

**Development of efficient cytochrome P450-dependent
whole-cell biotransformation reactions for steroid
hydroxylation and drug discovery**

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Publications resulting from this work

A. Manuscripts:

1. Derouet-Hümbert, E., Dragan, C. A., **Hakki, T.** and Bureik, M., 2007. ROS production by adrenodoxin does not cause apoptosis in fission yeast. *Apoptosis*. 12, 2135-2142.
2. **Hakki, T.** and Bernhardt, R., 2006. CYP17- and CYP11B-dependent steroid hydroxylases as drug development targets. *Pharmacol Ther.* 111, 27-52.
3. **Hakki, T.**, Zearo, S., Dragan, C. A., Bureik, M. and Bernhardt, R., 2008. Coexpression of redox partners increases the hydrocortisone (cortisol) production efficiency in CYP11B1 expressing fission yeast *Schizosaccharomyces pombe*. *J Biotechnol.* 133, 354-359.
4. **Hakki, T.**, Hübel, K., Waldmann, H. and Bernhardt, R., in preparation. The development of high throughput screening system for the discovery of human aldosterone synthase (CYP11B2) inhibitors.
5. Petric, S., **Hakki, T.**, Bernhardt, R., Cresnar, B. in preparation. Characterization and expression of progesterone-inducible cytochrome P450 genes in the zygomycete fungus *Rhizopus oryzae*.

B. Patent application

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Vier neue und spezifische Inhibitoren der humanen Aldosteronsynthase (Submitted)

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Abbreviations

| | |
|----------------------|---|
| 18-OH-B | 18-hydroxycorticosterone |
| 5-FOA | 5 -fluoroorotic acid |
| A | Area |
| ACE | Angiotensin-converting enzyme |
| ACTH | Adrenocorticotrophic hormone |
| AdR | Adrenodoxin reductase |
| Adx | Adrenodoxin |
| Adx ^{D113Y} | Adx substitution mutant containing Tyr instead of Asp at position 113 |
| Adx ^{S112W} | Adx substitution mutant containing Trp instead of Ser at position 112 |
| Adx ^{WT} | Adrenodoxin wild type |
| AGS | Adrenogenital syndrome |
| Aldo | Aldosterone |
| ampR | Ampicillin resistance gene |
| arh1 | Fission yeast ferredoxin reductase |
| ars1 | Autosomal replicating sequence |
| B | Corticosterone |
| CAH | Congenital adrenal hyperplasia |
| C _{inh} | Inhibitor concentration |
| CMO | Corticosterone methyl oxidase |
| CPR | Cytochrome P450 reductase |
| CRH | Corticotropin-releasing hormone |
| CYP11B1 | Steroid 11 β -hydroxylase, cytochrome P450c11 |
| CYP11B2 | Aldosterone synthase, cytochrome P450c11Aldo |
| Da | Dalton |
| DBH | Dopamine β - hydroxylase |
| DHEA | Dehydroepiandrosterone |
| DITIs | Disposable Tips |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DOC | 11-deoxycorticosterone |
| DTE | 1,4-Dithioerythritol |
| EMM | Edinburgh minimal medium |
| EPHESUS | The Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study |
| ET | Electron transfer |
| EtOH | Ethanol |
| etp1 ^{fd} | Adrenodoxin-like ferredoxin |
| F | Cortisol (Hydrocortisone) |
| <i>f</i> | Correction factor |
| FAD | Flavine adenine dinucleotide |
| FH-I | Familial hyperaldosteronism type I |

| | |
|--------------------------|---|
| FMN | Flavine mononucleotide |
| GRA | Glucocorticoid-remediable aldosteronism |
| GSH | Glucocorticoid-suppressible hyperaldosteronism |
| HPLC | High performance liquid chromatography |
| HPTLC | High performance thin-layer chromatography |
| HTS | High throughput screening system |
| IC ₅₀ | Concentration of inhibitor that gives 50% inhibition |
| <i>INH(P)</i> | The inhibition of the product production |
| <i>I_{radio}</i> | Intensity of the radioactive signal |
| IST | Internal standard |
| IZA | Inner zone antigen |
| kDa | kilodalton |
| LB | Luria-Bertani |
| LiAc | Lithium acetate |
| LOPAC | Library of pharmacologically active compounds |
| MAO | Monoamine oxidase |
| MeOH | Methanol |
| NaAc | Sodium acetate |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NC | Negative control |
| PC | Positive control |
| PEG | Polyethylene glycol |
| PMSF | Phenyl methyl sulfonyl fluoride |
| PNMT | Phenylethanolamine N-methyltransferase |
| pNMT | no message with thiamine promoter |
| RALES | Randomized Aldosterone Evaluation Study trial |
| RNA | Ribonucleic acid |
| ROI | Region of interest |
| RSS | 11-deoxycortisol |
| RT | Room temperature |
| <i>S. cerevisiae</i> | <i>Saccharomyces cerevisiae</i> |
| <i>S. pombe</i> | <i>Schizosaccharomyces pombe</i> |
| SAR | Structure-activity relationship |
| SCC | Side-chain cleavage |
| SDH | Steroid dehydrogenases |
| SDS-PAGE | Sodium dodecylsulfate polyacrylamid gel electrophoresis |
| SE | Standard error of the mean |
| TH | Tyrosine hydroxylase |
| YEA | Yeast extract and supplements |

Abstract

Cytochromes P450 play a vital role in the steroid biosynthesis in the human adrenal gland, e.g. the production of hydrocortisone and aldosterone by CYP11B1 and CYP11B2, respectively. The steroid hydroxylases of the CYP11B family are important targets for drug development. Since they are very closely related, the discovery of selective inhibitors has been a focus of interest. Furthermore, hydrocortisone is a precursor for drugs with high therapeutic potential. Therefore, the purpose of this work was the development of an efficient system for CYP11B-dependent whole-cell biotransformation to facilitate the bioproduction of hydrocortisone and the discovery of selective inhibitors. The present work shows clearly that hydrocortisone production can be dramatically enhanced (3.4-fold) by coexpression of the natural redox partners of CYP11B1. Moreover, a high production efficiency has been achieved by optimisation of the reaction conditions. Additionally, in the course of this work an automated screening technology plate-format has been developed using a CYP11B2-expressing fission yeast strain. Additionally, the conditions for HPLC analysis of steroids were optimised and a high throughput screening system for the discovery of CYP11B2 inhibitors has been established. The new screening system was successfully used for the investigation of a library of pharmacologically active compounds resulting in the identification of several novel potential inhibitors of CYP11B2.

Zusammenfassung

Cytochrome P450 spielen eine entscheidende Rolle in der Steroidbiosynthese in der menschlichen Nebenniere z. B. bei der Produktion von Hydrocortison durch CYP11B1 sowie von Aldosteron durch CYP11B2. Steroidhydroxylasen der CYP11B Familie stellen Ziele für die Entwicklung von Medikamenten dar. Wegen des hohen Verwandtschaftsgrades dieser Enzyme ist die Entdeckung selektiver Inhibitoren von großem Interesse. Darüber hinaus ist Hydrocortison eine Vorstufe für Arzneimittel mit großem therapeutischem Potenzial. Das Ziel der vorliegenden Arbeit war daher die Entwicklung eines effizienten Systems für die CYP11B-abhängige Ganzzellbiotransformation, um die Herstellung von Hydrocortison sowie die Identifizierung selektiver CYP11B2-Inhibitoren zu erleichtern. Die Hydrocortisonproduktion konnte in dieser Arbeit durch die Coexpression der natürlichen Redoxpartner von CYP11B1 und durch die Optimierung der Reaktionsbedingungen deutlich gesteigert werden. Für den Nachweis von selektiven CYP11B2-Inhibitoren wurde für die Spaltheife ein automatisches hintergrundarmes Hochdurchsatz-Screening-System, basierend auf Mikrotiterplatten und HPLC-Analyse entwickelt. Dieses Verfahren wurde anschließend erfolgreich für die Untersuchung einer Bibliothek pharmakologisch aktiver Komponenten benutzt, wobei neue potentielle Inhibitoren detektiert wurden.

Summary

Cytochromes P450 play a vital role in the steroid biosynthesis pathway in the human adrenal gland, exemplified by the production of the main glucocorticoid hydrocortisone (cortisol) from 11-deoxycortisone by CYP11B1, and the production of the most important human mineralocorticoid aldosterone from 11-deoxycorticosterone by CYP11B2. CYP11B-dependent steroid hydroxylases are drug development targets, and since they are very closely related enzymes, the discovery of selective inhibitors of each one has been a hot topic. Furthermore, hydrocortisone is a precursor for drugs with high therapeutic potential. Therefore, the purpose of this work was the development of efficient CYP11Bs-dependent whole-cell biotransformation reactions for the bioproduction of hydrocortisone and the discovery of selective inhibitors of CYP11B2.

For this reason, the corresponding mitochondrial electron transfer proteins (AdR and Adx) were coexpressed with CYP11B1 in fission yeast *Schizosaccharomyces pombe*. Moreover, two mutants of Adx were investigated and coexpressed with AdR to improve the electron transport to CYP11B1 to increase the bioproduction of hydrocortisone in recombinant fission yeast. This work shows clearly that hydrocortisone production can be dramatically enhanced (3.4- fold) by coexpressing the other components of the CYP11B1 electron transfer chain and by optimising the reaction conditions to achieve a high production efficiency on the laboratory level. The new fission yeast strain TH75 coexpressing the wild type Adx and AdR displays high production efficiency at an average of 9.7 μmol hydrocortisone / 10 ml test culture over a period of 72 hours, the highest value published to date for this biotransformation.

In addition, using a CYP11B2-expressing fission yeast strain, an automated screening technology plate-format has been developed. The new screening technology is a one-point HPLC-based assay that investigates compounds regarding of their inhibitory effect against CYP11B2 at concentration of 41.6 μM . Furthermore, the HPLC was further optimised, which enabled the separation of the closely related steroids 11-deoxycorticosterone (DOC) and corticosterone (B) within 2 minutes. Hence, a high throughput screening system has been established. The new screening system displayed high reproducibility and was validated in the presence of controls. In a next step, a library of pharmacologically active compounds was investigated using this new screening system, which reported novel potential inhibitors of CYP11B2. The new inhibitors were further validated and new selective inhibitors have been discovered. Since the use of these drugs is usually combined with unexplained hypotension and severe side effects, the ability of these compounds to inhibit CYP11B2 can explain to

some extent these side effects. Furthermore, the new inhibitors are “druggable” compounds that could be used either in the treatment of hyperaldosteronism-related diseases or as lead compounds that could further optimised in the field of drug development to achieve more safe and selective inhibitors of CYP11B2.

Although the screening system was developed and validated on the laboratory level, it displayed the ability to screen up to 600 compounds per week with the possibility to increase the capacity of the test (10-fold) up to 6000 compounds per week.

Taken together, the newly developed system is a robust screening system that can be applied to investigate libraries of existing drugs to find novel CYP11B2 inhibitors. This screening enables the reposition (recycling) of existing drugs, which can save costs and billions of dollars spend to develop new CYP11B2 inhibitors.

1. Introduction

1.1. Steroid hormones and cytochromes P450

Steroid hormone research began in a broader sense with the crystallisation of several sex steroid hormones in the years 1929-1935, of the glucocorticoids in 1935–1938, and finally of aldosterone in 1953. All of these hormones possess the basic parent cyclopentanophenanthrene ring structure provided by cholesterol, which is modified by an array of enzymes expressed at various levels and in numerous tissues throughout the body.

The enzymes involved in steroid hormone metabolism can be divided into three large groups: the cytochromes P450, reductases and steroid dehydrogenases (SDH), each of which exhibits important, biochemically distinct properties (Miller 1988; Lisurek and Bernhardt 2004; Hakki and Bernhardt 2006). P450 enzymes comprise a large family of highly conserved proteins that incorporate molecular oxygen into lipophilic substrates with the provision of reducing equivalents from the cofactor NAD(P)H (Figure 1.1).

Cytochrome P450 proteins in humans are enzymes that synthesise cholesterol, steroids, and other important endogenous substrates such as prostacyclins and thromboxane A₂, and degrade xenobiotics and drugs. They catalyse many types of reactions, but the most important one is hydroxylation. These enzymes are classified as mixed function oxidases or monooxygenases, because they incorporate one atom of molecular oxygen into the substrate and one atom into water (Figure 1.1.).

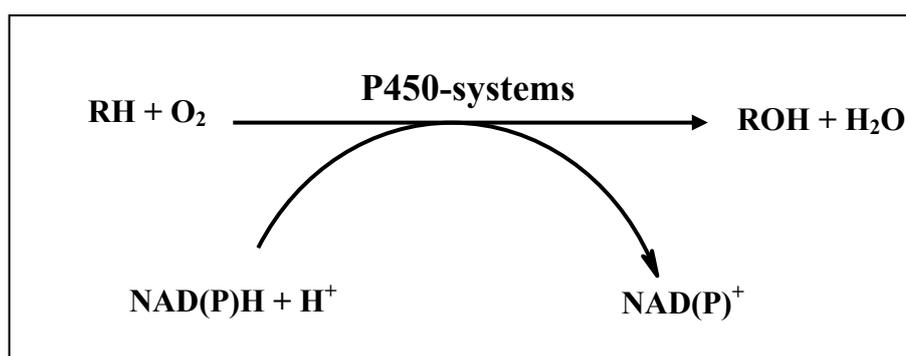


Figure 1.1. Reaction generally catalysed by cytochrome P450.

Cytochromes P450 are monooxygenases, which in contrast to dioxygenases catalyses the incorporation of a single atom of molecular oxygen into the substrate. The reduction equivalents needed for this reaction are provided by an external cofactor.

These reactions are essentially irreversible, not easily to be inhibited, and are so poised in the steroidogenic pathway that they determine the formation of each of the five major classes of

steroid hormones: progestagens, mineralocorticoids, glucocorticoids, androgens, and estrogens. To activate oxygen in the substrate binding pocket of P450s, electrons must be transferred from NAD(P)H to the P450. Although some P450s do not require any other protein component to achieve the reductive activation of molecular oxygen (Degtyarenko and Kulikova 2001), the vast majority of P450s performs the diverse range of chemical reactions after interaction with one or more redox partners to obtain the redox equivalents from electron transfer (ET) chains. A protein complex forms transiently between the P450 and the redox partner allowing the effective transfer of electrons. Although ten classes of P450 systems have been recently classified depending on the topology of the protein components involved in the electron transfer to the P450 enzyme (Hannemann *et al.*, 2007), there are only two redox protein systems involved in the steroid biosynthesis in mammals. One for the P450 enzymes anchored in the mitochondrial membrane and one for P450s located in the endoplasmic reticulum (microsomal compartment). The mitochondrial electron transfer chain consists of two components, a FAD containing flavoprotein, adrenodoxin reductase (AdR), and an iron-sulphur protein of the [2Fe-2S] ferredoxin type, adrenodoxin (Adx) (Figure 1.2) (Lambeth *et al.*, 1982; Hannemann *et al.* 2007).

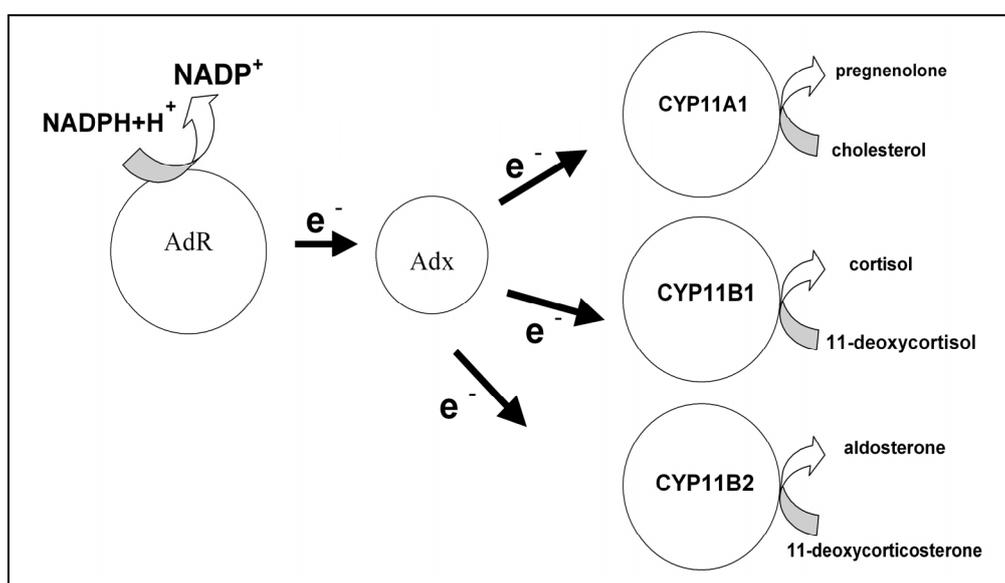


Figure 1.2. The mitochondrial steroid hydroxylase systems.

The electron transport chain for the adrenal mitochondrial steroid hydroxylases consisting of a [2Fe-2S] ferredoxin designated as adrenodoxin (Adx) and a FAD containing, NADPH-dependent ferredoxin reductase accordingly referred to as adrenodoxin reductase (AdR).

Microsomal P450s are supported by a single redox partner protein, the highly conserved FAD and FMN containing flavoprotein NADPH-cytochrome P450 reductase (CPR) (Black and

Coon 1987; Porter 1991) In some cases, also a third protein, cytochrome b5 is involved in modular protein–protein interaction and electron transport (Figure 1.3).

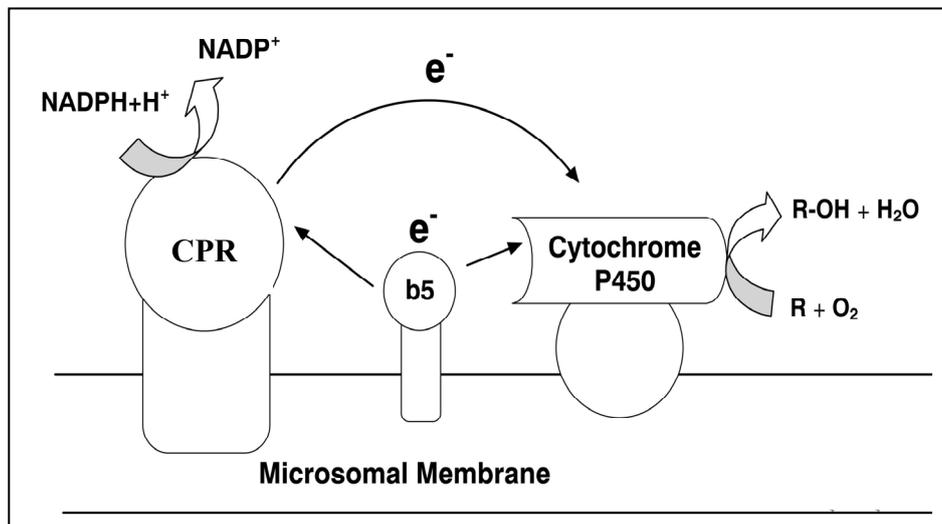


Figure 1.3. The microsomal steroid hydroxylase systems.

Microsomal hydroxylases receive the necessary electrons from an NADPH-dependent FAD- and FMN-containing reductase (CPR).

Thus, the subcellular location (Tamaoki 1973) and corresponding electron transfer or redox system also defines a subclassification of mitochondrial or microsomal cytochrome P450s involved in steroid synthesis, collectively known as the steroid hydroxylases. Within the mitochondrial class of steroid hydroxylases of most species, there are three functionally distinct P450 enzymes (Figure 1.4).

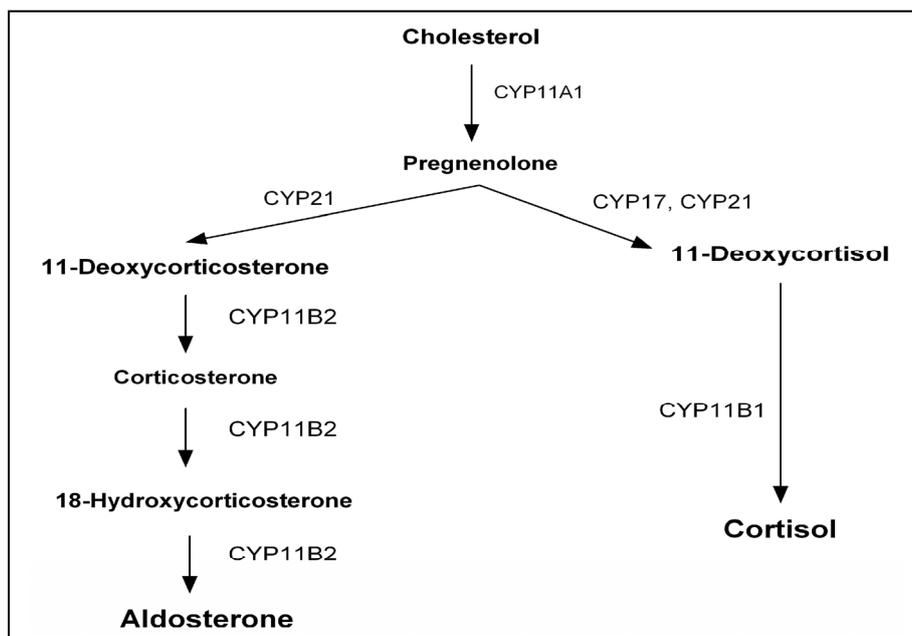


Figure 1.4. The role of cytochromes P450 in the biosynthesis of steroid hormones in the human adrenal cortex. The biosynthesis of all steroid hormones in the adrenal cortex starts with a reaction catalysed by CYP11A1, which cleaves the cholesterol side chain and forms pregnenolone. Other members of the CYP11 family (CYP11B1 and CYP11B2) are involved in the production of cortisol and aldosterone.

The first one, the cholesterol side-chain cleavage P450 (CYP11A, also known as P450_{sc}), utilizes cholesterol for the formation of pregnenolone, which is the universal precursor for all subsequent steroids. A second enzyme, which is cytochrome P450 steroid 11 β -hydroxylase (CYP11B1, also known as P450_{11 β} or P450c11) catalyses the last step in cortisol (hydrocortisone) biosynthesis (Figure 1.4). In addition, CYP11B1 catalyses the subsequent conversion of corticosterone to aldosterone in some species such as cow and pig, and therefore this enzyme is critical in mineralocorticoid metabolism in these animals. In humans, baboons, rats, mice, and guinea pigs, however, a third mitochondrial cytochrome P450, aldosterone synthase (CYP11B2, also known as P450_{aldo}), is encoded by another gene (CYP11B2), which has evolved by duplication of CYP11B1 to specifically catalyses aldosterone synthesis (Bureik *et al.*, 2002a).

The enzymes comprising the microsomal steroid hydroxylase group include three P450s involved in steroid hormone biosynthetic steps subsequent to CYP11A1 leading to both corticoid and sex steroid hormone synthesis.

CYP17 (17 α -hydroxylase/17,20-lyase, also known as P45017 α or P450c17) catalyses the 17-hydroxylation of pregnenolone and progesterone and the 17,20-lyase reaction of the corresponding 17-hydroxylated products. Progesterone and 17-hydroxyprogesterone are substrates for 21-hydroxylase cytochrome P450 (CYP21, also known as P450c21), which catalyses the formation of 11-deoxycorticosterone (DOC) and 11-deoxycortisol (RSS), intermediates in corticosterone and cortisol biosynthesis (Figure 1.4). Finally, the aromatase enzyme (CYP19, P450arom) is responsible for the aromatisation of ring A leading to estrogens. Mutations in steroid hydroxylase genes, or deficiencies of these enzymes, are responsible for several human diseases. Thus, congenital adrenal hyperplasia (CAH) (also known as adrenogenital syndrome; AGS) is mainly caused by defects of CYP21 (Migeon and Donohoue 1991; New 1992), although in 8-9% of the patients with CAH, CYP11B1 mutations are fault (Naganuma *et al.*, 1988; Migeon and Donohoue 1991; New 1992). Defects in aldosterone production caused by mutations in CYP11B2 lead to salt wasting and to failure to thrive (White 2004). Defects in the CYP17 gene exemplified by the 17-hydroxylase deficiency in which the production of sex steroids is absent, results in a compensatory increase in follicle-stimulating hormone and luteinizing hormone, comparable to menopausal levels. In humans, the CYP17 gene is expressed in the adrenal cortex, testes, and ovaries but not the placenta. The adrenals produce glucocorticoids, mineralocorticoids, and C-19 steroids. The gonads, on the other hand, predominantly produce the C-19 steroids and sex hormones. Thus, in patients with 17-hydroxylase deficiencies both adrenal and gonadal steroidogenesis

is impaired. In contrast, CYP17 overproduction leads to prostate cancer (Madigan *et al.*, 2003). Moreover, in some cases prostate cancer is stimulated by androgen production as breast cancer is by estrogens (Figure 1.5). Overproduction of cortisol can be one cause of Cushing's syndrome, which is a chronic glucocorticoid excess associated with substantial morbidity and mortality (Boscaro *et al.*, 2001; Fisher *et al.*, 2001). Overproduction of aldosterone has been shown to cause hypertension. Furthermore, hyperaldosteronism was found to be in 5 to 10% of all patients with hypertension (Young 2007) and congestive heart failure and fibrosis of the heart (Pitt *et al.*, 1999; Brilla 2000; Pitt *et al.*, 2001; Hakki and Bernhardt 2006). More recently, R-fadrozole, which has been reported as aldosterone synthase inhibitor showed the ability to reverse cardiac fibrosis in spontaneously hypertensive heart failure rats (Minnaard-Huiban *et al.*, 2008).

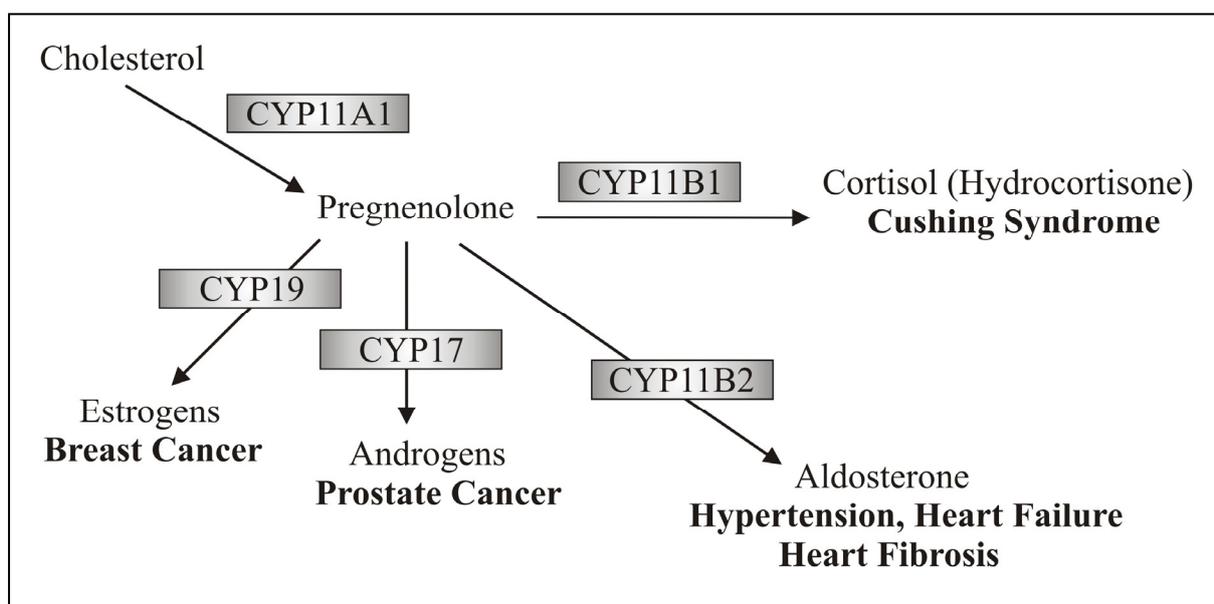


Figure 1.5. Steroid hydroxylases as drug development targets.

Steroid overproduction-related diseases can be treated or controlled by inhibiting the corresponding steroid hydroxylase.

During the past years it became obvious that cytochromes P450 not only play an exceptional role in drug and xenobiotics metabolism and in the biosynthesis of endogenous compounds, but also gain increasing importance as novel therapeutic targets for drug development (Baston and Leroux 2007; Schuster and Bernhardt 2007).

1.2. Human CYP11B1 and CYP11B2

1.2.1. General aspects

Human CYP11B1 and CYP11B2 are localised mainly in the adrenal cortex that consists of the zona glomerulosa and the zona fasciculata/reticularis, which differ from each other with regard to steroidogenic reactions catalysed by different cytochrome P450 isozymes. Glucocorticoids and adrenal androgens are synthesised in the zona fasciculata/reticularis, whereas aldosterone, the most potent natural mineralocorticoid, is synthesised in zona glomerulosa cells (Miller and Tyrell 1995). CYP11B1 is expressed at high levels in the zona fasciculata/reticularis of the adrenal cortex (Erdmann *et al.*, 1995a; Erdmann *et al.*, 1995b; Miller and Tyrell 1995) and produces cortisol, the principal human glucocorticoid. In contrast, aldosterone secretion (Miller and Tyrell 1995) and CYP11B2 expression (Pascoe *et al.*, 1995) take place at low levels in the zona glomerulosa of the adrenal cortex. Vinson argued that the zona glomerulosa in fact has many functions, including aldosterone synthesis, but is probably only a relatively poor de novo source of steroids. In vitro, CYP11B2 (aldosterone synthase) of the glomerulosa has the ability to use the products that arise from CYP11B1 activity in fasciculate cells (Vinson 2004) as substrates. This zonal distribution of expression has been further investigated with surgically removed human adrenal gland cells, showing a higher concentration of CYP11B1 in the zona fasciculata than in the zona reticularis (Mitani *et al.*, 1982). Furthermore, Mitani *et al.* identified the so-called “undifferentiated cell zone”, which could facilitate the exploration of molecular mechanisms for the differentiation and development of adrenocortical cells (Mitani *et al.*, 2003). Min *et al.* characterized the adrenal-specific inner zone antigen (IZA), which is a protein specifically expressed in the zona fasciculate/reticularis of the adrenal cortex, and reported the inhibition of adrenal steroidogenesis by the addition of an anti-IZA monoclonal antibody, and the adrenal steroidogenesis activation by IZA overexpression suggesting its importance in the steroidogenesis (Min *et al.*, 2004). CYP11B enzymes of other species have also been studied, and results indicated that bovine (Wada *et al.*, 1985), porcine (Yanagibashi and Hall 1986), and frog (Nonaka *et al.*, 1995) adrenal cortex syntheses of gluco- and mineralocorticoids are catalysed by a single enzyme, while in man (Kawamoto *et al.*, 1990a; Ogishima *et al.*, 1991), baboons (Hampf *et al.*, 1996; Brown *et al.*, 2002), rats (Matsukawa *et al.*, 1990), mice (Domalik *et al.*, 1991), and guinea pigs (Bülow *et al.*, 1996; Bülow and Bernhardt 2002) two distinct isoforms are involved in the formation of either mineralo- or glucocorticoids. The

reason for these interspecies differences is unknown. Enzymes with 11 β -hydroxylase activity have also been found in several fungi (Megges *et al.*, 1990); however, none of the genes for these enzymes has been cloned to date and their relation to the CYP11B family remains unclear.

The DNA sequence of the *CYP11B2* gene is about 95% identical to that of the *CYP11B1* gene in the coding regions and 90% identical in the introns. The 5' upstream region has, however, diverged considerably from that of *CYP11B1*, suggesting that this second gene, if expressed, may be regulated differently. Mornet *et al.* determined that the *CYP11B1* and *CYP11B2* genes both contain nine exons (Mornet *et al.*, 1989). The eight introns are identical in location to the introns of *CYP11A1*. The genes encoding the two human enzymes are arranged ~ 45 kb apart from each other on chromosome 8 (Chua *et al.*, 1987; Wagner *et al.*, 1991) and chimeric *CYP11B1/CYP11B2* genes that result from unequal crossing-over between these two genes have been found in patients suffering from familial hyperaldosteronism type I (FH-I; also called glucocorticoid-remediable hyperaldosteronism) and congenital adrenal hyperplasia (Lifton *et al.*, 1992; Pascoe *et al.*, 1992; MacConnachie *et al.*, 1998; Hampf *et al.*, 2001).

1.2.2. Physiological role of CYP11B1 and CYP11B2

Cortisol (Hydrocortisone) is the main glucocorticoid in humans. It regulates energy mobilisation and thus the stress response. In addition, it is involved in the immune response of the human body. It is formed by 11 β -hydroxylation of 11-deoxycortisol (RSS) (Figure 1.6) and is normally secreted 100-to 1000-fold in excess over aldosterone.

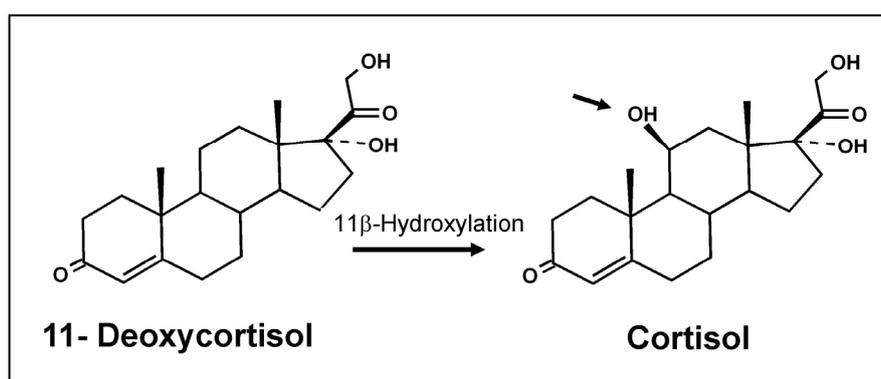


Figure 1.6. CYP11B1-dependent hydroxylation reaction.

Human CYP11B1 enzyme is a pure 11 β -hydroxylase, and catalyses the 11 β -hydroxylation reaction that produces cortisol (F) from 11-deoxycortisol (RSS).

Aldosterone, the most important human mineralocorticoid, is involved in the regulation of the salt and water household of the body and thus in the regulation of blood pressure. The terminal three steps in aldosterone biogenesis in humans are the 11 β -hydroxylation of 11-deoxycorticosterone (DOC) that leads to corticosterone (B), which is then 18-hydroxylated to yield 18-hydroxycorticosterone (18-OH-B) and finally oxidized to aldosterone (Figure 1.7).

DOC can also be first 18-hydroxylated to yield 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) followed by conversions to 18-OH-B and aldosterone, but in man this pathway is unlikely to be important (Fisher *et al.* 2001). Both CYP11B1 and CYP11B2 11 β -hydroxylate RSS and DOC in vitro (Kawamoto *et al.* 1990a; Curnow *et al.*, 1991; Denner *et al.*, 1995a); however, the human CYP11B1 enzyme is a pure 11 β -hydroxylase without 18-hydroxylase or 18-oxidase activity and is even unable to 11 β -hydroxylate 18-OH-DOC (Fisher *et al.* 2001).

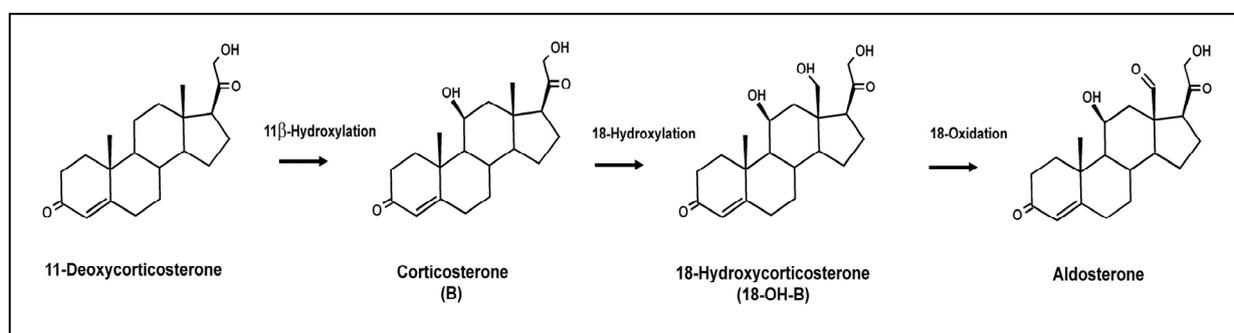


Figure 1.7. CYP11B2 converts 11-deoxycorticosterone via corticosterone and 18-OH corticosterone to aldosterone.

CYP11B2 11 β -hydroxylates 11-deoxycorticosterone (DOC) to yield corticosterone (B), which will then 18-hydroxylated to yield 18-hydroxycorticosterone and finally 18-oxidized to aldosterone.

CYP11B2 displays much weaker 11 β -hydroxylase activity towards RSS, but also 18-hydroxylates cortisol. The influence of several 18-hydroxylated steroids on human CYP11B1 and CYP11B2 activity was investigated (Fisher *et al.* 2001) using stably transfected V79 cells (Denner *et al.* 1995a; Denner *et al.*, 1995b). It was found that neither 18-hydroxycortisol nor 18-oxocortisol affected the efficiency of use of DOC or RSS as substrates by both enzymes, thus ruling out that these compounds contribute to lower 11 β -hydroxylase activity in glucocorticoid-suppressible hyperaldosteronism. In contrast, 18-OH-DOC significantly reduced the conversion rate of DOC to B and that of RSS to cortisol by both enzymes, while it increased the production rate of 18-OH-B and aldosterone by CYP11B2.

As mentioned above, there is a series of different diseases that are connected with changes in the steroid hormone production. Adrenogenital syndrome (AGS, CAH), which is a disorder affecting one in 14,000 patients, with mild forms of the disease occurring in one of every

100–1000 persons has been studied for many years (Cutler and Laue 1990; New 1992; Ohlsson *et al.*, 1998). The condition is caused by deficient synthesis of cortisol; most cases are related to 21-hydroxylase or 11 β -hydroxylase deficiency (White and Speiser 1994). The affected enzyme can be totally or partially impaired. The degree of enzyme insufficiency determines the severity of the condition (White and Speiser 1994). Steroid 11 β -hydroxylase deficiency, an autosomal recessive disorder, is the second most common cause of congenital adrenal hyperplasia (Zachmann *et al.*, 1983), and hypertension is a feature that often distinguishes this disorder from the steroid 21-hydroxylase deficiency causing virilizing adrenal hyperplasia due to the overproduction of 17-hydroxylated steroids. Before the identification and characterization of the CYP11B2 enzyme, it was thought that each of the last steps of aldosterone biosynthesis was catalysed by a separate enzyme, and in addition to the already known steroid 11 β -hydroxylase, the existence of an 18-hydroxylase called corticosterone methyl oxidase (CMO) type I and of an 18-hydroxysteroid dehydrogenase (CMO II) was postulated. Consequently, isolated deficiencies of aldosterone biosynthesis that are caused by CYP11B2 gene defects were (and still are) called CMO deficiencies. These disorders are clinically characterised by salt wasting, hyponatremia, and hyperkalemia, often presenting in infants with failure to thrive. Plasma renin activity is elevated, plasma aldosterone is low or undetectable, and the plasma levels of aldosterone precursors are elevated. While different types of gene aberrations in CYP11B1 and CYP11B2 including gene conversions, insertions, and deletions have been found in patients (Peters *et al.*, 1998; White 2004), the investigation of missense point mutations contributes most to our understanding of the structure–function relationship of these enzymes. More recently a novel missense mutation (L451F) caused by a T to C transition at position c.1351 in exon 8 was discovered in a newborn infant. This mutation showed complete aldosterone synthase deficiency type I. The L451F mutation is the first mutation found located immediately adjacent to the highly conserved heme-binding C450 of the cytochrome P450 (Nguyen *et al.*, 2008).

In normal physiology, aldosterone secretion is under the principal control of the renin–angiotensin system in a classical endocrine negative feedback loop. Renin is a proteolytic enzyme that is synthesized and stored by specialized cells in the wall of the afferent arteriole situated in the glomerulus of the kidney. These cells are anatomically and functionally associated with the cells in the wall of the distal convoluted tubules (the “macula densa”), and the whole structure is known as the juxtaglomerular apparatus. The release of renin activates a cascade system (Figure 1.8) in which renin cleaves a leucine–valine bond in the

hepatic α_2 -globulin, angiotensinogen, to form the decapeptide angiotensin I. This is subsequently converted by angiotensin-converting enzyme (ACE) to the octapeptide angiotensin II. ACE is a dipeptidyl carboxypeptidase that is found in high concentrations in pulmonary circulation; it is, however, also present in systemic vasculature and the kidney. Angiotensin II is a potent vasoconstrictor and can thus elevate blood pressure but it also stimulates aldosterone secretion, which leads to sodium retention and potassium loss. The major trigger for renin release is a decrease in perfusion pressure, and this may result from hemorrhage, hypotension, or a reduction in the extracellular fluid volume after sodium depletion. Negative feedback for aldosterone secretion is ensured; increased renin secretion increases angiotensin II and aldosterone levels, which will raise blood pressure and result in sodium retention. In turn, this will subsequently inhibit renin secretion maintaining homeostasis.

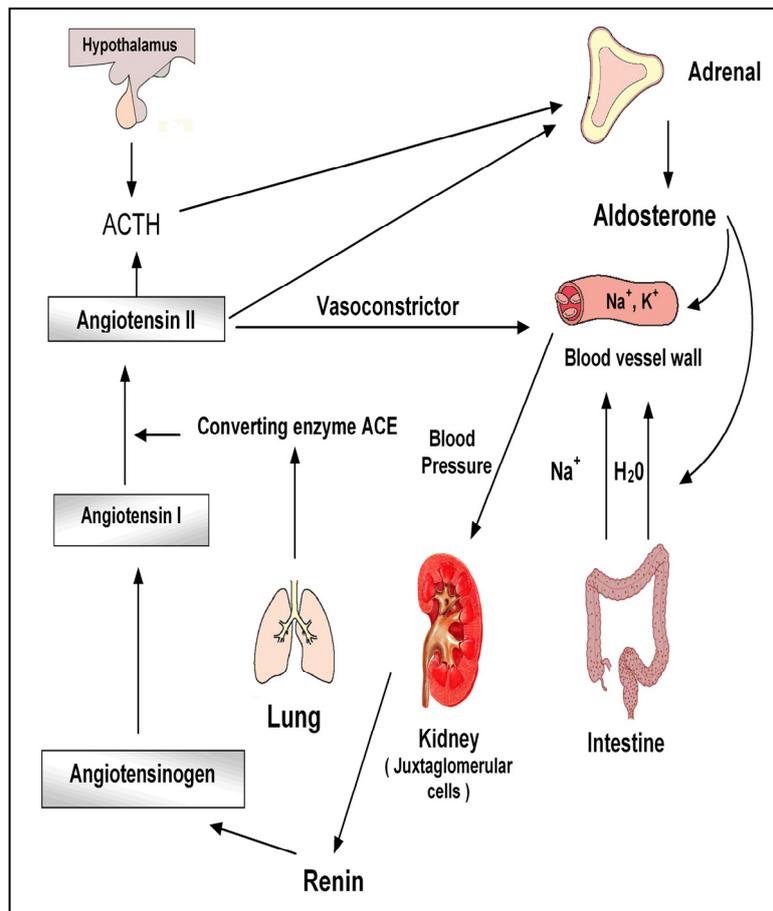


Figure 1.8. The renin–angiotensin–aldosterone system.

The release of renin activates a cascade system in which renin cleaves angiotensinogen to form angiotensin I and angiotensin II. Angiotensin II is a potent vasoconstrictor and can thus elevate blood pressure but it also stimulates aldosterone secretion, which leads to sodium retention and potassium loss.

1.2.3. Differences and similarities between CYP11B1 and CYP11B2

Human CYP11B1 and CYP11B2 are synthesised as 503 amino acids containing precursor proteins (Mornet *et al.* 1989; Kawamoto *et al.* 1990a; Kawamoto *et al.*, 1990b), both containing a 24-residue N-terminal mitochondrial targeting sequence, which is cleaved off after translocation into the mitochondrial matrix. In the mature enzymes, only 29 out of 479 residues are not identical, all of these residues seem to be located outside of the generally accepted substrate recognition sites (Gotoh 1992), and are spread throughout the protein (Böttner and Bernhardt 1996). Human mature CYP11B1 and CYP11B2 have apparent molecular masses of 50 (CYP11B1) and 48.5 (CYP11B2) kDa, respectively, and are bound to the inner mitochondrial membrane by as yet undefined protein segments (Ogishima *et al.* 1991). As mentioned above, human CYP11B1 and CYP11B2 are very similar in their primary sequence, but their catalytic properties are clearly different. To understand the structure–function relationships of these enzymes, which like all mitochondrial P450s so far have resisted all attempts at experimental structure determination, homology models were developed (Belkina *et al.*, 2001). Moreover, Böttner *et al.* suggested that the sequence spanned by amino acids 301 and 335 constitutes part of the substrate-binding site in CYP11B1 and CYP11B2 (Böttner and Bernhardt 1996; Böttner *et al.*, 1998). The effect of the C-terminal portions of both proteins was investigated as well, and it was found that diverging residues at positions 471, 472, 492, 493, and 494 were insignificant for the stereospecificity and regiospecificity of steroid hydroxylation (Böttner *et al.* 1998).

1.2.4. CYP11B1 and CYP11B2 modelling

Understanding the structure–function relationships of CYP11B enzymes requires information about their 3- dimensional structure. Protein structure determination by X-ray diffraction is often problematic in the case of membrane-bound proteins such as CYP11B1 and CYP11B2, and NMR structure determination is restricted to smaller proteins. Due to these reasons, no structure of a mitochondrial cytochrome P450 has been experimentally resolved so far. Only the structures of several bacterial cytochrome P450s and that of a few microsomal P450s solubilized by truncation and site-directed mutagenesis have been experimentally determined (for more information about the resolved 3-dimensional structures of cytochrome P450 visit: <http://www.expasy.org/>). Analysis of the structures revealed a conserved structural fold. Therefore, homology modelling studies of human CYP11B1 and CYP11B2 have been

performed. The models have been evaluated and used to explain the significance of a number of residues that were identified by mutagenesis studies or found in patients (Belkina *et al.* 2001). These models suggest that the main difference between the two proteins is the position of the heme. An angle of $\sim 20^\circ$ between the hemes of the two models has been observed, apparently dependent on the interaction of side chains forming the heme environment and the orientation of its binding loop (Figure 1.9).

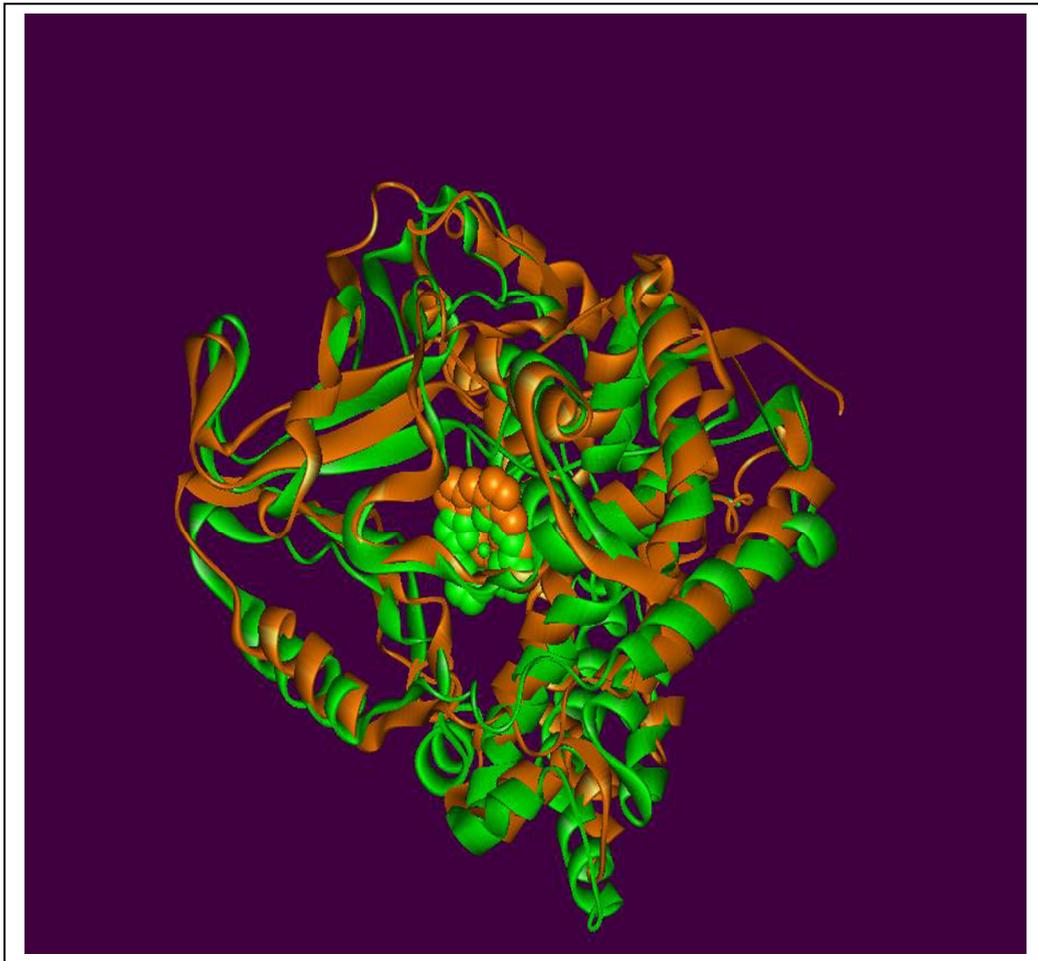


Figure 1.9. Superposition of the ribbon structures of the homology models of human CYP11B1 (green) and CYP11B2 (orange) (Belkina *et al.* 2001). While the overall structure is similar, the position of the hemes in both proteins is different.

In case of CYP11B1, one heme propionate group forms a hydrogen bond with Arg448 while the second one interacts with Arg384, whereas in CYP11B2 both heme propionate groups are involved in hydrogen bond interactions with Arg448. Both Arg384 and Arg448 have been found to be mutated in CYP11B1 of patients suffering from congenital adrenal hyperplasia (CAH) (White *et al.*, 1991; Curnow *et al.*, 1993; Nakagawa *et al.*, 1995); all known mutations in positions 384 and 448 led to a complete loss of enzyme activity, most probably due to destabilisation of the holoprotein. As a consequence of the different hydrogen bonding

network around Arg384, Arg448, and the heme propionates, the active site of CYP11B2 is predicted to be smaller than that of CYP11B1. The larger active site found in the CYP11B1 model correlates with the fact that the natural substrate of CYP11B1 (11-deoxycortisol) is larger than that of CYP11B2 (11-deoxycorticosterone) due to the presence of an additional 17 α -hydroxy group. Thus, from the structural point of view selective inhibition of one of the enzymes appears to be more probable than the extremely high homology of the protein primary sequences would suggest at first glance.

1.2.5. CYP11B1 and CYP11B2 as drug targets

As mentioned above, not only CYP11B1 and CYP11B2 deficiencies, but also abnormally increased plasma levels of aldosterone and cortisol are the cause of a variety of diseases like Cushing's syndrome which leads to chronic glucocorticoid excess. Elucidation of multiple pathogenetic mechanisms has greatly improved the management of this complex endocrine disorder. However, the syndrome is still associated with substantial morbidity and mortality (Boscaro *et al.* 2001; Fisher *et al.* 2001). Many drugs have been used in the treatment of pituitary-dependent Cushing's disease. They act at the hypothalamic–pituitary level and decrease corticotropin secretion, inhibit cortisol synthesis at adrenal level, or compete with cortisol at the receptor level. The neuromodulatory compounds used so far have shown real clinical efficacy only rarely when used as sole treatment, whereas inhibitors of steroid synthesis are effective in most cases in dose-dependent manner (Weber and Villarreal 1993; Engelhardt and Weber 1994; Hartmann *et al.*, 2002). Through their ability to correct hypercortisolism and its severe complications quickly, they are suitable for critical cases and in preparation for surgery, for patients treated with pituitary irradiation, and whenever a definitive treatment is delayed. The most common inhibitors of steroid biosynthesis in clinical use are mitotane, metyrapone, aminoglutethimide, etomidate, and ketoconazole (Gross *et al.*, 2007). While the mode of action of mitotane seems to be multifactorial, the other inhibitors of adrenal steroidogenesis that play an important role in the management of patients all reduce cortisol secretion by blocking one or more of the steroidogenic P450s. However, this mechanism of action has little selectivity and extra-adrenal effects are likely (Sonino and Boscaro 1999). For example, metyrapone treatment leads to increased concentrations of androgens (causing acne and hirsutism) and DOC (leading to hypokalaemia and oedema) as a consequence of CYP11B2 inhibition. Therefore, the development of highly selective CYP11B1 inhibitors would be a major improvement for the treatment of patients suffering

from Cushing's syndrome. The familial occurrence of primary aldosteronism was first described in 1966 by (Sutherland *et al.*, 1966), which reported a father and son with hypertension due to hyperaldosteronism that was resolved during treatment with dexamethasone. Subsequent studies confirmed an autosomal dominant mode of inheritance (New *et al.*, 1980). Today this disease is designated familial hyperaldosteronism type I [FH-I; also called glucocorticoid-suppressible hyperaldosteronism (GSH) or glucocorticoid-remediable aldosteronism (GRA)] in order to distinguish it from a non-glucocorticoidremediable form of familial hyperaldosteronism (FH-II) (Stowasser *et al.*, 1992). As mentioned above, the genetic basis for FH-I is a hybrid gene composed of 5' sequence (including regulatory sequences) derived from the *CYP11B1* gene fused to 3' sequence (which include most of the coding sequences) derived from the *CYP11B2* gene (Lifton *et al.* 1992). Like wild-type *CYP11B2*, the hybrid gene codes for an enzyme with aldosterone synthase activity, but its expression and consequently the production of aldosterone is regulated by the strong *CYP11B1* promoter (about 100- to 1000-fold stronger than the *CYP11B2* promoter) and by the Adrenocorticotrophic hormone (ACTH) (rather than angiotension II, the principal regulator of *CYP11B2* expression) by virtue of its *CYP11B1* regulatory sequences. In addition, chronic elevation of plasma aldosterone has also been diagnosed in other diseases such as adenoma, idiopathic hyperaldosteronism, as well as congestive heart failure or myocardial fibrosis (Brilla 2000; Tsybouleva *et al.*, 2004), and in cases of insufficient renal flow (Stowasser and Gordon 2001). Especially in congestive heart failure, elevated aldosterone levels lead to an increase in blood volume and may stimulate cardiac fibroblasts resulting in cardiac hypertrophy, myocardial fibrosis, ventricular arrhythmia, and other adverse effects (Ramires *et al.*, 1998; Lijnen *et al.*, 2000; Young and Funder 2000). Thus, the Randomised Aldosterone Evaluation Study trial (RALES) was done to determine whether the aldosterone antagonist spironolactone reduces mortality in patients with severe heart failure (Pitt *et al.* 1999). The study followed 1663 patients who had been diagnosed with severe heart failure for two years. During this study the patients, in addition to the standard therapy, were treated with either placebo or with 25 mg daily spironolactone. It was clearly demonstrated that the group treated with the aldosterone antagonist revealed a decreased risk of mortality of 30% and an improvement of the heart disease (Pitt *et al.* 1999). Since spironolactone is associated with severe side effects in the patients, the investigations have been repeated with another anti-mineralocorticoid, eplerenone, which is described to cause fewer side effects. The Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) trial investigated the benefits of using this drug and

demonstrated a reduced mortality in acute myocardial infarction by 15% and of sudden death by 21% (Pitt *et al.* 2001). Nevertheless, treatment with steroidal antihormones is still accompanied by severe side effects (MacFadyen *et al.*, 1997; Delyani 2000; Mantero and Lucarelli 2000; Soberman and Weber 2000; Pitt *et al.* 2001).

And thus, a promising pharmacological approach alternative to spironolactone, eplerenone, and to angiotensin-II antagonists (Thai *et al.*, 1999) might be the use of specific and selective CYP11B2 inhibitors. Thus, CYP11B1 and CYP11B2 comprise new targets for drug treatment and selective inhibitors of both enzymes are of high pharmacological interest.

1.2.6. General requirements for the development of CYP11B2 inhibitors

Although Zöllner *et al.* succeeded recently in the expression and purification of functional human CYP11B1 in *E. coli* (Zöllner *et al.*, 2008), human CYP11B2 has not been heterologous expressed so far in significant amounts. Hence, it is not possible to use pure CYP11Bs enzymes for the development of screening systems, and alternative systems have had to be developed. Thus, some clues toward the CYP11B reaction mechanism's and potential inhibition have only been drawn from data obtained from their animal counterparts, although differences between different organisms have to be taken into account (Wada *et al.* 1985; Yanagibashi and Hall 1986; Matsukawa *et al.* 1990; Domalik *et al.* 1991; Nonaka *et al.* 1995; Bülow *et al.* 1996), or by using recombinant mammalian cell cultures (Denner *et al.* 1995a; Denner *et al.* 1995b). The main challenges to develop specific and selective inhibitors of the two mitochondrial CYP11B enzymes are the following: (1) to overcome the high similarity of both proteins for producing inhibitors with sufficient selectivity; for example, inhibitors, which strongly bind to CYP11B1 and not to CYP11B2 and vice versa; and (2) to create convenient test systems for the analysis of the potential inhibitors. As mentioned before, the high similarity between CYP11B1 and CYP11B2 makes the development of selective inhibitors of each one a big challenge for pharmacists and chemists. Furthermore, the 3 dimensional structures are not available yet making the development of inhibitors difficult, but the computer models (Belkina *et al.* 2001; Ulmschneider *et al.*, 2005a; Ulmschneider *et al.*, 2005b; Roumen *et al.*, 2007) of both enzymes may help to design and synthesise new and efficient inhibitors.

1.2.7. Heterologous expression of CYP11B1 and CYP11B2 in stable cell cultures

In 1995, two cell lines that express human CYP11B1 and CYP11B2 in a stable and constitutive manner have been established in our group, the cells were derived from V79 Chinese hamster cells, designated V79MZh11B1 and V79MZh11B2, respectively (Denner *et al.* 1995a; Denner *et al.* 1995b). Interestingly, the recombinant V79 cells were able to support CYP11B1- and CYP11B2-dependent steroid conversion without additional heterologous expression of the corresponding electron donor system (AdR and Adx) in these non-steroidogenic lung fibroblast cells. As expected, metyrapone strongly inhibited CYP11B1 and to a lesser extent CYP11B2 activity when tested using these cell lines. When several pharmaceutically important azole derivatives were tested, it was shown for the first time that the inhibitory effect of fluconazole is minor compared with clotrimazole, ketoconazole, and miconazole (Denner and Bernhardt 1998). These results demonstrated the usefulness of V79MZh11B1 and V79MZh11B2 cell lines for investigating pharmaceutically important compounds for interference with human CYP11B1 and CYP11B2 activity. Spironolactone, which has been described as an inhibitor of rat aldosterone synthase (Weindel *et al.*, 1991), and bovine CYP11B1 (Cheng *et al.*, 1976) showed no inhibitory effect against human CYP11B2 using V79MZh11B2 cells even when used at high concentrations (Denner and Bernhardt 1998). This suggested that the rat enzyme is not an appropriate tool for the evaluation of inhibitors of the human enzyme. Furthermore, this finding indicated that the pharmacological activity of spironolactone is not caused by enzyme inhibition as had been speculated (Cheng *et al.* 1976) but is only due to its antagonistic property. Interestingly, the cell lines are still active and in use for testing potential inhibitors of CYP11B1 and CYP11B2 (Fisher *et al.* 2001; Ehmer *et al.*, 2002; Bureik *et al.*, 2004; Bureik *et al.*, 2005; Ulmschneider *et al.* 2005a; Ulmschneider *et al.* 2005b; Roumen *et al.* 2007).

