**, bearing a sulfoxy group in the side chain revealed the highest potency in the cytotoxicity assay, although they were less potent than **93** in the luciferase assay.

Taken together, the THIQ scaffold proved to be suitable for the development of "pure ER antagonists", while the ERα selectivity of the THIQ-based SERMs reported by others was retained.

Compounds **93** and **94** are characterized by relatively high antiestrogenic potencies in combination with ERα selectivities of 13 and 17 fold, respectively. The compounds are therefore appropriate tools for the selective blockade of the ERα in order to investigate the subtype specificity of cellular estrogen effects (cf. section D). Furthermore, the THIQs with sulfoxy side chain are interesting candidates as non-steroidal "pure ER antagonists" for the treatment of estrogen dependent breast cancer.

As a class of agents with a long history in our research group, 2-phenylindols from an in-house library were re-evaluated with respect to their binding affinities for human ERα and ERβ. Within a first series of isomeric 1-3-dimethyl-2-phenylindoles differing in the position of two hydroxy groups respectively, indoles **In3** and **In4** revealed remarkable binding affinities to ERβ. The RBA values of the two indoles and the endogenous ligand E2 were in the same order of magnitude. The indoles showed some selectivity for ERβ (4-5-fold) but in a much lower extent than 2-phenylbenzofurans or other ERβ selective ligands known from literature.

In a second series of 2-phenylindoles, the antiestrogen ZK119.010 revealed a high RBA value (25) and a 4-fold selectivity for ERα. **In8** as the amide analogue of ZK119.010, showed a very similar binding profile. These ERα-selectivities are noteworthy, but considerably lower than those of the tetrahydroisoquinoline-based antiestrogens synthesized within this project or those of 1,5-diphenylfuran-based ligands previously developed in our group.

6 Experimental

6.1 Chemistry

6.1.1 Marterials and General Methods

Chemicals and solvents

Chemicals and solvents were purchased from commercial suppliers and used without further purification if not otherwise noted.

Millipore water was used throughout for the preparation of HPLC eluents. Petroleum ether (PE) (40-60 °C) was distilled before use.

Column and thin layer chromatography

Thin layer chromatography was performed on Merck silica gel 60 F_{254} TLC aluminium plates. For column chromatography silica gel Geduran 60 (Merck, Darmstadt, Germany; 0.063-0.200 mm) was used.

Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded on a Bruker Avance 300 spectrometer ([^{1}H]: 300.13 MHz; [^{13}C]: 75.46 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. The following abbreviations are used for characterization of peaks: s = singlet; d = duplet; d(d) = duplet of duplet; t = triplet; d(t) = duplet of triplet; q = quartet; quin = quintet; sex = sextet; sept = septet; m = multiplet; br = broad; $^{(n)}$J = coupling constant over n bonds.

Mass spectrometry (MS)

Mass spectrometry analysis was performed in-house by the analytical laboratory. Low resolution mass spectra were recorded on a Finnigan ThermoQuest TSQ 7000 (ES-MS) and a Finnigan SSQ 710A (EI-MS 70 eV, CI-MS). High resolution mass spectrometry was performed on a LTQ Orbitrap Discovery (Thermo Fisher Scientific, Waltham, MA, USA).

Melting points

Melting points were determined with a Büchi 510 melting point apparatus and are uncorrected.

Elemental analysis

Elemental analyses of final benzofurans were performed in-house by the micro analytical laboratory

Preparative HPLC

Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Eurospher-100 C18, 250 × 32 mm, 5 µm, Knauer or Nucleodur-100 C18, 250 × 21 mm, 5µm, Macherey-Nagel, Düren, Germany) at a flow rate of 38 mL/min. Mixtures of acetonitrile and 0.1 % aq. TFA were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure (final pressure: 60 mbar) at 40 °C prior to lyophilization. Lyophilisation was done with a Christ alpha 2-4 LD equipped with a vacuubrand RZ 6 rotary vane vacuum pump (Christ, Osterode a. H., Germany).

Analytical HPLC

Analytical HPLC analysis was performed on a system from Merck, composed of a L-5000 controller, a 655A-12 pump, a 655A-40 autosampler and a L-4250 UV-VIS detector; the flow rate was 0.8 mL/min, the detection waveleangth 210 nm; all separations were run at 40 °C. Helium degassing was used throughout. If not otherwise indicated a Eurospher-100 C18 (250 × 4 mm, 5 µm) column (Knauer) served as RP-stationary phase. The analysis of the benzofurans was performed on a Nucleodur 100-5 C18 ec (250 × 4 mm, 5 µm) column (Macherey-Nagel).

Chiral HPLC

Chiral HPLC was performed on a system from Waters, composed of a 600s controller and pump, a Waters degasser, a temperature control module, a 717 plus autosampler and a 2487 UV-detector; the flow rate was 1 mL/min, the detection waveleangths were 210 and 276 nm. A Eurocel'01 (250 × 4.6 mm; 5µm; Knauer) analytic column served as chiral selector. Separations were performed in reversed phase mode with mixtures of methanol and 0.05 % aq. TFA as mobile phase at 35 °C.

6.1.2 Chemical Methods and Analytical Data

6.1.2.1 Synthesis of 2-Arylbenzofurans

6.1.2.1.1 Synthesis of Aliphatic Side Chains

1-Bromo-8-(pentylsulfanyl)octane (**1**)

Under nitrogen atmosphere pentanethiol (1.56 g, 15 mmol) in dry DMF (20 mL) was added dropwise to a suspension of sodium hydride (60% suspension in paraffin; 0.72 g, 18 mmol) in dry DMF (80 mL) and stirred till the gas evolution ceased. The resulting mixture was filled into a drop-ping funnel, slowly added to a DMF solution (35 mL; 50 °C) of 1,8-dibromooctane (15 g, 55 mmol) and stirred at this temperature for another two hours. Excess sodium hydride was decomposed by the addition of water and the product extracted with three portions of ethyl acetate. The organic extract was washed with water and brine and dried over Na_2SO_4. The solvent was removed *in vacuo*.

Unreacted starting material, desired product and the by-product 1,8-bis-(pentylsulfanyl)-octane were separated by column chromatography (SiO_2; PE/DCM 10:1, v/v). Staining of the TLC plates with iodine (1% on silica gel) reveals the starting material as pink spot and the two sulfur-containing products as yellow spots. The excess of starting material can be recovered quantitatively.

Light yellow oil; yield: 1.75 g (40%)

$C_{13}H_{27}BrS$ (295.36)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 6.9Hz; -C<u>H</u>$_3$), 1.20-1.50 (12H; m; -(C<u>H</u>$_2$)$_2$-CH$_3$, -(C<u>H</u>$_2$)$_4$-(CH$_2$)$_2$-Br), 1.51-1.65 (4H; m; -C<u>H</u>$_2$-CH$_2$-S-CH$_2$-C<u>H</u>$_2$-), 1.85 (2H; quin; ^3J = 6.9 Hz; Br-CH$_2$-C<u>H</u>$_2$-), 2.50 (4H; t; ^3J = 7.3 Hz; -C<u>H</u>$_2$-S-C<u>H</u>$_2$-), 3.40 (2H; t; ^3J = 6.9 Hz; Br-C<u>H</u>$_2$)

1-Bromo-9-(pentylsulfanyl)nonane (**2**)

Preparation from 1,9-dibromononane (10 g, 35 mmol) and pentanethiol (0.91 g, 8.75 mmol) following the procedure described for **1**.

Light yellow oil; yield: 800 mg (30%)

$C_{14}H_{29}BrS$ (309.35)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^{3}J = 6.9 Hz; -C$\underline{H}_3$), 1.30-1.50 (14H; m; -(C$\underline{H}_2$)$_2$-CH_3, -(C$\underline{H}_2$)$_5$-$(CH_2)_2$-Br), 1.51-1.65 (4H; m; -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-), 1.85 (2H; quin; ^{3}J = 7.0 Hz; Br-CH_2-C$\underline{H}_2$-), 2.50 (4H; t; ^{3}J = 7.3 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 3.40 (2H; t; ^{3}J = 7.0 Hz; Br-C$\underline{H}_2$-)

Ethyl 3-(pentylsulfanyl)propionate (**3**)

Under nitrogen atmosphere and at room temperature, pentanethiol (3.2 g, 31 mmol) in dry DMF (20 mL) was added dropwise to a suspension of sodium hydride (60% suspension in paraffin; 1.7 g, 42 mmol) in dry DMF (25 mL) and stirred till the gas evolution ceased. Then, a DMF solution (25 mL) of ethyl 3-bromopropionate (5 g, 28 mmol) was added dropwise and the resulting solution was stirred at this temperature for another two hours. Excess sodium hydride was decomposed by the addition of water and the product extracted with ethyl acetate (3 x 50 mL). The organic extract was washed with water and brine. After drying over Na_2SO_4 the solvent was removed *in vacu*o. The crude product was purified by column chromatography (SiO_2; PE/DCM 1:1; v/v).

Colourless oil; yield: 4.3 g (75%)

$C_{10}H_{20}O_2S$ (204.33)

[^{1}H]-NMR ($CDCl_3$):

δ (ppm) = 0.89 (3H; t; ^{3}J = 7.1 Hz; -$(CH_2)_4$-C$\underline{H}_3$), 1.26 (3H; t; ^{3}J = 7.1 Hz; -CH_2-C$\underline{H}_3$), 1.25-1.42 (4H; m; -(C$\underline{H}_2$)$_2$-CH_3), 1.53-1.63 (2H; m; C_3H_7-C$\underline{H}_2$-CH_2-S-), 2.52 (2H; t; ^{3}J = 7.4 Hz; -$(CH_2)_3$-C$\underline{H}_2$-S-), 2.58 (2H; t; ^{3}J = 7.4 Hz; -C$\underline{H}_2$-CO-), 2.77 (2H; t; ^{3}J = 7.4 Hz; -S-C$\underline{H}_2$-CH_2-CO), 4.11-4.18 (2H; q; ^{3}J = 7.1 Hz; -O-C$\underline{H}_2$-)

3-(Pentylsulfanyl)propionic acid (**4**)

Ethyl 3-(pentylsulfanyl)propionate (**3**) (4.3 g, 21 mmol) and potassium hydroxide (2.06 g, 37 mmol) in a mixture of EtOH/H_2O (3:1 v/v, 40 mL) were refluxed for 4 h. The solution was cooled to room temperature and concentrated. The remaining white residue was dissolved in water. This aqueous phase was washed with ether (30 mL) before it was acidified with conc. HCl. Then, the product was extracted from the aqueous phase with 3 portions of ether (3 x 30 mL). The combined organic extracts were washed with water and brine. After drying over Na_2SO_4 the solvent was evaporated.

Light yellow oil; yield: 3.7 g (100%)

$C_8H_{16}O_2S$ (176.27)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.89 (3H; t; ^3J = 7.1 Hz; -$(CH_2)_4$-C$\underline{H}_3$), 1.25-1.42 (4H; m; -$(C\underline{H}_2)_2$-CH_3), 1.53-1.63 (2H; quin; ^3J = 7.3 Hz; C_3H_7-C$\underline{H}_2$-CH_2-S-), 2.53 (2H; t; ^3J = 7.4 Hz; -$(CH_2)_3$-C$\underline{H}_2$-S-), 2.66 (2H; t; ^3J = 7.1 Hz; -C$\underline{H}_2$-COOH), 2.77 (2H; t; ^3J = 7.1 Hz; -S-C$\underline{H}_2$-CH_2-CO)

*N-Methyl-3-(pentylsulfanyl)propionamide (**6**)*

Oxalyl chloride (4 g, 31 mmol) was added dropwise to a stirred solution of 3-(pentylsulfanyl)propionic acid (**4**) in DCM (40 mL) and the mixture was stirred for 1 h at r.t.. The solvent and unreacted oxalyl chloride were then removed *in vacuo* to obtain crude 3-(pentylsulfanyl)propionic acid chloride (**5**). Crude **5** was added drop-wise to a solution of methylamine (40% in water; 20 mL, 210 mmol) and NaOH (1.7 g, 42 mmol) at -10 °C. After complete addition the reaction mixture was acidified with 15% HCl and extracted with DCM (3 x 50 mL). The combined organic phases were washed with water and brine, dried over Na_2SO_4 and the solvent was evaporated. The crude product was purified by column chromatography (SiO_2; DCM/ethyl acetate 1:1, v/v). TLC plates were stained with iodine (1% on silica gel) to reveal the product as yellow spot.

Orange oil; yield: 3.2 g (80%)

$C_9H_{18}NOS$ (189.32)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.83 (3H; t; ^3J = 7.1 Hz; -$(CH_2)_4$-C$\underline{H}_3$), 1.22-1.34 (4H; m; -$(C\underline{H}_2)_2$-CH_3), 1.47-1.57 (2H; quin; ^3J = 7.4 Hz; C_3H_7-C$\underline{H}_2$-CH_2-S-), 2.39 (2H; t; ^3J = 7.3 Hz; -$(CH_2)_3$-C$\underline{H}_2$-S-), 2.46 (2H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-CON), 2.70-2.79 (5H; m; -S-C$\underline{H}_2CH_2$CO, N-C$\underline{H}_3$)

N-Methyl-3-(pentylsulfanyl)propylamine (**7**)

Under dry nitrogen atmosphere, N-methyl-3-(pentylsulfanyl)propionamide **6** (1.5 g, 7.9 mmol) in dry THF (8 mL) was added dropwise to a suspension of $LiAlH_4$ (600 mg; 16 mmol) in dry THF (20 mL). The reaction mixture was refluxed for 3 h and then with cooling in an ice-water bath hydrolysed with water (20 mL) and sat. $NaHCO_3$ (10 mL). The organic components were extracted into ethyl acetate (3 x 25 mL) and this organic phase was washed with 3 portions of 2N HCl (3 x 25 mL). The combined acidic phases

were rebasified with 2N NaOH and extracted again with ethyl acetate (3 x 25 ml). The combined organic phases of the second extraction were washed with water and dried over Na_2SO_4. Finally the solvent was removed under reduced pressure.

Orange oil; yield: 500 mg (36%)

$C_9H_{21}NS$ (175.33)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.83 (3H; t; ^3J = 7.1 Hz; -$(CH_2)_4$-C<u>H</u>$_3$), 1.20-1.34 (4H; m; -$(C\underline{H}_2)_2$-CH_3), 1.47-1.57 (2H; quin; ^3J = 7.4 Hz; C_3H_7-C<u>H</u>$_2$-CH_2-S-), 1.67-1.77 (2H; quin; ^3J = 7.2 Hz; N-CH_2-C<u>H</u>$_2$-CH_2-S), 2.37 (3H; s; N-C<u>H</u>$_3$), 2.44 (2H; t; ^3J = 7.4 Hz; -$(CH_2)_3$-C<u>H</u>$_2$-S-), 2.50 (2H; t; ^3J = 7.3 Hz; N-$(CH_2)_2$-C<u>H</u>$_2$-S-), 2.61 (2H; t; ^3J = 7.0 Hz; N-C<u>H</u>$_2$)

5-Carboxypentyltriphenylphosphoniumbromide (**8**)

5-Bromovaleric acid (20.0 g, 110 mmol) and triphenylphosphine (26.2 g, 100 mmol) were mixed and heated to 85 °C. The gently stirred melt becomes solid after 1 h. The crude solid was dissolved in boiling chloroform/ethanol (20:1, v/v, 150 mL)). By addition of ether (200 mL) the pure product was precipitated.

White solid; yield: 39 g (90%)

$C_{23}H_{24}BrO_2P$ (433.31)

Melting point: 195 °C (Lit: 205-206 °C (Bhalerao et al., 1970))

[^{1}H] NMR (D_4-methanol):

δ (ppm) = 1.65-1.91 (4H; m; $(C\underline{H}_2)_2$-CH_2-COOH), 2.37 (2H; t; ^3J = 7.0 Hz; -CH_2-COOH), 3.39-3.53 (2H; m; -CH_2-P), 7.70-7.94 (15H; m; phenyl-<u>H)</u>

6.1.2.1.2 Preparation of Aryliodides and Arylbromides

Tetraethylammonium diacetoxyiodate (Doleschall and Toth, 1980)

Under dry nitrogen atmosphere tetraethylammoniumiodide (5 g, 19.4 mmol) and diacetoxyiodobenzene (6.25 g, 19.4 mmol) were dissolved in abs. chloroform (25 mL) and stirred for 2 h at room temperature. The reaction mixture was allowed to stand over night. Abs. ether (150 mL) was added to the clear brown solution and the mixture was cooled to 0 °C under stirring. A crystalline solid was filtered of quickly under a nitrogen

flush, washed with abs. ether and immediately dried over phosphorous pentoxide *in vacuo.*

Yellow crystals; yield: 10.2 g (88 %)

$C_{12}H_{26}NIO_4$ (375.25)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.41 (12 H; 3 × t; N-CH_2-C<u>H</u>$_3$), 1.98 (6H; s, CH_3-COO), 3.43 (8H; q; N-C<u>H</u>$_2$-CH_3)

2-Hydroxy-3-iodo-5-methoxybenzaldehyde (**10**)

Under nitrogen atmosphere at -15 °C tetraethylammonium diacetoxyiodate (900 mg, 2.4 mmol) was added to a stirred solution of 2-hydroxy-5-methoxybenzaldehyde (300 mg, 2.0 mmol) in abs. DCM (8 mL). After stirring for 14 h at this temperature a second portion of the iodination reagent was added (450 mg, 1.2 mmol). After a total reaction time of 16 h the reaction mixture was concentrated in vacuo and the residue was purified by column chromatography (SiO_2; PE/ethyl acetate 10/1, v/v).

Yellow solid; yield: 200 mg (36%)

Melting point: 98-100 °C (Lit: 102-104 °C (Hart and Mannino, 1996))

$C_8H_7IO_3$ (278.05)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 3.85 (3H; s; -O-C<u>H</u>$_3$), 7.10 (1H; s; phenyl-<u>H</u>), 7.60 (1H; s; phenyl-<u>H</u>), 11.35 (1H; s; C<u>H</u>O)

[^{13}C] NMR ($CDCl_3$):

δ (ppm) = 56, 85 (C-I), 117, 119, 133, 153, 154, 196

MS (CI, NH_3): m/z = 295.9 (100 % MNH_4^+), 278.9 (28%, MH^+)

3-Bromo-2-hydroxy -5-methoxybenzaldehyde (**11**)

Bromine (1.36 g, 0.44 mL, 8.5 mmol) was added to a stirred solution of sodium acetate (0.86 g, 10.5 mmol) and 2-hydroxy-5-methoxybenzaldehyde (1.0 g, 6.6 mmol) in glacial acetic acid (30 mL) and the reaction mixture was stirred for 1 h. The solvent was removed *in vacuo* and the residue was dissolved in DCM (50 mL). The solution was washed with water (2 × 25 mL), dried over Na_2SO_4 and evaporated. The crude product was crystallised from ethanol to obtain a first product fraction. The supernant was evaporated and from the residue a second product fraction was isolated by column chromatography (SiO_2; PE/ethyl acetate 10/1, v/v).

Yellow solid; yield: 0.95 g (62%)

Melting point: 106 °C (Lit: 108 °C (Evano et al., 2004))

$C_8H_7BrO_3$ (231.05)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 3.80 (3H; s; -O-C<u>H</u>$_3$), 7.00 (1H; d; ^{5}J = 3.0 Hz; phenyl-<u>H</u>), 7.40 (1H; d; ^{5}J = 3.0 Hz; phenyl-<u>H</u>), 11.11 (1H; s; C<u>H</u>O)

6.1.2.1.3 Unsuccessful Iodination Methods

Procedure a) (Collini et al., 2004)

Methyl-5-methoxysalicylate (0.91 g, 5.0 mmol) and KOH (0.28 g, 5.0 mmol) were dissolved in methanol (5 mL). A solution of iodine (1.27 g, 5.0 mmol) in methanol (7 mL) was droped into the mixture while heating to 50 °C. The mixture was stirred for 2 h at this temperature. The solvent was removed in vavuo and the product was purified by column chromatography (SiO_2; PE/DCM 1/2, v/v → DCM).

Procedure b) (Kometani et al., 1985)

Chloramin T (338 mg, 1.2 mmol) and NaI (180 mg, 1.2 mmol) were added to a solution of methyl-5-methoxysalicylate (182 mg, 1.0 mmol) in DMF (5 mL). After stirrig for 5 h the reaction mixture was diluted with water and acidified with conc. HCl. The formed precipitate was extracted into ethyl acetate. The organic phase was washed with a $Na_2S_2O_3$ solution and brine, dried over Na_2SO_4 and evaporated. By crystallisation from ethyl acetate a solid was isolated.

Procedures a) and b) yielded the biaryl product **12** as yellow solid

$C_{18}H_{18}O_8$ (362.33)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 3.80 (3H; s; -O-C<u>H</u>$_3$), 3.95 (3H; s; -COOC<u>H</u>$_3$), 7.15 (1H; s; phenyl-<u>H</u>), 7.30 (1H; s; phenyl-<u>H</u>)

[^{13}C] NMR ($CDCl_3$):

δ [ppm] = 52, 56, 112, 125, 127, 151, 154, 171

MS (EI): m/z = 362.2

6.1.2.1.4 Sonogashira Coupling Reaction

5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-carbaldehyde (**13**)

2-Hydroxy-3-iodo-5-methoxybenzaldehyde (**10**) (160 mg, 0.58 mmol), $Pd(PPh_3)_2Cl_2$ (12.0 mg, 0.017 mmol, 3 mol%), CuI (6.0 mg, 0.03 mmol, 5 mol%) and 4-ethinylanisol (86 mg, 0.65 mmol) were dissolved in a helium-degassed mixture of DMF (5 mL) and DEA (1 mL) and stirred for 2 h at 60 °C. The reaction mixture was then poured into a 0.1 N HCl solution (100 mL) and extracted with three portions of ethyl acetate (3 × 50 mL). The combined organic phases were dried over Na_2SO_4 and evaporated. From the obtained dark residue the product was isolated by column chromatography (SiO_2; PE/ethyl acetate 10/1, v/v). Final purification by recrystallisation from PE/ethyl acetate 10/1, v/v.

Pale yellow solid, yield: 100 mg (61 %)

Alternatively 2-hydroxy-3-bromo-5-methoxybenzaldehyde (**11**) (231 mg, 1.0 mmol), $Pd(PPh_3)_2Cl_2$ (21.0 mg, 0.03 mmol, 3 mol%), CuI (9.5 mg, 0.05 mmol, 5 mol%), NaI (100 mg, 0,65 mmol) and 4-ethinylanisol (165 mg, 1.25 mmol) were dissolved in a helium-degassed mixture of DMF (10 mL) and DEA (2 mL) and stirred for 14 h at 70 °C. The reaction was worked up as decribed above.

Pale yellow solid; yield: 130 mg (46 %)

$C_{17}H_{14}O_4$ (282.30)

Melting point: 127-128 °C (from PE/ethyl acetate) (Lit: 218-220 °C (Miller et al., 2003))

[^{1}H] NMR $(CDCl)_3$:

δ [ppm]) = 3.87 (3H; s; -O-C$\underline{H}_3$), 3.88 (3H; s; -O-C$\underline{H}_3$), 6.86 (1H; s; benzofuran-$\underline{H}^3$), 6.98-7.02 (2H; d; AA'BB'; ^{3}J = 8.8 Hz; phenyl-$\underline{H}$), 7.29-7.33 (2H; m; benzofuran-$\underline{H}^4$, -$\underline{H}^6$), 7.80-7.87 (2H; d; AA'BB'; ^{3}J = 8.8 Hz; phenyl-$\underline{H}$), 10.56 (1H; s; C$\underline{H}$O)

MS (EI): m/z = 282.1

6.1.2.1.5 Introduction of Side Chains

1-[5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]propane-1-ol (**14**)

Under nitrogen atmosphere Mg turnings (29 mg, 1.2 mmol) and ethylbromide (200 mg, 1.8 mmol) in dry THF (4ml) were activated with iodine and subsequently stirred at 50 °C for 1 h, until all the Mg was converted into the Gringard reagent. 5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-carbaldehyde (**13**) (282 mg, 1.0 mmol) in THF (1 mL) was added in small portions. After stirring for 2 h at 50 °C the reaction mixture was poured into a 0.1 N HCl solution (20 mL) and extracted with three portions of ethyl acetate (3 × 25 mL). The combined organic phases were washed with water and brine, dried over Na_2SO_4 and evaporated. The secondary alcohol was purified by column chromatography (SiO_2; PE/ethyl acetate 5:1, v/v).

Yellow solid, yield: 200 mg (64%)

$C_{19}H_{20}O_4$ (312.36)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 1.00 (3H; t; ^3J = 7.4 Hz; C$\underline{H}_3$-CH_2), 1.97-2.07 (2H; m; CH_3-C$\underline{H}_2$-), 3.85 (3H; s; -O-C$\underline{H}_3$), 3.86 (3H; s; -O-C$\underline{H}_3$), 5.13 (1H; t; ^3J = 6.5 Hz; Et-C$\underline{H}$-OH), 6.83 (1H; s; benzofuran-$\underline{H}^3$), 6.90-6.92 (2H; m; benzofuran-$\underline{H}^4$, -$\underline{H}^6$), 6.96-7.00 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-$\underline{H}$), 7.75-7.78 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-$\underline{H}$)

MS (EI): m/z = 312.2

1-[5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]butane-1-ol (**15**)

Preparation from propylbromide (150 mg, 1.2 mmol) and 5-methoxy-2-(4-methoxyphenyl)benzofuran-7-carbaldehyde (**13**) (200 mg, 0.71 mmol) following the procedure described for **14**. The product was purified by column chromatography (SiO_2; PE/ethyl acetate 5/1, v/v).

Yellow solid, yield: 140 mg (60%)

$C_{20}H_{22}O_4$ (326.39)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 0.98 (3H; t; ^3J = 7.3 Hz; C$\underline{H}_3$-CH_2-), 1.34-1.62 (2H; m; CH_3-C$\underline{H}_2$-) 1.90-2.02 (2H; m; -CH(OH)-C$\underline{H}_2$-), 3.85 (3H; s; -O-C$\underline{H}_3$), 3.86 (3H; s; -O-C$\underline{H}_3$), 5.22 (1H; t; ^3J = 6.6 Hz; C_3H_7-C$\underline{H}$-OH), 6.85 (1H; s; benzofuran-$\underline{H}^3$), 6.90-6.93 (2H; m; benzofuran-$\underline{H}^4$, -$\underline{H}^6$), 6.95-7.00 (2H; d;

AA'BB'; ^3J = 8.9 Hz; phenyl-H), 7.73-7.78 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-H)

MS (EI): m/z = 326.2

1-[5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]-9-(pentylthio)nonan-1-ol (**16**)

Preparation from 1-Bromo-8-(pentylsulfanyl)octane (**1**) (350 mg, 1.2 mmol) and 5-methoxy-2-(4-methoxyphenyl)benzofuran-7-carbaldehyde (**13**) (226 mg, 0.8 mmol) following the procedure described for **14**. The product was purified by column chromatography (SiO_2; PE/ethyl acetate 5:1, v/v).

Yellow oil; yield: 190 mg (47%)

$C_{30}H_{42}O_4S$ (498.72)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 0.90 (3H; t; ^3J = 7.1 Hz; CH_3-CH_2-), 1.20-1.64 (18H; m; CH_3-$(CH_2)_3$-; -S-CH_2-$(CH_2)_6$-) 1.91-2.02 (2H; q; ^3J = 7.0 Hz; -CH(OH)-CH_2-), 2.43-2.53 (4H; m; -CH_2-S-CH_2-), 3.85 (3H; s; -O-CH_3), 3.86 (3H; s; -O-CH_3), 5.22 (1H; t; ^3J = 6.6 Hz; C_3H_7-CH-OH), 6.83 (1H; s; benzofuran-H^3), 6.89-6.94 (2H; m; benzofuran-H^4, -H^6), 6.95-7.00 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-H), 7.73-7.79 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-H)

MS (CI, NH_3): m/z = 481.3 (100%, MH^+-H_2O), 516.2 (30%, M+NH_4^+)

1-[5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]-10-(pentylthio)decane-1-ol (**17**)

Preparation from 1-bromo-9-(pentylsulfanyl)nonane (**2**) (430 mg, 1.38 mmol) and 5-methoxy-2-(4-methoxyphenyl)benzofuran-7-carbaldehyde (**13**) (300 mg, 1.06 mmol) following the procedure described for **14**. The product was purified by column chromatography (SiO_2; PE/ethyl acetate 5:1, v/v).

Yellow oil; yield: 250 mg (45%)

$C_{31}H_{44}O_4S$ (512.74)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 0.89 (3H; t; ^3J = 7.1 Hz; CH_3-CH_2-), 1.20-1.64 (20H; m; CH_3-$(CH_2)_3$-; -S-CH_2-$(CH_2)_7$-) 1.92-2.03 (2H; q; ^3J = 7.0 Hz; -CH(OH)-CH_2-), 2.41-2.56 (4H; m (br); -CH_2-S-CH_2-), 3.85 (3H; s; -O-CH_3), 3.86 (3H; s; -O-CH_3), 5.20 (1H; t; ^3J = 6.6 Hz; C_3H_7-CH-OH), 6.83 (1H; s; benzofuran-H^3), 6.89-6.94 (2H; m; benzofuran-H^4, -H^6), 6.95-7.00 (2H; d;

AA'BB'; ^3J = 8.9 Hz; phenyl-H), 7.74-7.77 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-H)

MS (EI): m/z = 512.2

6.1.2.1.6 Dehydratation

(E)-5-Methoxy-2-(4-methoxyphenyl)-7-prop-1-enylbenzofuran (**18**)

1-[5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]propane-1-ol (**14**) (100 mg, 0.32 mmol) and p-toluenesulfonic acid (5.7 mg, 0.03 mmol) were dissolved in toluene (13 mL) and heated to 100 °C for 1 h. The reaction mixture was worked up by addition of 1N NaOH (10 mL) and extracted with three portions of ethyl acetate (3 × 20 mL). The combined organic phases were dried over Na_2SO_4 and evaporated. The product was purified by column chromatography (SiO_2; PE/ethyl acetate 10/1, v/v).

Yellow solid; yield: 70 mg (74 %)

Melting point: 116 °C (dec.)

$C_{19}H_{18}O_3$ (294.34)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 1.99-2.04 (3H; d; ^3J = 6.3 Hz; C$\underline{H}_3$-CH=CH-),3.84 (3H; s; -O-C$\underline{H}_3$), 3.86 (3H; s; -O-C$\underline{H}_3$), 6.61-6.70 (1H; d; ^3J = 15.9 Hz; C$\underline{H}$=CH-phenyl), 6.71-6.84 (3H; m; benzofuran-$\underline{H}^3$, -$\underline{H}^4$; -CH=C$\underline{H}$-phenyl), 6.86-6.90 (1H; d; ^5J = 2.5 Hz; benzofuran-$\underline{H}^6$), 6.96-7.02 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-H), 7.75-7.84 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-H)

MS (CI, NH_3): m/z = 295.2 (MH^+)

(E)-5-Methoxy-2-(4-methoxyphenyl)-7-(but-1-enyl)benzofuran (**19**)

Preparation from 1-[5-methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]butane-1-ol (**15**) (130 mg, 0.40 mmol) and p-toluenesulfonic acid (7.6 mg, 0.04 mmol) following the procedure described for **18**.

Yellow solid; yield: 80 mg (65 %)

Melting point: 121-122 °C (dec.)

$C_{20}H_{20}O_3$ (308.38)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 1.18 (3H; t; ^{3}J = 7.4 Hz; -CH_2-C$\underline{H}_3$), 2.30-2.42 (2H; quin; ^{3}J = 7.5 Hz; CH_3-C$\underline{H}_2$-CH), 3.85 (3H; s; -O-C$\underline{H}_3$), 3.87 (3H; s; -O-C$\underline{H}_3$), 6.61-6.69 (1H; d; ^{3}J = 16.0 Hz; -C$\underline{H}$=CH-phenyl), 6.71-6.84 (3H; m; benzofuran-$\underline{H}^3$, -$\underline{H}^4$; -CH=C$\underline{H}$-phenyl), 6.87-6.90 (1H; d; ^{5}J = 2.5 Hz; benzofuran-$\underline{H}^6$), 6.95-7.02 (2H; d; AA'BB'; ^{3}J = 8.9 Hz; phenyl-$\underline{H}$), 7.75-7.84 (2H; d; AA'BB'; ^{3}J = 8.9 Hz; phenyl-$\underline{H}$)

MS (EI): m/z = 308.2

(E)-5-Methoxy-2-(4-methoxyphenyl)-7-[9-(pentylthio)non-1-enyl]benzofuran (**20**)

Preparation from 1-[5-methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]-9-(pentylthio)-nonan-1-ol (**16**) (200 mg, 0.40 mmol) and p-toluenesulfonic acid (8 mg, 0.04 mmol) following the procedure described for **18**.

Yellow oil; yield: 90 mg (46%)

$C_{30}H_{40}O_3S$ (480.72)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 0.89 (3H; t; ^{3}J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 1.24-1.66 (16H; m; CH_3-(C$\underline{H}_2$)$_3$-; -S-CH_2-(C$\underline{H}_2$)$_5$-), 2.23-2.38 (2H; q; ^{3}J = 7.0 Hz; -CH=CH-C$\underline{H}_2$-), 2.44-2.56 (4H; m (br); -C$\underline{H}_2$-S-C$\underline{H}_2$-), 3.85 (3H; s; -O-C$\underline{H}_3$), 3.87 (3H; s; -O-C$\underline{H}_3$), 6.59-6.67 (1H; d; ^{3}J = 16.0 Hz; -C$\underline{H}$=CH-phenyl), 6.70-6.84 (3H; m; benzofuran-$\underline{H}^3$, -$\underline{H}^4$; -CH=C$\underline{H}$-phenyl), 6.87-6.89 (1H; d; ^{5}J = 2.5 Hz; benzofuran-$\underline{H}^6$), 6.95-7.02 (2H; d; AA'BB'; ^{3}J = 8.9 Hz; phenyl-$\underline{H}$), 7.76-7.84 (2H; d; AA'BB'; ^{3}J = 8.9 Hz; phenyl-$\underline{H}$)

MS (EI): m/z = 480.4

(E)-5-Methoxy-2-(4-methoxyphenyl)-7-[10-(pentylthio)dec-1-enyl]benzofuran (**21**)

Preparation from 1-[5-methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]-10-(pentyl-thiodecan)-1-ol (**17**) (200 mg, 0.39 mmol) and p-toluenesulfonic acid (8 mg, 0.04 mmol) following the procedure described for **18**.

Yellow oil; yield: 145 mg (75%)

$C_{31}H_{42}O_3S$ (494.73)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 0.89 (3H; t; ^{3}J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 1.24-1.67 (18H; m; CH_3-(C$\underline{H}_2$)$_3$-; -S-CH_2-(C$\underline{H}_2$)$_5$-), 2.27-2.37 (2H; q; ^{3}J = 7.0 Hz; -CH=CH-C$\underline{H}_2$-)

2.45-2.55 (4H; m (br); -C<u>H</u>$_2$-S-C<u>H</u>$_2$-), 3.85 (3H; s; -O-C<u>H</u>$_3$), 3.87 (3H; s; -O-C<u>H</u>$_3$), 6.59-6.67 (1H; d; ^{3}J = 16.0 Hz; -C<u>H</u>=CH-phenyl), 6.70-6.84 (3H; m; benzofuran-<u>H</u>^{3}, -<u>H</u>^{4}; -CH=C<u>H</u>-phenyl), 6.87-6.89 (1H; d; ^{5}J = 2.5 Hz; benzofuran-<u>H</u>^{6}), 6.95-7.02 (2H; d; AA'BB'; ^{3}J = 8.9 Hz; phenyl-<u>H</u>), 7.76-7.84 (2H; d; AA'BB'; ^{3}J = 8.9 Hz; phenyl-<u>H</u>)

6.1.2.1.7 Oxidation of the Side Chain Sulfur

(E)-5-Methoxy-2-(4-methoxyphenyl)-7-(9-pentylsulfonylnon-1-enyl)benzofuran (**22**)

A solution of meta-chloroperbenzoic acid (123 mg, 0.50 mmol) in chloroform (5 mL) was added dropwise to a solution of 5-methoxy-2-(4-methoxyphenyl)-7-[9-(pentylthio)non-1-enyl]-benzofuran (**20**) (100 mg, 0.21 mmol) in chloroform (20 mL). After stirring for 20 min at room temperature, the reaction mixture was poured into sat. $NaHCO_3$ solution (20 mL) and stirred for 15 min. The chloroform layer was separated using a separation funnel. After successively washing with $NaHCO_3$ solution, water and brine the organic phase was evaporated. Purification by column chromatography (SiO_2; PE/ethyl acetate 3:1, v/v).

Light yellow oil; yield: 50 mg (49%)

$C_{30}H_{40}O_5S$ (512.72)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 0.87 (3H; t; ^{3}J = 7.0 Hz; C<u>H</u>$_3$-CH$_2$-), 1.24-1.55 (12H; m; CH$_3$-(C<u>H</u>$_2$)$_2$-; -SO$_2$-(CH$_2$)$_2$-(C<u>H</u>$_2$)$_4$-), 1.73-1.83 (4H; m; -C<u>H</u>$_2$-CH$_2$-SO$_2$-CH$_2$-C<u>H</u>$_2$-), 2.25-2.32 (2H; q; ^{3}J = 6.9 Hz; -CH=CH-C<u>H</u>$_2$-), 2.85-2.92 (4H; m (br); -C<u>H</u>$_2$-SO$_2$-C<u>H</u>$_2$-), 3.80 (3H; s; -O-C<u>H</u>$_3$), 3.82 (3H; s; -O-C<u>H</u>$_3$), 6.55-6.62 (1H; d; ^{3}J = 16.0 Hz; -C<u>H</u>=CH-phenyl), 6.64-6.79 (3H; m; benzofuran-<u>H</u>^{3}, -<u>H</u>^{4}; -CH=C<u>H</u>-phenyl), 6.83-6.85 (1H; d; ^{5}J = 2.5 Hz; benzofuran-<u>H</u>^{6}), 6.93-6.96 (2H; d; AA'BB'; ^{3}J = 8.9 Hz; phenyl-<u>H</u>), 7.73-7.76 (2H; d; AA'BB'; ^{3}J = 8.9 Hz; phenyl-<u>H</u>)

MS (EI): m/z = 512.3

(E)-5-Methoxy-2-(4-methoxyphenyl)-7-(10-pentylsulfonyldec-1-enyl)benzofuran (**23**)

Preparation from 5-methoxy-2-(4-methoxyphenyl)-7-[10-(pentylthio)dec-1-enyl]-benzofuran (**21**) (50 mg, 0.10 mmol) and meta-chloroperbenzoic acid (34 mg, 0.2 mmol) as described for **22**.

Light yellow oil; yield: 40 mg (76%)

$C_{31}H_{42}O_5S$ (526.73)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 0.92 (3H; t; ^3J = 7.0 Hz; C<u>H</u>$_3$-CH$_2$-), 1.26-1.58 (14H; m; CH$_3$-(C<u>H</u>$_2$)$_2$-; -SO$_2$-(CH$_2$)$_2$-(C<u>H</u>$_2$)$_4$-), 1.78-1.88 (4H; m; -C<u>H</u>$_2$-CH$_2$-SO$_2$-CH$_2$-C<u>H</u>$_2$-) 2.29-2.36 (2H; q; ^3J = 6.9 Hz; -CH=CH-C<u>H</u>$_2$-), 2.89-2.95 (4H; m (br); -C<u>H</u>$_2$-SO$_2$-C<u>H</u>$_2$-), 3.85 (3H; s; -O-C<u>H</u>$_3$), 3.87 (3H; s; -O-C<u>H</u>$_3$), 6.59-6.67 (1H; d; ^3J = 16.0 Hz; -C<u>H</u>=CH-phenyl), 6.69-6.84 (3H; m; benzofuran-<u>H</u>^{3}, -<u>H</u>^{4}; -CH=C<u>H</u>-phenyl), 6.87-6.89 (1H; d; ^5J = 2.5 Hz; benzofuran-<u>H</u>^{6}), 6.97-7.00 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-<u>H</u>), 7.77-7.80 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-<u>H</u>)

MS (EI): m/z = 526.3

6.1.2.1.8 Final Cleavage of the Metoxy Protecting Groups

(E)-2-(4-hydroxyphenyl)-7-(prop-1-enyl)benzofuran-5-ol (**24**)

Under nitrogen atmosphere at -10 °C a solution of 5-methoxy-2-(4-methoxyphenyl)-7-(prop-1-enyl)benzofuran (**18**) in abs. DCM (1 mL) was added dropwise to a stirred solution of BBr_3 (1M stock solution in DCM, 0.85 mmol, 0.85 mL) in DCM (7 mL). After the adition the mixture was stirred for 90 min at room temperature. The reaction was terminated by slow addition of 10% $NaHCO_3$ solution at 0 °C. After dillution with water the mixture was extracted with three portions of ethyl acetate (3 × 30 mL) and the combined organic phases were evaporated. The product was purified by column chromatography (SiO_2; DCM/ethyl acetate 20/1, v/v) and finally crystallised from DCM.

White solid; yield: 10 mg (22%)

Melting point: 196 °C (dec.)

$C_{17}H_{14}O_3$ (266.29)

[^{1}H] NMR (D_6-DMSO):

δ [ppm]) = 1.94-2.00 (3H; d; ^3J = 6.3 Hz; C<u>H</u>$_3$-CH=CH-), 6.55-6.63 (1H; d; ^3J = 16.0 Hz; -C<u>H</u>=CH-phenyl), 6.65-6.78 (3H; m; benzofuran-<u>H</u>^{3}, -<u>H</u>^{4}; -CH=C<u>H</u>-phenyl), 6.86-6.92 (2H; d; AA'BB'; ^3J = 8.7 Hz; phenyl-<u>H</u>), 7.02 (1H; s; benzofuran-<u>H</u>^{6}), 7.69-7.75 (2H; d; AA'BB'; ^3J = 8.7 Hz; phenyl-<u>H</u>), 9.11 (1H; s; -O<u>H</u>), 9.83 (1H; s; -O<u>H</u>)

MS (EI): m/z = 226.3

HR-MS (EI): calculated: 266.0943

found: 266.0945

Analysis: calculated: C: 76.7 H: 5.3

found: C: 74.9 H: 5.5

HPLC: Gradient: 0-35 min: MeCN/H_2O 30/70 to 95/5; 35-40 min: 95/5

RT = 20.07 min; purity: 98%

(E)-2-(4-hydroxyphenyl)-7-(but-1-enyl)benzofuran-5-ol (**25**)

Preparation from 5-methoxy-2-(4-methoxyphenyl)-7-(but-1-enyl)benzofuran (**19**) (60 mg, 0.19 mmol) and BBr_3 (1M in DCM, 1.0 mmol, 1.0 mL) following the demethylation method described for **24**. The reaction mixture was stirred for 75 min.

White solid; yield: 40 mg (75%)

Melting point: 174 °C (dec.)

