Toxicological Evaluation of Azole Fungicides in Agriculture and Food Chemistry

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To my dear family

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1 Introduction and Background

1.1 Agricultural Fungicides

Infections of cultivated plants, their infestation by insects and rodents lead to a loss estimated at 10-20% of a year's crop in Europe. In tropical zones of e.g. Africa the loss can amount to more than 75%. Therefore crop protection agents are necessary to achieve high hygienic standards in foods and to ensure a sufficient supply of food [1]. In Europe fungal infections of crops are a severe threat especially to wheat, fruit and wine (grape) growing. In the United States (U.S.) a more moderate, dry climate is predominant, thus fungal infections of crops do not play such an important role [1].

According to the U.S. Environmental Protection Agency (EPA) approximately 215 000 tons of fungicides were sold worldwide in 2001. In the U.S. alone approximately 33 000 tons were sold (U.S. EPA 2005). A fourth of the world's production of fungicides falls to the group of azole compounds developed in the early 1970s. In 2003 one of the worlds leading producers in fungicides had a total revenue of € 1.168 billions with fungicides sold world wide. The second most top-selling product in 2003, with € 315 millions, contained the azole compound tebuconazole.

1.1.1 Azole Compounds

Azole fungicides show a broad antifungal activity and are either used to prevent or cure fungal infections [2]. The compounds are used on a wide variety of crops, ranging from exotic plants like bananas and papayas to different wheat crops and fruits like apples and plums [Appendix II German Regulation for Maximum Residue Levels in Food (RHmV); fields of application set by German authorities following § 18 a Abs. 1 PflSchG]. Azoles are applied directly on the crops and also as seed protection agents. Their antifungal activity is both topical and systemic.

According to their chemical structure azoles are grouped either into the class of imidazoles (Figure 1) or into the class of triazoles (Figure 2). The antifungal activity requires such a heterocyclic structure without changing the principal mode of action. Substitution at the heterocycle can vary and determines the physicochemical properties of the compounds, thereby affecting their activities: ability to penetrate into the fungal cell, affinity to the target, pharmacokinetic properties and pharmacological or toxicological effects on human cells [3]. Chemical structures of the 13 agriculturally

used fungicides, including most commercially important compounds, tested in this manuscript are given in Figure 1 and 2.

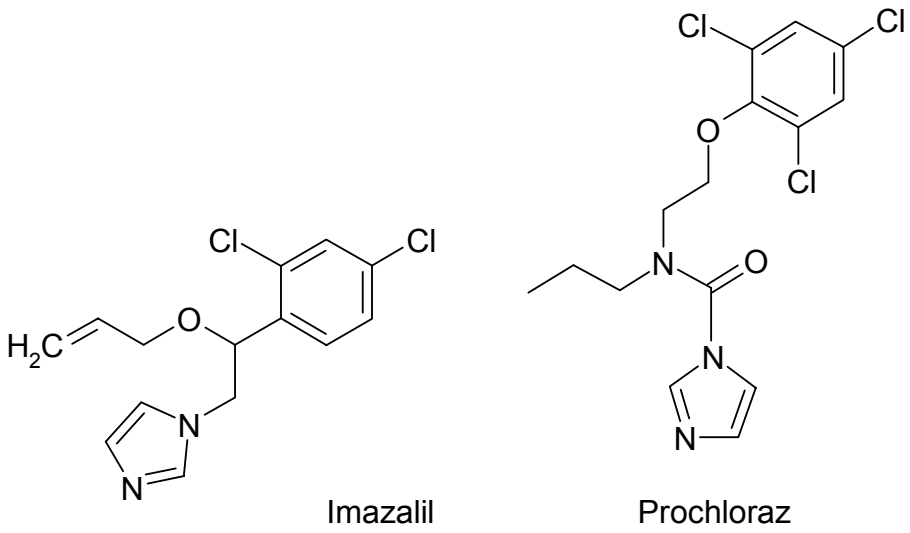


Figure 1: Chemical structures of agriculturally used fungicides, imidazoles, examined in this work.

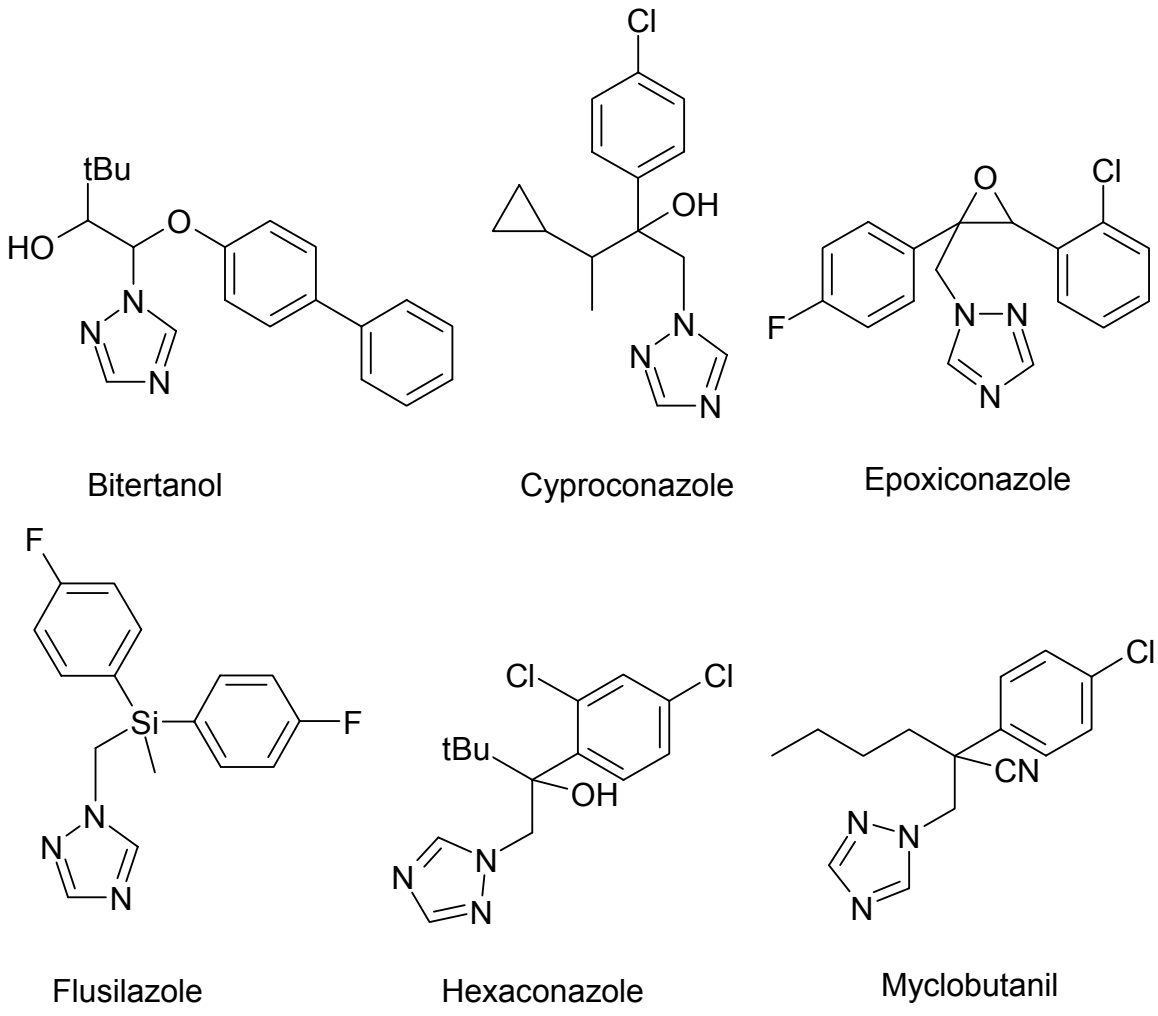


Figure 2: Chemical structures of agriculturally used fungicides, triazoles, examined in this work (tBu = tert. butyl).

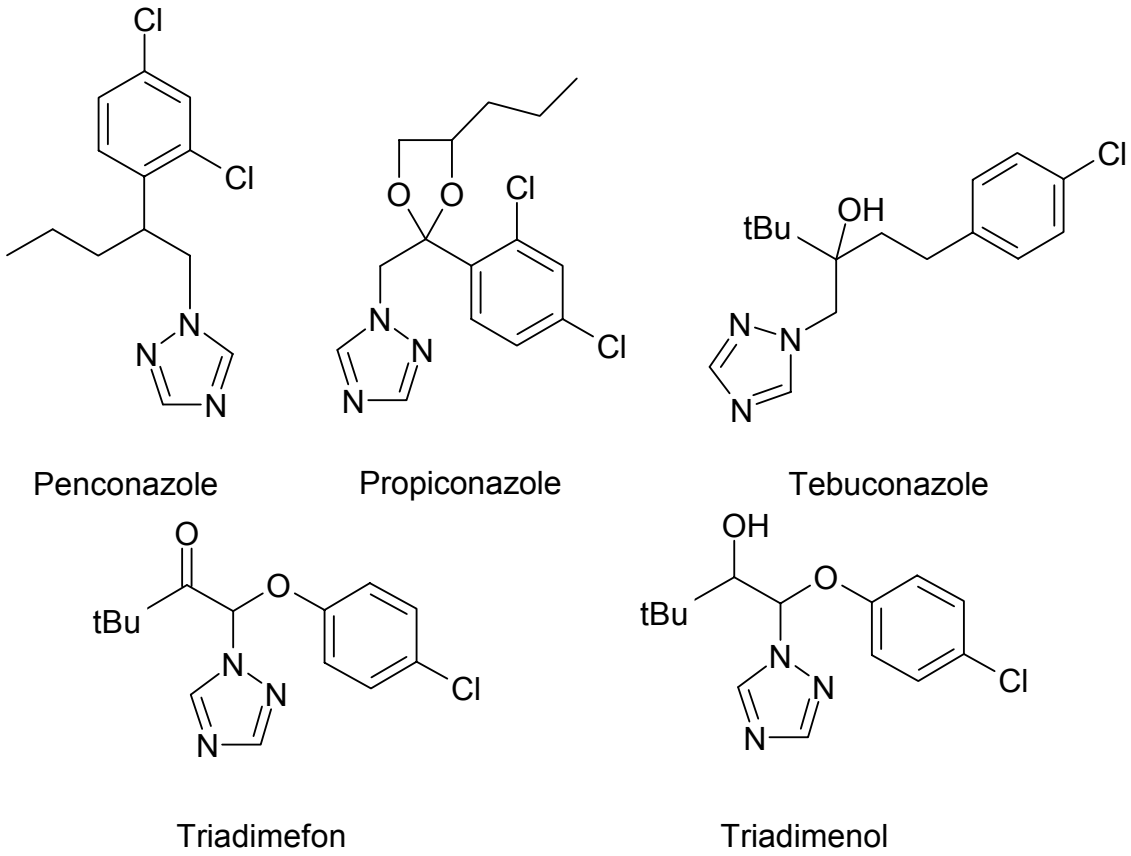
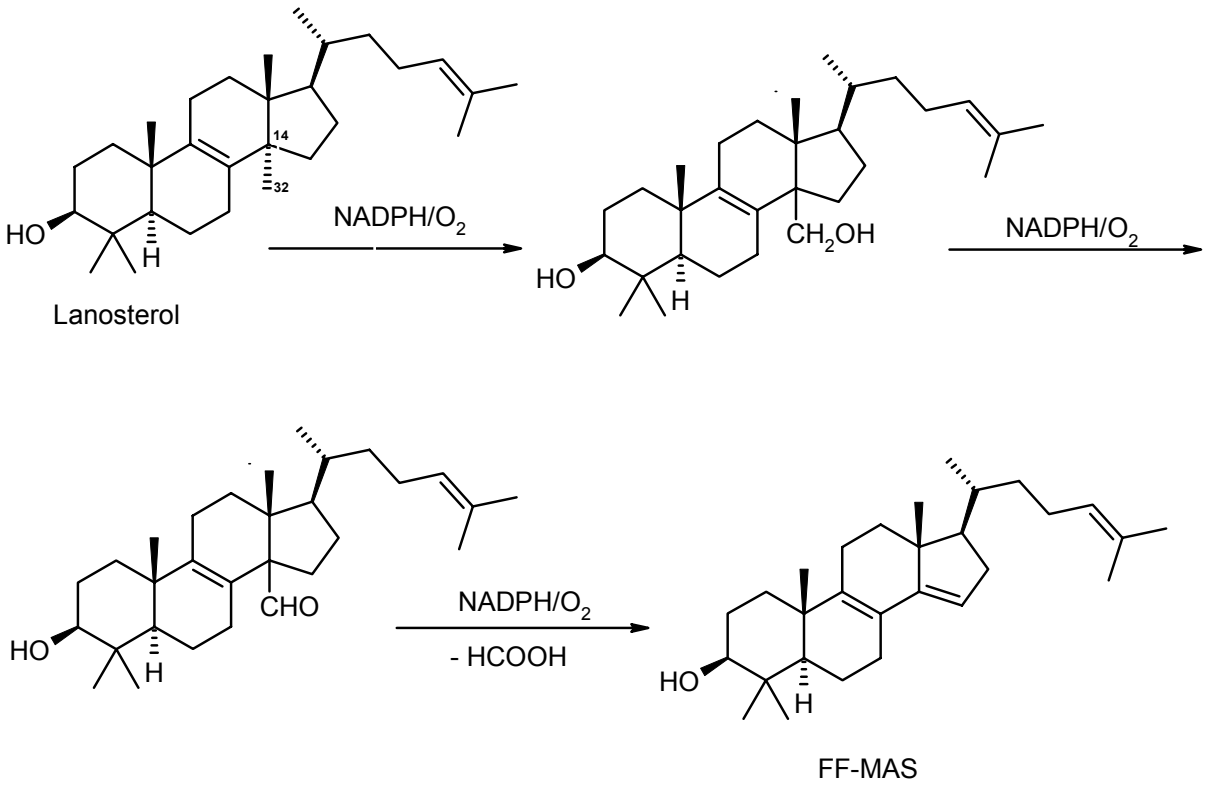


Figure 2 continued: Chemical structures of agriculturally used fungicides, triazoles, examined in this work (tBu = tert. butyl).

Antifungal activities are based on inhibition of lanosterol-14α-demethylase (CYP51) [4,5], a phylogenetically widely distributed and highly conserved cytochrome P450 monooxygenase found in mycobacteria, fungi, plants, animals and humans [6] [7,8]. CYP51 is a housekeeping enzyme expressed in virtually all animal cells [9]. Eukaryotic forms of CYP51 are membrane bound. The four substrates of the CYP51, lanosterol, 24,25-dihydrolanosterol, 24-methylenedihydrolanosterol and obtusifoliol are very lipophilic molecules and it is postulated that they enter the active site of the enzyme through the membrane [9].

In mammals and yeasts CYP51 catalyses the three-step oxidative removal of the methyl group # 32 of lanosterol to produce follicular fluid meiosis activating steroid (FF-MAS), an important step in sterol biosynthesis [10,11]. Each step requires one molecule each of O_2 and NADPH as shown in Scheme 1. The methyl group is first oxidised to the respective alcohol and then to the respective aldehyde. The final oxidation step leads to the removal of the methyl group as formic acid [9]. For fungi the later resulting ergosterol is an essential compound of the cell membrane. The

working principle of azoles as fungicides relies on the inhibition of CYP51, thus exposed fungi lack ergosterol, which leads to a collapse of the cell membrane.



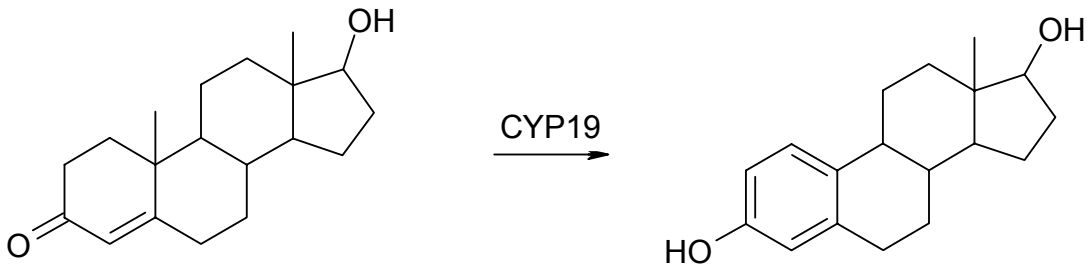
Scheme 1: Reaction catalysed by CYP51 (lanosterol-14α-demethylase); three step oxidative removal of the C_{14} methyl group.

Literature postulates that azole compounds bind to cytochrome P450 enzymes in a reversible, competitive manner [12] [4]. The π-electron rich nitrogen atom of the azole ring interacts with the central Fe^{3+} of the heme moiety of the cytochrome P450 enzyme [5]. This mechanism is not specific for fungal CYP51. Inhibition of other cytochrome P450 enzymes by azole compounds is reported in the literature and is held responsible for a variety of unwanted side-effects of azole compounds. Azole compounds are rather lipophilic and penetrate to the cytochrome P450 enzymes within the endoplasmatic reticulum. This lipophilic character in part is held responsible for their toxicological profile. Inhibition of non-target cytochrome P450 enzymes leads to toxicologically relevant changes in the liver and endocrine system [2]. Furthermore enzymes catalysing metabolism of xenobiotics in the liver are induced which is regarded a risk factor for the development of liver tumours in animal studies. This has been reported for e.g. cyproconazole, epoxiconazole and tebuconazole [2] [1] [13].

Azole compounds are resorbed orally by humans to a very different extent (25 - 95%). Due to their lipophilic character azoles can penetrate through the skin. The resorption kinetics depends on the compound, e.g. flusilazole penetrates to 5% within 8 hours, tebuconazole up to 60% within 24 hours.

Human lanosterol-14α-demethylase is one example for a cytochrome P450 enzyme inhibited by azoles where this is considered an unwanted side-effect [14]. In animals and humans, the downstream product of lanosterol-14α-demethylation, cholesterol, necessary for the synthesis of bile acids, mineral corticoids, glucocorticoids and sex steroids, can be supplemented with food intake [15]. However CYP51 is highly expressed in male germ cells, depending on the developmental stage. FF-MAS and the resulting testis meiosis activating steroids (T-MAS), the direct products of the CYP51 reaction of lanosterol act as ligands for nuclear receptors and are known to activate meiosis in ovaries and testes [16,17]. The accumulation of these meiosis activating sterols in the gonads suggests that they have a function beyond being intermediates in sterol biosynthesis. Inhibition of CYP51 in humans may therefore affect the endocrine system and is an unwanted side-effect of azoles [2].

CYP19 (aromatase) is another cytochrome P450 monooxygenase inhibited by azoles. Aromatase catalyses the cleavage of the methyl group at carbon 10 of the steroid ring system of androstenedione and testosterone to produce estrone and estradiol, respectively (Scheme 2) [18].



Testosterone Estradiol

Scheme 2: Three step oxidative removal of methyl group at C10 catalysed by aromatase (CYP19) to produce estradiol.

Similarly to the reaction described for CYP51 (Scheme 1) the three step reaction utilises three moles each of oxygen and NADPH. The methyl group is released from the androgen as formic acid.