1.2.8. Heterologous expression of CYP11B1 and CYP11B2 in yeast

It can be generally stated that mitochondrial cytochrome P450s are more difficult to express in microorganisms than their microsomal relatives. While some mitochondrial P450s like bovine CYP11A1 (Wada *et al.* 1985), rat CYP24 (Akiyoshi-Shibata *et al.*, 1994), and CYP27 (Pikuleva *et al.*, 1997) can be readily expressed in *Escherichia coli*, expression of human CYP11B2 in bacteria has not been successful so far and that of the rat counterparts was achieved with only a very low yield (Nonaka *et al.*, 1998).

Our group succeeded in the functional expression of human CYP11B2 and CYP11B1, using the fission yeast *Schizosaccharomyces pombe* (Bureik *et al.*, 2002b; Dragan *et al.*, 2005). The transformed yeasts displayed steroid hydroxylase activity *in vivo* without additional heterologous expression of the corresponding electron donor system (AdR and Adx) in these nonsteroidogenic cells. Our group found an adrenodoxin-like ferredoxin (etp1^{fd}) in this yeast, which was shown to be able to support substrate conversion of different cytochrome P450s (Bureik *et al.* 2002b; Schiffler *et al.*, 2004). Meanwhile etp1^{fd} has been cloned, isolated, and characterized. It was demonstrated to be able to transfer electrons to CYP11B1 and was shown to resemble the mammalian adrenodoxin in many aspects (Schiffler *et al.* 2004). Furthermore, our group confirmed recently the existence of a corresponding fission yeast ferredoxin reductase, which was characterised and called arh1 (Ewen *et al.*, 2008).

1.2.9. Inhibitors of CYP11B1 and CYP11B2

As described above, two systems for evaluating compounds with respect to their inhibitory effect on human CYP11Bs have been established in our group, the yeast system using recombinant *S. pombe* (Bureik *et al.* 2002b; Ehmer *et al.* 2002; Dragan *et al.* 2005), and the mammalian cell culture system using recombinant V79 cells (Denner *et al.* 1995a; Denner *et al.* 1995b). The availability of the recombinant yeast system allows a convenient and effective testing of potential CYP11B1 and CYP11B2 inhibitors. Since candidate compounds can be tested in the fission yeast system, a medium throughput screening system seems to be possible but has not been realized yet. Repeated steroid hydroxylation measurements testing several compounds with fission yeast strains expressing both CYP11B1 and CYP11B2 showed very good reproducibility of the inhibitory effects (Bureik *et al.* 2002b; Ehmer *et al.* 2002; Dragan *et al.* 2005).

Using both expression systems, it was clearly demonstrated that inhibitors can be identified with higher selectivity towards CYP11B2 or towards CYP11B1 (Denner and Bernhardt 1998; Ehmer *et al.* 2002), supporting the initial idea that although both proteins show an extremely high identity, the differences (as shown by the two models of CYP11B1 and CYP11B2) are nevertheless big enough to cause differences in the inhibitor binding. Moreover, the two testing systems were compared and evaluated and it was remarkable that all compounds that displayed an effective inhibitory effect in the cell culture assay were also active in the fission yeast system (Bureik *et al.* 2004).

Although several compounds were reported using these two testing systems as selective inhibitors of CYP11B2, the reported compounds were not ‘druggable’ compounds, which make their use as drugs or lead compounds difficult.

In conclusion, each one of the two test systems mentioned before possess advantages and disadvantages (Table 1.1). So far neither of them could be considered as high or even medium throughput screening system, as they are radioactive-dependent typical inhibition assays, in which the enzyme activity is monitored, and a multiple concentration-response curve is used to generate the IC₅₀ values. Using such multiple-concentrations assays at early (screening) stages of drug discovery is very time-and resource-intensive; therefore, further work is still needed to improve the fission yeast system for higher sensitivity and to allow medium or high throughput screening conditions.

Table 1.1. Comparison of the yeast and mammalian recombinant systems for the development and analysis of potential selective inhibitors

| System | Advantages | Disadvantages |
|-----------------|---------------------------------|------------------------------|
| V79 cells | Mammalian cell lines | Expensive |
| | Widely used for drug evaluation | |
| <i>S. pombe</i> | Low cost | Non mammalian cells |
| | Medium-high throughput possible | Cell wall can cause problems |

1.3. Fission yeast *Schizosaccharomyces pombe* as a model system

Fission yeast *Schizosaccharomyces pombe* is a unicellular eukaryote belonging to the Ascomycetes (Sipiczki 2000). P. Lindner first described it in 1893, and since it was originally isolated in millet beer from eastern Africa, the yeast was called pombe which means *beer* in Swahili, and it was called also fission yeast as it divides by fission as opposed to budding spores.

In comparison to baker’s yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), the whole genome of *S. pombe* is only slightly bigger in size (13.8 Mb), and is distributed between chromosomes I (5.7 Mb), II (4.6 Mb) and III (3.5 Mb) (Smith *et al.*, 1987), together with a 20 kb mitochondrial genome (Lang *et al.*, 1987). Fission yeast has only 4824 different genes (Wood *et al.*, 2002), which is significantly less than the number of genes in the human genome (about 23,000) (Pennisi 2003). It is also substantially lower than the 6200 different genes found in *S. cerevisiae*.

Fission yeast *S. pombe* is a harmless, rapidly growing eukaryote. The cells are cylindrical, oval or round, with a diameter of 3-4 μm and a length of up to 7-15 μm (Figure 1.10).

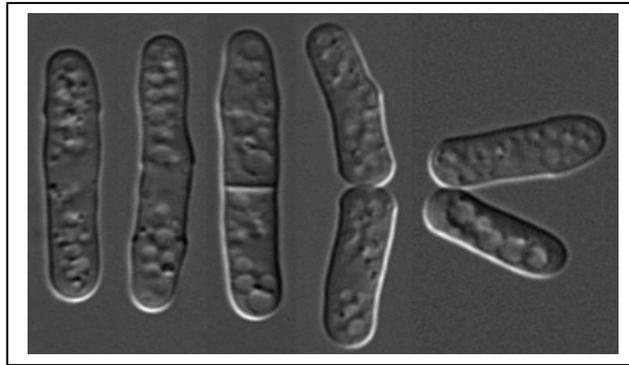


Figure 1.10. Picture of the fission yeast *Schizosaccharomyces pombe* from Steve's place (see http://www.steve.gb.com/science/model_organisms.html).

Fission yeast cells grow quickly, have a generation time between two and four hours and are easy to manipulate on the laboratory level, and since some features such as chromosome structure, cell cycle, and ribonucleic acid (RNA)-splicing are more similar between mammalian cells and *S. pombe* than between mammalian cells and *S. cerevisiae* (Moreno *et al.*, 1991; Box *et al.*, 2008; Miyoshi *et al.*, 2008; Takeda *et al.*, 2008), studying *S. pombe* gives the opportunity to understand what happens in mammalian system, which is more complex and experimentally more difficult to deal with.

Moreover, the works carried out in *S. pombe* have greatly improved our knowledge about the eukaryotic cell (Nurse 2000) and its regulation (Moser and Russell 2000), and added to many related areas, such as microtubule formation (Hagan and Petersen 2000), cellular morphogenesis (Brunner and Nurse 2000), stress response mechanisms (Toone and Jones 1998), and the response to deoxyribonucleic acid (DNA) damage (Zhou and Elledge 2000).

Furthermore, *S. pombe* is an interesting model for studying mitochondria (Chiron *et al.*, 2007), and has been reported to be an interesting host for recombinant expression of P450 (Yamazaki *et al.*, 1993; Bureik *et al.* 2002b; Dragan *et al.* 2005; Dragan *et al.*, 2006; Peters *et al.*, 2007). Therefore, this work will focus on the use of recombinant *S. pombe* as a whole-cell system to develop efficient P450-dependent biotransformation reactions for steroid hydroxylation and drug discovery.

1.4. Biotechnological applications of the 11 β -Hydroxylases

Hydrocortisone (cortisol) is an important starting molecule for the synthesis of drugs with potent anti-inflammatory or antiproliferative actions. The industrial synthesis of hydrocortisone and other glucocorticoids depends currently on hemi-synthesis, which involves multiple chemical and biotransformation reactions in order to introduce the functionally essential 11 β -hydroxy group directly into the steroid scaffold. Several decades ago, studies were carried out to identify 11 β -hydroxylating microorganisms and, as a result, several suitable species such as *Cunninghamella blakesleeana*, *Curvularia lunata* and *Cochliobolus lunatus* have been reported (Hanson *et al.*, 1953; Fried *et al.*, 1955; Zakelj-Mavric *et al.*, 1990).

The 11 β -position is axial and therefore more strongly hindered than the 11 α -position by 1,3-diaxial interactions with the C18- and C19-methyl groups and the 8 β -hydrogen atom. This can explain why the microbial steroid 11 β -hydroxylases generally proceed at lower yields and with more side-reactions (Megges *et al.* 1990). The 11 β -hydroxylases of *Curvularia lunata* is an extremely labile enzyme (Zuidweg 1968) and seems to catalyse in addition to the 11 β -hydroxylation, the 10 β and 14 α hydroxylation when 19-nortestosterone is used as substrate (Lin and Smith 1970). However, this enzyme was first purified and characterized in 1993. It showed a turnover rate of 207 nmol/min per nmol P450 (Suzuki *et al.*, 1993). *Cochliobolus lunatus* was shown to efficiently hydroxylate progesterone at both 11 β - and 14 α -positions into mono- and dihydroxy-products. In addition, this fungus displays other minor hydroxylation and side-chain cleavage reactions.

In the case of the 11 α -hydroxylase, a comparison of 11 β -hydroxylase on the basis of their specific activity was not possible, but comparative studies demonstrated *C. lunata* to be more effective than *Streptomyces fradia* and *C. blakesleeana*.

1.5. Aim of the work

To apply cytochrome P450s in biotechnology either whole-cell systems expressing the P450 isoforms of interest have to be developed or self-sufficient systems avoiding NAD(P)H regeneration have to be used. In general, whole-cell systems are used more often than (partly) isolated enzymes in biotechnological processes (Straathof *et al.*, 2002).

The main topic of this work consisted in the development of an efficient hydrocortisone-producing whole-cell system.

Hydrocortisone (Cortisol) is used as all other glucocorticoids as important anti-inflammatory agent and generally requires an 11 β -hydroxy group as a functionally essential entity. During the industrial synthesis of glucocorticoids, the microbiological introduction of the 11 β -hydroxy group into the steroid scaffold not only represents the most costly synthesis step, but also the step whereby most of the losses occur due to the formation of by-products (Dragan *et al.* 2005).

In recent years, it has been demonstrated that the fission yeast *Schizosaccharomyces pombe* is a very suitable model system for the investigation of P450 dependent steroid hydroxylases (Bureik *et al.* 2002a; Bureik *et al.* 2002b; Dragan *et al.* 2005; Dragan *et al.* 2006). During these studies, the construction of recombinant fission yeast strains that functionally express human CYP11B1 was reported. In these strains (named CAD1 (Bureik *et al.* 2004) and SZ1 (Dragan *et al.* 2005), respectively), 11 β -hydroxylation of RSS was accomplished without the need for coexpression of the other components of the mitochondrial P450 electron transfer chain (Adx and AdR).

While the hydrocortisone production efficiency using strain SZ1 is considerably higher than the values reported for production by other steroid 11 β -hydroxylation systems with recombinant microorganisms (e.g. those from bovine CYP11B1 expressed in baker's yeast *Saccharomyces cerevisiae* (Dumas *et al.*, 1996; Dragan *et al.* 2005)), all bioconversion activities published to date appear to still be not competitive enough for the consideration of their use for industrial applications. Therefore, the purpose of this work was to improve the efficiency of hydrocortisone bioproduction in the CYP11B1-expressing fission yeast. In this context, it is of special interest to determine whether the coexpression of the corresponding mitochondrial electron transfer partners (AdR, Adx) is capable of directly improving the 11 β -hydroxylation activity of CYP11B1-expressing fission yeast strains.

In addition to the wild type of Adx, two mutants that were previously reported by our group to have enhanced affinity for the cytochrome P450 (Schiffler *et al.*, 2001; Bichet *et al.*, 2007) were also included in this work to determine whether a higher 11 β -hydroxylation activity of CYP11B1 can be achieved by substituting Adx^{WT} with the Adx mutants.

The second part of this work consisted in the development of a medium or high throughput screening system for the discovery of aldosterone synthase inhibitors. These inhibitors can be used as lead compounds or drugs for the treatment of aldosterone overproduction-related diseases (Hakki and Bernhardt 2006; Baston and Leroux 2007; Schuster and Bernhardt 2007). As mentioned before (see subsection 1.2.9) two systems for evaluating compounds with respect to their inhibitory effect on human CYP11Bs have been developed in our group, the yeast system using recombinant *S. pombe* (Bureik *et al.* 2002b; Ehmer *et al.* 2002; Dragan *et al.* 2005), and the mammalian cell culture system using recombinant V79 cells (Denner *et al.* 1995a; Denner *et al.* 1995b). Although fission yeast test system allows a convenient and effective testing of CYP11Bs inhibitors, no screening system has been reported. Moreover, the established testing system is a radioactive-dependent typical inhibition assay, in which the enzyme activity is monitored, and a multiple concentration-response curve is used to generate the IC₅₀ value. Using such multiple-concentrations assays at early (screening) stages of drug discovery is very time- and resource-intensive; therefore, the target of this work was to develop a medium or a high throughput screening system (HTS) for the discovery of aldosterone synthase inhibitors.

To accomplish this goal, to develop and execute an efficient, rapid, and reproducible *S. pombe* screening assay, an automated screening technology plate-format had to be established. In this context, it is of special interest to optimise the steroid hydroxylation assay for the 96-well plate format and to determine whether a one-point assay can be developed and used instead of the multiple points assay. The use of one-point assay will increase the throughput of the screening system.

Additionally, the steroid detection method has to be investigated and optimised in order to allow the screening of large numbers of compounds and to achieve a high or even medium throughput screening system.

2. Materials & Methods

2.1. Materials

2.1.1. Microorganism growth media

All following media were prepared and sterilised by autoclaving on a liquid cycle (20 min at 121 °C) and stored in cold room. Solid form was obtained by setting up a 2% (w/v) agar concentration.

2.1.1.1. Growth media for *Escherichia coli* (*E. coli*)

- **LB medium (Luria-Bertani)**

25 g powder from Difco™ LB Broth, Miller (Luria-Bertani) (Becton, Dickinson and company) was dissolved in 1 L of distilled water and the pH was adjusted to 7.5 with drop NaOH or HCl.

LB formula per litter is shown in Table 2.1 below.

Table 2.1. Composition of LB medium

| | |
|-----------------|------|
| Tryptone | 10 g |
| Yeast extract | 5 g |
| Sodium chloride | 10 g |

When needed, ampicillin was added to a final concentration of 100 µg/ml.

- **SOC medium**

SOC is a suitable medium for use in the final step of cell transformation to obtain maximal transformation efficiency of *E. coli* (Hanahan 1983).

Table 2.2. Composition of SOC medium

| | |
|--------------------------------------|---------|
| Tryptone | 2.0 % |
| Yeast extract | 0.5 % |
| KCl | 2.5 mM |
| MgCl ₂ •6H ₂ O | 10.0 mM |
| Mg SO ₄ | 10.0 mM |
| NaCl | 10.0 mM |
| Glucose | 20.0 mM |

2.1.1.2. Growth media for *Schizosaccharomyces pombe* (*S. pombe*)

- **EMM (Edinburgh minimal medium)**

EMM is a minimal medium for the culturing of *S. pombe*, and is prepared with the following composition as shown in the Table below.

Table 2.3. Composition of EMM medium

| | |
|---|-------------------|
| Potassium hydrogen phthalate | 12.0 g (14.7 mM) |
| Na ₂ HPO ₄ | 8.8 g (15.5 mM) |
| NH ₄ Cl | 20.0 g (93.5 mM) |
| Glucose | 80.0 g (111.0 mM) |
| The pH must be adjusted to be 5.4- 5.8; subsequently the vitamins, minerals and salt have to be added as shown in the table below, and filled up to 4 L with distilled water. | |
| Salt stock (x50)* | 80.0 ml |
| Vitamin stock (x1000)* | 4.0 ml |
| Mineral stock (x10,000)* | 0.4 ml |

* For supplements, see appendix

For culturing of *S. pombe* the needed supplements have to be added for each strain (as shown in Tables 2.6, 3.6) with an end concentration of 0.01% (w/v) for each supplement. When needed, thiamine with final concentration of 5 μ M was added to suppress the *nmt1* promoter.

▪ **YEA: yeast extract medium and supplements**

YEA medium is a rich and complete medium for the culturing of *S. pombe*, and is prepared as shown below in the Table.

Table 2.4. Composition of YEA medium

| | |
|-------------------------------------|------------------------|
| Yeast extract | 5.0 g (0.5% w/v) |
| Glucose | 30.0 g (3.0% w/v) |
| adenine, histidine, leucine, uracil | 0.1 g each (0.01% w/v) |
| Distilled water | Up to 1.0 L |

▪ **2X YEA with 25 % glycerol**

This medium is used to prepare glycerol stock cultures from *S. pombe* strains, in order to freeze them by -80°C.

Table 2.5. Composition of 2X YEA with 25 % glycerol medium

| | |
|-------------------------------------|-------------|
| Yeast extract | 1.0 g |
| Glucose | 6.0 g |
| adenine, histidine, leucine, uracil | 0.02 g each |
| Distilled water | 75.0 ml |
| Glycerol | 25.0 ml |

2.1.2. Microorganisms

Microorganisms used in this work are summarised in Table 2.6 below.

Table 2.6. Microorganisms used in this work

| Name (Organism) | Genotype | Supplements | Reference |
|---|--|-------------------------------------|--|
| TOP10F ['] (<i>E. coli</i>) | F- mcrA Δ (mrr-hsdRMS-mcrBC)Φ80lacZΔ M15 Δ lacX74 recA1deoR araD139 Δ (araleu)7697 galU galK rpsL (Str ^R) endA1 nupG | - | US Patent 5,487,993 (Invitrogen; Carlsbad, CA) |
| MB164 (<i>S. pombe</i>) | NCYC2036/pINT5-CYP11B2 integrant | leucine | (Bureik <i>et al.</i> 2002b) |
| SZ1 (<i>S. pombe</i>) | h ⁻ ura4-dl18 leu1::pCAD1-CYP11B1 | leucine | (Dragan <i>et al.</i> 2005) |
| 1445 (<i>S. pombe</i>) | h- ade6.M210 leu1.32 ura4.dl18 his3.Δ1 | adenine, leucine, uracil, histidine | (Burke and Gould 1994) |

2.1.3. Plasmids

▪ pNMT1-TOPO[®]

Fission yeast vector pNMT1-TOPO (Invitrogen; Carlsbad, CA) was used for all subcloning steps for the development of AdR expression plasmids, and as a starting point for the construction of a new expression vector bearing two expression cassettes for Adx and AdR, respectively.

This vector uses the TOPO I ligation strategy and is mainly designed to be used for expression of cDNA in *S. pombe* under the control of the *nmt1* promoter (Maundrell 1990), which is the strongest known inducible promoter of *S. pombe* (Forsburg 1993). An autosomal replicating sequence (*ars1*) directs the high-copy maintenance in *S. pombe*. The LEU2 ORF from *S. cerevisiae* under the control of the *SV40* promoter allows auxotrophic selection in *leu1⁻* hosts. Biological amplification in *E. coli* is possible due to a pUC ORI and an ampicillin resistance ORF enables the selection of transformed *E. coli* colonies in the presence of ampicillin. An additional feature represents the opportunity of tagging the protein of interest with the Pk tag (Craven *et al.*, 1998) for immunologic detection and a hexahistidine (his6) tag

for purification with metal-chelating resins. Moreover, the addition of polyhistidine tags has sometimes a stabilising effect on the expressed protein (Nonaka *et al.* 1998). Further information can be extracted from Figure 2.1.

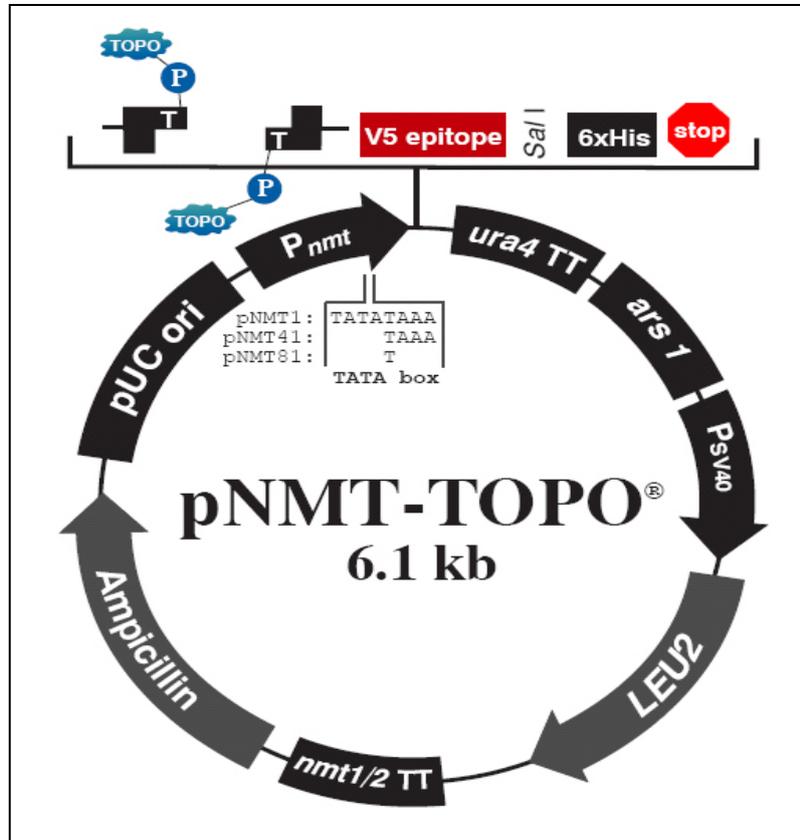


Figure 2.1. pNMT1-TOPO vector map (Invitrogen; Carlsbad, CA). Pk tag is the C-terminal peptide containing the V5 epitope, P_{nmt} is *nmt1* promoter, *S. pombe ars1* origin of replication for non-integrative high-copy maintenance of the plasmid in *S. pombe* cells, *S. cerevisiae LEU2* auxotrophic marker for selection of yeast transformants.

▪ pREP42 Pk C

A vector for the expression of tagged proteins in *Ura4⁻* *S. pombe* strains under the control of the *nmt1* promoter (Craven *et al.* 1998). An autosomal replicating sequence (*ars1*) directs the high-copy maintenance in *S. pombe*. The *Ura4* ORF allows auxotrophic selection in *Ura4⁻* hosts. Furthermore, this vector enables the tagging of the protein of interest with the Pk tag for immunologic detection (Figure 2.2).

Table 2.7. Adx expressing plasmids used in this work (Derouet-Hümbert *et al.* 2007)

| Plasmid | Insert | Selection marker |
|----------------------------|----------------------|------------------|
| pNMT1-Adx ^{WT} | Adx ^{WT} | <i>LEU2</i> |
| pNMT1-Adx ^{D113Y} | Adx ^{D113Y} | <i>LEU2</i> |
| pNMT1-Adx ^{S112W} | Adx ^{S112W} | <i>LEU2</i> |

2.1.4. Oligonucleotides

All primers used during this work were obtained from the company BioTeZ (Berlin-Buch, Deutschland) and purified via HPLC. The sequences as well as the purpose of each oligonucleotide used in this work are given in the appendix section.

5' fluorescence labelled oligonucleotides (fluorophore IR800) used for DNA sequencing with a LicorTM-DNA sequencer 4000 were purchased from MWG Biotech. The applied sequencing primers are also listed in the appendix section.

2.1.5. Library of pharmacologically active compounds (LOPAC)

A library of 1268 compounds from the LOPAC¹²⁸⁰ library (Library of pharmacologically active compounds) was obtained from SIGMA (Deisenhofen, Germany) and is shown in the appendix section. The library is a collection of high quality, innovative molecules that span a broad range of cell signalling and neuroscience areas.

The complexion of the investigated library reflects the most commonly screened targets in the field of drug discovery, and it contains marketed drugs, failed development candidates and “gold standards” that have well-characterised activities. These compounds are the result of lead optimisation efforts and thus, possess a great deal of value, having been rationally designed by structure activity relationship (SAR) studies. For more information about the LOPAC¹²⁸⁰ library see

<http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/LO1280>

2.1.6. Mega Block plates

Two different kinds of Mega Block plates from VWR (Darmstadt, Germany) were used as shown below in Figure 2.3.

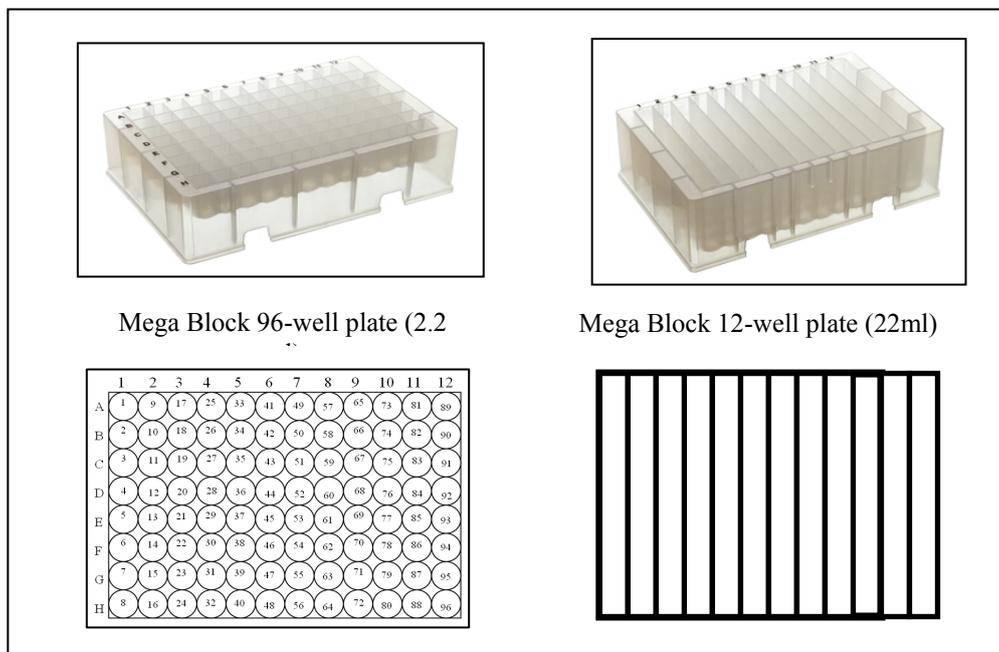


Figure 2.3. The Mega Block plates from VWR used during this work. The 96-well plate was used to perform the hydroxylation assay, whereas the 12-well plate was applied essentially for the preparation of the test as will be described in details below.

Since the 96-well plates are used to perform the steroid hydroxylation, an automated liquid handling system is needed to manipulate the 96-well plates. For this reason, the pipetting robot (Tecan Aquarius, Switzerland) (Figure 2.4) was used and several programs that enable the manipulation of plates were developed in this work.



Figure 2.4 The pipetting robot (Tecan Aquarius, Switzerland).

2.2. Methods

2.2.1. Molecular biology methods

Unless otherwise noted, all genetic methods applied during this work were carried out according to the standard methods as described previously (Sambrook and Russell 2001).

2.2.1.1. pNMT1- TOPO cloning

TOPO[®] Cloning depends on the DNA topoisomerase I enzyme, which functions both as a restriction enzyme and as a ligase. Its biological role is to cleave and rejoin DNA during replication. *Vaccinia* virus topoisomerase I specifically recognizes the pentameric sequence 5'-(C/T)CCTT-3' and forms a covalent bond with the phosphate group of the 3' thymidine. It cleaves one DNA strand, enabling the DNA to unwind. The enzyme then religates the ends of the cleaved strand and releases itself from the DNA (Shuman 1994). To harness the religating activity of topoisomerase, pNMT-TOPO[®] vector is provided linearized with topoisomerase I covalently bound to each 3' phosphate. This enables the vector to ligate DNA sequences with compatible ends (Figure 2.5) (Shuman 1994). In only five minutes at room temperature, the ligation is complete and ready for transformation into *E. coli*.

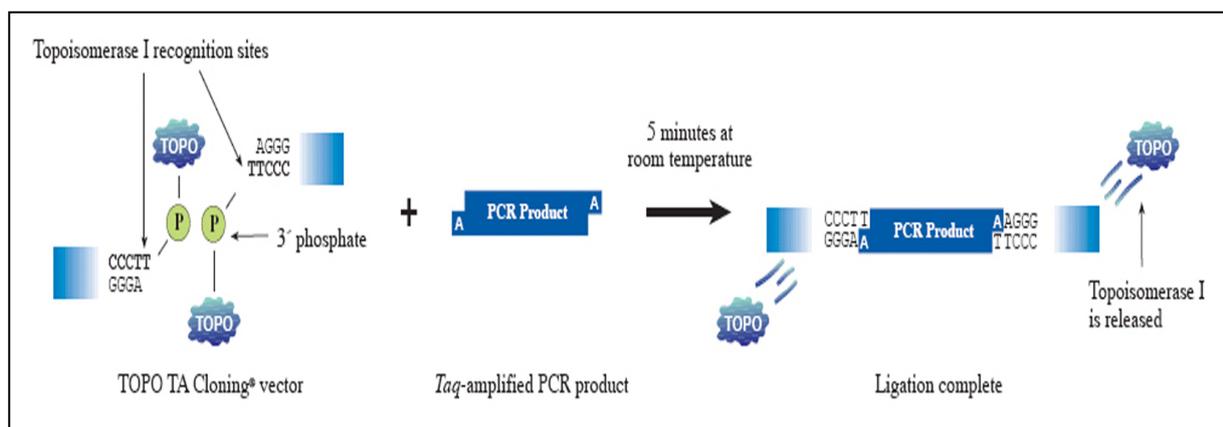


Figure 2.5. TOPO TA Cloning[®] of *Taq*-amplified DNA (Invitrogen; Carlsbad, CA).

The amplification of cDNA prior to cloning was carried out with PCR and the *PfuTurbo*[®] DNA Polymerase from Stratagene. Since an A-3' overhang is needed to perform the TOPO[®] cloning, the PCR product was then incubated at 72°C for ten minutes with the DNA

polymerase *Taq*, which has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR product.

The DNA was then analysed by agarose gel electrophoresis and precipitated as described in subsection 2.2.1.3, dissolved in water and used for the TOPO[®] cloning.

To perform the TOPO[®] cloning 4 µl from the DNA suspension was incubated with 1 µl pNMT1-TOPO[®] Vector and 1 µl salt solution for 5 minutes at room temperature, finally 2 µl from the reaction mixture was then used to transform *E. coli*. The transformation was carried out using the TOP10 chemically competent *E. coli*, and heat-shock method at 42°C for 30 seconds. The transformed cells were then resuspended in SOC medium, plated on ampicillin-containing LB medium plates and incubated at 37°C for 24 hours. Positive clones were identified and isolated after performing colony PCR.

2.2.1.2. Amplification of the human AdR

The AdR cDNA was PCR-amplified using *PfuTurbo*[®] DNA Polymerase from Stratagene. The compositions of a typical reaction as well as the applied PCR-conditions are shown below.

Sample composition:

| | |
|---------------------------------|---------|
| Pfu -polymerase Buffer (10x) | 5.0 µl |
| dNTP (5 mM) | 1.0 µl |
| Forward primer (10 µM) | 1.0 µl |
| Reverse primer (10 µM) | 1.0 µl |
| Template (100 ng/µl) | 1.0 µl |
| <i>Pfu</i> -polymerase (3 u/µl) | 0.5 µl |
| dest.H ₂ O | 40.5 µl |

PCR-program:

| Segment | Number of cycles | Temperature | Duration |
|---------|------------------|-------------|------------|
| 1 | 1 | 95°C | 5 minutes |
| 2 | 35 | 95°C | 30 seconds |
| | | 48°C | 30 seconds |
| | | 72°C | 4 minutes |
| 3 | 1 | 72°C | 10 minutes |

The PCR amplification product was then analysed on 1% (w/v) agarose gel, isolated from the gel and precipitated as shown below.

2.2.1.3. DNA electrophoresis and manipulation

Products from PCR and endonuclease reactions were analysed by agarose gel electrophoresis using agarose mass concentrations ranging from 0.7 % to 1.5 % (w/v) in 0.5X TBE. The Smart Ladder from Eurogentec (Liège, Belgium) was used as reference DNA.

The ethidium bromide stained DNA fragment of interest was then isolated from the agarose gel by cutting the band of interest. The band was then transferred to a bottom perforated 0.5 mL flask filled with saline treated glass wool (Supelco, Bellefonte, PA, USA). The 0.5 ml flask containing the gel and the glass wool was set on an empty 1.5 ml flask. This assembly was centrifuged at 8×10^3 g for 10 min at 4°C. Subsequently the DNA in the flow-through was precipitated with ethanol (EtOH) and sodium acetate (NaAc) (Sambrook and Russell 2001).

DNA concentration was determined spectroscopically by measuring the absorption at 260 nm. According to Hagemann (Hagemann 1990) 1 AU₂₆₀ corresponds to a DNA concentration of 50 µg/ml.

2.2.1.4. DNA restriction and ligation

Restriction endonucleases were from NEB (New England Biolabs, Beverly, MA, USA), Roche Diagnostics (Basel, Switzerland), and from Promega (Madison, WI, USA). All restriction reactions were performed according to the manufactures instructions in the recommended buffers. Double restrictions were simultaneously performed in the most appropriate buffers. Ligation of DNA fragments were performed using the commercially available T4 DNA ligase™ from NEB (New England Biolabs, Beverly, MA, USA). The ligation reactions were performed according to the manufactures instructions, at different molar ratios of linker to insert (Sambrook and Russell 2001).

2.2.1.5. Plasmid purification and DNA sequencing

Plasmid purification was performed using a commercially available kit from Macherey-Nagel (Nucleobond® midi plasmid preparation kit) according to the manufacturer's instructions.

After purification, the plasmid concentration was determined spectroscopically by measuring the absorption at 260 nm as mentioned before.

Correctness of all DNA inserts was verified by automatic sequencing using a slightly modified protocol of the dideoxynucleotide method developed by Sanger *et al.* (Sanger *et al.*, 1977). Primers used for DNA sequencing were 5' fluorescence labelled (MWG Biotech) enabling a laser-scan detection on an automated DNA sequencer (Licor™ 4000 DNA sequencer). PCRs were performed with the Thermo-Sequenase™ Cycle Sequencing Kit from Amersham according to the manufactures instructions. Mrs. Natalie Lenz or Mrs. Katharina Bompais thankfully carried out all DNA sequencing reactions being part of this work.

2.2.2. Microbiology methods

2.2.2.1. *E. coli* cultivation and transformation

Unless otherwise noted, *E. coli* cells were grown in LB medium (see 2.1.1.1) and incubated at 37°C and 200 rpm. Media used for the cultivation of transformed *E. coli* cells always contained 100 µg/ml ampicillin. Transformation of competent *E. coli* cells after addition of approximately 50 ng plasmid-DNA was performed via heat-shock (30 seconds at 42°C; (Sambrook and Russell 2001)). Chemically Competent *E. coli* cells were from invitrogen or generated following a protocol published by Sambrook *et al.* (Sambrook and Russell 2001).

2.2.2.2. *S. pombe* cultivation and transformation

The starting point for all *S. pombe* cultures was a fresh plate, which was prepared by streaking an agar plate containing the desired solid medium from a glycerol stock, and incubated at 30°C for approximately two to three days. The colony of interest was transferred to a 10 ml EMM medium containing the needed supplements (see Tables 2.6, 3.6) to prepare a pre-culture, which was incubated at 30°C and 180 rpm overnight. The pre-culture was then centrifuged and the pellet was used to inoculate a 100 ml fresh medium, this 100 ml main culture was then incubated under the same condition like the pre-culture.

In order to transform fission yeast *S. pombe*, a certain number of cells are needed to perform the transformation. For this reason, a culture of 100 ml was prepared as mentioned before and the cell density (δ_{cell}) was determined microscopically by using a haemocytometer for optical

counting. Cells were only used if $\delta_{\text{cell}} \in [5 \cdot 10^6 - 10^7]$ cells/ml, whereby the total number of cells used for transformation had to be 10^9 to $2 \cdot 10^9$ cells.

The following steps describe the process that was applied to make the *S. pombe* cells competent in order to perform the transformation. The first step comprised a centrifugation step at $3 \cdot 10^3$ g for 5 min and a washing step with 5 ml distilled water. After a second centrifugation step under the same condition as above, the cells were resuspended in 1 ml of 0.1 M LiAc pH 4.9 and transferred to a 1.5 ml flask. A volume of 100 μ l of the above cell suspension was used for each transformation. An amount of 10 μ g of DNA solution was added to each transformation. Following incubation at RT for 10 min, 260 μ l of 40% PEG 4000 in 0.1 M LiAc pH 4.9 solution were added and gently mixed with the cell suspension. After one hour incubation at 30°C and 10^3 rpm, 43 μ l DMSO was added, mixed and a heat shock was applied at 42°C for 5 min. Quickly, 500 μ l water was mixed with the cell suspension that was then centrifuged at $3 \cdot 10^3$ g for 5 min and washed again with 500 μ l water. After a second centrifugation step ($3 \cdot 10^3$ g, 5 min) the cells were resuspended in 500 μ l water and 100 μ l were streaked on the desired plate. The plates were then incubated at 30°C for 3-4 days. The resulted colonies were then analysed by colony PCR.

2.2.2.3. *ura4* gene disruption in *S. pombe*

A gene disruption process was carried out to disrupt the *ura4* gene in fission yeast SZ1, in order to create a new *S. pombe* strain that already express CYP11B1 (Dragan *et al.* 2005), and posses in addition to leucine, uracil as second auxotrophic marker. This process will enable then the transformation of a new strain with two plasmids at the same time.

The gene disruption was done essentially according to Akio Tohe-e (Toh-e 1995). The transformed yeast cells were selected depending on the new auxotrophic marker by multiple replica plating in the presence of 5-fluoroorotic acid (5-FOA), which generates a toxic metabolite in *ura4*⁺ strains (Boeke *et al.*, 1987).

The *Hind* III fragment containing the *ura4* disruption cassette was excised from the plasmid pAT539 and used as donor for the transformation of SZ1 from *ura4*⁺ to *ura4*⁻ (Toh-e 1995). The transformation of *S. pombe* (SZ1) was carried out as described in subsection 2.2.2.2, and transformed cells were then plated on EMM containing 0.01% leucine, 0.01% uracil and 1 mg/ml fluoroorotic acid (5-FOA) as selection factor (Boeke *et al.* 1987). Multiple replica plating was carried out to isolate the *ura4*⁻ fission yeast strain.

B2

| | |
|--|---------|
| Sorbitol | 1.2 M |
| KH ₂ PO ₄ , pH 7.4 | 20.0 mM |

Protein extraction buffer

| | | |
|-------------------|------------|--|
| Tris-HCl, pH 7.5 | 1.0 M | |
| MgCl ₂ | 1.0 M | |
| EDTA | 0.5 M | |
| DTT | 1.0 M | |
| IGEPAL CA-630 | 100% (w/v) | from Sigma [®] (Steinheim, Germany) |

2.2.3.2. SDS (Sodium dodecylsulfate) polyacrylamid gel electrophoresis and gel blotting

Separation of proteins according to their molecular mass was carried out using the Laemmli discontinuous gel electrophoresis (SDS-PAGE) method (Laemmli 1970). Gels used for the separation of Adx and AdR consisted of a 15 % acrylamide containing separation gel superimposed with a stacking gel (5 % acrylamide). Sample buffer composition was as described by Sambrook *et al.* (Sambrook and Russell 2001).

The reference protein mix for molecular weight identification was the pre-stained broad range protein marker purchased from NEB (New England Biolabs, Beverly, MA, USA).

Blotting of proteins separated on a SDS gel onto a nitrocellulose membrane (pore size 0.2 µm) was performed using a semi-dry electrophoretic unit from BIO-RAD[®] (München, Germany).

Blotting of mini gels (8*8 cm) was carried out for 15-30 min and 10-15 V, whereas middle gels (16*17cm) were blotted for 30-60 min and 15-25V according to the manufactures instructions.

2.2.3.3. Immunologic detection of proteins**▪ Antibodies**

The detection of proteins after blotting onto nitrocellulose was performed using a monoclonal anti-Pk tag antibody (MCA1360, Serotec; Oxford, UK) or polyclonal rabbit antibodies

(Biogenes; Berlin, Germany) raised against bacterially expressed Adx, and peroxidase-conjugated secondary antibodies (Dako; Glostrup, Denmark).

▪ Western Blotting

The nitrocellulose membrane bearing the transferred proteins (see 2.2.3.2) was blocked with Blotto1 for 1h. The primary antibody was added 1:10³ diluted for α -Pk tag and 1:2*10³ diluted for α -Adx, respectively, in TBST (Pk tag) for 2 h, in Blotto1 (Adx) for 45 min. After the binding reaction, three washing steps with TBST were followed by incubation with the secondary antibody, 1:500 diluted in TBST (Pk tag), Blotto1 (Adx) for 30 min. A second washing process was carried out twice to remove the excess secondary antibody.

TBST

| | |
|-----------------|--------------|
| Tris-Cl, pH = 8 | 10.00 mM |
| NaCl | 150.00 mM |
| Tween 20 | 0.05 % (v/v) |

The Blotto1 solution is TBST buffer with 1 % of lyophilised milk powder.

The staining of immunolabelled protein bands was carried out by chloronaphthol in presence of H₂O₂ via horseradish peroxidase.

The nitrocellulose membrane from the previous step above was washed twice with PBS. To perform the visualization of targeted proteins, 25 ml fresh PBS was added on the membrane, 10 mg of chloronaphthol was solved in 2 ml absolute ethanol and mixed with 10 μ l H₂O₂ (30 %) prior to be added on the membrane. After shaking for 10-30 minutes at room temperature, the targeted proteins became visible and membrane was then washed and scanned.

PBS, pH 7.3

| | |
|---|----------|
| NaCl | 137.0 mM |
| KCl | 2.7 mM |
| Na ₂ HPO ₄ .2H ₂ O | 8.0 mM |
| KH ₂ PO ₄ | 1.5 mM |

2.2.4. Steroid hydroxylation assays

The starting point for all bioconversion assays including IC_{50} determinations was fission yeast main culture set up as described in subsection 2.2.2.2. Main cultures with cell densities in the range of 10^7 to $5 \cdot 10^7$ cells/ml were used for bioconversion assays. Cells from the main culture were washed with EMM, centrifuged ($3 \cdot 10^3$ g, 5 min, 4°C) and resuspended in the appropriate assay medium as described in details below.

The bioconversion assays were initiated by adding the appropriate substrate. After shaking at 30°C, steroids were extracted with chloroform and measured.

2.2.4.1. Bioconversion assay in Erlenmeyer flasks

In order to follow the time course of the bioconversion process, the bioconversion assay was carried out in 300 ml wide-neck Erlenmeyer flasks covered by a cellulose-pot, where multiple sampling can be done.

A fission yeast cell suspension with cell density of 10^8 cells/ml was prepared using fresh EMM medium. A volume of 9.75 ml cell suspension was transferred to the Erlenmeyer and the substrate concentration was set up to 1 mM using 250 μ l from a 40 mM ethanolic steroid stock solution. The flask was then incubated at 30°C and 180 rpm, and multiple samples of 500 μ l were taken at defined time points and stored at -20°C until steroid extraction was carried out.

2.2.4.2. Bioconversion in modified 1.5 ml tubes

This bioconversion method is based on 500 μ l cultures. In contrast to the method described in 2.2.4.1, the use of such low-volume cultures allows only the sampling of one time point of the bioconversion period and was mainly designed to determine the IC_{50} values of CYP11Bs inhibitors.

The system requires a simple modification of a conventional 1.5 ml tube (Figure 2.6) in order to add an exhaust to the culture flask. A fission yeast cell suspension with cell density of $5 \cdot 10^7$ cells/ml was prepared using fresh EMM medium and steroid concentration was set to be 100 nM. The incubation was carried out at 30°C and 1400 rpm using a tube shaker (thermomixer). After the required assay period, the whole tip-tube content was extracted with chloroform (see subsection 2.2.4.3) or stored at -20°C until steroid extraction.

Inhibitors were dissolved in DMSO at different concentrations, and equal volumes were used in all cases (including controls). Final concentrations of inhibitors ranged from 100 nM to 25 μ M. Cells were pre-incubated with the respective inhibitor solutions for 15 min prior to the addition of 100 nM steroid substrate (11-deoxycortisol or 11-deoxycorticosterone in the case of CYP11B1 or CYP11B2, respectively). For the detection of CYP11Bs-dependent steroid bioconversion, 0.15 μ Ci [3 H] 11-deoxycortisol or 2.5 nCi [14 C] 11-deoxycorticosterone were added to each vial, respectively. After 6 h incubation at 30°C and 1400 rpm, steroids were extracted with chloroform. The detection of steroid bioconversion or inhibition was performed using the high performance thin-layer chromatography (HPTLC) as described below.

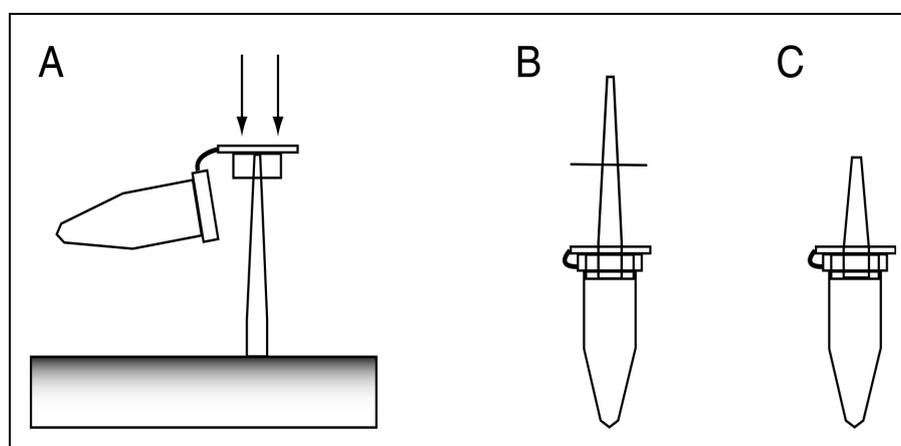


Figure 2.6. The construction of a modified 1.5 ml tube (tip-tube). This tip-tube format was developed by Dipl. Biol. Calin-Aurel Dragan.. The exhaust channel is made of a 200 μ l pipette tip that was pressed through the cap of the 1.5 ml tube (A). To avoid plastic material blocking the air pathway, a cut was done at the line indicated (B) to finally yield a tip-tube (C).

2.2.4.3. Steroid extraction

Samples (500 μ l) gained from bioconversion assays mentioned before were twice completely extracted with 500 μ l CHCl_3 except where indicated. After vigorous shaking, the aqueous phase was pulled out while the organic phase was dried under vacuum. An amount of 10 μ l of 10 mM internal standard steroid was given to the cell suspension prior to steroid extraction in case of subsequent non-radioactive HPLC-based quantification assay.

2.2.4.4. Steroid analysing methods

2.2.4.4.1. High performance liquid chromatography (HPLC)

After evaporation of the chloroform phase, the steroids were resuspended in acetonitril and separated on a Jasco reversed phase HPLC system (Tokyo, Japan) composed of an auto-sampler AS-2050 plus, pump PU-2080, gradient mixer LG-2080-02 and an UV-detector UV-2075 plus equipped with a reversed phase Nova-Pak® C18 60Ao 4 µm column from Waters (Milford, MA, USA). The column temperature was kept constant at 25°C with a peltier oven. The mobile phase used for steroid separation was a mixture of MeOH:H₂O(60:40) with flow velocity of 0.5 ml/min. Steroids were detected at 240 nm, and peak identification was done using the ChromPass software (V.1.7.403.1, Jasco), pure steroids (>99%) were used as standards to identify the peaks on HPLC and to construct calibration curve or as internal standard to normalize the steroid extraction efficiency.

2.2.4.4.2. High performance thin layer chromatography (HPTLC)

Extracted, dried, radioactive samples were dissolved in 10 µl chloroform and applied on the concentrating zone of an HPTLC silica gel 60 F254 plate (Merck, Darmstadt, Germany). The mobile phase for chromatography was CHCl₃:MeOH:H₂O (300:20:1). Radioactive decay signals were exposed to BAS-TR2040 (³H) or BAS-IIIS (¹⁴C) imaging plates (IP) from Fuji (Tokyo, Japan), and scanned with the BAS-2500 phosphoimager (BAS-2500, Fuji; Stamford, CT). Pure 10 mM steroid solutions dissolved in EtOH were used as reference substances for the identification of bands on the scanned IP.

Quantification data analysis procedures were performed using the open-source analysis software TINA v2.10g. The intensity (*I*) of a region of interest (ROI) on the imaging plate was reported in PSL (phosphostimulated luminescence) units, whereby the background exposure signal was subtracted from the raw PSL values prior to conversion calculations by the internal background quantification function. The intensity of the radioactive signal (*I*_{radio}) caused by a certain steroid is proportional to the amount of radioactively labelled steroid (*n*_{radio}).

2.2.4.5. Measuring of steroid bioconversion

Since, the steroids present in sample are chemically and physically very similar molecules, it was assumed that the relative loss of steroids during the extraction procedure is equal for all steroids. Therefore, the ratio of product formation can be calculated depending on the intensity signal (I) of steroid of interest as shown below.

$$R(\text{Pr oduct})\% = \frac{I(\text{Pr oduct})}{I(\text{Pr oduct}) + I(\text{Substrate})} * 100$$

The intensity signal of a steroid is the radioactive signal (I_{radio}) in the case of HPTLC or the peak Area (A) of a certain steroid in the chromatogram with dimension mV.min. in the case of non radioactive HPLC.

This kind of calculation displays the relative ratio of product formation and enables the direct comparison of different *S. pombe* strains and the investigation of the inhibitory effect of compounds compared with a negative control. Furthermore, a quantification assay was also applied to determine the hydrocortisone (Cortisol) production efficiency over time in the “hit” fission yeast strain developed during this work in comparison with the parental strain SZ1.

The quantification assay was carried out in Erlenmeyer flasks as described in subsection 2.2.4.1. Steroid extraction was carried out with chloroform in the presence of DOC as internal standard to normalize the steroid extraction efficiency. Therefore, the correction factor f , defined by the peak areas of the internal standard (IST) as

$$f := \frac{A_{\text{IST,non-extracted}}}{A_{\text{IST,sample}}}$$

was applied to correct the measured peak area of every detected steroid in a particular sample. The data analysis relies on the peak area as a function of molar amount that can be easily established on the used HPLC system by relating A to different n . The linear function was determined for hydrocortisone in the range $n \in [0.1, 10]$ nmol pure hydrocortisone (>99 %).

2.2.4.6. Measuring of the inhibition of steroid bioconversion (Determination of the IC₅₀ values)

The ratio of a certain steroid product P can be regarded as a function of the inhibitor concentration c_{inh} . The inhibition of the production of P ($\text{INH}(P)$) from the substrate is therefore defined as

$$INH(P) = 1 - \frac{R(P, c_{inh})}{R(P, c_{inh} = 0)}$$

Where $R(P, c_{inh} = 0)$ is the ratio of product in the control reaction without inhibitor. From the above formula one can clearly see that when $c_{inh} = 0$ or when the inhibitor shows no effect at all then $R(P, c_{inh} = 0) = R(P, c_{inh})$ for all c_{inh} and the inhibition is 0.

Multiplying $INH(P)$ by 100 displays the result in percent inhibition of the control reaction. The presentation of the data requires the following substitutions

$$y := INH(P),$$

$$x := \log(c_{inh}).$$

After inserting the data in a two dimensional scatter plot a function of the form

$$y = ax + b$$

is fitted by linear regression. The IC_{50} value is the inhibitor concentration $c_{inh,50}$ where $INH = 0.5$ for the production of P. Therefore we rearrange the equation above to

$$x = \frac{0.5 - b}{a}.$$

After back substitution and minor rearrangement we finally get

$$c_{inh,50} = 10^{\frac{0.5 - b}{a}}, \quad \text{Where } IC_{50} = c_{inh,50}.$$

2.2.5. Structure activity relationship (SAR) study

Structure activity relationship study was performed using the BenchwareHTS DataMiner (Tripos). Only the new selective CYP11B2 inhibitors defined in this work were included in this SAR study. SAR analysis being part of this work were thankfully carried out by Dr. Katja Hübel (Max-Planck-Institute of Molecular Physiology, Dortmund, Germany).

2.2.6. Statistical analysis

To evaluate the results; descriptive statistics were applied using the “Statistica” computer program. Moreover, *t*-test for independent samples was applied to evaluate the differences in means between two groups (fission yeast strains), whereas the correlation coefficient Pearson *r*, was applied to measure the relation between two or more variables. The results of these statistical tests are considered significant when $p < 0.05$. Furthermore, the *Z*'-Factor (Zhang *et al.*, 1999) was used for the evaluation and validation of the screening system developed in this work.

2.2.6.1. Descriptive statistics (Measures of variation)

Descriptive statistics were calculated separately for each variable to provide basic information as the mean, standard deviation as well as data about the shape of the distribution of the variable.

It is already known that standard deviation σ is a measure of the average deviation of measured values around the mean \bar{t} and is called in the case of single measurement “error of single measurement”. In practice, it is not interesting to know with which probability the result of a single measurement is within the range $\bar{t} \pm \sigma$, more important is the question of how reliable and reproducible is the mean \bar{t} , which was found with a series of measurements and which represents the result of measurement. For this reason the standard deviation of the mean, which is often called “standard error of the mean (SE)” will be used in this work to measure the standard deviation of the results. The standard deviation of mean say with which probability would the mean of a second measurement series found in a given interval around the mean found by the first measurement.

2.2.6.2. Statistical tests

2.2.6.2.1. *t*-test for independent samples

The *t*-test is the most commonly used method to evaluate the differences in means between two groups. For example, the *t*-test was used in this work to test for a difference in hydrocortisone bioproduction efficiency between fission yeast strain that coexpresses the

complete mitochondrial chain (CYP11B1+Adx+AdR) and the parental fission yeast strain that expresses only the cytochrome CYP11B1.

2.2.6.2.2. Correlation

Correlation is a measure of the relation between two or more variables. Correlation coefficients can range from -1.00 to +1.00. The value of -1.00 represents a perfect negative while a value of +1.00 represents a perfect positive correlation. A value of 0.00 represents a lack of correlation.

The most widely used type of correlation coefficient Pearson r was applied to determine the extent to which values of the two variables are "proportional" to each other. The value of correlation (i.e., correlation coefficient) does not depend on the specific measurement units used. Proportional means linearly related; that is, the correlation is high if it can be "summarised" by a straight line (sloped upwards or downwards).

This line is called the regression line or least squares line, because it is determined such that the sum of the squared distances of all the data points from the line is the lowest possible.

This test was applied in this work to investigate the correlation between the inhibition and concentration of inhibitor in different test media.

2.2.6.2.3. Z'-Factor of assay

To evaluate the robustness and reliability of the developed screening system the Z'-factor known as "screening window coefficient" (Zhang *et al.* 1999) that compares the assay's dynamic range to data variation was applied.

The Z'-factor was determined from the inhibition assays of ketoconazole, clotrimazole and miconazole against CYP11B2 in recombinant fission yeast. The calculation of Z'-factor was carried out using the following formula:

$$Z' = 1 - \frac{(3 * \sigma_{PC} + 3 * \sigma_{NC})}{|\bar{t}_{PC} - \bar{t}_{NC}|}$$

Where \bar{t} and σ are the mean and standard deviation of mean, respectively. PC refers to the positive control and NC to negative control.

A Z' -factor equal to 1 indicates a perfect assay whereas a Z' -factor above 0.5 indicates an excellent screening assay for whole cell systems (Zhang *et al.* 1999).

3. Results

The main topic of this work consisted in the improvement of hydrocortisone bioproduction using new recombinant fission yeast strains coexpressing additionally to CYP11B1 the corresponding electron transfer partners. In addition to this, a second project focussing on the CYP11B2-expressing fission yeast test system was carried out to develop a high throughput screening system for the discovery of selective CYP11B2 inhibitors.

In order to address each project separately the results presented in this section as well as the subsequent discussion were divided into independent sections with the following titles:

- Optimisation of steroid hydroxylation assay for the 96-well plate format
- Coexpression of the corresponding redox partners in the CYP11B1-expressing fission yeast *Schizosaccharomyces pombe*
- The development of a cell-based high throughput screening system for the discovery of human aldosterone synthase (CYP11B2) inhibitors

3.1. Optimisation of a steroid hydroxylation assay for the 96-well plate format

The goal of this part of work was to optimise the steroid hydroxylation assay in fission yeast described above in the Material and Methods section in order to use the 96-well plate to perform the assay in low-volume culture without the need to use radioactive-labelled substrates. This optimisation will give the opportunity to develop a screening assay that does not require the use of radioactively substances that need an especial area in the laboratory.