$C_{18}H_{16}O_3$ (280.32)

[^{1}H] NMR (D_6-DMSO):

δ [ppm]) = 1.12 (3H; t; ^3J = 7.4 Hz; C<u>H</u>$_3$-CH$_2$-), 2.25-2.37 (2H; quin; ^3J = 7.4 Hz; CH$_3$-C<u>H</u>$_2$-), 6.53-6.62 (1H; d; ^3J = 16.1 Hz; -C<u>H</u>=CH-phenyl), 6.67-6.79 (3H; m; benzofuran-<u>H</u>^{3}, -<u>H</u>^{4}; -CH=C<u>H</u>-phenyl), 6.86-6.92 (2H; d; ^3J = 8.7 Hz; phenyl-<u>H</u>), 7.02 (1H; s; benzofuran-<u>H</u>^{6}), 7.68-7.75 (2H; d; AA'BB'; ^3J = 8.7 Hz; phenyl-<u>H</u>), 9.11 (1H; s; -O<u>H</u>), 9.84 (1H; s; -O<u>H</u>)

MS (EI): m/z = 280.1

HR-MS (EI): calculated: 280.1099

found: 280.1104

Analysis: calculated: C: 77.12 H: 5.75

found: C: 75.89 H: 5.85

HPLC: Gradient: 0-35 min: MeCN/H_2O 30/70 to 95/5; 35-40 min: 95/5

RT = 22.56 min; purity: 98%

(E)-2-(4-hydroxyphenyl)-7-[9-(pentylthio)non-1-enyl]benzofuran-5-ol (**26**)

Preparation from 5-methoxy-2-(4-methoxyphenyl)-7-[9-(pentylthio)non-1-enyl]-benzofuran (**20**) (40 mg, 0.08 mmol) and BBr_3 (1M in DCM, 0.5 mmol, 0.5 mL) following the demethylation method described for **24**. The product was purified by column chromatography (SiO_2; DCM/ethyl acetate 30/1, v/v)

Light yellow solid; yield: 25 mg (69%)

Melting point: 121-122 °C

$C_{28}H_{36}O_3S$ (452.65)

[^{1}H] NMR (D_6-DMSO):

δ [ppm]) = 0.84 (3H; t; ^{3}J = 7.1 Hz; C$\underline{H}_3$-CH$_2$-), 1.20-1.55 (16H; m; CH$_3$-(C$\underline{H}_2$)$_3$-; -S-CH$_2$-(C$\underline{H}_2$)$_5$-), 2.25-2.32 (2H; q; ^{3}J = 6.7 Hz; -CH=CH-C$\underline{H}_2$-), 2.41-2.46 (4H; m (br); -C$\underline{H}_2$-S-C$\underline{H}_2$-), 6.52-6.61 (1H; d; ^{3}J = 16.0 Hz; -C$\underline{H}$=CH-phenyl), 6.63-6.76 (3H; m; benzofuran-$\underline{H}^3$, -$\underline{H}^4$; -CH=C$\underline{H}$-phenyl), 6.86-6.89 (2H; d; AA'BB'; ^{3}J = 8.7 Hz; phenyl-$\underline{H}$), 7.02 (1H; s; benzofuran-$\underline{H}^6$), 7.69-7.72 (2H; d; AA'BB'; ^{3}J = 8.7 Hz; phenyl-$\underline{H}$), 9.11 (1H; s; OH), 9.84 (1H; s; OH)

MS (EI): m/z = 452.3

HR-MS (EI): calculated: 452.2385

found: 452.2381

Analysis: calculated: C: 74.3 H: 8.0

found: C: 73.4 H: 8.5

HPLC: Gradient: 0-20 min: MeCN/H_2O 60/40 to 95/5; 20-40 min: 95/5

RT = 25.12 min; purity: 94%

(E)-2-(4-hydroxyphenyl)-7-[10-(pentylthio)dec-1-enyl]benzofuran-5-ol (**27**)

Preparation from 5-methoxy-2-[4-methoxyphenyl)-7-[10-(pentylthio)dec-1-enyl]-benzofuran (**21**) (80 mg, 0.16 mmol) and BBr_3 (1M in DCM, 0.9 mmol, 0.9 mL) following the demethylation method described for **24**. The product was purified by column chromatography (SiO_2; DCM/ethyl acetate 30/1, v/v)

Light yellow solid; yield: 40 mg (54%)

Melting point: 133-136 °C

$C_{29}H_{38}O_3S$ (466.68)

[^{1}H] NMR (D_6-DMSO):

δ [ppm]) = 0.84 (3H; t; ^{3}J = 7.1 Hz; C$\underline{H}_3$-CH$_2$-), 1.21-1.57 (18H; m; CH$_3$-(C$\underline{H}_2$)$_3$-; -S-CH$_2$-(C$\underline{H}_2$)$_5$-), 2.23-2.34 (2H; q; ^{3}J = 6.9 Hz; -CH=CH-C$\underline{H}_2$-), 2.40-2.48 (4H; m (br); -C$\underline{H}_2$-S-C$\underline{H}_2$-), 6.52-6.61 (1H; d; ^{3}J = 16.0 Hz; -C$\underline{H}$=CH-phenyl), 6.63-6.78 (3H; m; benzofuran-$\underline{H}^3$, -$\underline{H}^4$; -CH=C$\underline{H}$-phenyl), 6.84-6.92 (2H; d; AA'BB'; ^{3}J = 8.7 Hz; phenyl-$\underline{H}$), 7.02 (1H; s; benzofuran-

H^6), 7.67-7.74 (2H; d; AA'BB'; ^{3}J = 8.7 Hz; phenyl-H), 9.10 (1H; s; -OH), 9.84 (1H; s; -OH)

MS (EI): m/z = 466.1

HR-MS (EI): calculated: 466.2542
found: 466.2544

Analysis: calculated: C: 74.6 H: 8.2
found: C: 73.3 H: 8.3

HPLC: Gradient: 0-20 min: MeCN/H_2O 60/40 to 95/5; 20-40 min: 95/5
RT = 26.70 min; purity: 97%

(E)-2-(4-hydroxyphenyl)-7-[9-(pentylsulfonyl)non-1-enyl]benzofuran-5-ol (**28**)

Preparation from 5-methoxy-2-(4-methoxyphenyl)-7-[9-(pentylsulfonyl)non-1-enyl]-benzofuran (**22**) (50 mg, 0.1 mmol) and BBr_3 (1M in DCM, 0.5 mmol, 0.5 mL) following the demethylation method described for **24**. The product was purified by column chromatography (SiO_2; DCM/ethyl acetate 10/1, v/v). Final Purification by preparative HPLC (Stat. Phase: Nucleodur 100-5 C-18, 21 mm ec, mobile Phase: acetonitrile/H_2O 65/35)

Light yellow resin; yield: 20 mg (41%)

$C_{28}H_{36}O_5S$ (484.65)

[^{1}H] NMR (D_4-methanol):

δ [ppm]) = 0.92 (3H; t; ^{3}J = 7.0 Hz; CH$_3$-CH$_2$-) 1.28-1.61 (12H; m; CH$_3$-(CH$_2$)$_2$-; -SO$_2$-(CH$_2$)$_2$-(CH$_2$)$_4$-), 1.71-1.85 (4H; m; -CH$_2$-CH$_2$-SO$_2$-CH$_2$-CH$_2$-), 2.30-2.37 (2H; q; ^{3}J = 6.8 Hz; -CH=CH-CH$_2$-) 2.98-3.06 (4H; m (br); -CH$_2$-SO$_2$-CH$_2$-), 6.55-6.63 (1H; d; ^{3}J = 16.0 Hz -CH=CH-phenyl), 6.66-6.79 (3H; m; benzofuran-H^3, -H^4; -CH=CH-phenyl), 6.83 (1H; s; benzofuran-H^6), 6.85-6.91 (2H; d; AA'BB'; ^{3}J = 8.8 Hz; phenyl-H), 7.66-7.73 (2H; d; AA'BB'; ^{3}J = 8.8 Hz; phenyl-H)

MS (EI): m/z = 484.3

HR-MS (EI): calculated: 484.2283
found: 484.2272

HPLC: Gradient: 0-30 min: MeCN/H_2O 40/60 to 95/5; 30-40 min: 95/5
RT = 23.77 min; purity: 100%

(E)-2-(4-hydroxyphenyl)-7-[10-(pentylsulfonyl)dec-1-enyl]benzofuran-5-ol (**29**)

Preparation from 5-methoxy-2-(4-methoxyphenyl)-7-(10-(pentylsulfonyl)dec-1-enyl)-benzofuran (**23**) (40 mg, 0.08 mmol) and BBr_3 (1M in DCM, 0.4 mmol, 0.4 mL) following the demethylation method described for **24**. The product was purified by column chromatography (SiO_2; DCM/ethyl acetate 30/1, v/v)

Light yellow resin; yield: 15 mg (40%)

$C_{29}H_{38}O_5S$ (498.68)

[^{1}H] NMR (D_6-DMSO):

δ [ppm]) = 0.86 (3H; t; ^3J = 6.9 Hz; C$\underline{H}_3$-CH$_2$-), 1.23-1.55 (14H; m; CH$_3$-(C$\underline{H}_2$)$_2$-; -SO$_2$-(CH$_2$)$_2$-(C$\underline{H}_2$)$_5$-), 1.60-1.72 (4H; m; -C$\underline{H}_2$-CH$_2$-SO$_2$-CH$_2$-C$\underline{H}_2$), 2.26-2.32 (2H; q; ^3J = 6.9 Hz; -CH=CH-C$\underline{H}_2$-), 3.00-3.06 (4H; m (br), -C$\underline{H}_2$-SO$_2$-C$\underline{H}_2$-), 6.53-6.61 (1H; d; ^3J = 16.0 Hz; -C$\underline{H}$=CH-phenyl), 6.63-6.77 (3H; m; benzofuran-$\underline{H}^3$, -$\underline{H}^4$; -CH=C$\underline{H}$-phenyl), 6.85-6.91 (2H; d; AA'BB'; ^3J = 8.7 Hz; phenyl-$\underline{H}$), 7.02 (1H; s; benzofuran-$\underline{H}^6$), 7.67-7.74 (2H; d; AA'BB'; ^3J = 8.7 Hz; phenyl-$\underline{H}$), 9.11 (1H; s; -O$\underline{H}$), 9.84 (1H; s; -O$\underline{H}$)

MS (EI): m/z = 498.4

HR-MS (EI): calculated: 498.2440

found: 498.2451

HPLC: Gradient: 0-30min: MeCN/H_2O 40/60 to 95/5; 30-40 min: 95/5

RT = 25.44 min; purity: 99%

6.1.2.1.9 Introduction of a Bifunctional Side Chain in Position 7

(E)/(Z)-6-[5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]hex-5-enoic acid (**30**)

Under nitrogen atmosphere at -15 °C n-BuLi (15% in hexane, 1.63 mmol, 1.05 mL) was added to a stirred solution of HN(SiMe$_3$)$_2$ (287 mg, 1.78 mmol) in abs. THF (1mL). The mixture was allowed to warm up to room temperature and after 30 min this solution was droped into a suspension of 5-carboxypentyltriphenylphosphoniumbromide (**8**) (346 mg; 0.78 mmol) in abs. THF (3 mL) at -25 °C. The mixture was allowed to warm up slowly to -15 °C. Stirring for 1 h at this temperature resulted in a red suspension of phosphorous ylid **9**. A solution of 5-methoxy-2-(4-methoxyphenyl)benzofuran-7-carbaldehyde (**13**) (200 mg, 0.71 mmol) in abs. THF (2 mL) was added slowly to the mixture at -15 °C, while the red colour disappeared. After 1 h the reaction mixture was worked up by addition of 1N HCl solution (100 mL) followed by extraction with three portions of ethyl

acetate (3 × 50 mL). The combined organic phases were dried over Na_2SO_4 and evaporated. Pre-purification was performed by column chromatography (SiO_2; DCM /methanol 20/1, v/v). As the desired product **30** and the byproduct triphenylphosphinoxide have the same R_f value, a chromatographic separation was not achieved. The mixture was taken up in 0.5 N NaOH solution (100 mL) and stirred for 30 min. Triphenylphosphinoxide was filtered of as a yellow precipitate. The filtrate was washed with ether (30 mL) and acidified with 2N HCl solution to pH 1. The product was extracted with three portions of ethyl acetate (3 × 50 mL); the organic phase was dried over Na_2SO_4 and evaporated.

Pale yellow solid; yield: 120 mg (46 %), E/Z = 65/35 ([^{1}H] NMR)

$C_{22}H_{22}O_5$ (366.41)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 1.75-1.87 (0.74 H; q; ^{3}J = 7.5 Hz; -CH=CH-C<u>H</u>$_2$-, Z-isomer), 1.88-1.98 (1.26 H; q; ^{3}J = 7.4 Hz; -CH=CH-C<u>H</u>$_2$-; E-Isomer), 2.33-2.52 (4H; m; -(C<u>H</u>$_2$)$_2$-COOH), 3.83-3.88 (6H; m; -O-C<u>H</u>$_3$), 5.81-5.91 (0.33 H; d(t); ^3J_d = 11.6 Hz; ^3J_t = 7.3 Hz; -CH=C<u>H</u>-CH$_2$-, Z-isomer); 6.61-7.02 (6.65 H; m; benzofuran-<u>H</u>^{3}, -<u>H</u>^{4}, -<u>H</u>^{6}; AA'BB' phenyl-<u>H</u>, -CH=C<u>H</u>-CH$_2$- E-isomer (0.65 H), phenyl-C<u>H</u>=CH-CH$_2$-), 7.74-7.82 (2H; d; AA'BB'; ^{3}J = 8.9 Hz; phenyl-<u>H</u>)

MS (EI): m/z = 366.2

(E)/(Z)-6-[5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]-N-[3-(pentylthio)propyl]hex-5-enamide (**31**)

(E)/(Z)-6-[5-Methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]hex-5-enoic acid (**30**) (110 mg, 0.3 mmol), EDC (58 mg, 0.3 mmol), HOBT (48 mg, 0.3 mmol) and diisopropyl-ethylamine (39 mg, 0.3 mmol) were dissolved in DCM (5mL) and stirred for 5 min. N-Methyl-3-(pentylsulfanyl)propylamine (**7**) in DCM (0.5 mL) was added and the reaction mixture was stirred for 16 h at room temperature. DCM (30 mL) was added and the solution was washed with water (20 mL) and brine (20 mL), dried over Na_2SO_4 and evaporated. The product was purified by column chromatography (SiO_2; DCM/ethyl acetate 15/1, v/v).

Colourless oil; yield: 120 mg (78%); E/Z = 65/35 ([^{1}H] NMR)

$C_{31}H_{41}NO_4S$ (523.73)

[^{1}H] NMR ($CDCl_3$):

δ [ppm]) = 0.87 (3H; m; C<u>H</u>$_3$-CH$_2$-), 1.23-2.00 (10H; m; -(C<u>H</u>$_2$)$_3$-CH$_3$, CO-CH$_2$-C<u>H</u>$_2$-, -N-CH$_2$-C<u>H</u>$_2$-), 2.22-2.55 (8H; m; -C<u>H</u>$_2$-S-C<u>H</u>$_2$-, -C<u>H</u>$_2$-CH=CH-, -C<u>H</u>$_2$-CO-), 2.85, 2.93, 2.99 (3H; 3 × s; N-C<u>H</u>$_3$), 3.20-3.48 (2H; m; -C<u>H</u>$_2$-N), 3.85-3.87 (6H; m; -O-C<u>H</u>$_3$), 5.84-5.93 (0.35 H; d(t); ^3J_d = 11.4 Hz; ^3J_t = 7.2 Hz –CH=C<u>H</u>-CH$_2$-, Z-isomer), 6.62-7.00 (6.65 H; m; benzofuran-<u>H</u>^{3}, -<u>H</u>^{4}, -<u>H</u>^{6}; AA'BB' phenyl-<u>H</u>, phenyl-CH=C<u>H</u>-CH$_2$-, E-isomer (0.65 H), phenyl-C<u>H</u>=CH-CH$_2$-), 7.72-7.80 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-<u>H</u>).

MS (ES): m/z = 524.2 (MH^+)

HR-MS (EI): calculated: 523.2756

found: 523.2759

(E)-6-[5-Hydroxy-2-(4-hydroxyphenyl)benzofuran-7-yl]-N-[3-(pentylthio)propyl]hex-5-enamide (**32**)

Under nitrogen atmosphere at -18 °C a solution of (E)/(Z)-6-[5-methoxy-2-(4-methoxyphenyl)benzofuran-7-yl]-N-[3-(pentylthio)propyl]hex-5-enamid (**31**) (100 mg, 0.19 mmol) in abs. DCM (1 mL) was added dropwise over a period of 15 min to a stirred solution of BBr_3 (1M in DCM, 0.9 mmol, 0.9 mL) in DCM (7 mL). The reaction mixture turned dark immediately. After complete addition the reaction mixture was allowed to warm up to 10 °C and stirred for 2 h at this temperature. Then, at 0 °C, 10% $NaHCO_3$ solution was added and after dillution with water (30 mL) the mixture was extracted with three portions of ethyl acetate (3 × 50 mL). The organic phase was dried over Na_2SO_4 and evaporated. The product was purified by column chromatography (SiO_2; DCM/methanol 20/1, v/v).

Colourless oil; yield: 60 mg (64%); E/Z = 99/1 ([^{1}H] NMR)

$C_{29}H_{37}NO_4S$ (495.67)

[^{1}H] NMR (D_4-methanol):

δ [ppm]) = 0.82 (3H; m; C<u>H</u>$_3$-CH$_2$-), 1.19-1.94 (10H; m; -(C<u>H</u>$_2$)$_3$-CH$_3$, CO-CH$_2$-C<u>H</u>$_2$-, -N-CH$_2$-C<u>H</u>$_2$-), 2.26-2.54 (8H; m; -C<u>H</u>$_2$-S-C<u>H</u>$_2$-, -C<u>H</u>$_2$-CH=CH-, -C<u>H</u>$_2$-CO-), 2.88, 3.03 (3H; 2 × s; N-C<u>H</u>$_3$), 5.84-5.93 (0.01 H; d(t); ^3J_d = 11.4 Hz; ^3J_t = 7.2 Hz –CH=C<u>H</u>-CH$_2$-, Z-isomer), 6.59-6.83 (5H; m; benzofuran-<u>H</u>^{3}, -<u>H</u>^{4}, -<u>H</u>^{6}, phenyl-C<u>H</u>=C<u>H</u>-CH$_2$-, E-isomer), 6.86-6.88(2H; d; AA'BB'; ^3J = 8.7 Hz; phenyl-<u>H</u>), 7.69-7.72 (2H; d; AA'BB'; ^3J = 8.9 Hz; phenyl-<u>H</u>)

MS (ES): m/z = 495.1

HR-MS (EI): calculated: 495.2443

found: 495.2444

HPLC: Gradient: 0-25min: MeCN/H_2O 30/70 to 90/10; 25-40 min: 90/10

RT = 23.64 min; purity: 97%

(E)-2-(4-Hydroxyphenyl)-7-[6-[methyl[3-(pentylthio)propyl]amino]hex-1-enyl]benzofuran-5-ol (**33**)

Under dry nitrogen atmosphere a solution of (E)-6-[5-hydroxy-2-(4-hydroxyphenyl)-benzofuran-7-yl]-N-[3-(pentylthio)propyl]hex-5-enamide (**32**) (50 mg, 0.1 mmol) was added dropwise to a suspension of lithiumaluminiumhydride (12 mg, 0.3 mmol) in abs. THF (1 mL). The reaction mixture was stirred for 3 h at 60 °C. The excess of lithiumaluminiumhydride was decomposed by slow addition of water at 0 °C. The product was extracted with three portions of ethyl acetate (3 × 30 mL); the combined organic phases were dried over Na_2SO_4 and evaporated. The product was purified by column chromatography (SiO_2; ethyl acetate/methanol 7/1, v/v).

Yellow oil; yield: 40 mg (83 %); E:Z = 94:6 ([^{1}H] NMR)

$C_{29}H_{39}NO_3S$ (481.69)

[^{1}H] NMR (D_4-methanol):

δ [ppm]) = 0.88 (3H; t; ^3J =7.0 Hz; C$\underline{H}_3$-CH_2-), 1.22-1.41 (6H; m; -(C$\underline{H}_2$)$_2$-CH_3, -CH=CH-CH_2-C$\underline{H}_2$-), 1.45-2.81 (6H; m; -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-, -CH=CH-$(CH_2)_2$-C$\underline{H}_2$-) 2.34 (3H; s; N-C$\underline{H}_3$), 2.35-2.41 (2H; q; ^3J = 7.0 Hz; merged in N-C$\underline{H}_3$-peak; CH=CH-C$\underline{H}_2$-,), 2.40-2.50 (4H; m; -C$\underline{H}_2$-N-C$\underline{H}_2$-), 2.58, 2.60 (2 × 2H; 2 × t (merged); ^3J = 7.7 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 5.81-5.89 (0.06H; t(d); ^3J_d = 11.4 Hz; ^3J_t = 7.5 Hz; phenyl-CH=C$\underline{H}$-CH_2- Z-isomer), 6.59-6.83 (5H; m; CH (benzofuran-$\underline{H}^3$, -$\underline{H}^4$, -$\underline{H}^6$, phenyl-C$\underline{H}$=C$\underline{H}$-CH_2-, E-isomer), 6.85-6.88 (2H; d; AA'BB'; ^3J = 8.7 Hz; phenyl-$\underline{H}$), 7.69-7.72 (2H; d; AA'BB'; ^3J = 8.7 Hz; phenyl-$\underline{H}$)

MS (ES): m/z = 481.2

HR-MS (EI): calculated: 481.2651

found: 481.2637

HPLC: Gradient: 0-25min: MeCN/0.025 % TFA (aq) 20/80 to 90/10; 25-40 min: 90/10

RT = 20.14 min; purity: 97%

6.1.2.2 Synthesis of 2-Aryl-tetrahydroisoquinolin-6-ols

6.1.2.2.1 Synthesis of Side Chain Precursors

Ethyl 10-bromodecanoate (**34**)

10-bromodecanoic acid (1.0 g; 4.0 mmol) was dissolved in chloroform, ethanol (370 mg, 8.0 mmol) and conc. H_2SO_4 (20 mg) were added and the mixture was refluxed for 4 h using a distilling trap. The chloroform solution was then washed successively with water, sat. Na_2HCO_3 solution and brine. The chloroform layer was evaporated and the residue was destilled *in vacuo* (120 °C, 1 mbar).

Colourless oil; yield: 1.0 g (89%)

$C_{12}H_{23}O_2Br$ (279.21)

[^{1}H] NMR (CDCl3):

δ(ppm) = 1.23-1.46 (13H; m; -(C$\underline{H}_2$)$_5$ -, C$\underline{H}_3$), 1.61 (2H; quin; ^{3}J = 7.3 Hz; -C$\underline{H}_2$-CH$_2$-CO-), 1.79-1.89 (2H; quin; ^{3}J = 7.0 Hz; -C$\underline{H}_2$-CH$_2$-Br), 2.28 (2H; t; ^{3}J = 7.5 Hz; -C$\underline{H}_2$-CO-), 3.40 (2H; t; ^{3}J = 6.9 Hz; -C$\underline{H}_2$-Br), 4.08-4.15 (2H; q; ^{3}J = 7.1 Hz; -O-C$\underline{H}_2$-CH$_3$)

Ethyl 11-bromoundecanoate (**35**)

Preparation from 11-bromoundecanoic acid (5 g, 18.9 mmol) in the same way as described for **34**.

Colourless oil; yield: 5.2 g (94%)

$C_{13}H_{25}O_2Br$ (293.24)

[^{1}H] NMR (CDCl3):

δ(ppm) = 1.22-1.45 (15H; m; -(C$\underline{H}_2$)$_6$-, C$\underline{H}_3$), 1.60 (2H; quin; ^{3}J = 7.3 Hz; -C$\underline{H}_2$-CH$_2$-CO-), 1.79-1.89 (2H; quin; ^{3}J = 7.2 Hz; -C$\underline{H}_2$-CH$_2$-Br), 2.28 (2H; t; ^{3}J = 7.5 Hz; -C$\underline{H}_2$-CO-), 3.40 (2H; t; ^{3}J = 6.9 Hz; -C$\underline{H}_2$-Br), 4.08-4.15 (2H; q; ^{3}J = 7.1 Hz; -O-C$\underline{H}_2$-CH$_3$)

Ethyl-10-(pentylthio)decanoate (**36**)

Under nitrogen atmosphere pentanethiol (320 mg, 3.15 mmol) in dry DMF (5 mL) was added dropwise to a suspension of sodium hydride (60% suspension in paraffin; 172 mg, 4.3 mmol) in dry DMF (5 mL) and the mixture was stirred till the gas evolution ceased. Then ethyl-10-bromodecanoate (**34**) in abs. DMF (5 mL) was added slowly, and

the reaction mixture was heated to 60 °C for 2 h. Excess sodium hydride was decomposed by the addition of water and the product extracted with three portions of ethyl acetate. The organic extract was washed with water and brine and dried over Na_2SO_4. The solvent was removed *in vacuo*.

Purification by column chromatography (SiO_2; PE/ethyl acetate 50/1, v/v). Staining of the TLC plates with iodine (1% on silica gel) revealed the product as yellow spot.

Colourless oil; yield: 350 mg (40%)

$C_{17}H_{34}O_2S$ (302.52)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.89 (3H; t; ^3J = 7.0 Hz; -$(CH_2)_4$-C$\underline{H}_3$), 1.22-1.42 (17H; m; -(C$\underline{H}_2$)$_5$-$(CH_2)_2$-S-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-, -O-CH_2-C$\underline{H}_3$), 1.52-1.66 (6H; m; -C$\underline{H}_2$-CH_2-CO-, -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-), 2.27 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO), 2.49 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 4.08-4.15 (2H; q; ^3J = 7.1 Hz; O-C$\underline{H}_2$-CH_3)

Ethyl-11-(pentylthio)undecanoate (**37**)

Preparation from pentanethiol (1.95 g, 18.7 mmol), sodium hydride (60% suspension in paraffin; 612 mg, 25.5 mmol) and ethyl 11-bromoundecanoate (**35**) (5 g, 17.0 mmol) following the procedure described for **36**.

Colourless oil; yield: 2.70 g (50%)

$C_{18}H_{36}O_2S$ (316.53)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.88 (3H; t; ^3J = 7.1 Hz; -$(CH_2)_4$-C$\underline{H}_3$), 1.21-1.39 (19H; m; -(C$\underline{H}_2$)$_6$-$(CH_2)_2$-S-$(CH_2)_2$-((C$\underline{H}_2$)$_2$-, -O-CH_2-C$\underline{H}_3$), 1.51-1.65 (6H; m; -C$\underline{H}_2$-CH_2-CO-, -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-), 2.27 (2H; t; ^3J = 7.5Hz; -C$\underline{H}_2$-CO), 2.48 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 4.07-4.14 (2H; q; ^3J = 7.1 Hz; O-C$\underline{H}_2$-CH_3)

10-Pentylthiodecanoic acid (**38**)

Ethyl-10-(pentylthio)decanoate (**36**) (300 mg, 1 mmol) and KOH (112 mg, 2 mmol) were refluxed for 4 h in ethanol/water (3/1, v/v; 4 mL). The solvent was then removed *in vacuo* and the residue was dissolved in water. After acidification with 4N HCl solution the product was extracted with three portions of ether (3 × 50 mL). The organic extract

was dried over Na_2SO_4 and evaporated. The purity of the product was sufficient without further purification.

White solid; yield: 200 mg (73%)

$C_{15}H_{30}O_2S$ (274.46)

Melting point: 45-46 °C

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 7.1 Hz; -$(CH_2)_4$-C$\underline{H}_3$), 1.24-1.43 (14H; m; -(C$\underline{H}_2$)$_5$-$(CH_2)_2$-S-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-), 1.52-1.67 (6H; m; -C$\underline{H}_2$-CH_2-CO-, -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-), 2.34 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO), 2.49 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-)

11-Pentylthioundecanoic acid (**39**)

Preparation from ethyl-11-(pentylthio)undecanoate (**37**) (2.5 g, 7.9 mmol) and KOH (0.9 g, 16 mmol) following the procedure described for **38**.

White solid; yield: 2.0 g (88%)

$C_{16}H_{32}O_2S$ (288.49)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.89 (3H; t; ^3J = 7.1 Hz; -$(CH_2)_4$-C$\underline{H}_3$), 1.24-1.42 (16H; m; -(C$\underline{H}_2$)$_6$-$(CH_2)_2$-S-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-), 1.51-1.67 (6H; m; -C$\underline{H}_2$-CH_2-CO-, -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-), 2.34 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO), 2.49 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-)

10-Pentylthiodecanoic acid chloride (**40**)

Oxalyl chloride(120 mg, 1.2 mmol) was added to a solution of 10-pentylthiodecanoic acid (**38**) (220 mg, 0.8 mmol) in abs. DCM (2 mL) containing one drop of DMF. The solution was stirred for 1 h at room temperature. Solvent and excessive oxalyl chloride were removed *in vacuo* and the resulting oil was directly used for tetrahydroisoquinolin synthesis without further analysis.

Light yellow oil; yield: 230 mg (100%)

$C_{15}H_{29}OCl$ (292.91)

11-Pentylthioundecanoic acid chloride (**41**)

Preparation from oxalyl chloride (500 mg, 5.2 mmol) and 11-pentylthioundecanoic acid (**39**) (1 g, 3.5 mmol) in the same way as described for **40**.

Light yellow oil; yield: 1.03 g (96%)

$C_{16}H_{31}OCl$ (306.94)

Ethyl-11-(pentylsulfonyl)undecanoate (**42**)

Preparation from ethyl-11-(pentylthio)undecanoate (**37**) (370 mg, 1.17 mmol) and meta-chloroperbenzoic acid (70 %, 605 mg) following the procedure described for **22**. Purification by crystallisation from ethanol.

White solid; yield: 380 mg (93%)

$C_{18}H_{36}O_4S$ (348.54)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.92 (3H; t; ^3J = 7.0 Hz; -$(CH_2)_4$-C$\underline{H}_3$), 1.25-1.48 (19H; m; -(C$\underline{H}_2$)$_6$-$(CH_2)_2$-S-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-, -O-CH_2-C$\underline{H}_3$), 1.54-1.67 (2H; quin; -C$\underline{H}_2$-CH_2-CO-), 1.77-1.88(4H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-), 2.28 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO), 2.93 (4H; t; ^3J = 8.1 Hz; -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-), 4.08-4.15 (2H; q; ^3J = 7.1 Hz; O-C$\underline{H}_2$-CH_3)

11-Pentylsulfonylundecanoic acid (**43**)

Preparation from ethyl-11-(pentylsulfonyl)undecanoate (**42**) (350 mg, 1.0 mmol) and KOH (200 mg, 3.5 mmol) following the procedure described for **38**.

White solid; yield: 270 mg (84 %)

$C_{16}H_{32}O_4S$ (288.49)

[^{1}H] NMR ($CDCl_3$)

δ (ppm) = 0.92 (3H; t; ^3J = 7.0 Hz; -$(CH_2)_4$-C$\underline{H}_3$), 1.25-1.45 (16H; m; -(C$\underline{H}_2$)$_6$-$(CH_2)_2$-S-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-), 1.53-1.70 (2H; quin; ^3J = 7.2 Hz; -C$\underline{H}_2$-CH_2-CO-), 1.76-1.90 (4H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-), 2.33 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO), 2.91 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-)

11-Pentylsulfonylundecanoic acid chloride (**44**)

Preparation from oxalyl chloride (189 mg, 1.5 mmol) and 11-pentylsulfonyl-undecanoic acid (**43**) (300 mg, 0.94 mmol) following the procedure described for **40**.

Light yellow oil; yield: 290mg (100 %)

$C_{16}H_{31}O_3SCl$ (306.93)

7-Bromoheptanoic acid (**45**)

A solution of LiOH (400 mg, 16 mmol) in water (4 mL) was added to a stirred solution of etyl-7-bromoheptanoate (1.0 g, 4.22 mmol) in ethanol/THF (1/1, v/v; 8 ml) and stirred for 4 h at room temperature. Ethanol and THF were then removed *in vacuo* and the residue was diluted with water (50 mL). After washing with ether the aqueous solution was acidified with 4 N HCl solution to pH 1. The product was extracted with three portions of ethyl acetate (3 × 50 mL); the combined organic phases were dried over Na_2SO_4 and evaporated. The purity of the remaining product was sufficient for further use.

White solid; yield: 800 mg (91%)

$C_7H_{13}BrO_2$ (209.08)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.30-1.51 (4H; m; -(C$\underline{H}_2$)$_2$-(CH$_2$)$_2$-COOH), 1.60-1.70 (2H; quin, ^{3}J = 7.3 Hz; -C$\underline{H}_2$-CH$_2$-COOH), 1.81-1.91 (2H; quin; ^{3}J = 7.1 Hz; -C$\underline{H}_2$-CH$_2$-Br), 2.36 (2H; t; ^{3}J = 7.4 Hz; -C$\underline{H}_2$-COOH), 3.40 (2H; t; ^{3}J = 6.7 Hz; -C$\underline{H}_2$-Br)

7-Bromoheptanoic acid chloride (**46**)

Preparation from oxalyl chloride (476 mg, 3.75 mmol) and 7-bromoheptanoic acid (**45**) (300 mg, 0.94 mmol) following the procedure described for **40**.

Light yellow oil; yield: 550 mg (98%)

$C_7H_{12}BrOCl$ (227.53)

6.1.2.2.2 Synthesis of the N-Arylphenylethylamine Precursors

3-Benzyloxyphenylacetic acid (**47**)

Benzyl bromide (11.8 g, 69 mmol) and NaI (246 mg, 1.64 mmol) were added to a solution of 2-(3-hydroxyphenyl)acetic acid (10 g, 65.7 mmol) and KOH (9.2 g, 164 mmol) in Ethanol (250 mL). The reaction mixture was refluxed for 6 h. Then 0.5 N HCl solution (300 mL) was added and the product was extracted with three portions of ethyl acetate (300 ml, 2 × 100 mL); the combined organic phases were dried over Na_2SO_4 and evaporated. Purification by crystallisation from ethanol.

White crystals; yield: 13.4 g (84%)

Melting point: 119-120 °C (Lit: 136 °C (Rapson and Robinson, 1935))

$C_{15}H_{14}O_3$ (242.27)

[^{1}H] NMR (D_6 DMSO)

δ (ppm) = 3.36 (2H; s; -C<u>H</u>$_2$-COOH), 5.12 (2H; s; -O-C<u>H</u>$_2$-Ph), 6.82-6.95 (3H; m; phenyl-<u>H</u>), 7.21 (1H; t; ^{3}J = 8.8 Hz; phenyl-<u>H</u>), 7.32-7.50 (5H; m; phenyl-<u>H</u>)

2-[3-(benzyloxy)phenyl]-N-phenylacetamide (**49**)

Under nitrogen atmosphere at room temperature oxalyl chloride (4.76 g, 3.2 mL, 37.5 mmol) was added to a stirred solution of 3-benzyoxyphenylacetic acid (**47**) (6.06 g, 25 mmol) in DCM (50 mL) containing one drop of DMF. The mixture was stirred until the gas evolution ceased. The solvent and the excess of oxalyl chloride were removed *in vacuo*. The carboxylic acid chloride was dissolved in benzene (10 mL) and added to a stirred solution of aniline (3.5 g, 37.5 mmol) in benzene (250 mL) containing Na_2CO_3 (7.95 g, 75 mmol). The mixture was refluxed for 5 h and, after cooling to room temperature, carefully poured into a 0.5 N HCl solution (300 mL). The product was extracted with three portions of ethyl acetate (3 × 150 mL). The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated. The product was purified by crystallisation from acetonitrile.

Light yellow crystals, yield: 6.2 g (78%)

$C_{21}H_{19}NO_2$ (317.38)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) =3.71 (2H; s; -C<u>H</u>$_2$-CO), 5.08 (2H; s; -C<u>H</u>$_2$-OBn), 6.92-7.45 (14 H; phenyl-<u>H</u>)

MS (CI, NH_3): m/z = 318.1 (MH^+)

N,2-Bis(3-methoxyphenyl)acetamide (**50**)

Preparation by coupling of 3-methoxyphenylacetic acid chloride (prepared from 3 methoxyphenylacetic acid (5.0 g, 30 mmol) and oxalyl chloride (5.7 g, 45 mmol)) to meta-anisidine (5.54 g, 45 mmol). The procedure was the same as described for **49**. The product was pre-purified by column chromatography (SiO_2; PE/ethyl acetate 5/2 → 5/3, v/v). Final purification was achieved by crystallisation from PE/ethyl acetate 5/1, v/v.

White solid; yield: 7.3 g (89 %)

$C_{16}H_{16}NO_3$ (271.12)

Melting point: 75-77 °C

[^{1}H] NMR ($CDCl_3$):

δ (ppm) =3.68 (2H; s; -C$\underline{H}_2$-CO), 3.76, 3.80 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 6.61-6.66 (1 H; d(d); ^3J = 8.2 Hz; ^5J = 2.0 Hz; N-phenyl-$\underline{H}^4$), 6.83-6.93 (3H; m; 2-phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 7.12-7.19 (1H; t; ^3J = 8.1 Hz; 2-phenyl-$\underline{H}^5$), 7.22-7.93 (3H; m; N-phenyl-$\underline{H}^2$, -$\underline{H}^5$, -$\underline{H}^6$)

MS (EI): m/z = 271.1

N-Phenyl-2-[3-(benzyloxy)phenyl]ethylamine (**51**)

Under nitrogen atmosphere a solution of 2-[3-(benzyloxy)phenyl]-N-phenylacetamide (**47**) (2.0 g, 6.3 mmol) in abs. THF (6 mL) was added to a stirred suspension of $LiAlH_4$ (1.20 g, 31.5 mmol) in abs. ether (10 mL). The reaction mixture was gently refluxed for 2 h. Under ice cooling the excess of $LiAlH_4$ was hydrolysed with water (50 mL) and the product was extracted with ethyl acetate (3 × 30 mL). The organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. Purification by column chromatography (SiO_2; PE/ethyl acetate 20/1, v/v).

Yellow oil; yield: 1.55 g (81 %)

$C_{21}H_{21}NO$ (303.40)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 2.91 (2H; t; 3J = 7.0 Hz; -NH-C$\underline{H}_2$-), 3.42 (2H; t; ^3J = 7.0 Hz; phenyl-CH_2-), 5.08 (2H; s; -C$\underline{H}_2$-Ph), 6.62-6.65 (2H; d, ^3J = 8.5 Hz; N-phenyl-$\underline{H}^2$, -$\underline{H}^6$), 6.74 (1H; t; ^3J = 7.3 Hz; N-phenyl-$\underline{H}^4$), 6.85-6.90 (3H; m; 2-phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 7.18-7.48 (8H; m; phenyl-$\underline{H}$)

MS (CI, NH_3): m/z = 304.1 (MH^+)

N,2-Bis(3-methoxyphenyl)ethylamine (**52**)

Preparation from N,2-bis(3-methoxyphenyl)acetamide (**50**) (2.0 g; 7.38 mmol) and $LiAlH_4$ (0.83 g; 22 mmol) following the procedure described for **51**. Purification by column chromatography (SiO_2; PE/ethyl acetate 10/1 → 7/1, v/v).

Yellow-brownish oil; yield: 1.65 g (87 %)

$C_{16}H_{18}NO_2$ (257.14)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 2.90 (2H; t; ^3J = 7.0 Hz; -NH-C$\underline{H}_2$-), 3.40 (2H; t; ^3J = 7.0 Hz; -phenyl-C$\underline{H}_2$-CH_2-NH-), 3.78, 3.81 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 6.18 (1H; t; ^5J = 2.2 Hz; N-phenyl-H^2), 6.21-6.31 (2H; 2 × d(d) merged; ^3J = 8.1 Hz; ^5J =

2.2 Hz; N-phenyl-H^4, -H^6), 6.76-6.85 (3H; m; 2-phenyl-H^2, -H^4, -H^6), 7.06-7.12 (1H; t; ^3J = 8.1 Hz; N-phenyl-H^5), 7.21-7.28 (1H; t; ^3J = 7.6 Hz; 2-phenyl-H^5)

MS (EI): m/z = 257.2

6.1.2.2.3 Coupling of the Side Chains to the Amine Precursors

N-[3-(benzyloxy)phenylethyl]-N-phenylpropionamide (**53**)

Propionyl chloride (180 mg, 2.0 mmol) was added to a stirred mixture of N-phenyl-2-[3-(benzyloxy)phenyl]ethylamine (**51**) (400 mg, 1.3 mmol) and Na_2CO_3 (420 mg, 4.0 mmol) in abs. benzene (15 mL). The reaction mixture was refluxed for 6 h at 80 °C and, after cooling to room temperature, carefully poured into a 0.5 N HCl solution. (50 mL). The product was extracted into ethyl acetate (3 × 50 mL) and the combined extracts were washed with brine, dried over Na_2SO_4 and evaporated.

Purification by column chromatography (SiO_2; PE/ethyl acetate 5/1, v/v).

Colourless oil; yield: 400 mg (84 %)

$C_{24}H_{25}NO_2$ (359.46)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.05 (3H; t; ^3J = 7.5 Hz; -CH_2-C$\underline{H}_3$), 2.00-2.07 (2H; q; ^3J = 7.5 Hz; -C$\underline{H}_2$-CH_3) 2.86 (2H; t; ^3J = 7.9 Hz; -N-C$\underline{H}_2$-), 3.91 (2H; t; ^3J = 7.9 Hz; -phenyl-C$\underline{H}_2$-CH_2-N-), 5.02 (2H; s; -C$\underline{H}_2$-Ph), 6.76-6.84 (3H; m; 3-phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 7.05-7.08 (2H; d; ^3J = 7.0 Hz; N-phenyl-$\underline{H}^2$, -$\underline{H}^6$), 7.15-7.20 (1H; d(d); $^3J_1$ = 8.9 Hz; $^3J_2$ = 7.5 Hz; 3-phenyl-$\underline{H}^5$), 7.31-7.44 (8H; m; N-phenyl-$\underline{H}^3$, -$\underline{H}^4$, -$\underline{H}^5$, -phenyl-$\underline{H}$ [benzyloxy])

MS (CI, NH_3): m/z = 360.1 (MH^+)

N-[3-(benzyloxy)phenylethyl]-N-phenylbutyramide (**54**)

Preparation from butyryl chloride (210 mg, 2.0 mmol) and N-phenyl-2-[3-(benzyloxy)-phenyl)ethylamine (**51**) (400 mg, 1.3 mmol) following the procedure described for **53**.

Colourless oil; yield: 400 mg (84 %)

$C_{25}H_{27}NO_2$ (373.49)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.83 (3H; t; ^3J = 7.4 Hz; -CH_2-C$\underline{H}_3$), 1.54-1.66(2H; s; ^3J = 7.4 Hz; -C$\underline{H}_2$-CH_3), 1.97-2.05 (2H; q; ^3J = 7.4 Hz; -C$\underline{H}_2$-CH_2-CH_3), 2.86 (2H; t; ^3J = 7.9 Hz; -N-C$\underline{H}_2$-), 3.91 (2H; t; ^3J = 7.9 Hz; -phenyl-C$\underline{H}_2$-CH_2-N-), 5.02 (2H; s; -C$\underline{H}_2$-Ph), 6.77-6.82 (3H; m; 3-phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 7.03-7.08 (2H; d; ^3J = 6.8 Hz; N-phenyl-$\underline{H}^2$, -$\underline{H}^6$), 7.14-7.20 (1H; d(d); $^3J_1$ = 8.9 Hz; $^3J_2$ = 7.4 Hz; 3-phenyl-$\underline{H}^5$), 7.30-7.44 (8H; m; N-phenyl-$\underline{H}^3$, -$\underline{H}^4$, -$\underline{H}^5$, -phenyl-$\underline{H}$ [benzyloxy])

MS (CI, NH_3): m/z = 374.1 (MH^+)

N-[3-(benzyloxy)phenylethyl]-10-pentylthio-N-phenyldecanamide (**55**)

Preparation from 10-pentylthiodecanoic acid chloride (**40**) (230 mg, 0.8 mmol) and N-phenyl-2-[3-(benzyloxy)phenyl]ethylamine (**51**) (160 mg, 0.53 mmol) following the procedure described for **53**. Purification by column chromatography (SiO_2; PE/ethyl acetate 10/1, v/v).

Colourless resin; yield: 250 mg (85 %)

$C_{36}H_{49}NO_2S$ (559.84)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 7.1 Hz; -CH_2-C$\underline{H}_3$), 1.10-1.65 (20H; m; -(C$\underline{H}_2$)$_3$-CH_2-S-CH_2-(C$\underline{H}_2$)$_7$-), 2.00 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO), 2.46-2.52 (4H; 2 × t (merged); ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.86 (2H; t; ^3J = 7.9 Hz; -N-C$\underline{H}_2$-), 3.91 (2H; t; ^3J = 7.9 Hz; -phenyl-C$\underline{H}_2$-CH_2-N-), 5.02 (2H; s; -C$\underline{H}_2$-Ph), 6.77-6.82 (3H; m; 3-phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 7.04-7.07 (2H; d; ^3J = 8.0 Hz; N-phenyl-$\underline{H}^2$, -$\underline{H}^6$), 7.14-7.20 (1H; d(d); $^3J_1$ = 9.0 Hz; $^3J_2$ = 7.5 Hz; 3-phenyl-$\underline{H}^5$), 7.31-7.44 (8H; m; N-phenyl-$\underline{H}^3$, -$\underline{H}^4$, -$\underline{H}^5$, -phenyl-$\underline{H}$ [benzyloxy])

MS (EI): m/z = 559.3

N-[3-(benzyloxy)phenylethyl]-11-pentylthio-N-phenylundecanamide (**56**)

Preparation from 11-pentylthioundecanoic acid chloride (**41**) (1.02 g, 3.4 mmol) and N-phenyl-2-[3-(benzylox)phenyl]ethylamine (**51**) (708 mg, 2.33 mmol) following the procedure described for **53**. Purification by column chromatography (SiO_2; PE/ethyl acetate 10/1, v/v).