For agriculturally used fungicides inhibition of CYP19 is considered an unwanted side-effect [2]. Sexual development and adult sexual behaviour rely on tightly regulated sex steroid levels. Inhibition of aromatase can lead to disruption and imbalance. Testing for inhibition of aromatase activity *in vitro* is one of the Tier 1 screening assays proposed by the “Endocrine Disrupter Screening and Testing Advisory Committee” (EDSTAC) for the detection of potential endocrine disrupters.

1.1.2 Toxicological Profiles of Azole Fungicides

Results of *in vivo* studies of some azole fungicides are summarized in Appendix 1 with special focus on effects putatively connected to disturbed steroidogenesis [2]. In the allocation procedure for an “Acceptable Daily Intake” (ADI) by the Joint Food and Agriculture Organisation / World Health Organisation Meeting on Pesticide Residues (JMPR), these effects are included [2] [13]. Many of these fungicides affect the prostate, testis, uterus and ovaries as well as fertility, development and sexual behaviour. Table 1 shows a selection of studies, focussing on observed effects on the endocrine system.

Table 1: Selected toxicological effects on the endocrine system observed for the 13 azole compounds studied in this work.

Compound	Toxicological effects on the endocrine system observed in animal studies
Bitertanol	histopathologic changes in adrenal glands of rats (1.2 mg/kg b.w.) and dogs (≥ 81 mg/kg b.w.)
Cyproconazole	increased incidences of resorptions and dead foetuses (≥ 50 mg/kg b.w.)
Epoxiconazole	no data available
Flusilazole	reduced testosterone and estradiol levels in rats (14 days ≥ 20 mg/kg b.w.) induced Leydig cell tumours of testis in rats (2 years 31 mg/kg b.w.) fetotoxic and embryotoxic (developmental study ≥ 9 mg/kg b.w.)
Hexaconazole	induced Leydig cell tumours of testis in rats (2 years ≥ 4.7 mg/kg b.w.) histopathologic changes in adrenal glands of rats (≥ 2.5 mg/kg b.w.) fetotoxic (developmental study ≥ 25 mg/kg b.w.)
Imazalil	fetotoxic in rats, mice and rabbits (5-20 mg/kg b.w.)
Myclobutanil	effects on male reproductive system including reduced testes weight and testes atrophy ≥ 10 mg/kg b.w. (2 year rat study)
Penconazole	reduced testes weight with atrophic changes (1 year dog study ≥ 17 mg/kg b.w.)
Prochloraz	reduced testes and prostate weight (90 day dog study ≥ 7 mg/kg b.w.)
Propiconazole	reduced testes and epididymis weights in pups (reproduction study rat ≥ 21 mg/kg b.w.)
Tebuconazole	histopathologic changes in adrenal glands (1 year dog study 4.5 mg/kg b.w.)
Triadimefon	impairment of the sexual behaviour of males (reproductive rat study 77 mg/kg b.w.)
Triadimenol	reduced ovary weights (2 year rat study 144 mg/kg b.w.)

The overall view suggests a connection between inhibition of sterol 14α-demethylase and/or aromatase and disturbed steroidogenesis [2].

1.1.3 Workplace Exposure of Agricultural Workers

Pesticides can present a health risk during the production and agricultural application process [13]. Some epidemiological studies indicate a relationship between frequent and extended exposure to certain pesticides and the occurrence of certain disorders. Garry et al. reported in 1996 that professional pesticide appliers had significantly ($p < 0.001$) more children with an anomaly than did nonappliers of the same region [19]. Other epidemiological studies conclude conflicting results with regard to pesticide related health risks [20-23].

Risk assessment procedures in Europe for workers applying pesticides refer to scenarios of fieldworkers mixing, loading and spraying pesticides. Seed grain treated with pesticides, re-entry work such as working in greenhouses or harvester and

bystanders are also considered. To quantify the risk for a worker a “No Observed (Adverse) Effect Level” (NO(A)EL) is derived from animal studies. In the European Union the Acceptable Operator Exposure Level (AOEL) is defined as the NO(A)EL divided by a safety factor of 100. An exposure is considered acceptable when the exposure of the worker is below or equal to the AOEL. The exposure of workers can either be measured or it can be calculated by exposure models such as the German model developed by the Biological Research Centre for Agriculture and Forestry [24]. Exposure depends to a large amount on the application, the work performed, whether or not protective equipment is used and the concentration of the active ingredient in the pesticide formulation. These different influences were measured in real scenarios and integrated into the model calculations. Registration of newly developed pesticides is only granted when limit values for workers are met.

1.1.4 Pesticide Residues in and on Food

In the developed countries the risk posed to consumers, who are only exposed to pesticides via residues in and on food, is generally regarded as negligible [13]. The amounts taken up are much lower than the corresponding “Acceptable Daily Intakes” (ADI). Usually, the hazard assessments of pesticides by the Joint Food and Agriculture Organisation / World Health Organisation Meeting on Pesticide Residues (JMPR) are based on data of *in vitro* and animal studies providing no observable effect levels (NOEL) for delineation of individual ADIs in humans [2]. To determine the ADI the NOEL of the most sensitive species is divided by a safety factor of 100. Based on the ADI and residue levels in plants after application of recommended use levels, “Maximum Residue Levels” (MRLs) are allocated to individual foods by the authorities. Table 2 summarises ADI levels for the 13 azole compounds of this work. MRLs have been set for various food items. Table 2 gives the MRL of the food for which the highest residue is allocated with the respective food in brackets.

Table 2: Summary of the "Acceptable Daily Intake levels" (ADI) determined by the Joint Food and Agriculture Organisation / World Health Organisation Meeting on Pesticide Residues (JMPR). Values set by the German authorities (Bundesinstitut für Risikobewertung 2004) that differ are marked by *. "Maximum Residue Levels" allocated by the German authorities (Appendix II German Regulation for Maximum Residue Levels in Food (RHmV)). The highest possible residue is named with the respective food in brackets.

Compound	ADI [mg/kg bw]	MRL [mg/kg]
Bitertanol	0.01	3 (bananas, tomatoes)
Cyproconazole	0.01*.	0.1 (raw coffee)
Epoxiconazole	0.0032*	0.5 (bananas)
Flusilazole	0.001 / 0.002*	0.2 (pipfruit)
Hexaconazole	0.005	0.2 (strawberries)
Imazalil	0.03	5 (potatoes, pipfruit, citrus fruit)
Myclobutanil	0.03 / 0.025*	3 (citrus fruit)
Penconazole	0.03	0.5 (hops)
Prochloraz	0.01	10 (citrus fruit)
Propiconazole	0.04	0.5 (grapes)
Tebuconazole	0.03	30 (hops)
Triadimefon	0.03	10 (hops)
Triadimenol	0.05	10 (hops)

1.2 Azoles as Medical Agents

1.2.1 Antifungal Agents

Mycotic infections pose an increasing threat to public health. They range from skin thrush or infections of the mucous membrane, athlete's foot and infections of hand and toe nails, to serious systemic infections [25]. Opportunistic infections, such as aspergillosis, candidiasis and cryptococcosis, have emerged as major problems in cancer patients, transplant recipients and other immunocompromised individuals, including those with HIV/AIDS [26]. Classical infections, such as histoplasmosis and coccidioidomycosis, have appeared in new forms in these patients (Centers for Disease Control and Prevention, Department of Health and Human Services U.S.). As a growing group diabetics have a higher risk of suffering fungal infections due to inferior blood circulation, resulting in reduced immune defence. An increase in fungal infections is also due to the elevated use of antibiotics that disturb the biological balance between bacteria and fungi. Fungal infections of the vagina are probably promoted by the widespread use of oral contraceptives [25].

There has also been an upsurge in the number of cases where "harmless" environmental fungi - organisms that live in the soil, on plants, in compost heaps, or

on rotting food - that have been implicated as causes of serious illness or death in immunocompromised individuals (Centers for Disease Control and Prevention).

1.2.2 Azole Compounds

Azole compounds were introduced into the therapy of fungal infections of humans in the early 1970s, parallel to the first agriculturally used azoles (Bayer AG, Leverkusen, Germany). Figure 3 depicts the chemical structures of seven azoles used as antifungal agents in human medicine. Clotrimazole was the first compound on the market in 1971 and is used for dermal and vaginal infections in 1% topic formulations. Bifonazole and miconazole are also only used in topical formulations, however miconazole gels are indicated for infections of mucous membrane of the mouth (also for children), where parts of the dose could be ingested and become available systemically (German Summary of Product Characteristics). Ketoconazole was the first azole that could be applied orally.

Azoles act fungistatic due to the same mechanism as the compounds used as agricultural fungicides: inhibition of lanosterol-14α-demethylase in fungi pathogenic to humans. The resulting shortage of the essential cell membrane compound ergosterol and the inclusion of “false” steroids into the cell membrane disturb especially the function of membrane bound enzymes e.g. chitin synthase. *In vitro* fungicidal effects can be observed by the inclusion of azole compounds into the cell membrane resulting in an additional instability. Furthermore azoles inhibit mitochondrial enzymes resulting in elevated levels of peroxide derivates. In vivo, however, only fungistatic concentrations are reached [25].

1.2.2.1 Toxicokinetics

The orally active triazoles fluconazole, itraconazole and voriconazole have a favourable/reduced spectrum of adverse drug reactions, but differ in the spectrum of activity against pathogenous fungi. Itraconazole is especially active against aspergillus and histoplasma species; fluconazole and voriconazole are more effective against yeast species like candida and cryptococcus (German Summary of Product Characteristics) [25]. Orally active azole compounds are used to treat serious systemic fungal infections and infections of the skin and mucous membrane that can not be treated sufficiently by topic agents. Depending on the severity of the infection fluconazole and voriconazole can be applied intravenously.

The oral bioavailability, plasma protein binding and metabolism of azole compounds differ to a high degree. Table 3 gives an overview of the kinetic

parameters of the tested azole compounds used to treat fungal infections of humans (German Summary of Product Characteristics). Figure 3 gives the respective chemical structures of the azole compounds.

Table 3: Kinetic parameters of azole compounds used to treat fungal infections of humans (German Summary of Product Characteristics) [3] [25] [38].

Compound	Oral Bioavailability	Dosing	max. Plasma conc.	PPB[+]
Bifonazole		1% cream	0.051 µM	
Clotrimazole		1% cream	0.029 µM	
Fluconazole	>90%	200 mg/d i.v.[+] or p.o.[+]	9.8 µM	10 - 12%
Itraconazole	50 - 55%	2 x 200 mg/d p.o.	2.8 µM*	>99%
Ketoconazole	up to 75%	1-2 x 200 mg/day	6.6 µM	85 - 99%
Miconazole	25 - 30%	1% gel	2.4 µM[§] / 0.024 µM[$]	98%
Voriconazole	>90%	2 x 200 mg/day p.o. 4 mg/kg i.v.	5.7 µM	58%

* Concentrations in some organs can be higher than maximum plasma concentrations reached

[+] Plasma Protein Binding (PPB), intra veniously (i.v.), per oral (p.o.)

[§] oral dosing [$]vaginal dosing

Itraconazole, ketoconazole and voriconazole are extensively metabolised by cytochrome P450 enzymes including isoenzyme 3A4 (CYP3A4). Fluconazole is only metabolised to 10% whereas 80% of the dose is eliminated renally.

1.2.2.2 Adverse Drug Reactions

Azoles have a potential to interact with co-administered drugs. The inhibition of xenobiotic metabolising enzymes such as CYP3A4 by azoles can lead to a delayed elimination of a number of substances like ciclosporin, warfarin and ergotamine thus requiring a dose reduction for the duration of co-application. Vice versa, co-administered inhibitors of CYP3A4 e.g. HIV antiviral compounds can lead to elevated plasma levels of azole compounds. Inducers of CYP3A4 like carbamazepine or rifampicine can lead to accelerated metabolism of e.g. ketoconazole and itraconazole [25] [3].

Adverse effects described for azole compounds as medical agents are similar to those reported for azoles used agriculturally e.g. disturbances in the liver function. A rather seldom occurring but very severe adverse effect is acute liver failure that can be fatal. Due to the described teratogenicity the use of azole compounds during pregnancy and lactation is contraindicated (German Summary of Product

Characteristics). For higher dosing regimes a disturbance of the natural balance of steroid hormones has been reported. The unspecific binding and inhibition of a variety of cytochrome P450 enzymes is in part held responsible for these adverse effects.

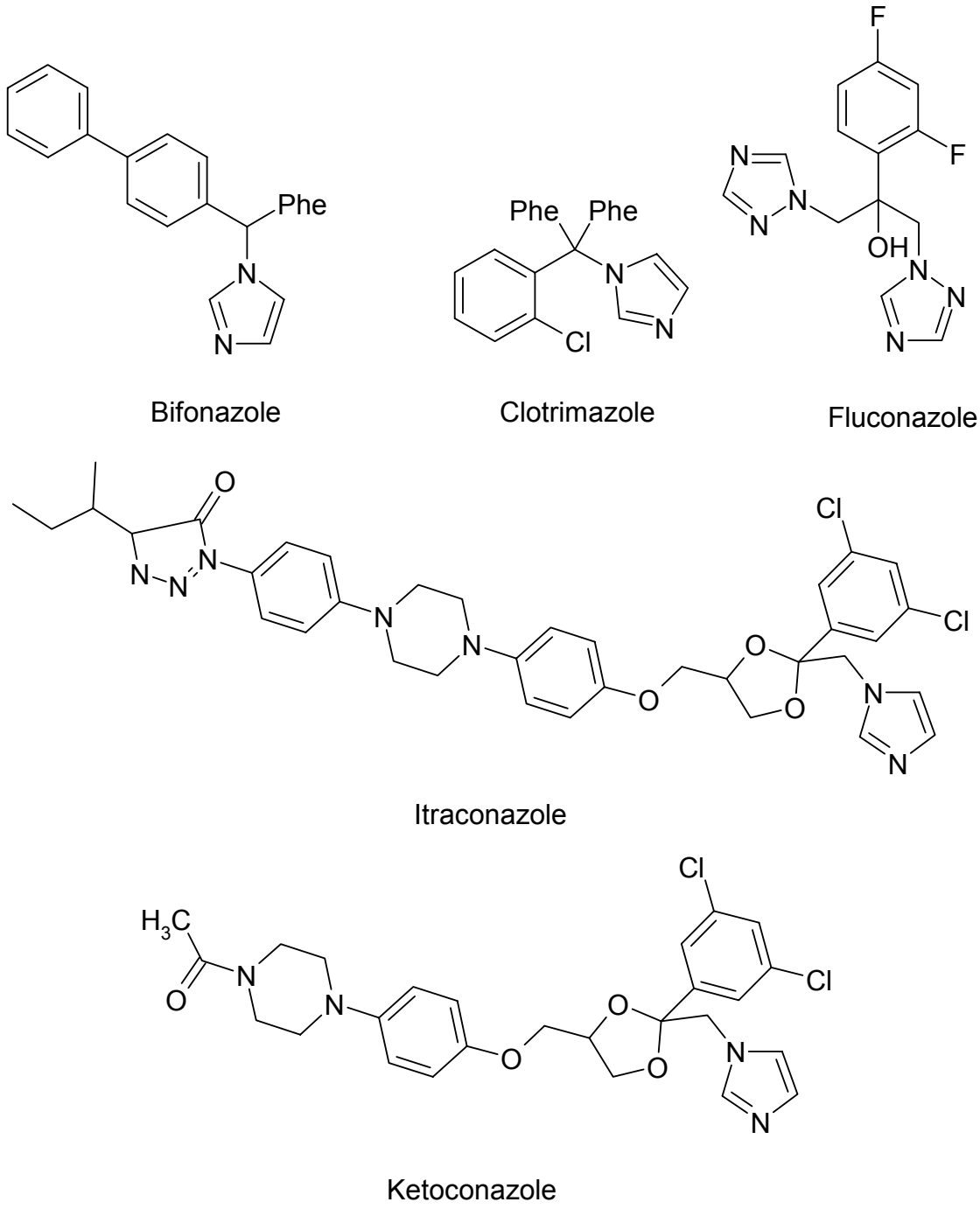
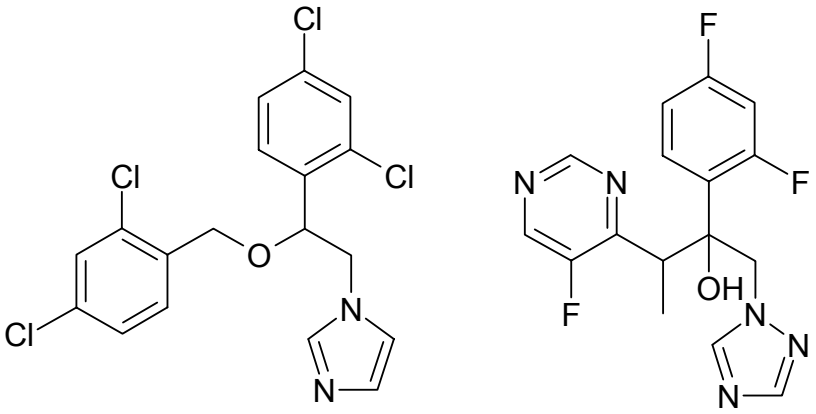


Figure 3: Chemical structures of seven azoles used as antifungal agents in human medicine (Phe = Phenylgroup).



Miconazole Voriconazole

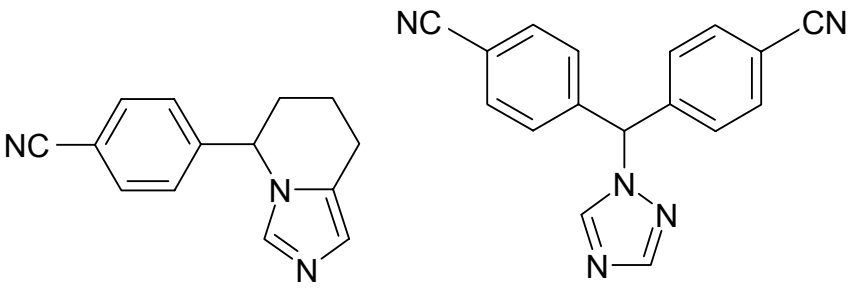
Figure 3 continued: Chemical structures of seven azoles used as antifungal agents in human medicine.

1.2.3 Azole Compounds in Chemotherapy

Aromatase (CYP19) is expressed in a wide variety of tissues such as ovaries, fat, muscle and mamma carcinoma. In the pre-menopause phase ovaries are the main source of estrogen production, in the post-menopause phase however estrogen is mainly produced outside the ovarian tissue.

Breast cancer is the most frequent malignant cancer form in women [25] [27]. In many cases growth of mamma carcinoma is stimulated by female sexual hormones. Thus the receptor status of steroid hormone receptors is of clinical importance. Growth of so called receptor positive tumours can be reduced by treatment with sexually opposite hormones or antiestrogens [28,29]. Inhibition of CYP19 and thus reduction of estrogen levels is the mechanistic basis for the use of azoles in breast cancer therapy [29].

Aromatase inhibitors are only indicated for the therapy of post-menopausal mamma carcinoma. Figure 4 shows the chemical structures of two azole compounds, fadrozole and letrozole used as chemotherapeutic agents. Both compounds bind aromatase in a reversible mode and are applied orally once a day, with dosing regimes of 2.5 mg/day. In an off-label use letrozole is used to treat boys with delayed puberty and short stature to induce growth [25]. Azoles inhibit the estrogen dependent bone maturation, leading to a delayed closure of the epiphysial growth plates.