3.1.1. Steroid bioconversion assay in modified 1.5ml tubes (tip-tube format)

The hydroxylation assay described in subsection 2.2.4 involved the use of radioactive labelled substrates when the bioconversion assay is carried out in low-volume culture (tip-tube format). Therefore, the aim of this part of work was the optimisation of the hydroxylation assay for low-volume cultures without the need to use radioactively labelled substrates. This optimisation was carried out using the CYP11B1-expressing fission yeast strain SZ1, and the tip-tube format described in subsection 2.2.4.2. The assay was designed to be carried out as described in subsection 2.2.4.2 with a cell density of 5×10^7 cells/ml and 100 nM non-

radioactive RSS as substrate. After 24 hours incubation at 30°C and 1400 rpm, the steroid extraction was carried out with chloroform and the HPLC technique was applied as described in subsection 2.2.4.4.1. The HPLC measurement displayed no steroid bioconversion under the conditions described before. In order to optimise the hydroxylation assay to get detectable conversion on HPLC, the substrate concentration was increased but no conversion was detected even after increasing the concentration by 1000-fold (from 100 nM to 100 µM) (Table 3.1). In a next step, the cell density of the assay culture was investigated and a 2-fold concentrated culture was investigated with different concentrations of the substrate as mentioned before. Increasing the cell density and the concentration of substrate to 10⁸ cells/ml and 100 µM, respectively, (Table 3.1) displayed detectable steroid bioconversion of RSS into F (Figure 3.1) in the tip-tube format.

Table 3.1. Steroid bioconversion parameters (tip-tube format)

| | | | | | | |
|--------------------------|----------------------------|-------------------|-------------------|-----------------|-----------------|-----------------|
| Test Volume | 500 µl | | | | | |
| Incubation time | 24 hours | | | | | |
| Shaking velocity | 1400 rpm (Thermo mixer) | | | | | |
| Cell density Cells/ml | 5*10 ⁷ | 5*10 ⁷ | 5*10 ⁷ | 10 ⁸ | 10 ⁸ | 10 ⁸ |
| Substrate concentration | 100 nM | 500 nM | 100 µM | 100 nM | 500 nM | 100 µM |
| Result | No conversion | No conversion | No conversion | No conversion | No conversion | Conversion |

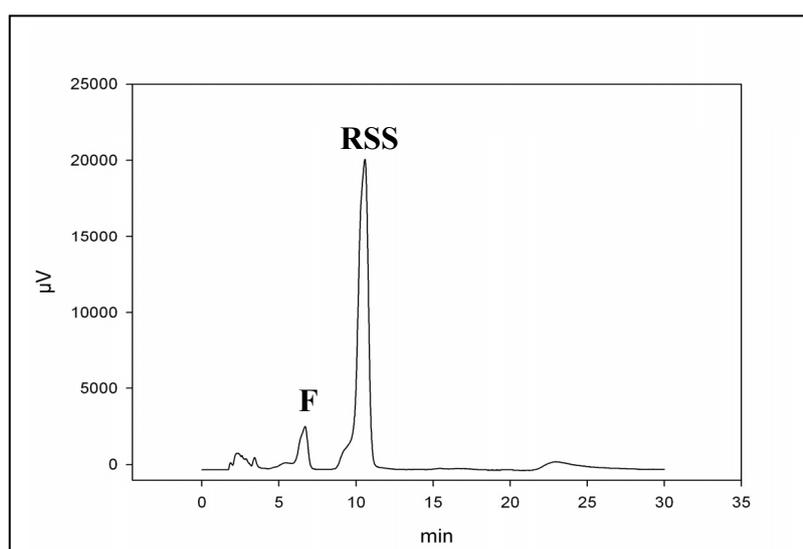


Figure 3.1. HPLC chromatogram of CYP11B1-dependent bioconversion of RSS into F carried out using the tip-tube format. The bioconversion was carried out in the tip-tube format using the CYP11B1-expressing strain SZ1 with cell density of 10⁸ cells/ml and 100 µM RSS. Steroids extraction was performed manually with chloroform.

This first optimisation step enabled the coupling of the tip-tube format (low-volume culture) with the HPLC technique to perform steroid hydroxylation assay in low-volume culture and to measure the steroid bioconversion efficiency using the HPLC technique.

3.1.2. Steroid bioconversion assay in 96-well plate

The hydroxylation assay parameters established in subsection 3.1.1 (Table 3.1) were the starting point to develop a hydroxylation assay in 96-well plates.

Since the hydroxylation assay will be performed in plates, an automated steroid extraction technology plate-format had to be developed. This automated technology should enable the manipulation of low-volume cultures on large scale and the performance of efficient steroid extraction in order to get detectable steroid bioconversion on the HPLC. For this reason, several protocols (Script) were developed during this work to perform the preparation of test plates and steroid extraction using the Aquarius 96 Multichannel pipetting robot (Tecan Aquarius, Switzerland).

The main idea behind using the Aquarius pipetting robot is to mix the culture with the chosen organic solvent, which should not be miscible with water in order to extract the steroids from the culture. This mixing process must be various enough to resemble a strong shaking effect with the ability to separate finally the organic phase in order to perform an efficient HPLC measurement. For the development of an Aquarius script that enables the extraction of steroids from a 96-well plate, two kinds of organic solvents that have different densities and boiling points were investigated. The high boiling range (114-117°C) of 4-Methyl-2-pentanone disables the fast evaporation of the solvent during the extraction process, which prevents any significant loss in the solvent volume. For this reason, it is expected heir to recover almost all the solvent that was added to extract the steroids after mixing the two phases using the pipetting robot. However the low density of 4-Methyl-2-pentanone (0.8) make it lighter than water and, as a result, it forms the upper phase above the culture after mixing the two phases. In contrast to this, chloroform has a boiling point of 61.2 °C and a density of 1.48, and, as a result, the solvent evaporation is higher in the case of chloroform whereas the high density of chloroform locates it under the aqueous phase. For this reason, it is important to determine the tip height at which the organic phase is dispensed to perform the mixing process and the height at which only the organic phase is aspirated to get pure organic phase in order to dray the steroids for the HPLC measurement.

Figure 3.2 below illustrates the different Z positions for a tube in a 96-well plate. This coordinates were taken into consideration to develop an Aquarius script to extract the steroids.

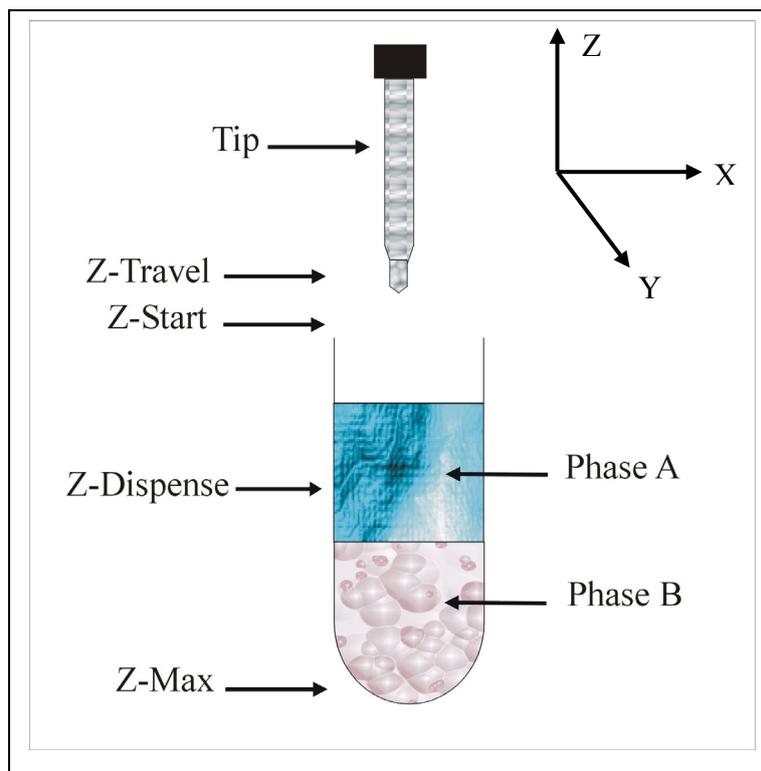


Figure 3.2. Z-positions for a tube.

Z-Travel is the height at which the tip moves from one position to another, Z-Start is usually slightly above the rim of the liquid container. Z-dispense is the tip height at which liquid is dispensed. Z-Max is the position in the lowest point of the well/tube of the rack and is the lowest possible position the tip is allowed to reach.

Since the test culture is incubated for 24 hours at 30°C, the volume of the aqueous phase (test culture) will decrease. This is due to the evaporation of water and, as a result, 4-Methyl-2-pentanone (Phase A, Figure 3.2) will display different Z positions above the aqueous phase after mixing them prior to perform the steroid extraction process. Hence, it is difficult in the case of an organic solvent, which is lighter than water to determine the tip height at which the organic phase is aspirated/dispensed to mix the two phases and the height at which only the organic phase is aspirated to get pure organic phase. For this reason, chloroform was chosen to develop the Aquarius script as it forms the lower phase (Phase B, Figure 3.2) and no changes in the Z position are expected. The Aquarius script developed in this work (Table 3.2) performs several aspirating and dispensing steps in order to mix the organic phase (chloroform) with the assay culture. For this reason, several types of programs that determine the liquid class were developed. These programs are sets of liquid handling parameters that specify speed, airgaps, tip height, etc. Furthermore, and as shown below in Table 3.2 the

Aquarius scrip performs the steroids extraction process on two levels. In the first level, the first volume of chloroform (400 μ l) added in Steps 6-9 is strongly mixed with the test culture for 50 times (steps 10-13). This strong mixing process resembles a various shaking effect that should be enough to extract almost all the steroids from the test culture. The strong mixing process is followed by a slow mixing process (steps 14-17) that enables the separation of the two phases by getting rid of the air bubbles formed between the two phases during the former mixing process. This first extraction level is ended by transferring 200 μ l organic phase on the HPLC 96-well plate. A second extraction process begins by step 26 in which the culture plate receives 200 μ l fresh chloroform. The second extraction process is also carried out like in the first level. Finally, 150 μ l organic phase will be transferred to the HPLC plate. This extraction program recovers 350 μ l organic phase from the 600 μ l chloroform added during the extraction process (Table 3.2).

Table 3.2. Steroid extraction script developed during this work. Red and blue sentences represent the liquid class programs and positions in the Tecan working area, respectively (Figure 3.3). For more information about the liquid class programs, see appendix

| Step | | | Purpose |
|------|------------|---|---|
| 1 | Get DITIs* | Grid1; site: 4 (TeMO_Diti_200 μ l) | Getting Tips |
| 2 | Begin loop | 5 times "Chloroform transfer" | |
| | 3 | Aspirate 200 μ l (Program 1) "Chloroform pool" | Filing up a 96-well plate with chloroform |
| | 4 | Dispense 200 μ l (Program 1) "Chloroform plate" | |
| 5 | End loop | "Chloroform transfer" | |
| 6 | Begin loop | 2 times "Chloroform transfer" | |
| | 7 | Aspirate 200 μ l (Program 1) "Chloroform plate" | Adding 400 μ l chloroform (1st time extraction) |
| | 8 | Dispense 200 μ l (Program 1) "Test plate" | |
| 9 | End loop | "Chloroform transfer" | |
| 10 | Begin loop | 50 times "Chloroform mixing" | |
| | 11 | Aspirate 200 μ l (Program 2) "Test plate" | Strong mixing to extract the steroids (Shaking-like effect) |
| | 12 | Dispense 200 μ l (Program 2) "Test plate" | |
| 13 | End loop | "Chloroform mixing" | |
| 14 | Begin loop | 20 times "Chloroform relaxing" | |

| | | | | | |
|----|----|-------------|--------------------------------|---|--|
| | 15 | | Aspirate | 200 μ l (Program 3) "Test plate" | Getting raid of the air bubbles formed between the organic and aqueous phases during the extraction process |
| | 16 | | Dispense | 200 μ l (Program 3) "Test plate" | |
| 17 | | End loop | "Chloroform relaxing" | | |
| 18 | | Wait timer | Timer 1: 300 sec | | |
| 19 | | Start timer | 1 | | |
| 20 | | Begin loop | 1 time "Transfer of extract" | | |
| | 21 | | Aspirate | 200 μ l (Program 1) "Test plate" | Checking if the 200 μ l organic phase pure is, unless the process can be stopped at this step and repeated by step14 |
| | 22 | | Dispense | 200 μ l (Program 1) "Test plate" | |
| | 23 | | Aspirate | 200 μ l (Program 1) "Test plate" | Transfer of 200 μ l to the HPLC plate |
| | 24 | | Dispense | 200 μ l (Program 1) "HPLC plate" | |
| 25 | | End loop | "Transfer of extract" | | |
| 26 | | | Aspirate | 200 μ l (Program 1) "Chloroform plate" | Adding 200 μ l chloroform (2nd time extraction) |
| 27 | | | Dispense | 200 μ l (Program 1) "Test plate" | |
| 28 | | Begin loop | 20 times "Chloroform mixing" | | |
| | 29 | | Aspirate | 200 μ l (Program 2) "Test plate" | Strong mixing to perform steroid extraction (Second time) |
| | 30 | | Dispense | 200 μ l (Program 2) "Test plate" | |
| 31 | | End loop | "Chloroform mixing" | | |
| 32 | | Begin loop | 10 times "Chloroform relaxing" | | |
| | 33 | | Aspirate | 200 μ l (Program 3) "Test plate" | Getting raid of the air bubbles formed between the organic and |
| | 34 | | Dispense | 200 μ l (Program 3) "Test plate" | |
| 35 | | End loop | | | |
| 36 | | Wait timer | Timer 2: 150 sec | | |

| | | | |
|----|-------------|---|---|
| 37 | Start timer | 2 | aqueous phases during the extraction process |
| 38 | | Aspirate 150 μ l (Program 1) "Test plate" | Checking if the 150 μ l organic phase pure is, unless the process can be stopped at this step and repeated by step 32 |
| 39 | | Dispense 150 μ l (Program 1) "Test plate" | |
| 40 | | Aspirate 150 μ l (Program 1) "Test plate" | Transfer of 150 μ l to the HPLC plate |
| 41 | | Dispense 150 μ l (Program 1) "HPLC plate" | |
| 42 | Drop DITIs | Grid1; site: 4 (TeMO_Diti_200 μ l) | |

*DITIs: Disposable Tip

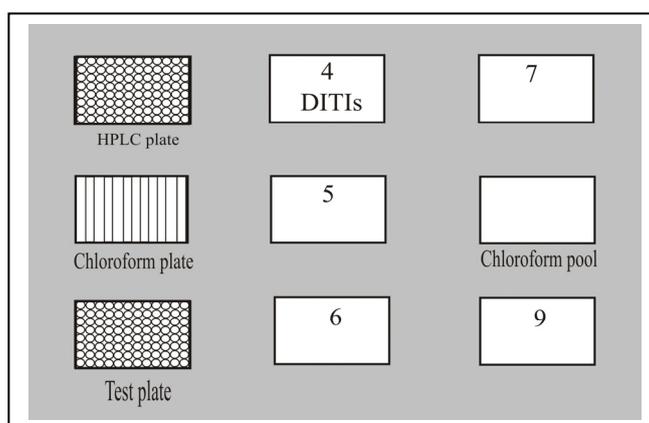


Figure 3.3. Tecan working area during the extraction process

The automated extraction program developed in this work performs the several tasks mentioned above (Table 3.2) in ca 20 min. To investigate the efficiency of this program, a test was carried out to determine the steroid extraction efficiency when a 500 μ l EMM with 100 μ M steroid concentration (RSS or DOC) is extracted automatically in comparison with the manual method.

Since the automated extraction program recovers only 350 μ l organic phase from the 600 μ l chloroform added during the extraction process, a 2 ml tube with 500 μ l EMM with steroid

concentration of 100 μM RSS or DOC was extracted manually with 600 μl chloroform (by shaking strongly for 3 min) whereas only 350 μl organic phase was transferred to be analysed on the HPLC. At the same time, another tube was extracted under the same conditions and the whole recoverable amount of chloroform (500 μl) was transferred to be analysed. After drying under vacuum, the steroids were resolved and measured with HPLC as described above in subsection 2.2.4.4.1.

Figure 3.4 below shows the original HPLC chromatograms of the extracted steroids, which were obtained using the automated method that was applied in a 96-well plate. It is clearly to notice that the peak areas of the extracted steroids do not display any significant difference between the several wells.

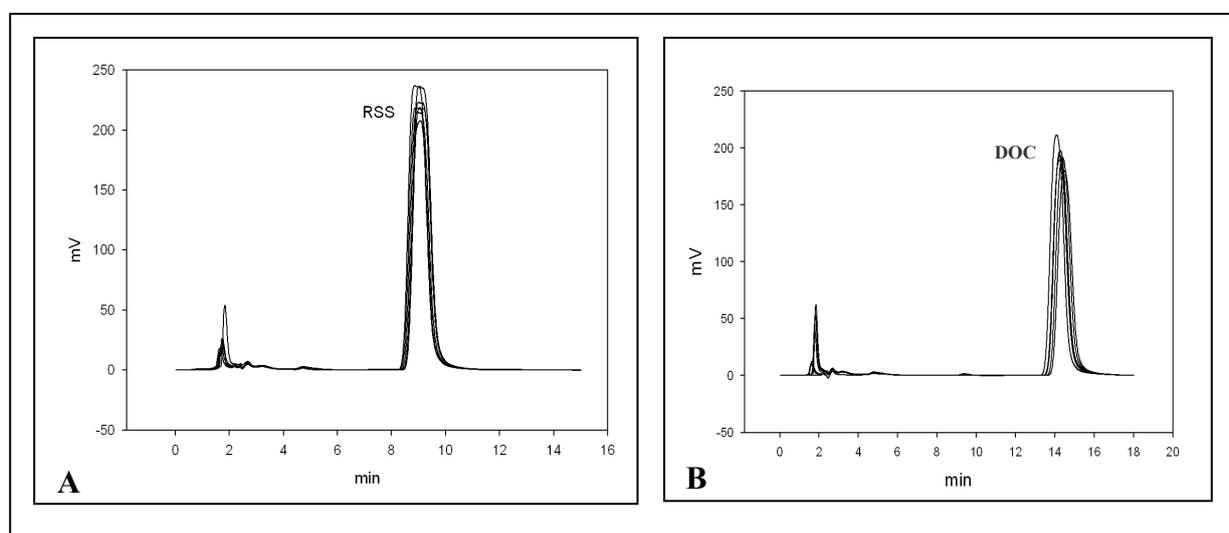


Figure 3.4. HPLC chromatograms of extracted steroids obtained using the automated extraction method applied in a 96-well plate.

96-well plate was filed with 500 μl EMM per well and steroid concentration was set to be 100 μM . Steroid extraction was carried out using the automated method described above. (A) Extracted RSS, (B) Extracted DOC.

Moreover, peak areas of the steroids obtained with the automated extraction method were compared with the steroid peak areas obtained with the manual method, as well as with the same amount of steroids that was given directly on the HPLC without extraction (Figure 3.5). It is clearly to notice that 350 μl of the organic phase in the automated method displays higher steroid content in comparison with the same volume of organic phase obtained using the manual extraction method after mixing with the same volume of chloroform. Moreover, the steroid amount recovered with 350 μl organic phase in the automated method is still more than the amount of steroid recovered with 500 μl organic phase in the manual method.

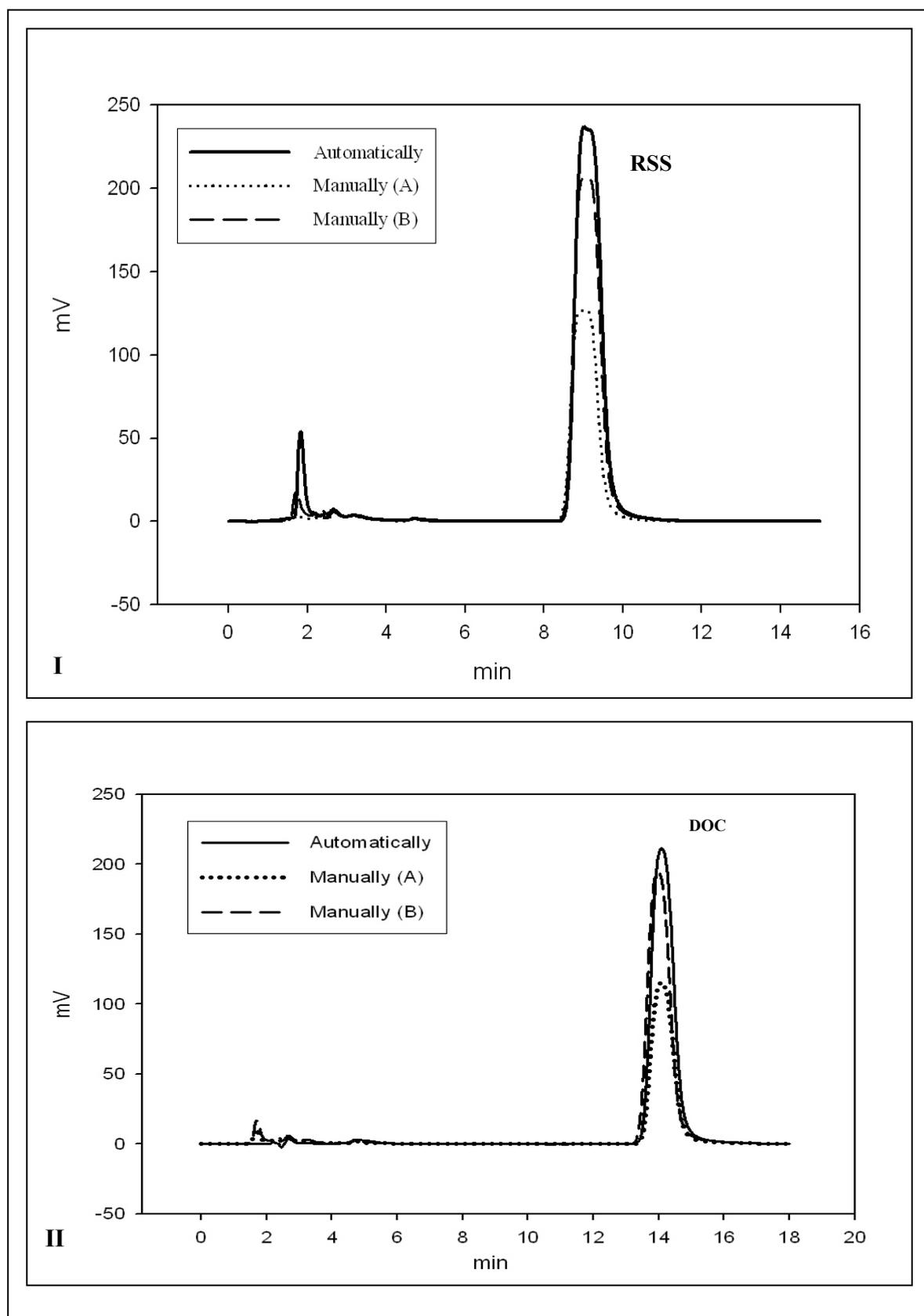


Figure 3.5. HPLC chromatograms of steroids extracted automatically or manually.

(I); RSS, (II); DOC. All samples were mixed with 600 μ l chloroform, whereas different volumes of the organic phase were then analysed on the HPLC (Automatically; 350 μ l, Manually (A); 350 μ l, Manually (B); 500 μ l).

These observations mean that the steroids extraction efficiency of the automated method is significantly higher than the manual method (Figure 3.6). Although the automated method displays high efficiency to extract steroids in comparison with the manual method, only a fraction and not all the organic phase will be transferred to the HPLC plate to be analysed. For this reason, the hydrocortisone ratio (R(F)) will be used to compare the 11 β -hydroxylation activity between the different samples (wells), as it presents proportionally the hydrocortisone production (see 2.2.4.5).

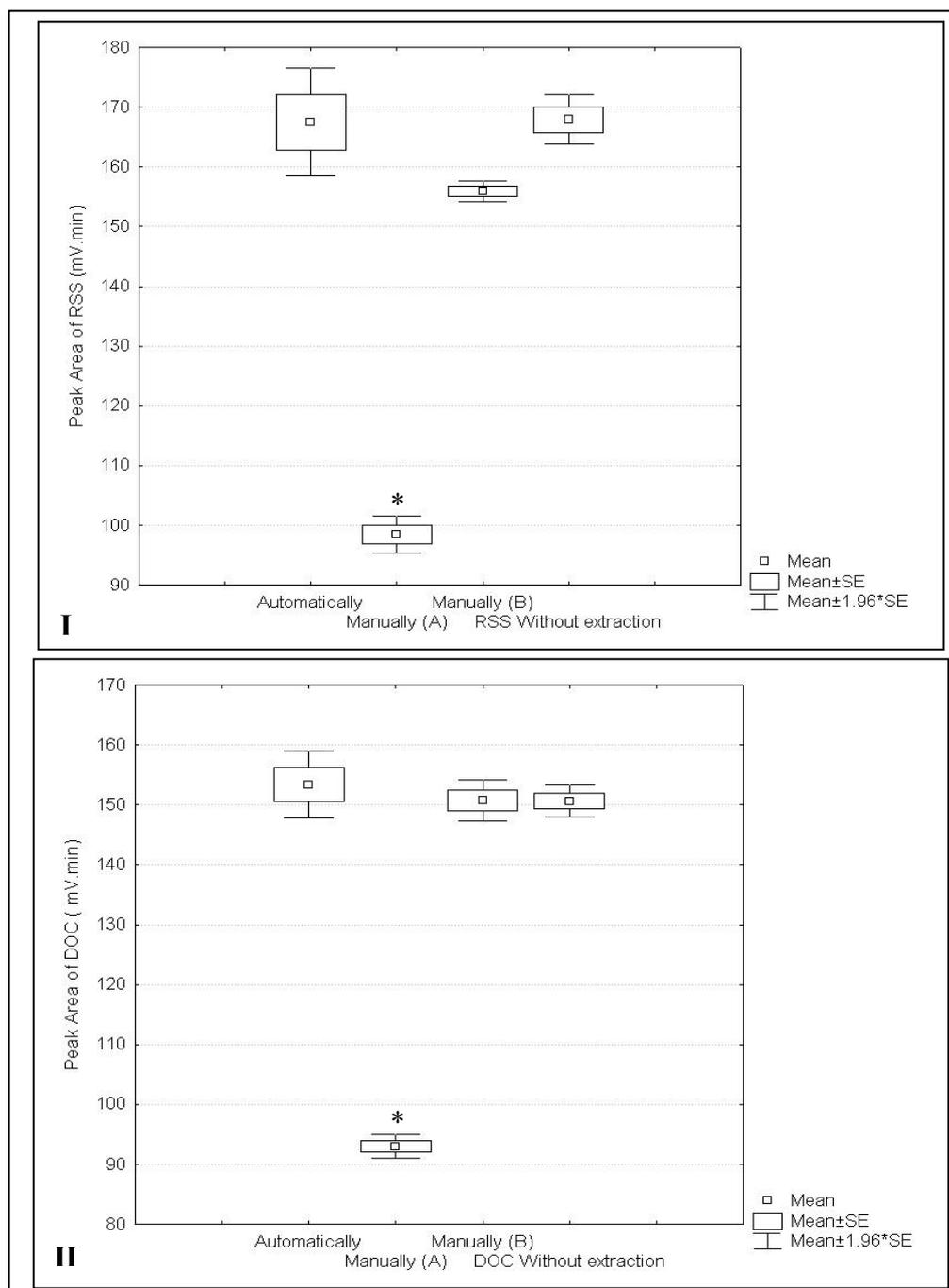


Figure 3.6. Direct comparison of the recovered steroids obtained using different extraction methods in comparison with the same amount of steroids that were given on the HPLC without extraction. (I); RSS, (II); DOC. The samples were mixed with 600 μ l chloroform, whereas different volumes of the organic phase were then analysed on the HPLC (Automatically; 350 μ l, Manually (A); 350 μ l, Manually (B); 500 μ l). Values presented as mean \pm standard error of mean. Asterisks above boxes indicate a significant difference to RSS without extraction ($p < 0.05$).

To validate the 96-well plate hydroxylation assay and the extraction method, the fission yeast strain SZ1 was investigated under the parameters established in subsection 3.1.1. The test was carried out in a 96-well plate, which was shaken at 30°C and 1400 rpm for 24 hours. The HPLC measurement did not show any detectable steroid bioconversion, whereas increasing the test volume and the shaking velocity to 600 μ l and 480 rpm, respectively, play an important role and detectable steroid bioconversion of RSS into F was achieved under these conditions (Table 3.3, Figures 3.7, 3.9).

Table 3.3. Steroid bioconversion parameters (96-well plat format)

| | |
|-------------------------------|------------------------------|
| Cell density (cells/ml) | 10 ⁸ |
| Substrate concentration (RSS) | 100 μ M |
| Test volume | 600 μl |
| Incubation time | 24 hours |
| Shaking (Incubator) | 480 rpm |

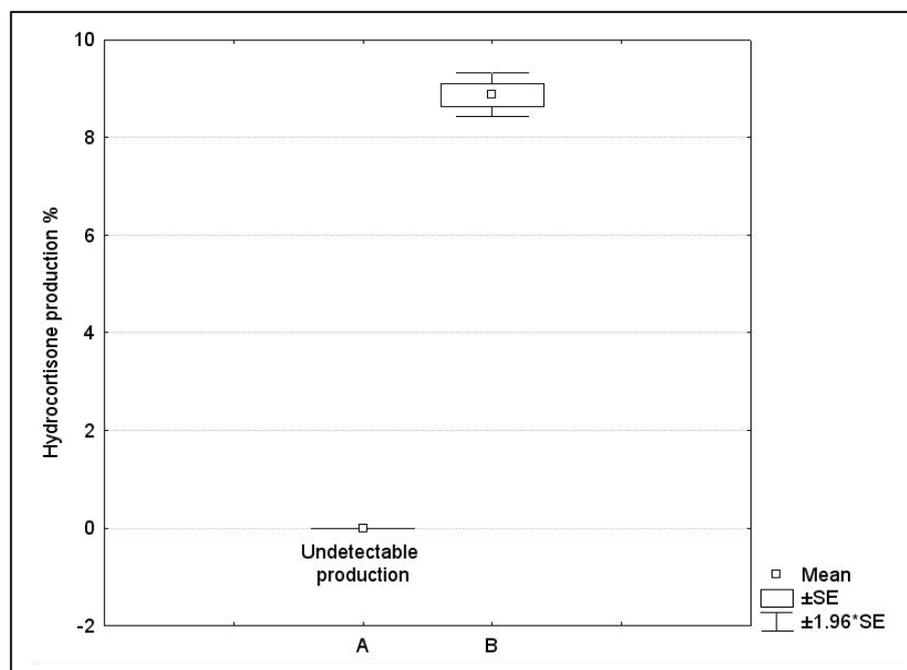


Figure 3.7. Steroid 11 β -hydroxylation activity of SZ1 in a 96-well plate format.

The test was carried out with 100 μ M RSS under different conditions. (A); 500 μ l test volume incubated at 1800 rpm for 24h. (B); 600 μ l test volume incubated at 480 rpm for 24h. Values were calculated from three independent experiments and are presented as mean \pm standard error of mean.

Furthermore, and since each assay plate contains 96 samples, the HPLC parameters described in subsection 2.2.4.4.1 had to be optimised in order to increase the throughput of the HPLC

assay. For this reason, the flow velocity of the mobile phase was increased in order to decrease the time needed for the separation of RSS and F.

Figure 3.8 below shows the separation of RSS and F using a mixture of MeOH:H₂O(60:40) as mobile phase with different flow velocities. Increasing the flow velocity from 0.5 to 1 ml/min decreased the retention times of RSS and F from 9.5 min and 6.7 min (Figure 3.1) to 5.32 min and 3.35 min, respectively (Figure 3.8 A). Moreover, RSS and F displayed with flow velocity of 1.2 ml/min retention times of 4.45 min and 2.8 min (Figure 3.8 B). Whereas increasing the flow velocity up to 1.5 ml/min decreased the time needed to separate RSS and F to less than 5 min (Figures 3.8 C, 3.9) increasing the throughput of the HPLC assay by more than 2-fold. Hence, these new HPLC parameters (Table 3.4) will be applied when a steroid bioconversion assay is carried out in a 96-well plate.

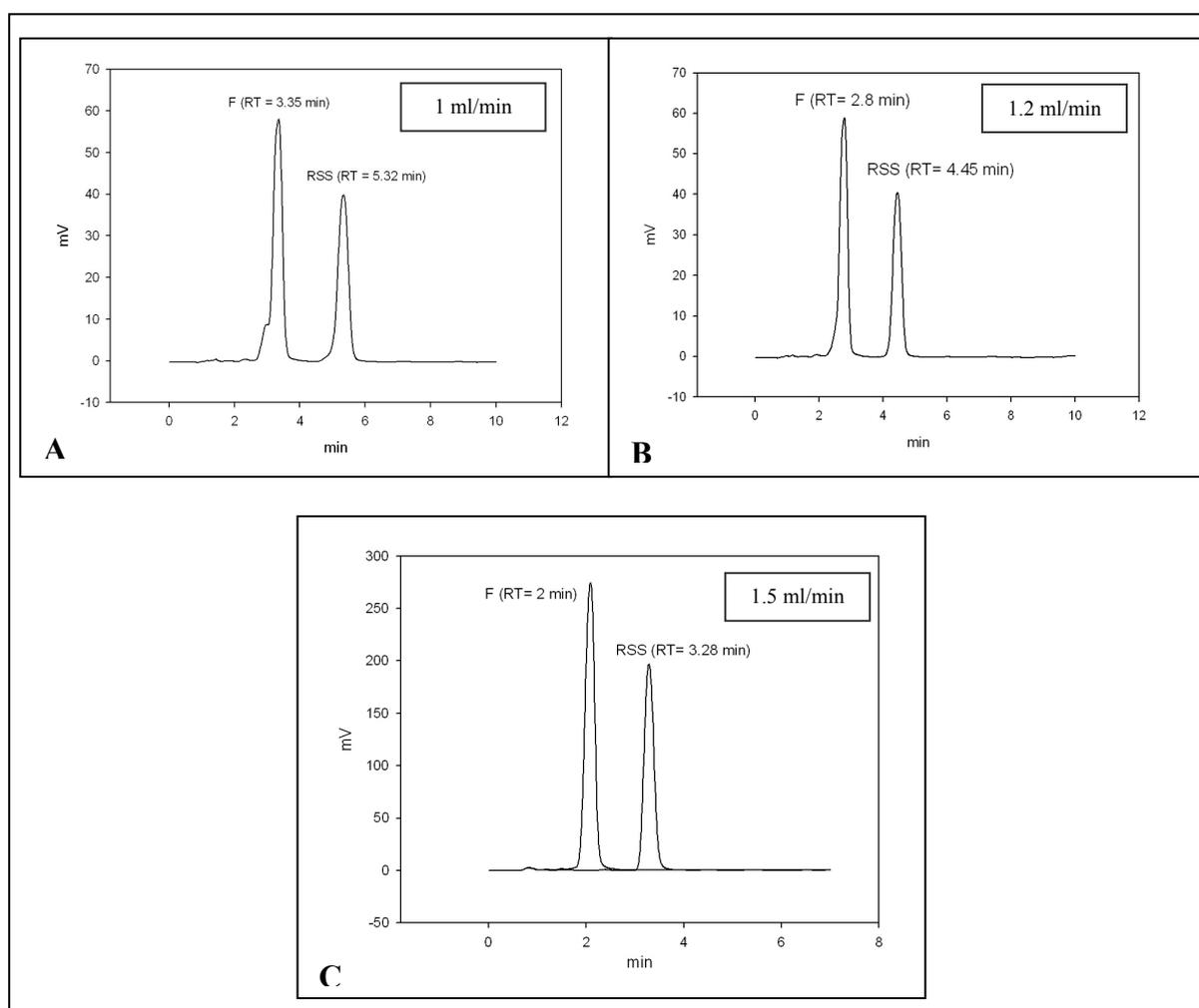
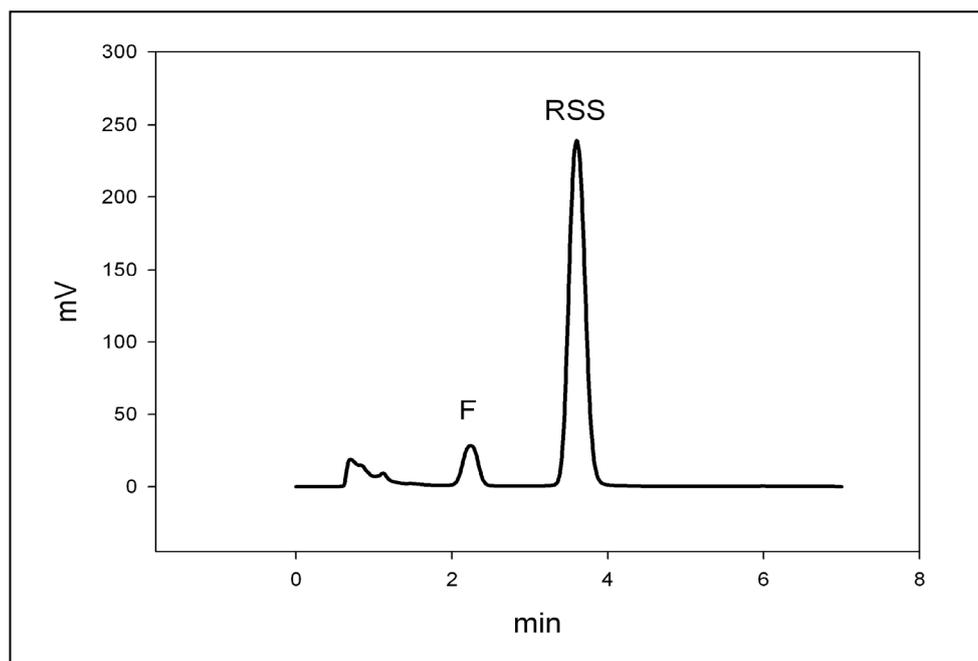


Figure 3.8. HPLC Chromatograms show the separation of RSS and F under different HPLC conditions.

The HPLC was carried out using a mixture of MeOH:H₂O(60:40) as mobile phase with different flow velocities (A; 1 ml/min, B; 1.2 ml/min, C; 1.5 ml/min).

Table 3.4. HPLC parameters to separate RSS and F in less than 5 min

| | |
|--------------------|-------------------------------|
| Mobile phase | MeOH:H ₂ O (60:40) |
| Flow velocity | 1.5 ml/min |
| Column temperature | 25 °C |
| Time per sample | < 5 min |

**Figure 3.9. HPLC chromatogram of the CYP11B1-dependent bioconversion of RSS into F carried out using the 96-well plate format.**

The bioconversion was carried out in 96-well plate, using the CYP11B1-expressing strain SZ1 with cell density of 10^8 cells/ml and 100 μ M RSS. Steroid extraction was carried out with chloroform using the pipetting robot as described above.

Moreover, the steroid hydroxylation assay in 96-well plate did not show any significant difference in the bioconversion efficiency between the several wells (*t*-test, $p < 0.05$) (As will be shown below in subsection 3.2.4). Therefore, this 96-well hydroxylation assay is an efficient screening tool to investigate and compare the steroid hydroxylation activity in the recombinant strains that will be developed during this work.

3.2. Coexpression of the corresponding redox partners in CYP11B1-expressing fission yeast *Schizosaccharomyces pombe*

As mentioned before, recombinant fission yeast strains that functionally express human CYP11B1 have been developed in our group (Bureik *et al.* 2002b; Dragan *et al.* 2005). Although these strains display 11 β -hydroxylation of RSS without the need for coexpression of Adx and AdR, the hydrocortisone production is considerably higher than the values reported by other steroid 11 β -hydroxylation systems, but still not competitive enough for industrial applications. Therefore, the purpose of this part of work was to coexpress the corresponding mitochondrial electron chain (Adx and AdR) to improve the activity of CYP11B1 and, as a result, the efficiency of hydrocortisone bioproduction at the laboratory level. In order to achieve this aim, two strategies were selected: one involved a gene disruption based method while the other involved the construction of an expression plasmid that could bear the Adx and the AdR expression cassette. Additionally, the Adx wild type was substituted by two Adx mutants, Adx^{S112W} (Schiffler *et al.* 2001) and Adx^{D113Y} (Bichet *et al.* 2007), in the coexpression strain which were suspected to further improve the electron transport chain and, as a result, the efficiency of steroid 11 β -hydroxylation and hydrocortisone production.

3.2.1. The Coexpression of AdR and Adx through two expression vectors (Strategy I)

Since Adx expression vectors used in the work are derived from the pNMT1- TOPO vector (see subsection 2.1.3), and since the latter possesses a *Leu2* gene from *S. cerevisiae* that complements functionally each *leu1* mutant strain and enables the selection of yeast transformants (Andreadis *et al.*, 1984), the transformation of the Adx plasmid in fission yeast strain SZ1 is possible through the (*leu1*⁻) auxotrophy. Since SZ1 contains only a single selection marker (*leu1*⁻), the creation of a second marker was a necessary prerequisite to accomplish the coexpression of AdR on a second plasmid.

3.2.1.1. *Ura4* gene disruption in *S. pombe* (SZ1) and the characterisation of the new strain

A gene disruption process was carried out to knockout the *ura4* gene inserted by the pCAD1-hCYP11B1 plasmid (Figure 3.10) in the fission yeast strain SZ1 (Dragan *et al.* 2005) in order to create a strain that expresses the human CYP11B1, and possesses *ura4⁻* and *leu1⁻* auxotrophies. The resulting strain then can be used as a host for the assembly of the human electron transfer chain. The *ura4* gene disruption was done according to Akio Tohe-e (Tohe-e 1995). The *Hind* III fragment containing the *ura4* disruption cassette was excised from the plasmid pAT539 and used as a donor for the transformation of SZ1 from *ura4⁺* to *ura4⁻*. Transformed cells were plated on agar plates containing EMM, leucine, uracil and 5-FOA. After three days incubation in the presence of 5-FOA, and replica plating on selective media, positive colonies were checked for the presence of selection marker.

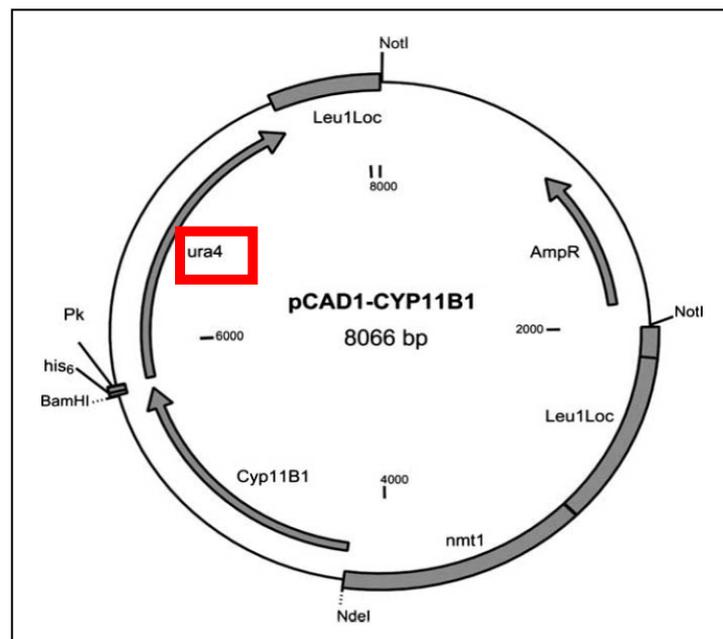


Figure 3.10. Vector map of pCAD1_CYP11B1. Leu1Loc: gene fragments of the *leu1* gene that serve as integration target sequences, *ura4*: ORF for orotidine monophosphate decarboxylase, complements *ura4.dl18* in *S. pombe* (Dragan *et al.*, 2005).

The transformation procedure yielded strain TH1, which was characterised as a new fission yeast strain that already expresses CYP11B1 and needs the addition of leucine and uracil to grow (Figure 3.11).

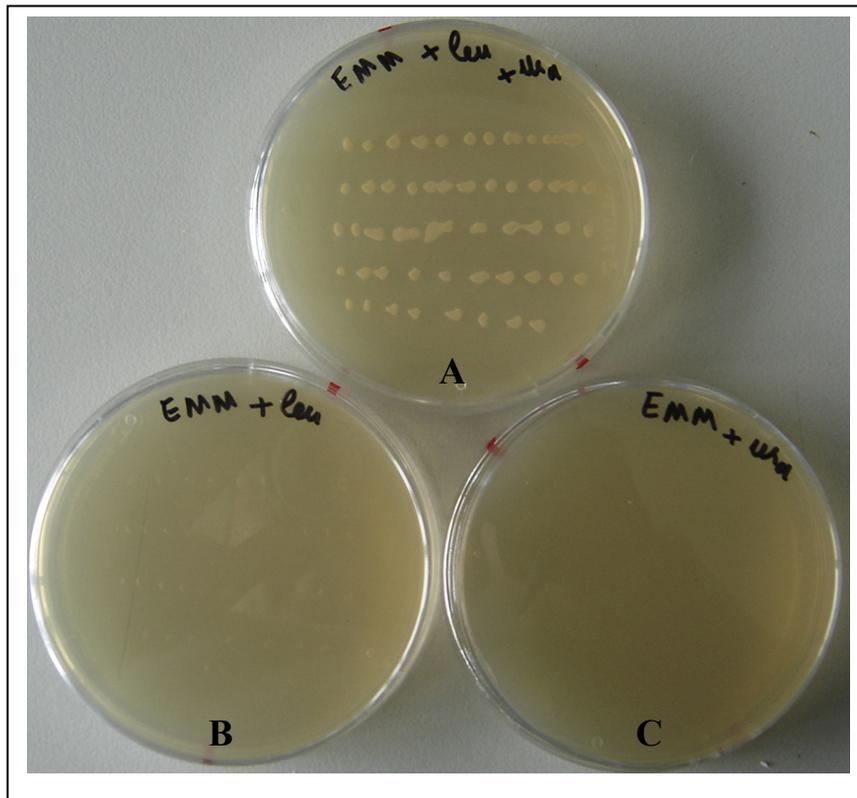


Figure 3.11. Fission yeast TH1 plated on different EMM plates.

The new fission yeast TH1 shows no growth without the addition of leucine and uracil to the medium (A; EMM + leucine + uracil, B; EMM+ leucine, C; EMM + uracil).

Furthermore, strain TH1 displayed the ability to grow in the presence of 1% 5-FOA in contrast to SZ1 that showed an altered phenotype in comparison with TH1 after incubation with 5-FOA (data not shown). The morphologic differences between SZ1 and TH1 could indicate that the *ura4* gene in TH1 is not functional, whereas the intact *ura4* gene in strain SZ1 generates toxic metabolites (Boeke *et al.* 1987) that could be responsible for the morphological changes of the cells after incubation with 5-FOA. Since the *ura4* gene is located near the cloned *CYP11B1* in strain SZ1 (Dragan *et al.* 2005) (Figure 3.10) further validation was carried out to investigate the CYP11B1 activity in TH1.

The CYP11B1 activity in the new *S. pombe* strain TH1 was investigated in comparison with the parental strain SZ1. The test was performed in the tip-tube format as described in subsection 2.2.4.2. The hydroxylation assay showed that strain TH1 still retains the 11 β activity of SZ1 and no significant differences were noticed ($p < 0.05$) (Figure 3.12). This observation indicates that the *CYP11B1* gene in TH1 is still intact rendering TH1 as a potential host for the construction of the electron transfer chain in fission yeast.

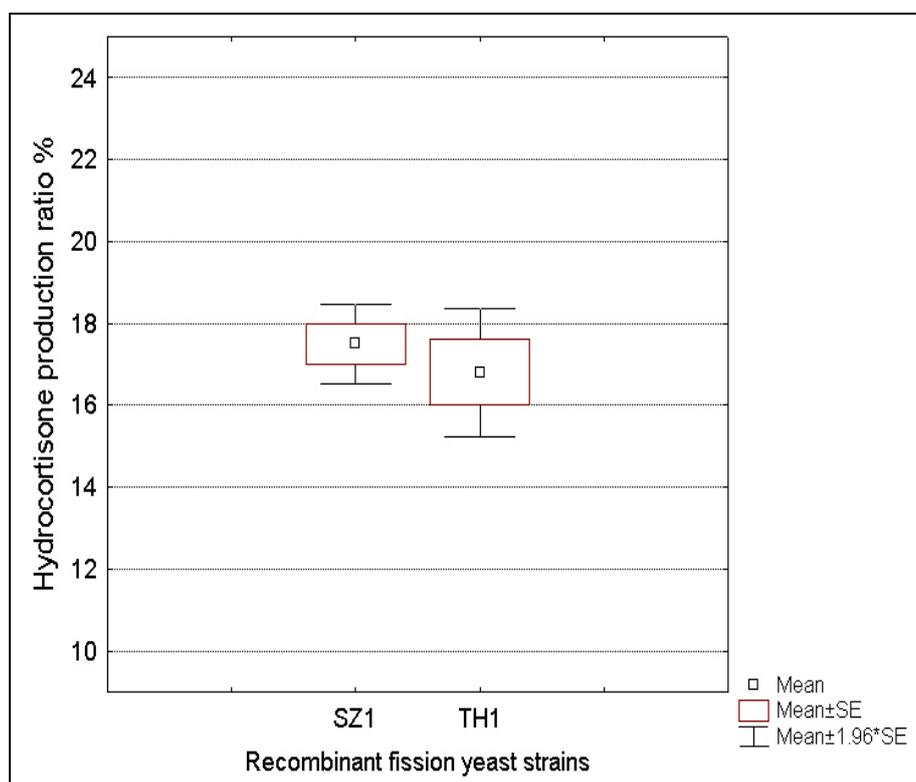


Figure 3.12. Steroid 11 β -hydroxylation activity of TH1 and SZ1.

The bioconversion assay was carried out in tip-tube with 100 nM RSS. Values were calculated from three independent experiments and are presented as mean \pm standard error of mean.

3.2.1.2. Construction of AdR expressing vector (pTH1)

The *Hsa*AdR cDNA was PCR-amplified with the primers Pr₁, Pr₂ that introduce *NdeI/XhoI* restriction sites (see the appendix), and sub-cloned into the pNMT1-TOPO vector as described in subsection 2.2.1.1. After transformation of *E. coli* and plasmid purification, the fragment was cut out using *NdeI* and *XhoI*. The *Hsa*AdR fragment was then cloned into the *NdeI/XhoI* -digested pREP42 Pk C vector to yield pTH1. The developed vector pTH1 was sequenced and it was shown that AdR revealed no alteration compared to the wild type sequence. In this way a vector for the expression of Pk tagged-AdR in *ura4⁻ S. pombe* strains is constructed, and will be used to transform the fission yeast strain TH1.

3.2.2. Construction of an AdR+Adx expressing vector pTH2 (Strategy II)

The aim of this cloning strategy was to develop an expression vector that coexpresses both, wild type Adx and AdR, under the control of the strong inducible *nmt1* promoter. The construction of this expression vector enables the coexpression of the complete mitochondrial

electron transfer partners through one single plasmid. For the construction of the Adx-AdR expression plasmid a cloning strategy was carried out as shown below in Figure 3.13. The $HsaAdR$ cDNA was PCR-amplified with the primers Pr_3, Pr_4 (see the appendix). The PCR product was isolated, purified and subsequently subcloned into the pNMT1-TOPO vector. After transformation of *E. coli*, a colony PCR was carried out to isolate the positive colonies with the correct orientation of the inserted *AdR* gene downstream the *nmt1* promoter. The colony PCR was performed using Primer Pr_4 as forward primer and primer Pr_6 as reverse primer.

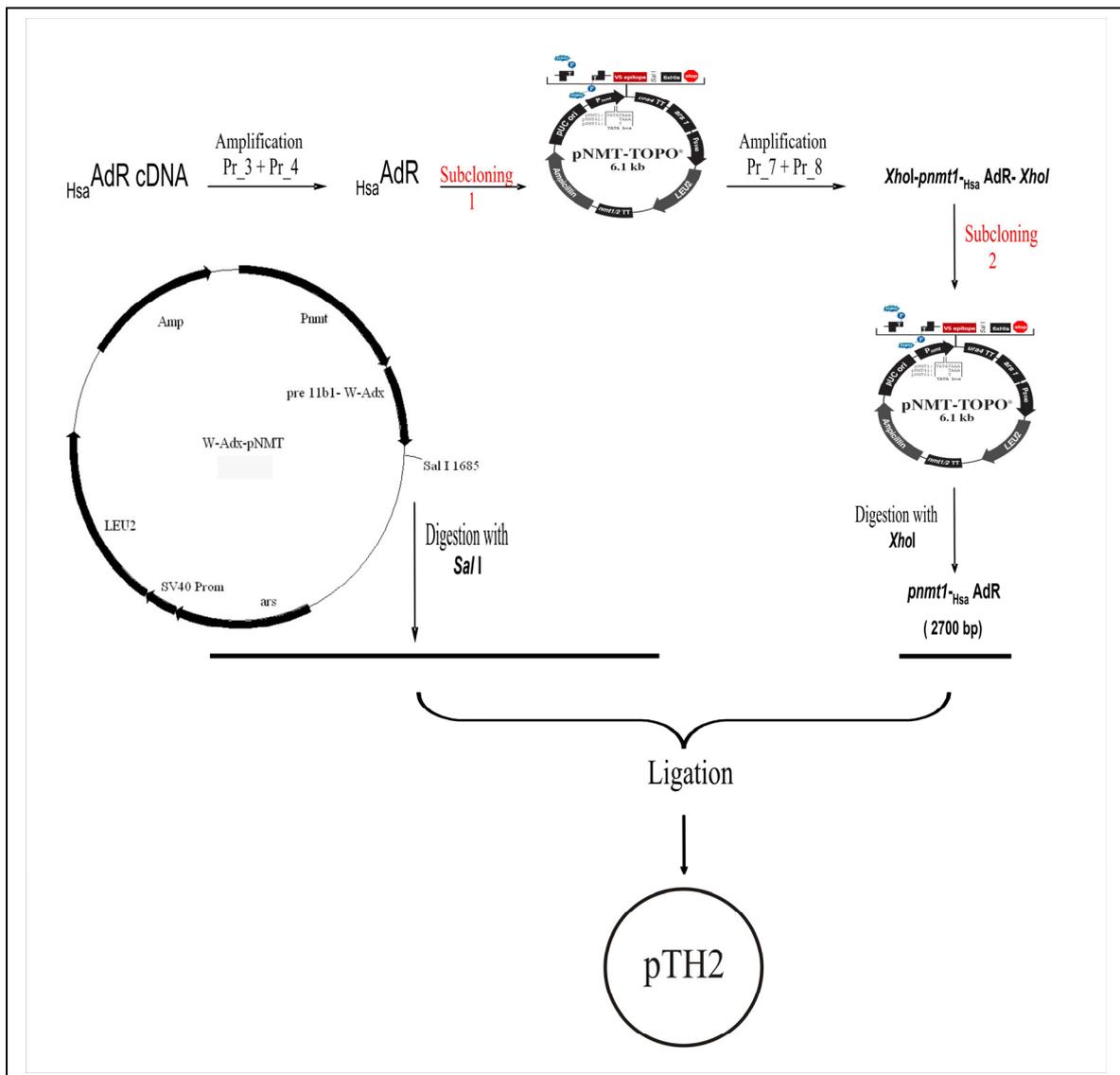


Figure 3.13. The cloning strategy used to create the Adx_AdR expression vector pTH2.

The $HsaAdR$ cDNA was PCR-amplified and subcloned in the PNMT1-TOPO, the resulting plasmid was then used as template to amplify the (*nmt1* promoter + AdR expression cassette) with two *XhoI* sites as overhangs. The *XhoI* digested fragment was then isolated and cloned in the *SalI* site in the pNMT1-Adx^{WT} to yield pTH2.

Subsequently the resulting plasmid from last step with the correct orientation was used as a template to PCR-amplify the (*nmt1* promoter + AdR expression cassette). For this target, primers Pr_7, Pr_8 were used that introduced 5' and 3' terminal *Xho*I sites. The amplified product was then subcloned in pNMT1-TOPO, and finally cloned into the *Sal*II site in pNMT1-Adx^{WT} to yield pTH2 (Figure 3.14), which accordingly allows the coexpression of Adx and Pk-tagged AdR in *leu1* hosts.

The developed vector pTH2 was validated by performing Adx colony PCR using primers Pr_9, Pr_10. Furthermore, sequencing of the *nmt1* promoter-AdR expression cassettes in pTH2 revealed no alteration compared to the wild type sequence. Restriction analysis of pTH2 confirmed the construction of the plasmid as shown in Figure 3.14 A, B.

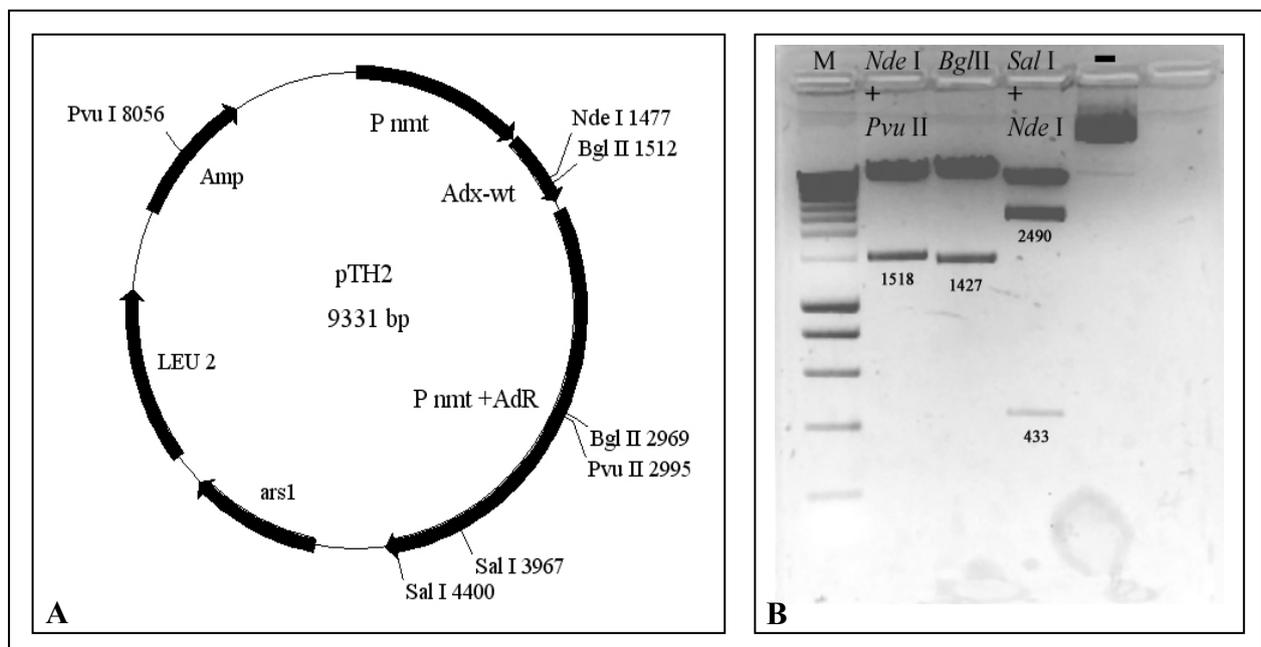


Figure 3.14. A: Vector map of pTH2. Relevant restriction sites are shown, P nmt: *nmt1* promoter. **B. Restriction analysis of pTH2 confirming the existence of the two expression cassettes.** *Nde*I and *Pvu*II are unique sites that cut in Adx and AdR, respectively, whereas *Bgl* II cuts in Adx and AdR as shown. Numbers at bands indicate the theoretical sizes calculated from the pTH2 vector map.