Colourless resin; yield: 1.00 g (80 %)

$C_{37}H_{51}NO_2S$ (573.87)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 7.1 Hz; -CH_2-C$\underline{H}_3$), 1.11-1.62 (22H; m; -($C\underline{H}_2$)$_3$-CH_2-S-CH_2-($C\underline{H}_2$)$_8$-), 2.01 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO), 2.46-2.52 (4H; 2 × t (merged); ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.86 (2H; t; ^3J = 7.9 Hz; -N-C$\underline{H}_2$-), 3.91 (2H; t; ^3J = 7.9 Hz; -phenyl-C$\underline{H}_2$-CH_2-N-), 5.02 (2H; s; -C$\underline{H}_2$-Ph), 6.76-6.82 (3H; m; 3-phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 7.02-7.08 (2H; d; ^3J = 8.0 Hz; N-phenyl-$\underline{H}^2$, -$\underline{H}^6$), 7.14-7.20 (1H; d(d); $^3J_1$ = 9.0 Hz; $^3J_2$ = 7.5 Hz; 3-phenyl-$\underline{H}^5$), 7.28-7.43 (8H; m; N-phenyl-$\underline{H}^3$, -$\underline{H}^4$, -$\underline{H}^5$, -phenyl-$\underline{H}$ [benzyloxy])

MS (CI, NH_3): m/z = 574.4 (MH^+)

N-[3-(benzyloxy)phenylethyl]-11-pentylsulfonyl-N-phenylundecanamide (**57**)

Preparation from 11-pentylsulfonylundecanoic acid chloride (**44**) (290 mg, 1.0 mmol) and N-phenyl-2-[3-(benzyloxy)phenyl]ethylamine (**51**) (200 mg, 0.7 mmol) following the procedure described for **53**. Purification by column chromatography (SiO_2; PE/ethyl acetate 3/1, v/v).

White solid; yield: 360 mg (85 %)

$C_{37}H_{51}NO_4S$ (605.35)

Melting point: 64-65 °C

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.08-1.66 (14H; m; -($C\underline{H}_2$)$_2$-$(CH)_2)_2$-SO_2-$(CH_2)_2$-($C\underline{H}_2$)$_5$-), 1.70-1.89 (4H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-), 2.00 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO), 2.80-2.99 (6H; m; ^3J = -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-, -N-C$\underline{H}_2$-), 3.90 (2H; t; ^3J = 7.8 Hz; -phenyl-C$\underline{H}_2$-CH_2-N-), 5.02 (2H; s; -C$\underline{H}_2$-Ph), 6.75-6.83 (3H; m; 3-phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 7.02-7.10 (2H; d; ^3J = 8.0 Hz; N-phenyl-$\underline{H}^2$, -$\underline{H}^6$), 7.14-7.20 (1H; d(d); $^3J_1$ = 9.0 Hz; $^3J_2$ = 7.5 Hz; 3-phenyl-$\underline{H}^5$), 7.28-7.43 (8H; m; N-phenyl-$\underline{H}^3$, -$\underline{H}^4$, -$\underline{H}^5$, -phenyl-$\underline{H}$ [benzyloxy])

MS (CI, NH_3): m/z = 606.2 (MH^+)

N-(3-Methoxyphenylethyl)-N-(3-methoxyphenyl)propionamide (**58**)

Preparation from propionyl chloride (270 mg, 2.9 mmol) and N,2-bis(3-methoxyphenyl)ethylamine (**52**) (500 mg, 1.9 mmol) following the procedure described for **53**. After work-up, the purity of the product was sufficient for further use.

Colourless oil; yield: 590 mg (97 %)

$C_{19}H_{23}NO_3$ (313.39)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.06 (3H; t; ^3J = 7.3 Hz; C$\underline{H}_3$-CH_2-), 2.00-2.14 (2H; q; 3J = 7.3 hz; CO-C$\underline{H}_2$-) 2.86 (2H; t; ^3J = 7.6 Hz; -NH-C$\underline{H}_2$-), 3.78, 3.79 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 3.89 (2H; t; ^3J = 7.0 Hz; phenyl-C$\underline{H}_2$-CH_2-NH-), 6.55 (1H; s; N-phenyl-$\underline{H}^2$), 6.62-6.80 (4H; m; N-phenyl-$\underline{H}^6$, phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 6.85-6.93 (1H; d(d); ^3J = 8.1 Hz; ^5J = 2.4 Hz; N-phenyl-$\underline{H}^4$), 7.12-7.20 (1H; t; ^3J = 8.3 Hz; phenyl-H^5), 7.27-7.33 (1H; t; merged in $CDCl_3$ peak; ^3J = 8.2 Hz; N-phenyl-$\underline{H}^5$)

N-(3-Methoxyphenylethyl)-N-(3-methoxyphenyl)-11-(pentylthio)undecanamide (**59**)

Preparation from 11-pentylthioundecanoic acid chloride (**41**) (890 mg, 2.9 mmol) and N,2-bis(3-methoxyphenyl)ethylamine (**52**) (500 mg, 1.9 mmol) following the procedure described for **53**. Purification by column chromatography (SiO_2; PE/ethyl acetate 5/1, v/v).

Colourless oil; yield: 920 mg (90 %)

$C_{32}H_{49}NO_3S$ (527.80)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.89 (3H; t; ^3J = 7.0 Hz; C$\underline{H}_3$-CH_2-), 1.12-1.43 (16 H; m; -(C$\underline{H}_2$)$_6$-$(CH_2)_2$-S-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-), 1,48-1.64 (6H; m; -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-, CO-CH_2-C$\underline{H}_2$-), 2.04 (2H; t; ^3J = 7.5 Hz; -CO-C$\underline{H}_2$-), 2.44-2.53 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.86 (2H; t; ^3J = 7.6 Hz; -N-C$\underline{H}_2$-), 3.76, 3.78 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 3.89 (2H; t; ^3J = 7.0 Hz; phenyl-C$\underline{H}_2$-CH_2-N-), 6.55 (1H; t; ^5J = 2.2 Hz; N-phenyl-H^2), 6.63-6.79 (4H; m; N-phenyl-$\underline{H}^6$, phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 6.85-6.91 (1H; d(d); ^3J = 8.1 Hz; ^5J = 2.0 Hz; N-phenyl-$\underline{H}^4$), 7.13-7.20 (1H; t; ^3J = 8.2 Hz; phenyl-H^5), 7.26-7.33 (1H; t; ^3J = 8.1 Hz; merged in $CDCl_3$ peak; N-phenyl-$\underline{H}^5$)

MS (CI, NH_3): m/z = 528.3 (MH^+)

N-(3-Methoxyphenylethyl)-N-(3-methoxyphenyl)-11-(pentylsulfonyl)undecanamide (**60**)

Preparation from 11-pentylsulfonylundecanoic acid chloride (**44**) (300 mg, 1.0 mmol) and N,2-bis(3-methoxyphenyl)ethylamine (**52**) (180 mg, 0.70 mmol) following the procedure described for **53**. Purification by column chromatography (SiO_2; PE/ethyl acetate 5/1, v/v).

Light yellow oil; yield: 320 mg (84 %)

$C_{32}H_{49}NO_5S$ (559.80)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.92 (3H; t; ^3J = 7.0 Hz; C$\underline{H}_3$-CH_2-), 1.12-1.62 (16H; m; -(C$\underline{H}_2$)$_5$-$(CH_2)_2$-SO_2-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-), 1,72-1.89 (4H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-), 2.04 (2H; t; ^3J = 7.5 Hz; -CO-C$\underline{H}_2$-), 2.81-2.97 (6H; m; -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-, -C$\underline{H}_2$-N-), 3.76, 3.78 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 3.89 (2H; t; ^3J = 7.0 Hz; phenyl-C$\underline{H}_2$-CH_2-N-), 6.55 (1H; t; ^5J = 2.0 Hz; N-phenyl-$\underline{H}^2$), 6.63-6.79 (4H; m; N-phenyl-$\underline{H}^6$, phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 6.85-6.91 (1H; d(d); ^3J = 8.1 Hz; ^5J = 2.0 Hz; N-phenyl-$\underline{H}^4$), 7.13-7.20 (1H; t; ^3J = 8.2 Hz; phenyl-H^5), 7.26-7.33 (1H; t; merged in $CDCl_3$ peak; ^3J = 8.1 Hz; N-phenyl-$\underline{H}^5$)

MS (CI, NH_3): m/z = 560.3 (MH^+)

6.1.2.2.4 Bischler Napiralski Cyclisation to the Tetrahydriisoquinolines

6-(Benzyloxy)-1-ethyl-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**61**)

N-[3-(Benzyloxy)phenylethyl]-N-phenylpropionamide (**53**) (380 mg; 1.06 mmol) was dissolved in $POCl_3$ (5 mL) and refluxed for 20 h. $POCl_3$ was destilled off *in vacuo* at 60 °C. Water (30 mL) and ether (30 mL) were added to the remaining resin and the mixture was stirred for 30 min at room temperature, until all of the material was dissolved. Then KI (830 mg; 5 mmol) was added to the water phase and the formed yellow precipitate was filtered off and dried *in vacuo*.

This dihydroisoquinolinium iodide was dissolved in methanol and $NaBH_4$ (80 mg, 2.1 mmol) was added in small portions under gas evolution. After stirring for 1 h at room temperature water was added and the product was extracted with ether (3 × 50 ml); the organic extract was dried over Na_2SO_4 and evaporated. Purification by column chromatography (SiO_2; PE/ethyl acetate 50/1, v/v).

Light yellow resin; yield: 260 mg (71 %)

$C_{24}H_{25}NO$ (343.46)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.00 (3H; t; ^3J = 7.4 Hz, C$\underline{H}_3$-CH_2-), 1.65-1.80 (1H; hept.; ^3J = 7.0 Hz; -CH-C$\underline{H}_2$-CH_3 1diastereotopic H), 1.90-2.02 (1H; hept.; ^3J = 7.0 Hz; -CH-C$\underline{H}_2$-CH_3 1diastereotopic H), 2.80-2.89 ´(1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.95-3.04 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotiopic H), 3.51-3.66 (2H; m; THIQ-$\underline{H}^3$), 4.52 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 5.05 (2H; s; -C$\underline{H}_2$-Ph), 6.70-6.89 (5H; m; N-phenyl-$\underline{H}^2$, -$\underline{H}^4$- $\underline{H}^6$, THIQ -$\underline{H}^5$, -$\underline{H}^7$), 7.03-7.06 (1H; d; ^3J = 8.2 Hz; THIQ-$\underline{H}^8$), 7.22-7.28 (2H, d(t); merged in $CDCl_3$-peak; ^3J_d = 7.3 Hz; ^5J_t = 2.0 Hz; N-phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.31-7.46 (5H; m; phenyl-$\underline{H}$)

MS (CI, NH_3): m/z = 344.1 (MH^+)

6-(Benzyloxy)-1-propyl-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**62**)

Preparation from N-[3-(benzyloxy)phenylethyl]-N-phenylbutyramide (**54**) (400 mg, 1.05 mmol) in $POCl_3$ (5 mL) followed by reduction with $NaBH_4$ (80 mg, 2.1 mmol). The procedure was the same as described for **61**.

Light yellow resin; yield: 250 mg (67 %)

$C_{25}H_{27}NO$ (357.49)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.95 (3H; t; ^3J = 7.3 Hz, C$\underline{H}_3$-CH_2-), 1.37-1.52 (2H; m; -C$\underline{H}_2$-CH_3), 1.60-1.73 (1H; hept.; ^3J = 7.0 Hz; -CH-C$\underline{H}_2$-C_2H_5 1diastereotopic H), 1.90-2.02 (1H; hept.; ^3J = 7.0 Hz; -CH-C$\underline{H}_2$- C_2H_5 1diastereotopic H), 2.78-2.87 ´(1H; m; THIQ-$\underline{H}^4$- 1 diastereotopic H), 2.94-3.04 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotiopic H), 3.57-3.62 (2H; m; THIQ-$\underline{H}^3$), 4.62 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 5.04 (2H; s; -C$\underline{H}_2$-Ph), 6.70-6.89 (5H; m; N-phenyl-$\underline{H}^2$, -$\underline{H}^4$- $\underline{H}^6$, THIQ -$\underline{H}^5$, -$\underline{H}^7$), 7.02-7.05 (1H; d; ^3J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.21-7.27 (2H, d(t); merged in $CDCl_3$-peak ; ^3J_d = 7.3 Hz; ^5J_t = 2.0 Hz; N-phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.31-7.46 (5H; m; phenyl-$\underline{H}$)

MS (CI, NH_3): m/z = 358.1 (MH^+)

6-(Benzyloxy)-1-[9-(pentylthio)nonyl]-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**63**)

N-[3-(Benzyloxy)phenylethyl]-10-pentylthio-N-phenyldecanamide (**55**) (240 mg; 0.43 mmol) was dissolved in $POCl_3$ (3 mL) and refluxed for 20 h. After evaporation of

$POCl_3$ water was added. The dihydroisoquinolinium salt was not soluble. The product was extracted into ethyl acetate (3 × 50 mL); the combined organic extracts were dried over Na_2SO_4 and evaporated. The remaining resin was directly reduced with $NaBH_4$ (42 mg, 1.1 mmol) in ethanol as described for **61**.

Colourless resin; yield: 100 mg (43 %)

$C_{36}H_{49}NOS$ (543.85)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.91 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.23-1.71 (21H; m; -(C$\underline{H}_2$)$_7$-CH_2-S-CH_2-(C$\underline{H}_2$)$_3$-, -CH-C$\underline{H}_2$- 1 diastereotopic H), 1.86-1.99 (1H; m; -CH-C$\underline{H}_2$- 1 diastereotopic H), 2.50 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.77-2.86 ´(1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.94-3.04 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotiopic H), 3.58-3.63 (2H; m; THIQ-$\underline{H}^3$), 4.59 (1H; t; ^3J = 6.9 Hz; -C$\underline{H}$-), 5.04 (2H; s; -C$\underline{H}_2$-Ph), 6.69-6.88 (5H; m; N-phenyl-$\underline{H}^2$, -$\underline{H}^4$- $\underline{H}^6$, THIQ -$\underline{H}^5$, -$\underline{H}^7$), 7.00-7.05 (1H; d; ^3J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.20-7.25 (2H, d(t); merged in $CDCl_3$-peak ; ^3J_d = 7.3 Hz; ^5J_t = 2.0 Hz; N-phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.31-7.46 (5H; m; phenyl-$\underline{H}$)

MS (CI, NH_3): m/z = 544.3 (MH^+)

6-(Benzyloxy)-1-[10-(pentylthio)decyl]-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**64**)

Preparation from N-[3-(benzyloxy)phenylethyl]-11-pentylthio-N-phenylundecanamide (**56**) (900 mg, 1.57 mmol); in $POCl_3$ (11 mL) followed by reduction with $NaBH_4$ (150 mg, 4.0 mmol). The procedure was the same as described for **63**.

Colourless resin; yield: 660 mg (75 %)

$C_{37}H_{51}NOS$ (557.87)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.91 (3H; t; ^3J = 7.1 Hz; -CH_2-C$\underline{H}_3$), 1.20-1.75 (23H; m; -(C$\underline{H}_2$)$_8$-CH_2-S-CH_2-(C$\underline{H}_2$)$_3$-, -CH-C$\underline{H}_2$- 1 diastereotopic H), 1.85-1.99 (1H; m; -CH-C$\underline{H}_2$- 1 diastereotopic H), 2.51 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.77-2.86 ´(1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.94-3.04 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotiopic H), 3.55-3.64 (2H; m; THIQ-$\underline{H}^3$), 4.59 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 5.04 (2H; s; -C$\underline{H}_2$-Ph), 6.68-6.89 (5H; m; N-phenyl-$\underline{H}^2$, -$\underline{H}^4$- $\underline{H}^6$, THIQ -$\underline{H}^5$, -$\underline{H}^7$), 7.01-7.04 (1H; d; ^3J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.21-7.26 (2H, d(t); merged in $CDCl_3$-peak ; ^3J_d = 7.3 Hz; ^5J_t = 2.0 Hz; N-phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.30-7.47 (5H; m; phenyl-$\underline{H}$)

MS (CI, NH_3): m/z = 558.2 (MH^+)

6-(Benzyloxy)-1-[10-(pentylsulfonyl)decyl]-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**65**)

Preparation from N-[3-(benzyloxy)phenylethyl]-11-pentylsulfonyl-N-phenylundecanamide (**57**) (200 mg, 0.33 mmol) in $POCl_3$ (3 ml) followed by reduction with $NaBH_4$ (31 mg; 0.83 mmol). The procedure was the same as described for **63**. The crude product was sufficiently pure without chromatographic purification.

White solid; yield: 180 mg (92 %)

$C_{37}H_{51}NO_3S$ (589.87)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.93 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.19-1.72 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-SO_2-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.73-1.98 (6H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-, -CH-C$\underline{H}_2$-), 2.51 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.77-2.87 ´(1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.88-3.05 (5H; m; THIQ-$\underline{H}^4$ 1 diastereotiopic H, -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-), 3.54-3.61 (2H; m; THIQ-$\underline{H}^3$), 4.58 (1H; t; ^3J = 6.9 Hz; -C$\underline{H}$-), 5.03 (2H; s; -C$\underline{H}_2$-Ph), 6.69-6.90 (5H; m; N-phenyl-$\underline{H}^2$, -$\underline{H}^4$- $\underline{H}^6$, THIQ -$\underline{H}^5$, -$\underline{H}^7$), 7.01-7.04 (1H; d; ^3J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.19-7.26 (2H, d(t); merged in $CDCl_3$-peak ; ^3J_d = 7.3 Hz; ^5J_t = 2.0 Hz; N-phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.30-7.47 (5H; m; phenyl-$\underline{H}$)

MS (CI, NH_3): m/z = 590.2 (MH^+)

1-Ethyl-6-methoxy-2-(3-methoxyphenyl)-1,2,3,4-tetrahydroisoquinoline (**66**)

Preparation from N-(3-methoxyphenylethyl)-N-(3-methoxyphenyl)propionamide (**58**) (500 mg, 1.6 mmol) in $POCl_3$ (12 mL) followed by reduction with $NaBH_4$ (150 mg, 4.0 mmol). The procedure was the same as described for **63**. Purification by column chromatography (SiO_2; PE/ethyl acetate 20/1, v/v).

Light yellow resin; yield: 310 mg (65%)

$C_{19}H_{23}NO_2$ (297.17)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.98 (3H; t; ^3J = 7.4 Hz; C$\underline{H}_3$-CH_2-), 1.62-1.77 (1H; m; -C$\underline{H}_2$-CH- 1 diastereotopic H), 1.87-2.02 (1H; m; -C$\underline{H}_2$-CH- 1 diastereotopic H), 2.79-2.91 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.93-3.05 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.46-3.60 (2H; m, THIQ-$\underline{H}^3$), 3.79, 3.80 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 4.49 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 6.25-6.33 (1H; d; ^3J

= 7.9 Hz; phenyl-H^4), 6.41 (1H; s; phenyl-H^2); 6.43-6.53 (1H; d; ^{3}J = 8.3 Hz; phenyl-H^6), 6.66-6.76 (2H; m; THIQ-H^5, -H^7), 6.98-7.06 (1H; d; ^{3}J = 8.3 Hz; THIQ-H^8), 7.11-7.20 (1H; t; ^{3}J = 8.2 Hz; phenyl-H^5)

MS (CI, NH_3): m/z = 298.1 (MH^+)

6-Methoxy-2-(3-methoxyphenyl)-1-[10-(pentylthio)decyl]-1,2,3,4-tetrahydroisoquinoline (**67**)

Preparation from N-(3-methoxyphenylethyl)-N-(3-methoxyphenyl)-11-(pentylthio)-undecanamide (**59**) (400 mg, 0.76 mmol) in $POCl_3$ (6 mL) followed by reduction with $NaBH_4$ (75 mg, 2.0 mmol). The procedure was the same as described for **63**. Purification by column chromatography (SiO_2; PE/ethyl acetate 30/1, v/v).

Colourless oil; yield: 330 mg (85%)

$C_{32}H_{49}NO_2S$ (511.80)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H, t; ^{3}J = 7.0 Hz; CH$_3$-CH$_2$-), 1.20-1.47 (18H; m; -(CH$_2$)$_7$-(CH$_2$)$_2$-S-(CH$_2$)$_2$-(CH$_2$)$_2$-), 1.50-1.71 (6H; m; -CH-CH$_2$-, -CH$_2$-CH$_2$-S-CH$_2$-CH$_2$-), 2.50 (4H; t; ^{3}J = 7.4 Hz; -CH$_2$-S-CH$_2$-); 2.77-2.89 (1H; m; THIQ-H^4 1 diastereotopic H), 2.92-3.05 (1H; m; THIQ-H^4 1 diastereotopic H), 3.47-3.63 (2H; m, THIQ-H^3), 3.78, 3.79 (2 × 3H; 2 × s; -O-CH$_3$), 4.57 (1H; t; ^{3}J = 7.0 Hz; -CH-), 6.26-6.31 (1H; d(d); ^{3}J = 8.0 Hz; ^{5}J = 2.1 Hz; phenyl-H^4), 6.40 (1H; t; ^{5}J = 2.3 Hz; phenyl-H^2), 6.45-6.51 (1H; d(d); ^{3}J = 8.2 Hz; ^{5}J = 2.2 Hz; phenyl-H^6), 6.65-6.74 (2H; m; THIQ-H^5, -H^7), 6.97-7.04 (1H; d; ^{3}J = 8.3 Hz; THIQ-H^8), 7.10-7.18 (1H; t; ^{3}J = 8.2 Hz; phenyl-H^5)

MS (CI, NH_3): m/z = 512.3 (MH^+)

6-Methoxy-2-(3-methoxyphenyl)-1-[10-(pentylsulfonyl)decyl]-1,2,3,4-tetrahydroiso-quinoline (**68**)

Preparation from N-(3-methoxyphenylethyl)-N-(3-methoxyphenyl)-11-(pentylsulfonyl)-undecanamide (**60**) (300 mg, 0.54 mmol) in $POCl_3$ (5 mL) followed by reduction with $NaBH_4$ (50 mg, 1.3 mmol). The procedure was the same as described for **63**. Purification by column chromatography (SiO_2; PE/ethyl acetate 5/1, v/v).

Yellow oil; yield: 200 mg (68%)

$C_{32}H_{49}NO_4S$ (543.80)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.92 (3H, t; ^3J = 7.0 Hz; C$\underline{H}_3$-CH_2-), 1.20-1.71 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-SO_2-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.75-1.98 (6H; m; -CH-C$\underline{H}_2$-, -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-), 2.77-2.88 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.89-3.04 (5H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H, -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-), 3.48-3.64 (2H; m, THIQ-$\underline{H}^3$), 3.78, 3.79 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 4.56 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 6.25-6.31 (1H; d(d); ^3J = 8.0 Hz; ^5J = 2.3 Hz; phenyl-$\underline{H}^4$), 6.39 (1H; t; ^5J = 2.3 Hz; phenyl-$\underline{H}^2$), 6.45-6.51 (1H; d(d); ^3J = 8.3 Hz; ^5J = 2.3 Hz; phenyl-$\underline{H}^6$), 6.65-6.74 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.98-7.03 (1H; d; ^3J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.10-7.17 (1H; t; ^3J = 8.2 Hz; phenyl-$\underline{H}^5$)

MS (CI, NH_3): m/z = 544.2 (MH^+)

N-[3-(benzyloxy)phenylethyl]-7-bromo-N-phenylheptanamide (**69**)

Preparation from N-phenyl-2-(3-benzyloxyphenyl)ethylamine (**51**) (500 mg, 1.7 mmol) and 7-bromoheptanoic acid chloride (550 mg, 2.5 mmol) following the procedure described for **53**. Purification by column chromatography (SiO_2; PE/ethyl acetate 5/1, v/v)

Light yellow oil; yield: 730 mg (89 %)

$C_{28}H_{32}BrNO_2$ (494.46)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.16-1.39 (4H; m; -(C$\underline{H}_2$)$_2$-(CH_2)$_2$-Br), 1.50-1.63 (2H, quin; ^3J = 7.5 Hz; -C$\underline{H}_2$-CH_2-CON-), 1.74-1.84 (2H; quin; ^3J = 7.2 Hz; Br-CH_2-C$\underline{H}_2$-), 2.02 (2H; t; ^3J = 6.8 Hz; -C$\underline{H}_2$-CON-), 2.86 (2H; t; ^3J = 7.9 Hz, N-C$\underline{H}_2$-), 3.35 (2H; t; ^3J = 6.8 Hz; -C$\underline{H}_2$-Br), 3.91 (2H; t; ^3J = 7.9 Hz; phenyl-C$\underline{H}_2$-), 5.02 (2H; s; -C$\underline{H}_2$-Ph), 6.76-6.83 (3H; m; phenyl-$\underline{H}^2$, $\underline{H}^4$, -$\underline{H}^6$), 7.04-7.07 (2H; d; ^3J = 6.7 Hz; N-phenyl-$\underline{H}^4$), 7.15-7.20 (1H; d(d); $^3J_1$ = 8.9 Hz, $^3J_2$ = 7.5 Hz; phenyl-H^5), 7.31-7.43 (8H; m; phenyl-H)

7-Bromo-N-(3-methoxyphenethyl)-N-(3-methoxyphenyl)heptanamide (**70**)

Preparation from N,2-bis(3-methoxyphenyl)ethylamine (**52**) (500 mg, 1.9 mmol) and 7-bromoheptanoic acid chloride (560 mg, 2.9 mmol) following the procedure described for **53**. Purification by column chromatography (SiO_2; PE/ethyl acetate 5/2, v/v)

Light yellow oil; yield: 800 mg (92 %)

$C_{23}H_{30}BrNO_3$ (448.39)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.10-1.48 (4H; m; -(C$\underline{H}_2$)$_2$-(CH$_2$)$_2$-Br), 1.50-1.65 (2H, quin; ^{3}J = 7.5 Hz; -C$\underline{H}_2$-CH$_2$-CON-), 1.73-1.85 (2H; quin; ^{3}J = 7.3 Hz; Br-CH$_2$-C$\underline{H}_2$-), 2.05 (2H; t; ^{3}J = 7.4 Hz; -C$\underline{H}_2$-CON-), 2.86 (2H; t; ^{3}J = 7.8 Hz, N-C$\underline{H}_2$-), 3.36 (2H; t; ^{3}J = 6.9 Hz; -C$\underline{H}_2$-Br), 3.77, 3.79 (2 × 3H; 2 × s; O-C$\underline{H}_3$), 3.90 (2H; t; ^{3}J = 7.9 Hz; phenyl-C$\underline{H}_2$-), 6.53-6.57 (1H; t; ^{5}J = 2.0 Hz; N-phenyl-$\underline{H}^2$), 6.63-6.79 (4H; m; N-phenyl-$\underline{H}^6$, phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 6.86-6.92 (1H; d(d); ^{3}J = 8.1 Hz; ^{5}J = 2.4 Hz; N-phenyl-$\underline{H}^4$), 7.13-7.21 (1H; t; ^{3}J = 8.2 Hz; phenyl-$\underline{H}^5$), 7.26-7.34 (1H; t; merged in $CDCl_3$-peak; ^{3}J = 8.1 Hz; N-phenyl-$\underline{H}^5$)

MS (CI, NH_3): m/z = 448.2 (100% ; MH^+), 450.2 (91%, MH+)

6-Benzyloxy-1-(6-chlorohexyl)-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**71a**) and *6-Benzyloxy-1-(6-bromohexyl)-2-phenyl-1,2,3,4-tetrahydroisoquinoline* (**71b**)

Preparation from N-(3-(benzyloxy)phenylethyl)-7-bromo-N-phenylheptanamide (**69**) (400 mg, 0.81 mmol) in $POCl_3$ (7 mL) followed by reduction with $NaBH_4$ (75 mg, 2.0 mmol). The procedure was the same as described for **63**. Purification by column chromatography (SiO_2; PE/ethyl acetate 25/1, v/v). The NMR-spectrum revealed the product as a mixture of **71a** (92 %) and **71b** (8%) which was used for nucleophilic substitution with appropriate amines.

Light yellow oil; yield: 300 mg (**71a**: 80%; **71b**: 7%)

71a: $C_{28}H_{32}ClNO$ (434.01)

71b: $C_{28}H_{32}BrNO$ (478.47)

[^{1}H] NMR ($CDCl_3$) (**71a**):

δ (ppm) = 1.32-1.51 (6H; m; -(C$\underline{H}_2$)$_3$-(CH$_2$)$_2$-Cl), 1.62-1.81 (3H; m; -C$\underline{H}_2$-CH$_2$-Cl, -CH-C$\underline{H}_2$- 1 diastereotopic H), 1.85-2.00 (1H; m; CH-C$\underline{H}_2$- 1 diastereotopic H), 2.77-2.86 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.94-3.04 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.39 (0.16H; 8%; t; ^{3}J = 6.8 Hz; -C$\underline{H}_2$-Br), 3.51 (1.84H; 92% t; ^{3}J = 6.7 Hz; -C$\underline{H}_2$-Cl), 3.55-3.61 (2H; m; THIQ-$\underline{H}^3$), 4.59 (1H; t; ^{3}J = 7.0 Hz; -C$\underline{H}$-), 5.04 (2H; s; -C$\underline{H}_2$-Ph), 6.70-6.88 (5H; m; N-phenyl-$\underline{H}^1$, -$\underline{H}^4$, -$\underline{H}^6$, phenyl-$\underline{H}^5$, -$\underline{H}^7$), 7.00-7.05 (1H; d; ^{3}J = 8.3 Hz; phenyl-$\underline{H}^8$), 7.21-7.27 (2H; d(d); merged in $DCCl_3$ peak; $^3J_1$ = 8.7 Hz; $^3J_2$ = 7.3 Hz; N-phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.30-7.46 (5H; m; phenyl-$\underline{H}$)

MS (EI): m/z = 434.2 (100% ; **71a**), 478.2 (2%, **71b**)

1-(6-Chlorohexyl)-6-methoxy-2-(3-methoxyphenyl)-1,2,3,4-tetrahydroisoquinoline (**72a**) and

1-(6-Bromohexyl)-6-methoxy-2-(3-methoxyphenyl)-1,2,3,4-tetrahydroisoquinoline (**72b**)

Preparation from 7-bromo-N-(3-methoxyphenethyl)-N-(3-methoxyphenyl)heptanamide (**70**) (400 mg, 0.90 mmol) in $POCl_3$ (7 mL) followed by reduction with $NaBH_4$ (90 mg, 2.4 mmol). The procedure was the same as described for **63**. Purification by column chromatography (SiO_2; PE/ethyl acetate 20/1, v/v). The NMR-spectrum revealed the product as a mixture of **72a** (85 %) and **72b** (15%). This mixture was directly used for nucleophilic substitution with appropriate amines.

Light yellow oil; yield: 340 mg (**72a**: 82%; **72b**: 14%)

72a: $C_{23}H_{30}ClNO_2$ (387.94)

72b: $C_{23}H_{30}BrNO_2$ (432.39)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.28-1.51 (6H; m; -(C$\underline{H}_2$)$_3$-(CH$_2$)$_2$-Cl), 1.58-1.81 (3H; m; -C$\underline{H}_2$-CH$_2$-Cl, -CH-C$\underline{H}_2$- 1 diastereotopic H), 1.84-2.00 (1H; m; CH-C$\underline{H}_2$- 1 diastereotopic H), 2.76-2.89 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.92-3.04 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.38 (0.31 H; 15%; t; ^{3}J = 6.8 Hz; -C$\underline{H}_2$-Br), 3.51 (1.69H; m; –C$\underline{H}_2$-Cl), 3.53-3.60 (2H; m; THIQ-$\underline{H}^3$), 3.78, 3.79 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 4.57 (1H; t; ^{3}J = 7.0 Hz; -C$\underline{H}$-), 6.25-6.33 (1H; d; ^{3}J = 7.9 Hz; phenyl-$\underline{H}^4$), 6.39 (1H; s; phenyl-$\underline{H}^2$), 6.45-6.52 (1H; d; ^{3}J = 8.1 Hz; phenyl-$\underline{H}^6$), 6.65-6.75 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.97-7.03 (1H; d; ^{3}J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.11-7.19 (1H; t; ^{3}J = 8.2 Hz; phenyl-$\underline{H}^5$)

MS (CI, NH_3): m/z = 388.2 (100% ; **72a**, MH^+), 432.2, 434.1 (7%, **72b**, MH^+)

6.1.2.2.5 Introduction of the Amine Functions

6-Benzyloxy-2-phenyl-1-[6-(pyrrolidin-1yl)hexyl]-1,2,3,4-tetrahydroisoquinoline (**73**)

Pyrrolidine (71 mg, 1.0 mmol) was added to the mixture of 6-benzyloxy-1-(6-chlorohexyl)-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**71a**, 92 %) and 6-benzyloxy-1-(6-bromohexyl)-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**71b**, 8%) (100 mg ; 0.23 mmol) in abs. Ethanol (2 mL). The reaction mixture was stirred for 72 h at 70 °C. After cooling to room temperature the mixture was poured into sat. NaHCO3 solution (50 mL) and the product was extracted with ethyl acetate (3 × 30 mL). The combined organic phases

were dried over Na_2SO_4 and evaporated. The product was purified by column chromatography.

Yellow oil; yield: 80 mg (75%)

$C_{32}H_{40}N_2O$ (468.67)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.27-1.72 (8H; m; -(C$\underline{H}_2$)$_4$-(CH$_2$)$_2$-pyrrolidine), 1.73-1.82 (4H; m; -pyrrolidine-$\underline{H}^3$, -$\underline{H}^4$,), 1.85-2.00 (2H; m; -C$\underline{H}_2$-CH$_2$-pyrrolidine), 2.37-2.42 (2H; t; ^{3}J = 7.6 Hz; -C$\underline{H}_2$-pyrrolidine), 2.44-2.52 (4H; m; pyrrolidine-H^2, -H^5), 2.77-2.86 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.93-3.03 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.51-3.64 (2H; m; THIQ-$\underline{H}^3$), 4.58 (1H; t; ^{3}J = 7.0 Hz; -C$\underline{H}$-), 5.03 (2H; s; -C$\underline{H}_2$-Ph), 6.68-6.88 (5H; m; N-phenyl-$\underline{H}^1$, -$\underline{H}^4$, -$\underline{H}^6$, phenyl-$\underline{H}^5$, -$\underline{H}^7$), 6.99-7.04 (1H; d; ^{3}J = 8.3 Hz; phenyl-$\underline{H}^8$), 7.19-7.26 (2H; d(d); merged in $DCCl_3$ peak; $^3J_1$ = 8.6 Hz; $^3J_2$ = 7.3 Hz; N-phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.29-7.46 (5H; m; phenyl-$\underline{H}$)

MS (CI, NH_3): m/z = 469.3 (MH+)

6-Methoxy-2-(3-methoxyphenyl)-1-(6-pyrrolidin-1-yl)hexyl-1,2,3,4-tetrahydroisoquinoline (**74**)

Preparation from the mixture of 1-(6-chlorohexyl)-6-methoxy-2-(3-methoxyphenyl)-1,2,3,4-tetrahydroisoquinoline (**72a**) and 1-(6-Bromohexyl)-6-methoxy-2-(3-methoxy-phenyl)-1,2,3,4-tetrahydroisoquinoline (**72b**) (200 mg, 0.51 mmol) and pyrrolidine (106 mg, 1.5 mmol) following the procedure described for **73**. Purification by column chromatography (SiO_2 ; DCM/methanol/TEA 80/19/1, v/v/v).

Yellow oil; yield: 105 mg (49 %)

$C_{27}H_{38}N_2O_2$ (422.29)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.23-1.72 (9H; m; -(C$\underline{H}_2$)$_4$-CH$_2$-pyrrolidine, -CH-C$\underline{H}_2$- 1 diastereotopic H), 1.78-1.95 (5H; m; -pyrrolidine-$\underline{H}^3$, -$\underline{H}^4$, -CH-C$\underline{H}_2$- 1 diastereotopic H), 2.44-2.53 (2H; t; ^{3}J = 7.9 Hz; -C$\underline{H}_2$-pyrrolidine), 2.55-2.65 (4H; m; pyrrolidine-$\underline{H}^2$, -$\underline{H}^5$), 2.76-2.87 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.91-3.04 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.47-3.63 (2H; m; THIQ-$\underline{H}^3$), 3.78, 3.79 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 4.56 (1H; t; ^{3}J = 7.0 Hz; -C$\underline{H}$-), 6.25-6.31 (1H; d(d); ^{3}J = 8.0 Hz; ^{5}J = 2.1 Hz; phenyl-$\underline{H}^4$), 6.39 (1H; t; ^{5}J = 2.3 Hz; phenyl-$\underline{H}^2$), 6.44-6.50 (1H; d(d); ^{3}J = 8.3 Hz; ^{5}J =

2.3 Hz; phenyl-H^6), 6.65-6.74 (2H; m; THIQ-H^5, -H^7), 6.97-7.02 (1H; d; ^{3}J = 8.3 Hz; THIQ-H^8), 7.10-7.17 (1H; t; ^{3}J = 8.2 Hz; phenyl-H^5)

MS (CI, NH_3): m/z = 423.2 (MH^+)

6-Methoxy-2-(3-methoxyphenyl)-1-(6-piperidin-1-yl)hexyl-1,2,3,4-tetrahydroisoquinoline (**75**)

Preparation from the mixture of 1-(6-chlorohexyl)-6-methoxy-2-(3-methoxyphenyl)-1,2,3,4-tetrahydroisoquinoline (**72a**) and 1-(6-bromohexyl)-6-methoxy-2-(3-methoxyphenyl)-1,2,3,4-tetrahydroisoquinoline (**72b**) (200 mg; 0.51 mmol) and piperidine (128 mg; 1.5 mmol) in abs. ethanol (5mL). The reaction mixture was heated for 10 min in a microwave (Biotage, Grenzach-Wyhlen, Germany)) (T = 180 °C; p = 18 bar). Work-up in the same way as described for **73**. Purification by column chromatography (SiO_2; DCM/methanol/TEA 90/9/1, v/v/v).

Yellow oil; yield: 200 mg (88%)

$C_{28}H_{40}N_2O_2$ (436.63)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.20-1.70 (14H; m; -(CH$_2$)$_4$-CH$_2$-piperidine, -CH-CH$_2$- 1 diastereotopic H, piperidine-H^3, -H^4, -H^5), 1.83-2.00 (1H; m; -CH-CH$_2$- 1 diastereotopic H), 2.26-2.35 (2H; t; ^{3}J = 7.8 Hz; -CH$_2$-piperidine), 2.36-2.49 (4H; s (br); piperidine-H^2, -H^6), 2.76-2.89 (1H; m; THIQ-H^4 1 diastereotopic H), 2.91-3.04 (1H; m; THIQ-H^4 1 diastereotopic H), 3.47-3.59 (2H; m; THIQ-H^3), 3.78, 3.79 (2 × 3H; 2 × s; -O-CH$_3$), 4.56 (1H; t; ^{3}J = 7.0 Hz; -CH-), 6.25-6.30 (1H; d(d); ^{3}J = 8.0 Hz; ^{5}J = 2.1 Hz; phenyl-H^4), 6.39 (1H; t; ^{5}J = 2.3 Hz; phenyl-H^2), 6.44-6.50 (1H; d(d); ^{3}J = 8.3 Hz; ^{5}J = 2.3 Hz; phenyl-H^6), 6.64-6.74 (2H; m; THIQ-H^5, -H^7), 6.96-7.02 (1H; d; ^{3}J = 8.3 Hz; THIQ-H^8), 7.10-7.17 (1H; t; ^{3}J = 8.2 Hz; phenyl-H^5)

MS (CI, NH_3): m/z = 437.3 (MH^+)

6-(6-Benzyloxy-2-phenyl-1,2,3,4-tetrahydroisoquinolin-1-yl)-N-methyl-N-[3-(pentylthio)propyl]hexan-1-amine (**76**)

Preparation from the mixture of 6-benzyloxy-1-(6-chlorohexyl)-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**71a**, 92 %) and 6-benzyloxy-1-(6-bromohexyl)-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**71b**, 8%) (150 mg ; 0.34 mmol), N-Methyl-3-(pentylthio)-propylamine (**7**) (90 mg, 0.5 mmol) and TEA (50 mg, 0.5 mmol) following the procedure

described for **73**. The reaction time was 7 days. Purification by column chromatography (SiO_2; DCM/methanol 25/1, v/v).

Brown-yellowish oil; yield: 150 mg (84 %)

$C_{37}H_{52}N_2OS$(572.89)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 1.30-1.50 (15H; m; -(C$\underline{H}_2$)$_4$-(CH_2)-N-, -(C$\underline{H}_2$)$_3$-CH_3, CH-C$\underline{H}_2$- 1 diastereotopic H), 1.73-1.83 (2H; quin; ^3J = 7.3 Hz; -N-CH_2-C$\underline{H}_2$-CH_2-S-) 1.86-2.00 (1H; m; -CH-C$\underline{H}_2$- 1 diastereotopic H), 2.24 (3H; s; -N-C$\underline{H}_3$), 2.35 (2H; t; ^3J = 7.5 Hz; -CH_2)$_5$-C$\underline{H}_2$-N), 2.43-2.55 (6H; m; -S-(CH_2)$_2$-C$\underline{H}_2$-N-, -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.76-2.87 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.93-3.03 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.55-3.60 (2H; m; THIQ-$\underline{H}^3$), 4.58 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 5.03 (2H; s; -C$\underline{H}_2$-Ph), 6.68-6.86 (5H; m; N-phenyl-$\underline{H}^1$, -$\underline{H}^4$, -$\underline{H}^6$, phenyl-$\underline{H}^5$, -$\underline{H}^7$), 6.99-7.04 (1H; d; ^3J = 8.3 Hz; phenyl-$\underline{H}^8$), 7.20-7.26 (2H; d(d); merged in $DCCl_3$ peak; $^3J_1$ = 8.6 Hz; $^3J_2$ = 7.3 Hz; N-phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.29-7.45 (5H; m; phenyl-$\underline{H}$)

MS (CI, NH_3): m/z = 573.5 (MH^+)

6.1.2.2.6 Alternative Route: Introduction of the Amine Function Prior to Cyclisation

N-(3-Methoxyphenylethyl)-N-(3-methoxyphenyl)-7-[methyl[3-(pentylthio)propyl]amino]-heptanamide (**77**)

Preparation from 7-bromo-N-(3-methoxyphenethyl)-N-(3-methoxyphenyl)heptanamide (**70**) (400 mg, 0.9 mmol), N-methyl-3-(pentylthio)propylamine (**7**) (160 mg, 0.9 mmol) and TEA (90 mg; 0.9 mmol) following the procedure described for **73**. Purification by column chromatography (DCM/methanol 20/1 → 10/1, v/v).

Light yellow oil; yield: 370 mg (76%)

$C_{32}H_{50}N_2O_3S$ (542.82)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 7.0 Hz; C$\underline{H}_3$-CH_2-), 1.16-1.41 (8H; m; -(C$\underline{H}_2$)$_2$-CH_3, CO-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.47-1.64 (6H;m; -S-CH_2-C$\underline{H}_2$-C_3H_7, CO-CH_2-C$\underline{H}_2$-, -C$\underline{H}_2$-CH_2-N-), 1.80-1.94 (2H; quin; ^3J = 7.0 Hz; -N-CH_2-C$\underline{H}_2$-CH_2-S-), 2.04 (2H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-CON-), 2.31-2.42 (3H; s (br); -N-C$\underline{H}_3$),

2.44-2.71 (8H; m; -C$\underline{H}_2$-S-C$\underline{H}_2$-, -C$\underline{H}_2$-N-C$\underline{H}_2$-) 2.85 (2H; t; ^{3}J = 7.8 Hz, phenyl-N-C$\underline{H}_2$-), 3.76, 3.79 (2 × 3H; 2 × s; O-C$\underline{H}_3$), 3.89 (2H; t; ^{3}J = 7.9 Hz; phenyl-C$\underline{H}_2$-), 6.55 (1H; t; ^{5}J = 2.0 Hz; N-phenyl-$\underline{H}^2$), 6.63-6.79 (4H; m; N-phenyl-$\underline{H}^6$, phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 6.86-6.92 (1H; d(d); ^{3}J = 8.3 Hz; ^{5}J = 2.4 Hz; N-phenyl-$\underline{H}^4$), 7.13-7.21 (1H; t; ^{3}J = 8.2 Hz; phenyl-$\underline{H}^5$), 7.26-7.33 (1H; t; merged in $CDCl_3$-peak; ^{3}J = 8.1 Hz; N-phenyl-$\underline{H}^5$)

MS (ES): m/z = 543.5 (MH^+)

6-[6-methoxy-2-(3-methoxyphenyl)-1,2,3,4-tetrahydroisoquinolin-1-yl]-N-methyl-N-[3-(pentylthio)propyl]hexan-1-amine (**78**)

Preparation from N-(3-methoxyphenylethyl)-N-(3-methoxyphenyl)-7-[methyl[3-(pentylthio)propyl]amino]heptanamide (**77**) (250 mg, 0.46 mmol) in $POCl_3$ (4 mL) followed by reduction with $NaBH_4$ (75 mg, 2.0 mmol). The procedure was the same as described for **63**. Purification by column chromatography (SiO_2; DCM/MeOH 20/1 v/v).