Fadrozole Letrozole

Figure 4: Chemical structure of two azole compounds used as chemotherapeutic agents.

Except for their use in chemotherapy fungicides, applied agriculturally or in humans, inhibition of CYP19 is considered a severe side-effect disturbing the natural balance of estrogens and androgens [2].

2 Objectives

In view of the effects on the endocrine system observed for azoles used as fungicides in animal studies and as medical agents in humans a possible explanation on a molecular basis was to be tested.

A favourable fungicide or antifungal drug should be a strong inhibitor of fungal CYP51 expressed as low IC_{50} values. In contrast human CYP51 and human CYP19 should not be inhibited by an azole fungicide or antifungal agent. This would be indicated by high IC_{50} values. The favourable cytostatic drug should show a high potency towards human CYP19 expressed as low IC_{50} values. Neither human CYP51 nor fungal CYP51 should be inhibited by a cytostatic drug thus high IC_{50} values should result for the two enzymes.

The aim of this work was to assess: are fungicides and antifungal drugs strong inhibitors of fungal CYP51? In return do they not inhibit human CYP51 and human CYP19? Do cytostatic drugs strongly inhibit human CYP19? And in return do they not inhibit human CYP51 or fungal CYP51?

To this end, incubation procedures and analytical methods to monitor product formation of human CYP19 and human CYP51 were established. Human CYP19 and human CYP51 had been provided by “Gentest Corporation”. To compare the specificity of azole fungicides for their therapeutic target enzyme, a fungal CYP51 was engineered in the baculovirus system.

The baculovirus system lent itself to be used for several reasons: the kinetic properties to be determined with the expressed enzyme can be compared to the ones determined with the human CYP51 and human CYP19 from “Gentest”. These two enzymes had also been expressed in the baculovirus system. Thus the baculovirus system was chosen in order to have a similar protein background and comparable conditions. *Candida albicans* CYP51 and *Candida tropicalis* oxidoreductase were co-expressed to create a functional cytochrome P450 enzyme system.

The exposure of the population to residues of azole fungicides used on grapes was assessed in a model case by determination of azole residues in wine samples. For this purpose an analytical procedure to determine azole concentrations in wine was also developed.

To classify risk exerted by the inhibitory potencies obtained IC_{50} values were compared to exposure scenarios for farmers applying fungicides and to blood levels reported for human drugs.

3 Inhibition of CYP19 (aromatase)

3.1 Dibenzylfluorescein as Substrate for CYP19

3.1.1 Introduction

In a first step the inhibitory potency of azole fungicides, used agriculturally or in human medicine, on the enzyme aromatase (CYP19) was to be assessed since many azoles inhibit not only the therapeutic target enzyme fungal CYP51. CYP19 catalyses, among other reactions, the demethylation of the methyl group at carbon 10 of androstenedione and testosterone to produce estrone and estradiol, respectively [30]. The reaction of testosterone to estradiol catalysed by aromatase is shown in Scheme 2. The concomitant reduction of estrogen levels is the mechanistic basis for the use of azoles in breast cancer therapy [29].

Inhibition of CYP19 by azoles had first been investigated for antifungal drugs, using human placental [31,32] or rat ovarian microsomes [33]. Fungicides used in agriculture were added to the list two years later [34]. Investigation of fungicides has recently been intensified [35,36], one reason being that inhibition of aromatase activity *in vitro* is one of the Tier 1 screening assays proposed by the “Endocrine Disrupter Screening and Testing Advisory Committee” (EDSTAC) for the detection of potential endocrine disrupters.

Interclass comparison between azoles used in medicine and agriculture is hampered by the fact that azoles used for the different applications have not so far been tested in one laboratory using the same assay. One of the aims of this work was to close this gap. Inhibition of CYP19 by antifungal chemicals is compared to antifungal drugs and cytostatic aromatase inhibitors, using an assay based on human recombinant CYP19 and a fluorescent pseudo-substrate, namely dibenzylfluorescein (DBF), that had recently been developed [37] and validated with antifungal drugs [38].

3.1.2 Materials and Methods

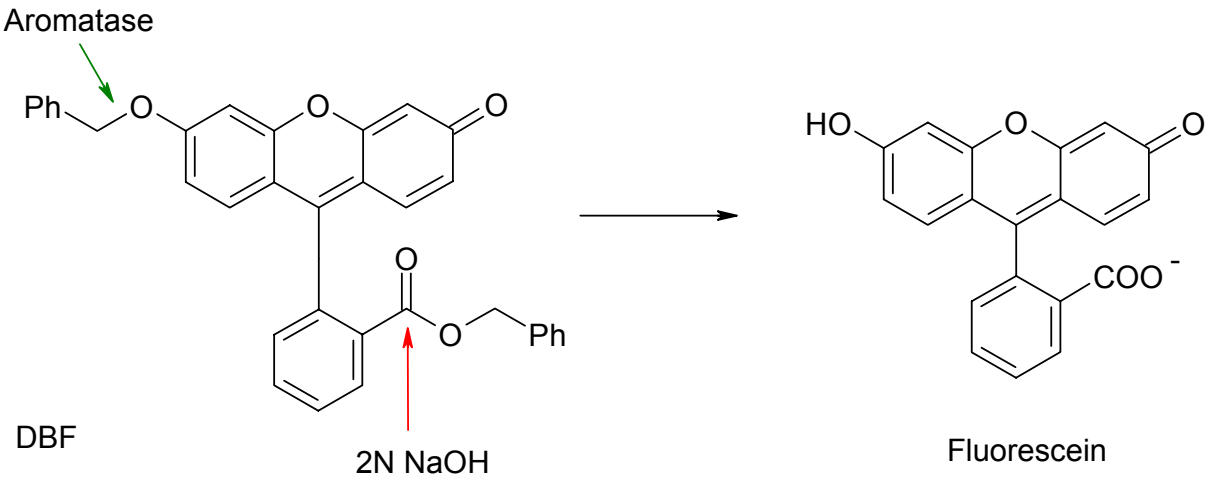
3.1.2.1 Chemicals and Reagents

Human aromatase enzyme (CYP19) co-expressed with human P450 reductase (baculovirus/insect cell-expressed) and the substrate dibenzylfluorescein (DBF) were from BD Gentest (Natutec, Frankfurt/Main). Bitertanol, cyproconazole, flusilazole, hexaconazole, imazalil, myclobutanil, penconazole, prochloraz, propiconazole, tebuconazole, triadimefon and triadimenol were from Dr. Ehrenstorfer

(Augsburg, Germany). Epoxiconazole was a kind gift from the Swiss Federal Research Station (Wädenswil, Switzerland). Fluconazole was a kind gift of Pfizer GmbH (Karlsruhe, Germany). Fadrozole and letrozole were kind gifts from Novartis Pharma AG (Basel, Switzerland).Clotrimazole was from Bufa (Uitgeest, The Netherlands). Bifonazole, ketoconazole, itraconazole, miconazole and all other chemicals were from Sigma/Fluka (Taufkirchen, Germany). Most azoles and DBF were dissolved and diluted in HPLC-grade ethanol, except for itraconazole and imazalil, where dimethylsulfoxide was used.

3.1.2.2 Inhibition Assay

A 1.1 mL reaction mixture containing 966 µL of 0.1 M potassium phosphate buffer (pH 7.4), 75 µL of a 100 mM glucose-6-phosphate solution, 15 µL of a 50 mM $NADP^+$ solution, 3.75 µL of a 0.1 U/µL glucose-6-phosphate dehydrogenase solution, 15 µL of a 33 mM magnesium chloride solution and 5 µL of one of the azole solutions was prepared. Finally 5 µL of the enzyme were added and the sample was preincubated at 37°C for 5 minutes. Reactions were started by addition of 5 µL 0.1 mM DBF, which resulted in a DBF concentration of 0.46 µM. The 0.1 mM DBF solution was freshly prepared by diluting a 1 mM DBF stock solution in methanol with water. Addition of 410 µL of 2 M NaOH after 30 min stopped the enzymatic reaction and resulted in a cleavage of the oxidised DBF to the fluorescent product fluorrescein. After 2 h at 37°C, the fluorescence was measured on a SFM 25 Fluorimeter from Kontron Instruments (Eching, Germany), at an excitation wavelength λ = 485 nm and an emission wavelength λ = 538 nm. The reaction catalysed by aromatase and the following production of fluorescein is illustrated in Scheme 3. Background fluorescence was determined by using a heat-denatured enzyme suspension (30 min at 100°C) incubated with either the maximum or the minimum azole concentration used in main assay. The mean was deducted from all sample values.



Scheme 3: Reaction on Dibenzylfluorescein (DBF) catalysed by aromatase and following production of fluorescein (Ph = Phenyl).

In a dose-finding pilot study, a dilution series based on concentration steps of factors of ten was used to define the concentration range to be tested in the main experiment. For the main experiments, the concentration range spanned at least three orders of magnitude with seven logarithmically spaced values and the expected IC_{50} at the geometric mean. All experiments were carried out in duplicate.

The Michaelis-Menten constant K_m for the substrate DBF was determined to be 0.19 µM, which is in accordance with the previously published value 0.188 µM [38]. The substrate-to-enzyme ratio was chosen to have zero-order kinetics for the 30 min period of incubation. A number of azoles were also tested at 5-fold higher substrate concentrations, in order to get information on the mode of the inhibitor-enzyme interaction.

3.1.2.3 Data Analysis

Plotting the net fluorescence against the $\log_{10}$ of the concentration of azole showed a symmetrical sigmoidal curve. Therefore, a log-probit function was used for data fitting. Four parameters were estimated for the combined replicate data sets: The fluorescence at inhibitor concentration 0 ("start") and inhibitor concentration ∞, ("maxinhib"), the concentration at half maximum inhibition ("IC_{50}") and the "slope" of the probit curve. Statistical analysis was performed by a nonlinear mixed-effects model fit by maximum likelihood, run on the free statistics software "R" (http://www.r-project.org). A random effect was attributed to the fluorescence at concentration 0, to account for differences between the replicates. Since the standard errors of the IC_{50}

estimates were obtained as logarithms of the concentration, they are given as geometric errors, i.e., as factors x/: rather than as ±.

3.1.3 Results

Azoles used for different purposes were tested for their potency to inhibit human CYP19 (aromatase), using the recently developed assay based on human recombinant CYP19 and dibenzylfluorescein as substrate [37]. The inhibitory potencies of all tested racemic azoles are shown in Table 4. The 13 antifungal azoles used in agriculture exhibited a range of IC_{50} values that spanned more than 750-fold from 0.047 µM (prochloraz) to 35 µM (hexaconazole). The seven antifungal agents used in human medicine spanned an even larger range of more than 7 000-fold, from 0.019 µM (bifonazole) to >138 µM (fluconazole). The two cytostatic drugs showed the lowest measured IC_{50} values (0.0076 and 0.015 µM), but the most potent azoles used as fungicides in agriculture or as antifungal agents came close to these values. The same assay had been used before with antifungal drugs [38]. The published IC_{50} values were marginally lower than the ones determined in this work, which may best be explained by the use of a lower substrate concentration (0.2 µM DBF, as opposed to 0.46 µM used here).

Table 4: Inhibition of human recombinant CYP19 (aromatase) by azoles used in agriculture and as drugs for humans.

Azole	IC_{50} [µM]	Standard error‡
Fungicides		
Bitertanol	>20* (24% inhibition)	
Cyproconazole	8.5	x/: 1.09
Epoxiconazole	1.44	x/: 1.65
Flusilazole	0.055	x/: 1.04
Hexaconazole	35	x/: 1.08
Imazalil (I)	0.072	x/: 1.32
Myclobutanil	0.47	x/: 1.07
Penconazole	0.85	x/: 1.17
Prochloraz (I)	0.047	x/: 1.06
Propiconazole	3.2	x/: 1.13
Tebuconazole	5.8	x/: 1.18
Triadimefon	17.5	x/: 1.10
Triadimenol	12.6	x/: 1.18
Antifungal agents		
Bifonazole (I)	0.019	x/: 1.19
Clotrimazole (I)	0.11	x/: 1.54
Fluconazole	>138* (16% inhibition)	
Itraconazole	>69* (33% inhibition)	
Ketokonazole (I)	5.6	x/: 1.23
Miconazole (I)	0.064	x/: 1.15
Voriconazole	>138* (40% inhibition)	
Cytostatic drugs		
Fadrozole (I)	0.0076	x/: 1.27
Letrozole	0.015	x/: 1.19

IC_{50} values indicate the concentration of azole required to reduce product fluorescence by a factor of two.

(I) designates an imidazole, all other azoles are triazoles.

‡The standard error is given as a factor because estimates of IC_{50} values and standard errors were given on a logarithmic scale.

*IC_{50} could not be determined due to solubility problems at high concentrations.

Figure 5 shows a representative duplicate dataset for the inhibition of CYP19 (aromatase) by miconazole. The statistical analysis was based on a nonlinear mixed-effects model, with the curve showing the combined fit. A mixed effects model allows for the analysis of replicate data in situations where one or more parameters have a random error that is unrelated to the independent variable (the concentration of inhibitor). This situation is often met in biological experiments where some fluctuation between experiments is unavoidable. Our independent duplicate samples often presented with different net fluorescence values at low concentration of inhibitor.

Therefore, a random effect was attributed to the respective parameter of the probit curve (the fluorescence at asymptotic concentration 0). For all azoles that could be tested without solubility problems the net fluorescence estimated for concentration ∞ was in no case significantly different from zero.

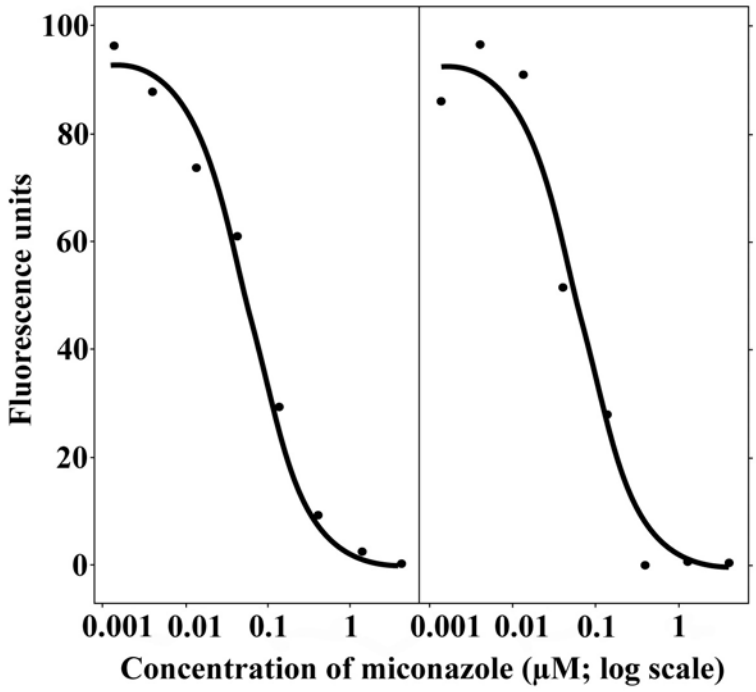


Figure 5: Representation of the duplicate dataset showing the inhibitory action of miconazole on human recombinant CYP19 (aromatase) as a function of the concentration. The curve represents the combined fit of the two independent assays to a log concentration-probit curve with a non-linear mixed-effects model.

3.1.4 Discussion

Inhibition of aromatase by azoles had been investigated before, using various assays and analyses. The first publication dates back 20 years when antifungal drugs were tested with human placental microsomes and tritiated androstenedione as substrate [31]. A potency ranking miconazole >clotrimazole >>ketoconazol was found, which was clearly reproduced in this work. All references included IC_{50} values so that this measure was used throughout. Inhibitory constants K_i had been determined in addition when the type of inhibition was a major question, e.g., in initial studies [31], in connection with structure-activity relationships [39], or when the comparison encompassed chemicals of various structural classes [36]. The comparison of IC_{50} values determined in different laboratories using the same assay

however is difficult, since the substrate concentration used influences the value of the IC_{50}.

3.1.4.1 Literature IC_{50} Values

Azoles used in agriculture had also been investigated before. In 1987, the first data on imazalil and prochloraz were published [34]. Using human placental microsomes and tritiated androstenedione as substrate, respective IC_{50} values of 0.15 and 0.7 were found. Only more than ten years later the list was extended with IC_{50} values of 0.04, 0.34, 6.5, 21 and 32 µM for imazalil, prochloraz, propiconazole, triadimenol and triadimefon, respectively [35] (Note that imazalil and prochloraz have to be interchanged in the respective abstract). In a later study that included pesticides of various chemical classes, IC_{50} values were determined in a human adrenocortical carcinoma cell line [36]. IC_{50} values of 0.1, 0.1, 5, 20, 50 and 55 µM, for imazalil, prochloraz, propiconazole, penconazole, tebuconazole and hexaconazole, respectively were found. This study included the determination of K_i values, which indicated imazalil and prochloraz to be potent mixed-type inhibitors, penconazole and propiconazole to be weak competitive inhibitors. The inhibition by tebuconazole and hexaconazole was attributed largely to cytotoxicity.

3.1.4.2 IC_{50} Values in this Manuscript

IC_{50} values determined in this work with the human recombinant enzyme and dibenzylfluorescein as substrate are within a factor of two of the concentrations published before, except that prochloraz was found to be as potent as imazalil and that penconazole and tebuconazole are more potent inhibitors than reported previously [36]. Differences may be due to inhibitor depletion by non-target enzymes present in the microsomal incubations reported in the literature. The differences are small, however, if the total range of IC_{50} values within the classes of fungicides and antifungal agents is considered. This also indicates that DBF can be used as a surrogate for the natural substrates if the concentration is chosen appropriately.

3.1.4.3 Toxicological Evaluation

It should be noted, however, that IC_{50} values determined with a fluorescent pseudo-substrate may be useful for a ranking of inhibitory potency but they should not directly be used for effect considerations, e.g., by comparison with blood levels reached in therapy.

In animal studies, for instance, azole fungicides induced weight changes in reproductive organs, histologic changes, as well as developmental and fertility effects. Even though the effects partly matched observed imbalances in sex steroid hormones due to inhibition of CYP19, a generally disturbed steroidogenesis due to inhibition of CYP51 should also be considered. Some azole pesticides have also been shown to act as estrogen agonists and androgen antagonists [40], which renders the situation even more complex.

Regarding food safety of azole fungicides residues in crops, the maximum residue limits (MRL) set by authorities assure protection of the general population. These MRLs warrant that acceptable daily intakes (ADI), derived from animal toxicity studies, are not exceeded (see Appendix to [2]). However, sexual development and adult sexual behavior rely on tightly regulated sex steroid levels during gestation [41] [42]. Under the assumption of a time window of particular sensitivity for developmental toxicity attributable to disturbed steroidogenesis, incidental exposures to azole residues in food above the ADI should be avoided. A similar *caveat* has to be expressed for toxicity to wildlife exposed to azole fungicides on the field as well as to agricultural workers handling the azoles incorrectly.

The classification of an azole as either fungicide or antifungal agent or cytostatic drug reflects the initial marketing purpose and blurs the biological significance of the common inhibitory actions on aromatase and CYP51. The finding that compounds designed to inhibit fungal CYP51 in agriculture or as antifungal drugs are almost as potent inhibitors of aromatase as cytostatic aromatase inhibitors questions the respective specificity and calls for further investigation of putative endocrine disruption in patients treated with azole antifungal agents and in humans and wildlife exposed to azole fungicides used in agriculture.