3.2.3. Coexpression of Adx and AdR in fission yeast

The Adx expressing plasmids described in subsection 2.1.3, and the new developed plasmids pTH1 and pTH2 (Table 3.5) were then used to transform CYP11B1-expressing strains as well as the control strain 1445 as shown in Table 3.6.

Table 3.5. Fission yeast expression plasmids used in this work

| Plasmid | Insert(s) | Selection marker | Reference |
|----------------------------|---------------------------------------|------------------|--------------------------------------|
| pNMT1-Adx ^{WT} | Adx ^{WT} | <i>LEU2</i> | (Derouet-Hümbert <i>et al.</i> 2007) |
| pNMT1-Adx ^{D113Y} | Adx ^{D113Y} | <i>LEU2</i> | (Derouet-Hümbert <i>et al.</i> 2007) |
| pNMT1-Adx ^{S112W} | Adx ^{S112W} | <i>LEU2</i> | (Derouet-Hümbert <i>et al.</i> 2007) |
| pTH1 | AdR ^{WT} | <i>ura4</i> | (Hakki <i>et al.</i> , 2008) |
| pTH2 | Adx ^{WT} + AdR ^{WT} | <i>LEU2</i> | (Hakki <i>et al.</i> 2008) |

Table 3.6. Fission yeast strains created in this work

| Name | Parental strain | Expression construct(s) | Expressed Protein(s) | Required supplement(s) |
|-------|-----------------|-----------------------------------|--|----------------------------|
| TH1 | SZ1 | pAT539 | CYP11B1 | leucine + uracil |
| TH2 | TH1 | pNMT1-Adx ^{WT} | CYP11B1 + Adx ^{WT} | uracil |
| TH3 | TH1 | pTH1 | CYP11B1 + AdR ^{WT} | leucine |
| TH4 | TH1 | pNMT1-Adx ^{WT} + pTH1 | CYP11B1 + Adx ^{WT} + AdR ^{WT} | none |
| TH6 | TH1 | pNMT1-Adx ^{S112W} + pTH1 | CYP11B1 + Adx ^{S112W} + AdR ^{WT} | none |
| TH7 | TH1 | pNMT1-Adx ^{D113Y} + pTH1 | CYP11B + Adx ^{D113Y} + AdR ^{WT} | none |
| TH75 | SZ1 | pTH2 | CYP11B + Adx ^{WT} + AdR ^{WT} | none |
| TH175 | 1445 | pTH2 | Adx ^{WT} + AdR ^{WT} | adenine, uracil, histidine |

Since the molecular mass of the Pk-tagged human AdR of 50 kDa could interfere with the very similar mass of the Pk-tagged human CYP11B1 (59 kDa), an approach was used to

confirm expression from the double cassette plasmid pTH2. A wild type *leu1⁻* fission yeast strain (1445) was transformed with pTH2 in order to eliminate the Pk signal background introduced by the CYP11B1 in SZ1. Western blot analysis using α -Pk and α -Adx antibodies to detect AdR and Adx, respectively, showed that pTH2 is able to coexpress AdR and Adx with mitochondrial localisation in fission yeast (Figure 3.15 A and B).

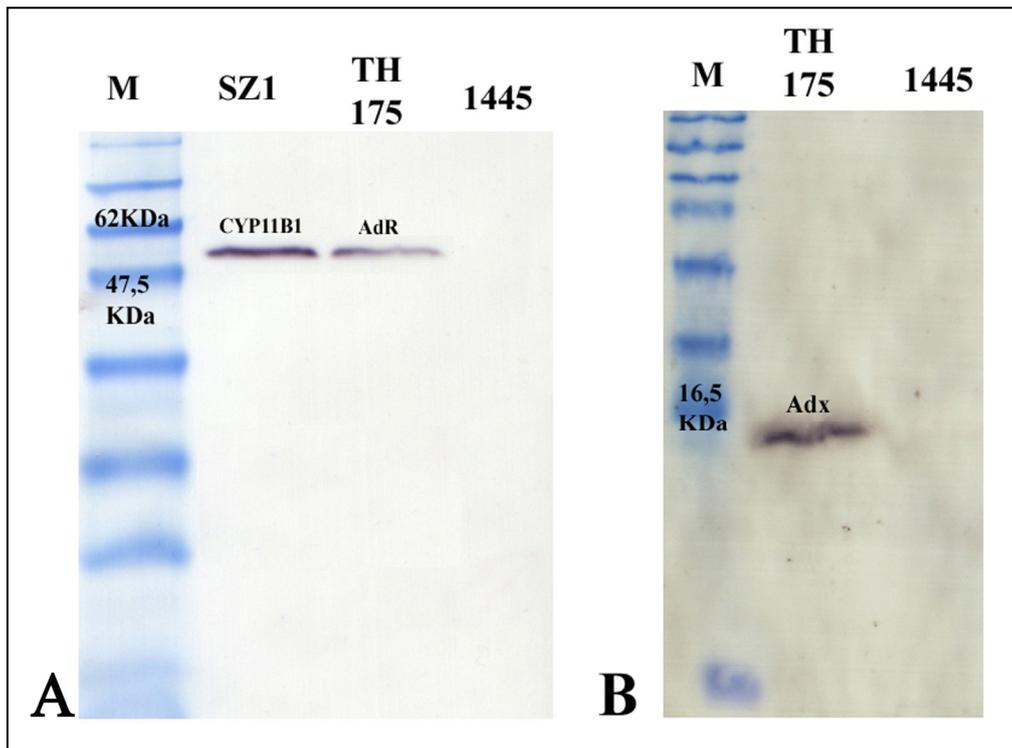


Figure 3.15. Detection of AdR^{WT} (A) and Adx^{WT} (B) in mitochondrial lysates of strain TH175 by Western blot analysis. Mitochondrial lysates of TH175 and its parental strain 1445 were separated by SDS/PAGE and blotted onto nitrocellulose membranes. Immunologic protein detection was carried out using α -Pk and α -Adx antibodies as described by materials and methods. M: Protein standard.

As expected, the signals could not be detected in the parental wild type strain 1445. The apparent molecular weight of AdR and Adx expressed from pTH2 are displayed at approximately 56 and 15 KDa and are in good agreement with the calculated masses of 50 and 13.8 KDa, respectively (Figure 3.15. A, B). For each of the Adx expressing plasmids used in this work, correct subcellular localisation of the expressed Adx in fission yeast strains was previously confirmed by Western blot analysis of mitochondrial protein lysates (Figure 3.16) (Derouet-Hümbert *et al.* 2007).

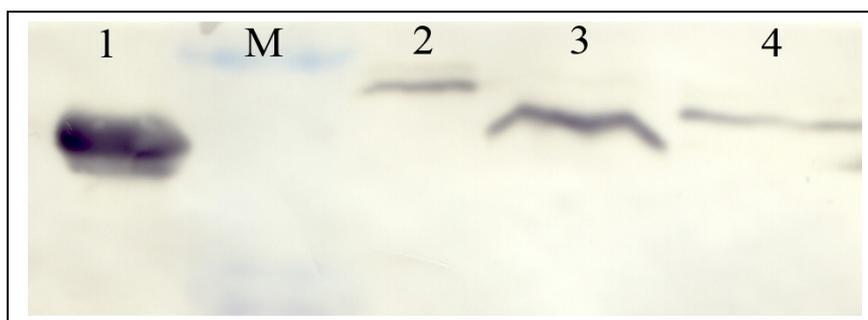


Figure 3.16. Immunological detection of adrenodoxin (Adx) expressed in *S. pombe*. Mitochondrial lysates were prepared and analyzed by SDS-PAGE and Western blot using an α -Adx antibody. Lane 1; purified mature bovine Adx (positive control), lane 2; Adx^{WT}-expressing fission yeast strain, lane 3; Adx^{S112W}-expressing fission yeast strain, lane 4; Adx^{D113Y}-expressing fission yeast strain.

Wild type of Adx (Adx^{WT}) migrates at a higher molecular weight than the positive control because it contains an additional mitochondrial localisation signal (Derouet-Hümbert *et al.* 2007). The calculated molecular weight of mutant Adx^{S112W} is lower (about 2.2 kDa) than that of Adx^{WT}, which is in good agreement with these results. Mutant Adx^{D113Y}, however, should theoretically have a slightly higher mass than Adx^{WT}, but migrates much faster during SDS-PAGE. The reason for this discrepancy is not known, but rarely other point mutations in Adx also cause a change in migration behavior during SDS-PAGE that cannot be explained by their mass difference (Hannemann, unpublished observation). Expression of the activated Adx mutants also did not lead to slower growth of the cells or to a pronounced phenotype (Derouet-Hümbert *et al.* 2007).

3.2.4. The 11 β -hydroxylation activity of CYP11B1 in the new recombinant fission yeast strains

The hydroxylation assay in the 96-well plate format developed in this work (subsection 3.1.2) was further validated. The validation was carried out to investigate the reliability of the assay. For this reason, the bioconversion efficiencies between the several wells in one plate were investigated for any differences. The test was carried out using the new fission strain TH4 with a cell density of 10⁸ cells/ml. The bioconversion was initiated by adding RSS (substrate) to give a final concentration of 100 μ M. After 24h shaking at 480 rpm and 30°C, steroids were extracted with chloroform, resolved in acetonitrile and analysed by HPLC as mentioned before in subsection 3.1.2.

Figure 3.17 below shows the bioconversion results of 66 wells. It is clearly to see that the majority of results belong to the range (Mean \pm 1.96*SD) which is an acceptable normal distribution for any measurement. Therefore, this 96-well hydroxylation assay is an efficient screening tool and can be applied to investigate the steroid hydroxylation activity in the recombinant strains developed during this work.

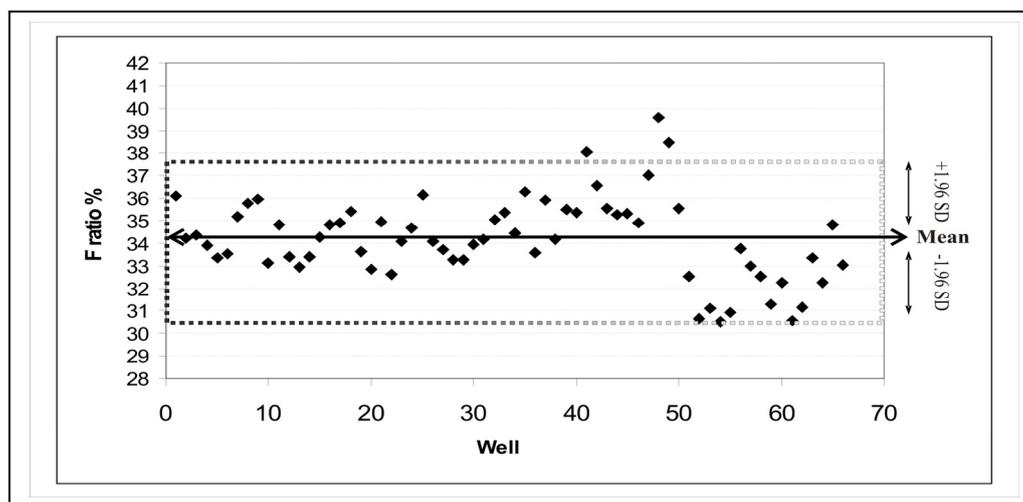


Figure 3.17. The biotransformation of RSS into F investigated in 66 wells using the newly developed hydroxylation assay.

The test was carried out in a 96-well plate using the recombinant fission yeast TH4. 66 wells were analysed and presented as hydrocortisone production ratio (F ratio).

3.2.4.1. Comparison of biotransformation activity of CYP11B1-expressing strains after coexpressing of the corresponding redox partners

To compare the activities of CYP11B1 in the newly developed fission yeast strains, the hydroxylation assay in 96-well plate format developed in this work (subsection 3.1.2) was applied. To perform the steroid hydroxylation assay, fission yeast cell suspensions with a cell density of 10^8 cells/ml were prepared. The bioconversion was initiated by adding RSS (substrate) to give a final concentration of 100 μ M. After 24h shaking at 480 rpm and 30°C, steroids were extracted with chloroform, resolved in acetonitrile and analysed by HPLC as mentioned before in subsection 3.1.2. Steroid 11 β -hydroxylation activity was investigated in all fission yeast strains, which coexpressed Adx and/or AdR and compared with the parental strain SZ1 (Figure 3.18). These experiments clearly demonstrate that coexpression of Adx^{WT} increases the biotransformation activity, with the hydrocortisone ratio increasing significantly from 12% in SZ1 to 25% in TH2 (*t*-test, $p < 0.05$). As expected, 11 β -hydroxylation activity in strain TH3 (CYP11B1 + AdR) did not show any improvement over the parental strain SZ1

due to the fact that Adx plays a central role in the electron transfer chain (Figures 1.2, 4.1). A further increase in steroid hydroxylation activity could, however, be achieved in the strain TH4, which coexpresses AdR, Adx^{WT} and CYP11B1, and displays a hydrocortisone production ratio of 40%. Since it was previously shown by our group that mutants of Adx are able to stimulate 11 β -hydroxylation, I attempted to further increase the biotransformation efficiency by substituting Adx^{WT} with the Adx mutants. The first mutant chosen was Adx^{S112W}, which lacks the 16 carboxy terminal amino acids and features a terminal tryptophane, leading to enhanced affinity for the cytochrome P450 and also to a lower redox potential (-334 mV) (Schiffler *et al.* 2001). The second mutant, Adx^{D113Y}, is a full-length Adx mutant with enhanced cytochrome P450 binding ability and a slightly changed redox potential (-298 mV) (Bichet *et al.* 2007). However, as can be seen in Figure 3.18, fission yeast strains TH6 and TH7 that coexpress AdR and either Adx^{S112W} or Adx^{D113Y} did not show any improvement in the 11 β -hydroxylation activity compared with the strain TH4. Moreover, strains TH6 and TH7 even displayed a slightly lower activity than the strain TH4.

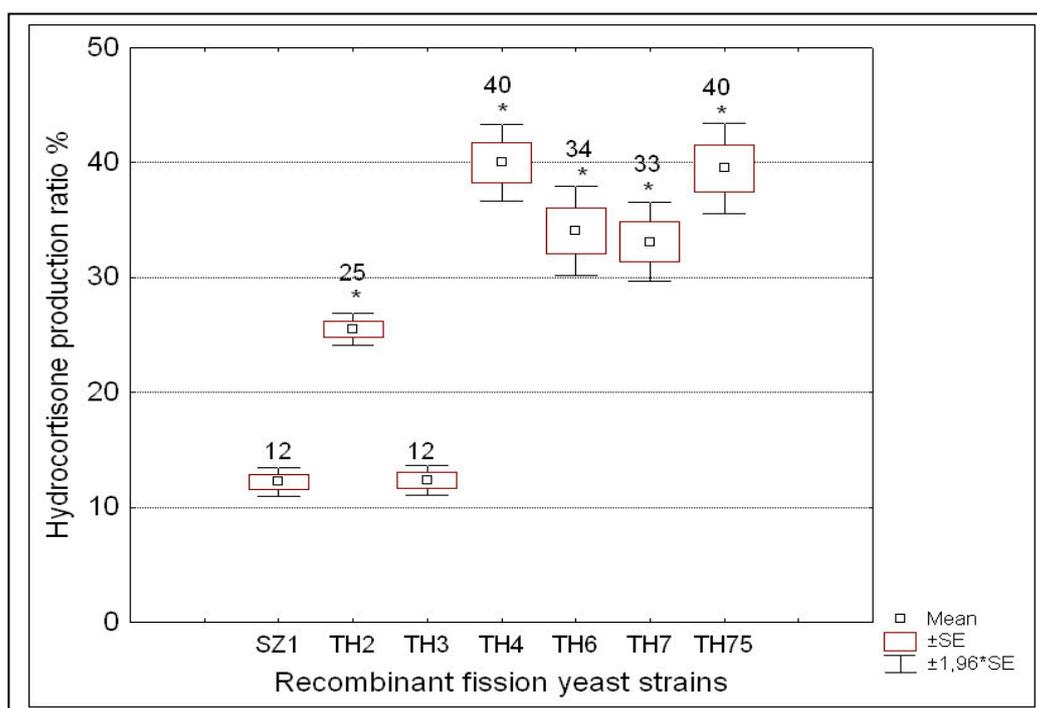


Figure 3.18. Direct comparison of the bioconversion rates of fission yeast strains coexpressing CYP11B1 and electron transfer proteins. SZ1 (CYP11B1), TH2 (CYP11B1+Adx^{WT}), TH3 (CYP11B1+AdR^{WT}), TH4 (CYP11B1+Adx^{WT}+AdR^{WT}), TH6 (CYP11B1+Adx^{S112W}+AdR^{WT}), TH7 (CYP11B1+Adx^{D113Y}+AdR^{WT}), TH75 [CYP11B1+(Adx^{WT}+AdR^{WT})]. Values were calculated from three independent experiments and are presented as mean \pm standard error of mean. Asterisks above boxes indicate a significant difference to the parental strain SZ1 (*t*-test, $p < 0.05$).

The second approach for the construction of a reconstituted CYP11B1 electron transfer chain in fission yeast involved the fusion of the Adx and the AdR expression cassettes on one

plasmid (pTH2). This approach bypasses the need for gene disruption and speeds up the process of strain generation by offering a more convenient way of adding two proteins at once. The coexpression of the wild type Adx and AdR from pTH2 in TH75 resulted in an 11 β -hydroxylation activity in the same range of the strain TH4, thereby confirming the functionality of pTH2 in fission yeast.

3.2.4.2. Quantification of hydrocortisone production in the novel strain TH75

3.2.4.2.1. Optimisation of the biotransformation parameters to achieve a high conversion rate

To achieve a high production rate of hydrocortisone using the fission yeast strain TH75, biotransformation conditions were investigated and optimised. The cell density displayed an important factor to influence the hydrocortisone production in this system, since a cell suspension from strain TH75 with a cell density of 10⁹ cell/ml displayed significant higher hydrocortisone production efficiency in comparison with low-density suspensions when investigated with 1 mM RSS (Figure 3.19).

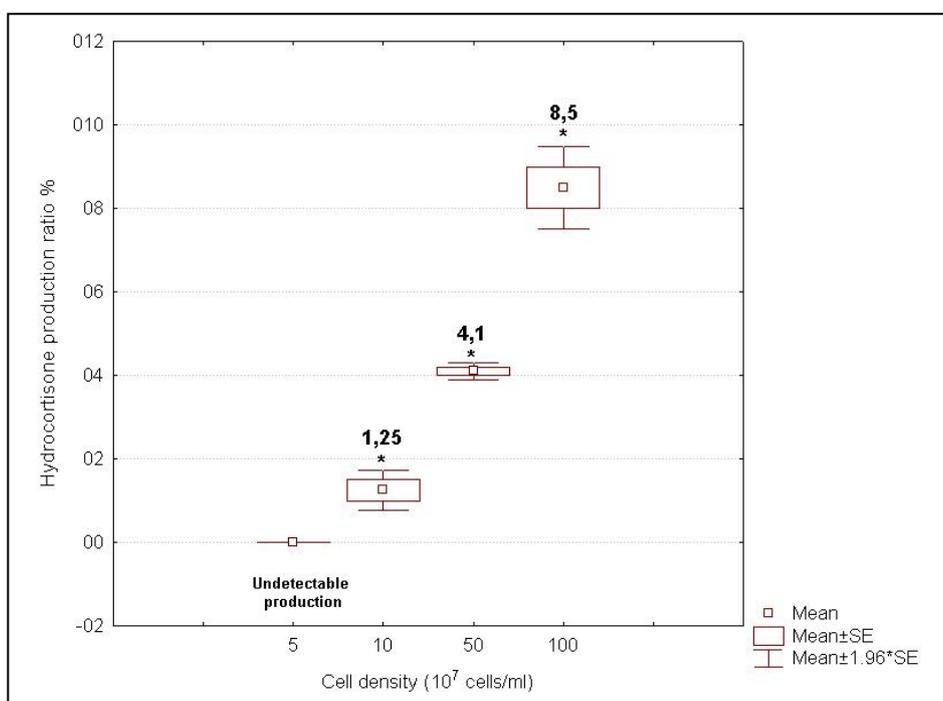


Figure 3.19. The influence of cell density of the recombinant fission yeast on the hydrocortisone production. The assay was carried out with 1 mM RSS, and different cell densities from TH75. Values were calculated from three independent experiments and are presented as mean \pm standard error of mean. Asterisks above boxes indicate a significant difference (*t*-test, $p < 0.05$).

Simultaneously increasing the substrate concentration up to 5 mM RSS increased the biotransformation efficiency, whereas 10 mM RSS displayed a negative effect and less hydrocortisone production was reported in comparison with 5 mM RSS (data not shown).

3.2.4.2.2. Hydrocortisone production efficiency in the fission yeast strain TH75

Hydrocortisone production efficiency was investigated in the new strain TH75 in comparison with the parental strain SZ1. The assay was designed to be carried out in 300-ml Erlenmeyer flasks with 10 ml assay culture under the optimal conditions (10^9 cells/ml with 5 mM RSS) reported in subsection 3.2.4.2.1. During shaking for 72 hours at 180 rpm and 30°C, samples of 500 μ l were extracted with chloroform at several time points after adding DOC as an internal standard to normalize the steroid extraction efficiency. A calibration curve was used to quantify the hydrocortisone amount produced over time. The calibration curve was constructed using pure hydrocortisone and displayed a high correlation between the amount of hydrocortisone injected on the HPLC and the corresponding peak areas. Depending on the internal standard and the hydrocortisone calibration curve, a quantitative measurement was carried out, and the hydrocortisone production was investigated over time in the two fission yeast strains.

The assay demonstrated clearly that the new fission yeast strain TH75 possesses higher 11 β -hydroxylation activity in comparison with the parental strain SZ1 (Figure 3.20), although no differences in the steroid extraction efficiency between the two investigated strains had occurred (Figure 3.21).

Additionally, the time course presented a steady increase in hydrocortisone production from the beginning up to maximum activity of 9.7 μ mol/10ml test culture measured after 72 hours, in contrast to the parental strain that produce 3.2 μ mol/10ml over the same time (Figure 3.20).

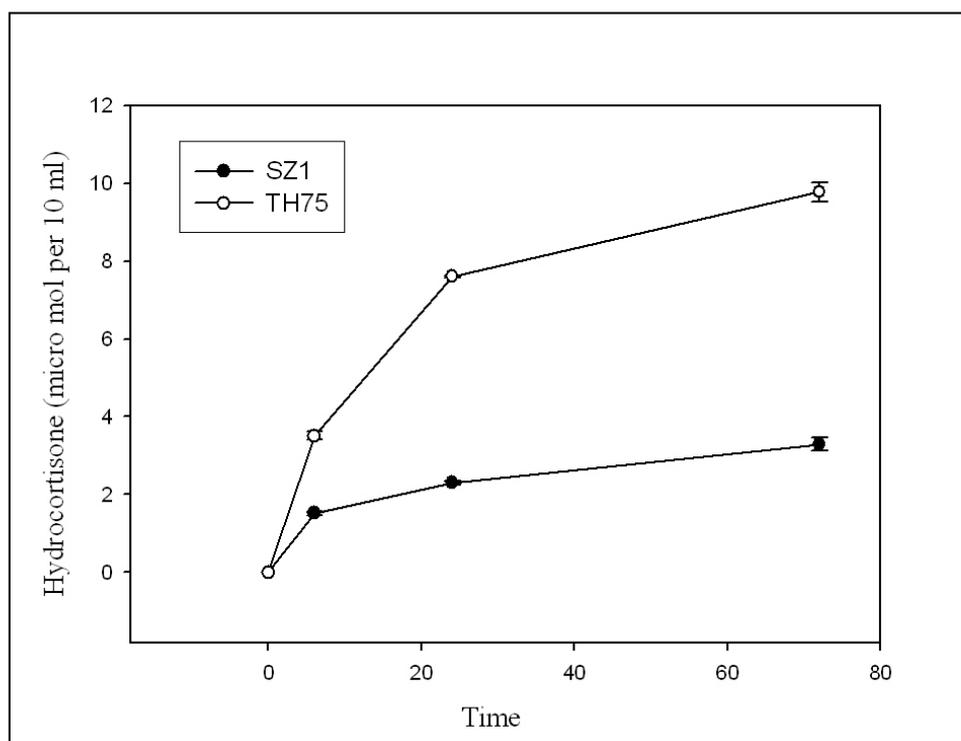


Figure 3.20. Time course of the hydrocortisone production efficiency by SZ1 (CYP11B1) and TH75 (CYP11B1 + Adx + AdR). Data shown are mean values for triplicate measurements and standard deviations are too small to be displayed. The assay was performed in 300-ml Erlenmeyer flasks with a cell density of 10^9 cells/ml with 10 ml assay culture and 5 mM RSS. During shaking for 72 hours at 180 rpm and 30°C, samples of 500 μ l were extracted with chloroform at several time points after adding DOC as an internal standard to normalize the steroid extraction efficiency.

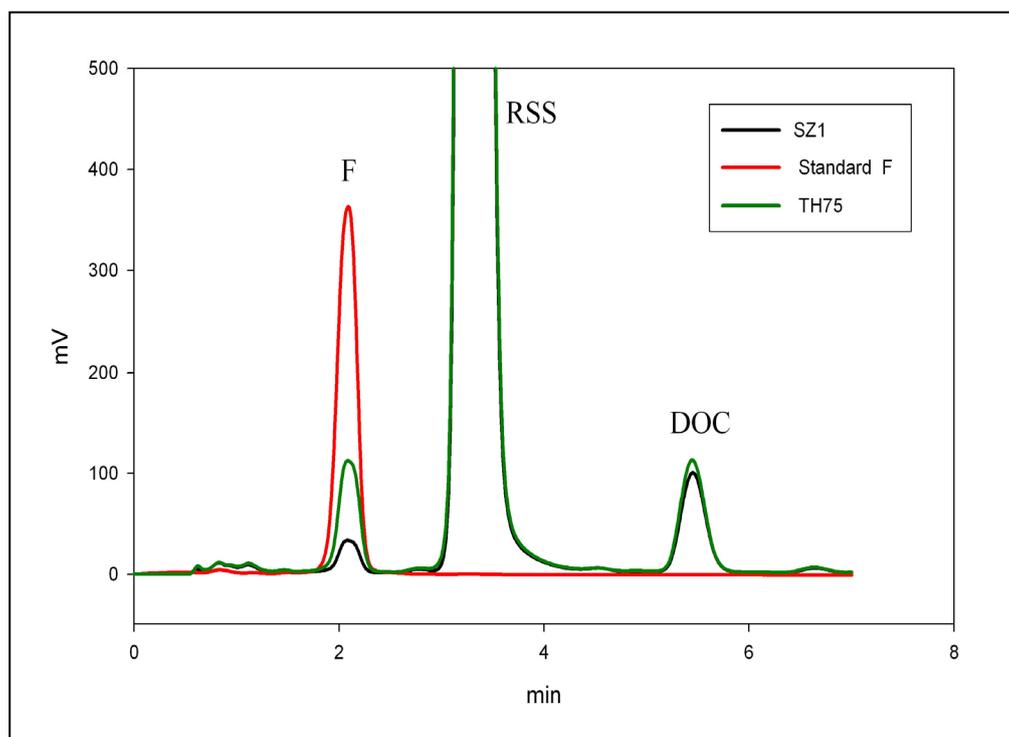


Figure 3.21. HPLC chromatograms of CYP11B1-dependent substrate conversion using the novel strain in comparison with the parental strain. Chromatograms were obtained from extracted samples after adding DOC as internal standard, black and green chromatograms represent the CYP11B1-dependent substrate conversions obtained with SZ1 and TH75 respectively, whereas the red chromatogram represents the pure hydrocortisone injected on HPLC under the same condition.

This demonstrates clearly that the 11β -hydroxylation activity in TH75 is significantly higher than in the parental strain SZ1.

3.3. Development of a cell-based high throughput screening system for the discovery of human aldosterone synthase inhibitors

In order to successfully develop and execute an efficient, rapid, and reproducible cell-based HTS assay in the field of drug discovery, one must have access to (1) automated screening technology plate-format, (2) detectable conversion and inhibition response.

3.3.1. Automated screening technology plate-format

The steroid bioconversion assay in the 96-well format established in this work (subsection 3.1.2) was adapted to develop a CYP11B2 activity screening system. Plate preparation and steroid extraction programs were applied as described before. Taken into consideration the technical limitations and the capacity of the available HPLC instrument; 192 samples (two 96-well plates) can be analysed per run, and since the HPLC parameters described in subsection 3.1.2 enable the separation of DOC and its hydroxylated products within 7 minutes (Figures 3.21, 3.22 A), the time needed to analyse 192 samples is 1344 min = 22.4 h. Hence, the HPLC parameters had to be further optimised to increase the throughput of the assay. For this reason, acetonitrile (ACN), which is more polar than methanol was used in a mixture with water as mobile phase. The new mobile phase was used to separate DOC and B and different flow velocities were investigated (Figure 3.22 B, C, D).

It is clearly shown that a mixture of ACN:H₂O (60:40) with flow velocity of 0.5 ml/min separates DOC and B within the same time that a mixture of MeOH:H₂O (60:40) needs to separate them with a higher flow velocity (1.2 ml/min). Increasing the flow velocity of ACN:H₂O mixture reduced the time needed to separate DOC and B (Figure 3.22 B, C, D).

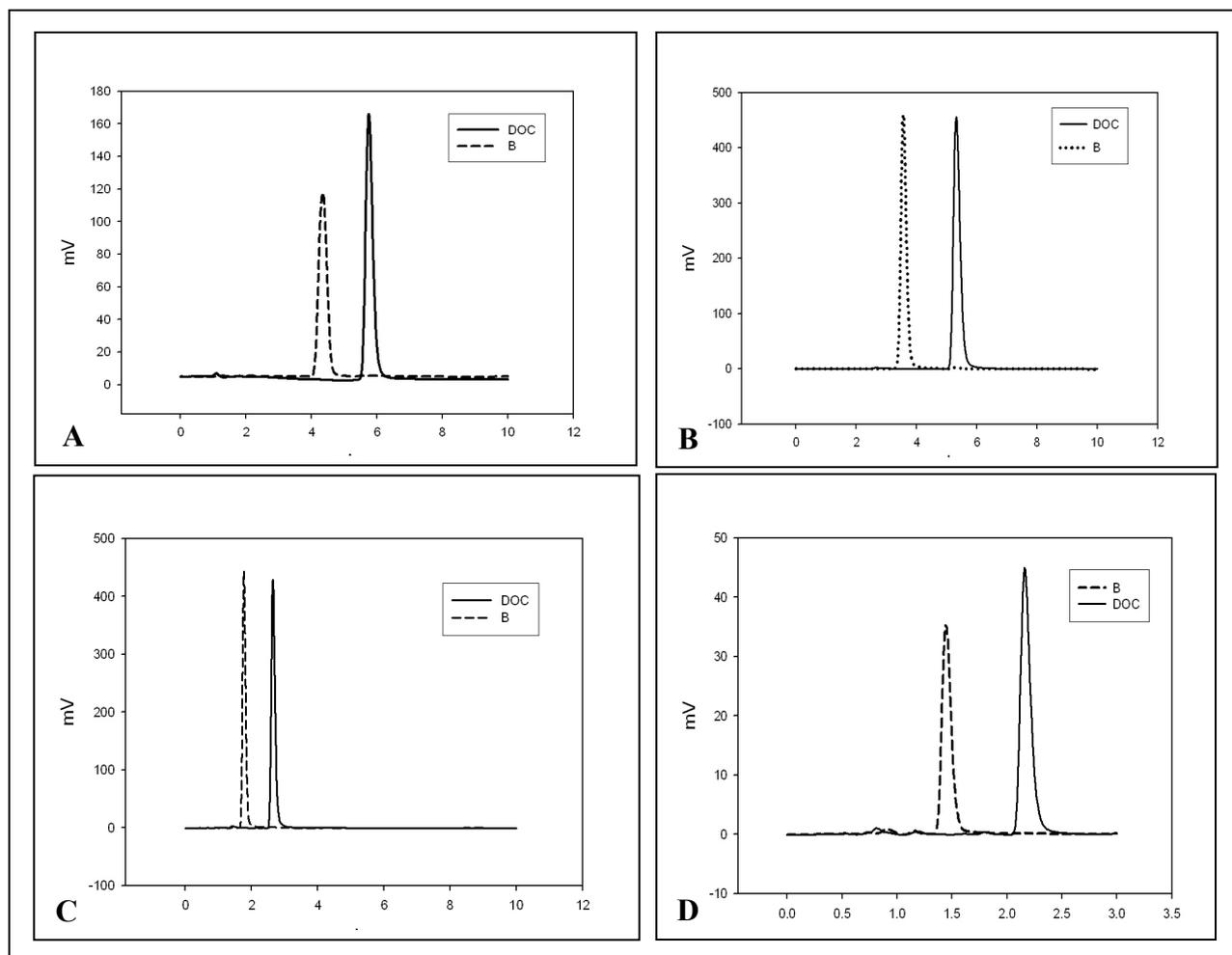


Figure 3.22. HPLC Chromatograms show the separation of DOC and B under different HPLC parameters. A; 1.2 ml/min MeOH:H₂O (60:40), B; 0.5 ml/min ACN:H₂O (60:40), C; 1 ml/min ACN:H₂O (60:40), D; 1.2 ml/min ACN:H₂O (60:40).

As a result, the mixture of ACN:H₂O (60:40) with a flow velocity of 1.2 ml/min displays an efficient separation of DOC and B within 3 min (Figure 3.22 D). This HPLC conditions (Table 3.7) decreased the time needed to analyse 196 samples from 22.4 h to 9.6 h, which increases the throughput of the HPLC by more than 2-fold.

Table 3.7. Optimised HPLC parameters to separate DOC and B within 3 min

| | |
|--------------------|------------------------------|
| Mobile phase | ACN:H ₂ O (60:40) |
| Flow velocity | 1.2 ml/min |
| Column temperature | 25 °C |
| Time per sample | 3 min |

3.3.2. Optimisation of the screening assay parameters to get detectable conversion/inhibition response

In order to get reproducible conversion/inhibition response using a whole-cell-based assay, several parameters should be taken into consideration and optimised to achieve a reliable screening assay. These parameters include the amount of enzyme, substrate concentration, incubation time and the optimal test medium needed to get reproducible conversion and inhibition. For this reason, and in order to estimate the optimal conditions where reproducible screening can be performed, the CYP11B2-expressing *S. pombe* strain MB164 was used to investigate the parameters mentioned above. Moreover, this strain has already been used to test compounds for their CYP11B2 inhibitory effect and several compounds were identified to inhibit CYP11B2 in recombinant fission yeast with different IC₅₀ values (Table 3.8).

Table 3.8. The IC₅₀ values of CYP11B2 inhibitors determined using recombinant *S. pombe* strain MB164 (Bureik *et al.* 2004)

| Compound | IC ₅₀ against CYP11B2 |
|--------------|----------------------------------|
| Clotrimazole | 0.20 µM |
| Ketoconazole | 3.50 µM |
| Miconazole | 5.60 µM |

As addressed below, several optimisation steps were carried out in the presence of the known inhibitors of CYP11B2 (Table 3.8) as positive controls, in addition to mock-treated control. The starting point was the setting of assay time (duration of incubation) needed to get detectable conversion and inhibition. Hence, three hours incubation was suggested for the conversion of DOC into B and for the detection of a possible inhibition. For this reason, 600

μl cell suspension from MB164 with cell density of 10^8 cell/ml was investigated with different concentrations of substrate and inhibitors. Furthermore, different test media were also investigated to estimate the optimal environment to get reproducible detection and inhibition in the presence of negative and positive controls.

- **Substrate concentration**

Although $100 \mu\text{M}$ substrate concentration described in subsection 3.1.2 displayed detectable conversion of DOC into B, no inhibition response was observed after incubation with even high concentrations of the well-known inhibitors of CYP11B2 (ketoconazole, clotrimazole). For this reason, the substrate concentration had to be reduced to a minimal level where detectable conversion and inhibition can take place and can be followed in the screening system. The investigation showed that $5 \mu\text{M}$ DOC is a minimal concentration that gives detectable conversion of DOC into B (Figure 3.23). Hence, the test will be carried out with $5 \mu\text{M}$ DOC as substrate. In a next step, the inhibitor concentration must be estimated to get reproducible inhibition in the present of the CYP11B2 inhibitors.

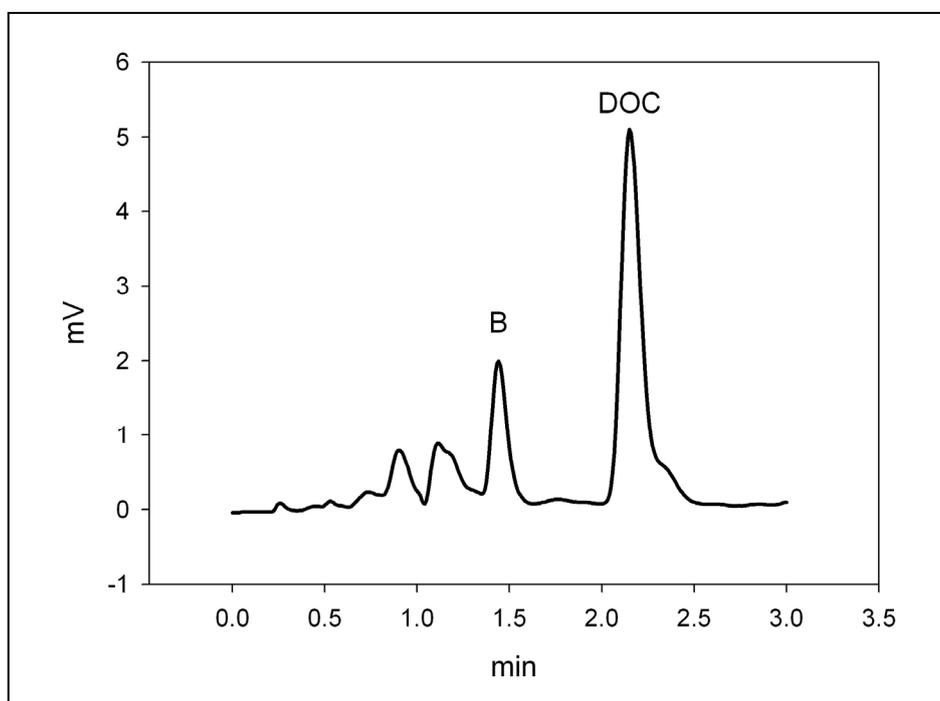


Figure 3.23. HPLC chromatogram of the CYP11B2-dependent bioconversion using the 96-well plate format.

The bioconversion was carried out in a 96-well plate using the CYP11B2-expressing fission yeast strain MB164 incubated with $5 \mu\text{M}$ Doc for three hours. Steroid extraction was carried out with chloroform using the pipetting robot as described before (see subsection 3.1.2).

- **Inhibitor concentration**

To estimate the inhibitor concentration that has to be applied in the screening system, the inhibition profile of the CYP11B2 inhibitor ketoconazole was investigated in different test media. Although fission yeast cells used for each bioconversion assay in this work are prepared from a main culture, in which we expect that the cells achieved the stationary phase, performing the bioconversion/inhibition assay in fresh growth medium can give the cells the opportunity to grow and to use the fresh added glucose, which could influence the inhibition profile of the inhibitor during the assay. Hence, the aim of this part of work was to investigate the influence of different test media on the inhibition profile of ketoconazole, and the correlation between the concentration of inhibitor and inhibition.

Fission yeast strain MB164 with cell density of 10^8 cells/ml was incubated in a 96-well plate with 5 μ M substrate (DOC). Ketoconazole was added to achieve final concentrations in the range from 5 to 60 μ M. Different test media including EMM, simple potassium phosphate buffer (50 mM) with different pH values (5.8, 7, 7.4) were investigated. After three hours incubation, steroid extraction and subsequent HPLC measurements were carried out as described before. The ketoconazole inhibition profile in EMM showed bad correlation between the inhibitor concentration and inhibition ($r = 0.548$). Furthermore, unexplained activation was observed with 5, 10, and 40 μ M ketoconazole (Figure 3.24 A). This result shows clearly that the inhibition profile of ketoconazole in EMM is not reliable to perform reproducible screenings under our test condition since the correlation between the inhibitor concentrations and inhibition is very low (Figure 3.24 A). In contrast, it is clearly shown that the inhibition profile of ketoconazole displays better correlation between inhibitor concentration and inhibition when the test is performed in a simple phosphate buffer (Figure 3.24 B, C, D). Although high correlations were observed in the different pH-variants of the simple buffer, the physiological pH (7.4) will be used as test medium to develop the screening assay during this work.

As mentioned before; the use of multiple-concentrations assay at early (screening) stages of drug discovery is very time- and resource-intensive, and when conducting the CYP11B2-inhibition assay in a high-throughput format to support early drug discovery, inaccuracies in IC_{50} values determination are less problematic, because we are trying to quickly identify strong inhibitors, and we are less concerned with minor inhibition. Furthermore, in the early stages of drug development, before the *in vivo* pharmacokinetics and pharmacodynamics are

known, CYP inhibition assay results are often interpreted in board terms and used to classify compounds into three categories as potent, moderate or weak inhibitors (Lin *et al.*, 2007).

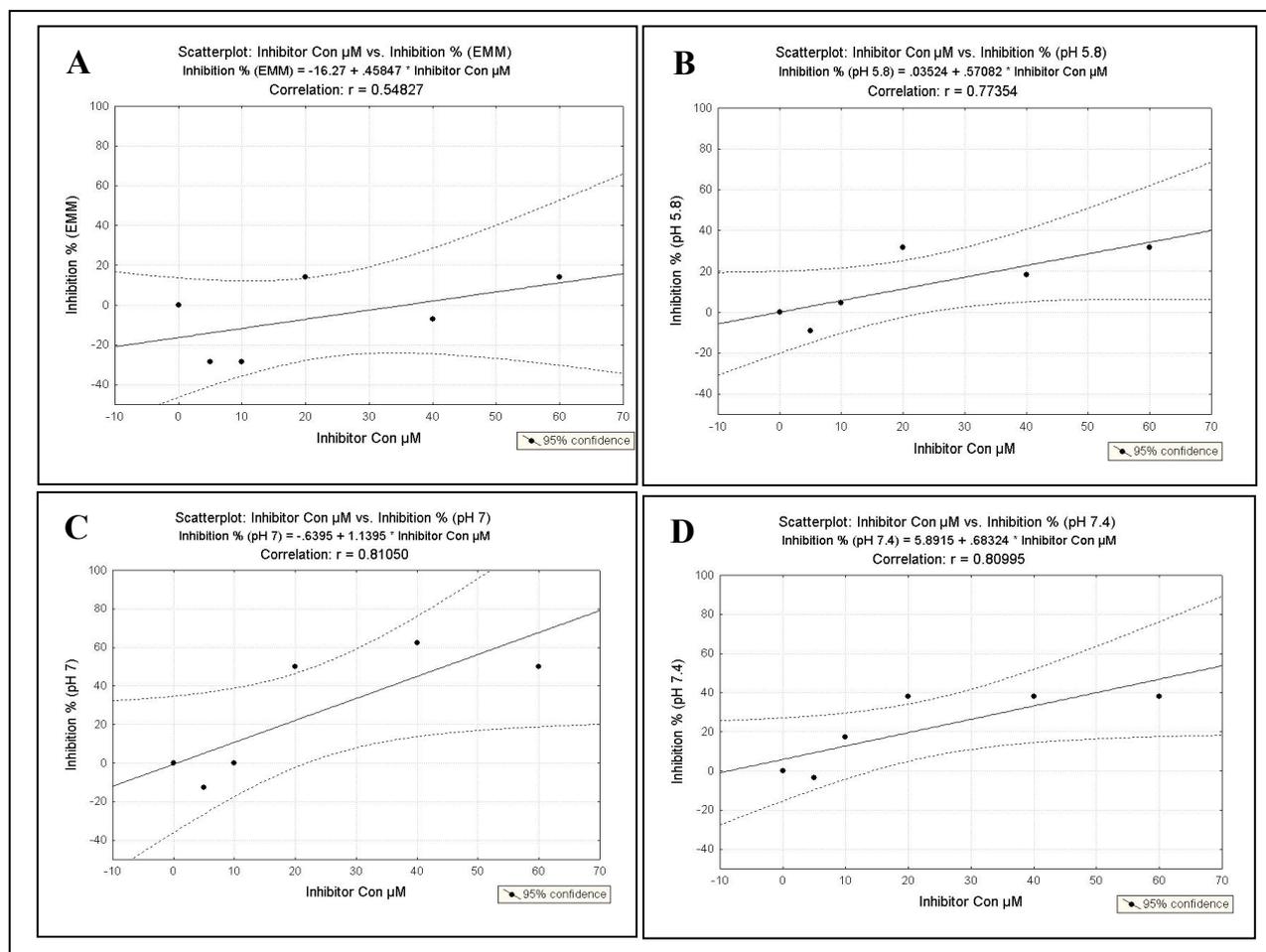


Figure 3.24. Correlation between the concentration of ketoconazole and inhibition in different test media. (A; EMM) [B, C, D; Potassium phosphate buffer 50 mM (B; pH 5.8), (C; pH 7), (D; pH 7.4)].

Therefore, the aim of this part of work was the development of a one-point method to estimate the CYP11B2 inhibitory profile. Bureik *et al.* reported the high inhibition effect of clotrimazole and ketoconazole against the expressed CYP11B2 in recombinant fission yeast, with IC_{50} values of $0.20 \mu\text{M}$ and $3.50 \mu\text{M}$, respectively, whereas miconazole was reported to inhibit CYP11B2 with a relatively higher IC_{50} value of $5.6 \mu\text{M}$ and to be a less potent inhibitor of CYP11B2 (Bureik *et al.* 2004). These observations enable the classification of these known inhibitors of CYP11B2 into two groups; potent inhibitors (clotrimazole and ketoconazole), and less potent inhibitors (miconazole).

To develop a one-point assay, a concentration of $41.6 \mu\text{M}$ ($10 \mu\text{l}$ from a 2.5 mM stock solution) of each inhibitor was tested under the conditions established above. A cell suspension from MB164 with a cell density of 10^8 cells/ml and final volume of $600 \mu\text{l}$ was

incubated in a 96-well plate in potassium phosphate buffer (50 mM, pH 7.4) as test medium with 5 μ M DOC as substrate. In addition to DMSO-mock treated samples; ketoconazole, clotrimazole and miconazole were added to achieve final concentrations of 41.6 μ M (10 μ l from 2.5 mM stock solutions in DMSO). After three hours incubation, steroid extraction and subsequent HPLC measurement were carried out as described before.

The CYP11B2 inhibitors displayed different inhibition profiles during this test. The mock-treated samples (DMSO) displayed CYP11B2 activity with B production ratio of 11.5 %, whereas the presence of miconazole decreased the CYP11B2 activity and B production ratio to 6.5 % showing 44 % inhibition. Furthermore, clotrimazole or ketoconazole displayed total inhibition (100 %) of CYP11B2 under the test conditions (Figure 3.25).

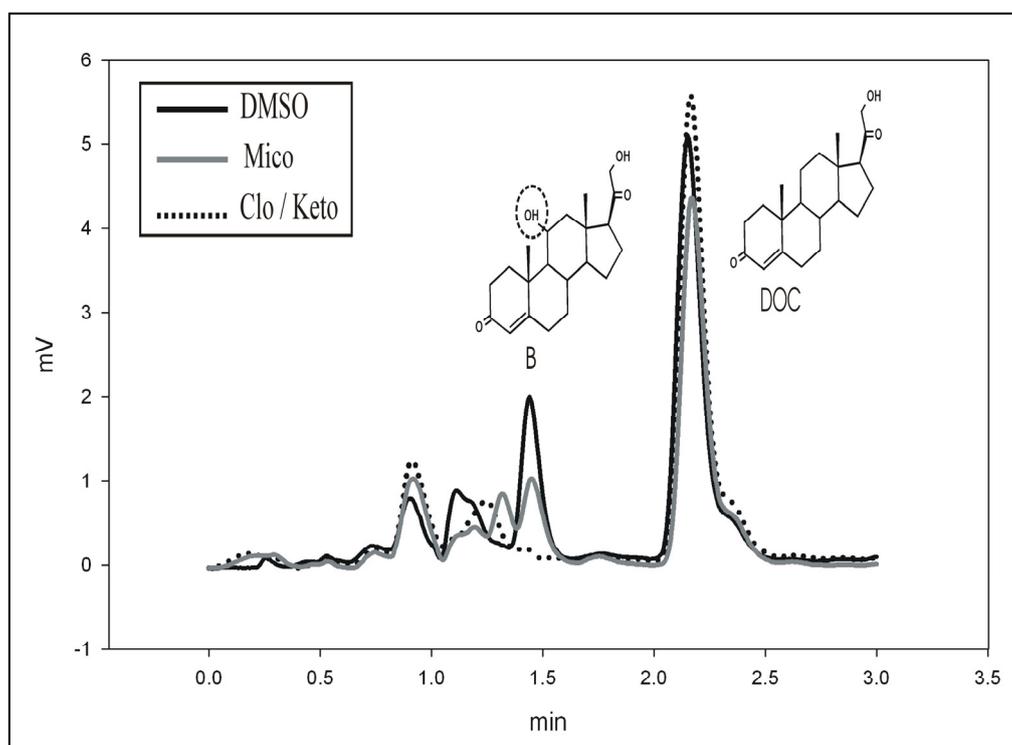


Figure 3.25. HPLC Chromatograms of the CYP11B2-dependent conversion of DOC into B using MB164 in the presence of positive and negative controls. The presence of clotrimazole (Clo) or ketoconazole (Keto) causes total inhibition of CYP11B2, whereas the less potent inhibitor miconazole (Mico) inhibits partially CYP11B2 under these test conditions.

These results confirm clotrimazole/ketoconazole and miconazole as potent and less potent inhibitors of CYP11B2, respectively. Moreover, these observations display a significant and logical correlation between the multiple-point assay reported before (Bureik *et al.* 2004) and the one-point assay developed in this work. Hence, and since clotrimazole/ketoconazole display total inhibition of CYP11B2 under these test conditions, each compound with similar

inhibition profile will be defined during the screening assay as clotrimazole-like inhibitor of CYP11B2, whereas compounds with less inhibition effect resembling the miconazole effect will be defined as miconazole-like inhibitors.

3.3.3. Proof of principle

For the validation of the newly developed screening system, a library of pharmacologically active compounds (LOPAC) was investigated as kindly provided by Prof. Herbert Waldmann (Max-Planck-Institute of Molecular Physiology, Dortmund, Germany). For controls, entire DMSO-treated and positive controls wells were incorporated in each plate (Figure 3.26) in order to have a kind of internal quality control along the screening process, and to enable the interpretation of results.

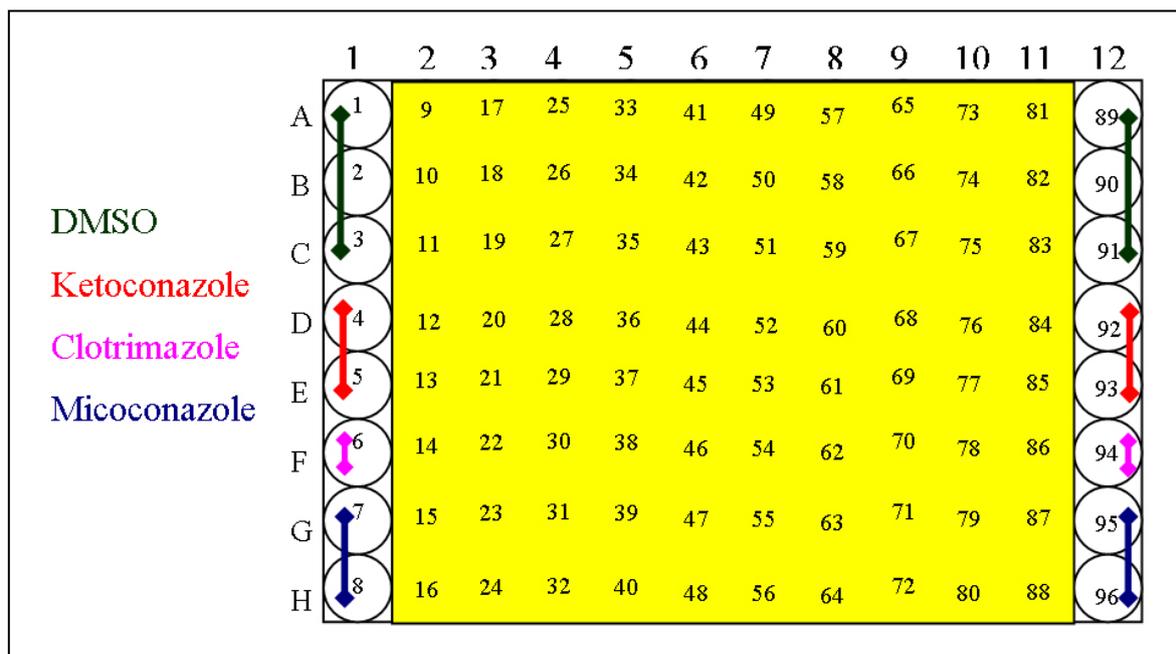


Figure 3.26. Schematic overview of the 96-well plate during the screening assay of the investigated library (LOPAC). The yellow area presents the wells with the investigated compounds (one compound per well); 2.5 mM of stock solution of the individual compounds was prepared in DMSO to be tested at once per plate. Furthermore, mock-treated wells (DMSO) and well-known inhibitors of CYP11B2 as positive controls were incorporated in each plate as shown in the Figure.

Moreover, further optimisation was carried out in order to increase the throughput of the HPLC system. This was achieved by using a 70 mm high-speed column packed with 3 μ m counterparts (reversed NUCLEODUR 100-3 C18) from MACHEREY-NAGEL (Düren, Germany). This column is shorter than the one mentioned by Materials and Methods, which decrease the time of separation whereas the smaller particles size packing allows the use of this short column for rapid separation without loss of resolution. This column decreased the

HPLC time needed to separate DOC and B from 3 min to 1.6 min per each sample (Figure 3.27) under the HPLC parameters mentioned in Table 3.7. This optimisation increased further the throughput of the HPLC and, as a result, the throughput of the developed screening system by 2-fold. An additional benefit in this rapid resolution column is the remarkable reduction of solvent consumption.

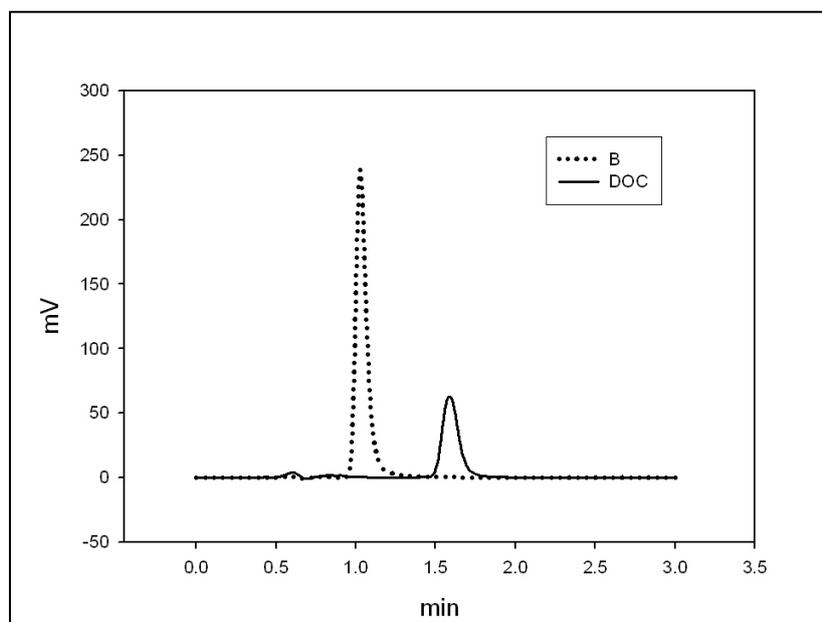


Figure 3.27. HPLC chromatograms show the separation of DOC and B using a high-speed column.

The HPLC separation was performed using pure steroids and the HPLC parameters mentioned in Table 3.7. The separation of DOC and B was done within 1.6 min, which increases the throughput of the HPLC system.

Cells from a main culture of MB164 were washed with EMM, centrifuged (3×10^3 g, 5 min, 4°C) and resuspended in 50 mM potassium phosphate buffer (pH 7.4) to cell density of 10^8 cells/ml with final volume of 600 μl of cell suspension per well. All assay steps were carried out using the pipetting robot Tecan. The assay was initiated by adding 10 μl from a 2.5mM stock solution of the investigated compound to give a final concentration of 41.66 μM . After 20 min shaking at 480 rpm and 30°C , the substrate (DOC) was added to give a final concentration of 5 μM (10 μl from a 0.3 mM stock solution in DMSO). Once again, the assay plate was shaken at 480 rpm and 30°C for three hours the steroids were extracted with chloroform using the extraction program mentioned in subsection 3.1.2 and dried under vacuum. The dried steroids were then dissolved in acetonitril and analysed by HPLC using the high-speed column mentioned above and the HPLC parameters described in Table 3.7.

Figure 3.28 below shows the HPLC separation of the CYP11B2-dependent bioconversion of DOC into B analysed using the high-speed column mentioned above. In contrast to Figure

3.27, the HPLC chromatogram in Figure 3.28 displays an unknown peak with retention time of 0.6 min. This peak was also detected when a 500 μ l water mixed with 20 μ l DMSO was extracted with chloroform and analysed with HPLC under the same condition (Figure 3.29).