Light yellow oil; yield: 200 mg (83%)

$C_{32}H_{50}N_2O_2S$ (526.82)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.89 (3H; t; ^{3}J = 7.0 Hz; C$\underline{H}_3$-CH_2-), 1.20-1.72 (15H; m; -(C$\underline{H}_2$)$_3$-CH_3, N-(CH_2)-(C$\underline{H}_2$)$_4$-, -CH-C$\underline{H}_2$- 1 diastereotopic H), 1.73-1.98 (3H; m; -S-CH_2-C$\underline{H}_2$-CH_2-N-, -CH-C$\underline{H}_2$- 1 diastereotopic H), 2.27 (3H; s; N-C$\underline{H}_3$), 2.33-2.43 (2H; t; ^{3}J = 7.5 Hz; N-C$\underline{H}_2$-), 2.45-2.57 (6H; m; N-C$\underline{H}_2$-; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.76-2.88 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.91-3.04 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.49-3.64 (2H; m; THIQ-$\underline{H}^3$), 3.78, 3.79 (2 × 3H; 2 × s; -O-C$\underline{H}_3$), 4.56 (1H; t; ^{3}J = 7.0 Hz; -C$\underline{H}$-), 6.25-6.31 (1H; d(d); ^{3}J = 7.9 Hz; ^{5}J = 2.1 Hz; phenyl-$\underline{H}^4$), 6.39 (1H; t; ^{5}J = 2.3 Hz; phenyl-$\underline{H}^2$), 6.45-6.50 (1H; d(d); ^{3}J = 8.3 Hz; ^{5}J = 2.3 Hz; phenyl-$\underline{H}^6$), 6.64-6.74 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.97-7.03 (1H; d; ^{3}J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.10-7.17 (1H; t; ^{3}J = 8.2 Hz; phenyl-$\underline{H}^5$)

MS (ES): m/z = 527.4 (MH^+)

6.1.2.2.7 Cleavage of the Benzyloxy Protecting Group

1-Ethyl-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**79**)

Concentrated HCl (3 mL) was added to a stirred solution of 6-(benzyloxy)-1-ethyl-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**61**) (200 mg, 0.58 mmol) in dioxane (4 mL) and the reaction mixture was heated for 4 h to 95 °C. After cooling to room temperature the mixture was neutralised carefully with sat. $NaHCO_3$ solution and diluted with water (25 mL). The product was extracted with ethyl acetate (3 × 30 mL); the combined organic extracts were washed with brine, dried over Na_2SO_4 and evaporated. The product was purified by column chromatography (SiO_2; PE/ethyl acetate 5/1, v/v).

Yellow resin; yield: 110 mg (75%)

$C_{17}H_{19}NO$ (253.34)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.99 (3H; t; ^3J = 7.4 Hz; C$\underline{H}_3$-CH_2-), 1.62-1.77 (1H; m; CH_3-C$\underline{H}_2$-CH- 1 diastereotopic H), 1.88-2.03 (1H; m; CH_3-C$\underline{H}_2$-CH- 1 diastereotopic H), 2.76-2.85 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.90-3.00 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.48-3.63 (2H; m; THIQ-$\underline{H}^3$), 4.50 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 6.60-7.67 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.72 (1H; t; ^3J = 7.2 Hz; phenyl-$\underline{H}^4$), 6.85-6.88 (2H; d; ^3J = 8.0 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$), 6.96-6.99 (1H; d; ^3J = 8.0 Hz; THIQ-$\underline{H}^8$), 7.22-7.27 (2H; d(d); merged in $CDCl_3$-peak; ^3J = 7.3 Hz; ^5J = 2.0 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$)

MS (EI): m/z = 224.1 (100%, $[M - C_2H_5]^+$), 253.1 (5%)

HPLC: Gradient: 0-25 min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 25-40 min: 95/5

RT = 16.18 min; purity: 91%

1-Propyl-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**80**)

Preparation from 6-(benzyloxy)-1-propyl-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**62**) (200 mg, 0.56 mmol) in dioxane (4mL) / conc. HCl (3 mL) following the procedure described for **79**.

Yellow resin; yield: 120 mg (80%)

$C_{18}H_{21}NO$ (267.37)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.94 (3H; t; ^3J = 7.4 Hz; C$\underline{H}_3$-CH_2-), 1.36-1.54 (2H; m; CH_3-C$\underline{H}_2$-), 1.58-1.70 (1H; m; CH_3-C$\underline{H}_2$-CH- 1 diastereotopic H), 1.85-1.97 (1H; m; CH_3-C$\underline{H}_2$-CH- 1 diastereotopic H), 2.75-2.84 (1H; m; THIQ-$\underline{H}^3$ 1 diastereotopic H), 2.90-3.00 (1H; m; THIQ-$\underline{H}^3$ 1 diastereotopic H), 3.46-3.64 (2H; m; THIQ-$\underline{H}^4$), 4.60 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 6.60-6.65 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.72 (1H; t; ^3J = 7.3 Hz; phenyl-$\underline{H}^4$), 6.85-6.89 (2H; d; ^3J = 8.0 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$), 6.95-6.99 (1H; d; ^3J = 8.1 Hz; THIQ-$\underline{H}^8$), 7.21-7.27 (2H; d(d); merged in $CDCl_3$-peak; ^3J = 7.3 Hz; ^5J = 2.0 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$)

MS (EI): m/z = 224.1 (100%, [M - C_3H_7] $^+$), 267.2 (4%)

HPLC: Gradient: 0-25 min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 25-40 min: 95/5

RT = 18.10 min; purity: 90%

1-(9-(Pentylthio)nonanyl)-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**81**)

Preparation from 6-(benzyloxy)-1-[9-(pentylthio)nonyl]-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**63**) (150 mg, 0.28 mmol) in dioxane (3 mL) / conc. HCl (2 mL) following the procedure described for **79**; the reaction mixture was stirred for 2 h. Purification by column chromatography (SiO_2; PE/ethyl acetate 10/1, v/v).

Light yellow oil; yield: 80 mg (63%)

$C_{29}H_{43}NOS$ (453.29)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 1.20-1.72 (21H; m; -(C$\underline{H}_2$)$_7$-CH_2-S-CH_2-(C$\underline{H}_2$)$_3$-; CH-CH_2- 1diastereotopic H), 1.83-1.99 (1H; m; -C$\underline{H}_2$-CH- 1 diastereotopic H), 2.47-2.53 (4H; m; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.75-2.84 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.90-3.00 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.50-3.63 (2H; m; THIQ-$\underline{H}^3$), 4.57 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 6.59-6.66 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.71 (1H; t; ^3J = 7.2 Hz; phenyl-$\underline{H}^4$), 6.82-6.89 (2H; d; ^3J = 8.2 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$), 6.94-6.99 (1H; d; ^3J = 8.1 Hz; THIQ-$\underline{H}^8$), 7.19-7.26 (2H; d(d); merged in $CDCl_3$-peak; $^3J_1$ = 8.8 Hz; $^3J_2$ = 7.2 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$)

MS (EI): m/z = 224.1 (100%, [M – $(CH_2)_9SC_5H_{11}$] $^+$), 453.2 (1.5%)

HR-MS (EI): calculated: 452.2987
found: 452.2975

HPLC: Gradient: 0-20 min: MeCN/0.025 % TFA (aq) 50/50; to 98/2; 20-40 min: 98/2
RT = 27.95 min; purity: 93%

1-(10-(Pentylthio)decyl)-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**82**)

Preparation from 6-(benzyloxy)-1-(10-(pentylthio)decyl)-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**64**) (200 mg, 0.36 mmol) in dioxane (4 mL) / conc. HCl (3 mL) following the procedure described for **79**; the reaction mixture was stirred at 95 °C for 2 h, becoming dark brown. Purification by column chromatography (SiO_2; PE/ethyl acetate 10/1, v/v).

Light yellow oil; yield: 145 mg (86%)

$C_{30}H_{45}NOS$ (467.32)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 1.20-1.70 (23H; m; -(C$\underline{H}_2$)$_8$-CH_2-S-CH_2-(C$\underline{H}_2$)$_3$-; CH-CH_2- 1diastereotopic H), 1.85-1.98 (1H; m; -C$\underline{H}_2$-CH- 1 diastereotopic H), 2.47-2.53 (4H; m; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.73-2.85 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.88-3.01 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.51-3.59 (2H; m; THIQ-$\underline{H}^3$), 4.57 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 6.58-6.66 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.71 (1H; t; ^3J = 7.2 Hz; phenyl-$\underline{H}^4$), 6.81-6.90 (2H; d; ^3J = 8.2 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$), 6.94-6.99 (1H; d; ^3J = 8.1 Hz; THIQ-$\underline{H}^8$), 7.19-7.26 (2H; d(d); merged in $CDCl_3$-peak; $^3J_1$ = 8.8 Hz; $^3J_2$ = 7.2 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$)

MS (CI, NH_3): m/z = 468.4 (MH^+)

1-(10-(Pentylsulfonyl)decyl)-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**83**)

Preparation from 6-(benzyloxy)-1-[10-(pentylsulfonyl)decyl]-2-phenyl-1,2,3,4-tetrahydroisoquinoline (**65**) (120 mg, 0.20 mmol) in dioxane (3 mL) / conc. HCl (1.5 mL) following the procedure described for **79**; the reaction mixture was stirred at 95 °C for 2 h, becoming dark brown. Purification by column chromatography (SiO_2; PE/ethyl acetate 3/1, v/v). Final purification by preparative HPLC (Eurospher; gradient: 0-7 min:MeCN/0.05 % TFA 50/50 to 98/2, 7-25 min: 98/2).

Yellow resin; yield: 12 mg (12%)

$C_{30}H_{45}NO_3S$ (499.75)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.92 (3H; t; ^3J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 1.17-1.70 (18H; m; -(C$\underline{H}_2$)$_7$-CH_2-SO_2-CH_2-(C$\underline{H}_2$)$_3$-), 1.73-1.98 (6H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-, -C$\underline{H}_2$-CH-), 2.75-2.99 (6H; m; -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-, THIQ-$\underline{H}^4$), 3.43-3.63 (2H; m; THIQ-$\underline{H}^3$), 4.55 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 6.60-6.76 (3H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$, phenyl-$\underline{H}^4$), 6.82-6.86 (2H; d; ^3J = 8.2 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$), 6.94-6.97 (1H; d; ^3J = 8.0 Hz; THIQ-$\underline{H}^8$), 7.20-7.26 (2H; d(d); merged in $CDCl_3$-peak; $^3J_1$ = 8.8 Hz; $^3J_2$ = 7.2 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$)

MS (EI): m/z = m/z = 224.1 (100%, $[M - (CH_2)_{10}SO_2C_5H_{11}]^+$), 499.1 (1.2%)

HR-MS (EI): calculated: 499.3120

found: 499.3106

HPLC: Gradient: 0-20 min: MeCN/0.025 % TFA (aq) 50/50 to 98/2; 20-40 min: 98/2

RT = 19.45 min; purity: 98%

2-Phenyl-1-(6-pyrrolidin-1-yl)hexyl)-1,2,3,4-tetrahydroisoquinolin-6-ol (**84**)

Preparation from 6-benzyloxy-2-phenyl-1-(6-(pyrrolidin-1yl)hexyl)-1,2,3,4-tetrahydro-isoquinoline (**73**) (70 mg, 0.20 mmol) in dioxane (1.5 mL) / conc. HCl (1 mL) following the procedure described for **79**; the reaction mixture was stirred at 95 °C for 2 h, becoming dark brown. Purification by column chromatography (SiO_2; DCM/ methanol/TEA 100/5/1 → 100/10/1, v/v/v). Final purification by preparative HPLC (Eurospher; gradient: 0-15 min:MeCN/0.05 % TFA 25/75 to 35/65).

Yellow oil; yield: 30 mg (53%)

$C_{25}H_{34}N_2O \cdot C_2HF_3O_2$ (492.57)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.16-1.62 (8H; m; CH-(C$\underline{H}_2$)$_4$-), 1.68-1.88 (6H; m; -C$\underline{H}_2$-CH_2-pyrrolidine, pyrrolidine-$\underline{H}^3$, -$\underline{H}^4$), 2.40 (2H; t; ^3J = 7.9 Hz, -C$\underline{H}_2$-pyrrolidine), 2.44-2.55 (4H; m; pyrrolidine-$\underline{H}^2$, -$\underline{H}^5$) 2.65-2.76 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.81-2.92 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.44-3.51 (2H; m; THIQ-$\underline{H}^3$), 4.48 (1H; t; ^3J = 6.9 Hz; -C$\underline{H}$-), 6.48-6.54 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.64 (1H; t; ^3J = 7.2 Hz; phenyl-$\underline{H}^4$), 6.74-6.80 (2H; d; ^3J = 8.1 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$), 6.83-6.87 (1H; d; ^3J = 8.9 Hz; THIQ-$\underline{H}^8$), 7.14-7.19 (2H; d; ^3J = 8.7 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$)

MS (EI): m/z = 224.1 (100%, $[M - (CH_2)_6NC_4H_8]^+$), 378.2 (1.5%)

HR-MS (EI): calculated: 378.2671
found: 378.2665

HPLC: Gradient: 0-30min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 30-40 min: 95/5
RT = 12.39 min; purity: 98%

1-[N-methyl-N-[3-(pentylthio)propyl]amino]hexyl-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**85**)

Preparation from 6-[6-(benzyloxy)-2-phenyl-1,2,3,4-tetrahydroisoquinolin-1-yl]-N-methyl-N-[3-(pentylthio)propyl]hexan-1-amine (**76**) (140 mg, 0.24 mmol) in dioxane (3 mL) / conc. HCl (1.5 mL) following the procedure described for **79**; the reaction mixture was stirred at 95 °C for 2.5 h,. Purification by column chromatography (SiO_2; DCM/ methanol 15/1, v/v/).

Orange oil; yield: 60 mg (52%)

$C_{30}H_{46}N_2OS$ (482.76)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.84 (3H; t; ^3J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 1.15-1.66 (15H; m; -(C$\underline{H}_2$)$_3$-CH_3, -CH-CH_2-(C$\underline{H}_2$)$_4$-, CH-C$\underline{H}_2$- 1 diastereotopic H), 1.72-1.90 (3H; m; -N-CH_2-C$\underline{H}_2$-CH_2-S- , CH-C$\underline{H}_2$- 1 diastereotopic H), 2.26 (3H; s; N-C$\underline{H}_3$), 2.36-2.54 (8H; m; -C$\underline{H}_2$-S-C$\underline{H}_2$-, -C$\underline{H}_2$-N-C$\underline{H}_2$-), 2.67-2.76 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.83-2.93 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.42-3.56 (2H; m; THIQ-$\underline{H}^3$), 4.49 (1H; t; ^3J = 6.9 Hz; -C$\underline{H}$-), 6.53-6.60 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.65 (1H; t; ^3J = 7.2 Hz; phenyl-$\underline{H}^4$), 6.76-6.82 (2H; d; ^3J = 8.1 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$), 6.84-6.90 (1H; d; ^3J = 8.9 Hz; THIQ-$\underline{H}^8$), 7.15-7.20 (2H; d(d); $^3J_1$ = 8.7 Hz; $^3J_2$ = 7.3 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$)

MS (EI): m/z = 224.1 (100%, [M – $(CH_2)_{10}N(CH_3)(CH_2)_3SC_5H_{11}$] $^+$), 482.2 (3.1%)

HR-MS (EI): calculated: 482.3331
found: 482.3326

HPLC: Gradient: 0-20 min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 20-30 min: 95/5
RT = 19.99 min; purity: 99%

1-[9-(Pentylsulfinyl)nonanyl]-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**86**)

At 10 °C a H_2O_2-solution (30%; 30 µL, 0.2 mmol) was added to a solution of 1-[9-(pentylthio)nonanyl]-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**81**) (30 mg, 0.056 mmol) in glacial acetic acid. The reaction mixture was allowed to warm up to room temperature and stirreed for 20 min. Then the solution was poured into sat. $NaHCO_3$ solution, followed by extraction with etyl acetate (3 × 30 mL). The organic extracts were washed with water and brine, driesd over Na_2SO_4 and evaporated. The product was purified by column chromatography (SiO_2; DCM/methanol 20/1, v/v).

Light yellow resin; yield: 30 mg (97%)

$C_{29}H_{43}NO_2S$ (469.29)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.92 (3H; t; ^3J = 7.1 Hz; C<u>H</u>$_3$-CH$_2$-), 1.15-1.94 (22H; m; -(C<u>H</u>$_2$)$_8$-CH$_2$-SO-CH$_2$-(C<u>H</u>$_2$)$_3$-), 2.55-2.97 (6H; m; -C<u>H</u>$_2$-SO-C<u>H</u>$_2$-, THIQ-<u>H</u>^{4}), 3.42-3.62 (2H; m; THIQ-<u>H</u>^{3}), 4.54 (1H; t; ^3J = 6.7 Hz; -C<u>H</u>-), 6.63-6.73 (3H; m; THIQ-<u>H</u>^{5}, -<u>H</u>^{7}, phenyl-<u>H</u>^{4}), 6.79-6.87 (2H; d; ^3J = 8.2 Hz; phenyl-<u>H</u>^{2}, -<u>H</u>^{6}), 6.90-6.96 (1H; d; ^3J = 8.0 Hz; THIQ-<u>H</u>^{8}), 7.19-7.26 (2H; d(d); merged in $CDCl_3$-peak; $^3J_1$ = 8.7 Hz; $^3J_2$ = 7.3 Hz; phenyl-<u>H</u>^{3}, -<u>H</u>^{5})

MS (EI): m/z = 224.1 (100%, $[M - (CH_2)_9SOC_5H_{11}]^+$), 452.2 (1.4%, $[M - OH]^+$), 469.2 (0.4%)

HR-MS (EI): calculated: 469.3015
found: 469.3003

HPLC: Gradient: 0-25min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 25-40 min: 95/5
RT = 24.30 min; purity: 98%

1-(10-(Pentylsulfinyl)decyl)-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**87**)

Preparation from 1-[10-(pentylthio)decyl]-2-phenyl-1,2,3,4-tetrahydroisoquinolin-6-ol (**82**) (130 mg, 0.28 mmol) and H_2O_2 (30%; 60 µL, 0.5 mmol) following the procedure described for **86**. Purification by column chromatography (SiO_2; DCM/methanol 20 /1, v/v).

Light yellow resin; yield: 120 mg (89%)

$C_{30}H_{45}NO_2S$ (483.75)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.87 (3H; t; ^3J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 1.10-1.90 (24H; m; -(C$\underline{H}_2$)$_9$-CH_2-SO-CH_2-(C$\underline{H}_2$)$_3$-), 2.53-2.93 (6H; m; -C$\underline{H}_2$-SO-C$\underline{H}_2$-, THIQ-$\underline{H}^4$), 3.37-3.60 (2H; m; THIQ-$\underline{H}^3$), 4.59 (1H; t; ^3J = 7.5 Hz; -C$\underline{H}$-), 6.60-6.68 (3H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$, phenyl-$\underline{H}^4$), 6.77-6.80 (2H; d; ^3J = 8.3 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$), 6.88-6.91 (1H; d; ^3J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.16-7.21 (2H; d(d); merged in $CDCl_3$-peak; $^3J_1$ = 8.7 Hz; $^3J_2$ = 7.3 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$)

MS (EI): m/z = 224.1 (100%, [M – $(CH_2)_{10}SOC_5H_{11}$] $^+$), 466.2 (2.2%, [M – OH]$^+$), 483.1 (0.7%)

HR-MS (EI): calculated: 483.3171
found: 483.3160

HPLC: Gradient: 0-30min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 30-40 min: 95/5
RT = 33.01 min; purity: 99%

6.1.2.2.8 Cleavage of the Methoxy Protecting Groups

1-Ethyl-2-(3-hydroxyphenyl)-1,2,3,4-tetrahydroisoquinolin-6-ol (**88**)

Preparation from 1-ethyl-6-methoxy-2-(3-methoxyphenyl)-1,2,3,4-tetrahydroisoquinoline (**66**) (100 mg, 0.33 mmol) and BBr_3 (1M stock solution in DCM; 1.7 mL; 1.7 mmol) following the demethylation-procedure described for **24**. The reaction mixture was stirred for 4 h at room temperature. The product was purified by column chromatography (SiO_2; PE/ethyl acetate 5/2, v/v) and finally by preperative HPLC (Eurospher; gradient: 0-15 min: acetonitrile/0.05 % TFA 55/45 to 80/20).

Yellow-brownish resin; yield: 40 mg (45 %)

$C_{17}H_{19}NO_2$ (269.14)

[^{1}H] NMR (D_4-methanol):

δ (ppm) = 0.95 (3H; t; ^3J = 7.4 Hz; C$\underline{H}_3$-CH_2-), 1.57-1.73 (1H; sept; ^3J = 7.2 Hz; -C$\underline{H}_2$-CH- 1 diastereotopic H), 1.80-1.97 (1H; sept; ^3J = 7.2 Hz; -C$\underline{H}_2$-CH- 1 diastereotopic H), 2.69-2.81 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 2.82-2.95 (1H; m; THIQ-$\underline{H}^4$ 1 diastereotopic H), 3.39-3.58 (2H; m, THIQ-$\underline{H}^3$), 4.42 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 6.10-6.16 (1H; d(d); ^3J = 8.0 Hz; ^5J = 2.1 Hz; phenyl-$\underline{H}^4$), 6.33 (1H; t; ^5J = 2.2 Hz;

phenyl-H^2), 6.34-6.40 (1H; d(d); ^{3}J = 8.3 Hz; ^{5}J = 2.2 Hz; phenyl-H^6), 6.54-6.66 (2H; m; THIQ-H^5, -H^7), 6.90-7.02 (2H; m; THIQ-H^8, phenyl-H^5)

MS (EI): m/z = 240.1 (100%, $[M - C_2H_5]^+$), 269.2 (8.3%)

HR-MS (EI): calculated: 269.1416
found: 269.1412

HPLC: Gradient: 0-25min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 25-40 min: 95/5
RT = 11.92 min; purity: 98%

2-(3-Hydroxyphenyl)-1-[10-(pentylthio)decyl]-1,2,3,4-tetrahydroisoquinolin-6-ol (**89**)

Preparation from 6-methoxy-2-(3-methoxyphenyl)-1-[10-(pentylthio)decyl]-1,2,3,4-tetrahydroisoquinoline (**67**) (120 mg ; 0.23 mmol) and BBr_3 (1M stock solution in DCM; 1.2 mL; 1.2 mmol) following the demethylation-procedure described for **24**. The reaction mixture was stirred for 3 h at room temperature. The product was purified by column chromatography (SiO_2; PE/ethyl acetate 5/2, v/v).

Colourless oil; yield: 75 mg (68 %)

$C_{30}H_{45}NO_2S$ (483.75)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^{3}J = 7.4 Hz; CH$_3$-CH$_2$-), 1.12-1.47 (18H; m; -(CH$_2$)$_7$-(CH$_2$)$_2$-S-(CH$_2$)$_2$-(CH$_2$)$_2$-), 1.50-1.69 (6H; m ;-CH$_2$-CH$_2$-S-CH$_2$-CH$_2$-; -CH-CH$_2$-), 2.45-2.54 (4H; t; ^{3}J = 7.4 Hz; -CH$_2$-S-CH$_2$-), 2.72-2.95 (2H; m; THIQ-H^4), 3.40-3.57 (2H; m, THIQ-H^3), 4.53 (1H; t; ^{3}J = 6.9 Hz; -CH-), 6.15-6.25 (1H; d; ^{3}J = 7.2 Hz; phenyl-H^4), 6.28-6.50 (2H; m; phenyl-H^2, -H^6), 6.57-6.66 (2H; m; THIQ-H^5, -H^7), 6.89-6.97 (1H; d; ^{3}J = 8.1 Hz; THIQ-H^8), 7.03-7.11 (1H; t; ^{3}J = 8.1 Hz; phenyl-H^5)

MS (CI, NH_3): m/z = 484.3 (MH^+)

2-(3-Hydroxyphenyl)-1-[10-(pentylsulfonyl)decyl]-1,2,3,4-tetrahydroisoquinolin-6-ol (**90**)

Preparation from 6-methoxy-2-(3-methoxyphenyl)-1-[10-(pentylsulfonyl)decyl]-1,2,3,4-tetrahydroisoquinoline (**68**) (150 mg, 0.28 mmol) and BBr_3 (1M stock solution in DCM; 1.4 mL; 1.4 mmol) following the demethylation-procedure described for **24**. The reaction mixture was stirred for 4 h at room temperature. The product was purified by column chromatography (SiO_2; PE/ethyl acetate 5/2, v/v) and finally by preperative HPLC (Eurospher; gradient: 0-12min: MeCN/0.05 % TFA (aq) 55/45 to 85/15).

Light yellow oil; yield: 75 mg (52%)

$C_{30}H_{45}NO_4S$ (515.75)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.91 (3H; t; ^3J = 7.1 Hz; C<u>H</u>$_3$-CH$_2$-), 1.10-1.48 (18H; m; -(C<u>H</u>$_2$)$_7$-(CH$_2$)$_2$-SO$_2$-(CH$_2$)$_2$-(C<u>H</u>$_2$)$_2$), 1.52-1.71 (1H; m; -CH-C<u>H</u>$_2$- 1 diastereotopic H), 1.72-1.97 (5H; m; -CH-C<u>H</u>$_2$- 1 diastereotopic H, -C<u>H</u>$_2$-CH$_2$-SO$_2$-CH$_2$-C<u>H</u>$_2$-), 2.75-3.00 (2H; m; THIQ-<u>H</u>^{4}, -C<u>H</u>$_2$-SO$_2$-C<u>H</u>$_2$-), 3.43-3.64 (2H; m, THIQ-<u>H</u>^{3}), 4.53 (1H; t; ^3J = 6.0 Hz; -C<u>H</u>-), 6.44-6.53 (1H; d; ^3J = 7.9 Hz; phenyl-<u>H</u>^{4}), 6.54-6.72 (4H; m; phenyl-<u>H</u>^{2}, -<u>H</u>^{6}, THIQ-<u>H</u>^{5}, -<u>H</u>^{7}), 6.83-6.92 (1H; d; ^3J = 8.4 Hz; THIQ-<u>H</u>^{8}), 7.04-7.15 (1H; t; ^3J = 8.1 Hz; phenyl-<u>H</u>^{5})

MS (ES): m/z = 516.4 (MH^+)

HR-MS (EI): calculated: 515.3069

found: 515.3057

HPLC: Gradient: 0-15 min: MeCN/0.025 % TFA (aq) 55/45 to 95/5; 15-30 min: 95/5

RT = 10.92 min; purity: 99%

2-(3-Hydroxyphenyl)-1-[6-(pyrrolidin-1-yl)hexyl)]-1,2,3,4-tetrahydroisoquinolin-6-ol (**91**)

Preparation from 6-methoxy-2-(3-methoxyphenyl)-1-[6-(pyrrolidin-1-yl)hexyl]-1,2,3,4-tetrahydroisoquinoline (**74**) (60 mg, 0.14 mmol) and BBr_3 (1M stock solution in DCM; 0.7 mL; 0.7 mmol) following the demethylation-procedure described for **24**. The reaction mixture was stirred for 4 h at room temperature. The product was purified by column chromatography (SiO_2; DCM/methanol/TEA 100/20/1, v/v/v) and finally by preperative HPLC (Eurospher; gradient: 0-12 min: MeCN/0.05 % TFA 55/45 to 85/15).

Yellow oil; yield: 48 mg (55 %)

$C_{25}H_{34}N_2O_2 \cdot C_2HF_3O_2$ (509.57)

[^{1}H] NMR (D_4-methanol):

δ (ppm) = 1.18-2.20 (10 H; m; -(C<u>H</u>$_2$)$_5$-CH$_2$-pyrrolidine), 2.87-3.19 (8H; m; -C<u>H</u>$_2$-pyrrolidine; pyrrolidine-<u>H</u>^{2}, -<u>H</u>^{5}; THIQ-<u>H</u>^{4}), 3.33-3.51 (2H; m; THIQ-<u>H</u>^{3}), 4.23 (1H; t; ^3J = 7.5 Hz; -C<u>H</u>-), 6.69-6.82 (3H; m; phenyl-<u>H</u>), 6.94-7.21 (4H; m; phenyl-<u>H</u>)

MS (CI, NH_3): m/z = 395.3 (MH^+)

HR-MS (EI): calculated: 394.2620
found: 394.2610
HPLC: Gradient: 0-25min: MeCN/0.025 % TFA (aq) 20/80 to 40/60; 25-30 min: 40/60 to 95/5; 30-40 min: 95/5
RT = 15.35 min; purity: 99%

2-(3-Hydroxyphenyl)-1-[6-(piperidin-1-yl)hexyl]-1,2,3,4-tetrahydroisoquinolin-6-ol (**92**)

Preparation from 6-methoxy-2-(3-methoxyphenyl)-1-[(6-piperidin-1-yl)hexyl]-1,2,3,4-tetrahydroisoquinoline (**75**) (150 mg; 0.34 mmol) and BBr_3 (1M stock solution in DCM; 1.7 mL; 1.7 mmol) following the demethylation-procedure described for **24**. The reaction mixture was stirred for 4 h at room temperature. The product was purified by column chromatography (SiO_2; DCM/methanol/TEA 100/20/1, v/v/v) and finally by crystallisation from DCM/methanol 20/1 v/v.

White solid; yield: 100 mg (72 %)

$C_{26}H_{36}N_2O_2$ (408.28)

Melting point: 176-178 °C

[^{1}H] NMR (D_4-methanol):

δ (ppm) = 1.28-1.97 (16H; m; -(C$\underline{H}_2$)$_5$-CH-, piperidine-$\underline{H}^3$, -$\underline{H}^4$, -$\underline{H}^5$), 2.65-3.28 (8H; m; -C$\underline{H}_2$-piperidine; piperidine-$\underline{H}^2$, -$\underline{H}^6$; THIQ-$\underline{H}^4$), 3.46-3.55 (2H; m; THIQ-$\underline{H}^3$), 4.55 (1H; t; ^{3}J = 6.9 Hz; -C$\underline{H}$-), 6.10-6.18 (1H; d(d); ^{3}J = 7.8 Hz; ^{5}J = 1.9 Hz; phenyl-$\underline{H}^4$), 6.34 (1H, t, ^{5}J = 2.2 Hz; phenyl-$\underline{H}^2$), 6.36-6.43 (1H; d(d); ^{3}J = 8.2 Hz; ^{5}J = 2.1 Hz; phenyl-$\underline{H}^6$), 6.52-6.62 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.91-7.03 (2H; m; THIQ-$\underline{H}^8$, phenyl-$\underline{H}^5$)

MS (CI, NH_3): m/z = 409.2 (MH^+)

HR-MS (EI): calculated: 408.2777
found: 408.2765

HPLC: Gradient: 0-20 min: MeCN/0.025 % TFA (aq) 20/80 to 50/50; 20-30 min: 50/50 to 95/5; 30-40 min: 95/5
RT = 13.62 min; purity: 98%

2-(3-Hydroxyphenyl)-1-[6-[N-methyl-N-[3-(pentylthio)propyl]amino]hexyl]-1,2,3,4-tetrahydroisoquinolin-6-ol (**93**)

Preparation from 6-[6-methoxy-2-(3-methoxyphenyl)-1,2,3,4-tetrahydroisoquinolin-1-yl]-N-methyl-N-[3-(pentylthio)propyl]hexan-1-amine (**78**) (100 mg; 0.19 mmol) and BBr_3

(1M stock solution in DCM; 1.0 mL; 1.0 mmol) following the demethylation-procedure described for **24**. The reaction mixture was stirred for 3 h at room temperature. The product was purified by column chromatography (SiO_2; DCM/methanol 10/1, v/v) and finally by preperative HPLC (Eurospher; gradient: 0-13 min: MeCN/0.05 % TFA (aq) 35/65 to 60/40).

Light yellow resin; yield: 50 mg (43 %)

$C_{30}H_{45}N_2O_2S \cdot C_2HF_3O_2$ (612.78)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.88 (3H; t; ^3J = 7.0 Hz; C$\underline{H}_3$-CH_2-), 1.14-1.63 (15H; m; -(C$\underline{H}_2$)$_3$-CH_3, N-(CH_2)-(C$\underline{H}_2$)$_4$-, -CH-C$\underline{H}_2$- 1 diastereotopic H), 1.72-1.98 (3H; m; -S-CH_2-C$\underline{H}_2$-CH_2-N-, -CH-C$\underline{H}_2$- 1 diastereotopic H), 2.38-2.62 (9H; m; N-C$\underline{H}_3$, 1 × -N-C$\underline{H}_2$-, -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.64-2.89 (4H; m; N-C$\underline{H}_2$-; THIQ-$\underline{H}^4$), 3.34-3.46 (2H; m; THIQ-$\underline{H}^3$), 4.44 (1H; t; ^3J = 7.0 Hz; -C$\underline{H}$-), 6.18-6.25 (1H; d; ^3J = 7.5 Hz; phenyl-$\underline{H}^4$), 6.30-6.35 (1H; d; ^3J = 8.3 Hz; phenyl-$\underline{H}^6$), 6.40 (1H; s; phenyl-$\underline{H}^2$), 6.55-6.66 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.76-6.84 (1H; d; ^3J = 8.1 Hz; THIQ-$\underline{H}^8$), 6.98-7.07 (1H; t; ^3J = 8.0 Hz; phenyl-$\underline{H}^5$)

MS (CI, NH_3): m/z = 498.2 (MH^+)

HR-MS (EI): calculated: 497.3202
found: 497.3196

HPLC: Gradient: 0-25min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 25-40 min: 95/5
RT = 18.40 min; purity: 99%

2-(3-Hydroxyphenyl)-1-[(10-pentylsulfinyl)decyl]-1,2,3,4-tetrahydroisoquinolin-6-ol (**94**)

Preparation from 2-(3-hydroxyphenyl)-1-[(10-pentylthio)decyl]-1,2,3,4-tetrahydroisoquinolin-6-ol (**89**) (60 mg; 0.12 mmol) and H_2O_2 (30%; 40 µL, 0.3 mmol) following the procedure described for **86**. Purification by column chromatography (SiO_2; DCM/methanol 20/1, v/v). The product was purified by column chromatography (SiO_2; DCM/methanol 25/1, v/v).

Light yellow resin; yield: 50 mg (83%)

$C_{30}H_{45}NO_3S$ (499.31)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.91 (3H; t; ^{3}J = 7.4 Hz; C$\underline{H}_3$-CH_2-), 1.08-1.96 (24H; m; -(C$\underline{H}_2$)$_9$-CH_2-SO-CH_2-(C$\underline{H}_2$)$_3$-), 2.55-2.88 (6H; m; ^{3}J = 7.4 Hz; -C$\underline{H}_2$-SO-C$\underline{H}_2$-, THIQ-$\underline{H}^4$), 3.35-3.57 (2H; m, THIQ-$\underline{H}^3$), 4.51 (1H; t; ^{3}J = 6.8 Hz; -C$\underline{H}$-), 6.17-6.48 (3H; m; phenyl-$\underline{H}^2$, -$\underline{H}^4$, -$\underline{H}^6$), 6.58-6.69 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.85-6.93 (1H; d; ^{3}J = 8.1 Hz; THIQ-$\underline{H}^8$), 7.00-7.09 (1H; t; ^{3}J = 8.2 Hz; phenyl-$\underline{H}^5$)

MS (EI): m/z = 499.3 (1.16%), 240.1 (100%; [M – $(CH_2)_{10}SOC_5H_{11}$

HR-MS (EI): calculated: 499.3120

found: 499.3107

HPLC: Gradient: 0-25 min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 25-40 min: 95/5

RT = 24.17 min; purity: 98%

6.1.2.3 Synthesis of N-trifluoroacetyl and N-Phenylsulfonyltetrahydroisoquinolin-6-ols

6.1.2.3.1 Synthesis of N-unsubstituated THIQs

O-(Ethoxycarbonyl)-3-methoxymandelonitrile (**95**) (Kashdan et al., 1982)

3-Methoxybenzaldehyde (20 g, 147 mmol) and ethyl chloroformate (17.6 g, 162 mmol) were dissolved in THF (30 mL) and the solution was cooled to 0 °C. A solution of KCN (10.6 g, 162 mmol) in water (40 mL) was added and the mixtute was stirred for 4 h at this temperature and another 16 h at room temperature. Then the reaction mixture was poured into water (200 mL) and the product was extracted with ether (3 × 100 mL). The combined organic extracts were washed with brine, dried over Na_2SO_4 and evaporated. The crude product was destilled *in vacuo* (bp: 115-120 °C; 0.3 mbar).

Colourless oil; yield: 26.5 g (75%)

$C_{12}H_{13}NO_4$ (235.08)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 1.33 (3H; t; ^{3}J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 3.83 (3H; s; -O-C$\underline{H}_3$), 4.20-4.37 (2H; m; 2 diastreotopic Hs; O-C$\underline{H}_2$-CH_3), 6.23 (1H; s; -C$\underline{H}$-CN), 6.96-7.02 (1H; d(d); ^{3}J = 8.3 Hz; ^{5}J = 2.5 Hz; phenyl-$\underline{H}^4$), 7.05 (1H; t; ^{5}J = 2.3 Hz; phenyl-$\underline{H}^2$), 7.08-7.13 (1H; d; ^{3}J = 8.0 Hz; phenyl-$\underline{H}^6$), 7.36 (1H; t; ^{3}J = 8.0 Hz; phenyl-$\underline{H}^5$)

2-(3-Methoxyphenyl)ethylamine (**96**) (Kashdan et al., 1982)

A solution of O-(ethoxycarbonyl)-3-methoxymandelonitrile (**95**) (5.8 g, 25 mmol) in abs. ethanol (75 mL) was added dropwise into a stirred mixture of Pd/C (0.38 g; 10 mol%) and conc. H_2SO_4 (1.75 mL; 32.5 mmol) while hydrogen was bubbling through the solution. Stirring and bubbling was continued for 12 h. Pd/C was filtered of and the solvent was destilled of *in vacuo*. The residue was taken up in water (100 mL) and the mixture was neutralised with 4M NaOH under ice.cooling. The organic components were extracted into ether (3 × 50 mL); the organic phase was washed with brine, dried over Na_2SO_4 and evaporated. The crude product was purified by Kugelrohr-destillation under reduced pressure (bp = 65-68 °C; 0.1 mbar).

Light yellow oil; yield: 1.2 g (32 %)

$C_9H_{13}NO$ (151.21)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 2.75 (2H; t; ^3J = 7.0 Hz; -C$\underline{H}_2$-NH_2), 2.93 (2H; t; ^3J = 7.1 Hz; -C$\underline{H}_2$-phenyl), 3.78 (3H; s; -O-C$\underline{H}_3$), 6.63-6.85 (3H; m; phenyl-$\underline{H}$), 7.20 (1H; t; ^3J = 8.0 Hz; phenyl-$\underline{H}^5$)

N-(3-Methoxyphenylethyl)benzamide (**97**)

Preparation from benzoyl chloride (170 mg; 1.2 mmol) and 2-(3-methoxyphenyl)-ethylamine (**96**) (151 mg; 1.0 mmol) following the procedure described for **53**. Purification by column chromatography (SiO_2; DCM/ethyl acetate 15/1, v/v).

Colourless resin; yield: 180 mg (70 %)

$C_{16}H_{17}NO_2$ (255.31)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 2.90 (2H; t; ^3J = 7.3 Hz; phenyl-C$\underline{H}_2$-), 3.69 (2H; t; ^3J = 7.3 Hz; -C$\underline{H}_2$-N), 3.80 (3H; s; -O-C$\underline{H}_3$), 6.78-6.87 (3H; m; phenyl-$\underline{H}$), 7.20-7.27 (1H; m; phenyl-$\underline{H}^5$), 7.36-7.55 (3H; m; -CO-phenyl-$\underline{H}$), 7.68-7.75 (2H; d; ^3J = 8.2 Hz; -CO-phenyl-$\underline{H}^2$, -$\underline{H}^6$)

MS (CI, NH_3): m/z = 256.2 (100%; MH^+), 273.3 (49%; MNH_4^+)

N-(3-Methoxyphenylethyl)-11-pentylthioundecanamide (**98**)

Preparation from 11-pentylthioundecanoic acid chloride (**41**) (300 mg; 1.0 mmol) and 2-(3-methoxyphenyl)ethylamine (**96**) (100 mg; 0.66 mmol) following the procedure

described for **53**. Purification by column chromatography (SiO_2; DCM/methanol 50/1, v/v).

Colourless oil; yield: 250 mg (90 %)

$C_{25}H_{41}NO_2S$ (421.22)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.89 (3H; t; ^3J = 7.1 Hz; C$\underline{H}_3$-CH_2-), 1.20-1.42 (16H; m; -(C$\underline{H}_2$)$_6$-$(CH_2)_2$-S-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-), 1.50-1.63 (6H; m; -CO-CH_2-C$\underline{H}_2$-, -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-), 2.11 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO-), 2.43-2.56 (4H; t; ^3J = 7.0 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-) 2.79 (2H; t; ^3J = 6.9 Hz; phenyl-C$\underline{H}_2$-), 3.47-3.56 (2H; d(t); $^3J_1$ = 6.8 Hz; $^3J_2$ = 6.1 Hz; -C$\underline{H}_2$-NH-), 3.80 (3H; s; -O-C$\underline{H}_3$), 6.72-6.80 (3H; m; phenyl-$\underline{H}$), 7.19-7.25 (1H; m; phenyl-$\underline{H}^5$)

MS (CI, NH_3): m/z = 422.2 (100%; MH^+), 439.4 (9%; MNH_4^+)

N-(3-Methoxyphenylethyl)-11-pentylsulfonylundecanamide (**99**)

Preparation from 11-pentylsulfonylundecanoic acid chloride (**44**) (600 mg; 1.8 mmol) and 2-(3-methoxyphenyl)ethylamine (**96**) (250 mg; 1.65 mmol) following the procedure described for **53**. Purification by crystallisation from toluene.

White crystals; yield: 520 mg (71 %)

Melting point: 109-110 °C

$C_{25}H_{43}NO_4S$ (453.68)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.92 (3H; t; ^3J = 7.0 Hz; C$\underline{H}_3$-CH_2-), 1.21-1.49 (16H; m; -(C$\underline{H}_2$)$_6$-$(CH_2)_2$-SO_2-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-), 1.52-1.64 (2H; m; -CO-CH_2-C$\underline{H}_2$-, -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-), 2.11 (2H; t; ^3J = 7.5 Hz; -C$\underline{H}_2$-CO-), 2.43-2.56 (4H; t; ^3J = 7.0 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-) 2.79 (2H; t; ^3J = 6.9 Hz; phenyl-C$\underline{H}_2$-), 2.89-2.97 (4H; t; ^3J = 8.1 Hz; -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-), 3.47-3.55 (2H; d(t); $^3J_1$ = 6.8 Hz; $^3J_2$ = 6.0 Hz; -C$\underline{H}_2$-NH-), 3.80 (3H; s; -O-C$\underline{H}_3$), 6.71-6.81 (3H; m; phenyl-$\underline{H}$), 7.19-7.26 (1H; t; merged in $CDCl_3$ peak; ^3J = 7.9 Hz; phenyl-$\underline{H}^5$)

MS (CI, NH_3): m/z = 454.2 (100%; MH^+), 471.2 (24%; MNH_4^+)

6-Methoxy-1-phenyl-1,2,3,4-tetrahydroisoquinoline (**100**)

N-(3-methoxyphenylethyl)benzamide (**97**) (180 mg, 0.7 mmol) was refluxed in $POCl_3$ (0.3 mL) / toluene (14 mL) for 16 h. Solvent and $POCl_3$ were removed in vacuo and the

residue was dissolved in DCM (25 mL). The solution was washed with sat. Na_2HCO_3 solution and brine, dried over Na_2SO_4 and evaporated. The crude 3-4-dihydroisoquinoline was directly reduced with $NaBH_4$ (200 mg; 5.2 mmol) in methanol (10 mL). The work-up procedure was the same as described for **63** with DCM as extraction-solvent. The crude product was purified by column chromatography (SiO_2; DCM/methanol/TEA 1000/50/1, v/v/v).