3.2 Testosterone as Substrate for CYP19

3.2.1 Introduction

The determination of inhibitory potencies described in the previous section (Chapter 3.1) based on the use of Dibenzylfluorescein (DBF) as substrate and the generation of a fluorescent product [43]. Fluorescence measurements have the advantage that no expensive radiolabeled compounds or equipments have to be used and for most probes fluorescent product formation can be monitored over time [44 190a]. However the actual fluorescent product of DBF, fluorescein, is only generated after termination of the reaction (Scheme 3) so that this advantage is not available. Finally, interpretation of these data was limited by the fact that DBF is not a natural substrate. Here, this gap of knowledge is closed and a list of IC_{50} values for the inhibition of human CYP19 by 21 azoles, using testosterone as substrate is presented.

In this work high performance liquid chromatography – tandem mass spectrometry (LC-MS/MS) with photospray ionisation was optimised to measure the formation of estradiol formed by CYP19 of testosterone. The method was developed and validated to meet analytical standards. Correlation of the IC_{50} values obtained with DBF and testosterone was analysed. The results are compared with literature data and are discussed for questions of putative endocrine disruption by interference of antifungal therapy with steroid synthesis in humans.

3.2.2 Materials and Methods

3.2.2.1 Chemicals and Reagents

Microsomes (“Supersomes”) containing human aromatase (CYP19) coexpressed with human cytochrome P450 reductase (baculovirus/insect cell expressed) and dibenzylfluorescein (DBF) were a kind gift from BD Gentest (Natutec/Frankfurt am Main/Germany). Internal standard, d_6-cholesterol, was from Sigma (Taufkirchen, Germany). Bitertanol, cyproconazole, flusilazole, hexaconazole, imazalil, myclobutanil, penconazole, prochloraz, propiconazole, tebuconazole, triadimefon and triadimenol were from Dr. Ehrenstorfer (Augsburg, Germany). Epoxiconazole was a kind gift from the Swiss Federal Research Station (Wädenswil, Switzerland). Fluconazole was kindly provided by Pfizer GmbH (Karlsruhe, Germany). Fadrozole and letrozole were gifts from Novartis Pharma AG (Basel, Switzerland). Clotrimazole was from Bufa (Uitgeest, The Netherlands). Bifonazole, ketoconazole,

itraconazole, miconazole and all other chemicals were from Sigma/Fluka (Taufkirchen, Germany). All solvents used were HPLC grade or of higher purity.

3.2.2.2 Inhibition Assay

A stock solution of testosterone (3.46 mM) was prepared in methanol. Stock solutions and dilution series of azoles were prepared in ethanol, except for itraconazole, miconazole and ketoconazole, where dimethylsulfoxide was used. Maximum concentration of organic solvents in the assay was 2%.

A mastermix containing 419 µL of 0.1 M potassium phosphate buffer (pH 7.4), 13 µL of a 50 mM $NADP^+$ solution, 50 µL of a 33 mM magnesium chloride solution, 17 µL of a 100 mM glucose-6-phosphate solution and 2 µL of a 0.1 U/µL glucose-6-phosphate dehydrogenase solution was prepared. this solution (80 µL) was mixed with 1 µL each of testosterone solution and azole dilution. The reaction mixture was preincubated for 5 min at 37°C, after that the reaction was started by addition of 2 µL of CYP19. After 40 min 84 µL of isopropanole containing d_6-cholesterol as internal standard (5 µM) were added to stop the reaction. Protein was removed by centrifugation at 15 000 g for 5 min. The supernatant was analysed by LC-MS/MS as described below. Concentrations of azoles were spaced logarithmically by a factor of $\sqrt{10}$ and covered at least three decades centered around an IC_{50} estimated from the DBF data (Chapter 3.1) [43]. Incubations were run in duplicates.

Deuteriated cholesterol was preferred to cholesterol as internal standard, to avoid interference by natural cholesterol present in the microsomal fractions containing the enzyme.

Apparent K_m and V_{max} for testosterone have been reported to be 0.043 µM and 8.4 min^{-1}, respectively [37]; the values for DBF are 0.188 µM and 0.32 min^{-1}, respectively (Chapter 3.1 and [37]). This indicates that CYP19 has a much higher substrate affinity to the substrate testosterone than to the pseudo-substrate DBF and much higher activity. In order to avoid substrate depletion the testosterone concentration used was much higher than the K_m (41 µM) and higher than the concentration of DBF (0.46 µM) used in the other assay.

3.2.2.3 Optimisation of Mass Spectrometry

The LC-APPI-MS/MS method (liquid chromatography-atmospheric pressure photoionisation-tandem mass spectrometry) was optimised for the analyte estradiol and the internal standard d_6-cholesterol, with respect to sensitivity, structural identification and quantification. An infusion experiment to examine ionisation and fragmentation patterns of estradiol and d_6-cholesterol was carried out. A syringe pump (Single Syringe Pump 11, Harvard Apparatus Inc., Holliston, USA) was used to provide a constant analyte infusion (300 µL/min) into the LC eluent via a T-connection. Analyte concentrations were chosen in the range of 5 - 100 ng/µL to obtain a constant signal in the Q1 scan mode. Basic source and MS parameters such as declustering potential (DP), focusing potential (FP), collision energy (CE) and exit potential (CXP) were optimised using the "quantitative optimisation" function of "Analyst 1.3.1."

3.2.2.4 HPLC

Ten µL of the supernatants obtained after centrifugation of the incubation mixtures were injected on a Symmetry Shield C_8 HPLC column (3.0 mm x 150 mm; 5 *µ*m; Waters, Eschborn, Germany) using an Agilent 1100 autosampler and an Agilent 1100 HPLC-pump (Waldbronn, Germany). The samples were separated by gradient elution with water (solvent A) and MeOH (solvent B) using the following conditions: 50% A isocratic for 1 min, linear to 2% A within 1 min and isocratic for 8 min at 2% A with a flow rate of 500 µL/min. Toluene was used as dopant with a flow rate of 50 µL/min and was delivered to the APPI source with a syringe pump and a 50 mL glass, gastight syringe (Hamilton Company, Reno, USA). Analyte detection was carried out in a two-period measurement comprising of 7.5 minutes measurement in negative ion mode for detection of estradiol followed by 3.5 minutes of positive ion mode measurement to detect d_6-cholesterol. To avoid ionisation interference and ion source contamination by the phosphate buffer in the sample, the first chromatographic fraction (0.1 - 3.0 min) was split away using a Valco Valve (VICI, Houston, Texas). The circuitry is shown in Figure 6. The external HPLC pump (Ti Series 1050 from Hewlett Packard, Waldbronn, Germany) was used to provide a permanent solvent flow into the APPI source during the initial split phase. This is necessary to obtain constant conditions of ionisation within the APPI source.

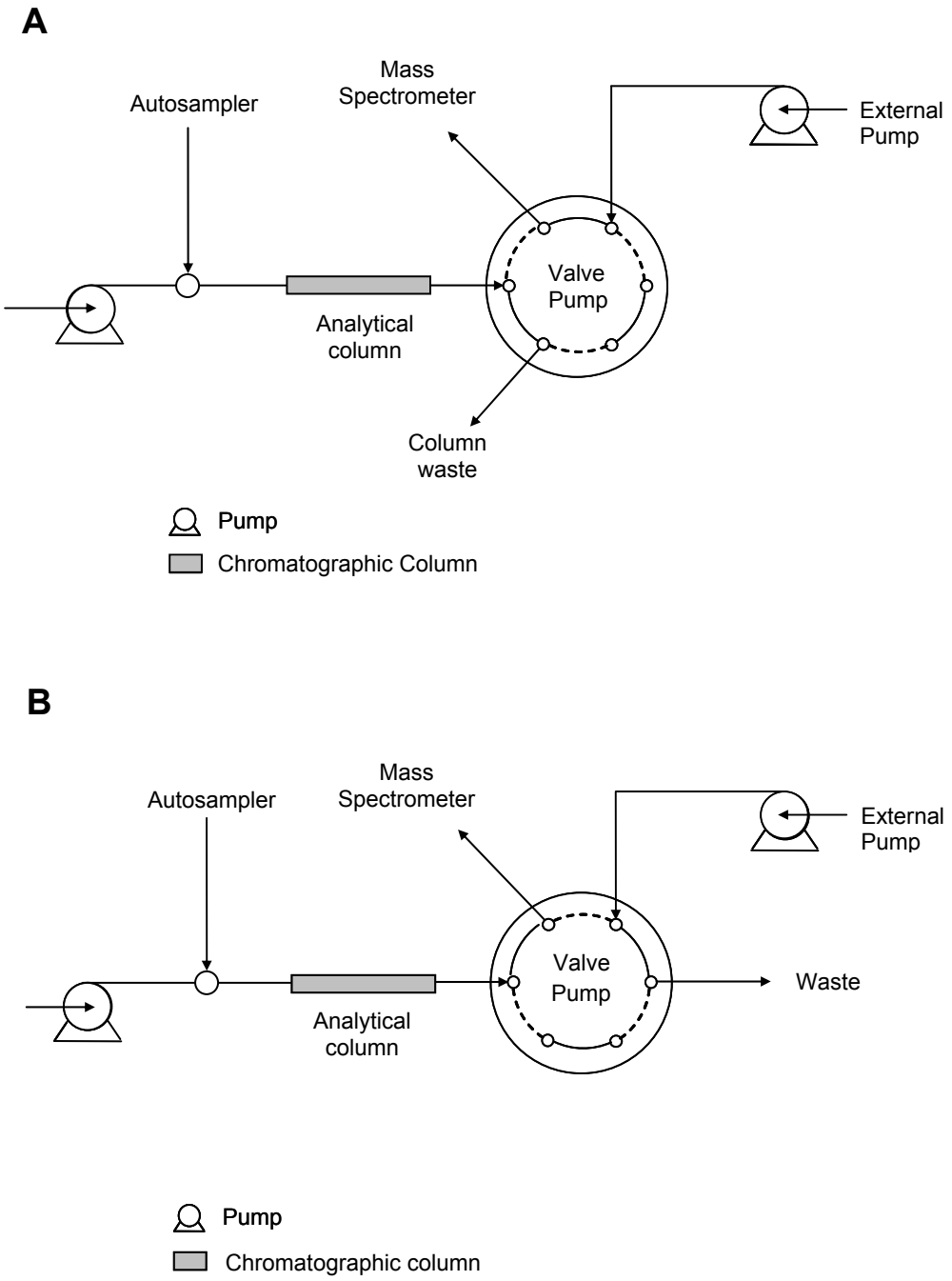


Figure 6: Sample clean up circuitry for HPLC: Step A: Sample loading and clean up step (0.1-3 min); step B: Elution (3-11 min).

3.2.2.5 MS Parameters

The HPLC system was coupled to a triple stage quadrupole mass spectrometer (API 3000, Applied Biosystems, Darmstadt, Germany) equipped with an atmospheric pressure photoionisation source. Estradiol was detected in negative ion mode, d_6-cholesterol in positive ion mode at a vaporizer temperature of 400°C and a source voltage (IS) of 2 000 V. Spectral data were recorded with N_2 as collision

gas (CAD = 4) in the multiple reaction monitoring (MRM) mode with a dwell time of 500 ms for each transition monitoring the following MS/MS ion-transitions.

Table 5: MS/MS-Transitions, declustering potential and collision energy used.

Transition $[M\text{-}H^+]^-$ →	**Compound**	**DP [V]**	**CE [V]**
271.2 → 144.9	Estradiol (quantifier)	-61	-54
271.2 → 182.9	Estradiol (qualifier)	-61	-56
Transition $[M+H^+\text{-}H_2O]^+$ →			**]**
375.4 → 167.5	d_6-Cholesterol (quantifier, internal standard)	36	31
375.4 → 95.2	d_6-Cholesterol (qualifier, internal standard)	36	55

3.2.2.6 Method Validation

The limit of detection (LOD s/n = 3) for estradiol was 0.05 ng/mL; the limit of quantification (LOQ s/n = 9) was 1 ng/mL, the dynamic range spanned from 1 to 10 000 ng/mL with a linear regression coefficient R = 0.998. The accuracy of individual samples in the calibration curve ranged from 83 to 116%, the precision was ≤ 7%. Analytical data for d_6-cholesterol were: LOD not determined, LOQ 0.1 ng/mL, dynamic range 0.1 to 5 000 ng/mL, linear regression coefficient 0.998, accuracy 81 to 111%, precision 7%. The negative ionisation mode was applied for estradiol since the phenolate anion produced is mesomerically stabilized and ionisation yields were considerably higher than in positive ion mode. This is consistent with literature for estradiol detection with electrospray – tandem mass spectrometry [45]. Figure 7 A shows the product-ion spectrum of estradiol in the positive ion mode, Figure 7 B in the negative ion mode and proposed fragmentation pattern. The product-ion spectrum of d_6-cholesterol is given in Chapter 4.1.

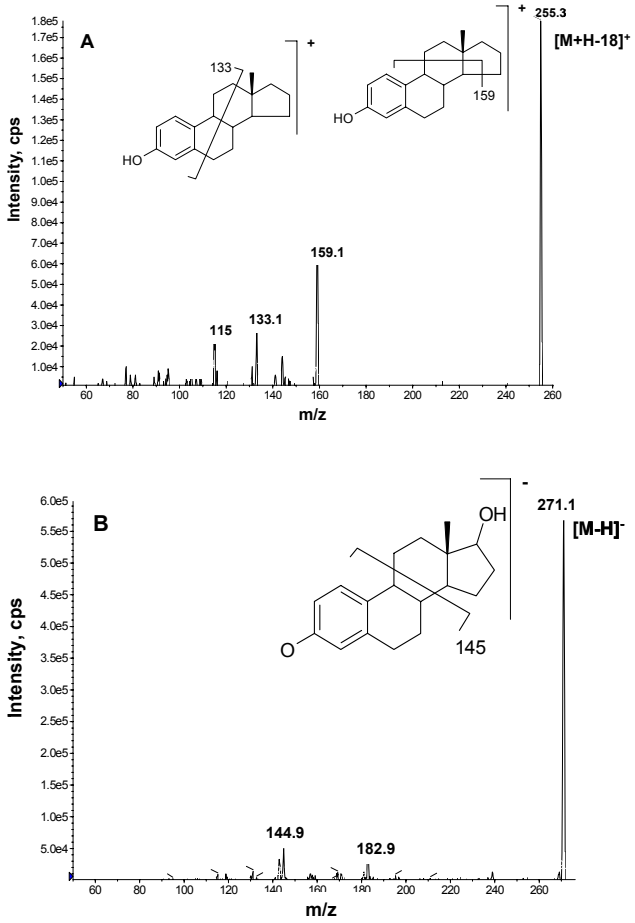


Figure 7: Product-ion spectra of estradiol in the positive ion mode (A) and in the negative ion mode (B) and proposed fragmentation patterns.

Estradiol had a retention time of 5.8 minutes, the internal standard d_6-cholesterol 9.3 minutes. Figure 8 shows chromatograms of incubations of CYP19 with (A) 1 000 µM, (B) 100 µM and (C) 1 µM hexaconazole as inhibitor, respectively.

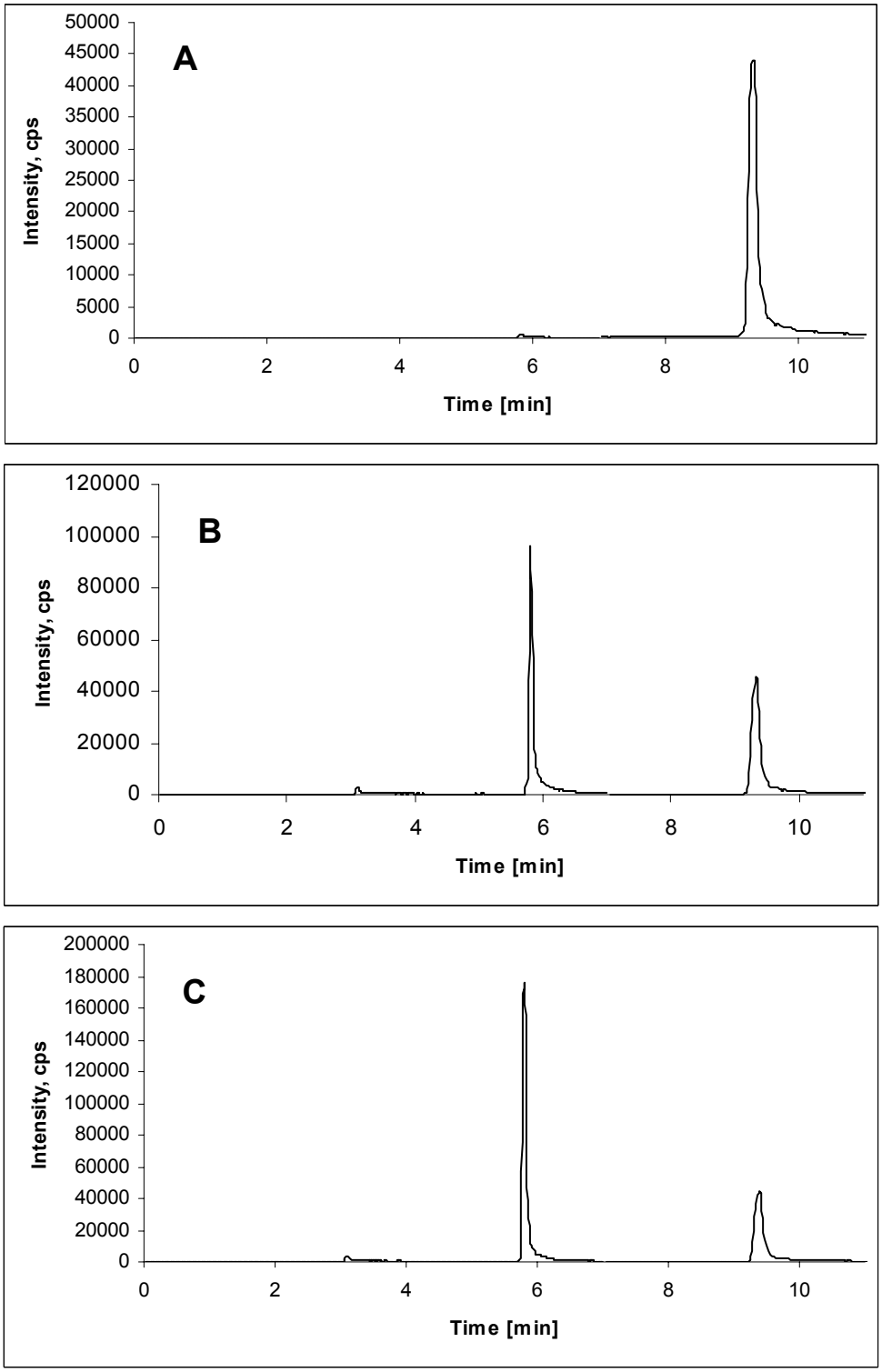


Figure 8: Separation of estradiol (5.8 min) and d_6-cholesterol (9.4 min) in incubations of testosterone with CYP19, using different concentrations of hexaconazole as inhibitor of CYP19. (A) 1 000 µM, (B) 100 µM and (C) 1 µM. Note the different scales for the y-axis.