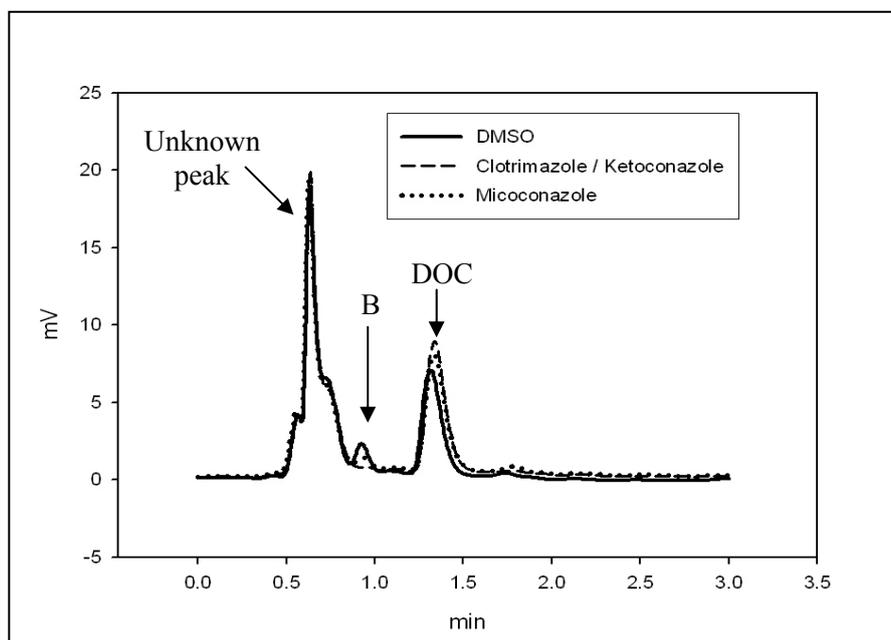


Figure 3.28. HPLC Chromatograms of the CYP11B2-dependent bioconversion of DOC into B analysed using the high-speed column. The bioconversion was carried out as described before using fission yeast strain MB164 in the presence of positive and negative controls. Unknown peak with retention time of 0.6 min was observed.

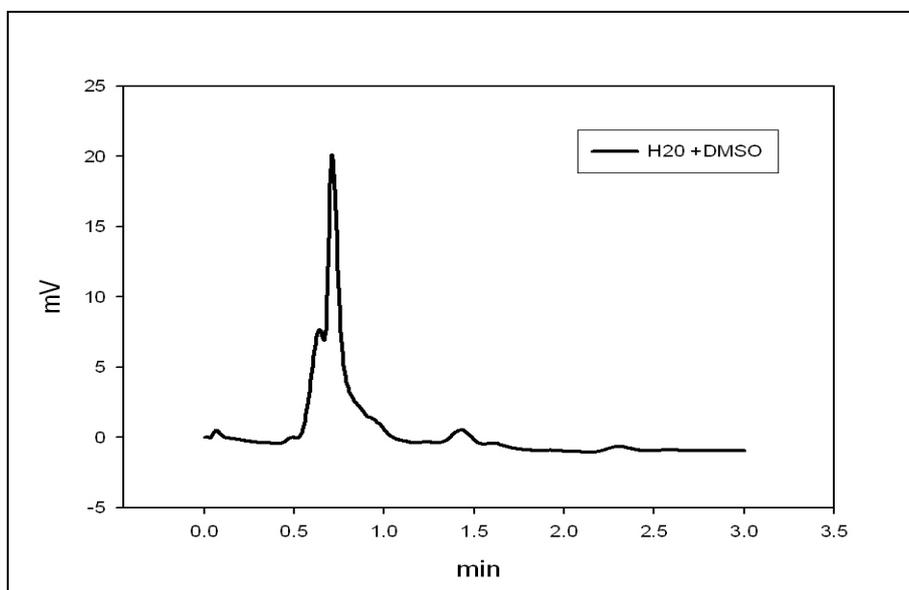


Figure 3.29. HPLC chromatogram of an extracted sample that consists of water and DMSO.

The chromatogram shows that the unknown peak reported in Figure 3.28 is a medium noise peak. This noise peak is obviously better separated using this high-speed column in comparison with the long column mentioned above by Materials and Methods.

The results of controls obtained from 32 independent assays using the one-point assay (41.6 μM) displayed reproducible results, and were statistically analysed using *t*-test ($p < 0.05$). The mock-treated samples (DMSO) displayed CYP11B2 activity with B production ratio of 11.5%, whereas the presence of miconazole decreased significantly ($p < 0.05$) the activity of CYP11B2 and B production ratio to 6.5% showing 44 % inhibition under our test condition. Furthermore, the presence of either clotrimazole or ketoconazole displayed total inhibition of CYP11B2 (100%) (Figure 3.30, Table 3.9).

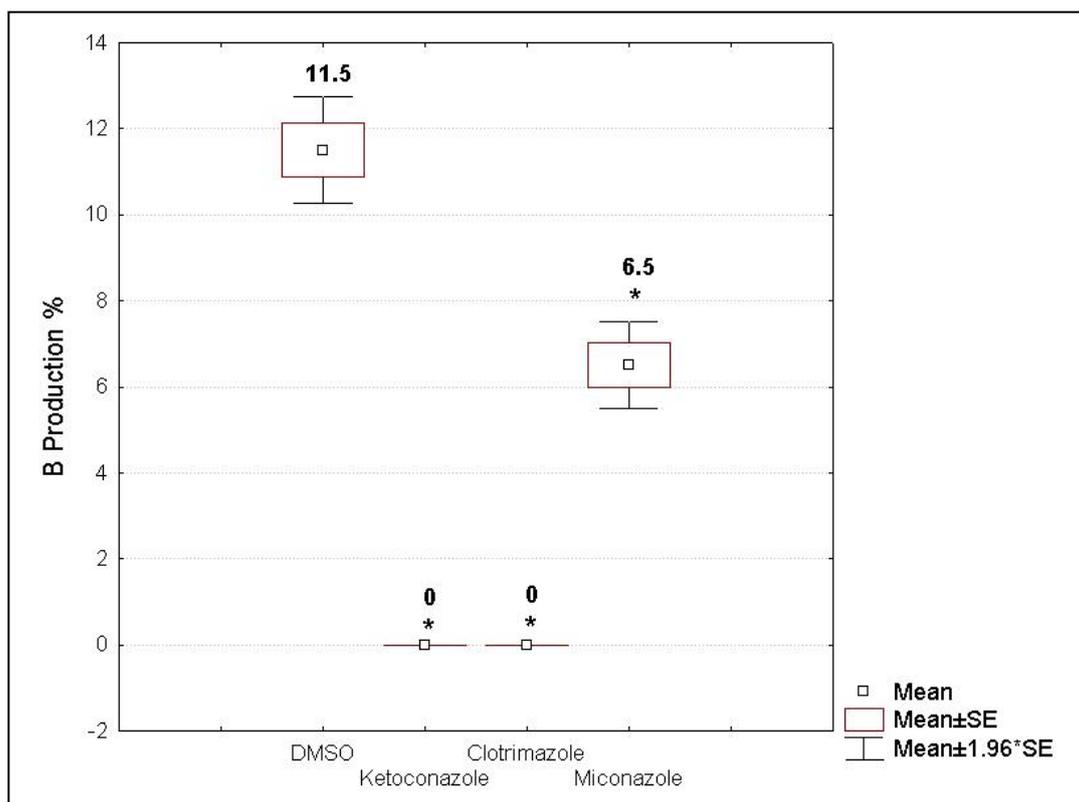


Figure 3.30 Direct comparisons of the CYP11B2-dependent conversion rates of DOC into B during the screening assay. Values were calculated from 32 independent experiments and are presented as mean \pm standard error of mean. Asterisks above boxes indicate a significant difference to the mock treated sample (DMSO) (*t*-test, $p < 0.05$).

Table 3.9. The inhibition profiles of the CYP11B2 inhibitors tested using the six-point inhibition assay and the one-point assay developed in this work

| Compound | IC ₅₀ against CYP11B2 (μM) ^a | B ratio (%) ^b | Inhibition (%) ^b |
|--------------|---|--------------------------|-----------------------------|
| Clotrimazole | 0.20 | 0 | 100 |
| Ketoconazole | 3.50 | 0 | 100 |
| Miconazole | 5.60 | 6.5 | 44 |

^a(Bureik *et al.* 2004)

^b This work

The Z' -factor of the screening system was determined from the inhibition assay for clotrimazole, ketoconazole and miconazole as positive controls and DMSO as negative control. The Z' -factor was calculated using the formula mentioned in subsection 2.2.6.2.3.

The screening assay gave a Z' -factor of 1.0 for clotrimazole, 1.0 for ketoconazole and 0.85 for miconazole, showing that the screening system is robust.

In a next step, the LOPAC library was tested on triplicate. Additionally to ketoconazole, clotrimazole already supplied in the library, the screening assay reported two clotrimazole-like inhibitors (e.g. Compound Co_TH1, Figure 3.31), whereas nine compounds were defined regarding to our definition as miconazole-like inhibitors (Table 3.10, Figure 3.32).

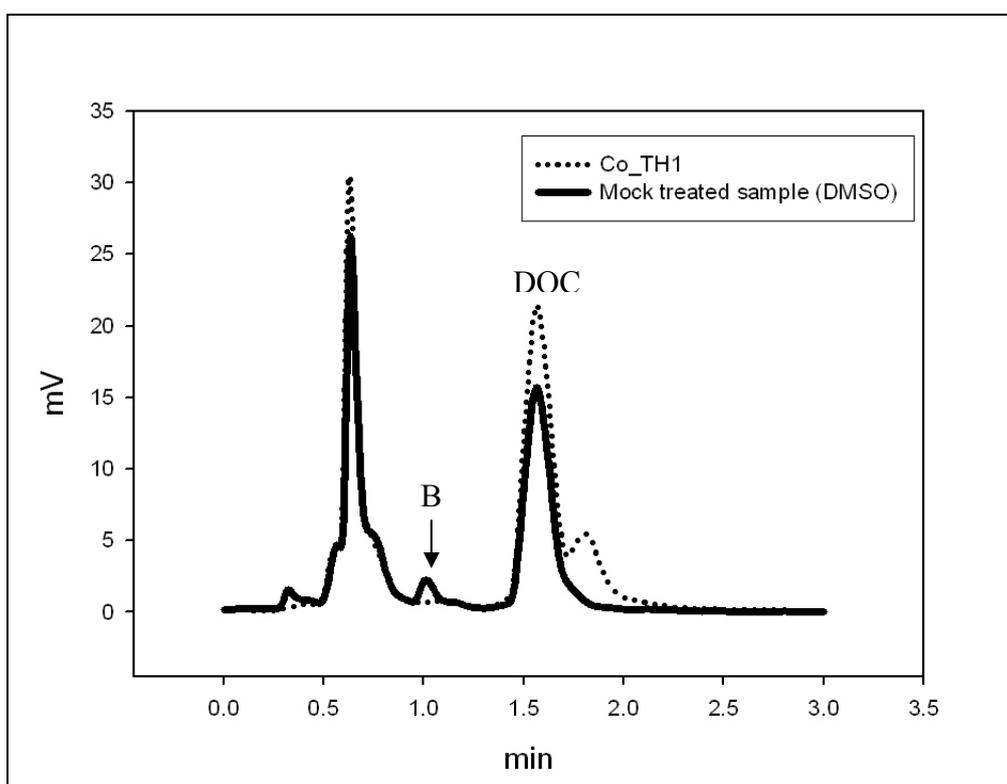


Figure 3.31. HPLC chromatograms of CYP11B2-dependent bioconversion in the presence of Co_TH1 during the screening assay.

Compound Co_TH1 displayed total inhibition of CYP11B2 under the test conditions. For this reason, Co_TH1 can be defined as clotrimazole-like inhibitor of CYP11B2.

Table 3.10. The new CYP11B2 inhibitors identified during the screening assay in this work

| Compound code in this work | Compound code by SIGMA® | Name | Screening assay result | Description |
|----------------------------|-------------------------|---|-----------------------------|---|
| Co_TH1 | A5791 | 4-Androsten-4-ol-3,17-dione | Clotrimazole-like inhibitor | Aromatase inhibitor |
| Co_TH2 | A9630 | 4-Androstene-3,17-dione | Miconazole-like inhibitor | Testosterone precursor and metabolite with androgenic activity |
| Co_TH3 | C3635 | DL-p-Chlorophenylalanine methyl ester hydrochloride | Miconazole-like inhibitor | Tryptophan hydroxylase inhibitor |
| Co_TH4 | E3380 | Ellipticine | Miconazole-like inhibitor | Cytochrome P450 (CYP1A1) and DNA topoisomerase II inhibitor |
| Co_TH5 | I0782 | Imazodan | Miconazole-like inhibitor | Selective phosphodiesterase II (PDEII) inhibitor |
| Co_TH6 | L3791 | Lamotrigine | Miconazole-like inhibitor | Anticonvulsant |
| Co_TH7 | V1889 | VER-3323 hemifumarate salt | Miconazole-like inhibitor | 5-HT _{2C} /5-HT _{2B} serotonin receptor agonist. |
| Co_TH8 | L131 | L-745,870 hydrochloride | Miconazole-like inhibitor | Selective D ₄ dopamine receptor antagonist |
| Co_TH9 | P6777 | Phenelzine sulfate salt | Miconazole-like inhibitor | Non-selective MAO-A/B inhibitor |
| Co_TH10 | P8765 | Ammonium pyrrolidinedithiocarbamate | Miconazole-like inhibitor | Prevents induction of nitric oxide synthase (NOS) by inhibiting translation of NOS mRNA |
| Co_TH11 | T7313 | 1-[2-(Trifluoromethyl)phenyl]imidazole | Clotrimazole-like inhibitor | Potent nitric oxide synthase (NOS) inhibitor |

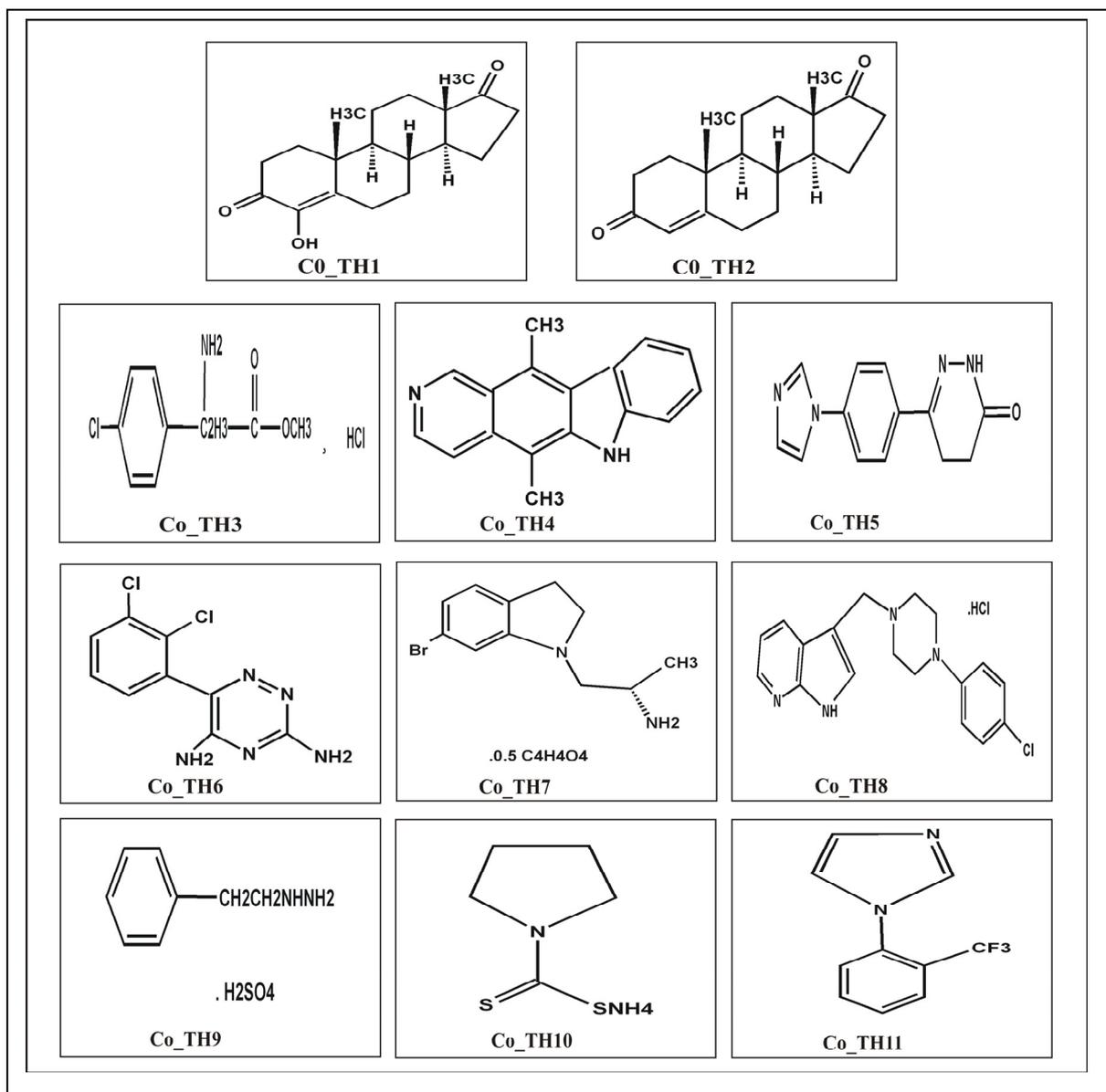


Figure 3.32. Structures of the new CYP11B2 inhibitors identified during the screening assay in this work.

Although fission yeast *S. pombe* has a cell wall, which could disable the transport of the investigated compound to the cell in the fission yeast test system, the new screening system reported in addition to ketoconazole, clotrimazole and miconazole eleven potential inhibitors of CYP11B2. These observations demonstrate clearly that these 14 compounds could pass the cell wall since they inhibited the mitochondria-localised CYP11B2 in the recombinant fission yeast although they have different molecular weight values (Figure 3.33). For this reason, it is clearly to say that the cell wall of fission yeast does not form any disadvantages in the fission yeast test system.

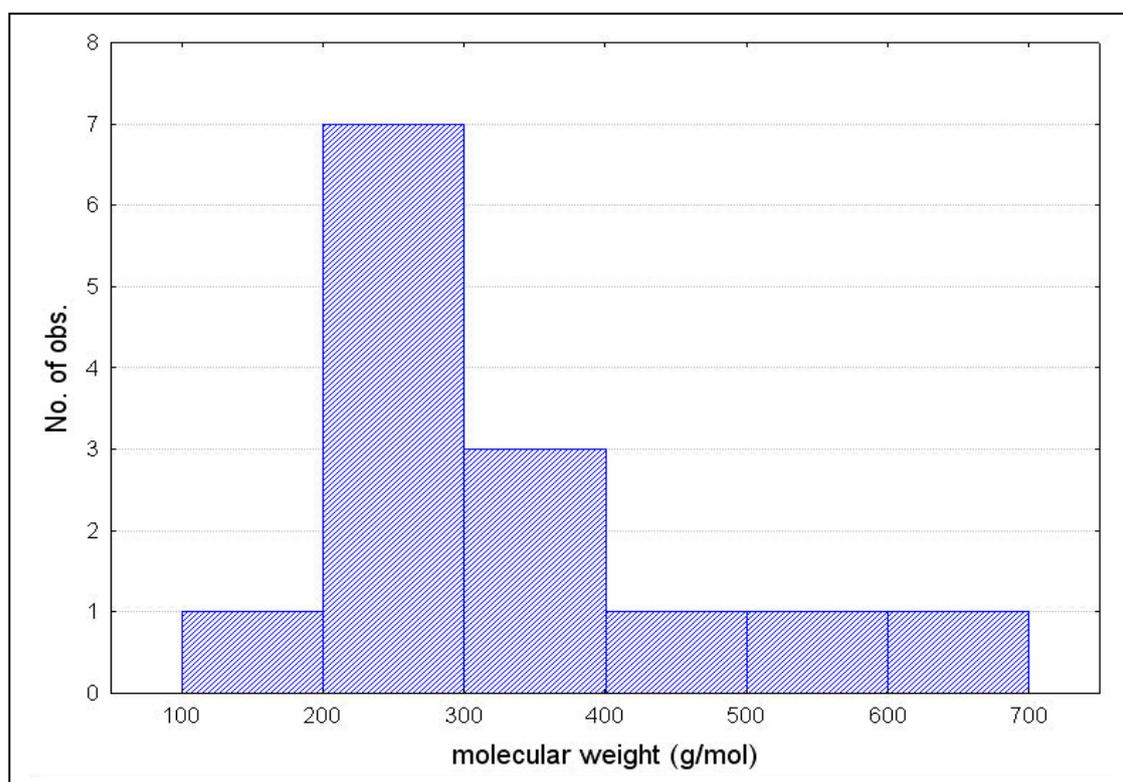


Figure 3.33. Distributions graph of the new inhibitors of CYP11B2 vs. their molecular weight.

3.3.4. Validation of the new CYP11B2 inhibitors identified during the screening assay

The potential inhibitors of CYP11B2 identified during the screening system (Table 3.10, Figure 3.32) were defined as active compounds (“hits”) and selected for further validation on the basis of commercial availability and clinical interest.

3.3.4.1. Toxicity in fission yeast

Since, the screening assay is an inhibition assay; further investigations had to be done to investigate if the detected inhibition is due to the inhibitory effect and not to the toxicity of the compound.

After incubation of fission yeast cultures with the different “hits” under the same conditions like in the screening assay, no morphological changes were observed (colour and shape). Moreover, a cell viability assay was carried out, and no significant changes ($p < 0.05$) were observed in comparison with mock-treated samples (Figure 3.34).

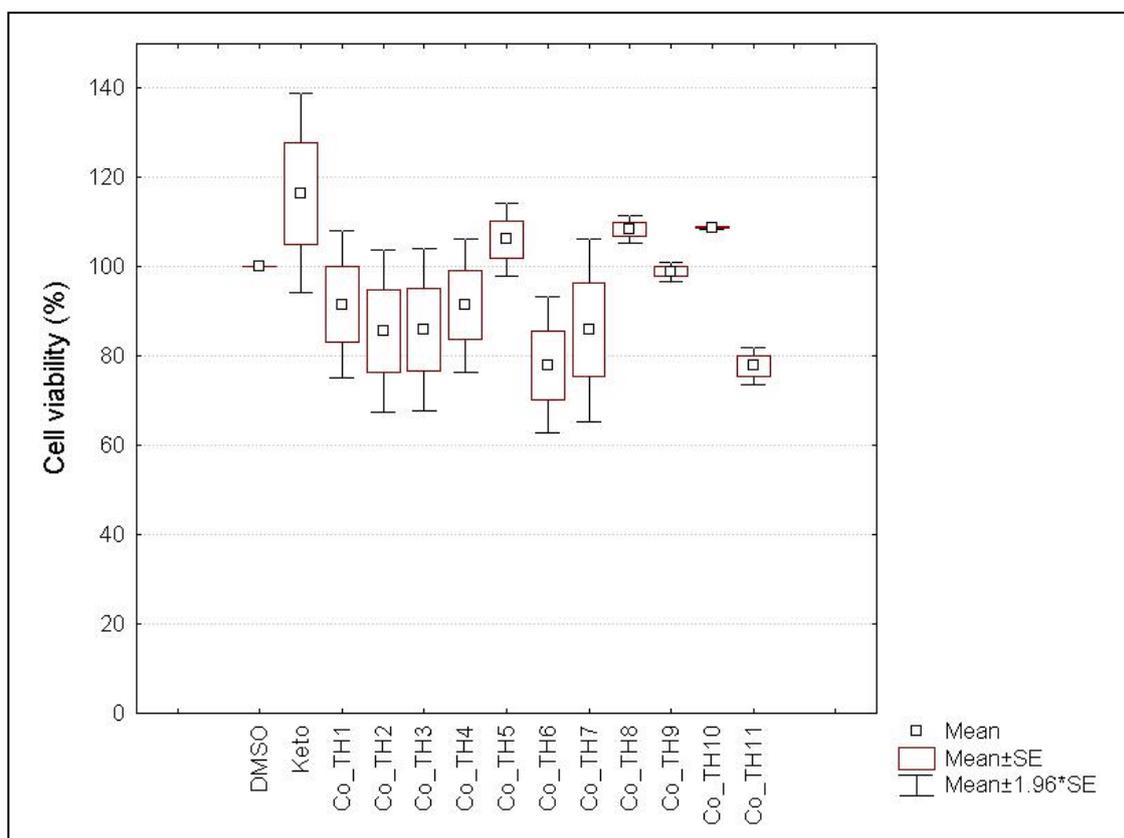


Figure 3.34. Cell viability shown as a percentage of negative control (DMSO).

The fission yeast cultures were incubated with 41.6 μM from each hit under the same condition like in the screening assay. Diluted samples were taken and plated on the desired plates. Colonies were calculated after 48 hours incubation at 30°C. Each *bar* represents the average of triplicate data points.

Although some compounds displayed less cell viabilities in comparison with the negative control, no significant differences were reported (*t*-test, $p < 0.05$). This result displays clearly that the “hits” are not toxic against the fission yeast cells during the screening assay. Hence, the detected inhibition is due to the inhibitory effect of these compounds under the test conditions. Further validation had to be done to investigate the selectivity of these compounds and to calculate the IC_{50} values against CYP11B2 and CYP11B1.

3.3.4.2. Determination of the IC_{50} values against CYP11B2 and CYP11B1

For the determination of the IC_{50} values, the six-point method described previously (Bureik *et al.* 2004) was applied. This radioactive assay possesses high sensitivity to test the effect of the compound of interest in low concentration ranges (100 nM – 25 μM) to determine the IC_{50} value in the presence of 100 nM substrate. This low concentration of the substrate does not allow a detectable bioconversion using HPLC, giving the radioactive method the advantage to perform the assay with low concentrations to determine the IC_{50} value. For this reason, fission yeast strains SZ1 and MB164 expressing human CYP11B1 and CYP11B2, respectively, were

used to perform the assay in the tip-tube format as described in subsection (2.2.4.2). The potassium phosphate buffer 50 mM (pH 7.4) was used as test medium, and the “hits” were tested with final concentrations ranging from 100 nM to 25 μ M. Radioactive substrate was applied to detect the steroid conversion and inhibition with a final concentration of 100 nM. Clotrimazole with 25 μ M was applied as positive control during the assay.

Repeated steroid hydroxylation measurements with both CYP11B2- or CYP11B1-expressing fission yeast systems, were carried out, and only highly correlative data sets ($R^2 > 0.75$) were used for the determination of the IC_{50} values. Comparing the inhibitor’s effect on the two enzymes using identical conditions is an appropriate strategy to evaluate their selectivity (Bureik *et al.* 2004). In this way, it was possible to identify highly selective inhibitors of CYP11B2 (Table 3.11).

Table 3.11. The inhibition profiles of the active compounds against CYP11B2 and CYP11B1 in the validation assay

| Compound code * | Name | Validation assay | |
|-----------------|---|--|---|
| | | CYP11B2 | CYP11B1 |
| Co_TH1 | 4-Androsten-4-ol-3,17-dione | $IC_{50} = 2.4 \mu\text{M}$ $R^2 = 0.92$ | - |
| Co_TH2 | 4-Androstene-3,17-dione | $IC_{50} = 3.11 \mu\text{M}$ $R^2 = 0.95$ | - |
| Co_TH3 | DL-p-Chlorophenylalanine methyl ester hydrochloride | $IC_{50} = 40 \mu\text{M}$ $R^2 = 0.90$ | - |
| Co_TH4 | Ellipticine | $IC_{50} = 8.9 \mu\text{M}$ $R^2 = 0.93$ | - |
| Co_TH5 | Imazodan | # | # |
| Co_TH6 | Lamotrigine | - | - |
| Co_TH7 | VER-3323 hemifumarate salt | - | - |
| Co_TH8 | L-745,870 hydrochloride | - | - |
| Co_TH9 | Phenelzine sulfate salt | $IC_{50} = 48 \mu\text{M}$ $R^2 = 0.75$ | - |
| Co_TH10 | Ammonium pyrrolidinedithiocarbamate | - | - |
| Co_TH11 | 1-[2-(Trifluoromethyl)phenyl]imidazole | $IC_{50} = 1.37 \mu\text{M}$ $R^2 = 0.97$ | $IC_{50} = 0.7 \mu\text{M}$ $R^2 = 0.85$ |

* In this work

(-) No significant inhibitory action was detectable under the conditions described

(#) Nonspecific inhibitory action was detected, and no IC_{50} value was calculated

The validation assay reported five selective inhibitors of CYP11B2 and two inhibitors of both, CYP11B2 and CYP11B1. Furthermore, four compounds showed no inhibitory effect against CYP11Bs under the validation assay conditions (Table 3.11). Co_TH1 and Co_TH11 reported as clotrimazole-like inhibitors in the screening assay, showed in the validation assay different inhibition profiles. Compound Co_TH1 showed selective inhibitory effect against CYP11B2, whereas Co_TH11 showed inhibitory effects against both isoforms of enzyme.

Interestingly, out of the nine compounds defined during the screening assay as miconazole-like inhibitors, four compounds showed selective inhibition against CYP11B2. For this reason, it is important to include all miconazole-like inhibitors defined during the screening assay into the validation assay when the screening is carried out to discover selective inhibitors of CYP11B2.

The new CYP11B2 inhibitors reported in this work are pharmacologically active compounds. Co_TH1 is already known as formestane (sold as Lentaron®) and described as an injectable steroidal aromatase inhibitor with significant activity against metastatic breast cancer (Wiseman and Goa 1996). Figure 3.35 below shows the autoradiographic detection of steroid hydroxylation activity in the case of increased concentrations of Co_TH1 in comparison with positive (clotrimazole) and negative (DMSO) controls.

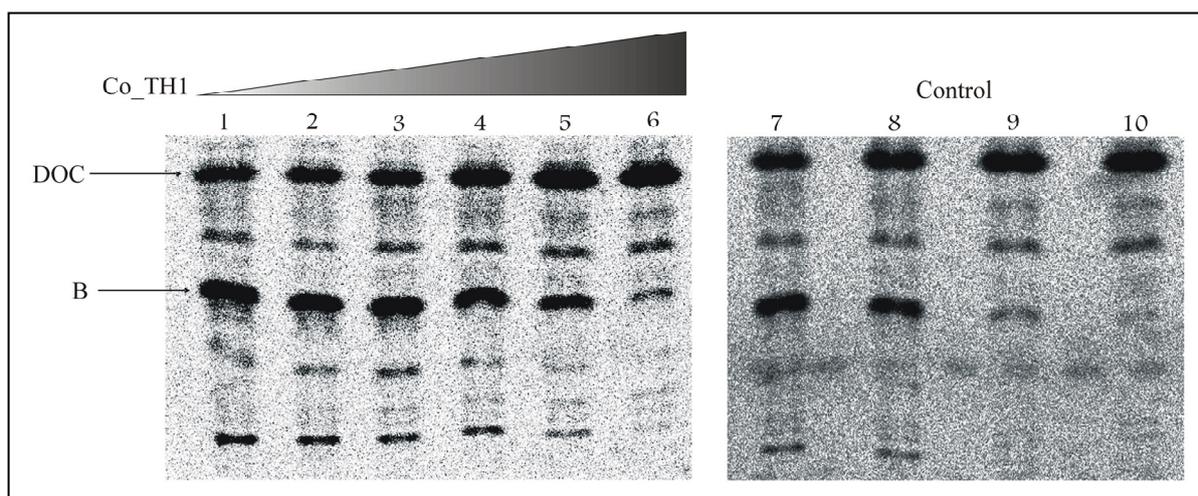


Figure 3.35. Autoradiographic detection of steroid hydroxylation activity. Steroid hydroxylation assay using strain MB164 and different concentrations of Co_TH1 was carried out as described in Section 2.2.4. Co_TH1 concentrations were as follows: (line 1) 100 nM; (line 2) 200 nM; (line 3) 500 nM; (line 4) 2 μ M; (line 5) 5 μ M; (line 6) 25 μ M; (lines 7, 8) mock-treated cells (DMSO); (line 9, 10) 25 μ M clotrimazole (positive control).

The plot of the CYP11B2 inhibition against the concentration of Co_TH1 shows a high correlation as shown in Figure 3.36 below, and was used to calculate the IC_{50} value for Co_TH1 against CYP11B2. This result displays that formestane (Co_TH1) inhibits selectivity CYP11B2 with an IC_{50} of 2.4 μM , whereas no significant inhibition was detected against CYP11B1.

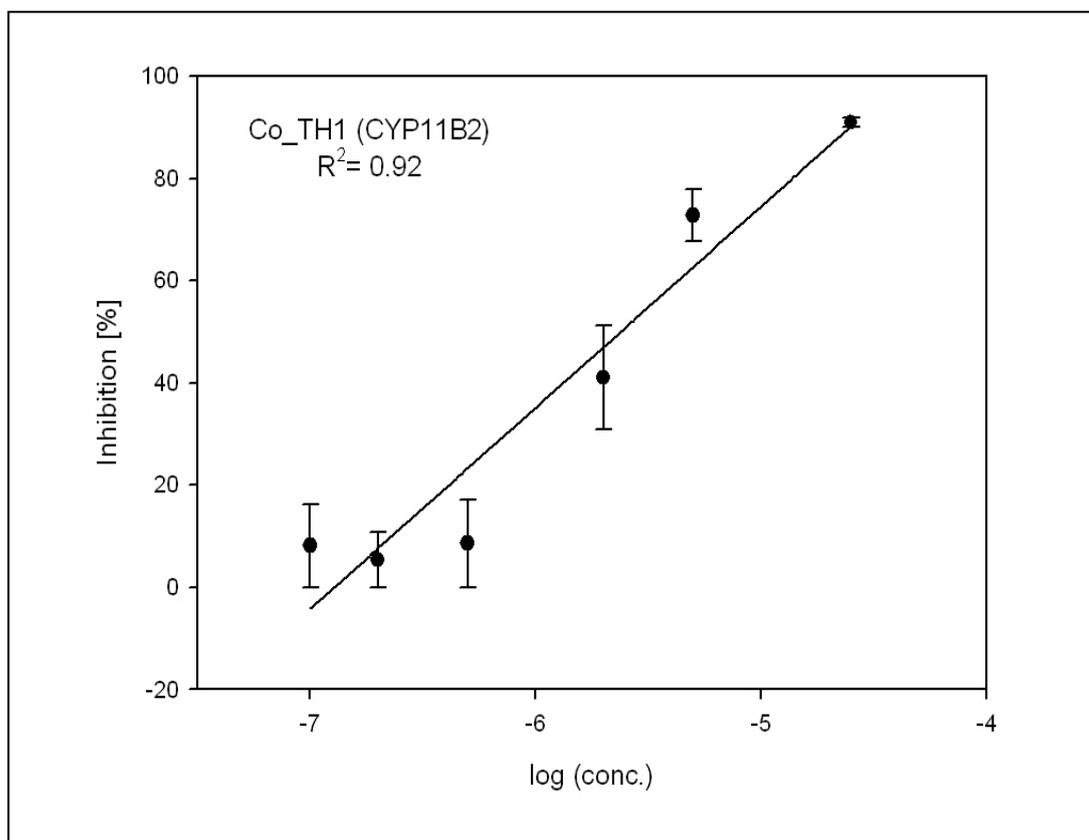


Figure 3.36. The inhibitory effect of Co_TH1 on the activity of CYP11B2.

The closely related compound Co_TH2, which is androstendion is a testosterone precursor and metabolite with androgenic activity. Interestingly, Co_TH1 and Co_TH2 were identified during the screening assay as clotrimazole-like and miconazole-like inhibitors of CYP11B2, respectively. These results were confirmed through the validation assay, where the miconazole-like inhibitor displayed relatively higher IC_{50} value (Co_TH2; $IC_{50} = 3.11 \mu\text{M}$) (Figure 3.37) in comparison with the clotrimazole-like inhibitor Co_TH1 ($IC_{50} = 2.4 \mu\text{M}$) (Figure 3.36). Although the difference between the two IC_{50} values is not very high, a difference has been reported that confirms the screening results.

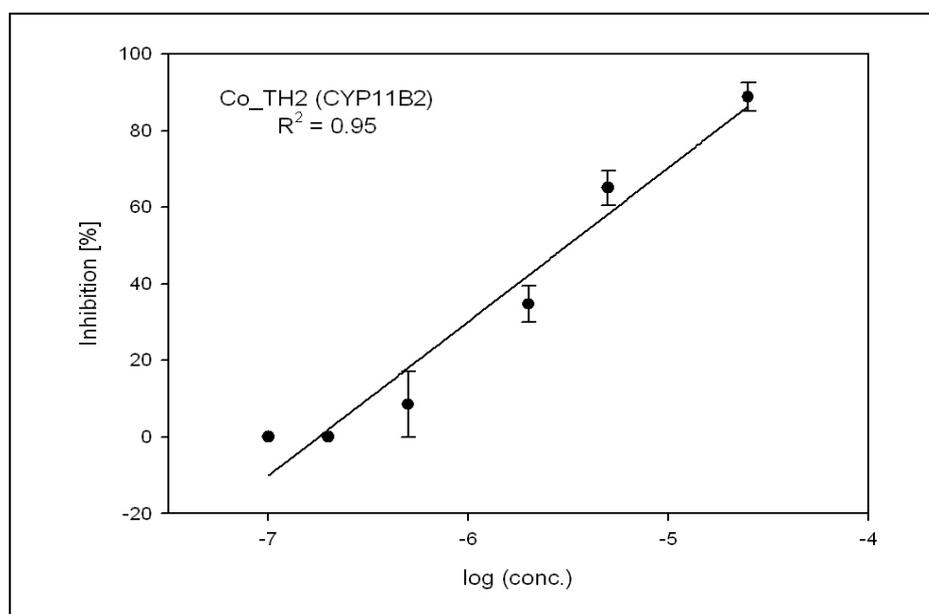


Figure 3.37. The inhibitory effect of Co_TH2 on the activity of CYP11B2.

Regarding the steroidal scaffold of 4-androsterone-4-ol-3, 17-dione the structure activity analysis revealed that the ketone in position 17 (D-ring) is beneficial for the activity, and an OH- residue at position 4 (A-ring) is necessary for potent inhibition of CYP11B2 (Figure 3.32). Although the nitric oxide synthase inhibitor Co_TH11 (1-[2-(Trifluoromethyl)phenyl]imidazole) was defined in the screening assay as clotrimazole-like inhibitor of CYP11B2, this compound displayed in the validation assay and in contrast to Co_TH1 a strong and unselective inhibition effect against CYP11B2 and CYP11B1 with IC_{50} values of 1.37 μ M and 0.7 μ M, respectively (Table 3.11, Figure 3.38).

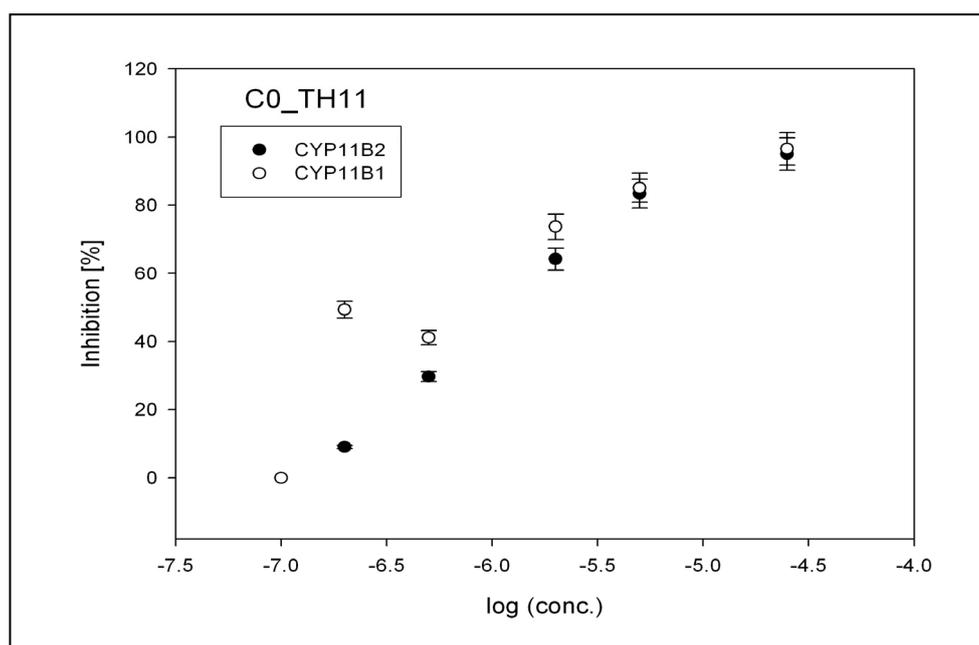


Figure 3.38. The inhibitory effect of Co_TH11 on the activity of CYP11B1 (opened symbols) and CYP11B2 (closed symbols).

In contrast to this, Co_TH3, Co_TH4 and Co_TH9 which were defined as miconazole-like inhibitors in the screening assay showed in the validation assay selective inhibition against CYP11B2 with IC₅₀ values of 40 µM, 8.9 µM and 48 µM respectively (Table 3.11).

The new CYP11B2 inhibitors defined in this work are pharmacologically active compounds which make them “druggable” lead compounds that could further optimised during the drug development process to achieve more selective and safe inhibitors of CYP11B2. Furthermore, some of these compounds are already commercial drugs and are applied clinically with unexplained side effects and severe complications. These unexplained complications could be explained to some extent because of their inhibitory effect against CYP11B2 as will be discussed in details below.

4. Discussion and Outlook

Since, the overall target of this work was the development of efficient P450-dependent whole-cell biotransformation reactions for steroid hydroxylation and drug discovery, the CYP11Bs-expressing fission yeast systems mentioned above were investigated and optimised in order to achieve this target.

The optimisation of the fission yeast systems was carried out on two different levels; the optimisation of the whole-cell system itself to increase the activity of the steroid hydroxylase and the optimisation of the hydroxylation assay parameters to achieve efficient reactions with biotechnological and pharmaceutical impacts.

4.1. Optimisation of the steroid hydroxylation assay for the 96-well plate format

Although fission yeast *Schizosaccharomyces pombe* was previously reported as an efficient host to express human CYP11Bs and to perform a whole-cell-based hydroxylation reaction (Bureik *et al.* 2002b; Bureik *et al.* 2004; Dragan *et al.* 2005), the ability to perform a CYP11B-dependent steroid hydroxylation assay in a 96-well plate has not yet been reported.

Performing the steroid hydroxylation assay in 96-well plate will give the opportunity to perform the assay on a relatively large scale in comparison with the tip-tube format or with Erlenmeyer flasks, which enables the comparison of the 11 β activity of several recombinant fission yeast strains at the same time. Furthermore, the plate-format method can be used to develop a screening system to check the steroid hydroxylation activity in a recombinant fission yeast strain under different conditions (inhibitor, medium, pH, etc.).

In order to perform a steroid hydroxylation assay in a 96-well plate a steroid bioconversion had to be achieved using a low-volume culture of fission yeast and a non-radioactive substrate, in which the steroid bioconversion can be measured with the HPLC. For this reason, the hydroxylation parameters were investigated and optimised to perform the reaction in low-volume culture in the tip-tube format and then in the 96-well plate format.

This work showed clearly the ability to get steroid bioconversion using a low-volume culture of the CYP11B1-expressing fission yeast and without the need to use radioactive-labelled substrate (Figure 3.1). Although the test was optimised for low-volume culture and carried out in the tip-tube format, no steroid bioconversion was detected when the test was performed in a 96-well plate. Taking into consideration the similarities and differences between the two test

formats (tip-tube and 96-well plate), it turns out that each format has its own test shape, which could influence the shaking process and aeration during the assay. Moreover, the tip-tube format assay was carried out using a thermomixer whereas the plate was shaken using an incubator. The investigations showed clearly the influence of the shaking velocity and assay volume on steroid bioconversion since increasing the shaking velocity to 480 rpm and assay volume to 600 μ l displayed detectable steroid bioconversion on the HPLC when the test was carried out in a 96-well plate (Figure 3.9). Furthermore, using the pipetting robot to manipulate the 96-well plates showed high efficiency to perform the hydroxylation assay on a relatively large scale in comparison with other test formats described above. The extraction program developed in this work (Table 3.2) enables the extraction of steroids from 96 samples in ca. 20 min, which is significantly shorter than the manual extraction process. In addition to this, and since no significant differences were observed between several wells, this hydroxylation assay provides an efficient screening tool to investigate the P450-dependent steroid hydroxylases in recombinant fission yeast strains (in the case of CYP11B1 (Dragan *et al.* 2005), CYP11B2 (Bureik *et al.* 2002b), CYP17 or CYP21 (Dragan *et al.* 2006)) and can be further optimised to develop a high or medium throughput screening system.

4.2. Coexpression of redox partners in CYP11B1-expressing fission yeast *Schizosaccharomyces pombe*

The biotechnological production of hydrocortisone is a complex process, which requires many different optimisation steps in order to significantly increase the product formation.

Although fission yeast *Schizosaccharomyces pombe* has been reported to be a very suitable model system for the investigation of P450 dependent steroid hydroxylases, hydrocortisone production efficiency using CYP11B1-expressing fission yeast strain SZ1 is not competitive enough for the consideration of its use for industrial applications.

As mentioned above and although the human CYP11B1 is able to accept electrons from the yeast Adx homologue, it has been demonstrated that the electron transfer to the cytochrome P450 can be rate limiting in various P450 systems (Grinberg *et al.*, 2000; Bernhardt 2006; Hannemann *et al.* 2007). For this reason, the target of this part of work was the improvement of the electron transfer pathway that supplies electrons to CYP11B1 in the recombinant fission yeast in order to increase the CYP11B1-mediated 11 β -hydroxylation activity to produce more hydrocortisone. Hence, the corresponding mammalian electron transfer partners (Adx and AdR) were coexpressed with CYP11B1, and different mutants of Adx were

investigated to achieve a recombinant fission yeast strain with the highest hydrocortisone bioproduction efficiency.

This work shows clearly that hydrocortisone production can be dramatically enhanced (3.4-fold) by coexpressing the other components of the CYP11B1 electron transfer chain and by optimising the reaction conditions to achieve high production efficiency on the laboratory level.

The CYP11B1-expressing fission yeast strains developed during this work were classified into four types according to the presence of the electron transfer proteins (Figure 4.1).

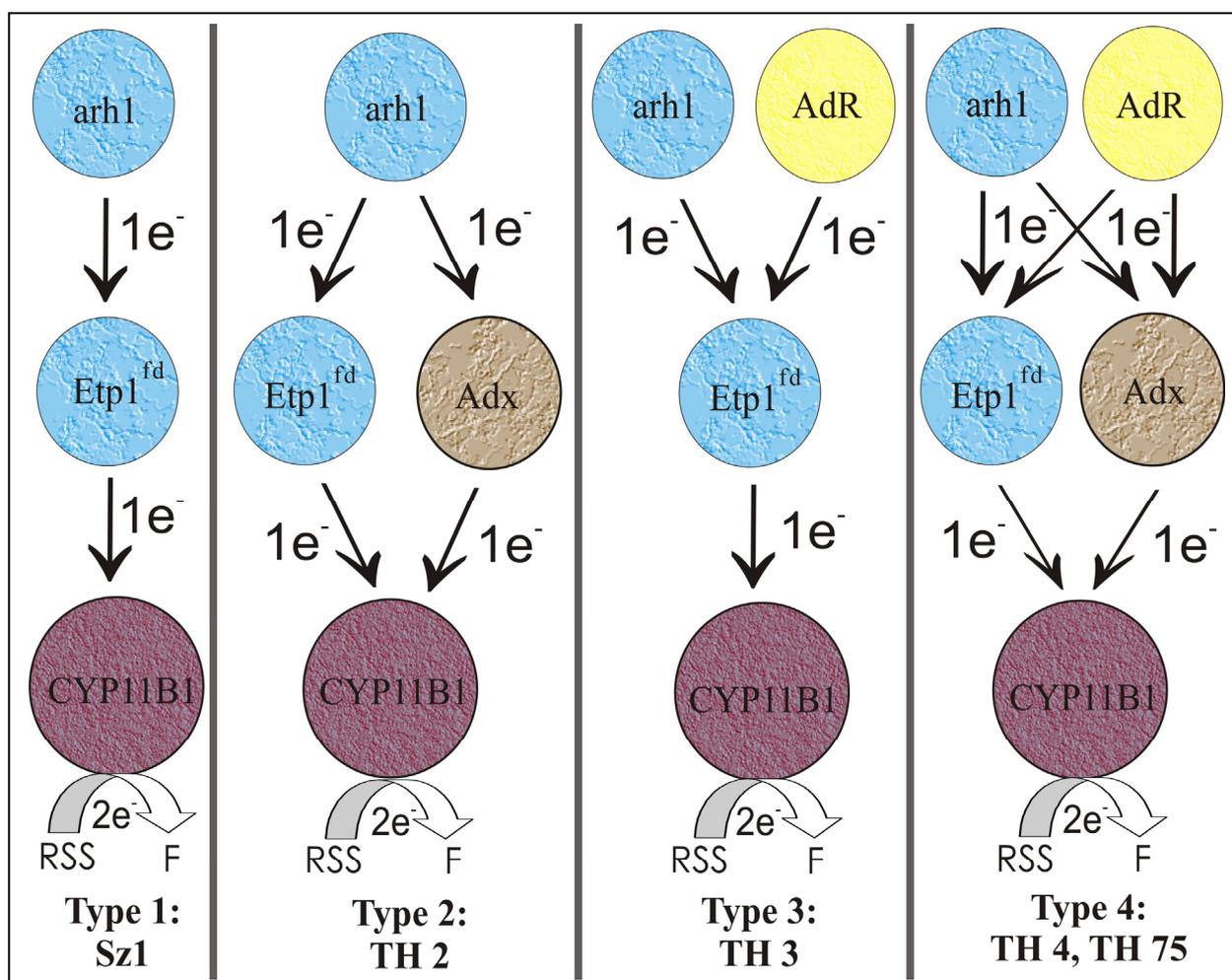


Figure 4.1. Schematic overview of the recombinant fission yeast types according to the availability of the electron transfer proteins in the fission yeast strains used in this study.

The fission yeast strain SZ1, which expresses the cytochrome CYP11B1 belongs to the first type, since this strain expresses only the P450. Nevertheless, this strain is capable of efficiently performing 11 β -hydroxylation reaction and produces 12% hydrocortisone under the test conditions mentioned before (Figure 3.18), which confirms earlier reports that the

P450 is supplied with reducing equivalents presumably from the endogenous electron transfer proteins *etp1^{fd}* (Bureik *et al.* 2002b; Dragan *et al.* 2005) and *arh1* (Ewen *et al.* 2008) (Figure 4.1, type I). Strain TH2 belongs to the second type in which the cytochrome CYP11B1 is heterologously coexpressed together with *Adx^{WT}*. This strain displayed an increased 11 β -hydroxylation activity compared to SZ1 (Type I electron transfer chain) with a hydrocortisone production of 25% (Figure 3.18) indicating a participation of *Adx* in the electron transfer to CYP11B1 also in fission yeast (Type II electron transfer chain). The third type implemented in the strain TH3 heterologously coexpresses *AdR^{WT}* and CYP11B1. The strain showed no increase in 11 β -hydroxylation activity compared to the SZ1 strain, which clearly demonstrated that *AdR^{WT}* alone can not improve the electron transfer efficiency in recombinant fission yeast. These findings could indicate a lack in cooperation between the heterologous *AdR* and the host ferredoxin *etp1^{fd}* in the presence of its putative natural host partner *arh1* or a maximum of electron transfer efficiency between the autologous redox partners (see Figure 4.1, compare type I and type III). Furthermore, it shows that the efficiency of substrate conversion depends mainly on the ferredoxin employed in the reaction (Ewen *et al.* 2008).

Strains TH4 and TH75 which coexpressed CYP11B1, *Adx^{WT}*, and *AdR^{WT}* (Type IV electron transfer chain) displayed a 3.4-fold higher activity (40% of initial RSS converted, Figure 3.18) with respect to the parental strain SZ1. In this way, TH75 is a highly efficient recombinant organism that can be used for the biotechnological conversion of RSS to hydrocortisone.

The investigations of *Adx* mutations that were assumed to improve the 11 β -hydroxylation activity in the context of a complete electron transfer chain could not further improve the hydrocortisone production compared to TH4 or TH75. This could be due to the distinct interaction forms between the different types of *Adx* and *AdR* in the yeast or to other limitations in the reaction process apart from the electron transfer.

Although human CYP11B1 in SZ1 was already optimised on the enzyme level and a site-directed mutagenesis was performed at positions 52 and 78 of CYP11B1, which showed that the presence of an isoleucine at position 78 increased the 11 β -hydroxylation activity (3.5-fold) (Hakki *et al.* 2008), the 11 β -hydroxylation activity could be further optimised (3.4-fold) by the coexpression of the corresponding electron transfer partners. These results demonstrated clearly the opportunity to optimise the steroid hydroxylases by optimising the whole-cell system itself. Moreover, and additionally to the coexpression of *Adx* and *AdR* optimising the bioconversion conditions enabled a high bioconversion efficiency on the laboratory level in comparison with the parental strain (Figure 3.20).

Thus it can be clearly demonstrated that the new fission yeast strain TH75 (Figure 4.2) coexpressing the complete electron transfer chain of the mitochondrial cytochrome CYP11B1 displays a significantly higher 11 β -hydroxylation activity than the parental strain SZ1. This new fission yeast strain TH75 displayed a high hydrocortisone production efficiency at an average of 9.7 μ mol hydrocortisone per 10ml test culture over a period of 72 hours (Figure 3.20), the highest value published to date for this biotransformation. Moreover, it can be expected that optimising the fermentation conditions in order to perform a sophisticated high-cell-density process can further enhance the efficiency of hydrocortisone bioproduction using TH75.

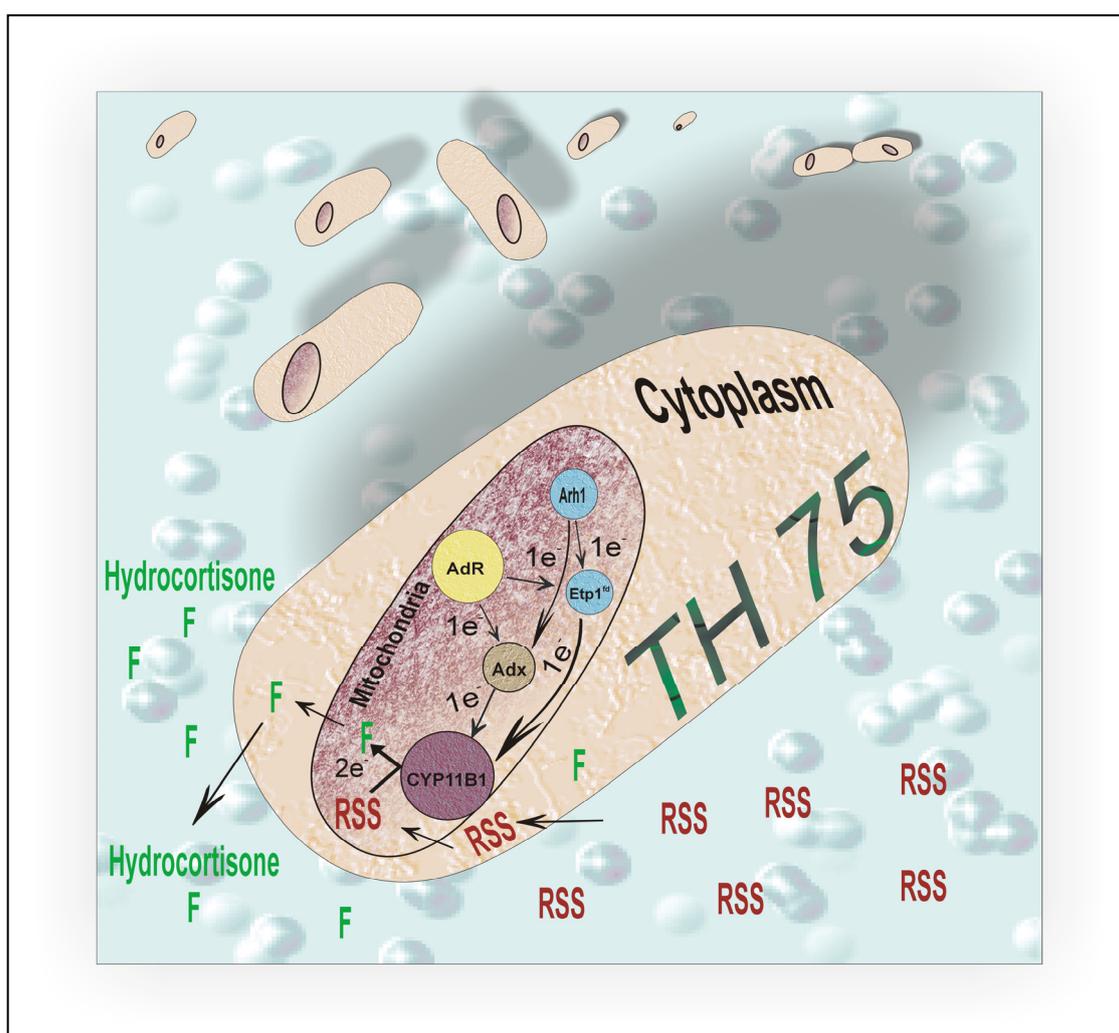


Figure 4.2. Schematic overview of the recombinant fission yeast strain TH75. The electron transfer proteins involved in the CYP11B1-dependent steroid hydroxylation in the fission yeast strain TH75.

Additionally, the newly developed vector pTH2 (Figure 3.14 A) has proven to be an important tool in combination with the integration vector pCAD1. Whereas the use of the integration vector pCAD1 enables the expression of a mitochondrial cytochrome of interest via chromosomal expression, the use of pTH2 enables the autosomal coexpression of the

complete mitochondrial electron transfer partners. This demonstrates an interesting way of functionally assembling the complete P450 system including the mitochondrial electron transfer chain by using only two expression vectors (pCAD1 and pTH2).

4.3. The development of a cell-based high throughput screening system for the discovery of human aldosterone synthase inhibitors

As mentioned before, the principal function of aldosterone is the maintenance of salt and water homeostasis and it therefore has a pivotal role in the regulation of blood pressure. It is unsurprising therefore that excessive aldosterone secretion has been reported in several cases of hypertension and has been correlated with higher mortality in congestive heart failure and fibrosis of the heart (Pitt *et al.* 1999; Brilla 2000; Pitt *et al.* 2001; Hakki and Bernhardt 2006). In addition to this, chronic elevation of aldosterone has also been implicated in adenoma, idiopathic hyperaldosteronism and insufficient renal flow (Stowasser and Gordon 2001). Although the use of aldosterone antagonists shows clinical benefit in the treatment of these diseases, it also leads to severe side effects like gynaecomastia and endocrinal dysregulation. Therefore, trials to inhibit the synthesis of aldosterone directly have been published (Denner *et al.* 1995b; Ehmer *et al.* 2002; Bureik *et al.* 2004; Ulmschneider *et al.* 2005a; Ulmschneider *et al.* 2005b; Hakki and Bernhardt 2006; Baston and Leroux 2007). Thus, CYP11B2 comprises a new target for drug treatment and selective inhibitors of the aldosterone producing CYP11B2 enzyme is of high pharmacological interest (Hakki and Bernhardt 2006; Baston and Leroux 2007; Schuster and Bernhardt 2007).

Although two systems (recombinant V79 cells, recombinant fission yeast) have been already established for evaluating compounds with respect to their inhibitory effect on human CYP11B1 and CYP11B2 (Bureik *et al.* 2004), neither of these two systems mentioned before could be considered as high or even medium throughput screening system. For this reason, the target of this part of work was the development of a high throughput screening system (HTS) for the discovery of aldosterone synthase inhibitors.