White solid; yield: 120 mg (72 %)

$C_{16}H_{16}NO$ (238.30)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 2.75-2.90 (1H; m; -C<u>H</u>$_2$-phenyl 1 diastereotopic H), 2.97-3.12 (2H; m; N-C<u>H</u>$_2$-C<u>H</u>$_2$-phenyl 1 diastereotopic H of each), 3.21-3.30 (1H; m; -C<u>H</u>$_2$-N- 1 diastereotopic H), 3.78 (3H; s; -O-C<u>H</u>$_3$), 5.06 (1H; s; -C<u>H</u>-phenyl-), 6.60-6.69 (3H; m; THIQ-<u>H</u>), 7.24-7.35 (5H; m; phenyl-<u>H</u>)

MS (CI, NH_3): m/z = 240.2 (MH^+)

6-Methoxy-1-[10-(pentylthio)decyl]-1,2,3,4-tetrahydroisoquinoline (**101**)

Preparation from N-(3-methoxyphenylethyl)-11-pentylthioundecanamide (**98**) (300 mg, 0.8 mmol) in $POCl_3$ (0.4mL) / toluene (15 mL) followed by reduction with $NaBH_4$ (200 mg; 5.2 mmol) in methanol (10 mL). The procedure was the same as described for **100**. Purification by column chromatography (SiO_2; DCM/methanol/TEA 1000/70/1, v/v/v).

White solid; yield: 260 mg (83 %)

$C_{25}H_{41}NOS$ (405.31)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^{3}J = 7.1 Hz; -CH$_2$-C<u>H</u>$_3$), 1.23-1.64 (22H; m; -(C<u>H</u>$_2$)$_8$-CH$_2$-S-CH$_2$-(C<u>H</u>$_2$)$_3$-), 1.70-1.92 (2H; m; -CH-C<u>H</u>$_2$-), 2.50 (4H; t; ^{3}J = 7.4 Hz; -C<u>H</u>$_2$-S-C<u>H</u>$_2$-), 2.74-2.97 (2H; m; -C<u>H</u>$_2$-phenyl 2 diastereotopic Hs), 3.01-3.10 (1H; m; -C<u>H</u>$_2$-NH- 1 diastereotopic H), 3.26-3.35 (1H; m; -C<u>H</u>$_2$-NH- 1 diastereotopic H), 3.78 (3H; s; -O-C<u>H</u>$_3$), 4.00-4.06 (1H; m; -C<u>H</u>-), 6.60-6.64 (1H; d; ^{5}J = 2.6 Hz; THIQ-<u>H</u>^{5}), 6.71-6.77 (1H; d(d); ^{3}J = 8.6 Hz; ^{5}J = 2.7 Hz; THIQ-<u>H</u>^{7}), 7.02-7.07 (1H; d; ^{3}J = 8.6 Hz; THIQ-<u>H</u>^{8})

MS (EI): m/z = 162.1 (100%, [M – $(CH_2)_{10}$-S-C_5H_{11}]$^+$), 405.4 (1%)

6-Methoxy-1-[(10-pentylsulfonyl)decyl]-1,2,3,4-tetrahydroisoquinoline (**102**)

Preparation from N-(3-methoxyphenylethyl)-11-pentylsulfonylundecanamide (**99**) (500 mg, 0.8 mmol) in $POCl_3$ (0.6mL) / toluene (25 mL) followed by reduction with $NaBH_4$ (280 mg; 7.3 mmol) in methanol (20 mL). The procedure was the same as described for **100**. Purification by column chromatography (SiO_2; DCM/methanol/TEA 1000/100/1, v/v/v).

Light yellow solid; yield: 450 mg (93 %)

Melting point: 55-56 °C

$C_{25}H_{43}NO_3S$ (437.68)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.92 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.23-1.50 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-SO_2-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.76-1.90 (6H; m; -CH-C$\underline{H}_2$-, -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-), 2.67-2.85 (2H; m; -C$\underline{H}_2$-phenyl 2 diastereotopic Hs), 2.91-3.04 (4H; m; -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-, -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.20-3.29 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.78 (3H; s; -O-C$\underline{H}_3$), 3.90-3.97 (1H; m; -C$\underline{H}$-), 6.60-6.63 (1H; d; ^5J = 2.6 Hz; THIQ-$\underline{H}^5$), 6.70-6.75 (1H; d(d); ^3J = 8.5 Hz; ^5J = 2.7 Hz; THIQ-$\underline{H}^7$), 7.02-7.07 (1H; d; ^3J = 8.6 Hz; THIQ-$\underline{H}^8$)

MS (CI, NH_3): m/z = 483.1 (MH^+)

6.1.2.3.2 Introduction of a N-Trifluoroaceyl Group and Cleavage of the Methoxy Protecting Group

2,2,2-Trifluoro-1-(6-methoxy-1-phenyl-1,2,3,4tetrahydroisoquinolin-2-yl)ethanon (**103**)

At 0 °C under nitrogen a solution of trifluoroacetic acid anhydride (132 mg, 0.63 mmol) in abs. DCM (1 mL) was added to a solution of 6-methoxy-1-phenyl-1,2,3,4-tetrahydro-isoquinoline (**100**) (100 mg, 0.63 mmol) in abs DCM (5 mL) / TEA (0.2 mL). The reaction mixture was stirred at this temperature for 1.5 h. Then water was added and the DCM-phase was separated. The aqueous phase was extracted with two portions of DCM; the combined organic phases were washed with water and brine, dried over Na_2SO_4 and evaporated. The product was purified by column chromatography (SiO_2; PE/ ethyl acetate 15/1, v/v).

Colourless resin; yield: 130 mg (92%

$C_{18}H_{16}F_3NO_2$ (335.32)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 2.76-2.85 (1H; m; -C$\underline{H}_2$-phenyl 1 diastereotopic H), 2.97-3.11 (1H; m; -C$\underline{H}_2$-phenyl 1 diastereotopic H), 3.37-3.49 (1H; m; -C$\underline{H}_2$-N- 1 diastereotopic H), 3.83-3.94 (1H; m; -C$\underline{H}_2$-N- 1 diastereotopic H), 3.77 (3H; s; -O-C$\underline{H}_3$), 6.66-6.70 (1H; m; -C$\underline{H}$-phenyl-), 6.72-6.77 (2H; m; THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.90-6.95 (1H; d; ^{3}J = 8.5 Hz; THIQ-$\underline{H}^8$), 7.14-7.28 (5H; m; phenyl-$\underline{H}$)

MS (CI, NH_3): m/z = 336.3 (9%; MH^+), 353.3 (100% MNH_4^+)

2,2,2-Trifluoro-1-[6-methoxy-1-[10-(pentylthio)decyl]-1,2,3,4-tetrahydroisoquinolin-2-yl]ethanone (**104**)

Preparation from 6-methoxy-1-[10-(pentylthio)decyl]-1,2,3,4-tetrahydroisoquinoline (**101**) (200 mg; 0.5 mmol) and trifluoroacetic acid anhydride (157 mg; 0.75 mmol) following the procedure described for **103**. Purification by column chromatography (SiO_2; PE/ ethyl acetate 20/1, v/v).

Colourless resin; yield: 170 mg (68%)

$C_{27}H_{42}F_3NO_2S$ (501.29)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = = 0.85 (3H; t; ^{3}J = 7.1 Hz; -CH_2-C$\underline{H}_3$), 1.13-1.41 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-S-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.46-1.61 (4H; m; -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-) 1.70-1.82 (2H; m; -CH-C$\underline{H}_2$-), 2.45 (4H; t; ^{3}J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.73-3.20 (2H; m; -C$\underline{H}_2$-phenyl 2 diastereotopic Hs), 3.51-3.61 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.90-4.10 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.74 (3H; s; -O-C$\underline{H}_3$), 5.41-5.47 (1H; m; -C$\underline{H}$-), 6.57-6.64 (1H; d; ^{5}J = 2.6 Hz; THIQ-$\underline{H}^5$), 6.71-6.76 (1H; d(d); ^{3}J = 8.5 Hz; ^{5}J = 2.6 Hz; THIQ-$\underline{H}^7$), 6.98-7.02 (1H; d; ^{3}J = 8.6 Hz; THIQ-$\underline{H}^8$)

MS (CI, NH_3): m/z = 502.2 (39%; MH^+), 519.3(100%; MNH_4^+)

2,2,2-Trifluoro-1-[6-methoxy-1-[10-(pentylsulfonyl)decyl]-1,2,3,4-tetrahydroisoquinolin-2-yl]ethanone (**105**)

Preparation from 6-methoxy-1-[10-(pentylsulfonyl)decyl]-1,2,3,4-tetrahydroisoquinoline (**102**) (80 mg; 0.2 mmol) and trifluoroacetic acid anhydride (58 mg; 0.27 mmol) following the procedure described for **103**. Purification by column chromatography (SiO_2; PE/ ethyl acetate 3/1, v/v).

White solid; yield: 80 mg (83%)

$C_{27}H_{42}F_3NO_4S$ (533.69)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = = 0.92 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.20-1.49 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-SO_2-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.75-1.90 (6H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-, -CH-C$\underline{H}_2$-), 2.78-3.09 (6H; m; -C$\underline{H}_2$-phenyl 2 diastereotopic Hs, -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-),), 3.54-3.67 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.78 (3H; s; -O-C$\underline{H}_3$), 3.95-4.06 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 5.45-5.52 (1H; m; -C$\underline{H}$-), 6.62-6.65 (1H; d; ^5J = 2.5 Hz; THIQ-$\underline{H}^5$), 6.75-6.81 (1H; d(d); ^3J = 8.5 Hz; ^5J = 2.6 Hz; THIQ-$\underline{H}^7$), 7.02-7.07 (1H; d; ^3J = 8.6 Hz; THIQ-$\underline{H}^8$)

MS (CI, NH_3): m/z = 534.2 (2.2%; MH^+), 551.2(100%; MNH_4^+)

2,2,2-Trifluoro-1-[6-hydroxy-1-phenyl-1,2,3,4-tetrahydroisoquinolin-2-yl]ethanon (**106**)

Preparation from 2,2,2-trifluoro-1-(6-methoxy-1-phenyl-1,2,3,4-tetrahydroisoquinolin-2-yl)ethanon (**103**) (100 mg, 0.3 mmol) and BBr_3 (1M stock solution in DCM; 0.9 mL; 0.9 mmol) following the demethylation procedure described for **24**. The reaction mixture was stirred for 2 h. Purification by column chromatography (PE/ethyl acetate 3/1 v/v)

Light yellow resin; yield: 60 mg (62 %)

$C_{17}H_{14}F_3NO_2$ (321.29)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 2.74-2.85 (1H; m; -C$\underline{H}_2$-phenyl 1 diastereotopic H), 2.95-3.13 (1H; m; -C$\underline{H}_2$-phenyl 1 diastereotopic H), 3.39-3.55 (1H; m; -C$\underline{H}_2$-N- 1 diastereotopic H), 3.88-4.00 (1H; m; -C$\underline{H}_2$-N- 1 diastereotopic H), 6.69-6.82 (3H; m; -C$\underline{H}$-phenyl, THIQ-$\underline{H}^5$, -$\underline{H}^7$), 6.89-6.95 (1H; d; ^3J = 8.5 Hz; THIQ-$\underline{H}^8$), 7.15-7.38 (5H; m; phenyl-$\underline{H}$)

MS (EI): m/z = 321.1

HPLC: Gradient: 0-25 min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 25-40 min: 95/5

RT = 20.63 min; purity: 99%

2,2,2-Trifluoro-1-[6-hydroxy-1-[10-(pentylthio)decyl]-3,4-dihydroisoquinolin-2-yl]ethanone (**107**)

Preparation from 2,2,2-trifluoro-1-[6-methoxy-1-[10-(pentylthio)decyl]-1,2,3,4-tetrahydroisoquinolin-2-yl]ethanone (**104**) (170 mg, 0.35 mmol) and BBr_3 (1M stock solution in DCM; 1 mL; 1.0 mmol) following the demethylation procedure described for **24**. The reaction mixture was stirred for 2 h. Purification by column chromatography (PE/ethyl acetate 5/1 v/v)

Colourless oil; yield: 80 mg (48%)

$C_{26}H_{40}F_3NO_2S$ (487.27)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = = 0.90 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.20-1.46 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-S-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.51-1.65 (4H; m; -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-) 1.70-1.90 (2H; m; -CH-C$\underline{H}_2$-), 2.50 (4H; t; ^3J = 7.4 Hz; -C$\underline{H}_2$-S-C$\underline{H}_2$-), 2.74-3.05 (2H; m; -C$\underline{H}_2$-phenyl 2 diastereotopic Hs), 3.53-3.66 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.94-4.04 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 5.42-5.50 (1H; m; -C$\underline{H}$-), 6.57-6.62 (1H; d; ^5J = 2.3 Hz; THIQ-$\underline{H}^5$), 6.67-6.74 (1H; d(d); ^3J = 8.3 Hz; ^5J = 2.6 Hz; THIQ-$\underline{H}^7$), 6.95-7.02 (1H; d; ^3J = 8.4 Hz; THIQ-$\underline{H}^8$)

MS (CI, NH_3): m/z = 403.2 (100%; [MNH_4 – C_5H_{11}]$^+$), 488.3 (10%; MH^+), 505.2 (41%; MNH_4^+)

2,2,2-Trifluoro-1-[6-hydroxy-1-[10-(pentylsulfonyl)decyl]-1,2,3,4-tetrahydroisoquinolin-2-yl]ethanone (**108**)

Preparation from 2,2,2-trifluoro-1-[6-methoxy-1-[10-(pentylsulfonyl)decyl]-1,2,3,4-tetrahydroisoquinolin-2-yl)ethanone (**105**) (60 mg, 0.11 mmol) and BBr_3 (1M stock solution in DCM; 0.35 mL; 0.35 mmol) following the demethylation procedure described for **24**. The reaction mixture was stirred for 2 h. Purification by column chromatography (PE/ethyl acetate 5/1 v/v)

Colourless resin; yield: 45 mg (79%)

$C_{26}H_{40}F_3NO_4S$ (519.66)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = = 0.92 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.18-1.50 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-SO_2-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.70-1.90 (6H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-, -CH-C$\underline{H}_2$-), 2.75-3.03 (6H; m; -C$\underline{H}_2$-phenyl 2 diastereotopic

Hs, -C$\underline{H}_2$-SO_2-C$\underline{H}_2$-), 3.55-3.67 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.93-4.04 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 5.41-5.49 (1H; m; -C$\underline{H}$-), 6.57-6.64 (1H; d; ^{5}J = 2.4 Hz; THIQ-$\underline{H}^5$), 6.67-6.73 (1H; d(d); ^{3}J = 8.3 Hz; ^{5}J = 2.6 Hz; THIQ-$\underline{H}^7$), 6.90-7.02 (1H; d; ^{3}J = 8.4 Hz; THIQ-$\underline{H}^8$)

MS (CI, NH_3): m/z = 520.3 (1%; MH^+), 537.2 (100%; MNH_4^+)

HR-MS (EI): calculated: 519.2630

found: 519.2622

HPLC: Gradient: 0-20 min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 40 min: 95/5

RT = 23.07 min; purity: 96%

6.1.2.3.3 Introduction of a Phenylsulfonyl Group and Cleavage of the Methoxy Protecting group

6-Methoxy-1-[10-(pentylthio)decyl]-2-(phenylsulfonyl)-1,2,3,4.tetrahydroisoquinoline (**109**)

Phenylsulfonylchloride (88 mg, 0.5 mmol) was added to a stirred suspension of 6-methoxy-1-[10-(pentylthio)decyl]-1,2,3,4-tetrahydroisoquinoline (**101**) (60 mg, 0.15 mmol) in THF (3 mL) / pyridine (2 mL) / TEA (120 µL) and the reaction mixture was stirred for 2 h at room temperature. Then the solvent was removed in vacuo and the residue was dissolved in DCM. The DCM solution was washed with brine; dried over Na_2SO_4 and evaporated. The product was purified by column chromatography (SiO_2; PE/ ethyl acetate 20/1 → 10/1, v/v).

Colourless oil; yield: 70 mg (85%)

$C_{31}H_{47}NO_3S_2$ (545.84)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^{3}J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.21-1.50 (18H; m; -(C$\underline{H}_2$)$_7$-$(CH_2)_2$-S-$(CH_2)_2$-(C$\underline{H}_2$)$_2$-), 1.52-1.82 (6H; m; -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-, -CH-C$\underline{H}_2$-), 2.47-2.55 (6H; m; C$\underline{H}_2$-phenyl, -C$\underline{H}_2$-S-C$\underline{H}_2$-), 3.39-3.51 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.71 (3H; s; -O-C$\underline{H}_3$), 3.80-3.90 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 4.87-4.95 (1H; m; -C$\underline{H}$-), 6.36-6.39 (1H; d; ^{5}J = 2.5 Hz; THIQ-$\underline{H}^5$), 6.66-6.71 (1H; d(d); ^{3}J = 8.5 Hz; ^{5}J = 2.6 Hz; THIQ-$\underline{H}^7$), 6.92-6.97 (1H; d; ^{3}J = 8.5 Hz; THIQ-$\underline{H}^8$), 7.27-

7.35 (2H; t; ^3J = 7.5 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.39-7.46 (1H; t; ^3J = 7.4 Hz; phenyl-$\underline{H}^4$), 7.68-7.73 (2H; d; ^3J = 7.9 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$)

MS (CI, NH_3): m/z = 461.2 (100%; $[MNH_4 - SC_5H_{11}]^+$), 546.2 (46%; MH^+), 563.2 (86%; MNH_4^+)

6-Methoxy-1-[10-(pentylsulfonyl)decyl]-2-(phenylsulfonyl)-1,2,3,4.tetrahydroisoquinoline (**110**)

Preparation from 6-methoxy-1-((10-pentylsulfonyl)decyl)-1,2,3,4-tetrahydroisoquinoline (**102**) (100 mg; 0.23 mmol) and phenylsulfonyl chloride (88 mg; 0.5 mmol) following the procedure described for **109**. The product was purified by column chromatography (SiO_2; PE/ ethyl acetate 5/1 → 2/1, v/v).

Colourless oil; yield: 120 mg (91%)

$C_{31}H_{47}NO_5S_2$ (577.29)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.92 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.20-1.52 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-SO_2-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.55-1.91 (6H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-, -CH-C$\underline{H}_2$-), 2.41-2.50 (2H; m; C$\underline{H}_2$-phenyl), 2.90-2.99 (4H; t; ^3J = 8.2 Hz; C$\underline{H}_2$-SO_2-C$\underline{H}_2$-), 3.39-3.51 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H),; 3.71 (3H; s; -O-C$\underline{H}_3$), 3.79-3.89 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 4.88-4.95 (1H; m; -C$\underline{H}$-), 6.35-6.38 (1H; d; ^5J = 2.6 Hz; THIQ-$\underline{H}^5$), 6.66-6.71 (1H; d(d); ^3J = 8.4 Hz; ^5J = 2.6 Hz; THIQ-$\underline{H}^7$), 6.92-6.97 (1H; d; ^3J = 8.5 Hz; THIQ-$\underline{H}^8$), 7.27-7.35 (2H; t; ^3J = 7.5 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.38-7.45 (1H; t; ^3J = 7.4 Hz; phenyl-$\underline{H}^4$), 7.67-7.72 (2H; d; ^3J = 7.9 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$)

MS (CI, NH_3): m/z = 438.1 (100%; $[MH - SO_2Ph]^+$), 578.2 (2.3%; MH^+), 595.2 (57%; MNH_4^+)

1-(10-(Pentylthio)decyl)-2-(phenylsulfonyl)-1,2,3,4-tetrahydroisoquinolin-6-ol (**111**)

Preparation from 6-methoxy-1-[10-(pentylthio)decyl]-2-(phenylsulfonyl)-1,2,3,4-tetrahydroisoquinoline (**109**) (80 mg, 0.15 mmol) and BBr_3 (1M stock solution in DCM; 0.45 mL; 0.45 mmol) following the demethylation procedure described for **24**. The reaction mixture was stirred for 2 h. Purification by column chromatography (PE/ethyl acetate 3/1 v/v)

Colourless resin; yield: 55 mg (69 %)

$C_{30}H_{45}NO_3S_2$ (531.81)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.90 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.20-1.50 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-S-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.51-1.83 (6H; m; -C$\underline{H}_2$-CH_2-S-CH_2-C$\underline{H}_2$-, -CH-C$\underline{H}_2$-), 2.39-2.56 (6H; m; C$\underline{H}_2$-phenyl, -C$\underline{H}_2$-S-C$\underline{H}_2$-), 3.37-3.50 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.78-3.89 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 4.85-4.93 (1H; m; -C$\underline{H}$-), 6.30-6.34 (1H; d; ^5J = 2.1 Hz; THIQ-$\underline{H}^5$), 6.57-6.64 (1H; d(d); ^3J = 8.3 Hz; ^5J = 2.4 Hz; THIQ-$\underline{H}^7$), 6.85-6.92 (1H; d; ^3J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.27-7.36 (2H; t; ^3J = 7.5 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.38-7.47 (1H; t; ^3J = 7.4 Hz; phenyl-$\underline{H}^4$), 7.66-7.74 (2H; d; ^3J = 7.3 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$)

MS (CI, NH_3): m/z = 447.2 (100%; [MNH_4 – SC_5H_{11}]$^+$), 532.2 (59%; MH^+), 549.2 (96%; MNH_4^+)

1-[10-(Pentylsulfonyl)decyl]-2-(phenylsulfonyl)-1,2,3,4-tetrahydroisoquinolin-6-ol (**112**)

Preparation from 6-methoxy-1-[10-(pentylsulfonyl)decyl]-2-(phenylsulfonyl)-1,2,3,4-tetrahydroisoquinoline (**110**) (80 mg, 0.14 mmol) and BBr_3 (1M stock solution in DCM; 0.45 mL; 0.45 mmol) following the demethylation procedure described for **24**. The reaction mixture was stirred for 3 h. Purification by column chromatography (DCM/methanol 30/1 v/v).

Colourless resin; yield: 50 mg (63 %)

$C_{30}H_{45}NO_5S_2$ (563.81)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.92 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.18-1.52 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-SO_2-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.58-1.91 (6H; m; -C$\underline{H}_2$-CH_2-SO_2-CH_2-C$\underline{H}_2$-, -CH-C$\underline{H}_2$-), 2.38-2.47 (2H; m; C$\underline{H}_2$-phenyl), 2.90-2.99 (4H; t; ^3J = 8.1 Hz; C$\underline{H}_2$-SO_2-C$\underline{H}_2$-), 3.38-3.52 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.76-3.89 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 4.85-4.93 (1H; m; -C$\underline{H}$-), 6.30-6.35 (1H; d; ^5J = 2.2 Hz; THIQ-$\underline{H}^5$), 6.58-6.64 (1H; d(d); ^3J = 8.3 Hz; ^5J = 2.4 Hz; THIQ-$\underline{H}^7$), 6.85-6.92 (1H; d; ^3J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.27-7.36 (2H; t; ^3J = 7.5 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.38-7.46 (1H; t; ^3J = 7.4 Hz; phenyl-$\underline{H}^4$), 7.66-7.74 (2H; d; ^3J = 7.9 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$)

MS (CI, NH_3): 564.2 (1%; MH^+), 581.3 (58%; MNH_4^+)

HR-MS (EI): calculated: 563.2739
found: 563.2727
HPLC: Gradient: 0-20min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 20-40 min: 95/5
RT = 22.95 min; purity: 96%

6.1.2.3.4 Oxidation of the Side Chain Sulfur to the Sulfoxide Function

2,2,2-Trifluoro-1-[6-hydroxy-1-[10-(pentylsulfinyl)decyl]-1,2,3,4-tetrahydroisoquinolin--2-yl]ethanone (**113**)

Preparation from 2,2,2-trifluoro-1-[6-hydroxy-1-[10-(pentylthio)decyl]-1,2,3,4-tetrahydro-isoquinolin-2-yl]ethanone (**107**) (60 mg, 0.12 mmol) and H_2O_2 (30%; 30µL, 0.25 mmol) following the procedure described for **86**. The product was purified by column chromatography (SiO_2; DCM/methanol 20/1, v/v).

Colourless resin; yield: 55 mg (91%)

$C_{26}H_{40}F_3NO_3S$ (503.27)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = = 0.91 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.18-1.54 (18H; m; -(C$\underline{H}_2$)$_7$-(CH$_2$)$_2$-SO-(CH$_2$)$_2$-(C$\underline{H}_2$)$_2$-), 1.69-1.89 (6H; m; -C$\underline{H}_2$-CH$_2$-SO-CH$_2$-C$\underline{H}_2$-, -CH-C$\underline{H}_2$-), 2.58-3.01 (6H; m; -C$\underline{H}_2$-SO-C$\underline{H}_2$-, -C$\underline{H}_2$-phenyl), 3.56-3.68 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.89-4.00 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 5.38-5.40 (1H; m; -C$\underline{H}$-), 6.59-6.63 (1H; d; ^5J = 2.0 Hz; THIQ-$\underline{H}^5$), 6.70-6.75 (1H; d(d); ^3J = 8.2 Hz; ^5J = 2.0 Hz; THIQ-$\underline{H}^7$), 6.93-6.99 (1H; d; ^3J = 8.4 Hz; THIQ-$\underline{H}^8$)

MS (CI, NH_3): 504.2 (100%; MH^+)
HR-MS (EI): calculated: 503.2681
found: 503.2676
HPLC: Gradient: 0-20 min: MeCN/0.025 % TFA (aq) 50/50 to 95/5; 20-40 min: 95/5
RT = 19.70 min; purity: 98%

1-[10-(Pentylsulfinyl)decyl]-2-(phenylsulfonyl)-1,2,3,4-tetrahydroisoquinolin-6-ol (**114**)

Preparation from 1-[10-(pentylthio)decyl]-2-(phenylsulfonyl)-1,2,3,4-tetrahydroiso-quinolin-6-ol (**111**) (50 mg, 0.09 mmol) and H_2O_2 (30%; 35 µL, 0.20 mmol) following the

procedure described for **86**. The product was purified by column chromatography (SiO_2; DCM/methanol 20/1, v/v).

Colourless resin; yield: 30 mg (67%)

$C_{30}H_{45}NO_4S_2$ (547.81)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 0.91 (3H; t; ^3J = 7.0 Hz; -CH_2-C$\underline{H}_3$), 1.14-1.53 (18H; m; -(C$\underline{H}_2$)$_7$-(CH_2)$_2$-SO-(CH_2)$_2$-(C$\underline{H}_2$)$_2$-), 1.58-1.88 (6H; m; -C$\underline{H}_2$-CH_2-SO-CH_2-C$\underline{H}_2$-, -CH-C$\underline{H}_2$-), 2.30-2.55 (2H; m; C$\underline{H}_2$-phenyl), 2.56-2.79 (4H; m; C$\underline{H}_2$-SO-C$\underline{H}_2$-), 3.42-3.54 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 3.68-3.79 (1H; m; -C$\underline{H}_2$-NH- 1 diastereotopic H), 4.83-4.92 (1H; m; -C$\underline{H}$-), 6.32-6.39 (1H; d; ^5J = 2.2 Hz; THIQ-$\underline{H}^5$), 6.58-6.66 (1H; d(d); ^3J = 8.3 Hz; ^5J = 2.0 Hz; THIQ-$\underline{H}^7$), 6.83-6.90 (1H; d; ^3J = 8.3 Hz; THIQ-$\underline{H}^8$), 7.28-7.36 (2H; t; ^3J = 7.7 Hz; phenyl-$\underline{H}^3$, -$\underline{H}^5$), 7.38-7.46 (1H; t; ^3J = 7.4 Hz; phenyl-$\underline{H}^4$), 7.67-7.74 (2H; d; ^3J = 7.7 Hz; phenyl-$\underline{H}^2$, -$\underline{H}^6$)

MS (CI, NH_3): 504.2 (100%; MH^+)

HR-MS (EI): calculated: 547.2790
found: 547.2783

HPLC: Gradient: 0-20 min: MeCN/0.025 % TFA (aq) 20/80 to 95/5; 20-35 min: 95/5
RT = 23.90 min; purity: 97%

6.1.2.4 Unsuccessful Approach to 1-Alkyl-2-aryltetrahydroisoquinolines

*6.1.2.4.1 Preparation of 6-Methoxy-2-(4-methoxyphenyl)-3,4-dihydroisoquinolin-1-on (**116**)*

6-Methoxy-3,4-dihydro-2H-isoquinolin-1-one (**115**)

At 60 °C NaN_3 (0.65 g; 10 mmol) was added slowly to a stirred solution of 5-methoxy-2,3-dihydroinden-1-one (1.62 g, 10 mmol) and conc. H_2SO_4 (6.0 mL, 110 mmol) in toluene (20 mL). A strong gas evolution started. After 1.5 h the gas evolution ceased. Then a second portion of NaN_3 (160 mg, 2.5 mmol) was added and the the mixtute was stirred for another hour at given temperature. The reaction mixture was cooled down and the toluene layer was discarded. The acidic phase was poured into ice water (150 mL) and neutralized with sat. $NaHCO_3$ solution. The organic components were

extracted with three portions of DCM (3. ×.100 mL); the combined organic extracts were dried over Na_2SO_4 and evaporated. Purification by column chromatography (SiO_2; DCM/methanol 20/1, v/v) followed by crystallisation from acetone.

Light yellow crystals; yield: 1.24 g (70%)

Melting point: 136 °C (Lit: 139-140 °C (Tomita et al., 1969))

$C_{10}H_{11}NO_2$ (177.20)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 2.96 (2H; t; ^3J = 6.6 Hz; DHIQ-H^3), 3.55 (2H; d(t); ^3J_t = 6.8 Hz; ^3J_d = 2.8 Hz; DHIQ-H^4), 3.85 (3H; s; -O-C$\underline{H}_3$), 6.69-6.72 (1H; d; ^3J = 2.5 Hz; DHIQ-$\underline{H}^5$), 6.83-6.89 (1H; dd; ^3J = 8.7 Hz; ^5J = 2.6 Hz; DHIQ-$\underline{H}^7$), 7.99-8.04 (1H; d; ^3J = 8.6 Hz; DHIQ-$\underline{H}^8$)

6-Methoxy-2-(4-methoxyphenyl)-3,4-dihydroisoquinolin-1-on (**116**)

A solution of 4-bromoanisol (1.25 g, 6.7 mmol), CuI (0.255 g, 1.34 mmol), K_2CO_3 (0.185 g, 1.34 mmol) and 6-methoxy-3,4-dihydro-2H-isoquinolin-1-one (**115**) (0.327 g, 1.34 mmol) in abs. DMF (2 mL) was stirred 70 h at 100 °C. After cooling to room temperature the reaction mixture was worked up by the addition of diluted NH_3 solution (100 mL) and the organic components were extracted with three portions of ethyl acetate (3 × 50 mL). The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated. Purification by column chromatography (SiO_2; DCM/methanol 80/1, v/v)

Light yellow crystals; yield: 280 mg (74 %)

Melting point: 132-133 °C

$C_{17}H_{17}NO_3$ (283.33)

[^{1}H] NMR ($CDCl_3$):

δ (ppm) = 3.09 (2H; t; ^3J = 6.5 Hz; DHIQ-H^3), 3.82 (3H; s; -O-C$\underline{H}_3$), 3.86 (3H; s; -O-C$\underline{H}_3$), 3.92 (2H; t; ^3J = 6.5 Hz; DHIQ-H^4), 6.69-6.72 (1H; d; ^5J = 2.5 Hz; DHIQ-$\underline{H}^5$), 6.85-6.90 (1H; d(d); ^3J = 9.0 Hz; ^5J = 2.5 Hz; DHIQ-$\underline{H}^7$), 6.90-6.95 (2H; d; AA'BB'; ^3J = 9.0 Hz; phenyl-$\underline{H}$), 7.25-7.30 (2H; d; AA'BB'; ^3J = 9.0 Hz; phenyl-$\underline{H}$), 8.07-8.12 (1H; d; ^3J = 8.6 Hz; DHIQ-$\underline{H}^8$)

*6.1.2.4.2 Attempted Grignard Reaction of 6-Methoxy-2-(4-methoxyphenyl)-3,4-dihydro-isoquinolin-1-on (**116**) with Alkylmagnesiumbromides.*

The Grignard reaction was performed as described for compound **14**. For ethylmagnesiumbromide and decylmagnesiumbromide no reaction with 3-4-dihydroisoquinolin-1-on **116** was observed (TLC).

6.1.2.5 Semipreparative Separation of Selected Tetrahydroisoquinolines by Chiral HPLC

Compounds **92**, **93** and **94**, obtained as racemates from chemical synthesis were submitted to a chiral HPLC separation to obtain the pure enantiomers on a semipreperative scale. The HPLC system used is listed under materials and general methods. The resolution (R_s) of the two peaks corresponding to the respective enantiomers was estimated according to:

$R_s = 2 \cdot (RT_2 - RT_1) / (w_{b1} + w_{b2})$, where $RT_2 > RT_1$ and $w_{b1,2}$ = baseline peak widths.

For this purpose 100 µL of a 50 µM solution of each compound (**92**-**94**) were injected. A Jasco CD1595 detector (Jasco, Gross-Umstadt, Germany) was used to assign CD signals to the enantiomers. For preparative separation, the pure enantiomers were accumulated by collecting the cerrespondung peaks from multiple injections. The injection volume for each preparative separation was 100 µL. Further separation conditions for each compound are depicted in table C14.
After separation the enantiomers were dissolved in ethanol p.a (200-400 µL); small samples of these stock solutions were submitted to an enantiomeric purity check by the chiral HPLC method mentioned above. Another sample of each stock solution was analyzed using a C-18 RP-HPLC column (Eurospher; see materials and methods) to determine the concentrations of the enantiomers in the prepared solutions. Solutions of the corresponding racemates with known concentrations were used as standards. The absolute concentrations were determined by peak-integration and comparison to the standards. Appropriate dillutionds of the stock solutions were then used in the binding assay.

Table C14: Conditions for semipreparative separation of enantiomers

Comp.	Gradient methanol/0.05% TFA	peaks	Concentration of injected solution
92	0 min: 40/60 20 min: 50/50	**92a**: 5.9 min **92b**: 9.5 min	0.5 mM
93	0 min: 65/35 25 min: 75/25	**93a**: 7.5 min **93b**: 11.5 min	1 mM
94	isocratic 90/10	**94a**: 6.3 min **94b**: 8.7 min	1 mM

*two successive separations

6.2 Pharmacology

6.2.1 Radiometric Binding Assay

Preparation of recombinant human estrogen receptor (ER) proteins

The recombinant full-length human receptor proteins ERα and ERβ (Invitrogen, Karlsruhe, Germany) were delivered as a solution in binding buffer in concentrations of 2600 nM and 1800 nM respectively. In order to prevent frequent freeze- and thaw cycles the whole protein solution was aliquoted in portions of 6.92 µL in the case of ERα and 10.0 µL in the case of ERβ and stored at –80 °C.

Prior to use the aliquots of ERα and ERβ were diluted up to 1mL with ER binding buffer (10 mM Tris-HCl pH 7.5, 10% glycerol, 2 mM DTT, 1% BSA) by gentle pipetting. Another dilution step (gentle pipetting, no vortexing to prevent protein aggregation) with ER binding buffer up to 20 mL provided the receptor concentration of 0.9 nM, that was used in the binding assay.

Preparation of HAP slurry

10 g of hydroxylappatite (HAP fast flow; Merck-Calbiochem, Darmstadt, Germany) was mixed vigorously with 60 mL of an equilibration buffer (50 mM Tris-HCl, pH 7.4). After 10 minutes the supernatant was decanted and the process repeated ten more times using 60 mL of the equilibration buffer. The HAP slurry was equilibrated at 4 °C

overnight before being adjusted to 50% (v/v) with the equilibration buffer. The slurry is stable at +4 °C over several months.

Preparation of compound solutions

For each compound a 10 mM stock solution in ethanol p.a. was prepared and stored at -20 °C. By 1/100 dilution 100 µM solutions in pure ethanol were prepared. For ≤10 µM concentrated solutions that were directly used in the assay, ER binding buffer containing maximal 10% (v/v) ethanol was used as solvent. All buffer containing solutions were prepared freshly on the day of the experiment.

The commercialy available [^{3}H]17β-estradiol stock solution (GE Healthcare, München, Germany) was diluted to a 200 nM solution in ethanol p.a. that was stored at -20 °C. For the assay the 200 nM solution was freshly diluted 1/100 in binding buffer.

The binding experiment

Each test compound and reference compound was tested twice (independently) in six different concentrations. The experiment was performed in triplicate for each concentration. As control for the concentration of the radioligand and as standard to calculate the relative binding affinity, a binding curve for 17β-estradiol (Sigma, München, Germany) was generated in each experiment.

The assay was performed in 1.5 mL cups (Sarstedt, Nümbrecht, Germany) according to scheme F1. The respective final concentrations of a test compound were achieved by appropriate dilution of the prepared solutions.

All reaction vessels were incubated for 16-20 hours at 4 °C with shaking. To bind the ligand-receptor-complex, 100 µL of HAP slurry was added to each vial. The suspensions were incubated 15 min while vortexing three times. After addiditon of 1mL wash buffer (40 mM Tris-HCl, pH 7.4; for ERα the buffer additionaly contained 100 mM KCl), the vials were vigorously vortexed and centrifuged at 4000 rpm for 10 min. The supernatant was discarded by suction. This washing step was once repeated. After the second wash the HAP pellet was resuspended in 400 µL of EtOH and pipetted into a minivial containing 3 mL of scintillation fluid (Roth, Karlsruhe, Germany). The bound radioactivity was counted in a LS6500 liquid scintillation beta counter (Beckmann Instruments, München, Germany).

Table C15: Pipetting scheme for the binding assay

solutions	Unspec. Binding [µL]	Control [µL]	Sample [µL]
binding buffer		50	n[a]
Testcompound[b]			50-n
0.9 nM receptor protein[c]	100	100	100
1µM 17β-estradiol[d]	50		
2nM [^{3}H]-17β-estradiol[e]	50	50	50

[1]n is the volume of binding buffer to make a defined final concentration of the testcompound and a volume of 200 µL; [b]variable concentrations; final concentrations by appropriate dilution; [c]final concentration: 0.45 nM; [d]final concentration: 250 nM; [e]final concentration: 0.5 nM

Data analysis

From the difference of the total binding and the unspecific binding the specific binding for each concentration of tested compounds was calculated.

For the determination of the IC_{50} values a logit-log-transformation for at least 4 datapoints in the range of [20 % ≤ (spec. bound radioactivity) ≤ 80 %] was performed.

The logit was calculated as follows:

$$\text{logit} = \log \{\text{displacement [\%]} / (100\% - \text{displacement [\%]})\}$$

The logit values were ploted semilogarithmic as a function of the concentration and linearized by linear regression. The IC_{50} values were derived from the intersections of the straight lines with the x-axis.

For estimation of the relative binding affinities (RBA) the IC_{50} values of tested compounds were compared with that of 17-β-estradiol:

$$\text{RBA} = IC_{50}\ (17\beta\text{-estradiol}) / IC_{50}\ (\text{test compound}) \cdot 100$$

6.2.2 Luciferase Assay

The MCF-7/2a cell line

The MCF-7/2a cell line is an estrogen receptor positive MCF-7 sub line that was stably transfected with the luciferase reporter plasmid 'EREwtc luc' (F. Hafner, University of Regensburg). The cells were used as a tool for the determination of estrogenic and antiestrogenic activities of test compounds *in vitro*.

Cell culture

The MCF-772a cell line was cultivated in Dublecco's modified eagle medium (DMEM, Invitrogen) without phenol red, supplemented with 5 vol% sterile FCS (25 mL), a solution of L-glutamine (Merck; 29.2 mg/mL in sterile PBS; 5 mL per 500 mL) and neomycin (Geneticin®, G-418; Merck; 175 µg / mL added from a 35 mg/mL solution in sterile PBS).

The cells grow as monolayer in 75 cm^2 culture flasks (Becton Dickinson, Franklin Lakes, NY, USA) in a humidified , 5% CO_2 containing atmosphere at 37 °C.

Once a week or shortly before the cells had grown confluent the cells were passaged 1/10 by routine trypsination using 3 mL of a trypsin/EDTA, solution (Boehringer, Mannheim, Germany) per culture flask.

Performance of the luciferase assay

Cells were seeded on 24 well plates (Becton Dickinson) in 0.5 mL of DMEM without FCS and incubated for 48 h or until the cells reached a density of about 40-50%.

The medium was removed by suction and replaced by fresh medium containing the corresponding concentrations of test compound (0.5 mL/well). In the antagonist mode the medium additionaly contained 1nM estradiol as stimulation agent (added as 1µM ethanolic stock solution to the used medium). The solutions of the test compounds in medium were prepared by 400-fold dilution of ethanolic solutions of the compounds. The assay was performed in triplicate for each concentration. For each assay a vehicle control (contained only the respective volume of solvent) and a 17β-estradiol (1nM) control was performed in triplicate. The potent antiestrogens fulvestrant (ICI 182.780; gift of Prof. Dr. M. Schneider; Schering AG, Berlin, Germany) and 4-hydroxytamoxifen (Sigma) were used as references for antiestrogenic activity.

After an incubation period of 48-50 hours, the medium was removed and washed twice with PBS (0.5 mL/well).

Cell lysis and luminescent measurement were performed by two alternative methods:

a) By using a commercial firefly luciferase asay kit (Biotium, Hayward, CA, USA) following the manufacturer's protocol
b) By using a self-prepared buffer system and commercially available D-luciferin (potassium salt; Molecular Imaging Products Company, Ann Arbor, MI, USA)

The majority of the assays were performed according to method b). Method a) mainly served as control for the optimization of method b).

The buffers and solutions for method b) were prepared as follows:

Lysis buffer: 25 mM Tricine (N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Sigma; pH 7.8); 10% (v/v) Glycerol; 2 mM ethyleneglycoltetraacetic acid (EGTA); 1% (v/v) Triton^{TM} X-100 (Serva, Heidelberg, Germany); 5 mM $MgSO_4 \times 7H_2O$, 1 mM dithiotreitol (DTT; Sigma; added directly before use).

Luciferase assay buffer: 25 mM Tricine (pH 7.8); 5 mM $MgSO_4 \times 7H_2O$; 2 mM EGTA; 2 mM ATP (Boehringer, Mannheim, Germany); When indicated, 50µM or 100 µM coenzyme A (CoA; Sigma) were supplemented.

D-Luciferin stock solution: A stock solution of 10 mg/mL was prepared in tricine buffer (pH 7.8) containing 10 mM DTT. Aliquots of 100 µL were stored at -70 °C

60 µL of lysis buffer was added to each well and incubated for 20 min under shaking. The unsoluble remains of the cells agglomerated and formed a pellet. 30 µL of the supernatant were pipetted into a polystyrene tube. A solution of D-luciferin in luciferase assay buffer (0.2 mg/mL; prepared by 1:50 dilution of the D-luciferin stock solution) was automaticly injected by a Lumat LB 9501 luminometer (Berthold, Bad Wildbad, Germany) and the luminescence was integrated over 10 s. The result is given in RLU (relative light units).

Normalisation of the luminescence by total protein content

The results of the luminescence measurement were corrected by the total protein content of each sample, which was quantified by Bradford's protein assay (Bradford, 1976).

95 µl millipore water and 5 µL of cell lysate were pipetted into a polystyrene cuvette (Sarstedt 67742) followed by the addition of 1 mL of Bradford dye reagent (Bio Rad Laboratories, Munich, Germany; 5-fold concentrate, dilluted with millipore water). After 10 min the UV-absorbance of each sample was measured at 595 nm in a UV

spectrophotometer Uvikon 930 (Kontron, Düsseldorf, Germany). To assign the absorption values to the corresponding protein contents a calibration curve using HSA (human serum albumin; Behringwerke, Marburg, Germany) standards in a range of 1-12 µg protein was recorded. 5µL of plain lysis buffer was added to each HSA-sample to exclude any adulteration by the buffer ingredients.

Data analysis

The luminescence was normalized by the protein content for each sample ($RLU/mg_{protein}$). The relative luciferase activity is given as the percent ratio of the respective corrected luciferase activity relative to the luciferase activity induced by 1 nM estradiol that is per definition 100%.

The IC_{50} values were derrived from the semilogarithmic plot of the relative luciferase activity as a function of the molar concentration.

6.2.3 Determination of Antiproliferative Activity

Human breast cancer cell lines

For the determination of antiproliferative activity of synthesized antiestrogens, two different human breast cancer cell lines were used:

- MCF-7 cells grow estrogen dependently and were therefore used to demonstrate estrogen receptor mediated antiproliferative effects of synthesized compounds.
- MDA-MB-231-cells grow hormone independently and were used to test the synthesized compounds with respect to unspecific cytotoxic or cytostatic effects.

Both cell lines were purchased from the American Type Culture Collection, (ATCC; Manassas, VA, USA)

Cell culture

Both cell lines were grown in 75 cm^2 culture flasks (Becton Dickinson) in a humidified, 5% CO_2 containing atmosphere at 37 °C. MCF-7 cells were cultivated in phenol red containing EMEM (Eagle's minimunm essential medium, Sigma), supplemented with 2.2 g of sodium bicarbonate per liter. MDA-MB-231 cells were cultivated in phenol red containing McCoy's 5A medium (Sigma) supplemented with 2.2 g of sodium bicarbonate per liter. In both cases 5 vol % of sterile FCS was added prior to usage.