3.2.2.7 Data Analysis

The area of the estradiol peak was normalized by the peak area of the internal standard. The resulting relative area was plotted against the $\log_{10}$ of the concentration of azole. The data were analysed as described in Chapter 3.1 and [43]. Four parameters were estimated by fitting the duplicate data sets to a probit curve: The estradiol relative area at azole concentrations 0 and ∞, the relative area at half maximum inhibition and the slope of the probit curve. Statistical analysis was performed by a nonlinear mixed-effects model fit by maximum likelihood run on the free statistics software "R" (http://www.r-project.org). A random error was attributed to the estradiol relative area at azole concentration 0, to account for differences between the duplicates for the asymptotic background measure. Spearman rank correlation was calculated using the statistics software “R”.

3.2.3 Results and Discussion

3.2.3.1 List of IC_{50} Values

Table 5 gives an overview of the potency of inhibition of recombinant human CYP19 (aromatase) by 21 azoles used as fungicides in agriculture or as antifungal or cytostatic drugs for human therapy. IC_{50} values determined with testosterone as substrate are given in column L. As expected from their therapeutic indication, the two cytostatic drugs fadrozole and letrozole were among the most potent inhibitors, with IC_{50} values of 0.66 and 0.13 µM, respectively. In the group of the 13 agriculturally used fungicides, IC_{50} values ranged more than 2 000-fold between 0.44 and 972 µM. Prochloraz was the most potent compound, with an IC_{50} in the range of those determined for the cytostatic drugs followed by imazalil and flusilazole. Bitertanol, triadimenol, tebuconazole, triadimefon and propiconazole were only weak inhibitors of aromatase. Of the six antifungal drugs tested bifonazole was the most potent. With an IC_{50} value of 0.24 µM this drug also classifies with the cytostatic drugs. The two older drugs clotrimazole and miconazole were also relatively potent, while the more recently developed drugs, ketoconazole and itraconazole were only weak inhibitors. Fluconazole showed no inhibition at 300 µM.

Table 6: Inhibition of recombinant human CYP19 (aromatase) by azoles used as fungicides in agriculture and as antifungal or cytostatic drugs in humans. New data and comparison with literature values. IC_{50} [µM] values indicate the concentration of azole required to reduce product formation by a factor of two.

Reference	**A**	**B**	**C / D**	**E**	**F / G**	**H**	**I / J**	**K**	**L**	**Ratio L/K**
Fungicides										
Bitertanol								>20	**>>300**	
Cyproconazole								8.5	**≈ 100**	
Epoxiconazole							4.5/2	1.44	**≈ 100**	
Flusilazole								0.055	**7.7**	140
Hexaconazole						55		9.9*	**96**	10
Imazalil (I)			---/0.15	0.04		0.1		0.072	**3.6**	50
Myclobutanil								0.47	**47**	100
Penconazole						20		0.85	**47**	55
Prochloraz (I)			---/0.7	0.34		0.1		0.047	**0.44**	9
Propiconazole				6.5		5		3.2	**199**	62
Tebuconazole						50		5.8	**609**	105
Triadimefon				32				17.5	**483**	28
Triadimenol				21				12.6	**972**	77
Antifungal drugs										
Bifonazole (I)		0.007			0.31/0.27			0.019	**0.24**	13
Clotrimazole (I)		0.018	1.8/1.8		0.43/0.35			0.11	**1.2**	11
Fluconazole		26.8						>140	**>>300**	
Itraconazole								>70	**≈ 100**	
Ketoconazole (I)	0.9	2.0	60/65		7.3/6			5.6	**281**	50
Miconazole (I)		0.036	0.6/0.6		0.47/0.45			0.064	**8.2**	128
Aromatase inhibitors	**M**	**N / O**	**P**	**Q**	**R**	**S**				
Fadrozole (I)	0.005	0.033/0.067	0.059	0.052	0.0054			0.0076	**0.66**	87
Letrozole	0.0115					0.002		0.015	**0.13**	9

(I) Imidazole; all other azoles are triazoles.

* IC_{50}-value corrected after publication

A:Stresser et al., 2000 baculovirus/insect cell-expressed aromatase supersomes and DBF as substrate [37]
B: Kragie et al., 2002 baculovirus/insect cell-expressed aromatase supersomes and DBF as substrate [38]
C: Mason et al., 1985 human placental microsomes and tritiated androstenedione as substrate [31]
D: Mason et al., 1987 human placental microsomes and tritiated androstenedione as substrate [34]
E: Vinggaard et al., 2000 human placental microsomes and tritiated androstenedione as substrate [35]
F: Ayub et al., 1988 human placental microsomes and tritiated androstenedione as substrate [32]
G: Ayub et al., 1988 human placental microsomes and tritiated testosterone as substrate [32]
H: Sanderson et al., 2002 H295R cells and tritiated androstenedione as substrate [36]
I: Heneweer et al., 2004 H295R cells and tritiated androstenedione as substrate [46]
J: Heneweer et al., 2004 R2C cells and tritiated androstenedione as substrate [46]
K:Trosken et al., 2004 baculovirus/insect cell-expressed aromatase supersomes and DBF as substrate Chapter 3.1 [43]
L:Chapter 3.2 baculovirus/insect cell-expressed aromatase supersomes and testosterone as substrate
M: Bhatnagar et al., 1990 human placental microsomes and ^{14}C-androstenedione as substrate [47]
N:Toma et al., 1996 purified aromatase from placental homogenate and androstenedione as substrate [48]
O:Toma et al., 1996 purified aromatase from placental homogenate and 7-ethoxycoumarin as substrate [48]
P: Auvray et al., 1999 human placental microsomes and tritiated androstenedione as substrate [49]
Q: Recanatini et al., 2001 human placental microsomes and tritiated testosterone as substrate [50]
R: Dukes et al., 1996 human placental microsomes and tritiated testosterone as substrate [51]
S: Miller et al., 1999 particulate fractions of breast cancers and tritiated androstenedione as substrate [52]

3.2.3.2 Comparison of Assays

The IC_{50} values obtained earlier with DBF as substrate (column K) (Chapter 3.1) [43] were lower than those obtained with testosterone by a mean factor of about 60. For the individual substances the ratios ranged between 9- and 140-fold (last column of Table 6). The fact that testosterone has higher apparent K_m- and V_{max}-values as compared to DBF and the high substrate concentration used in the testosterone assay in part explains the difference. While the numerical IC_{50} values differ markedly, the rank order of potencies determined with the two substrates does not change much. Figure 9 shows the correlation in a double logarithmic plot. The Spearman rank correlation coefficient σ calculated for the values given in columns K and L was 0.911 with a p of 2×10^{-16}. Bitertanol, fluconazole and itraconazole were not included in the calculation because of a lack of an IC_{50} value based on DBF.

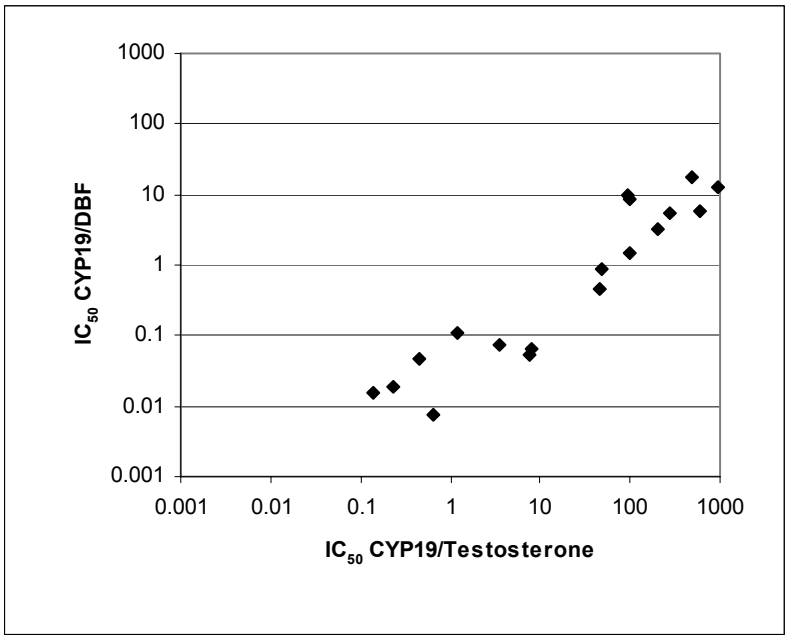


Figure 9: Correlation of inhibitory potencies of 18 azoles on recombinant human CYP19 (aromatase), using testosterone or dibenzylfluorescein (DBF) as substrates (x- or y-axis, respectively). IC_{50} values in µM concentration. Spearman's rank correlation σ = 0.91. Note the double-logarithmic dose scaling. Bitertanol, fluconazole and itraconazole were not included in this correlation analysis because of a lack of numerical IC_{50} estimates.

The DBF-based assay only requires a fluorescence measurement, while the LC-MS/MS assay uses much more expensive equipment. The high correlation of the IC_{50} values determined with the two different substrates indicates that the DBF assay can well be used for screening purposes. This holds not only for potency ranking but also in quantitative terms, if the ratio discussed above is taken into account and if a number of azoles are included as positive controls. It should be noted, however, that DBF is not specific for CYP19 as it is metabolised by a number of other cytochrome P450 enzymes, such as CYP3A4 or CYP2C8 [53]. DBF can therefore not be utilised for monitoring CYP19 activities in samples that contain other cytochrome P450 isoenzymes, e.g. placental microsomes. Another drawback of fluorescence detection is a potential spectrometric interference by inhibitors.

Up to now aromatase assays utilised a tritiated water release assay [54,55] and to a lesser extend ^{14}C-marked androgens [55], antibodies against estradiol [56], gas chromatography-mass spectrometry [57] or HPLC-UV detection [58]. Drawbacks of these methods are the use of expensive radiolabeled compounds, expensive antibodies or time consuming sample preparation. In comparison to the DBF and testosterone assays presented here the HPLC-UV assay utilises far more enzyme (25 µL compared to 5 µL/DBF and 2 µL/testosterone) [59].

The LC-MS/MS assay as developed here has a number of advantages. High analytical sensitivity and high selectivity for product measurement allow the use of very small amounts of enzyme. By monitoring the fragmentation estradiol and d_6-cholesterol can be detected unequivocally. Sample work-up is minimal. Testosterone is a physiological and specific substrate for CYP19 and the product formation is measured directly opposed to the assay based on the release of tritiated water. The assay can be used with other sources of enzyme, e.g. tissue fractions, since other CYPs do not convert testosterone to estradiol. Formation of 6-hydroxytestosterone by unspecific CYPs does not interfere with the LC-MS/MS detection, and reaction side products should not have a great influence on IC_{50} values, considering the high substrate concentration used. The assay could be adapted to a high throughput 96-well plate design since the incubation volume at the end of the assay is only 160 µL. Last not least, no interference of high concentrations of inhibitors with the LC-MS/MS detection has been noted.

3.2.3.3 Comparison with Literature Data

IC_{50} values for CYP19 inhibition that had been determined in other laboratories are also given in Table 6, with the respective references in the footnotes. Sources of enzyme included microsomes from placenta and a breast cancer specimen, cells expressing aromatase, and microsomes containing recombinant enzymes. Substrates used were DBF (columns A, B, K), 7-ethoxycoumarin (column O), or radiolabeled androstenedione or testosterone (all other columns of reference). Product formation was monitored by fluorescence or ^{3}H- and ^{14}C-measurements. The studies listed under A and B were based on the same assay the described study K, using DBF as substrate. The IC_{50} values determined for bifonazole and miconazole were within a factor of 2.5, while the differences for the other drugs were up to a factor of six. In view of the much larger span of IC_{50} values between the chemicals, this appears to be acceptable. In fact, potency ranking produced the same order. In other situations, inter-laboratory differences by up to a factor of 10 were observed even when the same assay was used [50,51].

Older studies on CYP19 inhibition were based on the use of tritiated androstenedione as substrate (columns C to J). In one study (F/G), both androstenedione and testosterone were used. The respective results show only minor differences for the IC_{50} values, which indicates that these androgens are interchangeable. Interlaboratory differences are much larger. Compared with data

generated in this work (column L), literature values are lower in most cases, but there are also examples of concordance (for bifonazole in F/G or for clotrimazole in C/D).

3.2.3.4 Confounding Factors

IC_{50} values can be influenced not only by the use of different substrates, but also by additional parameters, e.g. protein concentration or incubation time. Using microsomal proteins from human livers, animal livers and insect cells, it was shown that protein concentrations ≤ 0.1 mg/mL did not appreciably bind test compound in an unspecific manner [60]. In the assay described in this work protein concentrations were 0.11 mg/mL and 0.022 mg/mL with testosterone and DBF, respectively, so that this interference can be neglected.

Different incubation times had also been reported as confounders [61]. An up to 4-fold increase in IC_{50} values for the inhibition of CYP3A4 supersomes or human liver microsomes by ketoconazole was noted when the incubation time was increased from 4 to 40 minutes. The increase with time was consistent with a metabolic depletion of the inhibitor. Ketoconazole is a substrate for human CYP3A4. If metabolites are less inhibitory than the parent compound IC_{50} values increase with incubation time. If metabolites are more potent inhibitors than the parent compound a decrease in IC_{50} values with incubation time occurs.

CYP19 in the placenta has been shown to metabolise methadone, levo-alpha-acetylmethadol, 7-ethoxycoumarin, aflatoxin B1 and buprenorphine [48] [62-65]. No CYP19-dependent metabolism had been reported for azoles. On the contrary, azoles were used in the cited articles as probes to inhibit the monitored reactions. Thus, different incubation times or an effect of CYP19 on the concentration of inhibitor by metabolic elimination is not expected.

3.2.3.5 IC_{50} Values vs. Exposure to Fungicides

Urinary excretion of a hydroxylated metabolite of epoxiconazole had been measured after agricultural application. Exposure doses ≤ 100 µg (0.3 µmol) per person and day were estimated [24]. Assuming a body weight of 70 kg and an apparent volume of distribution of 1 liter per kg body weight, maximum plasma levels of 0.004 µM can be derived. This is by a factor of more than 20 000 below the IC_{50} value of epoxiconazole. Exposure to fungicides from residues in food items can show even larger margins of safety. For example, residues of seven azole fungicides were measured in wine samples. Tebuconazole showed highest levels of 30 ng/mL

(Chapter 5) [66]. Consumption of 500 mL wine would thus result in a dose of 15 µg, equivalent to 0.048 µmol. Using the same body weight and volume of distribution, a plasma level of 0.0005 µM results, which is by factor of nearly one million below the IC_{50} value of tebuconazole. Note that if the apparent volume of distribution is higher than 1 L/kg body weight, the safety factors are even larger.

3.2.3.6 IC_{50} Values vs. Exposure to Antifungal Drugs

For bifonazole maximum serum concentrations of 0.051 µM have been measured after dermal application of a 1% cream to infants with diaper rash [38]. Maximum plasma concentrations of 0.029 µM of clotrimazole were mentioned after application of a 1% cream formulation (German Summary of Product Characteristics). Itraconazole is applied orally and maximum plasma concentrations of 2.8 µM were reached after daily intake of 400 mg (German Summary of Product Characteristics). Ketoconazole is administered orally in doses of 200 to 400 mg per day. Maximum plasma concentrations after a single dose of 200 mg reached 6.6 µM [38]. After topical application of a 2% cream no plasma concentrations were reported (German Summary of Product Characteristics). For miconazole maximum serum concentrations reached 2.4 µM after a single oral dose of 1 000 mg (German Summary of Product Characteristics). A single vaginal dose of 1 200 mg resulted in maximum plasma levels of 0.024 µM [38]. For comparison to cytostatic drugs, letrozole in daily doses of 2.5 mg resulted in average maximum plasma levels of 0.129 µM (German Summary of Product Characteristics).

Ratios between the maximum plasma concentrations and the IC_{50} values for CYP19/testosterone were calculated on the basis of the values cited above and the values given in column L of Table 6 to be 0.21, 0.024, 0.028, 0.023, 0.29 and 0.0029 for bifonazole (dermal), clotrimazole (dermal), itraconazole (oral), ketoconazole (oral), miconazole (oral), and miconazole (vaginal). For letrozole, for which aromatase inhibition is the therapeutic principle, the respective value was 1. Compared with the analysis made above for exposure to fungicides used in agriculture, antifungal therapy with azoles appears to be more critical with respect to inhibition of aromatase.

3.2.4 Conclusions

The comparison between the IC_{50} values determined by the two assays rendered a rank correlation. Thus, although the absolute IC_{50} values differ by one to

two orders of magnitude and the natural substrate is not utilised, the values obtained with DBF give a good estimate of the inhibitory potencies of azole compounds.

Sexual development and adult sexual behavior rely on tightly regulated sex steroid levels. Inhibition of aromatase can lead to disruption and imbalance between androgens and estrogens. For letrozole the maximum plasma concentration corresponds to the IC_{50} value determined, which is favorable since CYP19 is the therapeutic target of azoles used as cytostatic drugs.

For the antifungal drugs bifonazole and miconazole, maximum plasma concentrations are within a factor of five of the IC_{50} values and thus quite close to a concentration producing 50 percent enzyme inhibition. On the other hand, a factor of at least 35 was estimated for the difference between the IC_{50} values and maximum plasma concentrations for clotrimazole, itraconazole, ketoconazole and miconazole (vaginal dosing).

For the orally applied azoles itraconazole and ketoconazole treatment during pregnancy is contraindicated (German Summaries of Product Characteristics). The same holds for vaginal applications of clotrimazole and miconazole (German Summaries of Product Characteristics). Problems could arise from the usage of miconazole gels for infections of mucous membrane of the mouth of children, were parts of the dose could be ingested and become available systemically. In Germany, bifonazole is only registered as topical cream, gel or solution, however not for vaginal application. As a consequence of the small safety factor between the IC_{50} value and the maximum plasma concentration, usage of bifonazole during pregnancy and in children should be reconsidered.

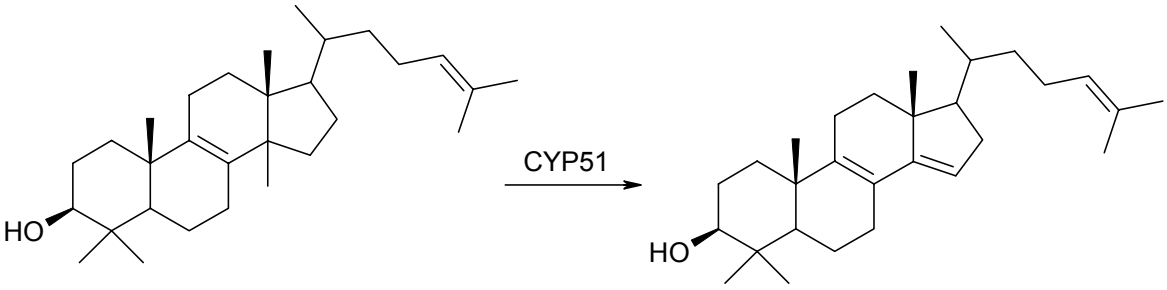
The quantitative considerations made above must obviously be viewed with caution. Plasma concentrations cannot be equated with concentrations near the enzyme. Pharmacokinetic considerations and duration of treatment must be considered case by case. Nevertheless, our data show differences in inhibitory potency by factors of >>1 000 for both classes, antifungal drugs and fungicides used in agriculture. Screening for CYP19 (aromatase) inhibition in an early stage of drug development can therefore help optimise the ratios between inhibitory potency and estimated exposure levels. In view of the manifold higher exposure doses associated with therapeutic use as compared with use in agriculture or as residues in food, careful analysis of the situation appears to be more urgent for antifungal drugs than for fungicides.