The development of the screening system was carried out on two levels; the development of a one point hydroxylation assay in fission yeast and the optimisation of the HPLC measurement in order to increase the throughput of the screening system.

Using the CYP11B2-expressing *S. pombe* system, a rapid, reliable and reproducible whole-cell-based HTS has been developed during this work. Furthermore, a new testing strategy has

been established to be applied in the field of drug discovery to discover CYP11B2 inhibitors for both academic and pharmaceutical purposes.

This new testing strategy consisted of a high throughput screening system followed by secondary validation assays for the further characterisation of the potential inhibitors of CYP11B2 (Figure 4.3).

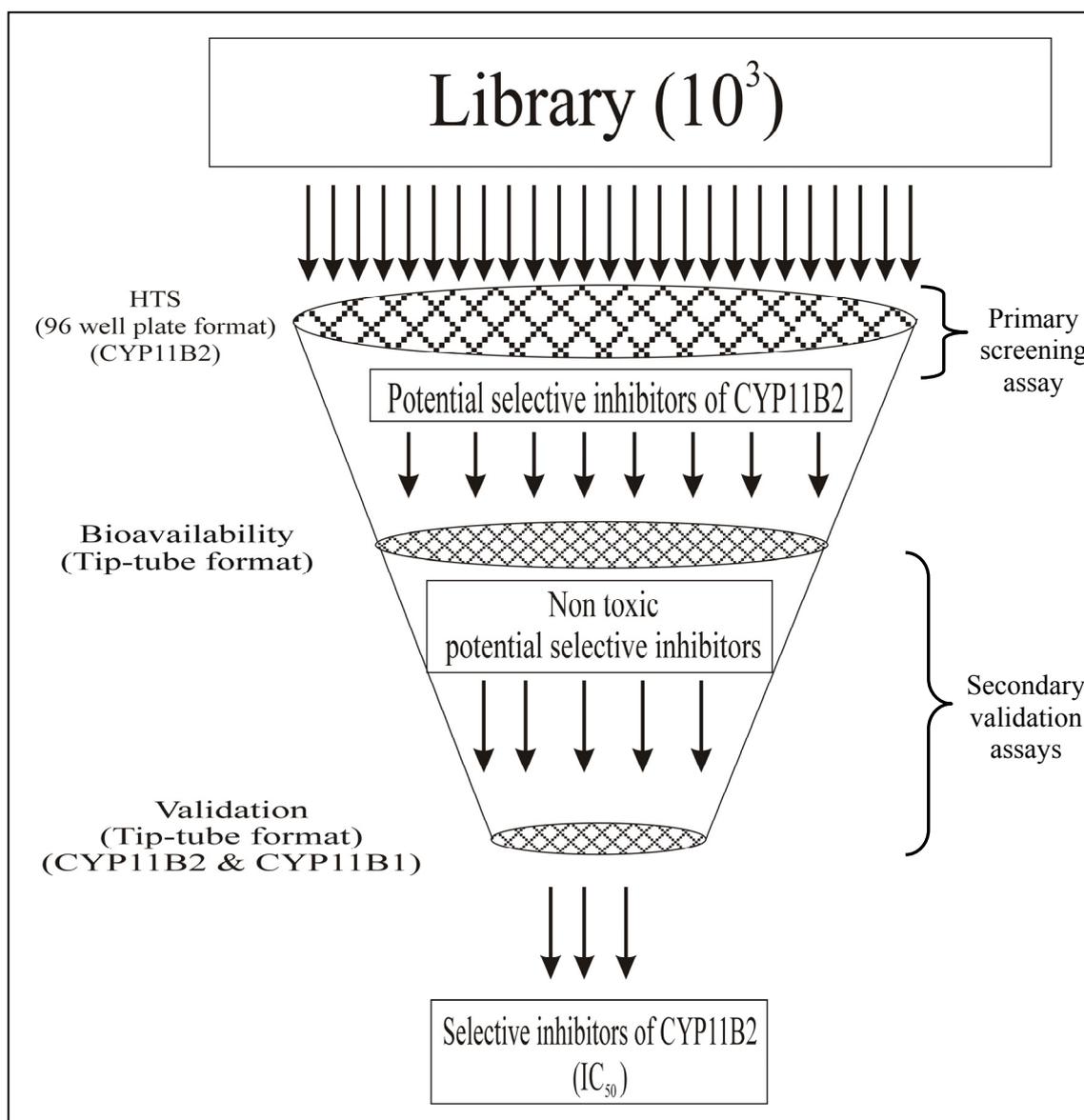


Figure 4.3. Schematic overview of the CYP11B2 testing strategy developed in this work. The new testing strategy consists of a primary screening assay, in which potential inhibitors will be investigated using the one point method to define the active compounds “hits. The hit will then go under secondary validation assays to investigate the toxicity of the compound and to define the selectivity against CYP11B2 and CYP11B1 using the multiple point method.

To develop a one-point whole-cell assay, the CYP11B2-expressing fission yeast strain MB164 was investigated in the presence of the known inhibitors of CYP11B2 (Table 3.8).

The target of these investigations was the determination of the optimal conditions for a one-point method, which gives reproducible results of conversion and inhibition in the presence of negative and positive controls.

The results demonstrated clearly that 5 μM DOC is an optimal concentration to get detectable conversion of DOC into B after three hours incubation in a 96-well plate (Figure 3.23). Moreover, test media were investigated to define the optimal medium in which the model inhibitor of CYP11B2 (ketoconazole) gives reproducible and highly correlated inhibition with its concentration. This investigation showed that simple potassium phosphate buffer (50 mM) is an optimal test medium to perform the screening assay (Figure 3.24). Although the different pH values showed high correlation between the concentration of ketoconazole and inhibition, the physiological pH 7.4 was chosen in order to test the compounds in a mammalian cell culture-resembling pH-environment.

Although the industry standard for initial CYP screening is 10 μM (Lin *et al.* 2007), the one-point assay developed in this work was designed to be carried out at 41.6 μM . This high concentration was chosen since this new developed screening assay is a whole cell-based assay and no information about the transport of compounds through the fission yeast cell wall is available. Moreover, it was assumed that the investigated compounds would be considered as uninteresting when no inhibition is detected even with this high concentration. Interestingly, this one-point method displayed reproducible results when the model inhibitors of CYP11B2 were tested. The mock- treated (DMSO) samples showed bioconversion ratio of 11.5%, whereas the presence of the CYP11B2 inhibitors showed significant inhibition profiles. The potent CYP11B2 inhibitors ketoconazole and clotrimazole showed total inhibition under the test conditions. Furthermore, the less potent CYP11B2 inhibitor miconazole showed 44% inhibition (Figure 3.30, Table 3.9). The results of controls are well correlated with the literature results reported before since significant differences between the potent and less potent inhibitors of CYP11B2 were noticed when tested using the one-point method. This one-point assay enables the classification of the CYP11B2 inhibitors (“hits”) defined during the primary screening into potent and less potent inhibitors of CYP11B2. For this reason, the controls were considered as internal quality control parameters along the screening process and were incorporated in each plate.

Moreover, the HPLC parameters were further optimised which enabled the separation of DOC and B within 2 min (Figures 3.27) reducing the solvent consumption and increasing the throughput of the HPLC by more than 2-fold.

4.4. Testing a library of pharmacologically active compounds using the developed screening system

Drug companies try to conserve efforts, years of time and money they have put into drugs that failed to reach the market for a variety of reasons like poor safety profile or unexplained side effects. However, recouping losses of billions of dollars spent to develop a drug could be simply done by finding a different disease to treat with it, or by taking drugs already existing on the market for one indication, and trying to find other possible indications to make more money with the existing drug, or in other words; recycling of existing drugs.

Although repositioning of existing drugs has appeared in the early 1990s, it has only existed in its current form since the beginning of the decade and only very few companies looked at existing drugs or retooled themselves to do repositioning sprouted up (Table 4.1).

Table 4.1. Selected long-standing pharmaceuticals that had been repositioned during or prior to 2004 (Ashburn and Thor 2004)

| Generic Name | Trade Name, Original Indication (originator) | Trade Name, Repositioned Indication (repositioner) |
|--------------|--|---|
| Celecoxib | Celebrex, osteoarthritis and rheumatoid arthritis (Pfizer) | Celebrex, familial adenomatous polyposis, colon & breast cancer |
| Minoxidil | trade name N/A, hypertension (Pharmacia & Upjohn) | Rogaine, hair loss (Pfizer) |
| Topiramate | Topamax, epilepsy (Johnson & Johnson) | trade name N/A, obesity (Johnson & Johnson) |
| Lidocaine | Xilocaine, local anesthesia (AstraZeneca) | trade name N/A, Oral corticosteroid-dependent asthma (Corus Pharma) |
| Bupropion | Wellbutrin, depression (GlaxoSmithKline) | Zyban, smoking cessation (GlaxoSmithKline) |
| Fluoxetine | Prozac, depression (Eli Lilly) | Sarafem, premenstrual dysphoria (Eli Lilly) |
| Duloxetine | Cymbalta, depression (Eli Lilly) | Duloxetine SUI, stress urinary incontinence (Eli Lilly) |

In fact, of the top 50 selling pharmaceuticals in 2004, 84% have had additional indications approved since their initial US licensure. For this reason, the target of this part of work was to screen a library of pharmacologically active compounds using the newly developed screening system. The screening was carried out to validate the system itself and to check if a

repositioning process can be carried out on these existing pharmaceuticals to add the inhibition of CYP11B2 as a new indication.

The results of controls displayed reproducible results using the one-point concentration method (41.6 μM), and showed significant differences between the controls and the mock-treated samples ($p < 0.05$). The mock-treated samples (DMSO) displayed CYP11B2 activity with B production ratio of 11.5%, whereas the presence of miconazole decreased significantly ($p < 0.05$) the activity of CYP11B2 and B production ratio to 6.5% showing 44% inhibition under the test conditions. Moreover, the presence of either clotrimazole or ketoconazole displayed total inhibition of CYP11B2 (100%). Hence, each compound with similar inhibition profile will be defined during this screening assay as clotrimazole-like inhibitor of CYP11B2, whereas compounds with less inhibition effect resembling the miconazole effect will be defined as miconazole-like inhibitors.

The results of control showed high reproducibility during the screening assay and confirmed the reliability of the screening system. Furthermore, the screening assay showed high robustness when the Z' -factor was calculated for the several known CYP11B2 inhibitors as positive controls and DMSO as negative control. The Z' -factor of 1.0 for clotrimazole and ketoconazole show that the screening assay is perfect to identify clotrimazole-like inhibitors, and is an excellent assay to identify miconazole-like inhibitors since the Z' -factor for miconazole was 0.85.

The investigated library contains 1268 proven pharmacologically active compounds (see appendix) and is distributed in different kinds of drug classes as shown in Figure 4.4.

Although 35% of the compounds in the library are inhibitors of different enzymes, only 13 compounds were reported during the screening assay as potential inhibitors of CYP11B2. The two novel clotrimazole-like inhibitors reported in this work belong to the inhibitors class, whereas the miconazole-like inhibitors belong to different classes (Table 3.10, Figure 4.4).

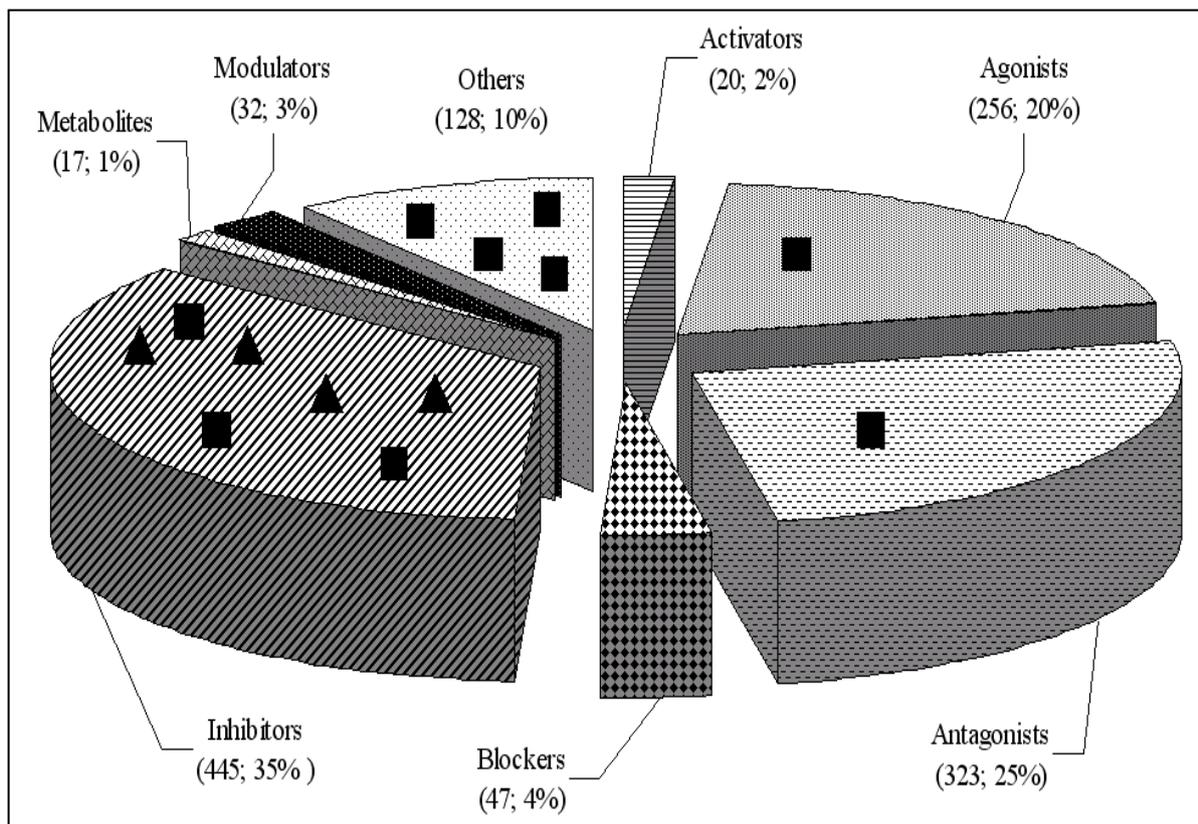


Figure 4.4. Pie chart depicting the composition of the investigated library.

The LOPAC library contains different classes of compounds. The screening assay reported four clotrimazole-like inhibitors, whereas nine miconazole-like inhibitors were also reported. The new inhibitors of CYP11B2 belong to different classes as shown (▲ clotrimazole-like inhibitor, ■ miconazole-like inhibitor).

Since the new CYP11B2 inhibitors are pharmacologically active compounds, the unexplained side effects associated with the therapeutic application of these drugs can be explained to some extent according to the results of this work.

Co_TH1 is formestane (sold as Lentaron®) and described as an injectable steroidal aromatase inhibitor with significant activity against metastatic breast cancer. In the clinical trials, formestane has been generally well tolerated following intramuscular administration at doses of up to 500 mg weekly (Goss *et al.*, 1986).

Previous studies showed that systemic adverse effects occurred in about 12% of patients following intramuscular drug administration (Coombes *et al.*, 1992). Many of these such as hot flushes, vaginal spotting and emotional lability were related to the mechanism of action of formestane i.e. estrogen suppression. Lethargy, rash, nausea, dizziness, indigestion, ataxia, cramps and facial swelling have also been reported with an incidence of <7% (Hoffken *et al.*, 1990). Moreover, it was reported recently that formestane treatment is also associated with changes in bile composition, which may predispose to gallstone formation (Czerny *et al.*,

2005). Our results display that formestane (Co_TH1) inhibits selectively CYP11B2 (IC_{50} : 2.4 μ M) (Figure 3.32). This observation could explain some of the side effects associated with formestane i.e. nausea and dizziness.

The closely related compound Co_TH2 is androstendion, which is a testosterone precursor and metabolite with androgenic activity. Interestingly Co_TH1 and Co_TH2 were identified during the primary screening assay as clotrimazole-like and miconazole-like inhibitors of CYP11B2, respectively. This result was confirmed through the validation assay, where the miconazole-like inhibitor displayed higher IC_{50} value (Co_TH2; IC_{50} = 3.11 μ M) in comparison with the clotrimazole-like inhibitor Co_TH1 (IC_{50} = 2.4 μ M). Regarding the steroidal scaffold of 4-androsterone-4-ol-3, 17-dione the structure activity analysis revealed that the ketone in position 17 (D-ring) is beneficial for activity, and an OH- residue at position 4 (A- ring) is necessary for potent inhibition of CYP11B2.

Since androstendion (Co_TH2) is a dehydroepiandrosterone (DHEA) metabolite, it might be of special interest that hormonal replacement therapy increasing DHEA will increase androstendion. Therefore, DHEA replacement performed as anti-aging therapy (Ohnaka and Takayanagi 2007), should take into consideration the possibility to develop salt depletion and unexplained hypotension.

The anti cancer drug Ellipticine (Co_TH4) identified during the primary assay as miconazole-like inhibitor of CYP11B2 showed selective inhibition of CYP11B2 with an IC_{50} of 8.9 μ M ($R^2 = 0.93$). Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) and several of its derivatives isolated from Apocyanaceae plants (i.e. *Ochrosia borbonica*, *Excavatia coccinea*) are alkaloids exhibiting significant antitumor and anti-HIV activities. Ellipticine and its more soluble derivatives (9-hydroxyellipticine, 9-hydroxy-N2-methylellipticinium, 9-chloro-N2-methylellipticinium and 9-methoxy-N2-methylellipticinium) exhibit promising results in the treatment of osteolytic breast cancer metastases, kidney sarcoma, tumors of brain and myeloblastic leukemia (Stiborova *et al.*, 2001). The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects and their complete lack of hematological toxicity (Auclair 1987).

Many suggestions were reported about the mechanisms of action that ellipticines follow as anticancer drugs, taken into consideration that the prevalent mechanisms of antitumor activities are (1) intercalation into DNA (Chu and Hsu 1992; Singh *et al.*, 1994), and (2) inhibition of DNA topoisomerase II activity (Monnot *et al.*, 1991; Fosse *et al.*, 1992).

Recently, it has been demonstrated that ellipticine covalently binds to DNA after being enzymatically activated. Cytochromes P450 (CYPs) are the major enzymes catalysing the ellipticine oxidation and its activation to more efficient metabolites forming DNA adducts (Aimova and Stiborova 2005). Furthermore, ellipticine was found to be a substrate of CYP1A1 and CYP1A2 (Auclair 1987; Frei *et al.*, 2002), and an inducer of several CYPs (Aimova *et al.*, 2007). On the other hand, this compound was previously reported to be a strong inhibitor of CYP1A1/2 (Auclair 1987). We found an inhibitory effect of ellipticine against the human CYP11B2. For this reason, it should be taken into consideration that the clinical application of ellipticine in the treatment of cancer could be associated with blood hypotension.

Compound Co_TH9 known as phenelzine (Sold as Nardil®) is a potent, irreversible inhibitor of monoamine oxidase (MAO)-A and -B, that has been used to treat depression since the late 1950s (Furst 1959). There has been a recent resurgence of interest in this drug class for patients with severe depression (Shelton Clauson *et al.*, 2004; Sokolski and Brown 2006).

Phenelzine toxicity is normally characterised by agitation, seizures, sweating, tachycardia and hypertension (Ciocatto *et al.*, 1972; Bhugra and Kaye 1986; Henry and Antao 1992), although hypotension has also been described (Linden *et al.*, 1984; Breheny *et al.*, 1986). Furthermore, the unexplained phenelzine-induced hypotension could be treated successfully with salt tablets (Munjack 1984). Moreover, several studies reported phenelzine-overdose induced complications, these complications include severe and unexplained hypotension, impaired left ventricular function and acute myocarditis, death was also reported and should be considered in patients who develop unexplained hypotension after phenelzine overdose (Linden *et al.* 1984; Waring and Wallace 2007).

Since it was found in this work that phenelzine inhibits the aldosterone synthase (CYP11B2) with an IC_{50} value of 48 μ M hence, the side effect of this drug concerning salt loss and hypotension can be explained with this result. As a result of CYP11B2 inhibition, the renin-angiotension system will be activated in a classical endocrine negative feedback loop, this activation will increase the amount of converted angiotension II (AT II), the potent vasoconstrictor, which elevate thus the blood pressure, and could be the reason for the hypertension usually characterizing phenelzine toxicity. Furthermore, AT II has a direct stimulating action on the corticotropin-releasing hormone (CRH) (Ganong 1993; Jezova *et al.*, 1998), which induces in addition to steroidogenesis the catecholamine synthesis via the induction of tyrosine hydroxylase (TH) (Dermitzaki *et al.*, 2007), dopamine β -hydroxylase

(DBH) and phenylethanolamine N-methyltransferase (PNMT) (Lima and Sourkes 1987). Moreover, CRH stimulates the catecholamine release and poses a trophic effect on chromaffin cells (Hoheisel *et al.*, 1998).

Thus, depending on the results of this work and the previous clinical observations mentioned above, it can be suggested that the unexplained hypotension after a massive phenelzine overdose is due to the CYP11B2 inhibition.

The massive overdose could inhibit totally the aldosterone synthase, and, as a result, aldosterone, the primary hormone responsible for Na⁺ retention by the kidney, will disappear causing hypotension. Moreover, the activation of the renin-angiotension system stimulates the corticotropin-releasing factor (CRF) and adrenocorticotrophic hormone (ACTH), which stimulate the adrenal gland, and since CYP11B2 is inhibited, more cortisol will be produced.

The increased CRH will enhance also the epinephrine synthesis through the stimulation of tyrosine hydroxylase and dopamine-β-hydroxylase. Moreover, the increased cortisol will increase the expression level of phenylethanolamine N-methyltransferase (PNMT) in chromaffin cells, enhancing also the synthesis of epinephrine (Isobe *et al.*, 2000; Wong 2006) (Figure 4.5).

Unlike many other hormones, epinephrine as well as the other catecholamines does not exert any negative feedback, and therefore the overdose of phenelzine increases the epinephrine synthesis, whether on the production level by enhancing indirectly the activity of the enzymes involved in catecholamine synthesis, or directly by inhibiting the monoamin oxidase enzymes involved in the epinephrine metabolism process (Figure 4.5).

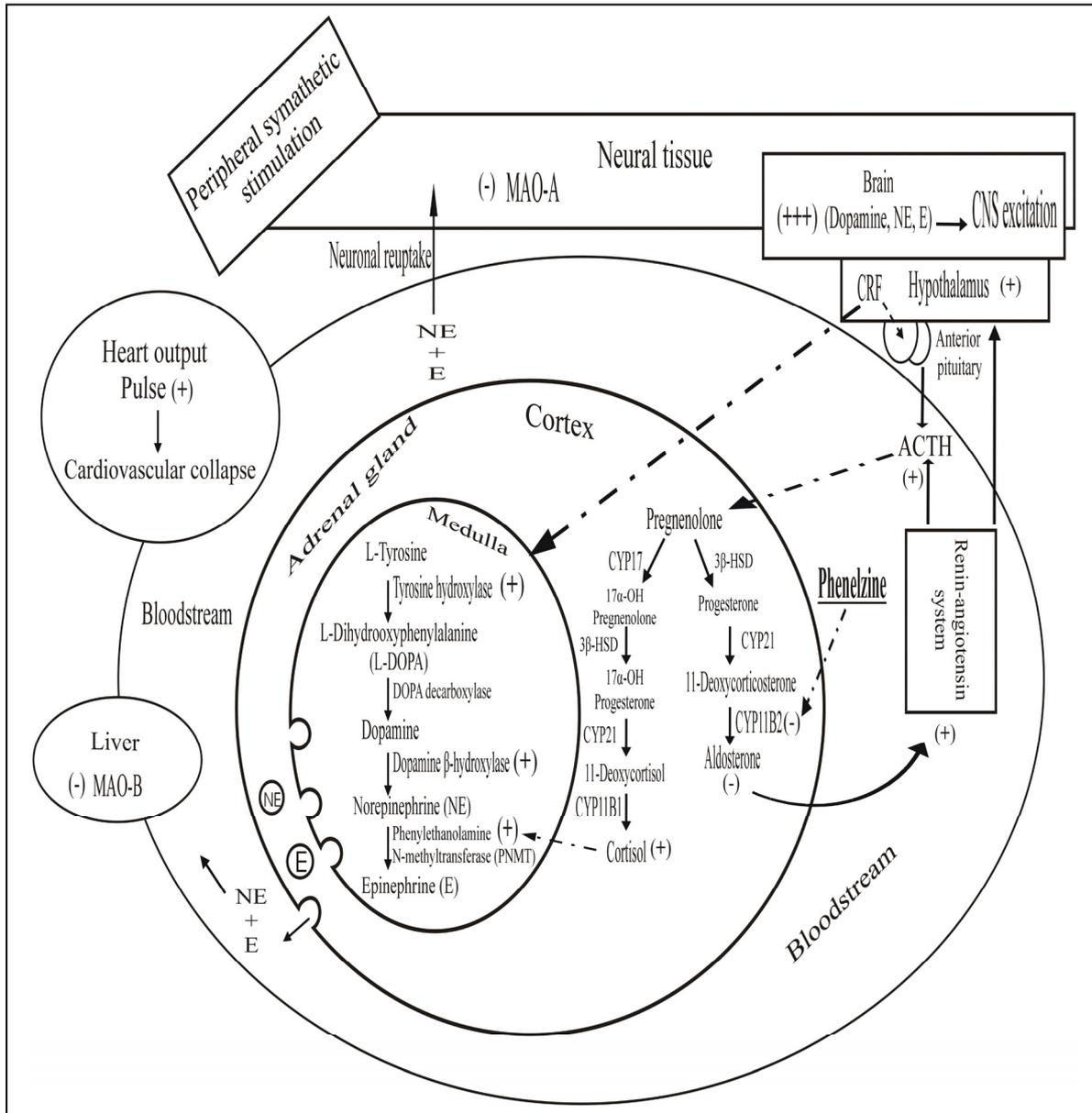


Figure 4.5. Schematic representation of catecholamine and adrenal hormone biosynthesis, and the speculated influence of an overdose of phenelzine (Co_TH9).

The synthesis of the catecholamines will increase which explains the increased levels of brain epinephrine and dopamine found in patients treated with MAO inhibitors prior to death (Bogdanski *et al.*, 1958; Green and Erickson 1962; Jones *et al.*, 1972). Furthermore, the high levels of catecholamines in brain seem to be responsible for the central nervous system excitation and peripheral sympathetic stimulation associated with increased pulse reported in MAO inhibitor overdose cases (Sandler 1959; Reid and Kerr 1969; Mackell *et al.*, 1979).

Although no study reported the aldosterone concentration in depressed patients receiving a phenelzine therapeutic course, depressed subjects were reported to display huge increase in aldosterone concentration in comparison with controls (Murck *et al.*, 2003). This could

minimize the influence of any aldosterone decrease in depressed patients receiving phenelzine. Whereas an overdose from phenelzine can lead to a total inhibition of CYP11B2 and to a dramatic decrease in the aldosterone concentration. These findings could play an important role in the management of depressed patients receiving phenelzine by monitoring the aldosterone concentration and blood pressure. Furthermore, phenelzine overdose-induced death could be prevented by rapid infusion of normal saline and aldosterone replacement therapy.

Finally, the tryptophan hydroxylase inhibitor Co_TH3 (4-Chloro-DL-phenylalanine methyl ester hydrochloride) defined during the screening assay as miconazole-like inhibitor, showed during the validation assay a selective inhibition of CYP11B2 with an IC_{50} of 40 μ M ($R^2=0.90$). In contrast, the nitric oxide synthase inhibitor Co_TH11 (1-[2-(Trifluoromethyl)phenyl]imidazole) defined during the screening assay as clotrimazole-like inhibitor displayed strong inhibition effect against both, CYP11B2 and CYP11B1 with IC_{50} values of 1.37 and 0.7 μ M, respectively (Table 3.11, Figure 3.38). It is, thus, not specific enough as a “lead” for the development of CYP11B2 inhibitors. For this reason, the miconazole-like inhibitors of CYP11B2 defined during the screening assay should be taken into consideration and included in the validation assays when the screening is carried out to discover selective inhibitors of CYP11B2.

Concluding, these results indicate that the new screening system developed in this work (Figure 4.3) is a robust screening system that can be applied to investigate libraries of existing drugs to find novel CYP11B2 inhibitors. This screening enables the reposition of existing drugs, which can save costs and billions of dollars spend to develop new CYP11B2 inhibitors.

Although the test was developed and validated on the laboratory level, it displayed the ability to screen up to 600 compounds per week. The throughput of the system can further increased by testing 10 compounds per well, which will increase the throughput of the system up to 6000 compounds per week.

Finally, this screening system can be modified and established for the use on the industrial level, especially when the required equipments are offered to enable the manipulation of large numbers of plates. Furthermore, the novel CYP11B2 inhibitors identified in this work are “druggable” compounds that can be repositioned to be used in the treatment of hyperaldosteronism-related diseases or as lead compounds that could further optimised in the field of drug development to achieve more safe and selective inhibitors of CYP11B2.

5. References

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6. Appendix

6.1. Contributions to international meetings

1. **July 2004:** Workshop “3000 and more Cytochromes P450: where to go now?” Saarbrücken, Germany.
2. **June 2007:** 15th International conference on Cytochromes P450, Bled, Slovenia.
Tarek Hakki, Silvia Zearo, Calin-Aurel Dragan, Matthias Bureik & Rita Bernhardt (Poster presentation)
3. **June 2008:** 9th International Symposium on Cytochrome P450 Biodiversity and Biotechnology, Nice, France
 - **Tarek Hakki**, Matthias Bureik & Rita Bernhardt (Poster presentation)
 - Spela Petric, Tarek Hakki, Rita Bernhardt & **Bronislava Cresnar** (Oral presentation)
4. **July 2008:** 17th International Symposium on Microsomes and Drug Oxidations, Saratoga Sprins, New York, USA.
 - Cornelia Virus, Tarek Hakki, Matthias Bureik, Michael Lisurek, Calin-Aurel Dragan, Benjamin Böttner, Iris Antes, Frank Hannemann and **Rita Bernhardt.** (Oral presentation)

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6.4. Materials and Methods

6.4.1. Stock solutions for EMM medium

o Vitamin stock solution (x1000)

| Content | Amount [g/L] | Final concentration |
|---------------------|--------------|---------------------|
| Sodium pantothenate | 1.00 | 4.2 mM |
| Nicotinic acid | 10.00 | 81.2 mM |
| Inositol | 10.00 | 55.5 mM |
| Biotin | 0.01 | 40.8 μ M |

o Salt stock solution (x50)

| Content | Amount [g/L] | Final concentration |
|--------------------------------------|--------------|---------------------|
| MgCl ₂ •6H ₂ O | 52.500 | 0.26 M |
| CaCl ₂ •2H ₂ O | 0.735 | 4.99 mM |
| KCl | 50.000 g | 0.67 M |
| Na ₂ SO ₄ | 2.000 | 14.10 mM |

o Mineral stock solution (x10,000)

| Content | Amount [g/L] | Final concentration |
|--------------------------------------|--------------|---------------------|
| H ₃ BO ₃ | 5.0 | 80.90 mM |
| MnSO ₄ | 4.0 | 23.70 mM |
| ZnSO ₄ •7H ₂ O | 4.0 | 13.90 mM |
| FeCl ₃ •6H ₂ O | 2.0 | 7.40 mM |
| H ₂ MoO ₄ | 1.6 | 2.47 mM |
| KI | 1.0 | 6.02 mM |
| CuSO ₄ •5H ₂ O | 0.4 | 1.60 mM |

| | | |
|-------------|------|----------|
| Citric acid | 10.0 | 47.60 mM |
|-------------|------|----------|

6.4.2. Oligonucleotides

All primers used during this work were obtained from the company BioTeZ (Berlin-Buch, Deutschland) and purified via HPLC. The sequences as well as the purpose of each oligonucleotide used in this work are given below:

| Code | Name | Purpose | Sequence (5'→3') |
|------|--|---|---|
| Pr_1 | PR(_{Hsa} AdR_cra ven) _{for} | Forward primer for the amplification of _{Hsa} AdR with <i>NdeI</i> restriction site | GGC GGT GGC CAT ATG GCT TCG CGC TGC TGG |
| Pr_2 | PR(_{Hsa} AdRXST OP_cra ven) _{rev} | Reverse primer for the amplification of _{Hsa} AdR with <i>XhoI</i> restriction site | GCC ACC GCC CTC GAG A GTG GCC CAG GAG GCG CAG |
| Pr_3 | 5'-hAdR | Forward primer for the amplification of _{Hsa} AdR | AGA GAG GGA TCC ATG GCT TCG CGC TGC TGG |
| Pr_4 | hSdR3PK | Reverse primer for the amplification of _{Hsa} AdR | GTG GCC CAG GAG GAG GCG CAG C |
| Pr_5 | NMT pombe Forward | Forward primer for the sequencing of cloned insert in pNMT1-TOPO vector | TTT CAA TCT CAT TCT CAC TTT CTG A |
| Pr_6 | URA4 pombe reverse | Reverse primer for the sequencing of cloned insert in pNMT1-TOPO vector | ACA AGG CAT CGA CTT TTT CAA TA |
| Pr_7 | PNMT1XhoI_FOR | Forward primer for the amplification of the <i>nmt1</i> promoter with <i>XhoI</i> restriction site | AGA GAG AGA CTC GAG GAC AGA ATA AGT CAT CAG CGG TTG |
| Pr_8 | ura4XhoI_REV | Reverse primer for the amplification of _{Hsa} AdR with <i>XhoI</i> restriction site | AGA GAG AGA CTC GAG ACA AGG CAT CGA CTT TTT CAA TA |

| | | | |
|-------|----------------|--|---|
| | | | |
| Pr_9 | K22-24Q (For) | Forward primer for the amplification of Adx (Colony PCR) | CAC TTT ATA AAC CGT GAT GGT GAA ACA TTA ACA ACC CAA GGA CAA ATT GGT GAC |
| Pr_10 | Adx S117A rück | Reverse primer for the amplification of Adx (Colony PCR) | CAT ATC AAT GGC CTC TCT GGC |
| Pr_11 | AdR_F | Forward primer for the sequencing of _{Hsa} AdR | ATG GCT TCG CGC TGC TGG CGC TG |
| Pr_12 | AdR_R | Reverse primer for the sequencing of _{Hsa} AdR | GTG GCC CAG GAG GCG CAG CAT CT |
| Pr_13 | AdR_F_+600 | Forward primer for the sequencing of _{Hsa} AdR | CAC CTG GAG GCC CTC CTT TTG TGC |

6.4.3. Library of pharmacologically active compounds

| Lab_code | mol weight Structure | Name | Class | Action |
|--------------|-------------------------|--|-----------------------|------------|
| Sigma_120693 | 195.22 | DL-alpha-Methyl-p-tyrosine | Neurotransmission | Inhibitor |
| Sigma_144509 | 213.24 | N-Phenylanthranilic acid | Cl- Channel | Blocker |
| Sigma_190047 | 373.23 | S(-)-p-Bromotetramisole oxalate | Phosphorylation | Inhibitor |
| Sigma_194336 | 153.61 | 5-Aminovaleric acid hydrochloride | GABA | Antagonist |
| Sigma_211672 | 129.16 | (±)-Nipecotic acid | GABA | Inhibitor |
| Sigma_246379 | 188.23 | Azelaic acid | DNA Metabolism | Inhibitor |
| Sigma_246557 | 196.68 | Tryptamine hydrochloride | Serotonin | Ligand |
| Sigma_265128 | 179.15 | 5-Fluoroindole-2-carboxylic acid | Glutamate | Antagonist |
| Sigma_291552 | 202.26 | 6-Methoxy-1,2,3,4-tetrahydro-9H-pyrido[3,4b]indole | Neurotransmission | Inhibitor |
| Sigma_861669 | 434.43 | S-(4-Nitrobenzyl)-6-thioguanosine | Adenosine | Inhibitor |
| Sigma_861804 | 432.00 | TMB-8 hydrochloride | Intracellular Calcium | Antagonist |
| Sigma_A 0152 | 94.12 | 4-Aminopyridine | K+ Channel | Blocker |
| Sigma_A 0257 | 676.83 | Atropine sulfate | Cholinergic | Antagonist |
| Sigma_A 0382 | 366.42 | Atropine methyl nitrate | Cholinergic | Antagonist |
| Sigma_A 0384 | 270.31 | Arcaïne sulfate | Glutamate | Antagonist |
| Sigma_A 0430 | 137.57 | 1-Aminocyclopropanecarboxylic acid hydrochloride | Glutamate | Agonist |
| Sigma_A 0500 | 59.07 | Acetamide | Biochemistry | Inhibitor |
| Sigma_A 0666 | 349.28 | N-(4-Aminobutyl)-5-chloro-2-naphthalenesulfonamide hydrochloride | Intracellular Calcium | Antagonist |
| Sigma_A 0760 | 101.11 | L-azetidine-2-carboxylic acid | Biochemistry | Inhibitor |
| Sigma_A 0779 | 281.57 | p-Aminoclonidine hydrochloride | Adrenoceptor | Agonist |
| Sigma_A 0788 | 136.15 | 3-aminobenzamide | Apoptosis | Inhibitor |
| Sigma_A 0937 | 319.27 | (±)-Norepinephrine (+)bitartrate | Adrenoceptor | Agonist |
| Sigma_A 0966 | 212.21 | 4-Amino-1,8-naphthalimide | Apoptosis | Inhibitor |
| Sigma_R 0875 | 608.69 | Reserpine | Serotonin | Inhibitor |
| Sigma_A 1260 | 187.71 | Amantadine hydrochloride | Dopamine | Releaser |
| Sigma_A 1755 | 420.43 | Aminophylline ethylenediamine | Adenosine | Antagonist |
| Sigma_A 1782 | 466.48 | S-(p-Azidophenacyl)glutathione | Multi-Drug Resistance | Modulator |
| Sigma_A 1784 | 440.42 | Aminopterin | Antibiotic | Inhibitor |
| Sigma_A 1824 | 218.26 | N-Acetyl-5-hydroxytryptamine | Melatonin | Precursor |
| Sigma_A 1895 | 422.35 | Aurintricarboxylic acid | Apoptosis | Inhibitor |
| Sigma_A 1910 | 183.10 | (±)-2-Amino-4-phosphonobutyric acid | Glutamate | Antagonist |
| Sigma_A 1977 | 361.53 | N-arachidonylglycine | Cannabinoid | Inhibitor |
| Sigma_A 2129 | 103.12 | GABA | GABA | Agonist |
| Sigma_A 2169 | 267.25 | 3'-Azido-3'-deoxythymidine | Immune System | Inhibitor |
| Sigma_A 2251 | 195.69 | Acetyl-beta-methylcholine chloride | Cholinergic | Agonist |
| Sigma_A 2385 | 244.21 | 5-azacytidine | DNA Metabolism | Inhibitor |
| Sigma_A 3085 | 299.77 | 5-(N-Ethyl-N-isopropyl)amiloride | Ion Pump | Blocker |

| | | | | |
|--------------|--------|--|-------------------------|------------|
| Sigma_A 3134 | 256.26 | 3-Aminopropionitrile fumarate | Multi-Drug Resistance | Substrate |
| Sigma_A 3145 | 270.24 | Apigenin | Cell Cycle | Inhibitor |
| Sigma_A 3539 | 175.62 | Gabaculine hydrochloride | GABA | Inhibitor |
| Sigma_A 3595 | 392.24 | AC 915 oxalate | Opioid | Ligand |
| Sigma_A 3711 | 326.44 | AA-861 | Leukotriene | Inhibitor |
| Sigma_A 3773 | 234.73 | 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride | Cholinergic | Inhibitor |
| Sigma_A 3846 | 402.51 | AL-8810 | Prostaglandin | Antagonist |
| Sigma_A 3940 | 134.14 | 1-Aminobenzotriazole | Multi-Drug Resistance | Inhibitor |
| Sigma_A 4147 | 161.16 | 3-Amino-1-propanesulfonic acid sodium | GABA | Agonist |
| Sigma_A 4393 | 303.79 | Apomorphine hydrochloride hemihydrate | Dopamine | Agonist |
| Sigma_A 4508 | 218.60 | O-(Carboxymethyl)hydroxylamine hemihydrochloride | Biochemistry | Inhibitor |
| Sigma_A 4562 | 294.15 | 5-(N,N-Dimethyl)amiloride hydrochloride | Ion Pump | Blocker |
| Sigma_A 4638 | 277.27 | Azathioprine | P2 Receptor | Inhibitor |
| Sigma_A 4669 | 225.21 | Acyclovir | Immune System | Inhibitor |
| Sigma_A 4687 | 341.84 | Amiprilose hydrochloride | Immune System | Modulator |
| Sigma_S 9318 | 465.80 | Sandoz 58-035 | Lipid | Inhibitor |
| Sigma_A 4910 | 169.07 | (±)-2-Amino-3-phosphonopropionic acid | Glutamate | Antagonist |
| Sigma_A 5006 | 174.20 | L-Arginine | Nitric Oxide | Precursor |
| Sigma_A 5157 | 225.18 | (±)-2-Amino-7-phosphonoheptanoic acid | Glutamate | Antagonist |
| Sigma_A 5282 | 197.13 | (±)-2-Amino-5-phosphonopentanoic acid | Glutamate | Antagonist |
| Sigma_A 5330 | 472.39 | L-732,138 | Tachykinin | Antagonist |
| Sigma_A 5376 | 180.16 | Acetylsalicylic acid | Prostaglandin | Inhibitor |
| Sigma_A 5585 | 299.77 | 5-(N-Methyl-N-isobutyl)amiloride | Ion Pump | Blocker |
| Sigma_A 5626 | 197.73 | Acetylthiocholine chloride | Cholinergic | Agonist |
| Sigma_A 5791 | 302.42 | 4-Androsten-4-ol-3,17-dione | Hormone | Inhibitor |
| Sigma_A 5879 | 281.01 | 2-(2-Aminoethyl)isothiourea dihydrobromide | Nitric Oxide | Inhibitor |
| Sigma_A 5909 | 313.83 | N-Acetylprocainamide hydrochloride | Na ⁺ Channel | Blocker |
| Sigma_T 9034 | 537.70 | Sodium Taurocholate | Multi-Drug Resistance | Modulator |
| Sigma_A 5922 | 214.22 | Amifostine | Cell Stress | Inhibitor |
| Sigma_A 6011 | 222.25 | Acetazolamide | Biochemistry | Inhibitor |
| Sigma_A 6134 | 236.11 | Arecoline hydrobromide | Cholinergic | Agonist |
| Sigma_A 6351 | 345.47 | A-315456 | Adrenoceptor | Antagonist |
| Sigma_G 8543 | 377.49 | GR 4661 | Serotonin | Agonist |
| Sigma_A 6566 | 265.72 | 2-Hydroxysaclofen | GABA | Antagonist |
| Sigma_A 6671 | 385.51 | Actinonin | Biochemistry | Inhibitor |
| Sigma_A 6770 | 454.45 | Methotrexate | DNA Metabolism | Inhibitor |
| Sigma_A 6883 | 384.32 | Atropine methyl bromide | Cholinergic | Antagonist |
| Sigma_A 6976 | 437.96 | Amperozide hydrochloride | Serotonin | Ligand |
| Sigma_A 7009 | 246.25 | Aminoguanidine hemisulfate | Nitric Oxide | Inhibitor |
| Sigma_A 7127 | 228.27 | Agmatine sulfate | Imidazoline | Agonist |
| Sigma_A 7148 | 208.09 | 4-Aminobenzamidine dihydrochloride | Biochemistry | Inhibitor |
| Sigma_A 7162 | 139.09 | 3-Aminopropylphosphonic acid | GABA | Agonist |
| Sigma_A 7250 | 163.20 | N-Acetyl-L-Cysteine | Glutamate | Antagonist |
| Sigma_A 7275 | 161.16 | L-2-aminoadipic acid | Glutamate | Inhibitor |

| | | | | |
|--------------|--------|--|-------------------------|----------------------|
| Sigma_A 7342 | 202.26 | N-Acetyltryptamine | Melatonin | Agonist - Antagonist |
| Sigma_A 7410 | 266.09 | Amiloride hydrochloride | Na ⁺ Channel | Blocker |
| Sigma_A 7655 | 266.34 | (±)-Atenolol | Adrenoceptor | Antagonist |
| Sigma_A 7755 | 292.47 | 5alpha-Androstane-3alpha,17beta-diol | Hormone | Metabolite |
| Sigma_A 7762 | 115.13 | L-allylglycine | Biochemistry | Inhibitor |
| Sigma_H-123 | 324.23 | H-9 dihydrochloride | Phosphorylation | Inhibitor |
| Sigma_A 7824 | 131.18 | 6-Aminohexanoic acid | Immune System | Inhibitor |
| Sigma_A 7845 | 322.26 | ATPO | Glutamate | Antagonist |
| Sigma_A 8003 | 136.11 | Allopurinol | Cell Stress | Inhibitor |
| Sigma_A 8404 | 313.87 | Amitriptyline hydrochloride | Adrenoceptor | Inhibitor |
| Sigma_A 8423 | 681.78 | Amiodarone hydrochloride | Adrenoceptor | Agonist |
| Sigma_A 8456 | 239.70 | 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride | Biochemistry | Inhibitor |
| Sigma_A 8598 | 261.67 | Ancitabine hydrochloride | DNA Metabolism | Inhibitor |
| Sigma_A 8676 | 285.82 | Alprenolol hydrochloride | Adrenoceptor | Antagonist |
| Sigma_A 8723 | 210.28 | Altretamine | DNA Metabolism | Inhibitor |
| Sigma_A 8762 | 195.22 | N-Acetyldopamine monohydrate | Dopamine | Precursor |
| Sigma_A 8835 | 110.55 | Aminoguanidine hydrochloride | Nitric Oxide | Inhibitor |
| Sigma_A 9013 | 566.42 | BW 284c51 | Cholinergic | Inhibitor |
| Sigma_A 9251 | 267.25 | Adenosine | Adenosine | Agonist |
| Sigma_A 9256 | 133.10 | L-Aspartic acid | Glutamate | Agonist |
| Sigma_A 9335 | 596.86 | Astaxanthin | Cell Stress | Inhibitor |
| Sigma_A 9345 | 272.69 | N-(4-Amino-2-chlorophenyl)phthalimide | Anticonvulsant | |
| Sigma_A 9501 | 329.21 | Adenosine 3',5'-cyclic monophosphate | Phosphorylation | Activator |
| Sigma_A 9512 | 319.27 | L(-)-Norepinephrine bitartrate | Adrenoceptor | Agonist |
| Sigma_A 9561 | 311.78 | 5-(N,N-hexamethylene)amiloride | Ion Pump | Inhibitor |
| Sigma_A 9630 | 286.42 | 4-Androstene-3,17-dione | Hormone | Precursor |
| Sigma_A 9657 | 232.28 | (±)-p-Aminoglutethimide | Biochemistry | Inhibitor |
| Sigma_A 9699 | 116.12 | (±)-HA-966 | Glutamate | Antagonist |
| Sigma_A 9755 | 290.45 | Androsterone | Hormone | |
| Sigma_A 9809 | 429.93 | Amsacrine hydrochloride | DNA Repair | Inhibitor |
| Sigma_A 9834 | 166.67 | (±)-AMT hydrochloride | Nitric Oxide | Inhibitor |
| Sigma_P 9623 | 365.84 | Paroxetine hydrochloride hemihydrate (MW = 374.83) | Serotonin | Inhibitor |
| Sigma_A 9899 | 299.81 | Antozoline hydrochloride | Imidazoline | Agonist |
| Sigma_A 9950 | 219.24 | Aniracetam | Glutamate | Agonist |
| Sigma_A-003 | 284.32 | 1,3-Diethyl-8-phenylxanthine | Adenosine | Antagonist |
| Sigma_A-013 | 336.33 | 8-(p-Sulfophenyl)theophylline | Adenosine | Antagonist |
| Sigma_A-022 | 392.44 | 1,3-Dipropyl-8-p-sulfophenylxanthine | Adenosine | Antagonist |
| Sigma_A-023 | 641.20 | 2-Methylthioadenosine triphosphate tetrasodium | P2 Receptor | Agonist |
| Sigma_A-024 | 294.27 | 5'-N-Methyl carboxamidoadenosine | Adenosine | Agonist |
| Sigma_P 0248 | 381.99 | PNU-37887A | K ⁺ Channel | Inhibitor |
| Sigma_A-129 | 313.79 | Amoxapine | Adrenoceptor | Inhibitor |
| Sigma_A-138 | 322.45 | Aminobenzotropine | Cholinergic | Ligand |
| Sigma_A-140 | 260.13 | Arecaidine propargyl ester hydrobromide | Cholinergic | Agonist |
| Sigma_A-142 | 266.34 | R(+)-Atenolol | Adrenoceptor | Antagonist |
| Sigma_A-143 | 266.34 | S(-)-Atenolol | Adrenoceptor | Antagonist |

| | | | | |
|--------------|--------|--|-------------------------|------------|
| Sigma_A-145 | 376.39 | 1-Allyl-3,7-dimethyl-8-p-sulfophenylxanthine | Adenosine | Antagonist |
| Sigma_A-155 | 173.17 | trans-(±)-ACPD | Glutamate | Agonist |
| Sigma_A-162 | 179.65 | 1-Amino-1-cyclohexanecarboxylic acid hydrochloride | Neurotransmission | Substrate |
| Sigma_A-164 | 292.21 | Alaproclate hydrochloride | Serotonin | Inhibitor |
| Sigma_P 9872 | 334.38 | Psora-4 | K ⁺ Channel | Inhibitor |
| Sigma_S 0568 | 304.78 | SB 200646 hydrochloride | Serotonin | Antagonist |
| Sigma_A-167 | 225.18 | D(-)-2-Amino-7-phosphonoheptanoic acid | Glutamate | Antagonist |
| Sigma_A-178 | 324.40 | Acetohexamide | Hormone | Releaser |
| Sigma_A-196 | 173.58 | SKF 97541 hydrochloride | GABA | Agonist |
| Sigma_A-201 | 101.11 | cis-4-Aminocrotonic acid | GABA | Agonist |
| Sigma_A-202 | 386.41 | N6-2-(4-Aminophenyl)ethyladenosine | Adenosine | Agonist |
| Sigma_A-206 | 238.34 | Agroclavine | Dopamine | Agonist |
| Sigma_A-230 | 127.14 | gamma-Acetylinic GABA | GABA | Inhibitor |
| Sigma_A-236 | 399.41 | AB-MECA | Adenosine | Agonist |
| Sigma_A-242 | 214.18 | Alloxazine | Adenosine | Antagonist |
| Sigma_A-243 | 145.12 | cis-Azetidine-2,4-dicarboxylic acid | Glutamate | Modulator |
| Sigma_A-244 | 145.12 | trans-Azetidine-2,4-dicarboxylic acid | Glutamate | Agonist |
| Sigma_A-252 | 189.73 | AGN 192403 hydrochloride | Imidazoline | Ligand |
| Sigma_A-254 | 221.21 | AIDA | Glutamate | Antagonist |
| Sigma_A-255 | 365.90 | A-77636 hydrochloride | Dopamine | Agonist |
| Sigma_A-263 | 228.25 | ATPA | Glutamate | Agonist |
| Sigma_A-265 | 785.06 | ARL 67156 trisodium salt | P2 Receptor | Inhibitor |
| Sigma_B 0385 | 408.93 | Beclomethasone | Hormone | |
| Sigma_B 0753 | 101.11 | 2,3-Butanedione monoxime | K ⁺ Channel | Blocker |
| Sigma_S 5192 | 380.49 | SB 222200 | Tachykinin | Antagonist |
| Sigma_B 1183 | 323.35 | 1-benzoyl-5-methoxy-2-methylindole-3-acetic acid | Multi-Drug Resistance | Inhibitor |
| Sigma_B 1266 | 108.10 | p-Benzoquinone | DNA Repair | Inhibitor |
| Sigma_B 1381 | 446.09 | 8-Bromo-cGMP sodium | Cyclic Nucleotides | Activator |
| Sigma_B 1552 | 317.18 | Bromo-enol lactone | Lipid | Inhibitor |
| Sigma_B 2009 | 121.14 | Benzamide | Apoptosis | Inhibitor |
| Sigma_B 2050 | 242.03 | 3-Bromo-7-nitroindazole | Nitric Oxide | Inhibitor |
| Sigma_B 2134 | 750.72 | (+)-Bromocriptine methanesulfonate | Dopamine | Agonist |
| Sigma_B 2292 | 241.25 | O6-benzylguanine | DNA Repair | Inhibitor |
| Sigma_B 2377 | 137.96 | N-Bromoacetamide | Na ⁺ Channel | Modulator |
| Sigma_B 2390 | 435.32 | (±)-Brompheniramine maleate | Histamine | Antagonist |
| Sigma_B 2417 | 356.22 | Benzamil hydrochloride | Ion Pump | Blocker |
| Sigma_B 2515 | 222.31 | L-Buthionine-sulfoximine | Multi-Drug Resistance | Inhibitor |
| Sigma_B 2640 | 222.31 | DL-Buthionine-[S,R]-sulfoximine | Multi-Drug Resistance | Inhibitor |
| Sigma_B 3023 | 364.42 | Bumetanide | Ion Pump | Inhibitor |
| Sigma_B 3501 | 153.61 | Betaine hydrochloride | Biochemistry | Metabolite |
| Sigma_B 3650 | 137.61 | Betaine aldehyde chloride | Cholinergic | Metabolite |
| Sigma_B 4555 | 320.37 | Benzoline oxalate | Imidazoline | Agonist |
| Sigma_B 4558 | 316.34 | BWB70C | Leukotriene | Inhibitor |

| | | | | |
|--------------|--------|--|--------------------------|--------------|
| Sigma_B 5002 | 307.10 | 5-Bromo-2'-deoxyuridine | DNA Metabolism | Inhibitor |
| Sigma_B 5016 | 403.01 | Bepridil hydrochloride | Ca ²⁺ Channel | Blocker |
| Sigma_B 5275 | 435.32 | (+)-Brompheniramine maleate | Histamine | Antagonist |
| Sigma_B 5399 | 213.67 | (±)-Baclofen | GABA | Agonist |
| Sigma_S 7067 | 331.35 | SB 202190 | Phosphorylation | Inhibitor |
| Sigma_B 5681 | 249.33 | Bay 11-7085 | Cell Cycle | Inhibitor |
| Sigma_B 5683 | 343.90 | Betaxolol hydrochloride | Adrenoceptor | Antagonist |
| Sigma_B 6506 | 156.62 | Benzamidine hydrochloride | Biochemistry | Inhibitor |
| Sigma_B 7005 | 392.47 | Betamethasone | Hormone | |
| Sigma_B 7148 | 421.97 | Buspirone hydrochloride | Serotonin | Agonist |
| Sigma_B 7283 | 293.71 | Benserazide hydrochloride | Biochemistry | Inhibitor |
| Sigma_B 7651 | 280.37 | Brefeldin A from <i>Penicillium brefeldianum</i> | Cytoskeleton and ECM | Inhibitor |
| Sigma_B 7777 | 430.55 | Budesonide | Hormone | |
| Sigma_B 7880 | 430.09 | 8-Bromo-cAMP sodium | Cyclic Nucleotides | Activator |
| Sigma_B 8262 | 403.54 | Benztropine mesylate | Cholinergic | Antagonist |
| Sigma_B 8279 | 278.35 | Ro 20-1724 | Cyclic Nucleotides | Inhibitor |
| Sigma_B 8385 | 344.84 | Bestatin hydrochloride | Biochemistry | Inhibitor |
| Sigma_B 8406 | 414.36 | Bretylium tosylate | Adrenoceptor | Blocker |
| Sigma_B 0936 | 244.27 | BRL 50481 | Phosphodiesterase | Inhibitor |
| Sigma_B 9308 | 417.56 | BP 897 | Dopamine | Agonist |
| Sigma_B 9647 | 333.14 | (E)-5-(2-Bromovinyl)-2'-deoxyuridine | Immune System | Inhibitor |
| Sigma_B 9929 | 443.42 | BRL 15572 | Serotonin | Antagonist |
| Sigma_B-003 | 408.59 | Chloroethylclonidine dihydrochloride | Adrenoceptor | Antagonist |
| Sigma_B-012 | 223.63 | 6-Fluoronorepinephrine hydrochloride | Adrenoceptor | Agonist |
| Sigma_B-015 | 481.48 | Bromoacetyl alprenolol menthane | Adrenoceptor | Antagonist |
| Sigma_B-016 | 397.92 | Benoxathian hydrochloride | Adrenoceptor | Antagonist |
| Sigma_B-019 | 340.30 | Phenoxybenzamine hydrochloride | Adrenoceptor | Blocker |
| Sigma_B-102 | 276.21 | Bupropion hydrochloride | Dopamine | Blocker |
| Sigma_B-103 | 462.30 | (-)-Bicuculline methbromide, 1(S), 9(R) | GABA | Antagonist |
| Sigma_B-112 | 356.30 | (±)-Bay K 8644 | Ca ²⁺ Channel | Agonist |
| Sigma_B-121 | 305.01 | Bromoacetylcholine bromide | Cholinergic | Ligand |
| Sigma_B-134 | 458.43 | BMY 7378 dihydrochloride | Serotonin | Agonist |
| Sigma_B-135 | 455.19 | R(+)-6-Bromo-APB hydrobromide | Dopamine | Agonist |
| Sigma_B-138 | 335.94 | BTCP hydrochloride | Dopamine | Blocker |
| Sigma_B-152 | 398.42 | N6-Benzyl-5'-N-ethylcarboxamidoadenosine | Adenosine | Agonist |
| Sigma_B-154 | 233.70 | BU224 hydrochloride | Imidazoline | Antagonist |
| Sigma_B-161 | 254.16 | B-HT 933 dihydrochloride | Adrenoceptor | Agonist |
| Sigma_B-168 | 397.99 | (±)-Butaclamol hydrochloride | Dopamine | Antagonist |
| Sigma_B-169 | 385.83 | BRL 37344 sodium | Adrenoceptor | Agonist |
| Sigma_B-173 | 346.39 | BRL 54443 maleate | Serotonin | Agonist |
| Sigma_B-175 | 322.86 | BW 723C86 | Serotonin | Agonist |
| Sigma_C 0253 | 304.22 | Chlorambucil | DNA | Intercalator |
| Sigma_C 0256 | 497.29 | Citicoline sodium | Lipid | Inhibitor |
| Sigma_C 0330 | 289.16 | Ciprofibrate | Transcription | Ligand |
| Sigma_C 0331 | 266.73 | 6-Chloromelatonin | Melatonin | Agonist |