Cytotoxicity assay

The antiproliferative activity of synthesized compounds and reference compounds fulvestrant (ICI 182.780) and 4-hydroxytamoxifen was determined by the crystal violet assay (Bernhardt et al., 1992).

Cells were seeded in 100 µL of the respective medium at a density of 10 (MDA-MB-231) or 15 (MCF-7) cells per microscopic field (320×, Diavert microscope, Leitz, Wetzlar, Germany) in 96 well flat bottomed microtitration plates (Greiner, Frickenhausen, Germany). After 48 hours the medium was carefully removed by suction and replaced by fresh medium (200 µL/well) containing different concentrations of test compounds, added as 1000 fold concentrated ethanolic solutions. Two vertical rows (16 wells) were arranged for one concentration of a test compound. Control wells (16 wells per plate) contained pure medium with 0.1 vol% ethanol. Positive controls contained 10 µM cisplatin or 10 nM vinblastin.

The cells of one untreated plate were fixed immediately after drug addition to determine the initial cell density. The cells of the treated plates were fixed after various incubation times. For fixation the culture medium was shaken off and replaced by 100 µL of a 2% glutardialdehyde solution (prepared by dillution of a 25% aqueous solution; Merck) in PBS. After 20 min the fixative was replaced by 180µL of PBS and the plates were stored at 4 °C.

At the end of the experiment the cells of all plates were stained simultaneously with 0.02 % crystal violet solution (N-hexamethylpararosanilin HCl in water, 100 µL per well, 25 min incubation). After decanting, remaining dye was removed by repeating washings with deionized water followed by an incubation step (20 min) with water at room temperature. Water was discarded and the plates were patted dry on a cellulose sheet. Cell bound dye was extracted by addition of 200 µL 70 % ethanol and incubated for 3 h at room temperature with permanent shaking. Absorbance was measured at 578 nm using a BioTEK EL309 autoreader (Bad Friedrichshall, Germany) and the average and standard deviation values were calculated. Absorbance values outside of the confinence interval (95%) were not considered for the calculations.

As unit for the growth inhibiting effect, the corrected T/C values were calculated according to:

$$T/C_{corr.}\ [\%] = (T-T_0) / (C-T_0) \cdot 100\ \%$$

T: optical density of tested cultures treated with a test compound

T_0: optical density of cells at the time of compound addition (initial cell density)

C: optical density of vehicle treated cultures (control)

7 References

Airth, R. L.; Rhodes, W. C. and McElroy, W. D., The function of coenzyme A in luminescence. *Biochim Biophys Acta* **1958,** 27, 519-532.

Arcadi, A.; Marinelli, F. and Cacchi, S., Palladium-catalyzed reaction of 2-hydroxyaryl and hydroxyheteroaryl halides with 1-alkynes: an improved route to the benzo[b]furan ring system. *Synthesis* **1986**, (9), 749-751.

Bernhardt, G.; Reile, H.; Birnbock, H.; Spruss, T. and Schonenberger, H., Standardized kinetic microassay to quantify differential chemosensitivity on the basis of proliferative activity. *J Cancer Res Clin Oncol* **1992,** 118, (1), 35-43.

Bhalerao, U. T.; Plattner, J. J. and Rapoport, H., Synthesis of dl-sirenin and dl-isosirenin. *J Am Chem Soc* **1970,** 92, (11), 3429-3433.

Biberger, C., 2-Phenylindole mit schwefelhaltigen Seitenketten. Synthese und biologische Charakterisierung neuer Estrogenantagonisten. Doctoral thesis, Universität Regensburg, Regensburg, **1996**

Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **1976,** 72, 248-254.

Brasier, A. R., Reporter System Using Firefly Luciferase. In: *Current Protocols in Molecular Biology*. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidmann, J.G., Smith, J.A. and Struhl, K., eds., Wiley-VCH: 1990, Vol. 1, 9.6.10-14.

Chesworth, R.; Zawistoski, M. P.; Lefker, B. A.; Cameron, K. O.; Day, R. F.; Mangano, F. M.; Rosati, R. L.; Colella, S.; Petersen, D. N.; Brault, A.; Lu, B.; Pan, L. C.; Perry, P.; Ng, O.; Castleberry, T. A.; Owen, T. A.; Brown, T. A.; Thompson, D. D. and DaSilva-Jardine, P., Tetrahydroisoquinolines as subtype selective estrogen agonists/antagonists. *Bioorg Med Chem Lett* **2004,** 14, (11), 2729-2733.

Collini, M. D.; Kaufman, D. H.; Manas, E. S.; Harris, H. A.; Henderson, R. A.; Xu, Z. B.; Unwalla, R. J. and Miller, C. P., 7-Substituted 2-phenyl-benzofurans as ER beta selective ligands. *Bioorg Med Chem Lett* **2004,** 14, (19), 4925-4929.

de Wet, J. R.; Wood, K. V.; DeLuca, M.; Helinski, D. R. and Subramani, S., Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol* **1987,** 7, (2), 725-737.

Ding, M. W.; Shi, D. Q.; Xiao, W. J.; Huang, W. F. and Wu, T. J., Studies on the Wittig reaction. XXII. A convenient synthesis of w-azolylalkyltriphenyl phosphonium salts

and their stereoselectivity in the Wittig reaction. *Phosphorus, Sulfur Silicon Relat Elem* **1995,** 102, (1-4), 59-63.

Doleschall, G. and Toth, G., The degradation of carboxylic acids into aldehydes. Regioselective α-acetoxylation of 1,2,4-triazolium salts with diacetoxyiodate(I) anion. *Tetrahedron* **1980,** 36, (11), 1649-1665.

Erber, S., Synthese und Testung mammatumorhemmender Derivate des 2-Phenylindols, -benzo[b]furans und -benzo[b]thiophens. Doctoral thesis, Universität Regensburg, Regensburg, **1989**

Evano, G.; Schaus, J. V. and Panek, J. S., A Convergent Synthesis of the Macrocyclic Core of Cytotrienins: Application of RCM for Macrocyclization. *Organic Letters* **2004,** 6, (4), 525-528.

Ford, S. R.; Buck, L. M. and Leach, F. R., Does the sulfhydryl or the adenine moiety of CoA enhance firefly luciferase activity? *Biochim Biophys Acta* **1995,** 1252, (2), 180-184.

Fraga, H.; Fernandes, D.; Fontes, R. and Esteves da Silva, J. C., Coenzyme A affects firefly luciferase luminescence because it acts as a substrate and not as an allosteric effector. *FEBS J* **2005,** 272, (20), 5206-5216.

Golob, T.; Liebl, R. and von Angerer, E., Sulfamoyloxy-substituted 2-phenylindoles: antiestrogen-based inhibitors of the steroid sulfatase in human breast cancer cells. *Bioorg Med Chem* **2002,** 10, (12), 3941-3953.

Hart, D. J. and Mannino, A., Synthesis of defucogilvocarcin V isosteres via MAD-mediated conjugate addition of carbanions to naphthoquinone ketals. *Tetrahedron* **1996,** 52, (11), 3841-3856.

Hoffmann, J. and Sommer, A., Steroid hormone receptors as targets for the therapy of breast and prostate cancer-recent advances, mechanisms of resistance, and new approaches. *J Steroid Biochem Mol Biol* **2005,** 93, (2-5), 191-200.

Horillo-Martinez, P.; Hultzsch, K. C.; Gil, A. and Branchadell, V., Base-catalyzed anti-Markovnikov hydroamination of vinylarenes - scope, limitations and computational studies. *Eur J Org Chem* **2007**, (20), 3311-3325.

Kashdan, D. S.; Schwartz, J. A. and Rapoport, H., Synthesis of 1,2,3,4-tetrahydroisoquinolines. *J Org Chem* **1982,** 47, (13), 2638-2643.

Kometani, T.; Watt, D. S. and Ji, T., Iodination of phenols using chloramine T and sodium iodide. *Tetrahedron Lett* **1985,** 26, (17), 2043-2046.

Kricka, L. J. and De Luca, M., Effect of solvents on the catalytic activity of firefly luciferase. *Arch Biochem Biophys* **1982,** 217, (2), 674-681.

Leichtl, S., Synthese und biologische Charakterisierung neuer Antiöstrogene vom 2-Phenylindol-, benzo[b]furan und benzo[b]thiophentyp. Doctoral thesis, Universität Regensburg, Regensburg, **1994**

Manas, E. S.; Unwalla, R. J.; Xu, Z. B.; Malamas, M. S.; Miller, C. P.; Harris, H. A.; Hsiao, C.; Akopian, T.; Hum, W. T.; Malakian, K.; Wolfrom, S.; Bapat, A.; Bhat, R. A.; Stahl, M. L.; Somers, W. S. and Alvarez, J. C., Structure-based design of estrogen receptor-beta selective ligands. *J Am Chem Soc* **2004,** 126, (46), 15106-15119.

Maryanoff, B. E.; Reitz, A. B. and Duhl-Emswiler, B. A., Stereochemistry of the Wittig reaction. Effect of nucleophilic groups in the phosphonium ylide. *J Am Chem Soc* **1985,** 107, (1), 217-226.

Meyer, T.; Koop, R.; von Angerer, E.; Schonenberger, H. and Holler, E., A rapid luciferase transfection assay for transcription activation effects and stability control of estrogenic drugs in cell cultures. *J Cancer Res Clin Oncol* **1994,** 120, (6), 359-364.

Miller, C. P.; Collini, M. D.; Kaufman, D. H.; Morris, R. L.; Singhaus, R. R., Jr.; Ullrich, J. W.; Harris, H. A.; Keith, J. C., Jr.; Albert, L. M. and Unwalla, R. J., Substituted 2-phenyl benzofurans active as estrogenic agents, and their preparation, pharmaceutical compositions, and use. PCT Int. Appl. WO 03051860, **2003**

Mortensen, D. S.; Rodriguez, A. L.; Carlson, K. E.; Sun, J.; Katzenellenbogen, B. S. and Katzenellenbogen, J. A., Synthesis and biological evaluation of a novel series of furans: ligands selective for estrogen receptor alpha. *J Med Chem* **2001,** 44, (23), 3838-3848.

Nagarajan, K.; Talwalker, P. K.; Kulkarni, C. L.; Shah, R. K.; Shenoy, S. J. and Prabhu, S. S., Antiimplantation agents: part II - 1,2-diaryl-1,2,3,4-tetrahydroisoquinolines. *Indian J Chem, Sect B: Org Chem Incl Med Chem* **1985,** 24B, (1), 83-97.

Rapson, W. S. and Robinson, R., Synthesis of substances related to the sterols. VII. *J Chem Soc* **1935**, 1533-1543.

Renaud, J.; Bischoff, S. F.; Buhl, T.; Floersheim, P.; Fournier, B.; Geiser, M.; Halleux, C.; Kallen, J.; Keller, H. and Ramage, P., Selective Estrogen Receptor Modulators with Conformationally Restricted Side Chains. Synthesis and Structure-Activity Relationship of ERα-Selective Tetrahydroisoquinoline Ligands. *J Med Chem* **2005,** 48, (2), 364-379.

Renaud, J.; Bischoff, S. F.; Buhl, T.; Floersheim, P.; Fournier, B.; Halleux, C.; Kallen, J.; Keller, H.; Schlaeppi, J.-M. and Stark, W., Estrogen Receptor Modulators: Identification and Structure-Activity Relationships of Potent ERα-Selective Tetrahydroisoquinoline Ligands. *J Med Chem* **2003,** 46, (14), 2945-2957.

Sugahara, M. and Ukita, T., A facile copper-catalyzed Ullmann condensation: N-arylation of heterocyclic compounds containing an -NHCO- moiety. *Chem Pharm Bull* **1997,** 45, (4), 719-721.

Tomita, M.; Minami, S. and Uyeo, S., The Schmidt reaction with benzocycloalkenones. *Journal of the Chemical Society [Section] C: Organic* **1969**, (2), 183-188.

von Angerer, E.; Biberger, C.; Holler, E.; Koop, R. and Leichtl, S., 1-Carbamoylalkyl-2-phenylindoles: relationship between side chain structure and estrogen antagonism. *J Steroid Biochem Mol Biol* **1994,** 49, (1), 51-62.

von Angerer, E.; Knebel, N.; Kager, M. and Ganss, B., 1-(Aminoalkyl)-2-phenylindoles as novel pure estrogen antagonists. *J Med Chem* **1990,** 33, (9), 2635-2640.

von Angerer, E.; Prekajac, J. and Strohmeier, J., 2-Phenylindoles. Relationship between structure, estrogen receptor affinity, and mammary tumor inhibiting activity in the rat. *J Med Chem* **1984,** 27, (11), 1439-1447.

Walter, G., Synthese und biologische Charakterisierung neuer Antiestrogene und Steroidsulfataseinhibitoren auf 2-Phenylindol- und Stilbenbasis. Doctoral thesis, Universität Regensburg, Regensburg, **2002**

Webster, J. J.; Chang, J. C.; Manley, E. R.; Spivey, H. O. and Leach, F. R., Buffer effects on ATP analysis by firefly luciferase. *Anal Biochem* **1980,** 106, (1), 7-11.

White, E. H.; Rapaport, E.; Hopkins, T. A. and Seliger, H. H., Chemi- and bioluminescence of firefly luciferin. *J Am Chem Soc* **1969,** 91, (8), 2178-2180.

Zimmermann, J., Furan- and pyran-based heterocycles as subtype-selective ligands of the estrogen receptor. Synthesis and biological characterisation. Doctoral thesis, Universität Regensburg, Regensburg, **2005**

Zimmermann, J.; Liebl, R. and von Angerer, E., 2,5-Diphenylfuran-based pure antiestrogens with selectivity for the estrogen receptor alpha. *J Steroid Biochem Mol Biol* **2005,** 94, (1-3), 57-66.

D Expression, Function and Cross-Talk of Estrogen and NPY Y_1 Receptors in Human Breast Cancer Cells

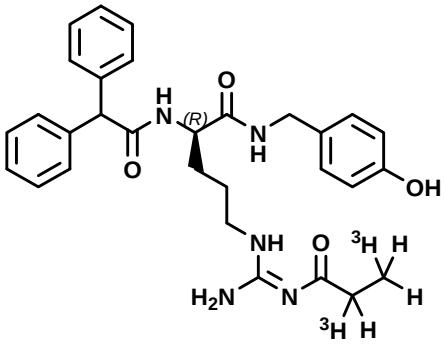
1 Introduction

NPY, a 36 amino acid peptide, is one of the most abundant peptides in the central and peripheral nervous system of mammals, involved in numerous (patho)physiological processes such as food intake, regulation of blood pressure, hormone secretion, anxiety and memory function (Pedrazzini et al., 2003).

In humans NPY exerts its biological effects by interaction with at least four distinct G-protein coupled receptors designated Y_1, Y_2, Y_4, and Y_5 (Michel et al., 1998). The NPY Y_1 receptor (Y_1R) subtype was the first NPY binding receptor to be cloned (Larhammar et al., 1992). Its constitutive expression and functionality in human erythroleukemia (HEL) cells (Motulsky and Michel, 1988) and in SK-N-MC neuroblastoma cells (Aakerlund et al., 1990) is well established. Y_1 and Y_2 receptors were recently reported to be expressed in several human cancers and were proposed as potential tumor markers (Körner and Reubi, 2007). Mammary carcinomas revealed a 85 % incidence of Y_1R expression, whereas Y_2R was shown to be the less expressed subtype (Reubi et al., 2001). An estrogen-induced expression of NPY Y_1R mRNA in MCF-7 breast cancer cells was shown in a differential screening study (Kuang et al., 1998). Later investigations confirmed the up-regulation of the Y_1R mRNA after estrogen treatment, and suggested a role of the Y_1R in cell signaling and proliferation (Amlal et al., 2006).

Derived from the *(R)*-argininamide BIBP3226 (Rudolf et al., 1994), [^{3}H]-UR-MK114 (cf. Figure D1) was recently synthesized and characterized as a highly potent and selective NPY Y_1R antagonist (Keller et al., 2008). The development of this novel Y_1R-selective tritium labeled antagonist enabled us to quantify Y_1R protein expression in a radioligand binding assay using adherent live cells. In the present study different subclones of MCF-7 breast cancer cells with varying estrogen receptor (ER) content were analyzed with respect to a possible correlation between ER and Y_1R expression. Furthermore, the influence of ER agonists and antagonists on expression of the functional Y_1R protein was investigated with focus on concentration-response relationships and ER subtype

specificity. The applicability of the Y_1R as endogenous gene reporter for the functional characterization of estrogens and antiestrogens was evaluated in MCF-7 cells.



[^{3}H]-UR-MK114

Figure D1: Structure of the NPY Y_1R-selective radioligand [^{3}H]-UR-MK114

To investigate the functional activity of the Y_1 receptor in MCF-7 breast cancer cells, the effect of NPY on intracellular calcium levels and on adenylyl cyclase activity were studied with appropriate assays. Further investigations were addressed to a possible Y_1R-mediated effect of NPY on the proliferation of MCF-7 cells and on ER-mediated transcriptional activity in a transfected MCF-7/2a subline expressing an estrogen-responsive luciferase reporter (see section C).

2 Results and Discussion

2.1 Characterization of Breast Cancer Cells with Respect to Antiestrogen Sensitivity, ER and NPY Y_1R Expression

Cultured MCF-7 breast cancer cells are known to show variable sensitivities against estrogen and antiestrogen treatment. Three different MCF-7 subclones (designated MCF-7 (a-c)) were separately cultivated in our laboratory, showing distinct differences in growth kinetics and response against the antiestrogen 4-hydroxytamoxifen. As the differential antiestrogen sensitivity was supposed to depend on the level of ER expression, and as Y_1R expression was estrogen-induced, we analyzed the three different MCF-subclones for expression of the Y_1R and ER proteins using appropriate radioligand binding assays.

The estrogen receptor content was determined from cytosols of breast cancer cells by a binding assay using the radioligand [^{3}H]-17β-estradiol. Representative curves for estrogen receptor saturation binding in the presence of increasing concentrations of [^{3}H]-17β-estradiol are depicted in Figure D2. ER content was estimated from the B_{max} values of the saturation curves and related to the total protein content of the corresponding cytosols.

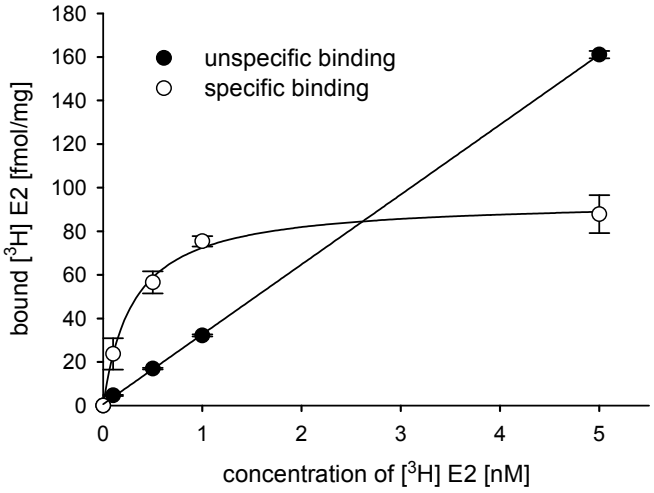


Figure D2: Representative saturation curves for unspecific and specific binding of [^{3}H]-17-β-estradiol to a cytosol of MCF-7 (a) breast cancer cells.

Growth kinetics of the three identically treated MCF-7 subclones and MDA-MB-231 cells in the presence and absence of 4-hydroxytamoxifen and the corresponding radiometrically determined ER contents are depicted in Figure D3.

MCF-7 (a) was identified as a subclone with strong response to 4-OH-tamoxifen treatment and the highest ER expression (95 fmol/$mg_{protein}$) among the studied MCF-7 subclones. The two other subclones, MCF-7 (b) and MCF-7 (c) revealed considerably decreased sensitivities against 4-hydroxytamoxifen, whereas the extent of drug resistance correlated with a decrease of the ER contents (30 and 46 fmol/mg, respectively). The latter MCF-7 subclones were also characterized by a faster growth of the untreated control cultures compared to the MCF-7 (a) subclone. As expected, in MDA-MB-231 cells the ER expression was below the limit of detection (10 fmol per mg of protein) and cell proliferation did not respond to 4-hydroxytamoxifen.

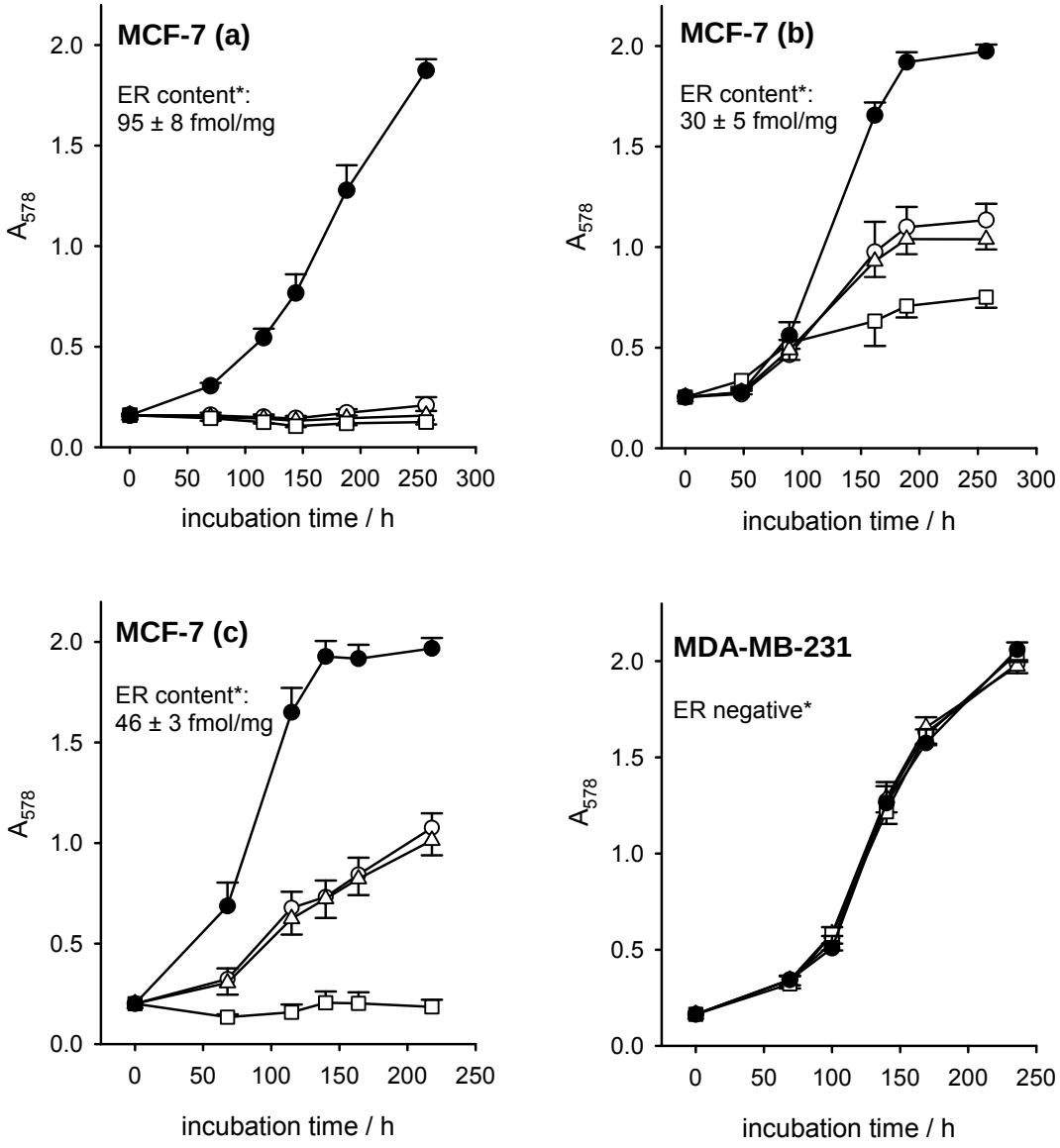


Figure D3: Growth kinetics of MCF-7 (subclones a-c) and MDA-MB-231 breast cancer cells in the presence of 4-hydroxytamoxifen (○ 10 nM; Δ 100 nM; □ 1 µM) compared to its vehicle (●).

*Cell densities were determined via absorbance at 578 nm (A_{578}) after crystal violet staining. Values represent means of at least 14 replicates ± standard deviations; Shown growth kinetics of each MCF-7 subclone are representative for two to three independently conducted experiments; *radiometricly determined from corresponding cytosols.*

As 17β-estradiol binds to ERα and ERβ with nearly the same dissociation constant (K_d), the radioligand binding experiments do not allow a discrimination between the expression of the ER subtypes. Therefore, ER subtype distribution in the studied MCF-7 (a-c) cells was analysed on the protein level by Western blot analysis using specific

antibodies and on the mRNA level by amplification of specific cDNA fragments within the ERα and ERβ genes after RT-PCR. Representative results are depicted in Figure D4.

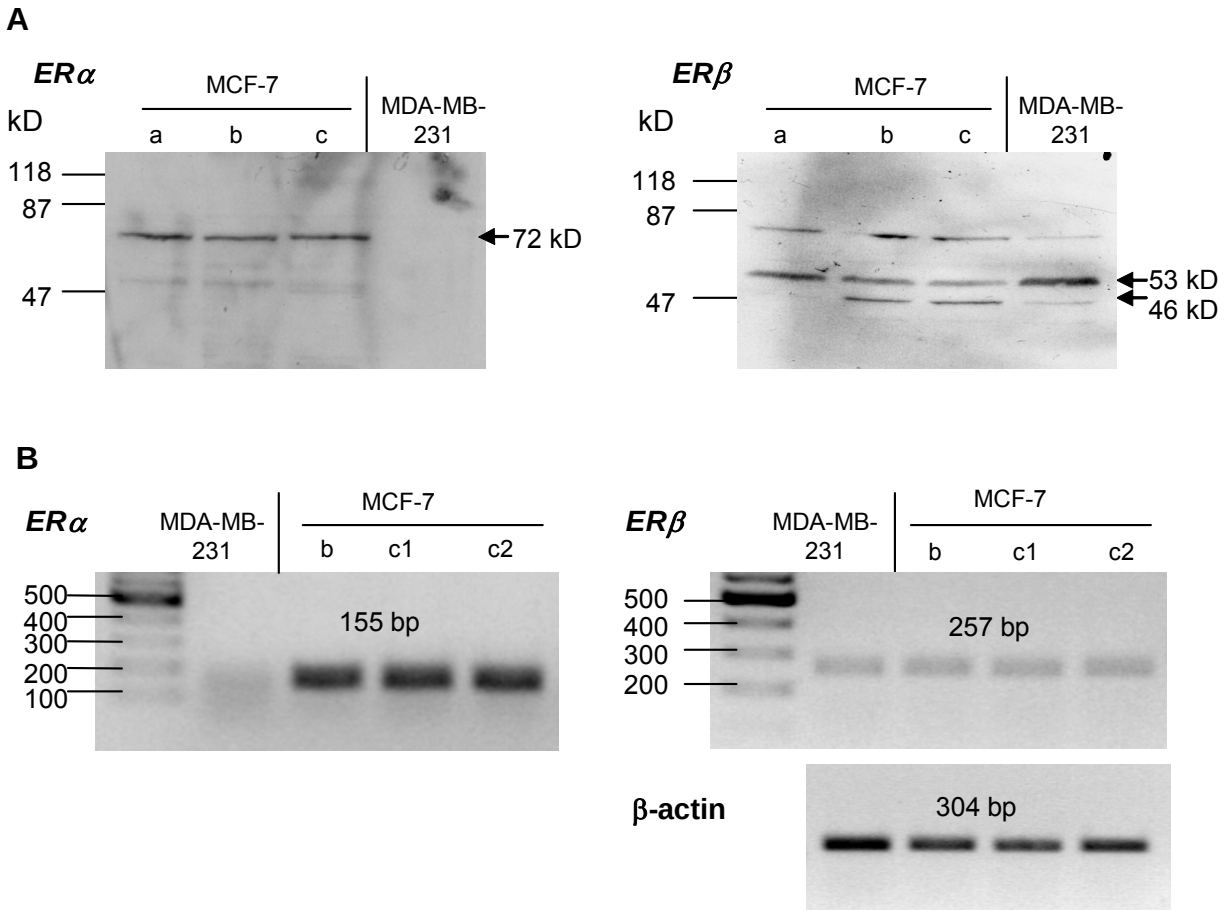


Figure D4: ER subtype distribution in MCF-7 and MDA-MB-231 breast cancer cells; A: Western Blot analysis of ERα and ERβ in different MCF-7 subclones (a-c) and MDA-MB-231 breast cancer cells; B: RT-PCR analysis of ERα and ERβ mRNA expression in different MCF-7 subclones and MDA-MB-231 cells; c1: MCF-7 (c) passage 50; c2: MCF-7 (c) passage 10; For MCF-7 (a) a similar result was obtained.

In Western blot analyses the antibody against ERα detected a single sharp band corresponding to the expected molecular weight of 72 kD in all MCF-7 subclones, whereas immunoreactivity was missing in MDA-MB-231 cells. The ERβ–selective antibody detected a band of the expected molecular weight (53 kD) in MCF-7 subclones and, surprisingly, also in homogenates of MDA-MB-231 cells. Consistently, the ERβ

was detected immunochemically by Filardo et al. in MDA-MB-231 cells using a different antibody (Filardo et al., 2000). In case of the MCF7 (b) and MCF-7 (c) subclones a second band corresponding to 46 kD became obvious, which might represent an isoform of ERβ (cf. Figure D4 (A)). The detection of a strong band in MDA-MB-231 cytosols corresponding to ERβ was in disagreement with the results of radioligand binding assays, where there was no specific binding of [^{3}H]-17β-estradiol. The question arises, why the ERβ was detected in a cell line, which is considered a prime example of ER-negative i. e. hormone insensitive breast cancer. A possible explanation is the expression of a non-functional ERβ maybe misfolded protein having an epitope, which is recognized by the antibody.

In accordance with Western blot analyses a high expression of the ERα mRNA was detected in MCF-7, but not in MDA-MB-231 cells. The strong band at the predicted position in the agarose gel indicates a specific cDNA fragment within the ERα gene after RT-PCR. On the contrary, weak bands indicate a low expression of the ERβ mRNA in all investigated cell lines and subclones. However, expression of the mRNA is not necessarily indicative of translation, correct folding and trafficking of a protein. A role of ERβ in estrogen signalling, in particular in cross-talk signalling with the Y_1R, that will be discussed in the following paragraphs cannot be excluded. Appropriate subtype selective ligands acting as agonists or antagonists at the estrogen receptor should give insight into ER subtype specific functions.

The expression of the intact Y_1R protein was determined by a radioligand binding assay on living cells that allows the simultaneous processing in the multiwell format. The recently developed non-peptidic BIBP3226 derived radioligand [^{3}H]-UR-MK114 used for this purpose has advantages over labeled peptides such as high stability towards enzymatic degradation, fast association kinetics and convenient non-expensive preparation (Keller et al., 2008). Typical curves for total, unspecific and specific binding of [^{3}H]-UR-MK114 to MCF-7 cells are shown in Figure D5 (A). The radioligand revealed no Y_1R specific binding sites in ER negative MDA-MB-231 (cf. Figure D5 (B)), HCC1806 and HCC1937 (data not shown) breast cancer cells.

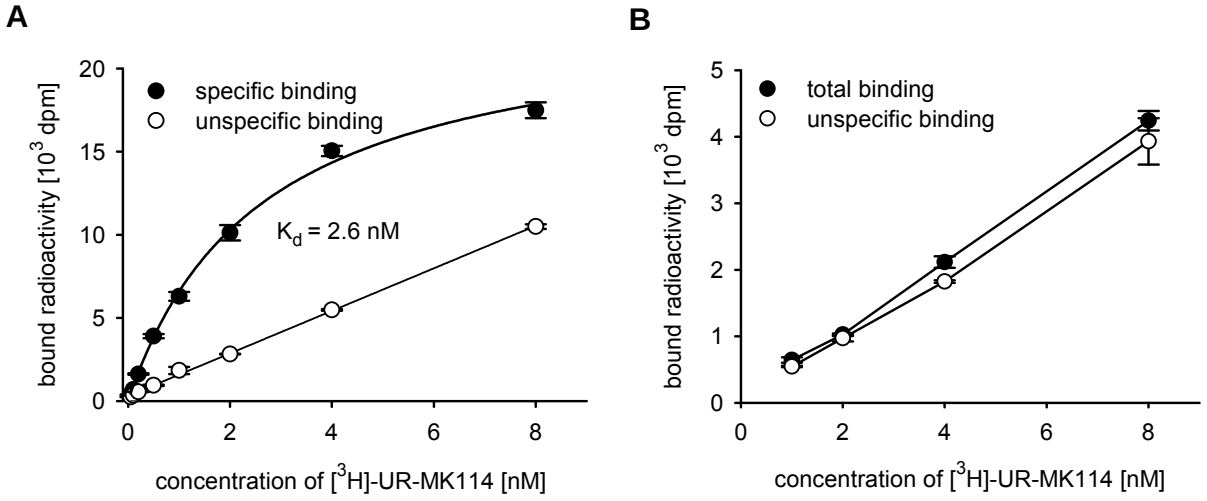


Figure D5: Representative curves for saturation binding of [^{3}H]-UR-MK114 to whole MCF-7 (A) and MDA-MB-231 (B) cells; *Values represent means of triplicates ± SEM.*

Table D1 summarizes the ER and Y_1R contents of the investigated cell lines obtained from radioligand binding assays and the relative ERα and ERβ subtype expression determined by densitometric analysis of the corresponding Western blots. A graphical overview of the expression profile of ERs and Y_1Rs by the investigated MCF-7 subclones a-c is given in Figure D6.

Table D1: Comparison of ER status, ER subtype expression and NPY Y_1R status in MCF-7 and MDA-MB-231 breast cancer cells

Cell line		ER status[a] [fmol/$mg_{protein}$]	ERα[b] % OD/mg	ERβ[b] % OD/mg	Y_1R status[c] [10^3 sites/cell]
MCF-7	a	95 ± 8	100	100	38 ± 10
	b	30 ± 5	70	69	98 ± 9
	c	46 ± 3	81	53	91 ± 4
MDA-MB-231		negative	negative	96	negative
SK-N-MC		n.d.	n.d.	n.d.	50[d]

Determined from [a]cytosol in a [^{3}H]-17β-estradiol saturation binding assay; [b]cytosol by Western blotting; OD/mg = optical density per mg of protein [c]intact cells in a radioligand binding assay using [^{3}H]-UR-MK114; [d](Entzeroth et al., 1995; Keller et al., 2008)

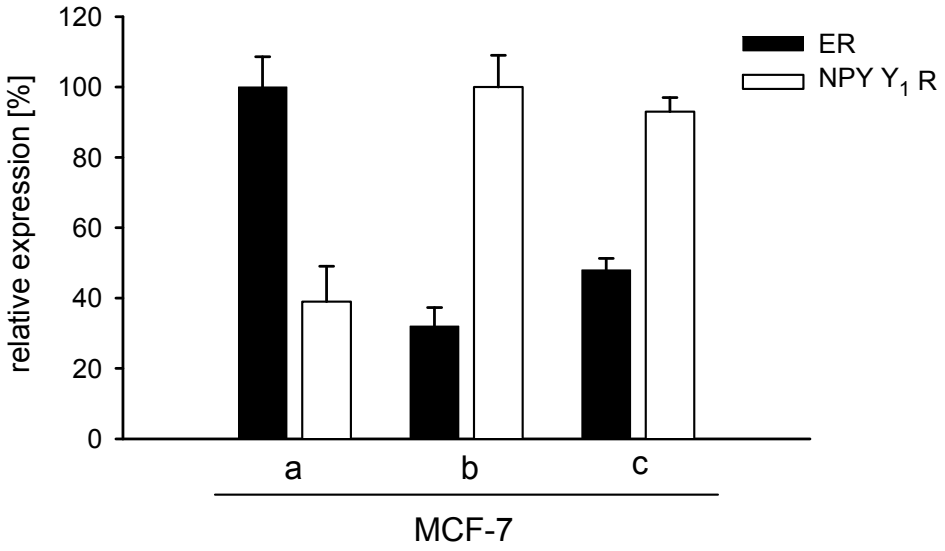


Figure D6: Comparison of the relative NPY Y_1 R basal expression and the total ER expression by MCF-7 (a-c) subclones.

Interestingly, the Y_1R expression appears to be inversely correlated with ER expression in identically cultured subclones of MCF-7 breast cancer cells: with approximately 40,000 sites per cell the basal Y_1R protein density in MCF-7 (a) cells was found to be in the same range as in SK-N-MC neuroblastoma cells (Entzeroth et al., 1995; Keller et al., 2008), whereas it was by more than a factor two higher in the antiestrogen insensitive MCF-7 (b) and MCF-7 (c) subclones (≈ 90,000-100,000 receptors per cell). Based on this result, future studies might reveal a possible link between tamoxifen resistance and Y_1R expression in hormone sensitive breast cancers.

2.2 Effect of (Anti)estrogens on Y_1R Expression in Human Breast Cancer Cells

Two previous reports suggest an estrogen induced Y_1R up-regulation in certain human breast cancer cells (Kuang et al., 1998; Amlal et al., 2006). By submitting (anti)estrogen pretreated cultured cells to a Y_1R radioligand binding assay we aimed to gain detailed information on estrogen responsiveness of the Y_1R expression on the level of the functional receptor protein, as previous data have been limited to the mRNA level. Our studies were especially focused on concentration response relationships and ER subtype specificity.

2.2.1 Characterization of the Estrogen-Induced Y_1R Up-regulation at the Protein Level

Figure D7 (A) shows representative saturation binding curves for the specific binding of the [^{3}H]-UR-MK114 to MCF-7 cells pretreated with E2 (1 nM) or its vehicle for 48 h. To facilitate the analysis of Y_1R regulation, the specifically bound radioactivity at a radioligand concentration of 12 nM was compared, whereupon the expression levels are given as a percentage of the control treated with 1 nM 17β-estradiol. At this radioligand concentration, the saturation curves reveal an approximation of the specifically bound radioactivity to the B_{max} value. The number of occupied binding sites at 12 nM is therefore representative for Y_1R expression.

An increase in Y_1R protein expression by somewhat more than 100 % was observed, when cells were treated wit 1 nM 17β-estradiol for 48 hours. The ratio between estrogen treated and untreated cells was not significantly increased when the time of incubation was prolonged to 72 hours (data not shown). Consequently, 45 to 50 hours were considered as an appropriate incubation period for the treatment of MCF-7 cells with (anti)estrogens in all following experiments. Such a time period is typical for genomic processes. In T-47-D breast cancer cells an up-regulation of the Y_1R after estrogen treatment occurred as well, but the basal expression was on a 20-fold lower level compared to MCF-7-cells (cf. Figure D7 B). For the MCF-7 and T-47-D cell lines our results are in accordance with the recently reported estrogen triggered Y_1R mRNA up-regulation (Amlal et al., 2006).

The strong difference between total and non-specific binding in autoradiography demonstrates a very high Y_1R density in sections of solid tumor xenografts established from MCF-7 (b) cells in nude mice (cf. Figure D7 (C)). An estrogen level comparable to that of premenopausal women was guaranteed by implantats, releasing 17β-estradiol to enable the growth of MCF-7 tumors in nude mice (Bernhardt et al., 1992) . The high Y_1R density in tumor xenografts from estrogen-substituted animals makes an estrogen-induced Y_1R up-regulation very likely to occur *in vivo* too.

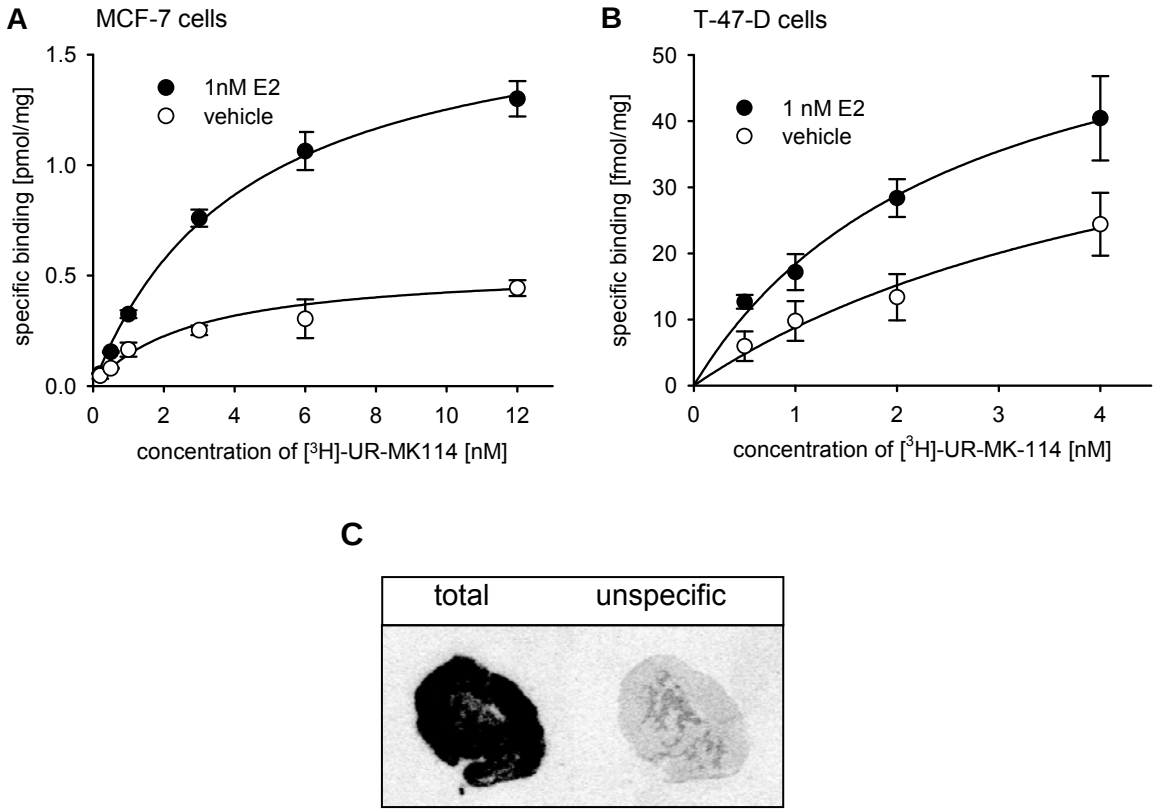


Figure D7: Saturation binding curves of [^{3}H]-UR-MK114 to MCF-7 (A, n = 2) and T47-D (B, n = 3) cells after preincubation with 1nM 17β-estradiol or its vehicle
C: Total and non-specific binding of [^{3}H]-UR-MK114 to adjacent tumor sections of a subcutaneous MCF-7 (b) mammary carcinoma from a NMRI (nu/nu) mouse with estrogen substitution.

The basal Y_1 R expression was compared when growing MCF-7 cells in two different culture media. Phenol red containing EMEM and phenol red free DMEM were either supplemented with normal fetal calf serum (FCS) or steroid depleted ct-FCS (charcoal treated FCS). As shown in Figure D8 (A) the basal Y_1R expression was significantly decreased in ct-FCS containing media compared to the respective untreated FCS containing media. The pH indicator phenol red was reported to bring along contaminants with weak estrogenic activity (Bindal et al., 1988) and might therefore contribute to basal Y_1R expression. In the present study the Y_1R baseline expression was not significantly different, when cells were maintained in phenol red free DMEM and EMEM with phenol red, respectively. Consequently an effect of phenol red contaminants on Y_1R expression was excluded, contrary to the finding of Amlal and coworkers (Amlal et al., 2006). The basal Y_1R expression is estrogen induced anyhow,

as it was significantly down-regulated to approximately 25% of the basal level when 17β-estradiol was co-incubated with the "pure ER antagonist" fulvestrant (100 nM) (cf. Figure D8 (B)).