4 Inhibition of CYP51 (Lanosterol-14α-demethylase)

4.1 Analytical Method to Detect Lanosterol

4.1.1 Introduction

Inhibition of CYP19 is not the only mode of endocrine disruption. In a second step the inhibitory potency of azole compounds used as agricultural fungicides, as fungicides in human medicine and as cytostatic drugs on human CYP51 was to be assessed. In mammals CYP51 catalyses the oxidative demethylation of lanosterol to FF-MAS (Scheme 4).



Lanosterol FF-MAS

Scheme 4: Demethylation of lanosterol to FF-MAS catalysed by CYP51 (lanosterol-14α-demethylase).

The resulting sterols of the CYP51 reaction are named meiosis activating sterols (MAS) and play an important role in meiosis in testis, sperm and follicle [67,68]. Therefore the inhibition of CYP51 by azoles may also influence the endocrine balance in mammals as previously suggested [2].

No assay for testing the inhibitory effect of azoles on human CYP51 comparable to that of human CYP19 is available. Such a method is necessary to test azoles applied as fungicides agriculturally or in human medicine for their inhibitory potency of human CYP51. It would further come to hand for the development of new substances, designed to inhibit specifically only fungal CYP51.

Four endogenous substrates of lanosterol-14α-demethylase are known: lanosterol, 24,25-dihydrolanosterol, 24-methylenedihydrolanosterol and obtusifoliol. Chemically the molecules are very closely related and differ only marginally in the side chain and in the number of methyl groups at C4. Most CYP51 enzymes from the different phyla will accept all four compounds as substrates, however some preferences are known: plant CYP51 exclusively demethylates obtusifoliol [69], in

animals lanosterol and 24,25-dihydrolanosterol are the substrates of choice and in fungi and yeast lanosterol and 24-methylenedihydrolanosterol are preferred [9]. Pseudo substrates for the 14α-demethylation reaction are not known up to now. Thus incubation procedures have to be performed with one of the natural substrates.

Lanosterol and its metabolites were predominantly quantitated directly or after derivatisation by gas chromatography mass spectrometry (GC-MS) [70,71]. If amounts of about 1 ng of each steroid were available, lanosterol and FF-MAS could be separated by reversed phase high performance liquid chromatography (HPLC) and detected using a diode array detector [72]. The detection of steroids like cholesterol and cholesterol oxides by HPLC mass spectrometry (HPLC-MS) using an atmospheric pressure chemical ionisation (APCI) source are published [73]. APCI has to be used instead of electrospray ionisation (ESI) since steroids are too lipophilic and exhibit no moieties for protonation or deprotonation processes typical for ESI (please also see Scheme 4). The distinction of ergosterol from lanosterol by APCI and tandem mass spectrometry was reported with a limit of detection in the low ng/mL range [74]. Here we present a LC-MS/MS method using an atmospheric pressure photoionisation source for quantitation of lanosterol and FF-MAS in the low ng/mL range. It was tested in inhibition experiments of azoles with a human recombinant CYP51 enzyme.

4.1.2 Materials and Methods

4.1.2.1 Chemicals and Reagents

Epoxiconazole was a kind gift from the Swiss Federal Research Station, Wädenswil, Switzerland. 4,4-Dimethyl-5α-cholesta-8,14,24-triene-3β-ol (FF-MAS) was a kind gift from NovoNordisk (Gentofte, Denmark). HPLC grade water was from Roth (Karlsruhe, Germany). All other chemicals were from Sigma/Fluka (Taufkirchen, Germany). All solvents used were HPLC grade or better.

Human lanosterol-14α-demethylase (CYP51, Accession No. U23942) co-expressed with human P450 reductase (baculovirus/insect cell-expressed) was a kind gift from BD Gentest. According to the manufacturer enzyme expression was carried out in spinner flasks of BTI-TN-5B1-4 insect cells. Infection time was for 48 hours. Membrane fraction from insect cells was prepared according to standard methods with slight modification [75].

4.1.2.2 Enzyme Inhibition Assay

Stock solutions of lanosterol (1 mM) and Triton X-100 (16 mg/mL) were prepared in isopropanol. Stock solutions and dilution series of azoles were prepared in ethanol. Lanosterol (11.9 µM) was incubated in 0.1 M potassium phosphate buffer (pH 7.4) containing 1.1 mM $NADP^+$, 2.8 mM glucose-6-phosphate, 0.34 U/mL glucose-6-phosphate dehydrogenase, 2.8 mM magnesium chloride, 1 µL of azole dilution and 2 µL of the Triton X- 100 stock solution in a total volume of 84 µL in all experiments. The reaction mixture was preincubated for 5 min at 37°C and the reaction was started by addition of the enzyme (0.1 µM CYP51). After 40 min at 37°C 80 µL of isopropanol containing internal standard d_6-cholesterol (5 µM) were added to stop the reaction. Protein was removed by centrifugation at 15 000 × g for 5 min. The supernatant was analysed by LC-MS/MS.

4.1.2.3 Optimisation of Mass Spectrometry Methods

Each method (LC-APCI-MS/MS, LC-APPI-MS/MS) was optimised separately with respect to sensitivity, analyte identification, and quantitative measurement. Infusion experiments to examine ionisation and fragmentation patterns of the analytes were carried out with both sources. A syringe pump (Single Syringe Pump 11, Harvard Apparatus Inc., Holliston, USA) was used to provide a constant analyte infusion (300 µL/min) into the LC eluent via a T-connection. Analyte concentrations were chosen in the range of 5 - 100 ng/µL to obtain a constant signal in the Q1 scan mode. Basic source and MS parameters such as declustering potential (DP), focussing potential (FP), collision energy (CE) and exit potential (CXP) were optimised using the “quantitative optimisation” function of “Analyst 1.3.1.” DP and CE values for the individual compounds are given in Table 7.

Table 7: MS/MS-transitions, Declustering Potential and Collision Energy used.

Transition $[M+H-H_2O]^+ \rightarrow$	Compound	DP [V]	CE [V]
409.5 → 109.1	Lanosterol (quantifier)	41	43
409.5 → 95.3	Lanosterol (qualifier)	41	51
375.4 → 167.5	d_6-Cholesterol (quantifier, internal standard)	36	31
375.4 → 95.2	d_6-Cholesterol (qualifier, internal standard)	36	55
393.5 → 68.9	FF-MAS (quantifier)	46	55
393.5 → 55.2	FF-MAS (qualifier)	46	63

4.1.2.4 Quantitation

To quantify lanosterol, FF-MAS and d_6-cholesterol 10 µL of the supernatants were injected on a Symmetry Shield C_8 HPLC column (3.0 mm x 150 mm; 5 μm; Waters, Eschborn, Germany) using an Agilent 1100 autosampler and an Agilent 1100 HPLC-pump. The samples were separated by gradient elution with water (solvent A) and MeOH (solvent B) using the following conditions: 50% A isocratic for 1 min, linear to 2% A within 1 min, and isocratic for 8 min at 2% A with a flow rate of 36 mL/h. For APPI measurements toluene was used as dopant with a flow rate of 2.5 mL/h. The dopant was delivered to the APPI source with a syringe pump and a 50 mL glas, gastight syringe (Hamilton Company, Reno, USA). The HPLC system was directly coupled to a triple stage quadrupole mass spectrometer (API 3000, Applied Biosystems, Darmstadt, Germany) equipped with an atmospheric pressure chemical ionisation or an atmospheric pressure photoionisation source. Analytes were detected in the positive ion mode at a vaporizer temperature of 400°C. For APCI a needle current of 3 and for APPI a source voltage (IS) of 2 000 was applied. Spectral data were recorded with N_2 as collision gas (CAD = 4) in the multiple reaction monitoring (MRM) mode with a dwell time of 500 ms for each transition monitoring the MS/MS ion-transitions given in Table 7.

Quantitation of FF-MAS was based on calibration curves obtained after addition of known amounts of FF-MAS (1 – 10 000 ng/mL) to incubation samples containing heat-deactivated CYP51. Calibration curves were calculated from seven duplicate data points using Analyst 1.3.1 (Applied Biosystems).

4.1.2.5 Data Evaluation

The concentration range to be tested in the main experiment was defined in a dose-finding study with inhibitor concentration steps of factors of ten.
For the main experiment, at least seven logarithmically spaced concentrations were used, with the expected IC_{50} at the median. Dose responses were run in duplicates. A full analytical method for the quantitation of amounts of FF-MAS produced for the described inhibition studies was developed.

The area of FF-MAS corrected by the internal standard (relative area) was plotted against the log (base 10) of the concentration of azole. Four parameters were estimated by fitting the two replicate area data sets to a probit curve: The FF-MAS relative area at azole concentration 0 and ∞, the relative area at half maximum inhibition, and the slope of the probit curve. Statistical analysis was performed by a nonlinear mixed-effects model fit by maximum likelihood run on the statistics software "R". A random error was attributed to the FF-MAS relative area at azole concentration 0, to account for differences between replicates for the extrapolated null measurement.

4.1.3 Results

4.1.3.1 Characterization of CYP51

According to the manufacturer membrane fractions of baculovirus expressed CYP51 were tested for the conversion of [^{3}H]-lanosterol to FF-MAS using previously described methods [76]. The turn-over number using 15 µM lanosterol was reported to be 0.9 pmol/min/pmol P450.

4.1.3.2 Identification and Quantitation of FF-MAS

Due to their lipophilic character the ionisation of lanosterol, FF-MAS and d_6-cholesterol was performed with atmospheric pressure chemical ionisation or atmospheric pressure photoionisation. Collision-induced dissociation (CID) of $[M+H^+–H_2O]^+$ of all compounds provided a number of characteristic fragmentations in spite of the low collision energies used (Figures 10 A-C).

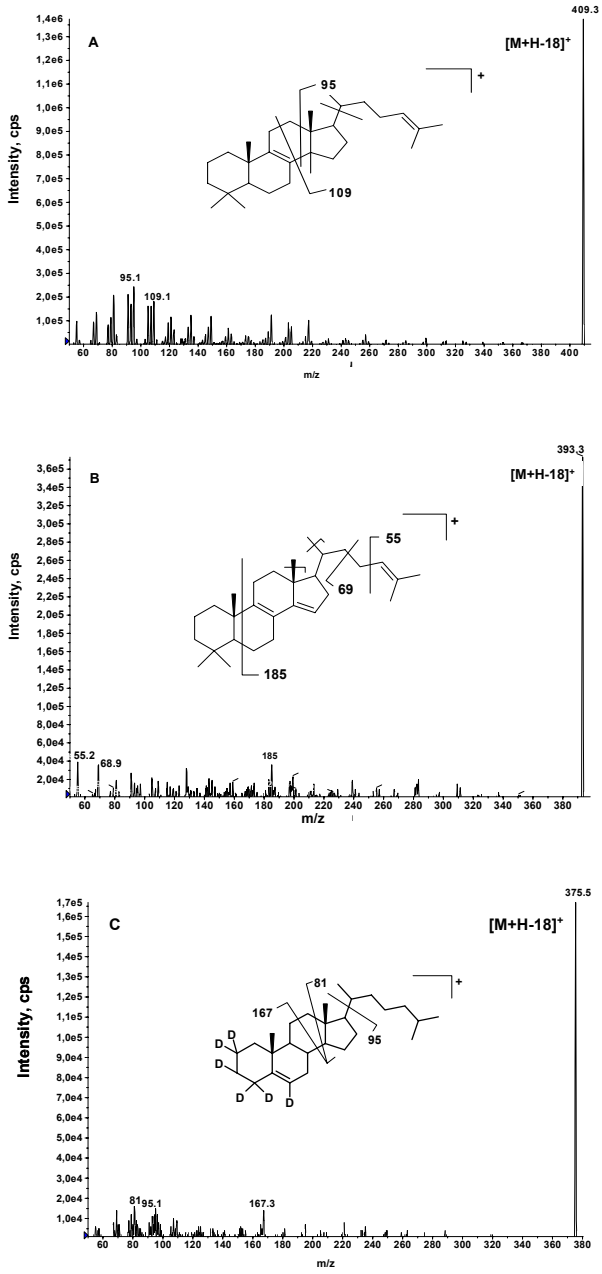


Figure 10: Product-ion spectra and proposed fragmentation of lanosterol (A), FF-MAS (B) and d_6-cholestrol (C).

For quantitation of FF-MAS in biological solutions, a method based on HPLC separation and multiple reaction monitoring mode was developed with the most intensive product-ions m/z 68.9 $[M\text{-}C_{24}H_{37}O]^+$ and m/z 55.2 $[M\text{-}C_{25}H_{39}]^+$ for FF-MAS and m/z 167.5 $[M\text{-}C_{15}H_{28}]^+$ for d_6-cholesterol used as internal standard. Calibration curves were linear in the range of 1 - 10 000 ng/mL with $R^2 \geq 0.999$. The accuracy for FF-MAS in incubation solution were within 100 ± 20% for n = 5 analyses of 10 ng/mL (Table 8).

Table 8: Analytical data of 3 steroids applying LC-APCI-MS/MS or LC-APPI-MS/MS.

		APCI	APPI
Lanosterol	LOD [ng/mL]	10	0.5
	LOQ [ng/mL]	50	5
	dynamic range [ng/mL]	50 – 1 000	5 – 10 000
	accuracy	80 – 120%	85 – 100%
	precision (10 x LOQ)	16%	4%
	regression factor	0.975	0.999
FF-MAS	LOD [ng/mL]	5	0.5
	LOQ [ng/mL]	10	1
	dynamic range [ng/mL]	10 – 1 000	1 – 10 000
	accuracy (10 x LOQ)	80 – 100%	94 – 115%
	precision (10 x LOQ)	14%	8%
	regression factor	0.998	0.999
d_6-Cholesterol	LOD [ng/mL]	n.d.	n.d.
	LOQ [ng/mL]	50	0.1
	dynamic range [ng/mL]	50 – 5 000	0.1 – 5 000
	accuracy (10 x LOQ)	91 – 119%	81 – 111%
	precision (10 x LOQ)	10%	7%
	regression factor	0.999	0.998

With the APPI source, the limit of quantitation (LOQ) could be reduced by a factor of 10 for lanosterol and FF-MAS (5 ng/mL and 1 ng/mL respectively) and by a factor of 500 for d_6-cholesterol (0.1 ng/mL) compared to APCI (Table 8). Thus, only very low amounts of about 8 pmol of the recombinant enzyme are required.

4.1.3.3 Inhibition of CYP51 by Azoles

Pilot inhibition studies were carried out with two azoles. Epoxiconazole is used as a fungicide, miconazole is used in human medicine to treat fungal infections e.g. of the skin or of the intestine. For epoxiconazole an IC_{50} value of 1.95 µM (range of 1 standard error: 0.048 - 0.068) and for miconazole a value of 0.057 (range of 1 standard error: 1.3 - 2.93) were calculated with n = 2. The testing of more azole compounds on human CYP51 is described under Chapter 4.3.

4.1.4 Discussion

4.1.4.1 Enzyme Assay

The described inhibition assay uses conditions that were optimised with respect to making comparison between different inhibitors possible. Substrate concentration was chosen to achieve high turn-over rates for lanosterol by CYP51 under optimal incubation conditions for CYP51 activity. Since lanosterol solubility is limited in aqueous systems and CYP51 activity decreases with increasing amounts of organic solvents a compromise had to be found. More than optimal amounts of organic solvent were needed to keep lanosterol reproducibly in solution. To limit the required amount of alcohol, suboptimal lanosterol concentrations were used.

This resulted in reproducible turn-over rates below K_m, necessitating a highly sensitive analytical method to detect FF-MAS as the product of the enzymatic reaction.

4.1.4.2 Steroid Analysis

Neutral sterols like lanosterol and FF-MAS were typically identified and quantitated in biological samples by GC-MS with or without derivatisation [77,78]. The use of GC-MS requires a time consuming sample work up to get volatile analytes and solvents. This is especially complicated for samples in an aqueous matrix. Therefore and due to the importance of sterols in medical applications, LC-MS based methods have to be developed to analyse sterols in biological samples. The same holds for the analysis of environmental water samples collected near farmlands and sewage plants, where sterols are discussed as important endocrine disrupters threatening the ecosystems [79].

4.1.4.3 Liquid-Chromatography Mass Spectrometry

The disadvantage of LC-MS for the analysis of neutral sterols is the poor ionisation efficiency by electrospray ionisation. Lanosterol, FF-MAS and d_6-cholesterol could not be ionised at concentrations below 100 ng/µL (data not shown). To improve ion formation in the ESI mode analytes have to be derivatised i.e. with electrophoric labels like pentafluorobenzylchloride which generally requires sample work up comparable to the GC-MS procedure. Another possibility is the use of sources which allow the ionisation of more lipophilic compounds like the atmospheric pressure chemical ionisation (APCI) source [80,81].

Previously, the atmospheric pressure photoionisation interface, independently developed by Bruins and Syage, was used for ionisation of corticosteroids by Greig et al. showing a higher sensitivity compared to ESI and APCI [82]. Greig et al. used a fourier transform ion cyclotron mass spectrometer (FTICRMS) which allows very sensitive analyses in the high resolution mode [82]

4.1.4.4 Fragmentation

Another possibility to get highest sensitivity with a more common mass selective detector is the use of a triple quadrupole mass spectrometer. Collision induced dissociation (CID) creates characteristic fragments that can be analysed in the third quadrupole. The product-ion spectra presented in Figure 10 are not very typical for CID spectra. Usually few characteristic fragment ions are produced by lower energies compared to electron impact (EI) spectra. In the case of steroids the fragment pattern is very similar to EI or to particle beam spectra of sterols as published by Byskov et al. [16]. CID spectra similar to ours have previously been published for 6β-OH-testosterone and hydrocortisone [83,84]. The fragments of the sterols presented in Figure 10 A-C are calculated as described for particle beam spectra of FF-MAS and lanosterol where a side chain fragmentation was observed [16]. The product-ion spectrum of ergosterol also shows a prominent m/z 69 identified as $[(CH_3)_2CHCH=CH]^+$ generated by CID of the side chain [85]. In addition, for sterols like estrone or testosterone a fragmentation in the C-ring of the steroid is reported after collision induced dissociation as suggested in Figure 10 A and 10 C [80] [86].

4.1.4.5 Photospray Ionisation

For the described application highest ionisation efficiency is achieved by the APPI source. In combination with the use of the multiple reaction monitoring (MRM) scan mode, which provides very low noise levels, the detection and quantitation in the low ng/mL range (0.1 - 5) is possible. These results are comparable to the electron capture (EC) derivatisation procedure for estrone [80]. For lanosterol, FF-MAS and d_6-cholesterol the use of the APPI source instead of the APCI source resulted in a 10 to 500 fold lower LOD and LOQ (Table 8). Greig et al. also reported a better signal to noise using APPI vs. APCI but did not provide quantitative data [82].