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| Sigma_C 0400 | 214.05 | Carmustine | DNA | Intercalator |
| Sigma_C 0424 | 352.87 | PK 11195 | GABA | Antagonist |
| Sigma_C 0625 | 180.16 | Caffeic Acid | Cell Stress | Inhibitor |
| Sigma_C 0737 | 369.47 | Cilostazol | Cyclic Nucleotides | Inhibitor |
| Sigma_C 0750 | 194.19 | Caffeine | Adenosine | Inhibitor |
| Sigma_C 0768 | 261.09 | Cyclophosphamide monohydrate | DNA | Intercalator |
| Sigma_C 0862 | 292.47 | CGP-7930 | GABA | Modulator |
| Sigma_C 0987 | 290.45 | CGP-13501 | GABA | Modulator |
| Sigma_C 1112 | 376.58 | CP55940 | Cannabinoid | Agonist |
| Sigma_C 1159 | 102.09 | L-Cycloserine | Sphingolipid | Inhibitor |
| Sigma_C 1172 | 361.29 | ML-9 | Phosphorylation | Inhibitor |
| Sigma_C 1251 | 290.28 | (+)-Catechin Hydrate | Cell Stress | Inhibitor |
| Sigma_C 1290 | 276.74 | Chlorpropamide | Hormone | Releaser |
| Sigma_C 1610 | 343.81 | 1-(4-Chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid | Multi-Drug Resistance | Inhibitor |
| Sigma_C 1671 | 352.33 | Chlorprothixene hydrochloride | Dopamine | Antagonist |
| Sigma_C 1754 | 184.08 | Choline bromide | Cholinergic | Substrate |
| Sigma_C 2137 | 707.25 | Ceramide | Phosphorylation | Inhibitor |
| Sigma_C 2235 | 252.19 | CB 1954 | DNA | Intercalator |
| Sigma_C 2321 | 255.15 | Carcinine dihydrochloride | Cell Stress | Inhibitor |
| Sigma_C 2505 | 346.47 | Corticosterone | Hormone | |
| Sigma_C 2538 | 371.26 | Carboplatin | DNA | Intercalator |
| Sigma_C 2755 | 360.45 | Cortisone | Hormone | |
| Sigma_C 2932 | 383.83 | Chelerythrine chloride | Phosphorylation | Inhibitor |
| Sigma_C 3010 | 320.05 | 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane | Hormone | Inhibitor |
| Sigma_C 3025 | 390.87 | (±)-Chlorpheniramine maleate | Histamine | Antagonist |
| Sigma_C 3130 | 402.49 | Cortisone 21-acetate | Hormone | |
| Sigma_C 3270 | 478.78 | Cephalosporin C zinc salt | Antibiotic | |
| Sigma_C 3353 | 422.36 | CGP-74514A hydrochloride | Phosphorylation | Inhibitor |
| Sigma_C 3412 | 416.95 | Cyproterone acetate | Hormone | Antagonist |
| Sigma_C 3635 | 250.13 | DL-p-Chlorophenylalanine methyl ester hydrochloride | Neurotransmission | Inhibitor |
| Sigma_C 3662 | 1202.64 | Cyclosporin A | Phosphorylation | Inhibitor |
| Sigma_C 3909 | 102.09 | D-Cycloserine | Glutamate | Agonist |
| Sigma_C 3912 | 493.80 | 8-(4-Chlorophenylthio)-cAMP sodium | Cyclic Nucleotides | Activator |
| Sigma_C 3930 | 687.71 | Calmidazolium chloride | Intracellular Calcium | Inhibitor |
| Sigma_G 5918 | 393.51 | GR 113808 | Serotonin | Antagonist |
| Sigma_C 4024 | 236.28 | Carbamazepine | Anticonvulsant | |
| Sigma_C 4042 | 217.29 | Captopril | Neurotransmission | Inhibitor |
| Sigma_C 4238 | 339.87 | CNS-1102 | Glutamate | Antagonist |
| Sigma_C 4382 | 182.65 | Carbachol | Cholinergic | Agonist |
| Sigma_C 4397 | 169.57 | Chlorzoxazone | Nitric Oxide | Inhibitor |
| Sigma_C 4418 | 153.16 | L-Cysteinesulfinic Acid | Glutamate | Ligand |
| Sigma_C 4479 | 299.35 | 9-cyclopentyladenine | Cyclic Nucleotides | Inhibitor |

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|--------------|--------|--|--------------------------|--------------|
| Sigma_C 4520 | 418.43 | Cephalothin sodium | Antibiotic | |
| Sigma_C 4522 | 252.34 | Cimetidine | Histamine | Antagonist |
| Sigma_C 4542 | 311.86 | Cyclobenzaprine hydrochloride | Serotonin | Antagonist |
| Sigma_C 4662 | 525.60 | Carbetapentane citrate | Opioid | Ligand |
| Sigma_C 4895 | 347.40 | Cephalexin hydrate | Antibiotic | |
| Sigma_C 4911 | 295.72 | Chlorothiazide | Biochemistry | Inhibitor |
| Sigma_C 4915 | 390.87 | (+)-Chlorpheniramine maleate | Histamine | Antagonist |
| Sigma_C 5020 | 476.49 | Cefazolin sodium | Antibiotic | |
| Sigma_C 5040 | 362.31 | Clemizole hydrochloride | Histamine | Antagonist |
| Sigma_C 5134 | 301.69 | 2-Chloroadenosine | Adenosine | Agonist |
| Sigma_C 5259 | 196.68 | Bethanechol chloride | Cholinergic | Agonist |
| Sigma_C 5270 | 368.53 | Cinnarizine | Ca ²⁺ Channel | Blocker |
| Sigma_C 5554 | 269.60 | 1-(3-Chlorophenyl)piperazine dihydrochloride | Serotonin | Agonist |
| Sigma_S 0693 | 286.36 | SB 204741 | Serotonin | Antagonist |
| Sigma_C 5793 | 598.55 | Ceftriaxone sodium | Antibiotic | |
| Sigma_C 5913 | 357.16 | 4-Chloromercuribenzoic acid | Biochemistry | Inhibitor |
| Sigma_C 5923 | 176.22 | (-)-Cotinine | Cholinergic | Metabolite |
| Sigma_C 5976 | 465.80 | CL 316,243 | Adrenoceptor | Agonist |
| Sigma_C 5982 | 256.69 | 7-Chloro-4-hydroxy-2-phenyl-1,8-naphthyridine | Adenosine | Antagonist |
| Sigma_C 6019 | 344.85 | Clotrimazole | K ⁺ Channel | Inhibitor |
| Sigma_C 6022 | 323.87 | Cyproheptadine hydrochloride | Serotonin | Antagonist |
| Sigma_C 6042 | 320.31 | 5'-(N-Cyclopropyl)carboxamidoadenosine | Adenosine | Agonist |
| Sigma_C 6048 | 493.52 | Cefmetazole sodium | Antibiotic | |
| Sigma_C 6305 | 326.83 | Clozapine | Dopamine | Antagonist |
| Sigma_C 6506 | 199.64 | (±)-p-Chlorophenylalanine | Neurotransmission | Inhibitor |
| Sigma_C 6628 | 515.87 | Chloroquine diphosphate | DNA | Intercalator |
| Sigma_C 6643 | 242.70 | Clofibrate | Lipid | Modulator |
| Sigma_C 6645 | 279.68 | Cytosine-1-beta-D-arabinofuranoside hydrochloride | DNA Metabolism | Inhibitor |
| Sigma_C 6862 | 438.79 | CB34 | Benzodiazepine | Ligand |
| Sigma_C 6895 | 367.81 | Cefaclor | Antibiotic | |
| Sigma_C 7005 | 102.09 | DL-Cycloserine | Sphingolipid | Inhibitor |
| Sigma_C 7041 | 317.22 | McN-A-343 | Cholinergic | Agonist |
| Sigma_C 7230 | 373.89 | N-(2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl)-3-methoxybenzamide | Dopamine | Agonist |
| Sigma_C 7255 | 225.20 | Cystamine dihydrochloride | Glutamate | Inhibitor |
| Sigma_C 7291 | 351.32 | Clomipramine hydrochloride | Serotonin | Inhibitor |
| Sigma_C 7522 | 523.63 | Calcimycin | Intracellular Calcium | |
| Sigma_C 7632 | 196.20 | Cantharidin | Phosphorylation | Inhibitor |
| Sigma_C 7861 | 405.31 | Citalopram hydrobromide | Serotonin | Inhibitor |
| Sigma_C 7897 | 266.56 | Clonidine hydrochloride | Adrenoceptor | Agonist |
| Sigma_C 7912 | 477.45 | Cefotaxime sodium | Antibiotic | |
| Sigma_C 7971 | 342.44 | Cilostamide | Cyclic Nucleotides | Inhibitor |
| Sigma_C 8011 | 183.12 | Chelidamic acid | Glutamate | Inhibitor |
| Sigma_C 8031 | 335.37 | N6-Cyclopentyladenosine | Adenosine | Agonist |
| Sigma_C 8088 | 214.22 | Cantharidic Acid | Phosphorylation | Inhibitor |

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|--------------|--------|--|----------------------|------------|
| Sigma_C 8138 | 355.33 | Chlorpromazine hydrochloride | Dopamine | Antagonist |
| Sigma_C 8145 | 554.54 | Cefsulodin sodium salt hydrate | Antibiotic | |
| Sigma_C 8221 | 284.31 | Caffeic acid phenethyl ester | Cell Cycle | Inhibitor |
| Sigma_C 8270 | 445.45 | Cephapirin sodium | Antibiotic | |
| Sigma_C 8395 | 349.41 | Cephadrine | Antibiotic | |
| Sigma_C 8417 | 313.07 | DSP-4 hydrochloride | Adrenoceptor | Neurotoxin |
| Sigma_C 8645 | 262.22 | Cinoxacin | Antibiotic | Inhibitor |
| Sigma_C 8759 | 260.34 | Carisoprodol | Neurotransmission | |
| Sigma_C 8773 | 294.18 | Centrophenoxine hydrochloride | Nootropic | |
| Sigma_C 8903 | 459.97 | Clemastine fumarate | Histamine | Antagonist |
| Sigma_C 9033 | 160.00 | beta-Chloro-L-alanine hydrochloride | Biochemistry | Inhibitor |
| Sigma_C 9510 | 110.11 | Pyrocatechol | Cell Cycle | Inhibitor |
| Sigma_C 9511 | 331.80 | Z-L-Phe chloromethyl ketone | Biochemistry | Inhibitor |
| Sigma_C 9611 | 247.25 | CPCCOEt | Glutamate | Antagonist |
| Sigma_C 9754 | 399.45 | Colchicine | Cytoskeleton and ECM | Inhibitor |
| Sigma_C 9758 | 274.25 | L-Canavanine sulfate | Nitric Oxide | Inhibitor |
| Sigma_C 9847 | 389.88 | Cyclothiazide | Glutamate | Agonist |
| Sigma_C 9901 | 349.39 | N6-Cyclohexyladenosine | Adenosine | Agonist |
| Sigma_C 9911 | 348.36 | (S)-(+)-Camptothecin | Apoptosis | Inhibitor |
| Sigma_C-007 | 362.92 | 10-(alpha-Diethylaminopropionyl)-phenothiazine hydrochloride | Biochemistry | Inhibitor |
| Sigma_C-008 | 287.14 | (+)-cis-Dioxolane iodide | Cholinergic | Agonist |
| Sigma_C-011 | 303.21 | OXA-22 iodide | Cholinergic | Agonist |
| Sigma_C-101 | 304.40 | 8-Cyclopentyl-1,3-dipropylxanthine | Adenosine | Antagonist |
| Sigma_C-102 | 248.29 | 8-Cyclopentyl-1,3-dimethylxanthine | Adenosine | Antagonist |
| Sigma_C-104 | 252.21 | (±)-CPP | Glutamate | Antagonist |
| Sigma_C-106 | 450.42 | CGS-12066A maleate | Serotonin | Agonist |
| Sigma_C-108 | 207.75 | 2-Cyclooctyl-2-hydroxyethylamine hydrochloride | Neurotransmission | Inhibitor |
| Sigma_C-117 | 319.32 | 5-Carboxamidotryptamine maleate | Serotonin | Agonist |
| Sigma_C-121 | 223.62 | 7-Chlorokynurenic acid | Glutamate | Antagonist |
| Sigma_C-125 | 315.80 | (±)-CGP-12177A hydrochloride | Adrenoceptor | Agonist |
| Sigma_C-126 | 226.23 | S-(-)-Carbidopa | Biochemistry | Inhibitor |
| Sigma_C-130 | 410.74 | (±)-Chloro-APB hydrobromide | Dopamine | Agonist |
| Sigma_C-141 | 535.99 | CGS-21680 hydrochloride | Adenosine | Agonist |
| Sigma_Y 0503 | 320.26 | Y-27632 dihydrochloride | Phosphorylation | Inhibitor |
| Sigma_C-144 | 248.12 | 1-(m-Chlorophenyl)-biguanide hydrochloride | Serotonin | Agonist |
| Sigma_C-145 | 629.56 | 2-Chloroadenosine triphosphate tetrasodium | P2 Receptor | Agonist |
| Sigma_C-147 | 271.41 | (+)-Cyclazocine | Opioid | Antagonist |
| Sigma_C-191 | 376.91 | Capsazepine | Vanilloid | Agonist |
| Sigma_C-192 | 273.74 | Chlormezanone | Neurotransmission | Modulator |
| Sigma_C-197 | 330.78 | 8-(3-Chlorostyryl)caffeine | Adenosine | Antagonist |
| Sigma_C-199 | 285.69 | CGS-15943 | Adenosine | Antagonist |
| Sigma_C-203 | 198.60 | 2-Chloro-2-deoxy-D-glucose | Biochemistry | Analog |
| Sigma_C-207 | 378.35 | 4'-Chloro-3-alpha-(diphenylmethoxy)tropane hydrochloride | Dopamine | Blocker |
| Sigma_C-223 | 252.75 | Cirazoline hydrochloride | Adrenoceptor | Agonist |
| Sigma_C-231 | 590.58 | CGP 20712A methanesulfonate | Adrenoceptor | Antagonist |

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| Sigma_C-237 | 159.14 | (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine | Glutamate | Agonist |
| Sigma_C-239 | 276.12 | CNQX disodium | Glutamate | Antagonist |
| Sigma_C-271 | 247.30 | CX 546 | Glutamate | Modulator |
| Sigma_C-277 | 544.74 | Chloro-IB-MECA | Adenosine | Agonist |
| Sigma_D 0411 | 381.86 | WB-4101 hydrochloride | Adrenoceptor | Antagonist |
| Sigma_D 0540 | 252.14 | DNQX | Glutamate | Antagonist |
| Sigma_D 0670 | 586.68 | Dihydroouabain | Ion Pump | Inhibitor |
| Sigma_D 0676 | 337.85 | Dobutamine hydrochloride | Adrenoceptor | Agonist |
| Sigma_D 1064 | 229.28 | Dihydrokainic acid | Glutamate | Blocker |
| Sigma_D 1260 | 418.30 | Decamethonium dibromide | Cholinergic | Agonist |
| Sigma_D 1262 | 887.49 | P1,P4-Di(adenosine-5')tetraphosphate triammonium | Biochemistry | Inhibitor |
| Sigma_D 1306 | 448.55 | Debrisoquin sulfate | Neurotransmission | Antihypertensive |
| Sigma_D 1413 | 224.22 | 2',3'-didehydro-3'-deoxythymidine | Immune System | Inhibitor |
| Sigma_D 1414 | 379.44 | Droperidol | Dopamine | Antagonist |
| Sigma_D 1507 | 247.68 | L-3,4-Dihydroxyphenylalanine methyl ester hydrochloride | Dopamine | Precursor |
| Sigma_D 1542 | 169.61 | 1,4-Dideoxy-1,4-imino-D-arabinitol | Phosphorylation | Inhibitor |
| Sigma_D 1791 | 348.24 | 2,4-Dinitrophenyl 2-fluoro-2-deoxy-beta-D-glucopyranoside | Biochemistry | Inhibitor |
| Sigma_D 1916 | 319.15 | D-ribofuranosylbenzimidazole | Transcription | Inhibitor |
| Sigma_D 2064 | 766.60 | Dequalinium analog, C-14 linker | Phosphorylation | Inhibitor |
| Sigma_D 2521 | 450.99 | Diltiazem hydrochloride | Ca ²⁺ Channel | Antagonist |
| Sigma_D 2531 | 352.32 | Dextromethorphan hydrobromide monohydrate | Glutamate | Antagonist |
| Sigma_S 0443 | 308.81 | SB 203186 | Serotonin | Antagonist |
| Sigma_D 2763 | 679.80 | Dihydroergotamine methanesulfonate | Serotonin | Antagonist |
| Sigma_D 2926 | 314.55 | Diphenyleneiodonium chloride | Nitric Oxide | Inhibitor |
| Sigma_D 3630 | 291.82 | Diphenhydramine hydrochloride | Histamine | Antagonist |
| Sigma_D 3634 | 86.09 | 2,3-Butanedione | Cytoskeleton and ECM | Inhibitor |
| Sigma_D 3648 | 172.19 | N,N,N',N'-Tetramethylazodicarboxamide | Cell Stress | Modulator |
| Sigma_D 3689 | 183.17 | (S)-3,5-Dihydroxyphenylglycine | Glutamate | Agonist |
| Sigma_D 3768 | 527.59 | Dequalinium dichloride | K ⁺ Channel | Blocker |
| Sigma_D 3775 | 388.47 | Doxylamine succinate | Histamine | Antagonist |
| Sigma_D 3900 | 302.85 | Desipramine hydrochloride | Adrenoceptor | Inhibitor |
| Sigma_D 4007 | 252.28 | 5,5-Diphenylhydantoin | Anticonvulsant | |
| Sigma_D 4268 | 238.72 | N [^] G,N [^] G-Dimethylarginine hydrochloride | Nitric Oxide | Inhibitor |
| Sigma_D 4434 | 288.86 | Clodronic acid | Cytoskeleton and ECM | Inhibitor |
| Sigma_D 4505 | 274.26 | Phenytoin sodium | Anticonvulsant | |
| Sigma_D 4526 | 315.85 | Doxepin hydrochloride | Adrenoceptor | Inhibitor |
| Sigma_P-152_a | 248.33 | S(-)-Pindolol | Adrenergic | Antagonist |
| Sigma_D 5290 | 183.21 | (-)-alpha-Methylnorepinephrine | Adrenoceptor | Agonist |
| Sigma_D 5294 | 677.63 | Dilazep hydrochloride | Adenosine | Inhibitor |
| Sigma_D 5385 | 180.17 | 1,7-Dimethylxanthine | Adenosine | Antagonist |
| Sigma_D 5439 | 218.21 | 2,3-Dimethoxy-1,4-naphthoquinone | Cell Stress | Modulator |
| Sigma_D 5564 | 178.15 | Daphnetin | Phosphorylation | Inhibitor |
| Sigma_D 5689 | 246.31 | DM 235 | Nootropic | |

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| Sigma_D 5766 | 113.16 | 5,5-Dimethyl-1-pyrroline-N-oxide | Cell Stress | Inhibitor |
| Sigma_D 5782 | 211.22 | 2',3'-dideoxycytidine | Immune System | Inhibitor |
| Sigma_D 5794 | 489.59 | Diacylglycerol Kinase Inhibitor II | Phosphorylation | Inhibitor |
| Sigma_D 5814 | 303.79 | Dihydropyridine hydrochloride | Dopamine | Agonist |
| Sigma_D 5886 | 203.67 | N-Methyl-dopamine hydrochloride | Dopamine | Agonist |
| Sigma_D 5891 | 318.20 | 1,1-Dimethyl-4-phenyl-piperazinium iodide | Cholinergic | Agonist |
| Sigma_P 9248 | 360.35 | PD 169316 | Phosphorylation | Inhibitor |
| Sigma_D 6035 | 437.48 | Disopyramide phosphate | K+ Channel | Modulator |
| Sigma_D 6140 | 501.32 | Demeclocycline hydrochloride | Antibiotic | |
| Sigma_D 6518 | 393.35 | Diethylenetriaminepentaacetic acid | Biochemistry | Inhibitor |
| Sigma_D 6899 | 318.14 | Diclofenac sodium | Prostaglandin | Inhibitor |
| Sigma_D 6908 | 301.52 | DL-erythro-Dihydrosphingosine | Phosphorylation | Inhibitor |
| Sigma_D 6940 | 209.72 | R(-)-Desmethyldeprenyl hydrochloride | Neurotransmission | Inhibitor |
| Sigma_D 7505 | 156.19 | 2,2'-Bipyridyl | Biochemistry | Inhibitor |
| Sigma_D 7644 | 339.48 | Disopyramide | Na+ Channel | Blocker |
| Sigma_D 7802 | 254.24 | Daidzein | Cell Cycle | Inhibitor |
| Sigma_D 7814 | 275.31 | Dubininine | Anticonvulsant | |
| Sigma_D 7909 | 345.96 | Dicyclomine hydrochloride | Cholinergic | Antagonist |
| Sigma_D 7910 | 215.04 | 3,4-Dichloroisocoumarin | Biochemistry | Inhibitor |
| Sigma_D 7938 | 297.62 | DBO-83 | Cholinergic | Agonist |
| Sigma_D 8008 | 336.56 | 7,7-Dimethyl-(5Z,8Z)-eicosadienoic acid | Lipid | Inhibitor |
| Sigma_D 8040 | 465.44 | (±) trans-U-50488 methanesulfonate | Opioid | Agonist |
| Sigma_D 8065 | 168.15 | Dephostatin | Phosphorylation | Inhibitor |
| Sigma_D 8190 | 425.11 | 3',4'-Dichlorobenzamil | Ion Pump | Inhibitor |
| Sigma_D 8296 | 252.23 | 3-deazaadenosine | Immune System | Inhibitor |
| Sigma_G 5168 | 312.46 | (Z)-Guggulesterone | Lipid Signaling | Antagonist |
| Sigma_D 8399 | 337.47 | Danazol | Hormone | Inhibitor |
| Sigma_D 8555 | 436.62 | N,N-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide | Benzodiazepine | Ligand |
| Sigma_D 8690 | 320.44 | (R,R)-cis-Diethyl tetrahydro-2,8-chrysenediol | Hormone | Antagonist |
| Sigma_S 5567 | 220.23 | SP600125 | Phosphorylation | Inhibitor |
| Sigma_D 9035 | 230.67 | Diazoxide | K+ Channel | Activator |
| Sigma_D 9128 | 168.15 | 3,4-Dihydroxyphenylacetic acid | Dopamine | Metabolite |
| Sigma_D 9175 | 336.24 | Dantrolene sodium | Intracellular Calcium | Inhibitor |
| Sigma_D 9190 | 231.08 | DCEBIO | K+ Channel | Activator |
| Sigma_D 9305_a | 199.64 | 1-Deoxynojirimycin hydrochloride | Biochemistry | Inhibitor |
| Sigma_D 9628 | 197.19 | L-3,4-Dihydroxyphenylalanine | Dopamine | Precursor |
| Sigma_D 9766 | 504.64 | Dipyridamole | Adenosine | Inhibitor |
| Sigma_D 9815 | 547.59 | Doxazosin mesylate | Adrenoceptor | Blocker |
| Sigma_D 9891 | 480.91 | Doxycycline hydrochloride | Antibiotic | |
| Sigma_D-002 | 260.13 | 6,7-ADTN hydrobromide | Dopamine | Agonist |
| Sigma_D-003 | 317.82 | R(-)-Apocodeine hydrochloride | Dopamine | Agonist |
| Sigma_D-027 | 331.85 | R(-)-Propyl-norapomorphine hydrochloride | Dopamine | Agonist |
| Sigma_D-029 | 364.24 | R(-)-2,10,11-Trihydroxyaporphine hydrobromide | Dopamine | Agonist |
| Sigma_D-030 | 392.30 | R(-)-2,10,11-Trihydroxy-N-propyl-noraporphine hydrobromide | Dopamine | Agonist |

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| Sigma_D-031 | 318.26 | Dipropyldopamine hydrobromide | Dopamine | Agonist |
| Sigma_D-033 | 397.99 | (+)-Butaclamol hydrochloride | Dopamine | Antagonist |
| Sigma_D-042 | 374.28 | R(-)-N-Allylnorapomorphine hydrobromide | Dopamine | Agonist |
| Sigma_D-044 | 308.34 | Amfonelic acid | Dopamine | Modulator |
| Sigma_I 9532 | 311.30 | Icilin | Neurotransmission | Agonist |
| Sigma_D-047 | 291.78 | (±)-SKF-38393 hydrochloride | Dopamine | Antagonist |
| Sigma_D-052 | 523.50 | GBR-12909 dihydrochloride | Dopamine | Inhibitor |
| Sigma_D-054 | 324.25 | R(+)-SCH-23390 hydrochloride | Dopamine | Antagonist |
| Sigma_D-101 | 357.62 | (±)-DOI hydrochloride | Serotonin | Agonist |
| Sigma_D-103 | 226.53 | (±)-2,3-Dichloro-alpha-methylbenzylamine hydrochloride | Neurotransmission | Inhibitor |
| Sigma_D-104 | 451.35 | 4-DAMP methiodide | Cholinergic | Antagonist |
| Sigma_D-108 | 250.30 | 1,3-Dipropyl-7-methylxanthine | Adenosine | Antagonist |
| Sigma_D-122 | 425.92 | Domperidone | Dopamine | Antagonist |
| Sigma_D126608 | 178.28 | Propofol | Cholinergic | Inhibitor |
| Sigma_D-127 | 407.47 | Dextrorphan D-tartrate | Glutamate | Antagonist |
| Sigma_D-129 | 399.32 | R(+)-Butylindazone | Ion Pump | Inhibitor |
| Sigma_E 2031 | 347.86 | Eliprodil | Glutamate | Antagonist |
| Sigma_D-131 | 200.11 | 3,5-Dinitrocatechol | Neurotransmission | Inhibitor |
| Sigma_D-132 | 403.48 | N,N-Dipropyl-5-carboxamidotryptamine maleate | Serotonin | Agonist |
| Sigma_D-133 | 231.04 | 6,7-Dichloroquinoxaline-2,3-dione | Glutamate | Antagonist |
| Sigma_D-134 | 218.22 | 3,7-Dimethyl-I-propargylxanthine | Adenosine | Antagonist |
| Sigma_D-138 | 258.06 | 5,7-Dichlorokynurenic acid | Glutamate | Antagonist |
| Sigma_D-142 | 394.34 | 4-Diphenylacetoxy-N-(2-chloroethyl)piperidine hydrochloride | Cholinergic | Antagonist |
| Sigma_D14204 | 172.32 | 1,10-Diaminodecane | Glutamate | Agonist (inverse) |
| Sigma_D-149 | 356.26 | Dihydro-beta-erythroidine hydrobromide | Cholinergic | Antagonist |
| Sigma_D-155 | 707.85 | Dihydroergocristine methanesulfonate | Dopamine | Agonist |
| Sigma_D1920-6 | 126.12 | 2,6-Diamino-4-pyrimidinone | Phosphorylation | Inhibitor |
| Sigma_D-193 | 218.63 | DL-alpha-Difluoromethylornithine hydrochloride | Angiogenesis | Inhibitor |
| Sigma_S 4443 | 277.33 | SCH-28080 | Ion Channels | Inhibitor |
| Sigma_D-206 | 264.80 | S(-)-DS 121 hydrochloride | Dopamine | Antagonist |
| Sigma_E 0137 | 223.27 | Vanillic acid diethylamide | Vanilloid | Agonist |
| Sigma_E 0381 | 344.84 | Epibestatin hydrochloride | Biochemistry | Inhibitor |
| Sigma_E 0516 | 287.36 | Etodolac | Prostaglandin | Inhibitor |
| Sigma_E 1279 | 248.31 | Enoximone | Cyclic Nucleotides | Inhibitor |
| Sigma_E 1383 | 588.57 | Etoposide | Apoptosis | Inhibitor |
| Sigma_E 1779 | 523.74 | ET-18-OCH3 | Lipid | Inhibitor |
| Sigma_E 1896 | 325.80 | Etazolate hydrochloride | Adenosine | Inhibitor |
| Sigma_C 8863 | 370.46 | 7-Cyclopentyl-5-(4-phenoxy)phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine | Phosphorylation | Inhibitor |
| Sigma_E 2375 | 553.58 | Emetine dihydrochloride hydrate | Apoptosis | Activator |
| Sigma_E 2387 | 308.30 | 5'-N-Ethylcarboxamidoadenosine | Adenosine | Agonist |
| Sigma_E 3132 | 357.41 | E-64 | Biochemistry | Inhibitor |
| Sigma_S 3567 | 359.73 | SB 415286 | Phosphorylation | Inhibitor |
| Sigma_E 3149 | 185.09 | S-Ethylisothiourea hydrobromide | Nitric Oxide | Inhibitor |

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|--------------|--------|---|-----------------------|------------|
| Sigma_E 3256 | 201.70 | Edrophonium chloride | Cholinergic | Inhibitor |
| Sigma_E 3263 | 252.75 | Efaroxan hydrochloride | Imidazoline | Antagonist |
| Sigma_E 3380 | 246.31 | Ellipticine | Cell Cycle | Inhibitor |
| Sigma_E 3520 | 274.18 | Ebselen | Leukotriene | Inhibitor |
| Sigma_E 3645 | 522.71 | rac-2-Ethoxy-3-hexadecanamido-1-propylphosphocholine | Phosphorylation | Inhibitor |
| Sigma_E 3770 | 550.77 | rac-2-Ethoxy-3-octadecanamido-1-propylphosphocholine | Phosphorylation | Inhibitor |
| Sigma_E 3876 | 125.13 | N-Ethylmaleimide | Biochemistry | Inhibitor |
| Sigma_E 4375 | 333.30 | (-)-Epinephrine bitartrate | Adrenoceptor | Agonist |
| Sigma_E 4378 | 380.35 | Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid | Biochemistry | Inhibitor |
| Sigma_E 4642 | 219.67 | (±)-Epinephrine hydrochloride | Adrenoceptor | Agonist |
| Sigma_E 7138 | 141.17 | Ethosuximide | Anticonvulsant | |
| Sigma_E 7649 | 186.17 | Endothall | Phosphorylation | Inhibitor |
| Sigma_E 7881 | 270.24 | Emodin | Phosphorylation | Inhibitor |
| Sigma_E 8375 | 275.35 | (-)-Physostigmine | Cholinergic | Inhibitor |
| Sigma_N 3911 | 434.20 | NBI 27914 | Neurotransmission | Antagonist |
| Sigma_E 8875 | 272.39 | beta-Estradiol | Hormone | |
| Sigma_E 9750 | 270.37 | Estrone | Hormone | |
| Sigma_E-002 | 226.24 | Methyl beta-carboline-3-carboxylate | Benzodiazepine | Agonist |
| Sigma_E-006 | 225.25 | N-Methyl-beta-carboline-3-carboxamide | GABA | Antagonist |
| Sigma_E-007 | 314.34 | Methyl 6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate | Benzodiazepine | Agonist |
| Sigma_E-100 | 334.38 | (-)-Eseroline fumarate | Cholinergic | Inhibitor |
| Sigma_E-101 | 377.31 | S(-)-Eticlopride hydrochloride | Dopamine | Antagonist |
| Sigma_E-111 | 361.40 | (S)-ENBA | Adenosine | Agonist |
| Sigma_E-114 | 313.83 | erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride | Adenosine | Inhibitor |
| Sigma_E-140 | 609.73 | Ergocristine | Dopamine | Agonist |
| Sigma_F 0778 | 238.25 | Felbamate | Glutamate | Antagonist |
| Sigma_F 0881 | 538.71 | Fusidic acid sodium | Cell Cycle | Inhibitor |
| Sigma_F 1016 | 384.27 | Fenoterol hydrobromide | Adrenoceptor | Agonist |
| Sigma_F 1553 | 345.80 | S-(+)-Fluoxetine hydrochloride | Serotonin | Inhibitor |
| Sigma_F 1678 | 345.80 | R-(-)-Fluoxetine hydrochloride | Serotonin | Inhibitor |
| Sigma_F 2802 | 434.42 | Fluvoxamine maleate | Serotonin | Inhibitor |
| Sigma_F 2927 | 327.36 | 1-(4-Fluorobenzyl)-5-methoxy-2-methylindole-3-acetic acid | Multi-Drug Resistance | Inhibitor |
| Sigma_F 3764 | 275.24 | Furegrelate sodium | Phosphorylation | Inhibitor |
| Sigma_F 4303 | 592.12 | Fiduxosin hydrochloride | Adrenoceptor | Antagonist |
| Sigma_F 4381 | 330.75 | Furosemide | Ion Pump | Inhibitor |
| Sigma_F 4646 | 183.18 | p-Fluoro-L-phenylalanine | Neurotransmission | Substrate |
| Sigma_F 4765 | 510.45 | Fluphenazine dihydrochloride | Dopamine | Antagonist |
| Sigma_F 6020 | 360.84 | Fenofibrate | Transcription | Agonist |
| Sigma_F 6145 | 296.80 | Fenspiride hydrochloride | Adrenoceptor | Antagonist |
| Sigma_F 6300 | 303.30 | Flumazenil | Benzodiazepine | Antagonist |
| Sigma_F 6426 | 307.35 | Foliosidine | Anticonvulsant | |
| Sigma_F 6513 | 179.22 | Fusaric acid | Dopamine | Inhibitor |

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| Sigma_F 6627 | 130.08 | 5-Fluorouracil | Cell Cycle | Inhibitor |
| Sigma_F 6777 | 474.40 | Flecainide acetate | Na ⁺ Channel | Blocker |
| Sigma_F 6800 | 386.68 | Fenoldopam bromide | Dopamine | Agonist |
| Sigma_F 6886 | 410.51 | Forskolin | Cyclic Nucleotides | Activator |
| Sigma_F 6889 | 337.45 | Famotidine | Histamine | Antagonist |
| Sigma_F 7927 | 506.56 | FSCPX | Adenosine | Antagonist |
| Sigma_F 8175 | 358.55 | Farnesylthiosalicylic acid | G protein | Antagonist |
| Sigma_F 8257 | 477.43 | Flunarizine dihydrochloride | Ion Pump | Blocker |
| Sigma_F 8791 | 246.20 | 5-fluoro-5'-deoxyuridine | DNA Metabolism | Inhibitor |
| Sigma_F 8927 | 420.40 | Flupirtine maleate | Glutamate | Antagonist |
| Sigma_F 9397 | 276.22 | Flutamide | Hormone | Inhibitor |
| Sigma_F 9427 | 501.67 | Fexofenadine hydrochloride | Histamine | Antagonist |
| Sigma_F 9552 | 804.90 | Formoterol | Adrenoceptor | Agonist |
| Sigma_F 9677 | 384.26 | Felodipine | Ca ²⁺ Channel | Blocker |
| Sigma_F-100 | 475.59 | Fluspirilene | Dopamine | Antagonist |
| Sigma_F-114 | 507.45 | cis-(Z)-Flupenthixol dihydrochloride | Dopamine | Antagonist |
| Sigma_F-124 | 260.25 | Furafylline | Biochemistry | Inhibitor |
| Sigma_F-131 | 347.42 | FPL 64176 | Ca ²⁺ Channel | Activator |
| Sigma_F-132 | 345.80 | Fluoxetine hydrochloride | Serotonin | Inhibitor |
| Sigma_D 8816 | 268.36 | N-(3,3-Diphenylpropyl)glycinamide | Glutamate | Blocker |
| Sigma_G 0639 | 494.01 | Glibenclamide | K ⁺ Channel | Blocker |
| Sigma_G 0668 | 395.47 | GW2974 | Phosphorylation | Inhibitor |
| Sigma_G 1043 | 282.56 | Guanfacine hydrochloride | Adrenoceptor | Agonist |
| Sigma_G 2128 | 183.59 | L-Glutamic acid hydrochloride | Glutamate | Agonist |
| Sigma_G 2536 | 255.24 | Ganciclovir | Cell Cycle | Inhibitor |
| Sigma_G 3126 | 146.15 | L-Glutamine | Glutamate | Agonist |
| Sigma_G 3416 | 699.61 | Guanidinylnaltrindole di-trifluoroacetate | Opioid | Antagonist |
| Sigma_G 4788 | 398.19 | Guanidinoethyl disulfide dihydrobromide | Nitric Oxide | Inhibitor |
| Sigma_G 5668 | 495.58 | GW1929 | Transcription | Agonist |
| Sigma_G 6416 | 520.95 | GW5074 | Phosphorylation | Inhibitor |
| Sigma_G 6649 | 270.24 | Genistein | Phosphorylation | Inhibitor |
| Sigma_G 6793 | 502.77 | GW7647 | Transcription | Agonist |
| Sigma_G 7788 | 189.17 | alpha-Guanidinoglutaric acid | Nitric Oxide | Inhibitor |
| Sigma_G 8134 | 891.54 | Gallamine triethiodide | Cholinergic | Antagonist |
| Sigma_G 9659 | 487.52 | GBR-12935 dihydrochloride | Dopamine | Inhibitor |
| Sigma_G-002 | 163.61 | Isoguvacine hydrochloride | GABA | Agonist |
| Sigma_G-007 | 163.61 | Guvacine hydrochloride | GABA | Inhibitor |
| Sigma_G-017 | 267.08 | (±)-AMPA hydrobromide | Glutamate | Agonist |
| Sigma_G-019 | 195.02 | Muscimol hydrobromide | GABA | Agonist |
| Sigma_G-110 | 291.14 | Guanabenz acetate | Adrenoceptor | Agonist |
| Sigma_G-111 | 240.24 | gamma-D-Glutamylaminomethylsulfonic acid | Glutamate | Antagonist |
| Sigma_G-117 | 445.54 | Glipizide | K ⁺ Channel | Blocker |
| Sigma_G-119 | 329.79 | GYKI 52466 hydrochloride | Glutamate | Antagonist |
| Sigma_G-120 | 295.34 | GYKI 52895 | Dopamine | Inhibitor |
| Sigma_G-133 | 530.41 | GR-89696 fumarate | Opioid | Agonist |
| Sigma_G-154 | 171.24 | Gabapentin | Anticonvulsant | |

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|--------------|--------|--|-------------------|------------|
| Sigma_H 0126 | 356.26 | DL-Homatropine hydrobromide | Cholinergic | Antagonist |
| Sigma_H 0131 | 198.18 | (±)-Vanillylmandelic acid | Adrenoceptor | Metabolite |
| Sigma_H 0627 | 248.28 | 6-Hydroxymelatonin | Melatonin | Metabolite |
| Sigma_H 0879 | 362.19 | Hexamethonium bromide | Cholinergic | Antagonist |
| Sigma_H 1252 | 182.18 | 4-Hydroxy-3-methoxyphenylacetic acid | Dopamine | Metabolite |
| Sigma_H 1377 | 454.52 | MHPG piperazine | Adrenoceptor | Metabolite |
| Sigma_H 1384 | 109.15 | Hypotaourine | Cell Stress | Inhibitor |
| Sigma_H 1512 | 375.87 | Haloperidol | Dopamine | Antagonist |
| Sigma_H 1753 | 196.64 | Hydralazine hydrochloride | Neurotransmission | Inhibitor |
| Sigma_H 1877 | 134.57 | 4-Imidazolemethanol hydrochloride | Histamine | Inhibitor |
| Sigma_H 2138 | 273.29 | Hexamethonium dichloride | Cholinergic | Antagonist |
| Sigma_H 2270 | 484.53 | Hydrocortisone 21-hemisuccinate sodium | Hormone | |
| Sigma_H 2380 | 213.19 | 6-Hydroxy-DL-DOPA | Adrenoceptor | Neurotoxin |
| Sigma_H 2775 | 149.10 | DL-threo-beta-hydroxyaspartic acid | Glutamate | Inhibitor |
| Sigma_H 3146 | 330.34 | Hydroxytacrine maleate | Cholinergic | Inhibitor |
| Sigma_H 4001 | 362.47 | Hydrocortisone | Hormone | |
| Sigma_L 4408 | 42.39 | Lithium Chloride | Neurotransmission | Inhibitor |
| Sigma_H 4759 | 297.74 | Hydrochlorothiazide | Biochemistry | Inhibitor |
| Sigma_S 8817 | 396.45 | SB 218795 | Neurotransmission | Antagonist |
| Sigma_H 5257 | 246.22 | Hispidin | Phosphorylation | Inhibitor |
| Sigma_H 5752 | 330.47 | 17alpha-hydroxyprogesterone | Hormone | Metabolite |
| Sigma_H 6036 | 386.45 | 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole | Hormone | Agonist |
| Sigma_H 6892 | 206.27 | 1-(4-Hydroxybenzyl)imidazole-2-thiol | Dopamine | Inhibitor |
| Sigma_H 7250 | 184.07 | Histamine dihydrochloride | Histamine | Agonist |
| Sigma_H 7258 | 182.23 | Harmane | Imidazoline | Agonist |
| Sigma_H 7278 | 250.26 | NG-Hydroxy-L-arginine acetate | Nitric Oxide | Metabolite |
| Sigma_H 7779 | 391.56 | Retinoic acid p-hydroxyanilide | Cell Cycle | Inhibitor |
| Sigma_H 8034 | 388.43 | HE-NECA | Adenosine | Agonist |
| Sigma_H 8125 | 191.62 | L-Histidine hydrochloride | Histamine | Precursor |
| Sigma_H 8250 | 328.30 | (±)-8-Hydroxy-DPAT hydrobromide | Serotonin | Agonist |
| Sigma_H 8502 | 189.64 | Dopamine hydrochloride | Dopamine | Agonist |
| Sigma_H 8627 | 76.06 | Hydroxyurea | DNA Metabolism | Inhibitor |
| Sigma_H 8645 | 383.40 | (+)-Hydrastine | GABA | Antagonist |
| Sigma_H 8653 | 328.30 | (±)-7-Hydroxy-DPAT hydrobromide | Dopamine | Agonist |
| Sigma_H 8759 | 302.35 | MHPG sulfate potassium | Adrenoceptor | Metabolite |
| Sigma_H 8876 | 191.19 | 5-Hydroxyindolacetic acid | Serotonin | Metabolite |
| Sigma_H 9002 | 289.38 | L-Hyoscyamine | Cholinergic | Antagonist |
| Sigma_H 9003 | 110.11 | Hydroquinone | Leukotriene | Inhibitor |
| Sigma_B 8433 | 243.29 | BU99006 | Imidazoline | Ligand |
| Sigma_H 9382 | 211.09 | 3-Hydroxybenzylhydrazine dihydrochloride | Biochemistry | Inhibitor |
| Sigma_H 9523 | 212.68 | Serotonin hydrochloride | Serotonin | Agonist |
| Sigma_L 2167 | 402.45 | L-165,041 | Lipid Signaling | Agonist |
| Sigma_H 9772 | 220.23 | 5-Hydroxy-L-tryptophan | Serotonin | Precursor |
| Sigma_H 9876 | 69.49 | Hydroxylamine hydrochloride | Neurotransmission | Inhibitor |
| Sigma_H 9882 | 152.15 | 4-Hydroxybenzhydrazide | Biochemistry | Inhibitor |
| Sigma_H-108 | 574.36 | Hemicholinium-3 | Cholinergic | Blocker |
| Sigma_H-120 | 329.81 | HA-1004 hydrochloride | Phosphorylation | Inhibitor |

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| Sigma_H-121 | 364.30 | H-7 dihydrochloride | Phosphorylation | Inhibitor |
| Sigma_H-127 | 386.03 | Hexahydro-sila-difenidol hydrochloride, p-fluoro analog | Cholinergic | Antagonist |
| Sigma_H-128 | 198.10 | Histamine, R(-)-alpha-methyl-, dihydrochloride | Histamine | Agonist |
| Sigma_H-135 | 210.25 | 5-hydroxydecanoic acid sodium | K+ Channel | Blocker |
| Sigma_H-140 | 328.30 | R-(+)-8-Hydroxy-DPAT hydrobromide | Serotonin | Agonist |
| Sigma_H-168 | 328.30 | R-(+)-7-Hydroxy-DPAT hydrobromide | Dopamine | Agonist |
| Sigma_G 6043 | 524.59 | GR 125487 sulfamate salt | Serotonin | Antagonist |
| Sigma_I 0154 | 428.30 | IEM-1460 | Glutamate | Inhibitor |
| Sigma_I 0157 | 230.31 | Ibudilast | Cyclic Nucleotides | Inhibitor |
| Sigma_I 0375 | 162.58 | Imidazole-4-acetic acid hydrochloride | GABA | Antagonist |
| Sigma_I 0404 | 277.28 | Indirubin-3'-oxime | Phosphorylation | Inhibitor |
| Sigma_N 1786 | 310.39 | NSC 95397 | Phosphorylation | Inhibitor |
| Sigma_I 0782 | 240.27 | Imazodan | Cyclic Nucleotides | Inhibitor |
| Sigma_I 1149 | 184.96 | Iodoacetamide | Biochemistry | Inhibitor |
| Sigma_I 1392 | 313.81 | HA-100 | Phosphorylation | Inhibitor |
| Sigma_I 1637 | 412.37 | Ipratropium bromide | Cholinergic | Antagonist |
| Sigma_I 1656 | 497.51 | Idarubicin | DNA Metabolism | Inhibitor |
| Sigma_I 1899 | 358.18 | 2-Iodomelatonin | Melatonin | Agonist |
| Sigma_S 2318 | 431.39 | SB 228357 | Serotonin | Antagonist |
| Sigma_I 2279 | 374.68 | IMID-4F hydrochloride | K+ Channel | Blocker |
| Sigma_I 2760 | 361.35 | R(-)-Isoproterenol (+)-bitartrate | Adrenoceptor | Agonist |
| Sigma_I 2764 | 452.74 | ML-7 | Phosphorylation | Inhibitor |
| Sigma_I 2765 | 158.11 | (±)-Ibotenic acid | Glutamate | Agonist |
| Sigma_I 2892 | 801.00 | Ifenprodil tartrate | Glutamate | Blocker |
| Sigma_I 3639 | 335.42 | Isotharine mesylate | Adrenoceptor | Agonist |
| Sigma_I 3766 | 256.26 | Isoliquiritigenin | Cyclic Nucleotides | Activator |
| Sigma_I 4883 | 206.29 | (±)-Ibuprofen | Prostaglandin | Inhibitor |
| Sigma_I 5531 | 348.45 | IIK7 | Melatonin | Agonist |
| Sigma_I 5627 | 247.72 | (±)-Isoproterenol hydrochloride | Adrenoceptor | Agonist |
| Sigma_I 5879 | 222.25 | 3-Isobutyl-1-methylxanthine | Adenosine | Inhibitor |
| Sigma_I 6138 | 240.69 | Idazoxan hydrochloride | Imidazoline | Ligand |
| Sigma_I 6391 | 364.30 | 1-(5-Isoquinolinylsulfonyl)-3-methylpiperazine dihydrochloride | Phosphorylation | Inhibitor |
| Sigma_I 6504 | 247.72 | (-)-Isoproterenol hydrochloride | Adrenoceptor | Agonist |
| Sigma_I 7016 | 364.30 | 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride | Phosphorylation | Inhibitor |
| Sigma_I 7378 | 357.80 | Indomethacin | Prostaglandin | Inhibitor |
| Sigma_I 7379 | 316.88 | Imipramine hydrochloride | Serotonin | Blocker |
| Sigma_I 7388 | 179.14 | Isoxanthopterin | Cell Stress | Metabolite |
| Sigma_I 7627 | 277.22 | Iproniazid phosphate | Neurotransmission | Inhibitor |
| Sigma_I 8005 | 361.35 | S(+)-Isoproterenol (+)-bitartrate | Adrenoceptor | |
| Sigma_I 8021 | 223.70 | L-N6-(1-Iminoethyl)lysine hydrochloride | Nitric Oxide | Inhibitor |
| Sigma_I 8250 | 307.09 | 3-Iodo-L-tyrosine | Neurotransmission | Inhibitor |
| Sigma_I 8768 | 209.68 | L-N5-(1-Iminoethyl)ornithine hydrochloride | Nitric Oxide | Inhibitor |

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|--------------|--------|---|-----------------------|------------|
| Sigma_I 8898 | 875.12 | Ivermectin | Cholinergic | Modulator |
| Sigma_I 9531 | 280.76 | Imiloxan hydrochloride | Adrenoceptor | Antagonist |
| Sigma_I 9778 | 548.66 | CR 2945 | Cholecystokinin | Antagonist |
| Sigma_I 9890 | 648.26 | m-Iodobenzylguanidine hemisulfate | Apoptosis | Activator |
| Sigma_I-106 | 206.29 | S(+)-Ibuprofen | Prostaglandin | Inhibitor |
| Sigma_I-114 | 392.46 | p-Iodoclonidine hydrochloride | Adrenoceptor | Agonist |
| Sigma_I-117 | 357.24 | R(+)-IAA-94 | Cl- Channel | Inhibitor |
| Sigma_I-119 | 328.67 | Indatraline hydrochloride | Dopamine | Inhibitor |
| Sigma_I-120 | 339.65 | Iofetamine hydrochloride | Neurotransmission | Analog |
| Sigma_I-122 | 501.84 | ICI 204,448 hydrochloride | Opioid | Agonist |
| Sigma_I-127 | 313.87 | ICI 118,551 hydrochloride | Adrenoceptor | Antagonist |
| Sigma_I-135 | 332.06 | Imetit dihydrobromide | Histamine | Agonist |
| Sigma_I-138 | 161.16 | 1,5-Isoquinolinediol | Apoptosis | Inhibitor |
| Sigma_M 1818 | 312.84 | Molindone hydrochloride | Dopamine | Antagonist |
| Sigma_I-146 | 510.29 | IB-MECA | Adenosine | Agonist |
| Sigma_I-151 | 426.90 | Indomethacin morpholinylamide | Cannabinoid | Ligand |
| Sigma_I-160 | 364.83 | 3-(1H-Imidazol-4-yl)propyl di(p-fluorophenyl)methyl ether hydrochloride | Histamine | Antagonist |
| Sigma_I18008 | 129.16 | Isonipecotic acid | GABA | Agonist |
| Sigma_J 4252 | 327.43 | JWH-015 | Cannabinoid | Agonist |
| Sigma_J-102 | 307.40 | JL-18 | Dopamine | Antagonist |
| Sigma_K 0250 | 213.24 | Kainic acid | Glutamate | Agonist |
| Sigma_K 1003 | 531.44 | Ketoconazole | Multi-Drug Resistance | Inhibitor |
| Sigma_K 1136 | 376.41 | Ketorolac tris salt | Prostaglandin | Inhibitor |
| Sigma_K 1751 | 254.29 | Ketoprofen | Prostaglandin | Inhibitor |
| Sigma_K 1888 | 376.50 | K 185 | Melatonin | Antagonist |
| Sigma_K 2628 | 425.51 | Ketotifen fumarate | Histamine | Antagonist |
| Sigma_K 3375 | 189.17 | Kynurenic acid | Glutamate | Antagonist |
| Sigma_K 3888 | 327.18 | Kenpaullone | Phosphorylation | Inhibitor |
| Sigma_K 4262 | 377.53 | Karakoline | Cholinergic | Antagonist |
| Sigma_L 0258 | 363.80 | L-701,324 | Glutamate | Antagonist |
| Sigma_L 0664 | 246.31 | loxoprofen | Prostaglandin | Inhibitor |
| Sigma_L 1011 | 364.88 | Labetalol hydrochloride | Adrenoceptor | Antagonist |
| Sigma_L 1415 | 582.79 | L-162,313 | Neurotransmission | Agonist |
| Sigma_L 1788 | 256.78 | Lidocaine N-methyl hydrochloride | Na+ Channel | Blocker |
| Sigma_L 2037 | 242.28 | beta-Lapachone | Apoptosis | Activator |
| Sigma_L 2411 | 452.55 | LY-367,265 | Serotonin | Antagonist |
| Sigma_L 2536 | 430.95 | LY-310,762 hydrochloride | Serotonin | Antagonist |
| Sigma_L 2540 | 591.24 | L-368,899 | Neurotransmission | Antagonist |
| Sigma_L 2906 | 387.82 | Lomefloxacin hydrochloride | Antibiotic | Inhibitor |
| Sigma_L 3791 | 256.10 | Lamotrigine | Anticonvulsant | |
| Sigma_L 4376 | 373.93 | alpha-Lobeline hydrochloride | Cholinergic | Agonist |
| Sigma_L 4762 | 513.51 | Loperamide hydrochloride | Opioid | Ligand |
| Sigma_L 4900 | 321.17 | Lonidamine | Cell Stress | Inhibitor |
| Sigma_L 5025 | 270.21 | Leflunomide | Immune System | Inhibitor |
| Sigma_V 1889 | 626.39 | VER-3323 hemifumarate salt | Serotonin | Agonist |

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|-------------------|--------|---|--------------------------|--------------|
| Sigma_L 5647 | 270.81 | Lidocaine hydrochloride | Na ⁺ Channel | Modulator |
| Sigma_L 5783 | 343.31 | Lidocaine N-ethyl bromide quaternary salt | Na ⁺ Channel | Antagonist |
| Sigma_L 8397 | 337.42 | L-Leucinethiol, oxidized dihydrochloride | Biochemistry | Inhibitor |
| Sigma_L 8401 | 290.41 | LE 300 | Dopamine | Antagonist |
| Sigma_L 8533 | 369.37 | Lansoprazole | Ion Pump | Inhibitor |
| Sigma_L 8539 | 327.90 | L-687,384 hydrochloride | Opioid | Agonist |
| Sigma_L 8789 | 360.01 | LFM-A13 | Phosphorylation | Inhibitor |
| Sigma_N 0287 | 564.58 | NNC 55-0396 | Ca ²⁺ Channel | Inhibitor |
| Sigma_L 9539 | 373.86 | L-655,240 | Thromboxane | Antagonist |
| Sigma_L 9664 | 382.89 | Loratadine | Histamine | Antagonist |
| Sigma_L 9756 | 240.76 | (-)-Tetramisole hydrochloride | Phosphorylation | Inhibitor |
| Sigma_L 9787 | 341.37 | L-655,708 | Benzodiazepine | Ligand |
| Sigma_L 9908 | 343.81 | LY-294,002 hydrochloride | Phosphorylation | Inhibitor |
| Sigma_L-106 | 445.91 | Loxapine succinate | Dopamine | Antagonist |
| Sigma_L-107 | 500.60 | LY-53,857 maleate | Serotonin | Antagonist |
| Sigma_L-109 | 481.40 | Lorglumide sodium | Cholecystokinin | Antagonist |
| Sigma_L-110 | 414.47 | LY-278,584 maleate | Serotonin | Antagonist |
| Sigma_P 0618_a | 312.25 | cis(+/-)-8-OH-PBZI hydrobromide | Dopamine | Agonist |
| Sigma_L-119 | 598.49 | L-703,606 oxalate | Tachykinin | Antagonist |
| Sigma_L-121 | 433.51 | Levallorphan tartrate | Opioid | Antagonist |
| Sigma_L-122 | 338.46 | S-(-)-Lisuride | Dopamine | Agonist |
| Sigma_L-131 | 363.29 | L-745,870 hydrochloride | Dopamine | Antagonist |
| Sigma_L-133 | 527.67 | L-750,667 trihydrochloride | Dopamine | Antagonist |
| Sigma_L-134 | 391.48 | Linopirdine | Cholinergic | Releaser |
| Sigma_L-135 | 340.86 | L-741,626 | Dopamine | Antagonist |
| Sigma_L-137 | 439.83 | L-733,060 hydrochloride | Tachykinin | Antagonist |
| Sigma_M 0763 | 336.26 | Metoclopramide hydrochloride | Dopamine | Antagonist |
| Sigma_M 0814 | 335.23 | R(-)-Me5 | Na ⁺ Channel | Antagonist |
| Sigma_M 1022 | 307.44 | Dihydrocapsaicin | Vanilloid | Agonist |
| Sigma_M 1275 | 252.25 | (-)-Naproxen sodium | Prostaglandin | Inhibitor |
| Sigma_M 1387 | 118.57 | 4-Methylpyrazole hydrochloride | Biochemistry | Inhibitor |
| Sigma_M 1404 | 301.33 | Nocodazole | Cytoskeleton and ECM | Inhibitor |
| Sigma_M 1514 | 280.28 | N-omega-Methyl-5-hydroxytryptamine oxalate salt | Serotonin | Ligand |
| Sigma_M 1559 | 278.14 | Moxonidine hydrochloride | Adrenoceptor | Agonist |
| Sigma_M 1692 | 398.42 | MRS 1845 | Ca ²⁺ Channel | Inhibitor |
| Sigma_D 9305_b | 199.64 | 1-Deoxynojirimycin hydrochloride | Biochemistry | Inhibitor |
| Sigma_M 1809 | 399.56 | MRS 1523 | Adenosine | Antagonist |
| Sigma_M 2011 | 305.21 | Melphalan | DNA Metabolism | Intercalator |
| Sigma_M 2398 | 520.60 | Metaproterenol hemisulfate | Adrenoceptor | Agonist |
| Sigma_M 2525 | 300.83 | Mianserin hydrochloride | Serotonin | Antagonist |
| Sigma_M 2537 | 390.52 | Mevastatin | Antibiotic | Inhibitor |
| Sigma_M 2547 | 266.30 | 8-Methoxymethyl-3-isobutyl-1-methylxanthine | Cyclic Nucleotides | Inhibitor |
| Sigma_M 2692 | 494.08 | MK-886 | Leukotriene | Inhibitor |
| Sigma_M 2727 | 215.73 | Mexiletene hydrochloride | Na ⁺ Channel | Blocker |