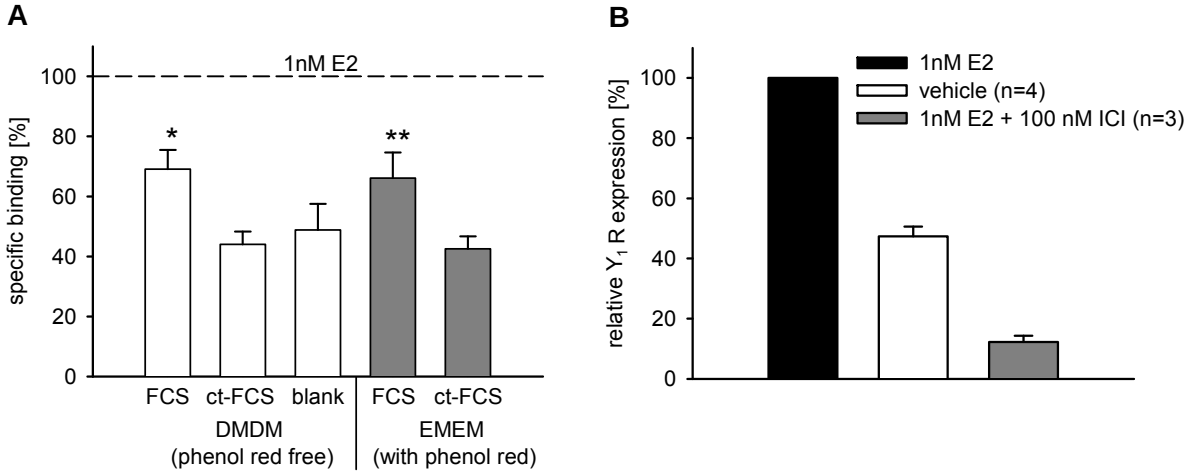


Figure D8: A: Dependence of the basal NPY Y_1R expression by MCF-7 (b) cells from culture medium and supplemented fetal calf serum (FCS or steroid depleted ct-FCS)

*In case of blank DMEM cells were grown in ct-FCS containing medium and estradiol or its vehicle was added in FCS-free medium; *p<0.01 compared with DMEM + ct-FCS and blank DMEM; ** p< 0.01 compared with EMEM + ct-FCS (n= 4; mean ± SD).*

B: Effect of the "pure antagonist" fulvestrant (ICI) on estrogen induced Y_1R expression: fulvestrant co-incubated with 17β-estradiol for 48 h effects a down-regulation of the Y_1R beyond the baseline expression in EMEM+ct-FCS.

*Values represent mans of n independent experiments ± SEM, each conducted at least in triplicate; *p<0.001 compared to vehicle.*

An inhibition of the intrinsic ER activity by potent ER antagonists was also observed in the luciferase assay as described in paragraph C.3.3.2. As the presence of estrogenic compounds in the medium supplements ct-FCS and phenol red was excluded, we suggest a ligand-independent ER activation mechanism being responsible for the relatively high baseline Y_1R expression. Ligand independent ER activation can be mediated by a number of cross-talk signaling pathways including proteinkinase A and C or growth factor mediated pathways. In previous studies it was shown that full ER antagonists such as fulvestrant are capable of blocking many cross-talk activation cascades (Driggers and Segars, 2002).

2.2.2 Concentration Dependent Y_1R Induction by ER Agonists and its Inhibition by ER Antagonists: The Y_1R as an Endogenous Gene Reporter for (Anti)estrogenic Activity in MCF-7 Cells

The experiments described in this paragraph aimed on the detailed investigation of estrogen-induced effects on Y_1R expression in MCF-7 cells concerning concentration response relationships and ER subtype specificity.

MCF-7 breast cancer cells grown in 48 well plates were simultaneously treated with different concentrations of various (anti-)estrogens for 45-50 hours. For each set of identically treated cells total and unspecific Y_1R radioligand binding were determined by applying 12 nM [^{3}H]-UR-MK114 alone or in combination with a high excess of pNPY, respectively. This processing allowed the analysis of a relative high number of (anti)estrogen pretreated cell cultures in one assay with respect to its relative expression of Y_1Rs. To exclude adulterations of the determined Y_1R expression due to anti-proliferative effects of antiestrogens or growth-stimulating effects of estrogenic agents, all specific binding values were normalized by the total protein content derived from an independently performed protein assay (Bradford). Figure D9 shows concentration-response curves for the relative Y_1R induction by a selection of ER agonists.

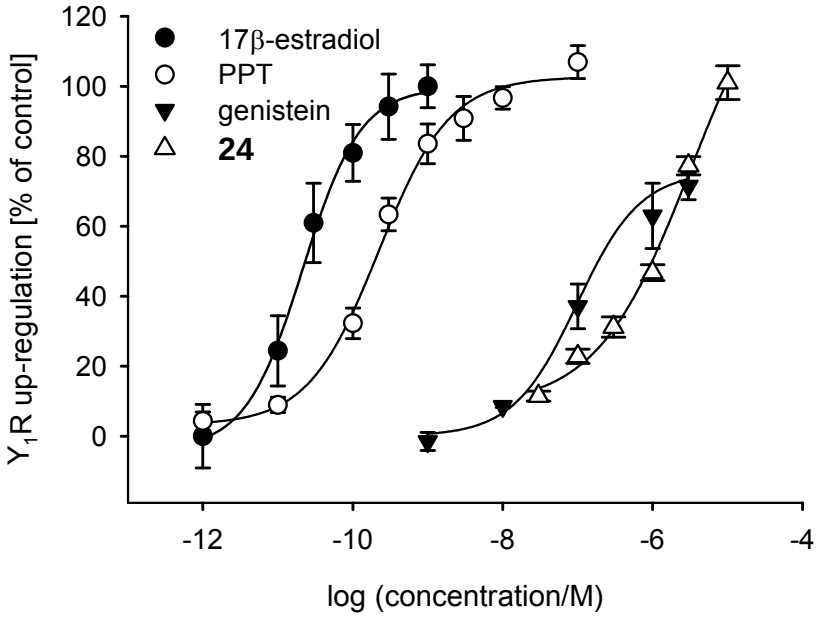


Figure D9: Concentration–response curves for the induction of the NPY Y_1 receptor by various ER agonists above the basal level

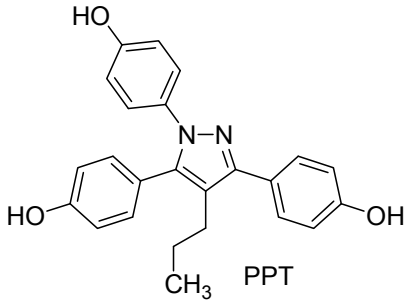
The Y_1R up-regulation induced by 1 nM estradiol was set to 100 %.

17β-estradiol was applied in the picomolar to nanomolar concentration-range, showing a sigmoidal concentration–response relationship with an EC_{50} value of approximately 20 pM. The maximum Y_1R protein expression was observed in the sub-nanomolar range of 17β-esteradiol, whereas a biphasic mechanism was excluded as no further effects were detected up to 50 nM (data not shown). This is the first time that an up-regulation of the Y_1R at physiologically relevant concentrations of 17β-estradiol has been demonstrated at the protein level. These results are in accordance with the work of Amlal et al. (Amlal et al., 2006) reporting an elevation of Y_1R mRNA expression albeit at supra-physiological estradiol concentrations (10 and 100 nM). The EC_{50} value determined via Y_1R up-regulation is in the same range as published data that were determined via progesterone receptor mRNA up-regulation in MCF-7 cells (44 pM; c.f. (Allan et al., 2001)) and by a gene reporter (lucifease) assay in our laboratory (40 pM).

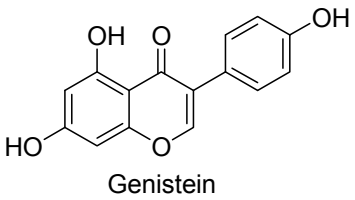
In further experiments, appropriate agonists were used as pharmacological tools, providing information on the subtype specificity of observed effects. The high efficacy and potency of the ERα selective agonist PPT suggests a predominant role of the ERα subtype in Y_1R regulation, as PPT is devoid of any activity at ERβ (Stauffer et al., 2000). The EC_{50} value is in good agreement with that reported for ERα from a co-transfection assay (≈ 0.1 nM cf. (Stauffer et al., 2000)). Genistein, a phytoestrogen, was previously reported to be a full agonist at ERα and a partial (50 %) agonist at ERβ thereby preferably binding to the ERβ subtype (Barkhem et al., 1998). Genistein up-regulated the Y_1R by 70% with an EC_{50} value of 100 nM. This result matches with the reported data for ERα rather than ERβ, underlining that Y_1R induction is ERα specific. The ERβ selective benzofuran **24** synthesized within the scope of this thesis (cf. paragraph C.2) showed 100 % Y_1R induction compared to 17β-estradiol, but only at micromolar concentrations. As compound **24** exhibited a high binding affinity for ERβ the observed effect is most likely mediated by ERα.

EC_{50} values and efficacies for Y_1R up-regulation by the investigated ER agonists are summarized in Table D2.

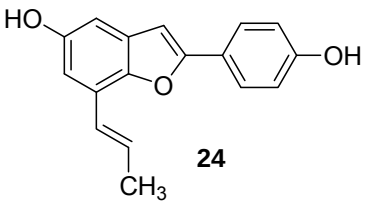
Table D2: EC_{50} values and intrinsic activities for the Y_1R up-regulation by various ER agonists



PPT



Genistein



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Comp.	Selecticity ERα/ERβ[a]	EC_{50} [nM] Y_1R up-regulation	Efficacy	EC_{50} [nM] references
E2	1	0.016 ± 0.006[b]	100	0.044[c]; 0.04[d]
PPT	400[e]	0.25 ± 0.03[b]	107	ERα: 0.1 nM; ERβ: >1 μM[e]
Genistein	0.05	98	72	ERα: 38; ERβ: 9[f]
24	0.03	1100	100	>1000[d]

[a]referred to relative binding affinity (RBA) for recombinant ERα and ERβ [b]mean of two independent determinations ± SEM; [c](Allan et al., 2001); [d]determined by the luciferase assay using the MCF-7/2a subline; [e](Stauffer et al., 2000); [f](Barkhem et al., 1998)

The observed effects of selective agonists suggest a dominant role of the ERα subtype in Y_1R regulation, but a contribution of ERβ to this signaling cannot be totally excluded from these experiments. One uncertain factor is the relatively high basal expression of the Y_1R that was shown to be strongly decreased by the unselective ER antagonist fulvestrant (cf. Figure D8). We assumed a ligand independent ER activation mechanism being responsible for this basal Y_1R induction that might be mediated by both ERα and ERβ. Therefore, the effect of the moderately ERα selective 1,2,3,4-tetrahydro-isoquinoline (THIQ)-based “pure antagonists” synthesized within the scope of this thesis (cf. section C) was investigated.

The representative curves for the inhibition of the estradiol-induced (1 nM) Y_1R protein expression by selected ER antagonists are depicted in Figure D10. Table D3 summarizes the IC_{50} values determined from the concentration dependent inhibition of the 17β-estradiol-induced Y_1R expression.

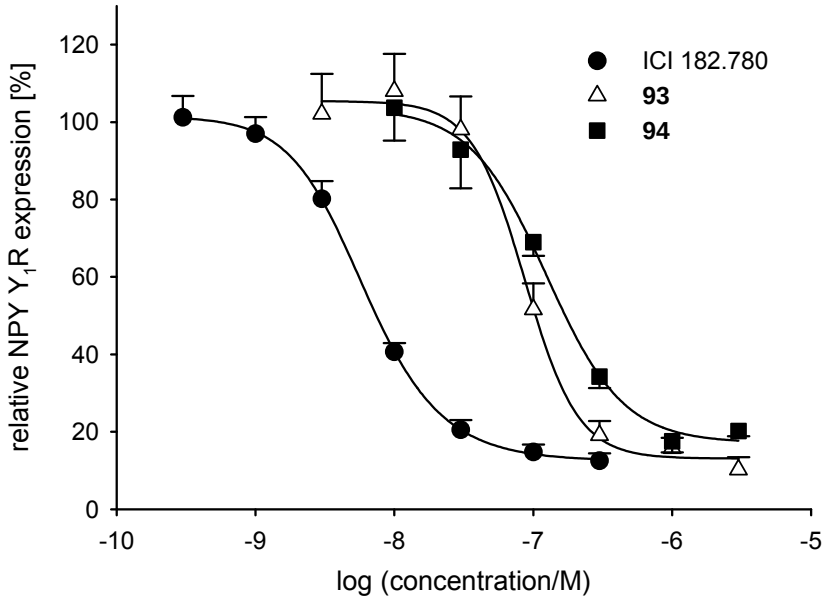
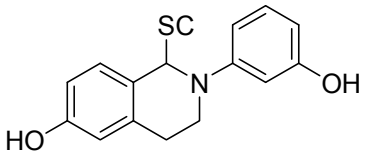


Figure D10: Concentration–response curves for the inhibition of the 17β-estradiol (1nM) mediated Y_1R expression by selected antiestrogens.

Each value represents Y_1R specific binding (mean of at least tiplicates) that was normalized by the corresponding protein content (mean of at least 6 control wells) ± propargated error. The Y_1R content in presence of 1nM 17β-estradiol was set to 100%

Table D3: IC_{50} values of ER antagonists for the inhibition of the estrogen-tinduced Y_1R expression in MCF-7 cells compared to inhibition of luciferase activity in MCF-7/2a cells.



Compound	SC[a]	Selectivity[b] ERα/ERβ	Y_1R expr. IC_{50} [nM][c]	Luciferase IC_{50} [nM][d]
fulvestrant		0.70	4.7 ± 1.3	10
93	$-(CH_2)_6-N(CH_3)-(CH_2)_3-S-C_5H_{11}$	13	92 ± 5	160
94	$-(CH_2)_{10}-SO-C_5H_{11}$	17	103 ± 22	470

[a]side chain; [b]referred to RBA values for human recombinant receptors; [c]means of two independent determinations ± SEM; [d]determined in transfected MCF-7/2a cells

As a potent, non-selective ER antagonist, fulvestrant (ICI 182.80) strongly inhibited the Y_1R expression to 15 % of the maximum (1 nM E2 triggered) level with an IC_{50} value of 5 nM when co-incubated with 1 nM E2. This result is in accordance with data obtained in a gene reporter (luciferase) assay with the transfected MCF-7/2a subline in our laboratory (IC_{50} = 10 nM).

The ERα-selective THIQ-based antagonists **93** and **94** inhibited the Y_1R expression to the same extent as fulvestrant revealing ERα as the main subtype mediating a ligand independent as well as a 17β-estradiol-induced Y_1R basal expression. The IC_{50} values of the THIQs are in the range of 100 nM.

Generally, the EC_{50} and IC_{50} values obtained from the radioligand binding assay are in agreement with literature data or values obtained from a gene reporter (luciferase) assay in our laboratory. The results suggest the exploitation of the Y_1R both as a tumor marker and as an endogenous, easily detectable gene reporter for the determination of (anti)estrogenic activity of new compounds in MCF-7 cells, a standard model in breast cancer research.

Several estrogen-responsive genes, such as the progesterone receptor (PR) gene that are constitutively expressed in MCF-7 cells have been used as endogenous gene reporters of estrogenic activity in previous studies (Jorgensen et al., 2000; Allan et al., 2001; Lim et al., 2005). Methods based on the detection of endogenous estrogen responsive genes on mRNA level - such as RT-PCR - are poorly applicable in screenings of compound libraries, which is a major drawback compared to artificial gene reporters such as the luciferase expression system established in our workgroup.

Within the present study the Y_1R was characterized as the first GPCR that is estrogen responsively overexpressed by MCF-7 cells and can be addressed by a small labeled ligand. Being located in the plasma membrane, the Y_1R protein can be quantified by a robust and fast radioligand binding assay on whole cells. By processing in multiwell (e.g. 48-well) plates, the method is applicable for screenings providing a throughput of several hundred samples. The radioligand [^{3}H]-UR-MK114 is economic in preparation and use, as low concentrations (12 nM) are sufficient for saturating the Y_1R binding sites. These aspects, and the fact that obtained EC_{50} and IC_{50} values were reproducible and match with data from established gene reporter assays, suggest the endogenous Y_1R expression system as an alternative to artificial estrogen responsive expression systems such as the luciferase reporter. The work with non-transfected MCF-7 cells offers further advantages such as easy availability of the cells, higher genetic stability

and possibly higher physiological relevance of the obtained results compared to engineered cells.
Nevertheless, further experiments are necessary for the validation of the assay and its establishment as a general accepted functional test system for (anti)estrogens. Alternatively to the radiolabeling approach, fluorescently labeled Y_1R ligands recently developed in our workgroup (to be reported) might also be useful tools for the fast quantification of estrogen induced Y_1R expression in MCF-7 cells.

2.3 Functional Characterization of the NPY Y_1 Receptor in MCF-7 Cells

To investigate the functionality of the Y_1R expressed in MCF-7 breast cancer cells, the coupling of the receptor to calcium mobilization and inhibition of adenylylcyclase (AC) activity was determined by appropriate assays established in our workgroup.
Furthermore, effects of NPY on MCF-7 cell proliferation and on ER mediated transcriptional activity were investigated in the crystal violet assay and the luciferase gene reporter assay, respectively.

2.3.1 NPY Y_1R- Mediated Mobilization of Intracellular Calcium

The intracellular calcium level was determined by a fluorescent calcium assay established for HEL cells in our workgroup using the selective calcium chelator fura-2 (Gessele, 1998).
With 10 nM porcine NPY (pNPY) an increase in the intracellular calcium level was induced by a factor of four. In the presence of the Y_1R antagonist BIBP3226 the signal was abrogated by ≈ 80 %, showing the Y_1R specificity of the signaling. As expected, the calcium signal was strongly reduced after receptor down-regulation by fulvestrant. Interestingly, in spite of a marked up-regulation of the Y_1R, the effect did not respond to pre-incubation with 17β-estradiol (cf. Figure D11). This might be due to a receptor reserve after the estrogen induced up-regulation or a limitation by the intracellular calcium stores.

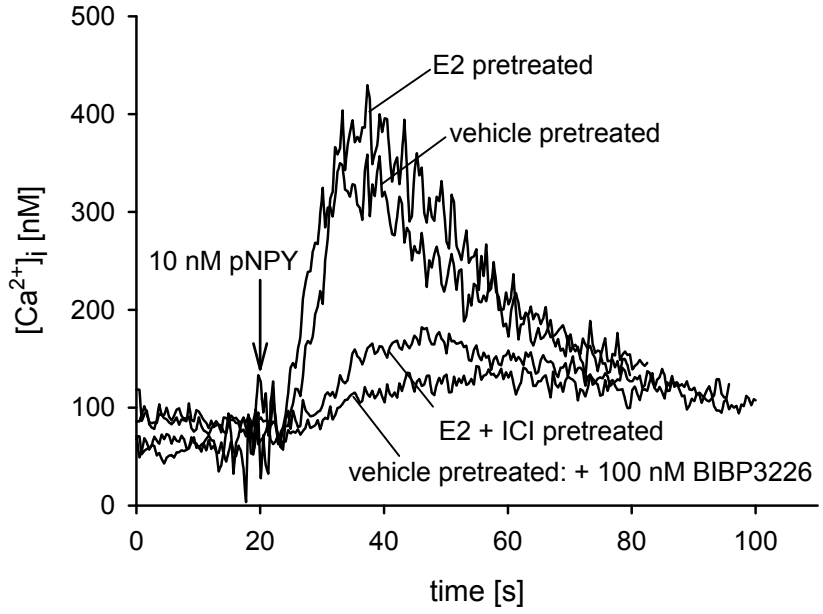


Figure D11: Intracellular calcium release after stimulation with 10 nM pNPY in MCF-7 cells preatreated with estradiol, estradiol + fulvestrant (ICI) or their vehicle; a similar result was obtained in another independent experiment.

2.3.2 NPY-Induced Inhibition of Adenylyl Cyclase (AC) Activity

Adenylyl cyclase (AC) catalyses the transformation of adenosin triphosphate (ATP) to the second messenger 3'-5'-cyclic adenosine monophosphate (cAMP). Therefore, the level of intracellular cAMP is a monitor of AC activity. In MCF-7 cells the cAMP content was determined by an enzymatic assay (Sugiyama and Lurie, 1994) that was established in our workgroup by Dr. Gessele (Gessele, 1998). The assay relies on the equimolar formation of NADPH during the last step of an enzymatic reaction sequence consuming cAMP from cell extracts. Finally, the NADPH concentration can be measured flurimetrically and allows the stoichiometric calculation of the cAMP amount consumed in the enzymatic reaction. Experimental and theoretical details on this method are described by Gessele.

MCF-7 breast cancer cells were submitted to the enzymatic cAMP assay to explore the functional coupling of the Y_1R to the inhibition of AC activity. As the intracellular cAMP content is normally very low, the AC had to be stimulated by the activators IBMX and forskolin in order to enable a detectable NPY induced inhibition of cAMP formation.

Figure D12 shows the concentration-dependent increase in the cAMP level after forskolin treatment and its inhibition by pNPY in MCF-7 cells.

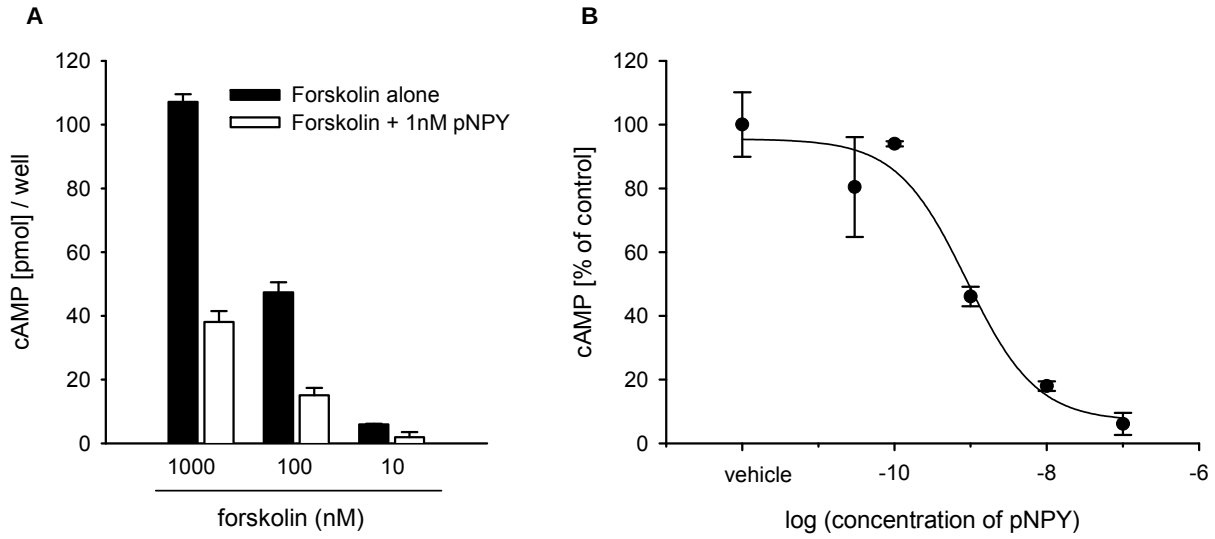


Figure D12: A: cAMP formation in MCF-7 cells after stimulation of the cells with different concentrations of forskolin and IBMX and inhibition with 1nM pNPY; B: concentration-response curve for the inhibition of adenylyl cyclase by pNPY; cells were stimulated with 1 µM forskolin; the concentration of IBMX was 50 µM throughout.

The concentration dependent inhibition of AC activity by pNPY revealed an IC_{50} value of approximately 1 nM, which is in agreement with data obtained from SK-N-MC cells. This finding demonstrates a functional coupling of the Y_1R to the cAMP pathway. Due to limited availability of certain enzymes, the assay was inapplicable for extensive investigations. Future studies using an alternative cAMP assay might show the Y_1R specificity of the signaling with help of selective antagonists (BIBP3226 and derivatives). Investigations of (anti)estrogen induced effects on the Y_1R mediated cAMP response are another interesting perspective.

2.3.3 Effect of NPY on Cell Proliferation and Estrogen Receptor Activity

The high expression and functionality of the Y_1R in MCF-7 breast cancer cells gives rise to speculations on a possible role of NPY on tumor growth. Recently, published data

suggest an involevement of NPY in the growth of Y_1R expressing cancer cell lines. NPY was reported to inhibit the proliferation of SK-N-MC cells (Reubi et al., 2001; Kitlinska et al., 2005), of certain prostate cancer cells (Ruscica et al., 2006) and MCF-7 cells (Amlal et al., 2006). Growth stimulating effects were observed in another Y_1R expressing prostate cancer cell line (Ruscica et al., 2006) and - contrary to the findings of Kitilinska et al. and Reubi et al. - in SK-N-MC cells (Shorter and Pence, 1997).

By the kinetic crystal violet assay we observed no significant effects of pNPY on the proliferation of MCF-7 cells at concentrations of 10 and 100 nM, respectively. At these pNPY concentrations full receptor occupation and maximum functional response was guaranteed. The inconsistent effects of NPY on cell proliferation obtained by different laboratories might be due to different experimental conditions. In the present study - contrary to Amlal et al. - the supplemented serum was not inactivated to guarantee an environment closer to physiological situation. Growth kinetics were compared in the presence or absence of 17β-estradiol coming to a similar result. Representative curves are depicted in Figure D13 (A).

Amlal et al. report an inhibition of the estrogen-induced MCF-7 cell proliferation by NPY. This finding suggests a direct impact of the Y_1R mediated cytoplasmic signaling on ER activity. Y_1R signaling might cross-talk with the ER activated transcription through the PKC/MAPK pathway, which has been described as one possible mechanism of ligand independent ER phosphorylation.

To explore such an indirect Y_1R-mediated effect on the activation state of the ER, MCF-7/2a cells expressing the estrogen responsive luciferase gene reporter were used as a tool to monitor ER mediated transcriptional activity (cf. section C). By radioligand binding, the Y_1R protein expression by the MCF-7/2a subline was confirmed (data not shown). To investigate the putative signaling cross-talk between the Y_1R and the ER, MCF-7/2a cells were treated with pNPY at concentrations of 10 and 100 nM for 48 h. As shown in Figure D13 (B) NPY neither affected basal lucifease activity nor the activity of the 17β-estradiol stimulated cells.

The data obtained from the present experiment do not support the existence of a link between the Y_1R-mediated signaling and the transcriptional activity of the ER. In agreement with this result, NPY did not influence the ER-dependent proliferation of MCF-7 cells.

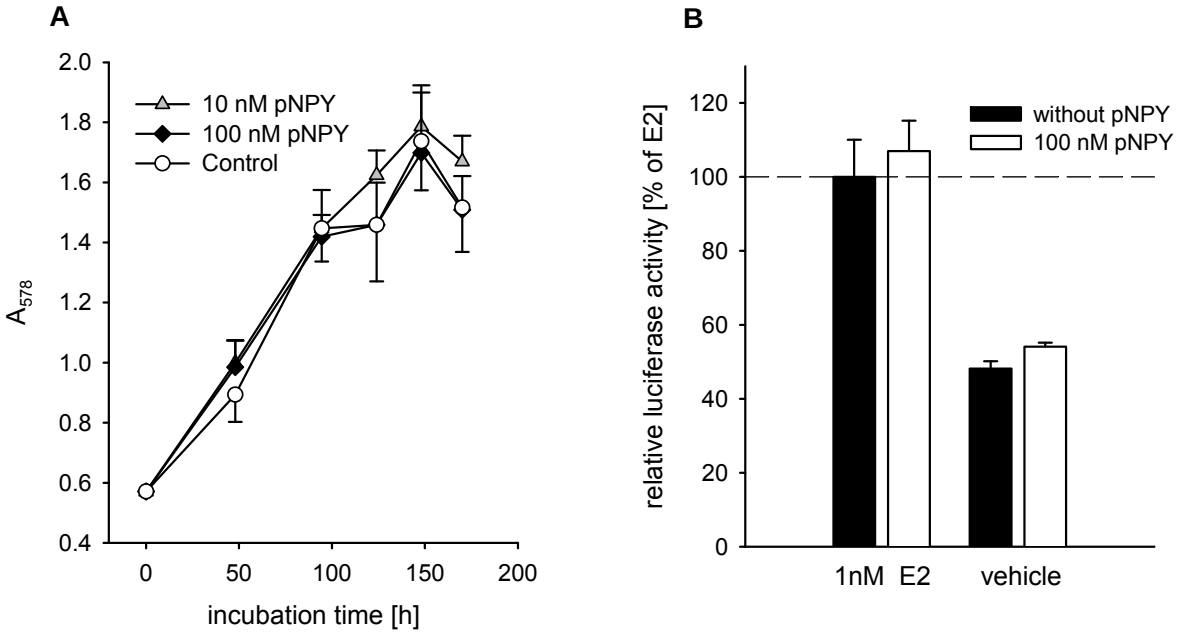


Figure D13: A: Growth kinetics of MCF-7 (b) cells in presence or absence of pNPY; 1 nM estradiol was present to maximize the Y_1 R expression and proliferation rate; a similar result was obtained in absence of estradiol;
B: Luciferase activity in estradiol-stimulated and unstimulated MCF-7/2a cells in presence or absence of pNPY; 100 nM pNPY revealed no activating or inhibiting effect on the estrogen receptor; a similar result was obtained for 10 nM pNPY.

3 Conclusion and Outlook

The novel radiolabeling and functional characterization of the Y_1R revealed a high functional expression of the G protein coupled receptor in MCF-7 breast cancer cells. Y_1R incidence was exclusively observed in ER positive breast cancer cells, whereas in different subclones of MCF-7 cells the Y_1R expression correlated inversely with ER content and antiestrogen sensitivity. The study provides the first evidence of an estrogen induced up-regulation of the Y_1R protein in intact MCF-7 cells. The Y_1R protein expression showed a maximum after pretreatment with 17β-estradiol at physiologically relevant concentrations and was down-regulated by the full ER antagonist fulvestrant (ICI182.780) to a sub-basal level. Y_1R induction was shown to be exclusively mediated by ERα: a potent ERα–selective agonist induced Y_1R expression to 100 % of 17β-

estradiol with a sub-nanomolar EC_{50} value and ERα–selective full antagonists, synthesized within the scope of this thesis, inhibited Y_1R expression to the same extent than fulvestrant. The Y_1R turned out to be an endogenous gene reporter for estrogen receptor activity in MCF-7 breast cancer cells. EC_{50} and IC_{50} values for the induction or inhibition of the Y_1R expression by selected (anti)estrogens were reproducible and in agreement with data obtained by established gene reporter assays. This was the basis to suggest the Y_1R radioligand binding assay as a fast and robust method for the functional screening of (anti)estrogens. The Y_1R was shown to be functionally coupled to mobilization of intracellular calcium and inhibition of AC activity, whereas cross-talk involving NPY was neither observed on cell proliferation nor on the activation state of the ER in MCF-7 cells.

Implications of the Y_1R in cellular functions, such as phosphorylation of Erk1/2, transactivation of tyrosine kinases or effects on downstream transcription factors other than the ER have not been described for MCF-7 breast cancer cells. The investigation of such effects in future studies might shed light on the role of the G protein coupled receptor in breast cancer cell biology.

Due to the high Y_1R receptor density, MCF-7 tumors are favorable as a model for *in vivo* imaging using recently developed Y_1R selective PET ligands (to be reported). Comparing the Y_1R densities in MCF-7 tumors from mice with and without estradiol substitution or after antiestrogen administration might substantiate estrogen-induced Y_1R up-regulation *in vivo*.

The NPY Y_1R was recently reported to be expressed by commonly used prostate cancer (PCa) cell lines (Ruscica et al., 2006). In future studies, Y_1R expression by the PCa lines LNCaP, PC3 and DU145, established in our laboratory, can be exactly quantified at the level of functional protein by radioligand binding using [^{3}H]-UR-MK114 and compared to values for SK-N-MC and MCF-7 cells determined within the present study. By analogy with its estrogen responsiveness in ER positive breast cancer cells, a putative androgen-induced regulation of the Y_1R expression in androgen receptor positive LNCaP cells is an interesting subject for coming investigations.

4 Experimental

4.1 General

Drugs and pharmacological tools:

The Y_1 receptor radioligand [^{3}H]-UR-MK114 and the Y_1R antagonist BIBP3226 were synthesized by Dr. M. Keller. pNPY was synthesized by Dr. C. Cabrele. PPT was purchased form Tocris (Ellisville, MO, USA); Genistein was from Roth (Karlsruhe); 17β-estradiol and 4-hydroxytamoxifen were purchased from Sigma. Fulvestrant (ICI 182.780) was a gift of Dr. M. R. Schneider (Schering AG, Berlin).

Cell culture:

T-47-D cells (ATCC) were grown in RPMI medium supplemented with 10 % FCS and insulin (0.01 mg/L). MCF-7 and MDA-MB-231 cells were cultivated as described in section C.

Preparation of dextran charcoal treated (ct) FCS:

A suspension of 5.0 g of charcoal (Norit A) in 100 mL of 10 mM Tris buffer (pH 7.4) was stirred at 0 °C for 4 h and allowed to stand over night at 4 °C. Particles on the surface were removed by suction. 50 mg of dextran 60 were added and this suspension was stirred for 15 min. The suspension was split into two aliquots and centrifuged at 5000 rpm for 15 min. The supernatant was discarded. One pellet was re-suspended in 250 mL of FCS, stirred at 0 °C for 3 h and finally centrifuged at 9500 rpm for 15 min. The supernatant was decanted onto the second charcoal pellet and incubated with stirring for 1 h at 56 °C to reach complete inactivation of the serum. After centrifugation (9500 rpm; 15 min) the supernatant FCS was filtered through a 0.2 µm membrane filter for sterilization.

4.2 Radiometric Analysis of the Estrogen Receptor Expression

4.2.1 Cytosol Preparation

The MCF-7- (3 different subclones) and MDA-MB-321 cells were grown in 175-cm^2 culture flasks. When cells were confluent, the medium was removed and cells were harvested after trypsination from 8-10 flasks. The pooled cell suspensions were centrifuged at 1200 rpm for 7 min. The pellet was washed twice with PBS and suspended in 4-5 mL of TED-Mo-buffer (10 mM Tris-HCl pH 7.4, 10 mM Na_2MoO_4 (Sigma), 1 mM EDTA, 1 tablet of EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland) per 100 mL). Cells were lysed using an ultrasonic cell disrupter B15 (Branson, Danbury, CT, USA; 3 × 10 cycles, 10-20 s.) under ice cooling. The suspension was centrifuged for 20 min at 5000 rpm. The supernatant cell extract was decanted carefully and stored at -70 °C. It was used for protein determination, radiometric binding and Western blot analysis.

The protein content of the cytosols was determined in appropriate dilutions by Bradford's protein assay (Bradford dye reagent, Bio Rad Laboratories) following the manufacturer's protocol. The UV-absorbance was measured at 595 nm in a UV spectrophotometer Uvikon 930 (Kontron, Düsseldorf, Germany). Protein contents were assigned to the absorption values by a calibration curve using HSA (human serum albumin; Behringwerke, Marburg, Germany) standards in a range of 1-12 µg of protein.

4.2.2 Performance of the [^{3}H]-17β-Estradiol Binding Assay

The [^{3}H]-17β-estradiol binding assay was performed in 1.5 mL reaction vessels (Eppendorf, Hamburg, Germany) under ice cooling. The radioligand was incubated with 100 µL- samples of the respective cytosol diluted to a final volume of 500 µL in Tris-buffer (10 mM Tris-HCl, pH 7.5). [^{3}H]-17β-estradiol was added as 5-fold concentrate in Tris-buffer to obtain a final concentration range of 0.1-5 nM. For the determination of the non-specific binding, the radioligand was displaced from the specific binding sites by an excess (1 µM) of non-labelled 17β-estradiol. For each concentration, total and nonspecific binding were determined in triplicate, respectively. The samples were incubated for 16-20 h at 4°C under shaking.

Unbound radioactivity was removed by the dextran coated charcoal (DCC) method. For this purpose 0.5 mL of a suspension containing 0.8 % charcoal (Norit A; Serva) and 0.008 % dextran 60 (Serva) were added to each sample followed by incubation for 30 min at 4 °C with shaking. After centrifugation (10 min at 4000 rpm) 200 µL of the supernatant were pipetted into a minivial containing 3 mL of liquid scintillator (Rothiszint^{TM} eco plus; Roth, Karlsruhe, Germany). The bound radioactivity was counted in a LS6500 liquid scintillation beta counter (Beckmann Instruments, München, Germany).

The specifically bound radioactivity (total binding minus unspecific binding) was plotted as a function of the concentration. The data points were fitted to one site saturation binding using the PC-software Sigma Plot version 9. The estrogen receptor status (fmol/mg$_{protein}$) was derived from the B_{max} of the radioligand binding (fmol) that was divided by the protein content (mg) from Bradford's protein assay.

4.3 Analysis of ERα and ERβ Expression

4.3.1 Western Blot

Immunoblotting protocol

40 µL aliquots of the cytosols (preparation see above) were supplemented with10 µL of 5-fold sample buffer (0.2 M Tris-HCl, pH 6.8, 2 0% Glycerol, 10 % SDS; 10 mM DTT, 0.05 % bromphenol blue) and heated to 100 °C for 3 min. Total protein was resolved by 12 % SDS page (5-10 µL per lane, 1.5 h, 150-200 V) and electrophoretically transferred onto nitrocellulose (system from Peqlab, Erlangen, Germany; conditions: 1,5 h, 1 mA/cm^2) in transfer buffer (20 mM Tris-HCl, pH 8.3, 192 mM glycerol, 20 % methanol). A pre-stained protein marker (Fermentas #SM0441, St. Leon-Rot, Germany) was used as molecular mass standard. The membranes were treated with PBS containing 5 % milk powder (Roth) for 1 h to prevent non specific binding. After washing with PBS containing 0.05 % Tween 20 (PBS-T; 3 × 5 min) the membranes were incubated with specific antibodies against ERα (NB200-374, Novus Biologicals, Littleton, CO, USA; dilution: 1:5000) or ERβ (AB3576, Abcam, Cambridge, UK; dilution: 1:1000), respectively at 4 °C over night in PBS-T containing 5 % milk powder. After washing the membranes with PBS-T (3 × 5 min) they were incubated with an anti rabbit HRP-conjugated secondary antibody (A9169, Sigma, dilution: 1:50,000) in PBS-T containing

5 % milk powder for 1 h at room temperature followed by washing once more with PBS-T (3 × 5 min). Chemoluminesce detection was performed using an Amersham ECL™ detection kit (GE Healthcare).

Data Analysis

The X-ray films were scanned and analyzed with a system from Bio Rad Laboratories consisting of a GS 710 scanner and the corresponding software Quantity one. After densitometric analysis the optical density of each band was quantified and corrected by the corresponding protein content of the cytosol ($OD/mg_{protein}$).

4.3.2 RNA Analysis by Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

RNA Isolation

MCF-7 subclones and MDA-MB-231 cells were seeded on 75-cm^2 culture flasks in the respective medium (see cell culture) and were grown to 70-90 % confluency. All materials used for the RNA isolation were RNAse-free or treated with DEPC water (0.1 % DEPC (Fluka, Steinheim, Germany) in Millipore water). Total mRNA of each cell type was isolated using a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration of the mRNA was determined photometrically at 260 nm according to the following relation: A_{260} = 40 µg/mL.

Reverse Transcription

cDNA was prepared by reverse transcription according to the following protocol:
1 µg of RNA and 1 µL of oligo (dT_{12}) primer solution (100 µM; MWG, Ebersberg, Germany) were added in 10 µL of autoclaved DEPC water. The solution was heated for 5 min to 70 °C in a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). Then, with cooling on ice, 1 µL of MLV-reverse transcriptase (200 U/µL; Invitrogen;), 2 µL of dNTP mix (2 mM; MBI Fermentas), 4 µL of 5 × first strand buffer (Invitrogen), 2 µL of DTT (Roche), and 1 µL of autoclaved DEPC water were added. The samples were incubated for 1 h at 37 °C in the thermocycler, followed by an inactivation step for 2 min at 95 °C. The cDNA samples were stored at -20 °C.

Polymerase Chain Reaction (PCR)

For the amplification of specific DNA fragments, 2 µL of each cDNA solution were supplemented with the respective primers (1 pmol/µL solution in milipore water; for β-actin primer pair: 1 µL; others 2 µL) and 8 µL of PCR-Master Mix (Eppendorf) and finally filled up with Millipore water to a total volume of 20 µL. cycling parameters were: 1) initial denaturation: 95 °C, 2 min; 2) denaturation: 95 °C, 30 s; 3) annealing: 55 °C, 30 s; 4) extension: 72 °C, 90 s; 5) final extension: 72 °C, 2 min; 6) hold: 4°C; steps 2 – 4 were repeated 34 times. Primers were synthesized by MWG. The sequences are shown in table D4.

Table D4: Sequences of the used primers for PCR

gene	sense primer	antisense primer	amplified product length
β-actin	5'-CGG GAT CCC CAA CTG GGA C-3'	5'-GGA ATT CTG GCG TGA GGG A-3'	304 bp
ERα[a]	5'-AGC ACC CAG TGA AGC TAC T-3'	5'-TGA GGC ACA CAA ACT CCT-3'	155 bp
ERβ[b]	5'-GGC CGA CAA GGA GTT GGT A-3'	5'-AAA CCT TGA AGT AGT TGC CAG GAG C-3'	257 bp

[a](de Cremoux et al., 2002); [b](Speirs et al., 1999)

Agarose gel electrophoresis

2 % agarose (pegGold Universal-Agarose; peqlab) gels were prepared in TAE buffer (44.5 mM Tris-base, 44.5 mM acetic acid, 1 mM EDTA). To visualize DNA 0.4 µg/mL ethidiumbromide (Janssen Chimica, Beerse, Belgium) was added.

4 µL of 6 × gel loading dye buffer were added to each PCR reaction mixture and 12 µL of each sample were pipetted per pocket. As reference the peqGold DNA ladder (Peqlab) mix was prepared according to the manufacturer's instructions. Electrophoresis was performed at 90 V for 45 min or until the tracking dye moved at least 2/3 of the gel length. DNA bands were visualized by illumination with UV light at 254 nm (Gel Doc 2000; Bio Rad Laboratories). Quantity one software was used for data analysis.

4.4 Analysis of NPY Y_1 Receptor Protein Expression

4.4.1 General Protocol for the Whole Cell Y_1R Radioligand Binding Assay

All NPY Y_1receptor binding assays using [^{3}H]-UR-MK114 were performed in 24-well plates (Becton Dickinson) or 48-well plates (Nunc) on whole cells.
The cells were seeded into multiwell plates as a homogenous suspension in 500 µL (24 well) or 300 µL (48 well) of the respective culture medium. Cells were cultivated for 3-5 days or until they reached confluency. The medium was removed by suction and the cells were washed with ice-cold 10 mM HEPES buffer (pH 7.4) supplemented with 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl{\cdot}2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$ and 25 mM $NaHCO_3$. This HEPES buffer additionally supplemented with 1 % BSA served as binding buffer. The cells were incubated with the radioligand (added as appropriate concentrate, 25 µL/well) in a total volume of 250 µL (24 well plates) or 150 µL (48 well plates) of binding buffer respectively. The unspecific binding was determined by incubation of the radioligand in the presence of a 300 fold excess of pNPY. Total and unspecific binding were determined at least in triplicate for each concentration. After an incubation period of 20 min with gentle shaking the binding buffer was removed by suction and the non bound radioactivity was washed away twice with the HEPES wash buffer (BSA free). Cells were lysed in 300µL of a solution consisting of 8M urea, 3 M acetic acid and 1 % Triton^{TM} X-100. After an incubation period of 30 min, the lysates were transferred completely into a minivial containing 3 mL of scintillation fluid and the tritium activity was counted in a Beckmann LS 6500 beta counter.

4.4.2 Determination of the NPY Y_1 Receptor Status

For the determination of the NPY Y_1 receptor status of the investigated cell lines, the radioligand was added in various concentrations. K_D and B_{max} values were determined by one site saturation binding fit (Sigma Plot Software).
The number of binding sites (B_{max}) was related to the cell number, which was determined from six identically treated control wells by counting with a Neubauer improved hemocytometer.

4.4.3 Effect of (Anti)estrogens on Y_1R Expression

Assay protocol

Cells were seeded into 48 well plates and grown in ct-FCS containing medium (see general methods) until they had reached 70-80 % confluency. 45-50 h before the Y_1R binding assay was performed, the medium was removed by suction and replaced by fresh (ct-FCS containing) culture medium (0.3 mL/well) containing the (anti)estrogens in the respective concentrations (by dilution of a 1000 fold concentrate in ethanol). Antiestrogens were added in multiple concentrations in the presence of 1nM 17β-estradiol as stimulating agent. At least six wells per set were processed for each (anti)estrogen concentration. All plates were prepared in duplicate as two identical sets.

One set of multiwell plates was used for the Y_1R radioligand binding assay to quantify Y_1R expression. If not otherwise noted, [^{3}H]-UR-MK114 was added in a concentration of 12 nM. For each group of identical wells (n= 6-8), one half was used for the determination of the total binding (radioligand alone), the other half for the determination of the unspecific binding (radioligand plus 300-fold excess of pNPY).