In contrast to FTICRMS, the APPI-MRM method is useful for quantitation in a wide dynamic range (Table 8). Compared to EC-APCI, only a simple sample work up

with a precipitation of proteins by centrifugation is necessary. This cleaning step is efficient to get highest sensitivity, reproducibility and accuracy better than 100 ± 20% at LOQ for all analytes tested so far.

For the use of the APPI-MS/MS method in biological matrix incubations with lanosterol and d_6-cholesterol were performed using a NADPH generating system and a human recombinant lanosterol-14α-demethylase (CYP51). With this method inhibitory activity of azoles on CYP51 could be determined (Figure 11). The quantitation of FF-MAS in supernatants of incubations shows clearly that the baculovirus expressed CYP51 forms FF-MAS with a similar turn-over number of 0.1 pmol product/min x pmol protein.

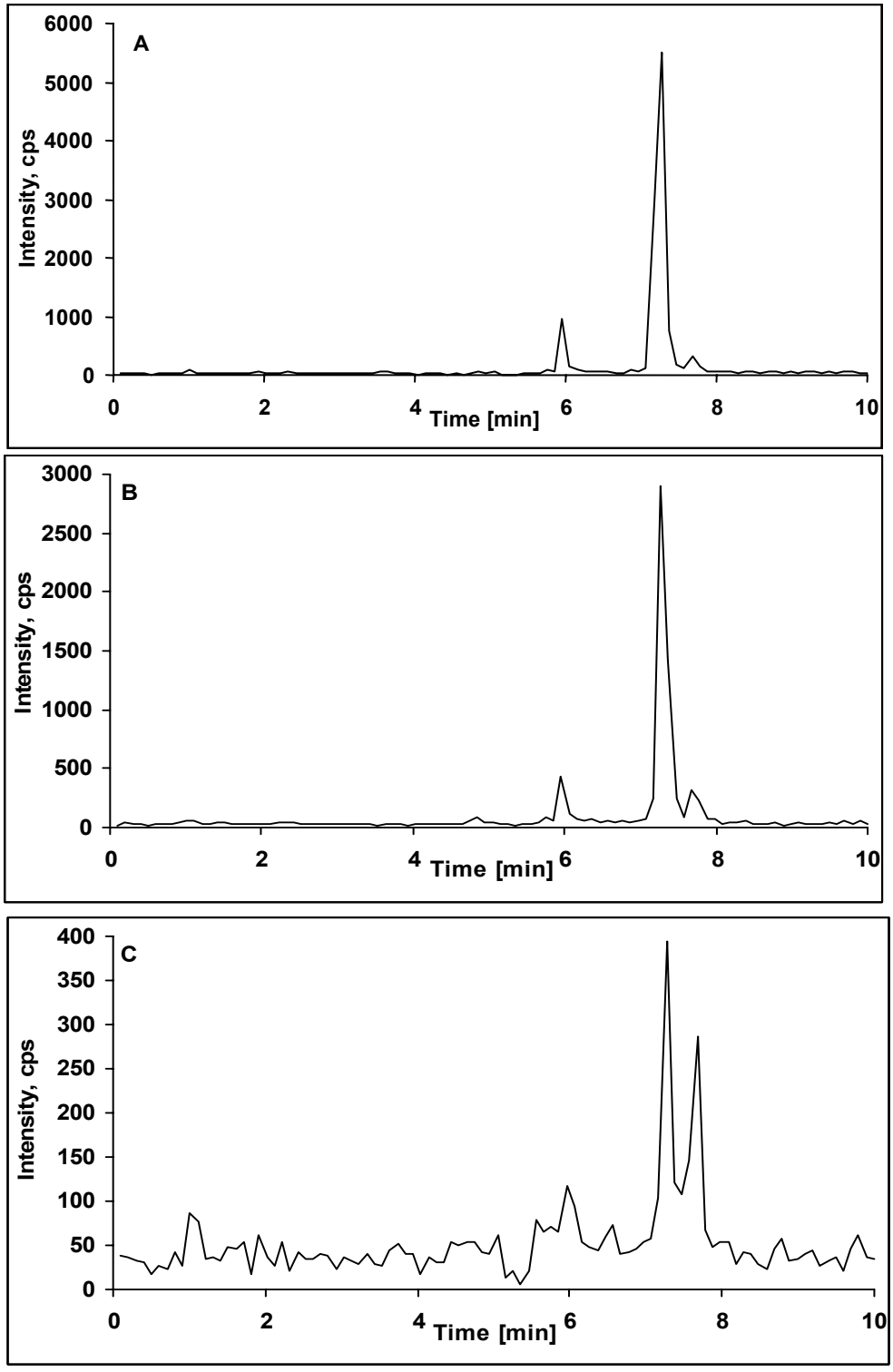


Figure 11: Separations of human CYP51 incubation performed with different concentrations of the human CYP51 inhibitor epoxiconazole (0.01 µmol A, 3 µmol B, 100 µmol C).

4.1.4.6 Analytical Method

To perform inhibition experiments of CYP51 by azoles a low LOQ and a wide dynamic range for FF-MAS is required to get suitable data to calculate IC_{50} values of azole derivatives. The concentrations of FF-MAS were in a dynamic range of 1 to 10 000 ng/mL for FF-MAS determined by calibration curves. Only the ratio of the peak areas of FF-MAS and d_6-cholesterol ($\Rightarrow$ relative peak area) were used for calculation of IC_{50} values. This was possible, since a linear correlation between relative areas and concentration could be proven by the measurement of calibration curves (n = 3).

Due to the structural similarity of lanosterol with FF-MAS a completely resolved separation by HPLC of the analytes was not achieved. However an unequivocal distinction of both substances was possible taking advantage of the specificity of the triple quadrupole mass spectrometer as detector. With the presented LC-APPI-MS/MS method a chromatographic separation is not required since the characteristic MRM transitions are different for FF-MAS, lanosterol and d_6-cholesterol. This results in short run times of < 10 minutes. If high concentrations of one analyte were injected, the two other substances were not detectable, clearly demonstrating that no artefacts were formed during the ionisation process and the standards were highly purified. A matrix effect that resulted in a reduction of peak areas was not observed. In connection with a well plate autosampler a fully automated assay with high sample throughput will be possible.

Due to its sensitivity, the method is not only applicable for the described assay using recombinant CYP51 but also for biological systems with lower CYP51 activity e.g. in microsomes or fungi. Furthermore, no expensive radioactive substrate is needed to perform this assay and analytes may be quantitated in every kind of sample like blood or urine. The LC-APPI-MS/MS method should also be useful for other sterols which have to be quantitated i.e. in river water near sewage plants or pastures of cattles for ecotoxicological risk assessments of a putative endocrine disrupting potential.

4.2 Production of Candida albicans CYP51

4.2.1 Introduction

So far the focus of this work lay on the unwanted side effects of antifungal agents on human CYP19 and human CYP51 enzymes. Now the specificity of these compounds on a fungal lanosterol-14α-demethylase was to be assessed. Since such a fungal enzyme is not available commercially it had to be engineered especially for this purpose.

Both human CYP19 and human CYP51 obtained from BD Gentest Corporation had been co-expressed with the corresponding human oxidoreductase in the baculovirus system. Cytochrome P450 enzymes are membrane bound enzymes requiring the presence of a NADPH-oxidoreductase to be catalytically active. To obtain a functional enzyme the CYP can be expressed alone and reconstituted later with the oxidoreductase or the CYP is co-expressed with the oxidoreductase on one virus construct [87-89].

To assess the inhibitory potency of azoles on a fungal CYP51, *Candida albicans* CYP51 and *Candida tropicalis* cytochrome P450 oxidoreductase were co-expressed in the baculovirus system. *Candida tropicalis* oxidoreductase was chosen instead of *Saccharomyces cerevisiae* oxidoreductase since sequence alignment showed higher homology. The identical system to the one used to express the two enzymes described beforehand was chosen to have a comparable protein background in the incubation procedures.

4.2.1.1 Baculovirus Protein Expression System

The rod shaped baculoviruses are formed in insects e.g. *Spodoptera frugiperda.* The virus DNA comprises of a double stranded covalently closed circular DNA of approximately 80 - 200 kbp. For long term survival the viruses are packed in so called polyhedra or occlusion bodies. The polyhedrin-matrix comprises of the protein polyhedrin that makes up to 50% of the total protein mass at very late stages of infection.

The virus can multiply under cell culture conditions, the virus particles then occur as non-occluded viruses (also called virions or budded virus) without forming polyhedrin occlusion bodies. Insect cell lines e.g. *Spodoptera frugiperda* cells (Sf9) can be cultured at 27°C and infected by recombinant baculoviruses to produce heterologous proteins. This setup will be later referred to as the baculovirus system.

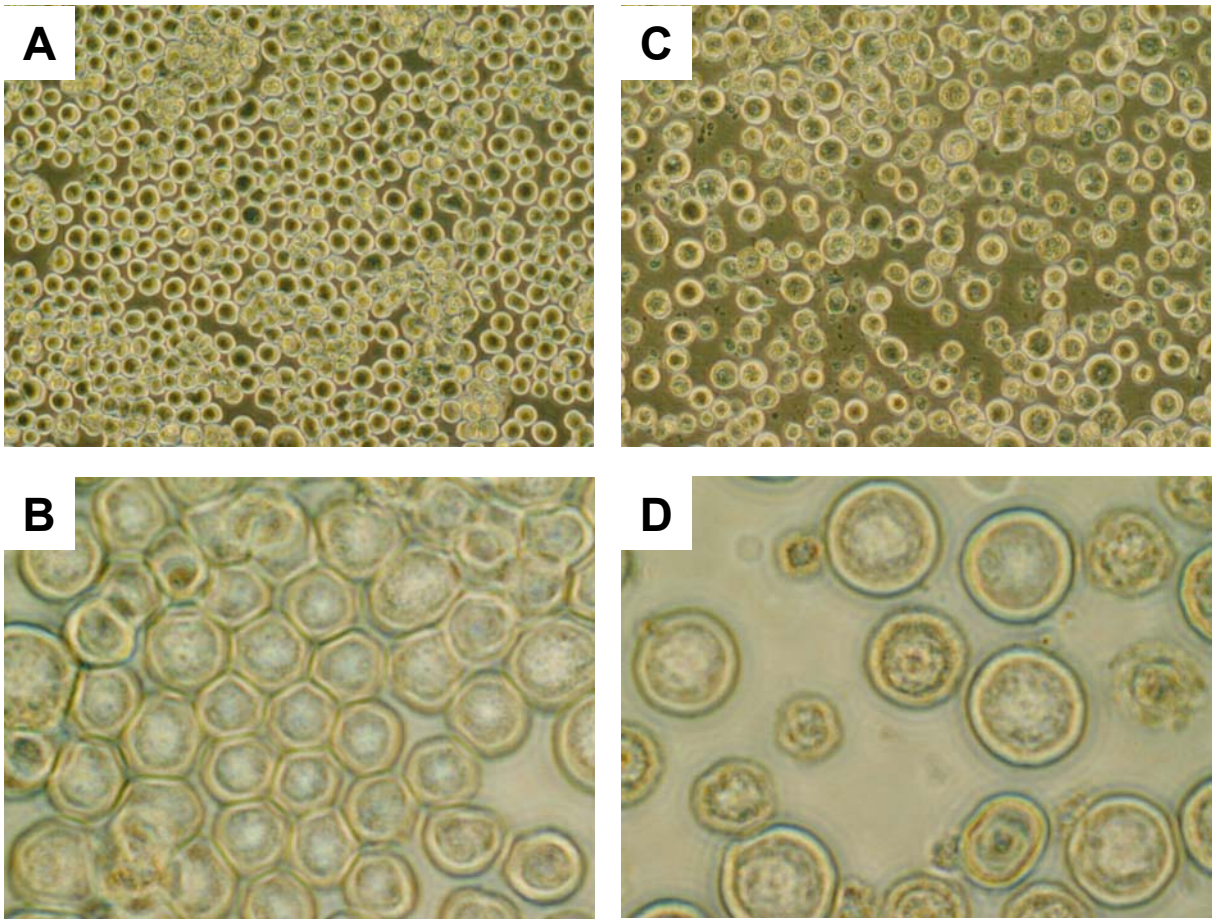
The baculovirus system has several advantages. Heterologous genes can be expressed under the polyhedrin promoter, one of the strongest promoters known. Protein expression occurs at very late stages of infection, expression levels are likely to be high. The baculovirus system is an eukaryotic system, thus prerequisites for a biological active protein such as proper protein folding and post translational modifications are given. The substitution of unusual analoges into proteins is possible e.g. heme groups into cytochrome P450 enzymes. The baculovirus genome can accommodate large DNA constructs making it possible to insert more than one construct into the genome. Literature reports of a number of cytochrome P450 enzymes expressed in the baculovirus system, either directly co-expressed with cytochrome P450 oxidoreductase or expressed alone and later reconstituted with the separately expressed oxidoreductase.

Expression levels of 500 mg/mL of foreign proteins can be achieved [87]; therefore it usually outperforms other eukaryontic expression systems. Cells are cultured at 27°C thus temperature sensitive proteins can be expressed more easily than in a system requiring 37°C. The Bac-to-Bac® Baculovirus Expression System utilises a baculovirus entry vector that is accessible to direct cloning thus recombinant viruses do not have to be selected.

Still, the baculovirus system has some drawbacks. Compared to bacterial expression systems it is far more costly concerning both equipment and consumables and more time consuming to set up and to perform the experiment. Post translational modifications are not always identical to the ones formed in the original species.

The engineering of the enzymes is described in short: the desired genes were cloned into the baculovirus entry vector pFastBac Dual that can harbour two genes simultaneously. The gene coding for the *Candida albicans* CYP51 was cloned under the polyhedrin promoter, the gene encoding the *Candida tropicalis* oxidoreductase was cloned under the p10 promoter. The genes were then transposed into the bacterial bacmid that comprises of viral genome. The recombinant bacmid DNA is isolated and transfected into Sf9 cells. The virus is generated and amplified in the insect cells by secondary infection. Since the genes of interest are cloned directly into the viral genome, recombination is not necessary. Protein expression then occurs by infection of insect cells with the engineered virus. Cells are harvested by centrifugation when showing signs of late to very late infection e.g. an increase in cell

diameter and size of cell nuclei as shown in Pictures 1 A-D. Cells are disintegrated by a potter homogenizer and subjected to sequential differential centrifugation at 10 000 × g and 100 000 × g.



Picture 1A and B: Not infected insect cells 4 x objective and 10 x objective, respectively.

Picture 1C and D: Infected insect cells showing late signs of infection 4 x objective and 10 x objective, respectively.

4.2.2 Materials and Methods

4.2.2.1 Apparatus

Sequence Alignment program	MegAlign GATC Biotech (Karlsruhe, Germany)
Centrifuge	Eppendorf Centrifuge 5417R (Hamburg, Germany)
Centrifuge	Megafuge 1.0 R Heraeus (Hanau, Germany)
Ultracentrifuge	L8-M Ultracentrifuge with Ti 70 rotor Beckmann Counter (Krefeld, Germany)
Gel Documentation System	Gel Doc 2000 Bio-Rad Laboratories (München, Germany)
Laminar Flow bench	Antair BSK Heraeus (Osterode, Germany)
Lightmicroscope	Diavert Leitz (Stuttgart, Germany)
Lightmicroscope	Olympus CH-2
Quarzcuevetes	Hellma Precision Quarzcuvettes 10 mm (Müllheim, Germany)
Orbital shaking platform	
Tissue Homogeniser	Potter S Homogeniser with 60 ml vessels, teflon and glass pestles Sartorius (Göttingen, Germany)
Thermocycler	Eppendorf Mastercycler gradient (Hamburg, Germany)
UV/VIS Spectrometer	Ultrospec 2000 / Pharmacia Biotech (Uppsala, Schweden)

4.2.2.2 Chemicals

Agarose, dNTPs, potassium acetate, potassium chloride and SDS were from Carl Roth (Karlsruhe, Germany). Carbonmonoxide gas was from Linde (Nürnberg, Germany). All other chemicals were from Sigma (Steinheim, Germany). All chemicals used were of the highest purity grade.

4.2.2.3 Buffers and Bacterial-Sera

DNA loading buffer 10x: 10 mM Tris HCl (pH 7.6), 10 mM EDTA, 0.015% bromphenolblue, 10% glycerin
Luria-Bertani Medium (LB medium) pH 7.0: 10 g/L bacto-tryptone; 5 g/L bacto-yeast extract; 10 g/L NaCl
SOC pH 7.0 : 20 mM glucose; 20 g/L bacto-tryptane; 5 g/L bacto-yeast extract; 0.5 g/L NaCl; 2.5 mM KCl; 0.01 M $MgCl_2$
Solution 1: 50 mM glucose; 25 mM TrisCl (pH 8.0); 10 mM EDTA (pH 8.0)
Solution 2: 0.2 N NaOH; 1% SDS
Solution 3: 60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml H_2O
TAE pH 8.0: 0.04 M Tris-acetate; 1 mM EDTA
TE pH 8.0 containing RNAse: 10 mM TrisCl (pH 8.0); 1 mM EDTA (pH 8.0); 20 µg/mL DNAase-free pancreatic RNAase
All buffers and solutions were prepared according to Molecular Cloning-A Laboratory manual 2nd edition (Sambrook, Fritsch, Maniatis).

4.2.2.4 Cellculture-Media and Sera

Cellfectin	Invitrogen (Karlsruhe, Germany)
FCS (Fetal Calf Serum)	PAA Laboratories (Cölbe, Germany)
SF 3 Baculo Express ICM	Biozol (München, Germany)
TNM-FH Insect Medium	BD Biosciences (Whoburn, USA)

4.2.2.5 Other Materials

Cloned Pfu Polymerase and corresponding buffer	Stratagene (Heidelberg, Germany)
DNA *Candida albicans* CYP51	Prof. M. Waterman (Vanderbilt University Nashville, USA) (pCW / C.albCYP51 [90]) EMBL-EBI X13296
DNA *Candida tropicalis* NADPH-Cytochrome P-450 Oxidoreductase	Dr. Sanglard (University of Lausanne, Switzerland) (pTS1 [91]) EMBL-EBI M35199
DNA Marker peqGOLD Range Mix DNA Ladder (0.1 mg/mL)	peqlab (Erlangen, Germany)
Bac-to-Bac® Baculovirus Expression System	Invitrogen (Karlsruhe, Germany)
Gelextraction Kit	Quiagen (Hilden, Germany)
HiSpeed Midi Kit	Quiagen (Hilden, Germany)
pFastBac™ Dual Expression Vector	Invitrogen (Karlsruhe, Germany)
Plasmid Midi Kit	Quiagen (Hilden, Germany)
Restriction enzymes and corresponding buffers	New England Biolabs (Frankfurt a.M., Germany)
Shrimp Alkaline Phosphatase and corresponding buffer	Fermentas (St. Leon Roth, Germany)
T4 Ligase and corresponding buffer	New England Biolabs (Frankfurt a.M., Germany)
Taq Polymerase and corresponding buffer	New England Biolabs (Frankfurt a.M., Germany)
Zero Blunt® TOPO® PCR Cloning Kit	Invitrogen (Karlsruhe, Germany)

Competent DH10 B bacteria were provided by the workgroup of Prof. Arand.
Max Efficiency DH10 bacteria were from Invitrogen (Karlsruhe, Germany).