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|--------------|--------|--|--------------------|------------|
| Sigma_M 2776 | 455.52 | Methylergonovine maleate | Dopamine | Antagonist |
| Sigma_M 2901 | 242.24 | Molsidomine | Nitric Oxide | Donor |
| Sigma_M 2922 | 278.24 | 3-Methyl-6-(3-[trifluoromethyl]phenyl)-1,2,4-triazolo[4,3-b]pyridazine | Benzodiazepine | Agonist |
| Sigma_M 3047 | 259.22 | Mizoribine | DNA Metabolism | Inhibitor |
| Sigma_M 3127 | 278.37 | S-Methylisothiurea hemisulfate | Nitric Oxide | Inhibitor |
| Sigma_M 3184 | 437.37 | MG 624 | Cholinergic | Antagonist |
| Sigma_M 3262 | 147.13 | N-Methyl-D-aspartic acid | Glutamate | Agonist |
| Sigma_M 3281 | 245.71 | alpha-Methyl-DL-tyrosine methyl ester hydrochloride | Neurotransmission | Inhibitor |
| Sigma_M 3315 | 514.49 | MJ33 | Lipid | Inhibitor |
| Sigma_M 3668 | 403.53 | Metergoline | Serotonin | Antagonist |
| Sigma_U-106 | 519.43 | (-)-cis-(1S,2R)-U-50488 tartrate | Neurotransmission | Ligand |
| Sigma_M 3778 | 308.64 | Clorgyline hydrochloride | Neurotransmission | Inhibitor |
| Sigma_M 3808 | 459.30 | MRS 2179 | P2 Receptor | Antagonist |
| Sigma_M 3935 | 373.39 | Meloxicam sodium | Prostaglandin | Inhibitor |
| Sigma_M 4008 | 302.24 | Morin | Cell Stress | Inhibitor |
| Sigma_M 4145 | 209.25 | Minoxidil | K+ Channel | Activator |
| Sigma_M 4531 | 318.14 | Meclofenamic acid sodium | Prostaglandin | Inhibitor |
| Sigma_M 4659 | 211.23 | Milrinone | Cyclic Nucleotides | Inhibitor |
| Sigma_M 4796 | 209.20 | (±)-alpha-Methyl-4-carboxyphenylglycine | Glutamate | Antagonist |
| Sigma_M 4910 | 198.10 | 1-Methylhistamine dihydrochloride | Histamine | Metabolite |
| Sigma_M 5154 | 315.84 | Moxisylyte hydrochloride | Adrenoceptor | Antagonist |
| Sigma_M 5171 | 265.33 | S-Methyl-L-thiocitrulline acetate | Nitric Oxide | Inhibitor |
| Sigma_M 5250 | 232.28 | Melatonin | Melatonin | Agonist |
| Sigma_M 5379 | 180.23 | L-Methionine sulfoximine | Glutamate | Inhibitor |
| Sigma_M 5391 | 684.83 | (±)-Metoprolol (+)-tartrate | Adrenoceptor | Antagonist |
| Sigma_M 5435 | 229.71 | 6-Methyl-2-(phenylethynyl)pyridine hydrochloride | Glutamate | Antagonist |
| Sigma_M 5441 | 568.56 | Mibefradil dihydrochloride | Ca2+ Channel | Blocker |
| Sigma_M 5501 | 281.27 | N6-Methyladenosine | Adenosine | Agonist |
| Sigma_M 5560 | 232.60 | (S)-MAP4 hydrochloride | Glutamate | Antagonist |
| Sigma_M 5644 | 521.10 | (±)-Methoxyverapamil hydrochloride | Ca2+ Channel | Antagonist |
| Sigma_M 5685 | 276.29 | Metrazoline oxalate | Imidazoline | Ligand |
| Sigma_M 6191 | 276.68 | GW9662 | Transcription | Inhibitor |
| Sigma_M 6316 | 486.53 | MRS 1754 | Adenosine | Antagonist |
| Sigma_M 6383 | 302.42 | 2-methoxyestradiol | Hormone | Metabolite |
| Sigma_M 6500 | 113.61 | Cysteamine hydrochloride | Somatostatin | Depleter |
| Sigma_M 6517 | 517.08 | alpha,beta-Methylene adenosine 5'-triphosphate dilithium | P2 Receptor | Agonist |
| Sigma_M 6524 | 247.72 | Methoxamine hydrochloride | Adrenoceptor | Agonist |
| Sigma_M 6545 | 517.41 | Mitoxantrone | DNA Metabolism | Inhibitor |
| Sigma_M 6628 | 226.71 | O-Methylserotonin hydrochloride | Serotonin | Agonist |
| Sigma_M 6680 | 218.54 | Se-(methyl)selenocysteine hydrochloride | Cell Cycle | Inhibitor |
| Sigma_M 6690 | 382.46 | MDL 28170 | Cell Cycle | Inhibitor |
| Sigma_M 6760 | 318.24 | Myricetin | Phosphorylation | Inhibitor |
| Sigma_M 7033 | 248.28 | NG-Monomethyl-L-arginine acetate | Nitric Oxide | Inhibitor |
| Sigma_M 7065 | 375.90 | MK-912 | Adrenoceptor | Agonist |

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|--------------|--------|---|-------------------|------------|
| Sigma_M 7277 | 211.22 | (±)-3-(3,4-dihydroxyphenyl)-2-methyl-DL-alanine | Neurotransmission | Inhibitor |
| Sigma_M 7684 | 461.21 | MRS 2159 | P2 Receptor | Antagonist |
| Sigma_G 5793 | 534.06 | GR 127935 hydrochloride | Serotonin | Antagonist |
| Sigma_D 8941 | 402.44 | 2,6-Difluoro-4-[2-(phenylsulfonylamino)ethylthio]phenoxyacetamide | Glutamate | Agonist |
| Sigma_M 8046 | 429.61 | Mifepristone | Hormone | Antagonist |
| Sigma_M 8131 | 195.22 | L-alpha-Methyl-p-tyrosine | Neurotransmission | Inhibitor |
| Sigma_S 1068 | 337.81 | SB-215505 | Serotonin | Antagonist |
| Sigma_M 8878 | 82.11 | 1-Methylimidazole | Prostaglandin | Inhibitor |
| Sigma_M 9020 | 203.76 | Mecamylamine hydrochloride | Cholinergic | Antagonist |
| Sigma_M 9125 | 297.85 | Methapyrilene hydrochloride | Histamine | Antagonist |
| Sigma_M 9292 | 215.77 | Memantine hydrochloride | Glutamate | Antagonist |
| Sigma_M 9440 | 168.15 | Me-3,4-dephostatin | Phosphorylation | Inhibitor |
| Sigma_M 9511 | 492.96 | Minocycline hydrochloride | Cell Cycle | Inhibitor |
| Sigma_M 9651 | 313.87 | Maprotiline hydrochloride | Adrenoceptor | Inhibitor |
| Sigma_M 9656 | 338.26 | H-8 dihydrochloride | Phosphorylation | Inhibitor |
| Sigma_M-001 | 334.42 | Proglumide | Cholecystokinin | Antagonist |
| Sigma_M-104 | 209.72 | (±)-Muscarine chloride | Cholinergic | Agonist |
| Sigma_M-105 | 728.77 | Methocramine tetrahydrochloride | Cholinergic | Antagonist |
| Sigma_M-107 | 337.38 | (+)-MK-801 hydrogen maleate | Glutamate | Antagonist |
| Sigma_M-108 | 337.38 | (-)-MK-801 hydrogen maleate | Glutamate | Antagonist |
| Sigma_M-109 | 306.32 | 2-Methyl-5-hydroxytryptamine maleate | Serotonin | Agonist |
| Sigma_M-110 | 306.32 | alpha-Methyl-5-hydroxytryptamine maleate | Serotonin | Agonist |
| Sigma_M-116 | 365.84 | Metolazone | Ion Pump | Inhibitor |
| Sigma_M-120 | 396.57 | Metaphit methanesulfonate | Opioid | Antagonist |
| Sigma_M-129 | 211.22 | L-alpha-Methyl DOPA | Biochemistry | Inhibitor |
| Sigma_M-137 | 469.54 | Methysergide maleate | Serotonin | Antagonist |
| Sigma_M-140 | 196.68 | Methylcarbamylocholine chloride | Cholinergic | Agonist |
| Sigma_M-149 | 452.66 | Methiothepin mesylate | Serotonin | Antagonist |
| Sigma_M-152 | 539.24 | 2-Methylthioadenosine diphosphate trisodium | P2 Receptor | Agonist |
| Sigma_M-153 | 397.97 | Mesulergine hydrochloride | Dopamine | Agonist |
| Sigma_M-166 | 280.67 | MDL 26,630 trihydrochloride | Glutamate | Agonist |
| Sigma_Z 4626 | 367.92 | ZM 39923 hydrochloride | Phosphorylation | Inhibitor |
| Sigma_M-184 | 206.63 | 3-Morpholinopyridone hydrochloride | Nitric Oxide | Donor |
| Sigma_M-187 | 293.84 | 3-Methoxy-morphanin hydrochloride | Glutamate | Antagonist |
| Sigma_M-204 | 578.88 | p-MPPI hydrochloride | Serotonin | Antagonist |
| Sigma_M-216 | 376.20 | MDL 105,519 | Glutamate | Antagonist |
| Sigma_M-225 | 371.40 | Metrifudil | Adenosine | Agonist |
| Sigma_M-226 | 507.44 | p-MPPF dihydrochloride | Serotonin | Antagonist |
| Sigma_M-231 | 391.90 | (-)-3-Methoxynaltrexone hydrochloride | Opioid | Antagonist |
| Sigma_N 0630 | 282.22 | Niflumic acid | Prostaglandin | Inhibitor |
| Sigma_N 1016 | 308.31 | Nimesulide | Prostaglandin | Inhibitor |
| Sigma_N 1392 | 298.35 | Nialamide | Neurotransmission | Inhibitor |
| Sigma_N 1530 | 354.41 | Nomifensine maleate | Dopamine | Inhibitor |
| Sigma_N 1771 | 734.73 | nor-Binaltorphimine dihydrochloride | Opioid | Antagonist |
| Sigma_N 2001 | 303.20 | Neostigmine bromide | Cholinergic | Inhibitor |
| Sigma_N 2034 | 256.35 | CR 2249 | Glutamate | Agonist |

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|--------------|---------|--|-----------------|--------------|
| Sigma_N 2255 | 419.42 | S-(4-Nitrobenzyl)-6-thioinosine | Adenosine | Inhibitor |
| Sigma_N 3136 | 377.87 | Naltrexone hydrochloride | Opioid | Antagonist |
| Sigma_N 3398 | 220.25 | S-Nitroso-N-acetylpenicillamine | Nitric Oxide | Donor |
| Sigma_N 3510 | 327.13 | Niclosamide | Antibiotic | |
| Sigma_N 3529 | 474.40 | NAN-190 hydrobromide | Serotonin | Antagonist |
| Sigma_N 4034 | 234.62 | NCS-356 | GABA | Agonist |
| Sigma_N 4148 | 336.33 | S-Nitrosoglutathione | Nitric Oxide | Donor |
| Sigma_N 4159 | 242.25 | NCS-382 | GABA | Antagonist |
| Sigma_N 4382 | 254.22 | Nalidixic acid sodium | Antibiotic | Inhibitor |
| Sigma_N 4396 | 393.91 | Nalbuphine hydrochloride | Opioid | Antagonist |
| Sigma_N 4779 | 300.32 | 5-Nitro-2-(3-phenylpropylamino)benzoic acid | Cl- Channel | Blocker |
| Sigma_N 4784 | 1505.10 | NF449 octasodium salt | G protein | Antagonist |
| Sigma_N 5023 | 302.37 | Nordihydroguaiaretic acid from Larrea divaricata (creosote bush) | Leukotriene | Inhibitor |
| Sigma_N 5260 | 462.41 | (-)-Nicotine hydrogen tartrate salt | Cholinergic | Agonist |
| Sigma_N 5501 | 219.20 | NG-Nitro-L-arginine | Nitric Oxide | Inhibitor |
| Sigma_N 5504 | 246.74 | Naphazoline hydrochloride | Adrenoceptor | Agonist |
| Sigma_N 5636 | 119.08 | 3-Nitropropionic acid | Cell Stress | Toxin |
| Sigma_N 5751 | 269.69 | NG-Nitro-L-arginine methyl ester hydrochloride | Nitric Oxide | Inhibitor |
| Sigma_N 7127 | 219.67 | (±)-Normetanephrine hydrochloride | Adrenoceptor | Metabolite |
| Sigma_N 7261 | 299.85 | Nortriptyline hydrochloride | Adrenoceptor | Inhibitor |
| Sigma_N 7505 | 833.36 | NADPH tetrasodium | Nitric Oxide | Cofactor |
| Sigma_N 7510 | 516.00 | Nicardipine hydrochloride | Ca2+ Channel | Antagonist |
| Sigma_N 7634 | 346.34 | Nifedipine | Ca2+ Channel | Antagonist |
| Sigma_N 7758 | 363.84 | Naloxone hydrochloride | Opioid | Antagonist |
| Sigma_N 7778 | 163.14 | 7-Nitroindazole | Nitric Oxide | Inhibitor |
| Sigma_N 7904 | 363.42 | NS 521 oxalate | Glutamate | Modulator |
| Sigma_N 7906 | 369.25 | 2-(alpha-Naphthoyl)ethyltrimethylammonium iodide | Cholinergic | Inhibitor |
| Sigma_N 8403 | 175.15 | 6-Nitroso-1,2-benzopyrone | Transcription | Inhibitor |
| Sigma_N 8534 | 317.23 | Nilutamide | Hormone | Inhibitor |
| Sigma_N 8652 | 1162.89 | NF 023 | P2 Receptor | Antagonist |
| Sigma_N 8659 | 272.70 | Nimustine hydrochloride | DNA | Intercalator |
| Sigma_N 8784 | 168.15 | Norcantharidin | Phosphorylation | Inhibitor |
| Sigma_N 9007 | 449.89 | Noscapine hydrochloride | Opioid | Ligand |
| Sigma_N 9765 | 548.60 | (+)-Nicotine (+)-di-p-toluoyl tartrate | Cholinergic | Agonist |
| Sigma_N-115 | 450.97 | Naltrindole hydrochloride | Opioid | Antagonist |
| Sigma_S 6319 | 342.70 | Sertraline hydrochloride | Serotonin | Inhibitor |
| Sigma_N-142 | 386.88 | NO-711 hydrochloride | GABA | Inhibitor |
| Sigma_N-144 | 360.37 | Nitrendipine | Ca2+ Channel | Antagonist |
| Sigma_N-149 | 418.45 | Nimodipine | Ca2+ Channel | Antagonist |
| Sigma_N-151 | 307.82 | Nisoxetine hydrochloride | Adrenoceptor | Blocker |
| Sigma_N-153 | 335.88 | Nylidrin hydrochloride | Adrenoceptor | Agonist |
| Sigma_N-154 | 217.28 | N6-Cyclopentyl-9-methyladenine | Adenosine | Antagonist |
| Sigma_N-156 | 511.60 | Naltriben methanesulfonate | Opioid | Antagonist |
| Sigma_N-158 | 465.42 | Naftopidil dihydrochloride | Adrenoceptor | Antagonist |
| Sigma_B 9305 | 368.48 | BW 245C | Prostanoids | Agonist |

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|----------------|--------|---|-----------------------|------------|
| Sigma_N-165 | 445.52 | Naloxone benzoylhydrazone | Opioid | Agonist |
| Sigma_N-170 | 362.23 | NS-1619 | K+ Channel | Activator |
| Sigma_N-176 | 723.70 | Naloxonazine dihydrochloride | Opioid | Antagonist |
| Sigma_N-183 | 380.25 | NBQX disodium | Glutamate | Antagonist |
| Sigma_N-211 | 269.06 | NS 2028 | Cyclic Nucleotides | Inhibitor |
| Sigma_O 0250 | 189.64 | (±)-Octopamine hydrochloride | Adrenoceptor | Agonist |
| Sigma_O 0383 | 325.54 | N-Oleylethanolamine | Sphingolipid | Inhibitor |
| Sigma_O 0877 | 261.24 | Oxolinic acid | Antibiotic | Inhibitor |
| Sigma_O 0886 | 298.35 | Olomoucine | Phosphorylation | Inhibitor |
| Sigma_O 1008 | 282.47 | Oleic Acid | Phosphorylation | Activator |
| Sigma_O 2378 | 296.84 | Oxymetazoline hydrochloride | Adrenoceptor | Agonist |
| Sigma_O 2751 | 111.03 | Sodium Oxamate | Biochemistry | Inhibitor |
| Sigma_O 2881 | 393.96 | Oxybutynin Chloride | Cholinergic | Antagonist |
| Sigma_O 3011 | 158.16 | Oxiracetam | Nootropic | |
| Sigma_O 3125 | 584.67 | Ouabain | Ion Pump | Inhibitor |
| Sigma_O 3636 | 187.16 | ODQ | Cyclic Nucleotides | Inhibitor |
| Sigma_O 3752 | 305.85 | Orphenadrine hydrochloride | Cholinergic | Antagonist |
| Sigma_T 5575 | 249.33 | TG003 | Cell Cycle | Inhibitor |
| Sigma_O 8757 | 361.38 | Ofloxacin | Antibiotic | |
| Sigma_O 9126 | 760.80 | Oxotremorine sesquifumarate salt | Cholinergic | Agonist |
| Sigma_O 9387 | 426.57 | Oxatomide | Immune System | Modulator |
| Sigma_S 3442 | 371.23 | SB 216763 | Phosphorylation | Inhibitor |
| Sigma_O 9637 | 293.33 | Oxaprozin | Prostaglandin | Inhibitor |
| Sigma_O-100 | 322.19 | Oxotremorine methiodide | Cholinergic | Agonist |
| Sigma_O-111 | 460.98 | (±)-Octoclothepein maleate | Dopamine | Antagonist |
| Sigma_P 0130 | 314.47 | Progesterone | Hormone | |
| Sigma_P 0359 | 299.50 | Palmitoylethanolamide | Cannabinoid | Agonist |
| Sigma_P 0453 | 244.25 | Piceatannol | Phosphorylation | Inhibitor |
| Sigma_P 0547 | 592.69 | Pentamidine isethionate | Glutamate | Antagonist |
| Sigma_P 0618_b | 312.25 | cis-(±)-8-OH-PBZI hydrobromide | Dopamine | Agonist |
| Sigma_P 0667 | 248.32 | Parthenolide | Serotonin | Inhibitor |
| Sigma_P 0778 | 248.33 | Pindolol | Adrenoceptor | Antagonist |
| Sigma_P 0878 | 185.07 | O-Phospho-L-serine | Glutamate | Antagonist |
| Sigma_P 0884 | 295.81 | (±)-Propranolol hydrochloride | Adrenoceptor | Antagonist |
| Sigma_P 1061 | 389.97 | SKF-525A hydrochloride | Multi-Drug Resistance | Inhibitor |
| Sigma_P 1675 | 602.60 | Picrotoxin | GABA | Antagonist |
| Sigma_P 1726 | 187.16 | 4-Phenyl-3-furoxanarbonitrile | Nitric Oxide | Donor |
| Sigma_P 1784 | 278.31 | Pentoxifylline | Cyclic Nucleotides | Inhibitor |
| Sigma_P 1793 | 461.56 | Pimozide | Dopamine | Antagonist |
| Sigma_P 1801 | 277.24 | L-Glutamic acid, N-phthaloyl- | Glutamate | Agonist |
| Sigma_P 1918 | 732.69 | Pancuronium bromide | Cholinergic | Antagonist |
| Sigma_P 2016 | 334.50 | 3- α ,21-Dihydroxy-5- α -pregnan-20-one | GABA | Modulator |
| Sigma_P 2116 | 185.23 | Pirfenidone | Immune System | Inhibitor |

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|--------------|--------|--|--------------------------|--------------|
| Sigma_P 2278 | 256.27 | 1,3-Dimethyl-8-phenylxanthine | Adenosine | Antagonist |
| Sigma_P 2607 | 353.89 | PRE-084 | Opioid | Agonist |
| Sigma_P 2738 | 694.37 | PPNDS tetrasodium | P2 Receptor | Antagonist |
| Sigma_P 2742 | 217.29 | PD 404,182 | Biochemistry | Inhibitor |
| Sigma_P 3510 | 375.86 | Papaverine hydrochloride | Cyclic Nucleotides | Inhibitor |
| Sigma_P 3520 | 538.60 | Pentolinium di[L(+)-tartrate] | Cholinergic | Antagonist |
| Sigma_P 4015 | 235.33 | 1-Phenyl-3-(2-thiazolyl)-2-thiourea | Dopamine | Inhibitor |
| Sigma_T 9567 | 210.30 | Thiolactomycin | Antibiotic | Inhibitor |
| Sigma_P 4394 | 300.06 | Cisplatin | DNA | Intercalator |
| Sigma_P 4405 | 414.42 | Podophyllotoxin | Cytoskeleton and ECM | Inhibitor |
| Sigma_S 1693 | 241.25 | SU 9516 | Cell Cycle | Inhibitor |
| Sigma_P 4509 | 436.08 | Palmitoyl-DL-Carnitine chloride | Phosphorylation | Modulator |
| Sigma_P 4532 | 385.43 | R(-)-N6-(2-Phenylisopropyl)adenosine | Adenosine | Agonist |
| Sigma_P 4543 | 166.20 | Valproic acid sodium | Anticonvulsant | |
| Sigma_P 4651 | 320.89 | Promethazine hydrochloride | Histamine | Antagonist |
| Sigma_P 4668 | 312.42 | Praziquantel | Antibiotic | |
| Sigma_P 4670 | 377.92 | Propafenone hydrochloride | K ⁺ Channel | Blocker |
| Sigma_P 5052 | 332.49 | 5alpha-Pregnan-3alpha-ol-11,20-dione | GABA | Modulator |
| Sigma_P 5114 | 305.37 | Pempidine tartrate | Cholinergic | Antagonist |
| Sigma_P 5295 | 142.16 | Piracetam | Glutamate | Modulator |
| Sigma_P 5396 | 182.02 | Phosphomycin disodium | Antibiotic | |
| Sigma_P 5514 | 401.47 | Pyrilamine maleate | Histamine | Antagonist |
| Sigma_P 5654 | 331.35 | Piroxicam | Prostaglandin | Inhibitor |
| Sigma_P 5679 | 194.19 | 3-n-Propylxanthine | Adenosine | Antagonist |
| Sigma_P 6126 | 203.67 | Phenylephrine hydrochloride | Adrenoceptor | Agonist |
| Sigma_P 6402 | 403.98 | Perphenazine | Dopamine | Antagonist |
| Sigma_P 6500 | 138.17 | Pentylentetrazole | Neurotransmission | Modulator |
| Sigma_P 6503 | 244.72 | (+)-Pilocarpine hydrochloride | Cholinergic | Agonist |
| Sigma_P 6628 | 271.28 | Pilocarpine nitrate | Cholinergic | Agonist |
| Sigma_P 6656 | 320.89 | Promazine hydrochloride | Dopamine | Antagonist |
| Sigma_P 6777 | 234.28 | Phenelzine sulfate | Neurotransmission | Inhibitor |
| Sigma_P 6902 | 356.43 | Pheniramine maleate | Histamine | Antagonist |
| Sigma_P 6909 | 140.03 | Phosphonoacetic acid | DNA | Inhibitor |
| Sigma_P 7083 | 166.22 | (-)-Perillic acid | G protein | Inhibitor |
| Sigma_P 7136 | 123.12 | Pyrazinecarboxamide | Antibiotic | |
| Sigma_P 7295 | 218.26 | Primidone | Anticonvulsant | |
| Sigma_P 7340 | 427.03 | (±)-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride | Sphingolipid | Inhibitor |
| Sigma_P 7412 | 424.33 | Pirenzepine dihydrochloride | Cholinergic | Antagonist |
| Sigma_P 7505 | 161.08 | Putrescine dihydrochloride | Glutamate | Agonist |
| Sigma_P 7561 | 377.47 | Phentolamine mesylate | Adrenoceptor | Antagonist |
| Sigma_P 7780 | 376.95 | Propionylpromazine hydrochloride | Dopamine | Antagonist |
| Sigma_P 7791 | 419.87 | Prazosin hydrochloride | Adrenoceptor | Antagonist |
| Sigma_P 7912 | 274.28 | Phloretin | Ca ²⁺ Channel | Blocker |
| Sigma_P 8013 | 195.69 | Pargyline hydrochloride | Neurotransmission | Inhibitor |
| Sigma_P 8139 | 616.84 | Phorbol 12-myristate 13-acetate | Phosphorylation | Activator |

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|---------------|--------|--|-------------------------|------------|
| Sigma_P 8227 | 444.26 | 1,3-PBIT dihydrobromide | Nitric Oxide | Inhibitor |
| Sigma_P 8293 | 562.67 | Protoporphyrin IX disodium | Cyclic Nucleotides | Activator |
| Sigma_P 8352 | 444.26 | 1,4-PBIT dihydrobromide | Nitric Oxide | Inhibitor |
| Sigma_P 8386 | 308.38 | Phenylbutazone | Prostaglandin | Substrate |
| Sigma_P 8477 | 376.42 | Picotamide | Thromboxane | Antagonist |
| Sigma_P 8511 | 169.66 | Tranlycypromine hydrochloride | Neurotransmission | Inhibitor |
| Sigma_P 8688 | 295.81 | (S)-Propranolol hydrochloride | Adrenoceptor | Blocker |
| Sigma_P 8765 | 164.29 | Ammonium pyrrolidinedithiocarbamate | Nitric Oxide | Modulator |
| Sigma_P 8782 | 173.17 | (±)-cis-Piperidine-2,3-dicarboxylic acid | Glutamate | Agonist |
| Sigma_P 8813 | 299.85 | Protriptyline hydrochloride | Adrenoceptor | Blocker |
| Sigma_P 8828 | 410.60 | Pergolide methanesulfonate | Dopamine | Agonist |
| Sigma_P 8852 | 195.22 | 6(5H)-Phenanthridinone | Transcription | Inhibitor |
| Sigma_P 8887 | 318.50 | 5alpha-Pregnan-3alpha-ol-20-one | GABA | Modulator |
| Sigma_P 8891 | 448.40 | Propantheline bromide | Cholinergic | Antagonist |
| Sigma_P 9159 | 165.21 | Piperidine-4-sulphonic acid | GABA | Agonist |
| Sigma_P 9178 | 606.10 | Prochlorperazine dimaleate | Dopamine | Antagonist |
| Sigma_P 9233 | 414.42 | Piribedil maleate | Dopamine | Agonist |
| Sigma_P 9297 | 713.72 | Paromomycin sulfate | Antibiotic | |
| Sigma_P 9375 | 180.21 | 1,10-Phenanthroline monohydrate | Biochemistry | Inhibitor |
| Sigma_P 9391 | 271.79 | Procainamide hydrochloride | Na ⁺ Channel | Antagonist |
| Sigma_P 9547 | 256.78 | Prilocaine hydrochloride | Na ⁺ Channel | Blocker |
| Sigma_P 9689 | 306.37 | Propentofylline | Adenosine | Inhibitor |
| Sigma_P 9708 | 377.92 | (S)-(-)-propafenone hydrochloride | Adrenoceptor | Blocker |
| Sigma_P 9797 | 261.12 | Pyridostigmine bromide | Cholinergic | Inhibitor |
| Sigma_P 9879 | 272.78 | Procaine hydrochloride | Na ⁺ Channel | Blocker |
| Sigma_P-101 | 358.36 | 2-Phenylaminoadenosine | Adenosine | Agonist |
| Sigma_P-102 | 255.79 | R(+)-3PPP hydrochloride | Dopamine | Agonist |
| Sigma_P-103 | 255.79 | S(-)-3PPP hydrochloride | Dopamine | Agonist |
| Sigma_P-105 | 345.92 | (±)-PPHT hydrochloride | Dopamine | Agonist |
| Sigma_P-106 | 167.64 | 3-Phenylpropargylamine hydrochloride | Dopamine | Inhibitor |
| Sigma_P-107 | 371.40 | N6-2-Phenylethyladenosine | Adenosine | Agonist |
| Sigma_P-108 | 343.34 | N6-Phenyladenosine | Adenosine | Agonist |
| Sigma_P-118 | 249.64 | Phaclofen | GABA | Antagonist |
| Sigma_P-119 | 480.45 | (±)-Pindobind | Adrenoceptors | Ligand |
| Sigma_P-120 | 177.21 | 1-Phenylbiguanide | Serotonin | Agonist |
| Sigma_S 3317 | 284.32 | SKF 94836 | Calcium Signaling | Inhibitor |
| Sigma_P-126 | 393.47 | Pirenperone | Serotonin | Antagonist |
| Sigma_I 0658 | 311.34 | IC 261 | Phosphorylation | Inhibitor |
| Sigma_P-152_b | 248.33 | S(-)-Pindolol | Serotonin | Agonist |
| Sigma_P-154 | 245.33 | Pinacidil | K ⁺ Channel | Activator |
| Sigma_P-162 | 418.53 | Pregnenolone sulfate sodium | GABA | Antagonist |
| Sigma_P-178 | 599.31 | PPADS | P2 Receptor | Antagonist |
| Sigma_P-183 | 285.77 | S(+)-PD 128,907 hydrochloride | Dopamine | Agonist |
| Sigma_P-203 | 401.83 | Phenamyl methanesulfonate | Na ⁺ Channel | Inhibitor |
| Sigma_P-204 | 335.30 | Phenylbenzene-omega-phosphono-alpha-amino acid | Glycine | Antagonist |

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|--------------|--------|---------------------------------------|-------------------------|------------|
| Sigma_P-209 | 361.20 | Phthalamoyl-L-glutamic acid trisodium | Glutamate | Agonist |
| Sigma_P-215 | 267.29 | PD 98,059 | Phosphorylation | Inhibitor |
| Sigma_P-216 | 285.77 | (±)-PD 128,907 hydrochloride | Dopamine | Agonist |
| Sigma_P-233 | 450.50 | PD 168,077 maleate | Dopamine | Agonist |
| Sigma_S 9692 | 371.46 | SU 6656 | Phosphorylation | Inhibitor |
| Sigma_P63204 | 167.12 | Quinolinic acid | Glutamate | Antagonist |
| Sigma_Q 0125 | 302.24 | Quercetin dihydrate | Cyclic Nucleotides | Inhibitor |
| Sigma_Q 0875 | 746.93 | Quinidine sulfate | Na ⁺ Channel | Antagonist |
| Sigma_Q 1004 | 445.43 | Quipazine dimaleate | Serotonin | Agonist |
| Sigma_Q 1250 | 746.93 | Quinine sulfate | K ⁺ Channel | Antagonist |
| Sigma_Q 2128 | 189.13 | (+)-Quisqualic acid | Glutamate | Agonist |
| Sigma_Q 3251 | 472.89 | Quinacrine dihydrochloride | Neurotransmission | Inhibitor |
| Sigma_Q 3504 | 235.67 | Quazinson | Cyclic Nucleotides | Inhibitor |
| Sigma_Q-102 | 255.79 | (-)-Quinpirole hydrochloride | Dopamine | Agonist |
| Sigma_Q-107 | 459.46 | Quipazine, N-methyl-, dimaleate | Serotonin | Agonist |
| Sigma_Q-109 | 374.36 | Quipazine, 6-nitro-, maleate | Serotonin | Inhibitor |
| Sigma_Q-110 | 319.28 | Quinelorane dihydrochloride | Dopamine | Agonist |
| Sigma_Q-111 | 292.25 | (±)-Quinpirole dihydrochloride | Dopamine | Agonist |
| Sigma_R 0500 | 346.47 | Cortisolone | Hormone | Precursor |
| Sigma_R 0758 | 323.82 | Ritodrine hydrochloride | Adrenoceptor | Agonist |
| Sigma_R 1402 | 510.06 | Raloxifene hydrochloride | Hormone | Modulator |
| Sigma_R 2625 | 300.44 | Retinoic acid | Apoptosis | Activator |
| Sigma_R 2751 | 786.36 | Ruthenium red | Ion Pump | Inhibitor |
| Sigma_R 3255 | 300.44 | 13-cis-retinoic acid | Transcription | Regulator |
| Sigma_R 3277 | 287.32 | Rutaecarpine | K ⁺ Channel | Blocker |
| Sigma_R 4152 | 296.84 | Ropinirole hydrochloride | Dopamine | Agonist |
| Sigma_R 5010 | 228.25 | Resveratrol | Prostaglandin | Inhibitor |
| Sigma_R 5523 | 335.45 | REV 5901 | Leukotriene | Antagonist |
| Sigma_R 5648 | 516.55 | Rottlerin | Phosphorylation | Inhibitor |
| Sigma_R 6152 | 500.47 | Ranolazine dihydrochloride | Lipid | Inhibitor |
| Sigma_R 6520 | 275.35 | Rolipram | Cyclic Nucleotides | Inhibitor |
| Sigma_R 7150 | 375.94 | Ro 25-6981 hydrochloride | Glutamate | Antagonist |
| Sigma_R 7385 | 587.48 | Phosphoramidon disodium | Biochemistry | Inhibitor |
| Sigma_R 7772 | 354.46 | Roscovitine | Phosphorylation | Inhibitor |
| Sigma_R 8875 | 394.43 | Rotenone | Cell Stress | Modulator |
| Sigma_R 8900 | 406.89 | Ro 8-4304 | Glutamate | Antagonist |
| Sigma_R 9525 | 270.72 | RX 821002 hydrochloride | Adrenoceptor | Antagonist |
| Sigma_R 9644 | 244.21 | Ribavirin | Cell Cycle | Inhibitor |
| Sigma_R-101 | 350.87 | Ranitidine hydrochloride | Histamine | Antagonist |
| Sigma_R-103 | 477.58 | Ritanserlin | Serotonin | Antagonist |
| Sigma_R-104 | 390.91 | Rauwolscine hydrochloride | Adrenoceptor | Antagonist |
| Sigma_R-106 | 235.11 | Ro 16-6491 hydrochloride | Neurotransmission | Inhibitor |
| Sigma_R-107 | 301.77 | Ro 41-1049 hydrochloride | Neurotransmission | Inhibitor |
| Sigma_R-108 | 277.21 | Ro 41-0960 | Neurotransmission | Inhibitor |
| Sigma_R-115 | 840.11 | Reactive Blue 2 | P2 Receptor | Antagonist |

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|--------------|--------|-----------------------------------|--------------------------|------------|
| Sigma_R-116 | 234.20 | Riluzole | Glutamate | Antagonist |
| Sigma_R-118 | 410.50 | Risperidone | Dopamine | Antagonist |
| Sigma_R-121 | 497.33 | S(+)-Raclopride L-tartrate | Dopamine | Antagonist |
| Sigma_S 4692 | 514.54 | Sobuzoxane | Gene Regulation | Inhibitor |
| Sigma_R-134 | 180.25 | Rilmenidine hemifumarate | Imidazoline | Agonist |
| Sigma_D 7815 | 317.39 | R(-)-Denopamine | Adrenoceptor | Agonist |
| Sigma_R-140 | 381.29 | Ro 04-6790 dihydrochloride | Serotonin | Antagonist |
| Sigma_S 0278 | 308.83 | (±)-Sotalol hydrochloride | Adrenoceptor | Antagonist |
| Sigma_S 0441 | 287.75 | SB-366791 | Vanilloid | Antagonist |
| Sigma_S 0501 | 261.92 | Sodium nitroprusside dihydrate | Nitric Oxide | Releaser |
| Sigma_S 0752 | 167.21 | (±)-Synephrine | Adrenoceptor | Agonist |
| Sigma_S 0758 | 314.37 | Sulfaphenazole | Multi-Drug Resistance | Inhibitor |
| Sigma_S 1316 | 808.99 | Seglitide | Somatostatin | Agonist |
| Sigma_S 1438 | 372.42 | Sulindac sulfone | Prostaglandin | Inhibitor |
| Sigma_S 1441 | 612.75 | Cortexolone maleate | Dopamine | Antagonist |
| Sigma_S 1563 | 232.15 | SKF 86466 | Adrenoceptor | Antagonist |
| Sigma_S 1688 | 248.16 | SR 57227A | Serotonin | Agonist |
| Sigma_S 1875 | 384.27 | (-)-Scopolamine hydrobromide | Cholinergic | Antagonist |
| Sigma_S 2064 | 352.75 | SC-560 | Prostaglandin | Inhibitor |
| Sigma_S 2201 | 111.53 | Semicarbazide hydrochloride | Neurotransmission | Inhibitor |
| Sigma_S 2250 | 380.40 | (-)-Scopolamine methyl nitrate | Cholinergic | Antagonist |
| Sigma_S 2381 | 464.13 | DL-Stearoylcarnitine chloride | Phosphorylation | Inhibitor |
| Sigma_S 2501 | 254.63 | Spermidine trihydrochloride | Glutamate | Ligand |
| Sigma_S 2812 | 449.64 | SNC80 | Opioid | Agonist |
| Sigma_S 2816 | 398.73 | SKF 83959 hydrobromide | Dopamine | Agonist |
| Sigma_S 2876 | 348.19 | Spermine tetrahydrochloride | Glutamate | Antagonist |
| Sigma_S 2941 | 350.26 | SKF 75670 hydrobromide | Dopamine | Agonist |
| Sigma_S 3065 | 331.76 | SC 19220 | Prostaglandin | Antagonist |
| Sigma_S 3066 | 328.23 | SKF 89626 | Dopamine | Agonist |
| Sigma_S 3191 | 419.15 | SKF 83565 hydrobromide | Dopamine | Agonist |
| Sigma_S 3313 | 419.35 | SB 204070 hydrochloride | Serotonin | Antagonist |
| Sigma_O 2139 | 417.64 | N-Oleoyldopamine | Neurotransmission | Ligand |
| Sigma_S 3378 | 416.58 | Spirolactone | Hormone | Antagonist |
| Sigma_S 4063 | 348.27 | SCH-202676 hydrobromide | G protein | Modulator |
| Sigma_S 4250 | 105.09 | D-Serine | Glutamate | Agonist |
| Sigma_S 5013 | 576.71 | Albuterol hemisulfate | Adrenoceptor | Agonist |
| Sigma_S 5890 | 367.79 | Sanguinarine chloride | Ion Pump | Inhibitor |
| Sigma_S 6633 | 215.21 | N-Succinyl-L-proline | Neurotransmission | Inhibitor |
| Sigma_S 6879 | 299.50 | Sphingosine | Phosphorylation | Inhibitor |
| Sigma_S 7389 | 388.96 | SB 269970 hydrochloride | Serotonin | Antagonist |
| Sigma_S 7395 | 431.94 | Spiperone hydrochloride | Dopamine | Antagonist |
| Sigma_S 7690 | 370.41 | SR 2640 | Leukotriene | Antagonist |
| Sigma_S 7771 | 341.43 | (-)-Sulpiride | Dopamine | Antagonist |
| Sigma_S 7809 | 402.93 | SKF 96365 | Ca ²⁺ Channel | Inhibitor |
| Sigma_S 7882 | 440.38 | (-)-Scopolamine,n-Butyl-, bromide | Cholinergic | Antagonist |
| Sigma_S 7936 | 330.41 | SB 205384 | GABA | Modulator |

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|--------------|---------|---|----------------------------|------------|
| Sigma_S 8010 | 341.43 | (±)-Sulpiride | Dopamine | Antagonist |
| Sigma_C 7238 | 592.78 | CV-3988 | Cytokines & Growth Factors | Antagonist |
| Sigma_S 8139 | 356.42 | Sulindac | Prostaglandin | Inhibitor |
| Sigma_S 8251 | 361.31 | Succinylcholine chloride | Cholinergic | Antagonist |
| Sigma_S 8260 | 239.32 | Salbutamol | Adrenoceptor | Agonist |
| Sigma_S 5068 | 603.76 | Salmeterol xinafoate | Adrenoceptor | Agonist |
| Sigma_S 8442 | 238.29 | SU 5416 | Phosphorylation | Inhibitor |
| Sigma_S 8502 | 398.30 | (-)-Scopolamine methyl bromide | Cholinergic | Antagonist |
| Sigma_S 8567 | 264.33 | SU 4312 | Phosphorylation | Inhibitor |
| Sigma_S 8688 | 415.49 | SR 59230A oxalate | Adrenoceptor | Antagonist |
| Sigma_B 5559 | 391.77 | BRL 52537 hydrochloride | Neurotransmission | Agonist |
| Sigma_S 9066 | 371.91 | SKF 89976A hydrochloride | GABA | Inhibitor |
| Sigma_S 9186 | 213.24 | SIB 1757 | Glutamate | Antagonist |
| Sigma_S 9311 | 195.27 | SIB 1893 | Glutamate | Antagonist |
| Sigma_S-003 | 248.76 | 1-(1-Naphthyl)piperazine hydrochloride | Serotonin | Antagonist |
| Sigma_S-006 | 545.53 | Ketanserin tartrate | Serotonin | Antagonist |
| Sigma_S-008 | 228.72 | 1-(2-Methoxyphenyl)piperazine hydrochloride | Serotonin | Agonist |
| Sigma_S-009 | 349.40 | PAPP | Serotonin | Agonist |
| Sigma_S-103 | 379.46 | Spiroxatrine | Serotonin | Agonist |
| Sigma_S-106 | 368.23 | SR-95531 | GABA | Antagonist |
| Sigma_S-143 | 370.68 | (±)-6-Chloro-PB hydrobromide | Dopamine | Agonist |
| Sigma_S-145 | 248.22 | SKF 91488 dihydrochloride | Histamine | Inhibitor |
| Sigma_S 2671 | 1429.19 | Suramin hexasodium | P2 Receptor | Antagonist |
| Sigma_S-153 | 205.22 | SQ 22536 | Cyclic Nucleotides | Inhibitor |
| Sigma_S-154 | 237.22 | Sepiapterin | Nitric Oxide | Cofactor |
| Sigma_S-159 | 413.47 | R(-)-SCH-12679 maleate | Dopamine | Antagonist |
| Sigma_S-168 | 376.30 | (±)-SKF 38393, N-allyl-, hydrobromide | Dopamine | Agonist |
| Sigma_S-174 | 337.25 | SDZ-205,557 hydrochloride | Serotonin | Antagonist |
| Sigma_S-180 | 328.80 | SB 206553 hydrochloride | Serotonin | Antagonist |
| Sigma_S-201 | 557.10 | SB 224289 hydrochloride | Serotonin | Antagonist |
| Sigma_T 0254 | 204.23 | L-Tryptophan | Serotonin | Precursor |
| Sigma_T 0318 | 327.34 | Tranilast | Leukotriene | Inhibitor |
| Sigma_T 0410 | 364.89 | Tiapride hydrochloride | Dopamine | Antagonist |
| Sigma_T 0625 | 125.15 | Taurine | Glycine | Agonist |
| Sigma_T 0780 | 480.10 | Thiothixene hydrochloride | Dopamine | Antagonist |
| Sigma_T 0891 | 270.35 | Tolbutamide | Hormone | Releaser |
| Sigma_T 1132 | 296.54 | Tetraethylthiuram disulfide | Biochemistry | Inhibitor |
| Sigma_T 1443 | 402.07 | TCPOBOP | Transcription | Agonist |
| Sigma_T 1505 | 342.36 | Tetraisopropyl pyrophosphoramidate | Biochemistry | Inhibitor |
| Sigma_T 1512 | 240.76 | Tetramisole hydrochloride | Phosphorylation | Inhibitor |
| Sigma_T 1516 | 337.94 | Trihexyphenidyl hydrochloride | Cholinergic | Antagonist |
| Sigma_T 1633 | 179.18 | Theophylline | Adenosine | Antagonist |
| Sigma_T 1694 | 101.11 | (E)-4-amino-2-butenoic acid | GABA | Agonist |
| Sigma_T 1698 | 288.50 | Tetradecylthioacetic acid | Transcription | Agonist |
| Sigma_T 2057 | 441.96 | Trequinsin hydrochloride | Cyclic Nucleotides | Inhibitor |

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|--------------|--------|--|-------------------------|------------|
| Sigma_T 2067 | 316.47 | Tyrphostin AG 879 | Phosphorylation | Inhibitor |
| Sigma_T 2265 | 165.71 | Tetraethylammonium chloride | Cholinergic | Antagonist |
| Sigma_T 2408 | 311.41 | Tolazamide | Hormone | Releaser |
| Sigma_T 2528 | 548.66 | Terbutaline hemisulfate | Adrenoceptor | Agonist |
| Sigma_T 2879 | 173.64 | 4-Hydroxyphenethylamine hydrochloride | Dopamine | Agonist |
| Sigma_T 2896 | 388.89 | Triflupromazine hydrochloride | Dopamine | Antagonist |
| Sigma_T 3146 | 410.52 | Trimipramine maleate | Serotonin | Inhibitor |
| Sigma_T 3434 | 294.31 | Tyrphostin AG 490 | Phosphorylation | Inhibitor |
| Sigma_T 3757 | 348.49 | TTNPB | Transcription | Ligand |
| Sigma_L 3040 | 522.61 | L-765,314 | Adrenoceptor | Antagonist |
| Sigma_T 4143 | 253.27 | Triamterene | Na ⁺ Channel | Blocker |
| Sigma_T 4182 | 315.76 | Tyrphostin AG 1478 | Phosphorylation | Inhibitor |
| Sigma_T 4264 | 236.75 | Tetrahydrozoline hydrochloride | Adrenoceptor | Agonist |
| Sigma_T 4318 | 280.29 | Tyrphostin AG 494 | Phosphorylation | Inhibitor |
| Sigma_T 4376 | 351.85 | N-p-Tosyl-L-phenylalanine chloromethyl ketone | Biochemistry | Inhibitor |
| Sigma_T 4425 | 314.17 | (6R)-5,6,7,8-Tetrahydro-L-biopterin hydrochloride | Neurotransmission | Cofactor |
| Sigma_T 4443 | 308.34 | Tyrphostin AG 527 | Phosphorylation | Inhibitor |
| Sigma_T 4500 | 180.17 | Theobromine | Adenosine | Antagonist |
| Sigma_T 4512 | 304.26 | (±)-Taxifolin | Cell Stress | Inhibitor |
| Sigma_T 4568 | 308.34 | Tyrphostin AG 528 | Phosphorylation | Inhibitor |
| Sigma_T 4680 | 423.90 | Terazosin hydrochloride | Adrenoceptor | Antagonist |
| Sigma_T 4693 | 448.44 | Tyrphostin AG 537 | Phosphorylation | Inhibitor |
| Sigma_T 4818 | 322.37 | Tyrphostin AG 555 | Phosphorylation | Inhibitor |
| Sigma_T 5193 | 308.34 | Tyrphostin AG 698 | Phosphorylation | Inhibitor |
| Sigma_T 5318 | 304.31 | Tyrphostin AG 808 | Phosphorylation | Inhibitor |
| Sigma_T 5515 | 781.46 | Thio-NADP sodium | Intracellular Calcium | Blocker |
| Sigma_T 5568 | 308.34 | Tyrphostin AG 835 | Phosphorylation | Inhibitor |
| Sigma_T 5625 | 206.33 | (±)-alpha-Lipoic Acid | Cell Stress | Coenzyme |
| Sigma_T 6031 | 253.32 | DL-Thiorphan | Neurotransmission | Inhibitor |
| Sigma_T 6050 | 264.20 | Tulobuterol hydrochloride | Adrenoceptor | Agonist |
| Sigma_T 6154 | 408.33 | Trazodone hydrochloride | Serotonin | Inhibitor |
| Sigma_T 6318 | 216.20 | Tyrphostin AG 34 | Phosphorylation | Inhibitor |
| Sigma_T 6376 | 394.44 | Triamcinolone | Hormone | Agonist |
| Sigma_T 6394 | 432.50 | S(-)-Timolol maleate | Adrenoceptor | Antagonist |
| Sigma_T 6692 | 423.34 | N,N,N-trimethyl-1-(4-trans-stilbenoxy)-2-propylammonium iodide | Cholinergic | Antagonist |
| Sigma_T 6764 | 314.86 | Triprolidine hydrochloride | Histamine | Antagonist |
| Sigma_T 6943 | 236.23 | Tyrphostin AG 112 | Phosphorylation | Inhibitor |
| Sigma_T 7040 | 184.20 | Tyrphostin 1 | Phosphorylation | Inhibitor |
| Sigma_T 7165 | 186.17 | Tyrphostin 23 | Phosphorylation | Inhibitor |
| Sigma_T 7188 | 284.73 | TFPI hydrochloride | Nitric Oxide | Inhibitor |
| Sigma_T 7254 | 369.31 | Na-p-Tosyl-L-lysine chloromethyl ketone hydrochloride | Cyclic Nucleotides | Inhibitor |
| Sigma_T 7290 | 202.17 | Tyrphostin 25 | Phosphorylation | Inhibitor |
| Sigma_T 7313 | 212.18 | 1-[2-(Trifluoromethyl)phenyl]imidazole | Nitric Oxide | Inhibitor |
| Sigma_T 7402 | 853.93 | Taxol | Cytoskeleton and ECM | Inhibitor |

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|--------------|--------|---|-------------------------|------------|
| Sigma_T 7508 | 300.83 | Tetracaine hydrochloride | Na ⁺ Channel | Modulator |
| Sigma_T 7540 | 220.25 | Tyrphostin 47 | Phosphorylation | Inhibitor |
| Sigma_T 7665 | 268.23 | Tyrphostin 51 | Phosphorylation | Inhibitor |
| Sigma_T 7692 | 665.68 | T-1032 | Cyclic Nucleotides | Inhibitor |
| Sigma_T 7697 | 437.19 | I-OMe-Tyrphostin AG 538 | Phosphorylation | Inhibitor |
| Sigma_T 7822 | 297.27 | Tyrphostin AG 538 | Phosphorylation | Inhibitor |
| Sigma_T 7883 | 290.32 | Trimethoprim | Antibiotic | Inhibitor |
| Sigma_T 7947 | 255.36 | Tomoxetine | Adrenoceptor | Inhibitor |
| Sigma_T 8067 | 620.07 | T-0156 | Cyclic Nucleotides | Inhibitor |
| Sigma_T 8160 | 314.21 | 3-Tropanyl-3,5-dichlorobenzoate | Serotonin | Antagonist |
| Sigma_T 8516 | 480.43 | Trifluoperazine dihydrochloride | Dopamine | Antagonist |
| Sigma_T 8543 | 266.47 | D-609 potassium | Lipid | Inhibitor |
| Sigma_T 9025 | 407.04 | Thioridazine hydrochloride | Dopamine | Antagonist |
| Sigma_T 9033 | 650.77 | Thapsigargin | Intracellular Calcium | Releaser |
| Sigma_T 9177 | 215.17 | Tyrphostin AG 126 | Phosphorylation | Inhibitor |
| Sigma_T 9262 | 563.65 | Tamoxifen citrate | Phosphorylation | Inhibitor |
| Sigma_T 9652 | 471.69 | Terfenadine | Histamine | Antagonist |
| Sigma_T 9778 | 284.36 | Tropicamide | Cholinergic | Antagonist |
| Sigma_T-101 | 176.60 | THIP hydrochloride | GABA | Agonist |
| Sigma_T-103 | 445.89 | Trifluoperidol hydrochloride | Dopamine | Antagonist |
| Sigma_T-104 | 320.82 | 3-Tropanyl-indole-3-carboxylate hydrochloride | Serotonin | Antagonist |
| Sigma_T-112 | 304.40 | Tracazolate | GABA | Modulator |
| Sigma_T-113 | 426.30 | 3-Tropanylindole-3-carboxylate methiodide | Serotonin | Antagonist |
| Sigma_T-122 | 443.40 | Telenzepine dihydrochloride | Cholinergic | Antagonist |
| Sigma_T-123 | 408.52 | Thioperamide maleate | Histamine | Antagonist |
| Sigma_T-144 | 258.24 | (±)-Thalidomide | Cytoskeleton and ECM | Inhibitor |
| Sigma_T-165 | 340.47 | R(+)-Terguride | Dopamine | Agonist |
| Sigma_T-173 | 191.25 | Thiocitrulline | Nitric Oxide | Inhibitor |
| Sigma_T-182 | 282.39 | Tyrphostin A9 | Phosphorylation | Inhibitor |
| Sigma_T-200 | 161.14 | TPMPA | GABA | Antagonist |
| Sigma_U 1508 | 361.53 | U-75302 | Leukotriene | Agonist |
| Sigma_U 4125 | 448.13 | Uridine 5'-diphosphate sodium | P2 Receptor | Agonist |
| Sigma_U 5882 | 726.92 | U-74389G maleate | Cell Stress | Inhibitor |
| Sigma_U 6007 | 593.65 | U-83836 dihydrochloride | Cell Stress | Inhibitor |
| Sigma_U 6756 | 464.65 | U-73122 | Lipid | Inhibitor |
| Sigma_S 5317 | 613.69 | SKF 95282 dimaleate | Histamine | Antagonist |
| Sigma_U 7500 | 138.13 | 4-Imidazoleacrylic acid | Histamine | Inhibitor |
| Sigma_U-100 | 423.95 | Urapidil hydrochloride | Adrenoceptor | Antagonist |
| Sigma_U-101 | 401.51 | Urapidil, 5-Methyl- | Adrenoceptor | Antagonist |
| Sigma_U-103 | 356.51 | U-69593 | Opioid | Agonist |
| Sigma_U-104 | 292.14 | UK 14,304 | Adrenoceptor | Agonist |
| Sigma_U-105 | 521.51 | U-62066 | Opioid | Agonist |
| Sigma_U-108 | 301.84 | S(-)-UH-301 hydrochloride | Serotonin | Antagonist |
| Sigma_U-109 | 301.84 | R(+)-UH-301 hydrochloride | Serotonin | Agonist |

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|--------------|---------|--|--------------------------|------------|
| Sigma_U-110 | 405.80 | (+)-trans-(1R,2R)-U-50488 hydrochloride | Opioid | Agonist |
| Sigma_U-111 | 405.80 | (-)-trans-(1S,2S)-U-50488 hydrochloride | Opioid | Agonist |
| Sigma_U-115 | 455.56 | U-101958 maleate | Dopamine | Antagonist |
| Sigma_U-116 | 393.48 | U-99194A maleate | Dopamine | Antagonist |
| Sigma_U-120 | 380.50 | U0126 | Phosphorylation | Inhibitor |
| Sigma_V 1377 | 909.07 | Vinblastine sulfate salt | Cytoskeleton and ECM | Inhibitor |
| Sigma_V 4629 | 491.08 | (±)-Verapamil hydrochloride | Ca ²⁺ Channel | Modulator |
| Sigma_V 5888 | 371.40 | VUF 5574 | Adenosine | Antagonist |
| Sigma_V 6383 | 350.46 | Vinpocetine | Cyclic Nucleotides | Inhibitor |
| Sigma_V 8138 | 1485.75 | Vancomycin hydrochloride from <i>Streptomyces orientalis</i> | Antibiotic | |
| Sigma_V 8261 | 129.16 | (±)-gamma-Vinyl GABA | GABA | Inhibitor |
| Sigma_V 8879 | 923.06 | Vincristine sulfate | Cytoskeleton and ECM | Inhibitor |
| Sigma_V 9130 | 293.41 | N-Vanillylnonanamide | Vanilloid | Ligand |
| Sigma_V-100 | 295.86 | (±)-Vesamicol hydrochloride | Cholinergic | Inhibitor |
| Sigma_X 3628 | 366.74 | XK469 | Apoptosis | Inhibitor |
| Sigma_W 1628 | 428.44 | Wortmannin from <i>Penicillium funiculosum</i> | Phosphorylation | Inhibitor |
| Sigma_W 4262 | 250.17 | 1400W dihydrochloride | Nitric Oxide | Inhibitor |
| Sigma_W 4761 | 808.66 | WB 64 | Cholinergic | Ligand |
| Sigma_W-102 | 522.63 | (R)-(+)-WIN 55,212-2 mesylate | Cannabinoid | Agonist |
| Sigma_W-104 | 438.58 | WIN 62,577 | Tachykinin | Antagonist |
| Sigma_W-105 | 199.17 | S(-)-Willardiine | Glutamate | Agonist |
| Sigma_W-108 | 538.65 | WAY-100635 maleate | Serotonin | Antagonist |
| Sigma_W-110 | 325.06 | S-5-Iodowillardiine | Glutamate | Agonist |
| Sigma_X 1251 | 256.80 | Xylazine hydrochloride | Adrenoceptor | Agonist |
| Sigma_X 3253 | 794.86 | Xamoterol hemifumarate | Adrenoceptor | Agonist |
| Sigma_X 6000 | 280.84 | Xylometazoline hydrochloride | Adrenoceptor | Agonist |
| Sigma_X-103 | 428.50 | Xanthine amine congener | Adenosine | Antagonist |
| Sigma_Y 3125 | 390.91 | Yohimbine hydrochloride | Adrenoceptor | Antagonist |
| Sigma_Y-101 | 395.93 | YS-035 hydrochloride | Ca ²⁺ Channel | Blocker |
| Sigma_Y-102 | 304.35 | YC-1 | Cyclic Nucleotides | Activator |
| Sigma_Z 0878 | 271.28 | Zaprinast | Cyclic Nucleotides | Inhibitor |
| Sigma_Z 2001 | 234.21 | Zonisamide sodium | Anticonvulsant | |
| Sigma_Z 3003 | 268.22 | Zardaverine | Cyclic Nucleotides | Inhibitor |
| Sigma_Z-101 | 390.15 | Zimelidine dihydrochloride | Serotonin | Inhibitor |

6.4.4. Liquid class programs

- Program 1 (20-201 μ l)

| | Aspiration | Dispensing |
|--|--|--|
| Speed (μl/s) | 100 | 100 |
| Delay (ms) | 500 | 200 |
| System Trailing Air gap (μl) | 0 | |
| Leading air gap (μl) | 15 | |
| Trailing air gap (μl) | 10 | |
| Aspiration/Dispensing position | z-max \pm offset, with tracking 0 mm, x: center | z-max \pm offset, no tracking 0 mm, x: center |
| Retract tip to | z-start 0 mm | z-start 0 mm |
| Retract speed (mm/s) | 100 | 100 |

- Program 2 (20-201 μ l)

| | Aspiration | Dispensing |
|--|--|---|
| Speed (μl/s) | 200 | 200 |
| Delay (ms) | 500 | 200 |
| System Trailing Air gap (μl) | 0 | |
| Leading air gap (μl) | 15 | |
| Trailing air gap (μl) | 0 | |
| Aspiration/ Dispensing position | z-max \pm offset, with tracking 0 mm, x: center | z-dispense \pm offset, no tracking -10 mm, x: center |
| Retract tip to | z-start 0 mm | z-start 0 mm |
| Retract speed (mm/s) | 42 | 42 |

- Program 3 (20-201 μ l)

| | Aspiration | Dispensing |
|--|--|--|
| Speed (μl/s) | 100 | 100 |
| Delay (ms) | 500 | 200 |
| System Trailing Air gap (μl) | 0 | |
| Leading air gap (μl) | 15 | |
| Trailing air gap (μl) | 10 | |
| Aspiration/ Dispensing position | z-max \pm offset, with tracking 0 mm, x: center | z-max \pm offset, no tracking -10 mm, x: center |
| Retract tip to | z-start 0 mm | z-start 0 mm |
| Retract speed (mm/s) | 100 | 100 |

▪ Program 3 (7.5-15 μ l)

| | Aspiration | Dispensing |
|--|--|--|
| Speed (μl/s) | 10 | 20 |
| Delay (ms) | 200 | 200 |
| System Trailing Air gap (μl) | 0 | |
| Leading air gap (μl) | 10 | |
| Trailing air gap (μl) | 1 | |
| Aspiration/ Dispensing position | z-max \pm offset, no tracking 0 mm, x: center | z-max \pm offset, no tracking 0 mm, x: center |
| Retract tip to | z-start 0 mm | z-start 0 mm |
| Retract speed (mm/s) | 5 | 42 |

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“Ich denke niemals an die Zukunft.

Sie kommt früh genug“

Albert Einstein

Affidavit

I hereby swear in lieu of an oath that I have independently prepared this thesis and without using other aids than those stated. The data and concepts taken over from other sources or taken over indirectly are indicated citing the source. The thesis was not submitted so far either in Germany or in another country in the same or a similar form in a procedure for obtaining an academic title.

Place, date

(signature)