The second set of plates was used as control to normalize the specifically bound radioactivity by the protein content. For this purpose the cells of the control wells were lysed by a lysis buffer (50-100 µL, volume depends on the protein concentration) consisting of 25 mM tricine (pH 7.8), 10 % glycerol, 1 % Triton^{TM} X-100 and 1 mM DTT for 30 min with shaking. 5 µL of each lysate were submitted to Bradford's Protein assay at appropriate dilution.

Data Analysis

The specifically bound radioactivity from the Y_1R binding assay was related to the protein content from Bradford's protein assay (dpm/$mg_{protein}$). The error in the term dpm/$mg_{protein}$ was calculated by error propargation from the corresponding SEMs. For calculation of the relative Y_1R expression, the normalized bound radioactivity (dpm/mg) at a given (anti)estrogen concentration was related to the normalized bound radioactivity (dpm/mg) of the 1nM estradiol-stimulated control (per definition 100 %).

4.4.4 Autoradiography

Autoradiography was performed according to a protocol recently reported by Keller et al. (Keller et al., 2008):
Subcutaneous MCF-7 tumors were established in female NMRI (nu/nu) mice bearing an estrogen depot (Bernhardt et al., 1992) by subcutaneous injection of a cell suspension in culture medium without FCS (4 Mio cells/50 µL, 171st in vitro passage). The tumor was taken from the animal 48 h prior to experiment, immediately frozen in Tissue-Tek with the help of dry ice, and stored at -78 °C. Cryosections (12 µm) were obtained at -16 °C with a 2800 Frigocut E freezing microtome (Reichert-Jung/Leica, Germany). Two adjacent sections were mounted on a microscopic slide (Superfrost Plus, 75 × 25 × 1 mm), put 1 min into a chamber of 100% humidity and then carefully covered with binding buffer or fixed for 20 s in an alcoholic formaldehyde fixative (40 mL of 37 % formaldehyde, 360 mL of 95 % ethanol and 0.2 g calcium acetate). The binding buffer was removed (after a period of less than 60 min under cooling) by putting the slides uprightly on a paper towel (≈ 1 min). For total binding the sections were covered with binding buffer (about 800 to 1000 µL for one slide) containing [^{3}H]-UR-MK114 (3 nM) and for non-specific binding with binding buffer containing [^{3}H]-UR-MK114 (3 nM), and pNPY (300 nM). The sections were incubated at room temperature (22 - 25 °C) for a period of 8 min. After incubation the binding buffer was removed, the slides were immerged three times into buffer split to 3 vessels (4 °C, 10 s) and finally rinsed with distilled water (4 °C, 3 s). The slides were put uprightly on a paper towel for 1 min and then dried in horizontal position in a desiccator over P_4O_{10}. The slides were set in close contact with a tritium sensitive screen (PerkinElmer, 192 × 125 mm) using an X-ray film cassette and stored in a dark room for 10 d. The autoradiographic image was generated from the tritium screen using a phosphorimager (Cyclone Storage Phosphor System, Packard).

4.5 Functional assays

4.5.1 Spectrofluorimetrric Calcium Assay

The spectrofluorimetric Ca^{2+} assay with the fluorescent Ca^{2+} indicator fura-2 was performed by analogy with a protocol established for HEL cells in our laboratory (Müller

et al., 1997; Gessele, 1998). MCF-7 cells were incubated with 1 nM 17β-estradiol, 17β-estradiol plus 100 nM fulvestrant (ICI 182.780) or the respective vehicle 45 h prior to the assay. Cells were grown to 70-80 % confluency, trypsinized and resuspended in FCS containing medium for trypsine inactivation. Cells were counted, centrifuged at 300 g for 5 min and resuspended at 1.3 million cells /mL in loading buffer containing 120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 25 mM HEPES and 10 mM glucose. 0.75 mL of this cell suspension were added to 0.25 mL of loading suspension consisting of 20 mg/mL BSA, 0.025% pluronic^{TM} F-127 (Invitrogen-Molecular Probes, Paisley, UK; 20 % stock solution in DMSO) and 1 µM fura-2/AM (Invitrogen-Molecular Probes; 1mM in anhydrous DMSO) in loading buffer to obtain a cell density of 1 million cells/mL. The cells were incubated for 30 min in the dark, centrifuged and re-suspended in the same volume of loading buffer. To achieve complete intracellular cleavage of the AM ester, cells were incubated for additional 30 min in the dark, washed twice with loading buffer and re-suspended at a density of 1 million cells/mL.

For the measurement, 1 mL of the cell suspension was transferred into a cuvette containing 1 mL of loading buffer under stirring. The baseline was recorded for 20 seconds before 10 nM pNPY was added to trigger the calcium signal. For inhibition of the calcium release, the antagonist BIBP 3226 was added in a concentration of 100 nM one minute before the addition of pNPY. Instrument settings were λ_{ex} = 340 and 380 nm (alterating) with slit = 10 nm and λ_{em} = 510 nM with slit = 10 nm. Stirring was low and temperature was 25°C.

The ratio R of fluorescence intensity at 510 nm after excitation at 340 and 380 nm was used for the calculation of the calcium concentration according to the Grynkiewicz equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}] = K_D \cdot (R - R_{min}) / (R_{max} - R) \cdot SFB$$

K_D: dissociation constant of the fura-2-Ca^{2+} complex

R_{max}: fluorescence ratio in presence of saturating Ca^{2+} concentration (determined after the addition of 10 µL of digitonin solution (2% in water; Sigma), which caused lysis of the cells)

R_{min}: ratio in absence of free Ca^{2+}, caused by addition of 50 µL of EGTA solution (600 mM in 1M tris buffer, pH 8.7) to lysed cells

SFB: correction factor; ratio of the fluorescence intensity (λ_{ex} = 380 nM, λ_{em} = 510 nM) of the Ca^{2+} free and Ca^{2+} saturated dye.

4.5.2 Enzymatic Determination of Intracellular 3',5'-cyclic AMP (cAMP)

The 3',5'-cAMP concentration in MCF-7 cells was determined in a multi step enzymatic reaction sequence as described by Dr. C. Gessele (Gessele, 1998) for SK-N-MC cells. In the enzymatic reaction sequence cAMP was first transformed into ATP in two steps. ATP quantitatively phosphoryalated fructose to fructose 6-phosphat, which was enzymaticly isomerized to glucose-6-phosphat. Glucose 6-phospat was oxidized by $NADP^+$ and the amount of equimolar formed NADPH was flurimetricly measured.
MCF-7 cells were seeded on 6-well plates and treated as described by Gessele. Intracellular cAMP was triggered by co-incubation with IBMX (50µM) and forskolin (0.01-1 µM). NPY was added in the given concentrations in order to obtain a concentration response curve for the inhibition of the forskolin / IBMX induced cAMP accumulation. All steps were performed according to this established protocol using enzymes and reagents from commercial suppliers given in the protocol. After the last reaction step, the mixtures were centrifuged and 200 µL of the supernatants were pipetted into a 96 well plate and flurescence was measured in a GENios Pro™ plate reader (Tecan, Salzburg, Austria; parameters: bottom 4 × 4, number of reads: 10, λ_{ex} = 340 nm; λ_{em} = 485 nm). Assays were run in triplicate throughout. From a standard curve generated with known NADPH concentrations the fluorescence intensities were assigned to the corresponding NADPH amounts and finally the cAMP amount (pmol) was derived from the stoichiometry of the reaction sequence (for details see protocol (Gessele, 1998)).

4.5.3 Effect of NPY on Cell Proliferation and ER Mediated Transcriptional Activity

Antiestrogen sensitivity and effects of NPY on MCF-7 cell proliferation were studied in the kinetic chemosensivity assay following the protocol descriped in Section C. The effect of NPY on ER mediated transcriptional activity was studied by the luciferase assay with MCF-7/2a cells using the luciferase assay kit (see Section C).

5 References

Aakerlund, L.; Gether, U.; Fuhlendorff, J.; Schwartz, T. W. and Thastrup, O., Y1 receptors for neuropeptide Y are coupled to mobilization of intracellular calcium and inhibition of adenylate cyclase. *FEBS Lett* **1990,** 260, (1), 73-78.

Allan, G. F.; Hutchins, A.; Liu, X. and Clancy, J., Induction of the progesterone receptor gene in estrogen target cells monitored by branched DNA signal amplification. *Steroids* **2001,** 66, (9), 663-671.

Amlal, H.; Faroqui, S.; Balasubramaniam, A. and Sheriff, S., Estrogen up-regulates neuropeptide Y Y1 receptor expression in a human breast cancer cell line. *Cancer Res* **2006,** 66, (7), 3706-3714.

Barkhem, T.; Carlsson, B.; Nilsson, Y.; Enmark, E.; Gustafsson, J. and Nilsson, S., Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol Pharmacol* **1998,** 54, (1), 105-112.

Bernhardt, G.; Spruß, T. and Rustler, M., Comparison of MCF-7 and ZR-75-1 cell lines as models for studying hormone dependent human breast cancer in nude mice. In: *Immunodeficient Mice in Oncology. Contrib. Oncol.* Fiebig, H. and Berger, D. (eds.), Karger, Basel, **1992**, Vol. 42, 128-130.

Bindal, R. D.; Carlson, K. E.; Katzenellenbogen, B. S. and Katzenellenbogen, J. A., Lipophilic impurities, not phenolsulfonphthalein, account for the estrogenic activity in commercial preparations of phenol red. *J Steroid Biochem* **1988,** 31, (3), 287-293.

de Cremoux, P.; Tran-Perennou, C.; Elie, C.; Boudou, E.; Barbaroux, C.; Poupon, M. F.; De Rycke, Y.; Asselain, B. and Magdelenat, H., Quantitation of estradiol receptors alpha and beta and progesterone receptors in human breast tumors by real-time reverse transcription-polymerase chain reaction. Correlation with protein assays. *Biochem Pharmacol* **2002,** 64, (3), 507-515.

Driggers, P. H. and Segars, J. H., Estrogen action and cytoplasmic signaling pathways. Part II: the role of growth factors and phosphorylation in estrogen signaling. *Trends Endocrinol Metab* **2002,** 13, (10), 422-427.

Entzeroth, M.; Braunger, H.; Eberlein, W.; Engel, W.; Rudolf, K.; Wienen, W.; Wieland, H. A.; Willim, K. D. and Doods, H. N., Labeling of neuropeptide Y receptors in SK-N-MC cells using the novel, nonpeptide Y1 receptor-selective antagonist [3H]BIBP3226. *Eur J Pharmacol* **1995,** 278, (3), 239-242.

Filardo, E. J.; Quinn, J. A.; Bland, K. I. and Frackelton, A. R., Jr., Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* **2000,** 14, (10), 1649-1660.

Gessele, K., Zelluläre testsysteme zur pharmakologischen Charakterisierung neuer Neuropeptid Y-Rezeptorantagonisten. Doctoral thesis, Universität Regensburg, Regensburg, **1998**

Jorgensen, M.; Vendelbo, B.; Skakkebaek, N. E. and Leffers, H., Assaying estrogenicity by quantitating the expression levels of endogenous estrogen-regulated genes. *Environ Health Perspect* **2000,** 108, (5), 403-412.

Keller, M.; Pop, N.; Hutzler, C.; Beck-Sickinger, A. G.; Bernhardt, G. and Buschauer, A., Guanidine-Acylguanidine Bioisosteric Approach in the Design of Radioligands: Synthesis of a Tritium-Labeled N(G)-Propionylargininamide ([(3)H]-UR-MK114) as a Highly Potent and Selective Neuropeptide Y Y(1) Receptor Antagonist. *J Med Chem* **2008,** 51, (24), 8168-8172.

Kitlinska, J.; Abe, K.; Kuo, L.; Pons, J.; Yu, M.; Li, L.; Tilan, J.; Everhart, L.; Lee, E. W.; Zukowska, Z. and Toretsky, J. A., Differential effects of neuropeptide Y on the growth and vascularization of neural crest-derived tumors. *Cancer Res* **2005,** 65, (5), 1719-1728.

Körner, M. and Reubi, J. C., NPY receptors in human cancer: a review of current knowledge. *Peptides* **2007,** 28, (2), 419-425.

Kuang, W. W.; Thompson, D. A.; Hoch, R. V. and Weigel, R. J., Differential screening and suppression subtractive hybridization identified genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line. *Nucleic Acids Res* **1998,** 26, (4), 1116-1123.

Larhammar, D.; Blomqvist, A. G.; Yee, F.; Jazin, E.; Yoo, H. and Wahlested, C., Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. *J Biol Chem* **1992,** 267, (16), 10935-10938.

Lim, Y. C.; Desta, Z.; Flockhart, D. A. and Skaar, T. C., Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. *Cancer Chemother Pharmacol* **2005,** 55, (5), 471-478.

Michel, M. C.; Beck-Sickinger, A.; Cox, H.; Doods, H. N.; Herzog, H.; Larhammar, D.; Quirion, R.; Schwartz, T. and Westfall, T., XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol Rev* **1998,** 50, (1), 143-150.

Motulsky, H. J. and Michel, M. C., Neuropeptide Y mobilizes Ca2+ and inhibits adenylate cyclase in human erythroleukemia cells. *Am J Physiol* **1988,** 255, (6 Pt 1), E880-885.

Müller, M.; Knieps, S.; Gessele, K.; Dove, S.; Bernhardt, G. and Buschauer, A., Synthesis and neuropeptide Y Y1 receptor antagonistic activity of N,N-disubstituted omega-guanidino- and omega-aminoalkanoic acid amides. *Arch Pharm (Weinheim)* **1997,** 330, (11), 333-342.

Pedrazzini, T.; Pralong, F. and Grouzmann, E., Neuropeptide Y: the universal soldier. *Cell Mol Life Sci* **2003,** 60, (2), 350-377.

Reubi, J. C.; Gugger, M.; Waser, B. and Schaer, J. C., Y(1)-mediated effect of neuropeptide Y in cancer: breast carcinomas as targets. *Cancer Res* **2001,** 61, (11), 4636-4641.

Rudolf, K.; Eberlein, W.; Engel, W.; Wieland, H. A.; Willim, K. D.; Entzeroth, M.; Wienen, W.; Beck-Sickinger, A. G. and Doods, H. N., The first highly potent and selective non-peptide neuropeptide Y Y1 receptor antagonist: BIBP3226. *Eur J Pharmacol* **1994,** 271, (2-3), R11-13.

Ruscica, M.; Dozio, E.; Boghossian, S.; Bovo, G.; Martos Riano, V.; Motta, M. and Magni, P., Activation of the Y1 receptor by neuropeptide Y regulates the growth of prostate cancer cells. *Endocrinology* **2006,** 147, (3), 1466-1473.

Shorter, N. A. and Pence, J. C., Retinoic acid-induced regulation of neuropeptide Y receptor expression and function in the neuroepithelioma line SK-N-MC. *J Pediatr Surg* **1997,** 32, (5), 721-723.

Speirs, V.; Malone, C.; Walton, D. S.; Kerin, M. J. and Atkin, S. L., Increased expression of estrogen receptor beta mRNA in tamoxifen-resistant breast cancer patients. *Cancer Res* **1999,** 59, (21), 5421-5424.

Stauffer, S. R.; Coletta, C. J.; Tedesco, R.; Nishiguchi, G.; Carlson, K.; Sun, J.; Katzenellenbogen, B. S. and Katzenellenbogen, J. A., Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *J Med Chem* **2000,** 43, (26), 4934-4947.

Sugiyama, A. and Lurie, K. G., An enzymatic fluorometric assay for adenosine 3':5'-monophosphate. *Anal Biochem* **1994,** 218, (1), 20-25.

E Summary

The estrogen receptor (ER), a member of the nuclear receptor family, is an established prediction factor in breast cancer with respect to successful hormonal therapy. The class of membrane bound receptors recently gained increasing interest in breast cancer diagnostics and treatment. Especially neuropeptide Y (NPY) receptors, members of the family of G-protein coupled receptors, have become a topic in breast cancer research in recent years, as the Y_1 receptor (Y_1R) subtype was found to be expressed by the majority (85 %) of human mammary carcinomas. This thesis aimed at the investigation of the functional expression and cross-talk between ERs and Y_1Rs in human breast cancer cells.

Selective "pure antagonists" of the ERα and β subtypes are useful pharmacological tools for the characterization of ER subtype specific cellular and physiological effects, such as cross-talk between ERα or β and the Y_1R, which is the subject of this thesis. 2-Phenylbenzofurans are known as ERβ-selective agonists from recent publications and previous work of our group, whereas substituents in position 7 of the benzofuran core were reported to be favorable with respect to selectivity. Aiming at ERβ–selective "pure antagonists", functionalized aliphatic side chains were introduced into position C7 of the benzofuran core. A 7-formylbenzofuran building block was synthesized for this purpose by a Sonogashira coupling reaction, starting from appropriate arylhalide and acetylene precursors. Simple alkyl and long "monofunctional" or "bifunctional" side chains were linked to the 7-formyl group by a reaction sequence involving a Grignard or a Wittig reaction, respectively.

Benzofuran **24,** bearing a small 1-propenyl substituent in C7 position, revealed high affinity to ERβ (RBA = 34) and >30-fold selectivity over ERα. **24** was shown to be an ER agonist in the luciferase gene reporter assay. Compounds, substituted with aliphatic side chains, comprising a thioether or sulfone group or a combination of a thioether and an amine function, revealed strongly decreased receptor affinities and selectivities. In the luciferase assay, these compounds were either weak antagonists or inactive.

To obtain ERα–selective antagonists, a library of 2-aryl-tetrahydroisoquinolin-6-ols (THIQs), substituted with different functionalized aliphatic side chains in position C1,

was synthesized. The THIQs were built up from appropriate phenylethylamine precursors, that were attached to the corresponding carboxylic acid chlorides of the side chains. The resulting amides were transformed into THIQs by a Bischler Napiralski ring closure reaction.

ER affinities of synthesized THIQs strongly depended on the nature of the side chain in position C1, whereas a 3'-hydroxy function at the *N*-phenyl ring was favorable for ERα–subtype selectivity. THIQs, containing side chains bearing a tertiary amine group at a distance of 6 atoms from the heterocyclic core, revealed binding affinities to ERα in the same order of magnitude (RBA ≈ 10) as determined for the potent ER antagonists fulvestrant (ICI 182.780) and 4-hydroxytamoxifen. After resolution of selected racemic THIQs into the respective enantiomers by chiral HPLC, the eutomers showed 3- to 6-fold higher ER binding affinity compared to the corresponding distomers. ERα–selectivities of 13- and 17-fold over ERβ were observed for compounds **93** and **94** with an amine and thioether containing "bifunctional" chain or a side chain bearing a sulfoxy function in combination with a 3'-hydroxy group, respectively. Both compounds exerted full antagonism in the luciferase assay with IC_{50} values in the sub-micromolar range, being appropriate pharmacological tools for blocking ERα–subtype specific cellular effects. Furthermore, THIQ **94,** containing a sulfoxide side chain, was a potent inhibitor of the proliferation of estrogen responsive MCF-7 breast cancer cells, suggesting further investigation of its value in the hormonal therapy of breast cancer.

ER and Y_1R protein expression by different subclones of MCF-7 breast cancer cells showing differential sensitivities against antiestrogen treatment were quantified by radioligand binding assays. For this purpose, Y_1Rs were labeled by the selective, high-affinity radioligand [^{3}H]-UR-MK114, recently developed in our workgroup. Basal expression of Y_1Rs by MCF-7 cells varied (40,000 to 100,000 binding sites per cell) and was inversely correlated with ER expression in vitro. In agreement with published results at the mRNA level, the Y_1R protein was up-regulated by 100 % after treatment of the cells with 17β-estradiol at physiological concentration (EC_{50} = 20 pM). An estrogen-induced Y_1R expression was also found in T-47-D breast cancer cells, but on a 20-fold lower level compared to MCF-7 cells. The incidence of Y_1Rs was exclusively observed in ER-positive breast cancer cells: Three ER-negative lines (MDA-MB-231, HCC1806 and HCC1937) revealed no Y_1R-specific binding.

The potent, highly ERα-selective agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole ("propylpyrazole triol", PPT), up-regulated the Y_1R by 100 % with an EC_{50} value of 0.25 nM, indicating a predominant role of ERα in Y_1R induction. The "pure ER antagonist" fulvestrant abrogated the 17β-estradiol-induced Y_1R expression in a concentration-dependent manner (IC_{50} = 5 nM) to 25 % of the basal level. The THIQ-based moderately ERα-selective antagonists **93** and **94,** synthesized within the scope of this thesis, down-regulated the Y_1R expression to the same extent as fulvestrant. Estrogen-induced Y_1R protein expression proved to be a useful endogenous reporter for the quantification of (anti)estrogenic activity of estrogen receptor ligands.

Functional coupling of the Y_1R to both, mobilization of intracellular calcium and inhibition of adenylyl cyclase activity, was demonstrated in MCF-7 cells by triggering intracellular calcium transients and suppression of forskolin-stimulated cAMP synthesis with NPY. However, there was neither an effect of NPY treatment on the proliferation of MCF-7 cells nor on ER-mediated transcriptional activity.

According to the results of this thesis, the Y_1R-mediated signaling cascade is not involved in downstream processes involved in tumor growth, that might be addressed in the therapy of breast cancer. Nonetheless, the Y_1R is a useful endogenous gene reporter for the quantification of ERα–specific (anti)estrogenic effects in whole-cell radioligand binding assays and a potential target for diagnostic imaging of breast cancer metastases.

F Appendix

1 Expression and Function of Histamine Receptors in MCF-7 and MDA-MB-231 Breast Cancer Cells

Histamine exerts its various functions through four histamine receptor (HR) subtypes H_1-H_4. The role of histamine in the biology and growth of malignant tumors has been discussed for a long time. For example in the mid 1990s, histamine was suggested as an autocrine growth factor, regulating breast cancer cell proliferation via H_1 and H_2 receptors (Cricco et al., 1994; Davio et al., 1995), and a potential use of H_2R antagonists in the treatment breast cancer was proposed (Davio et al., 1996). Very recently, the H_4R was reported to mediate growth inhibition of MDA-MB-231 breast cancer cells, whereas after stimulation of the H_3R an increased cell proliferation was observed (Medina et al., 2008).

In the present study the incidence of the histamine receptor subtypes H_2, H_3 and H_4 in MCF-7 and MDA MB-231 breast cancer cells was explored by radiochemical biding assays using appropriate radioligands, distinctly addressing the respective receptor subtypes. Data on the expression of functional histamine receptors by human breast cancer cells from binding assays have not been available up to date. This study aimed on the exploration of the role of the different histamine receptor subtypes in the biology of breast cancer.

1.1 Investigation of Histamine H_2, H_3, and H_4 Receptor Expression by Radioligand Binding in MCF-7 and MDA-MB-231 Cells

To detect H_2R expression, the commercially available radioligand [^{3}H]-tiotidine ($K_D \approx 30$ nM) was used. The unspecific binding was determined by displacement of the tritiated ligand from specific binding sites located in the plasma membranes by the H_2R selective antagonist ranitidine. A statistically significant difference between total and unspecific binding of [^{3}H]-tiotidine was observed in MDA-MB-231 cells, but not in MCF-7 cells. H_2R-specific binding increased with higher concentrations of the radioligand (cf. Figure

Ap1). The experiment was devised for the qualitative determination of the H_2R protein. As specific binding was only a samll fraction of nonspecific binding, the number of H_2R specific binding sites appears to be rather low.

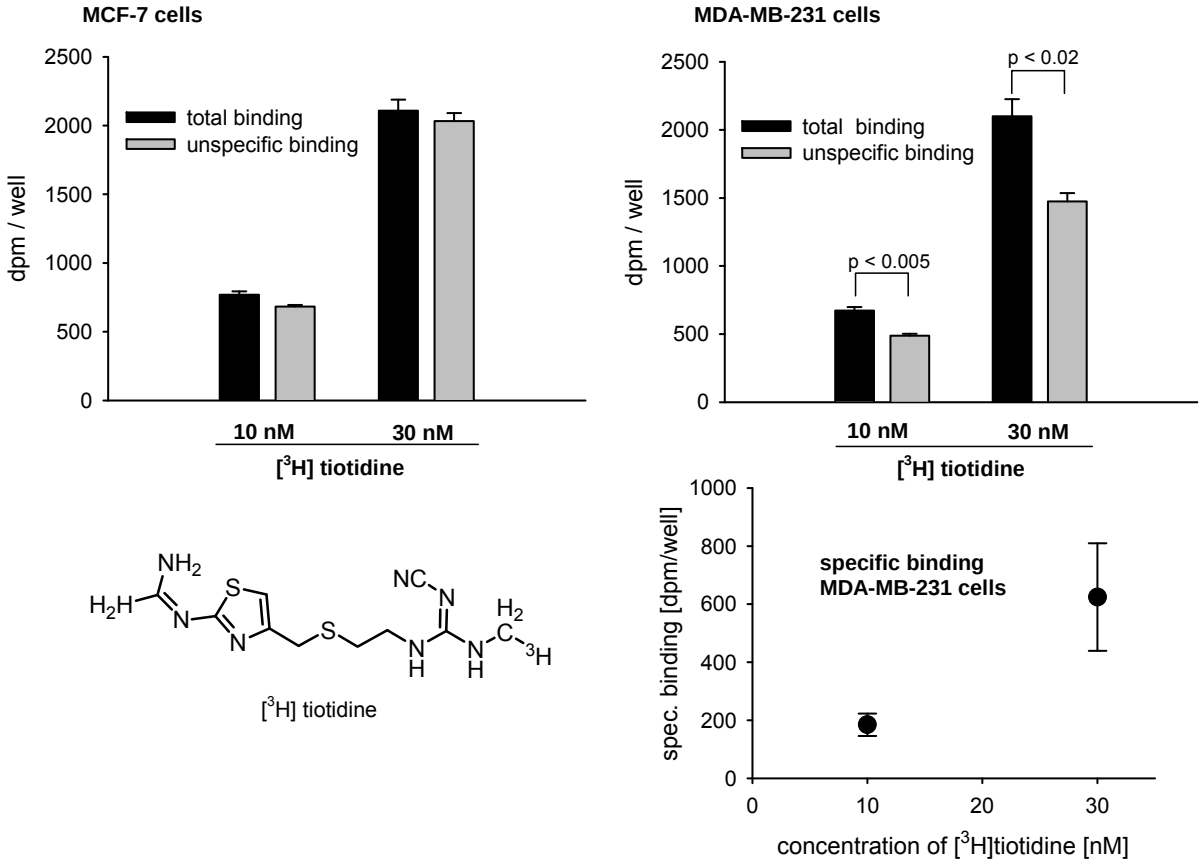


Figure F1: Binding of the H_2R selective radioligand [^{3}H]-tiotidine to whole MCF-7 and MDA-MB-231 cells. Unspecific binding was determined by displacement of the radioligand by the H_2R selective antagonist ranitidine.
Values represent means of triplicates ± SEM; p values were determined by Student's t-test; p<0.05 was considered as statistically significant.

The tritiated high affinity H_3 and H_4R ligand [^{3}H]-UR-PI-294 was recently developed by Dr. Igel in our research group (Igel et al., 2008). To discriminate between H_3 and H_4R specific binding the radioligand was successively displaced from the receptors by the H_3R selective agonist UR-PI-97 (Igel, 2008) or the H_4R selective antagonist JNJ7777120, respectively.

The H_3/H_4 R radioligand [^{3}H]-UR-PI-294 at a concentration of 10 nM showed no specific binding to MCF-7 cells. In the case of MDA-MB-231 cells total binding was significantly decreased after replacement of the radioligand by histamine and the H_3R selective

compound UR-PI-97. There was neither a statistically significant difference between the bound radioactivity in the presence of the radioligand alone nor in combination with the H_4R antagonist JNJ7777120 (cf. Figure Ap2). The pharmacological profile of [^{3}H]-UR-PI-294 at the H_3R and the H_4R (Igel et al., 2008) suggests an occupation of nearly all existing binding sites at the applied concentration of 10 nM. Thus, the result of the binding experiments suggests a poor expression of the H_3R by MDA-MB-231 cells, whereas in contrast to literature (Medina et al., 2008), H_4Rs were not detected on MDA-MB-231 cells.

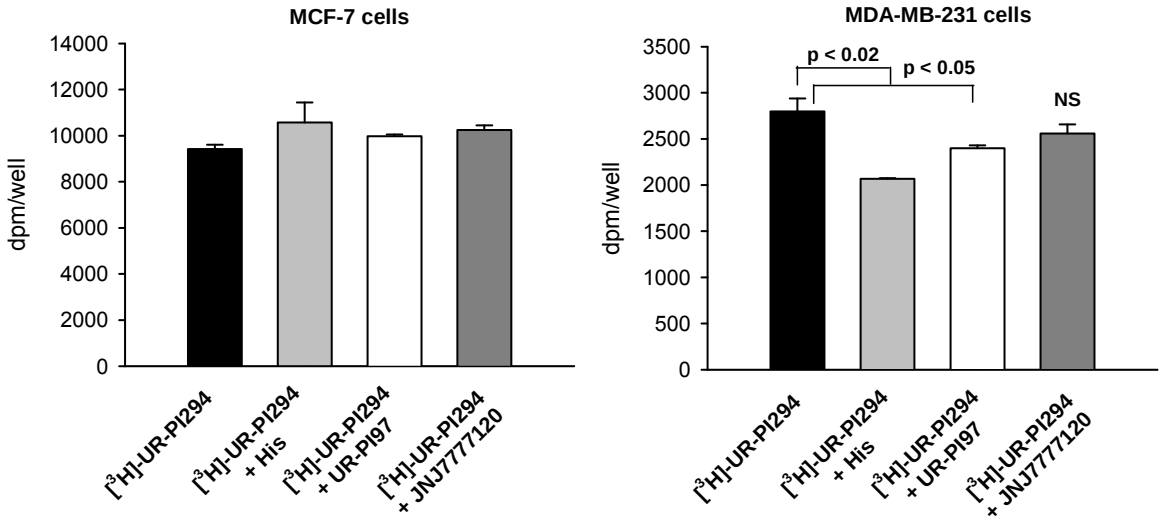


Figure F2: Binding of the H_3/H_4 R selective radioligand [^{3}H]-UR-PI-294 to MCF-7 and MDA-MB-231 breast cancer cells.

Values represent means of triplicates ± SEM; His: histamine; NS: not significant; p values were determined by Student's t-test; p<0.05 was accepted as statistically significant; a similar result was obtained in an additional independently performed experiment.

H_4R mRNA expression in MCF-7 and MDA-MB-231 cells was investigated by D. Schnell at the department of Pharmacology and Toxicology using a routine RT-PCR method. A strong band at the expected product size of 650 was detected in MDA-MB-231 cells but not in MCF-7 cells. The high mRNA expression found by RT-PCR-analysis agrees with a recently published report (Medina et al., 2006), but does not correlate with the result of the binding experiments, where no significant H_4R specific binding was detected. This might be due to either absent translation of the H_4R mRNA or misfolding or impaired trafficking of the receptor protein.

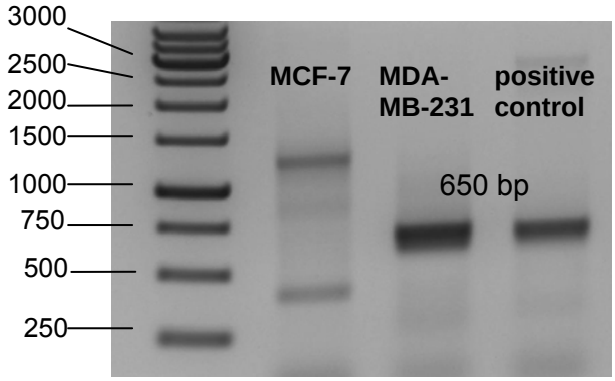


Figure F3:
mRNA expression by MCF-7 and MDA-MB-231 breast cancer cells. RT-PCR was conducted using a specific primer pair to give a 650 bp fragment of the H_4R cDNA. Positive control: cDNA generated from SF-9 insect cells, stably expressing the recombinant hH_4R.

1.2 Studies on the Role of Histamine in Breast Cancer Cell Proliferation

Histamine and H_4R agonists have recently been reported to induce a decreased proliferation of MDA-MB-231 cells (Medina et al., 2008). According to the binding experiments described above, H_2, H_3 or H_4R mediated effects in MDA-MB-231 are unlikely, as binding sites were very only present in the case of H_2 and H_3R in minute amounts or lacking as in case of H_4Rs. The impact of histamine receptors on the growth of MDA-MB-231 and MCF-7 breast cancer cells was extensively investigated in our research group in the mid 1990s (Bernhardt, unpublished results).

None of the growth kinetics experiments revealed any significant effect of a variety of histamine receptor ligands (agonists and antagonists) on the proliferation of the investigated cell lines. Figure Ap4 shows representative curves for the growth kinetics of MDA-MB-231 cells in the presence of histamine or the H_2R selective antagonist ranitidine.

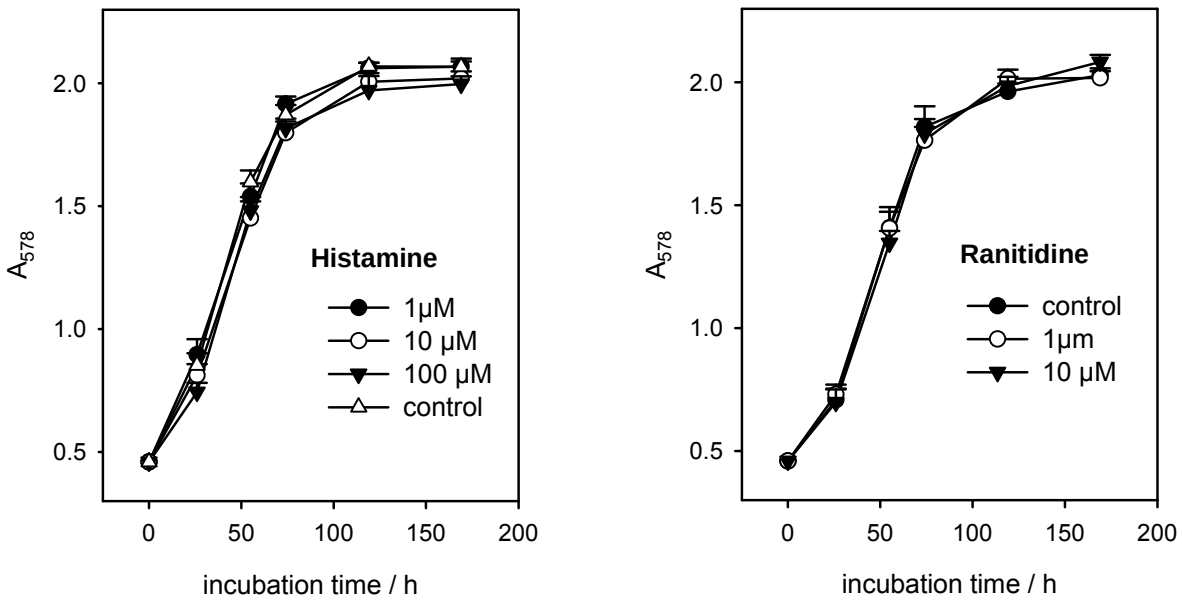


Figure F4: Growth kinetics of MDA-MB-231 cells in the presence of histamine or the H_2R antagonist ranititine compared to vehicle control; Crystal violet chemosensivity assay *Conditions: Mc Coy's 5A medium + 3% ct FCS*

Taken together, MCF-7 cells proved to be negative for all investigated H_2-H_4 receptor subtypes, as specific radioligand binding was not observed.

MDA-MB-231 cells revealed poor expression of H_2 and H_3 receptors, while in the case of the H_4R, there was no statistically significant difference between total and non-specific binding. H_4R mRNA expression was found in the case of MDA-MB-231 cells, but the gene appears not to be translated into a functional receptor protein. Regarding the low expression of histamine H_2 and H_3 receptors by MDA-MB-231 cells, the relevance of these receptors in cell biology, in particular in cell proliferation appears to be unlikely. In accordance with the result of the present binding studies, neither histamine nor any other agonist and antagonist influenced cell proliferation of MDA-MB-231 and MCF-7 breast cancer cells.

1.3 Experimental

Materials:

[^{3}H]-tiotidine was purchased from PerkinElmer (Waltham, MA, USA); histamine dihydrochloride was purchased from Alfa Aesar (Karlsruhe, Germany); ranitidine was purchased from Sigma; [^{3}H] UR-PI-294 (Igel et al., 2008) and UR-PI-97 (Igel, 2008)

were synthesized in house by Dr. Igel. JNJ7777120 was a gift from Dr. R. Thurmond (Department of Immunology, Johnson & Johnson Pharmaceutical R&D, San Diego, CA, USA).

Biding experiments:

All binding experiments were performed in whole cell assays in 24-well plates using the respective radioligands in appropriate dilutions. The protocol was adopted from the Y_1R radioligand binding assay as described in section D. The incubation period was 60 min in all experiments guaranteeing full receptor occupation. The concentration of [^{3}H]-UR-PI 294 was 10 nM. [^{3}H]-Tiotidine was applied at concentrations of 10 and 30 nM. For the determination of the unspecific binding [^{3}H]-tiotidine was displaced from receptor binding by a 1000-fold excess of the H_2R selective antagonist ranitidine. In the case of [^{3}H]-UR-PI-294 the radioligand was successively displaced by histamine (100 µM), UR-PI-97 (10 µM) and JNJ7777120 (10 µM), respectively.

RT-PCR analysis:

RNA analysis was performed by D. Schnell at the department of Pharmacology and Toxicology at the University of Regensburg following a routine protocol (Preuß, 2007). Total RNA was extracted from MDA-MB-231 and MCF-7 cells using an RNeasy Kit (Qiagen) following the manufacturer's protocol. Corresponding cDNA was generated by MMLV reverse transcriptase (Invitrogen). In a subsequent PCR a specific DNA fragment was generated by using the following primers: sense: 5'-GCC ATC ACA TCA TTC TTG GAA TTC GTG ATC CCA GTC-3'; antisense: 5'-GAT CCT CTA GAT TAG TGA TGG TGA TGA TGG TGA GAA GAT ACT GAC CG-3'; amplified product: 650 bp.

Proliferation assay:

The growth kinetics of MDA-MB-231 cells in presence of histamine and ranitidine was determined in the crystal violet assay as described in section C.

1.4 References

Cricco, G. P.; Davio, C. A.; Martin, G.; Engel, N.; Fitzsimons, C. P.; Bergoc, R. M. and Rivera, E. S., Histamine as an autocrine growth factor in experimental mammary carcinomas. *Agents Actions* **1994,** 43, (1-2), 17-20.

Davio, C.; Mladovan, A.; Shayo, C.; Lemos, B.; Baldi, A. and Rivera, E., Histamine receptors in neoplastic transformation: studies in human cell lines. *Inflamm Res* **1996,** 45 Suppl 1, S62-63.

Davio, C. A.; Cricco, G. P.; Bergoc, R. M. and Rivera, E. S., H1 and H2 histamine receptors in N-nitroso-N-methylurea (NMU)-induced carcinomas with atypical coupling to signal transducers. *Biochem Pharmacol* **1995,** 50, (1), 91-96.

Igel, P., Synthesis and structure-activity relationships of NG-acylated arylalkylguanidines and related compounds as histamine receptor ligands: searching for selective H4R agonists. Doctoral thesis, Universität Regensburg, Regensburg, **2008**

Igel, P.; Schnell, D.; Bernhardt, G.; Seifert, R. and Buschauer, A., Tritium-Labeled N(1)-[3-(1H-imidazol-4-yl)propyl]-N(2)-propionylguanidine ([(3)H]UR-PI294), a High-Affinity Histamine H(3) and H(4) Receptor Radioligand. *ChemMedChem* **2008**.

Medina, V.; Cricco, G.; Nunez, M.; Martin, G.; Mohamad, N.; Correa-Fiz, F.; Sanchez-Jimenez, F.; Bergoc, R. and Rivera, E. S., Histamine-mediated signaling processes in human malignant mammary cells. *Cancer Biol Ther* **2006,** 5, (11), 1462-1471.

Medina, V.; Croci, M.; Crescenti, E.; Mohamad, N.; Sanchez-Jimenez, F.; Massari, N.; Nunez, M.; Cricco, G.; Martin, G.; Bergoc, R. and Rivera, E., The role of histamine in human mammary carcinogenesis: H3 and H4 receptors as potential therapeutic targets for breast cancer treatment. *Cancer Biol Ther* **2008,** 7, (1), 28-35.

Preuss, H., Species-selective Interactions of Histamine H2 Receptors with Guanidine-type Agonists: Molecular Modelling, Site-directed Mutagenesis and Pharmacological Analysis. Doctoral thesis, Universität Regensburg, Regensburg, **2007**

2 Chemosensitivity of Triple Negative Human Breast Cancer Cells

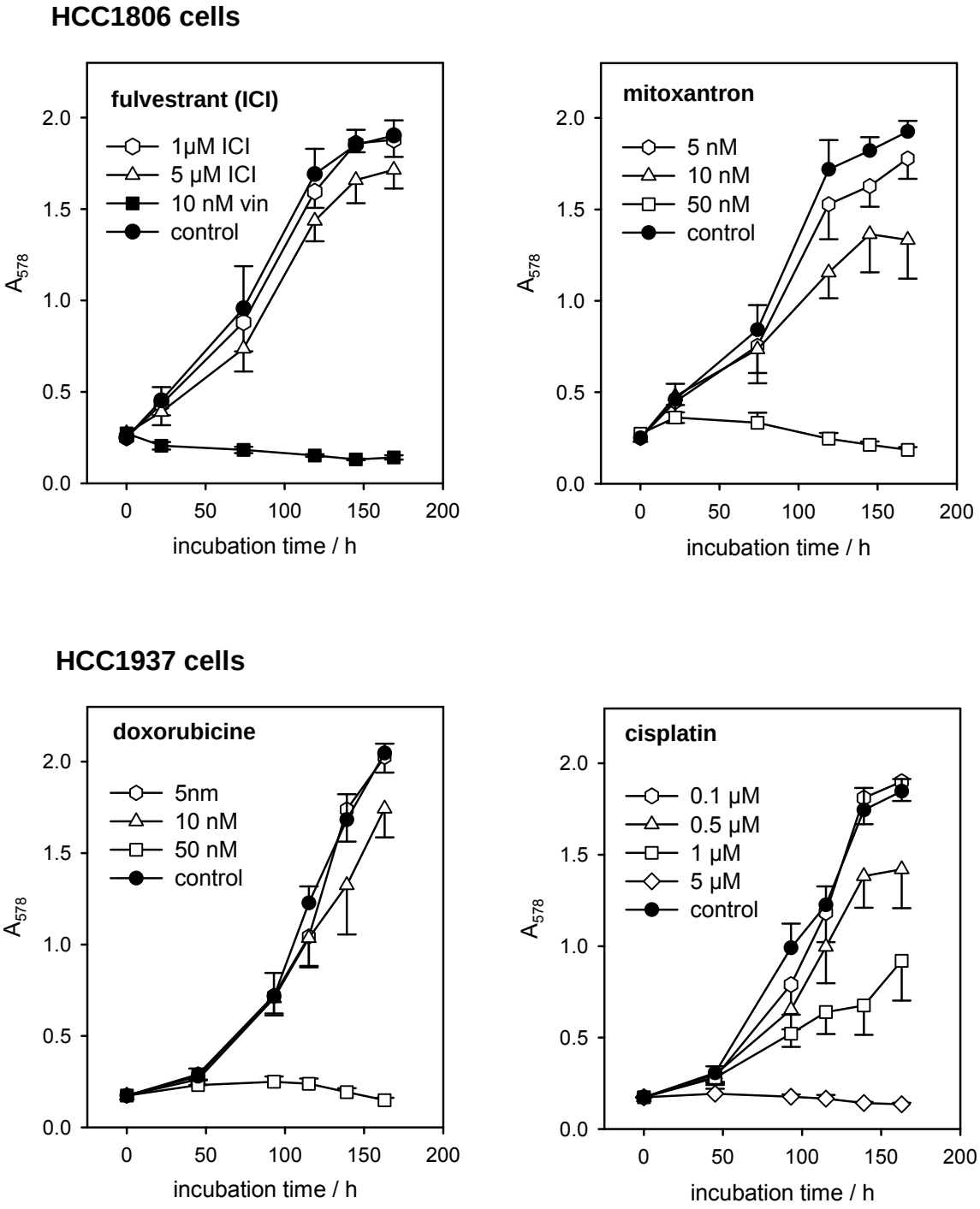


Figure F5: Growth kinetics of triple negative HCC1806 and HCC 1937 breast cancer cells in the presence of various cytostatics and the antiestrogen fulvestrant (ICI 182.780); Crystal violet chemosensivity assay; conditions: RPMI+10% FCS;
vin: vinblastin; "triple negative" refers to expression of ER, PR and HER2

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