4.2.3 Procedures

4.2.3.1 Agarose Gel Electrophoresis

DNA fragments were separated following the "*Agarose Gel Electrophoresis*" protocol from Molecular Cloning-A Laboratory Manual 2nd edition (Sambrook, Fritsch, Maniatis). In short: Agarose was melted in 0.5 x TAE buffer and agarose gels were casted in concentrations of 1, 1.2 and 1.5%. 0.5 x TAE was used as electrophoresis buffer. DNA samples and DNA Marker peqGOLD Range Mix DNA Ladder (0.1 mg/mL) were diluted with sterile water and DNA loading buffer and loaded into the slots of the agarose gel. DNA fragments were then separated by applying 40-80 V for 40 min. After electrophoresis DNA bands were visualised using ethidiumbromide (30 min in 0.5 µg/mL ethidiumbromide in TAE) and fluorescence detection was performed at 590 nm emission wavelength on the Gel Documentation System. The agarose gel concentrations utilised are quoted individually.

4.2.3.2 Transformations

All transformations were performed according to Molecular Cloning-A Laboratory Manual 2nd edition (Sambrook, Fritsch, Maniatis). Competent DH 10 B *E.coli* cells, stored at -70°C, were thawed at room temperature and kept on ice. DNA was added to the cells; the contents were gently mixed and stored on ice for 30 min. The cells were transferred to a 42°C warm water bath for 90 sec and cooled on ice, and then one millilitre of sterile LB medium was added to the culture. The cells were incubated for 1 h at 37°C and 220 rotations per minute (rpm) to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. 100 µL of the bacterial suspension were plated on LB agar plates containing 100 µg/mL ampicillin and incubated at 37°C over night. Single colonies appeared after 12 h and transformants were picked and amplified over night in 2 mL or 100 mL cultures of LB medium (quoted individually) containing 100 µg/mL ampicillin, at 37°C and 220 rpm.

4.2.3.3 DNA Isolation from Bacterial Cells

The 2 mL bacterial suspensions were centrifuged at 20 000 × g (≡ 13 000 rpm) (Centrifuge 5417R / Eppendorf) for one minute. Vector DNA was extracted following the protocol *small-scale preparations of plasmid DNA/Lysis by alkali* from Molecular Cloning-A Laboratory Manual 2nd edition (Sambrook, Fritsch, Maniatis). The bacterial pellet was resuspended in 150 µL of solution 1. The suspension was carefully mixed with 350 µL of solution 2 and incubated at room

temperature for 5 minutes. After addition of 250 µL solution 3, the mixture was incubated on ice for 10 minutes and for two minutes at room temperature. Cell debris was pelleted at 13 000 rpm ≡ 20. 000 × g for 10 minutes at 4°C. 700 µL of the supernatant were mixed with an equal volume of isopropyl alcohol and DNA was pelleted at 13 000 rpm ≡ 20 000 × g for 10 minutes at 4°C. The pellet was washed in 70% ethanol and left to dry. Finally, the pellet was resuspended in 50 µL of TE-RNAse buffer and incubated for 15 min to remove RNA. Bacterial 100 mL suspensions were centrifuged for 10 min at 4°C at 4 000 rpm ≡ 500 × g (Megafuge 1.0 R Heraeus). Plasmid DNA from 100 mL cultures was isolated using the Plasmid Midi Kit from Quiagen (Hilden, Germany) according to the manufacturers' instructions.

4.2.3.4 Restriction Analysis

Restriction enzymes recognize and cut DNA only at palindrome sequences, resulting in characteristic patterns of DNA fragments. Restriction analysis was performed for 1 h at 37°C according to the manufacturer's instruction unless otherwise stated.

4.2.3.5 DNA Purification from Agarosegels

DNA fragments were excised from the respective agarose gels and purified using the gel-extraction kit from Quiagen (Hilden, Germany).

4.2.3.6 DNA Dephosphorylation

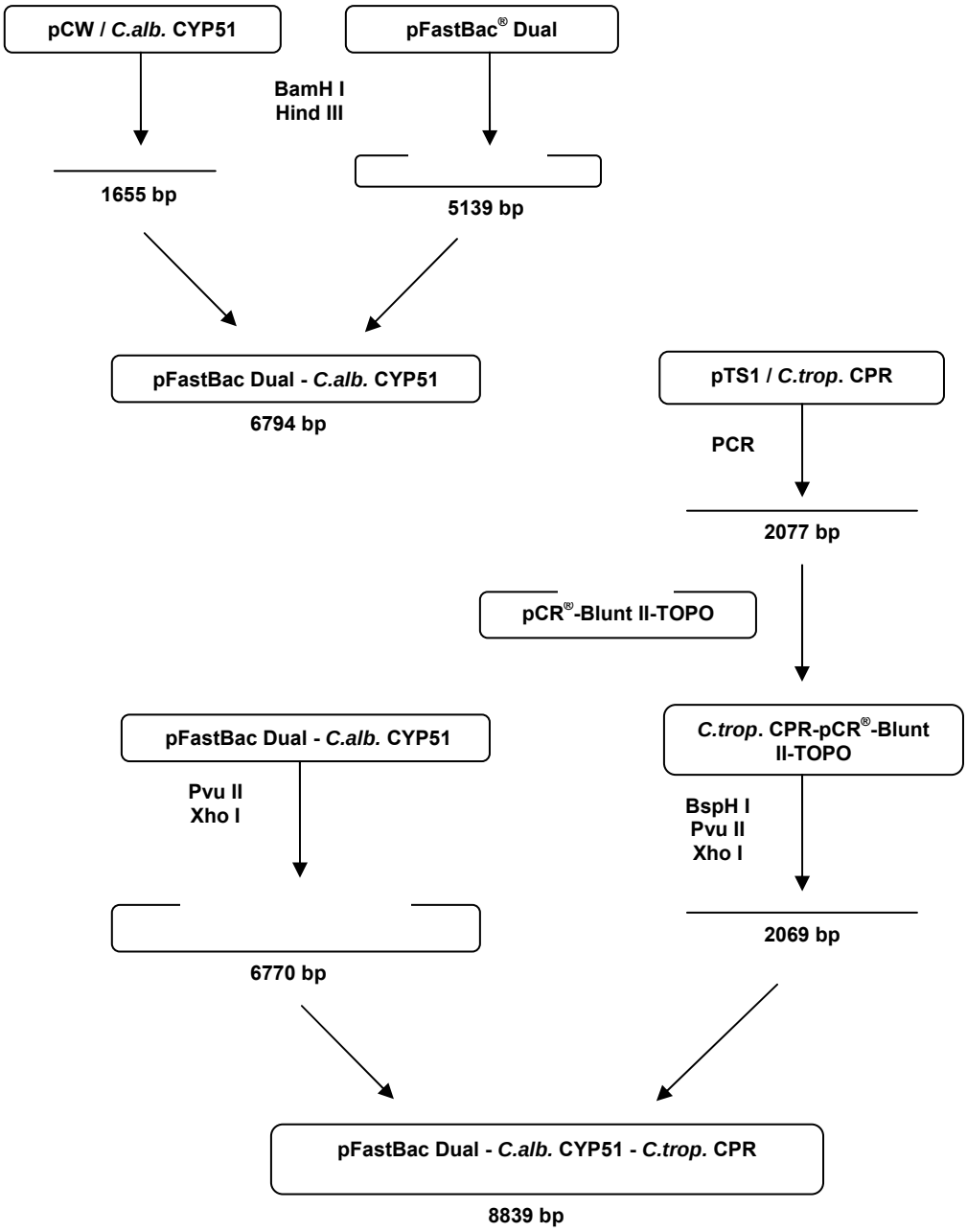
To minimize recirculation and self-ligation of linearised DNA vectors, the 5'-phosphate groups are removed. DNA fragment ends were dephosphorylated with shrimp alkaline phosphatase for 30 min at 37°C according to the manufacturer's instructions.

4.2.3.7 DNA Ligation

Ligation of foreign DNA into a linearised plasmid vector is achieved by formation of new bonds between the 5'-phosphate and 3'-hydroxyl groups catalysed by the bacteriophage enzyme T4-ligase. When dephosphorylated plasmid DNA is used only two new phosphodiester bonds result. The two single strand nicks in the vector DNA are repaired after transformation of the vector into competent bacteria. DNA fragments were ligated with T4 ligase according to the manufacturers' instructions.

4.2.3.8 Cloning and Protein Expression Procedure

Scheme 5 illustrates the cloning procedure of the recombinant donor plasmid harbouring cDNA of *Candida tropicalis* oxidoreductase and *Candida albicans* CYP51. Scheme 6 depicts the production of the recombinant bacmid, virus particles and protein.



Legend:

bp: basepairs

***C.Trop.* CPR: DNA encoding *Candida tropicalis* Cytochrome P450 Oxidoreductase**

***C.alb.* CYP51: DNA encoding *Candida albicans* Cytochrome P450 Isoenzyme 51**

pCR®-Blunt II-TOPO

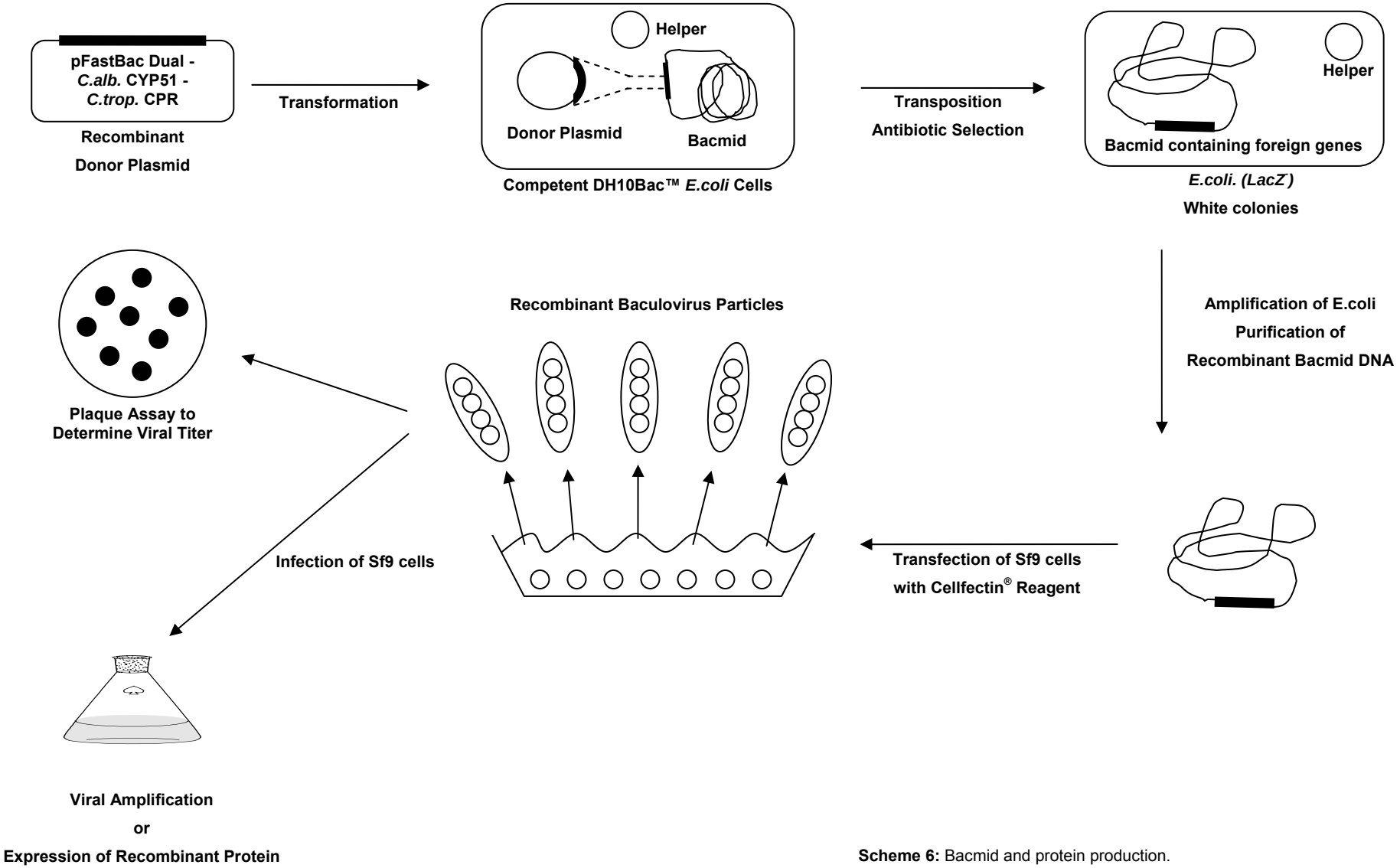
PCR: Polymerase Chain Reaction

pFastBac Dual: Donor Plasmid

pCW: plasmid containing DNA encoding *Candida albicans* Cytochrome P450 Isoenzyme 51

pTS1: plasmid containing DNA encoding *Candida tropicalis* Cytochrome P450 Oxidoreductase

Scheme 5: Cloning procedure of the recombinant donor plasmid.



Scheme 6: Bacmid and protein production.

4.2.4 Vector DNA Amplification

Escherichia coli DH 10 B cells were transformed with each expression plasmid, pTS1, pCW *C.alb* CYP51 and pFastBac™ Dual to produce a sufficient amount of DNA for the following cloning procedure. DNA concentration was determined by UV spectrometry at λ = 260 nm and 280 nm to be 0.22, 0.24 and 1.4 µg/µL for pCW *C.alb* CYP51, pTS1 and pFastBac™ Dual respectively.

4.2.5 Construction of the pFastBac™ Dual - *Candida albicans* CYP51 - *Candida tropicalis* Oxidoreductase Vector

4.2.5.1 Construction of pFastBac™ Dual - *Candida albicans* CYP51

The 1655 bp DNA fragment encoding the *Candida albicans* CYP51 was excised from the pCW *C.alb* CYP51 vector using BamH I and Hind III restriction enzymes. The pFastBac™ Dual vector (5139 bp) was linearised following the same protocol. The DNA fragments were separated by gel electrophoresis on a 1% agarose gel and visualised by ethidiumbromide staining (Figure 12).

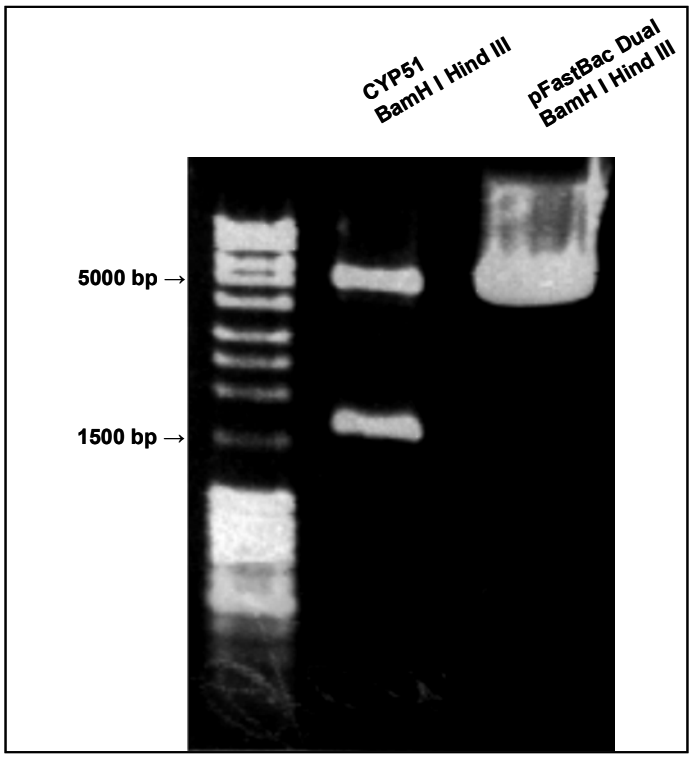


Figure 12: DNA-gel electrophoresis on 1% agarose: Restriction of *Candida albicans* CYP51 (lanosterol-14α-demethylase) DNA (1655 bp) and linearised pFastBac Dual vector (5139 bp).

The bands corresponding to the 1655 bp and 5139 bp fragments were excised from the gel and purified using the gel-extraction kit from Quiagen (Hilden, Germany). To avoid autoligation of the vector DNA fragment ends of the pFastBac™ Dual vector were dephosphorylated with shrimp alkaline phosphatase.

4.2.5.2 Ligation of CYP51 and pFastBac™ Dual Vector

The DNA fragment encoding the *Candida albicans* CYP51 was ligated into the pFastBac™ Dual vector with T4 ligase. Competent Escherichia coli strain DH 10 B cells were transformed with the ligation probe and transformants were selected on LB plates containing 100 µg/mL ampicillin. Selected transformants were cultured over night in 2 mL LB medium containing 100 µg/mL ampicillin at 37°C and 220 rpm.

The successful ligation of the CYP51 DNA fragment into the pFastBac™ Dual Vector was confirmed by digestion with the restriction enzymes Bgl II and Xba I, separation by gel electrophoresis (1% agarose) and ethidiumbromide staining (Figure 13).

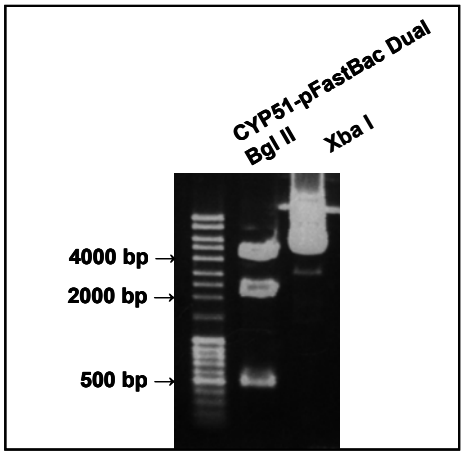


Figure 13: DNA-gel electrophoresis (1% agarose) of the restriction analysis of pFastBac Dual-*Candida albicans* CYP51 with Bgl I: 470 bp, 2143 bp, 4181 bp and Xba I: 6794 bp.

4.2.5.3 Amplification of *Candida tropicalis* Oxidoreductase DNA by PCR

The 2077 bp DNA fragment encoding the *Candida tropicalis* oxidoreductase was amplified from the pTS 1 vector by polymerase chain reaction (PCR) with Cloned Pfu polymerase according to the manufacturers' instructions. The forward primer was constructed to include an Xho I restriction site and the reverse primer was constructed to include a Pvu II restriction site. Successful amplification was confirmed by gel electrophoresis (1% agarose) and ethdiumbromide staining (Figure 14).

PCR Conditions

Primer forward Reductase (CPR-Xho If) (MWG, Ebersberg, Germany) c = 0.1 µg/µL
5'-ATC GCT CGA GTC ATG GCA TTA GAT AAG-3'
Primer reverse Reductase (CPR-Pvu IIr) (MWG, Ebersberg, Germany) c = 0.1 µg/µL
5'-TAA TCA GCT GTT CAT ATT TTC CTT ACC AG-3'

sterile H_2O	36 µL	Melting Temperature	95°C / 30 sec
DNA c = 0.24 µg/µL	1 µL	Annealing Temperature	60°C / 30 sec
dNTP's	5 µL	Elongation Temperature	70°C / 2 min 30 sec
Primer each	1 µL	→ 30 Cycles	
Cloned Pfu Buffer	5 µL	ultimate Elongationstep	70°C / 10 min
Cloned Pfu Polymerase	1 µL		
∑	50 µL		

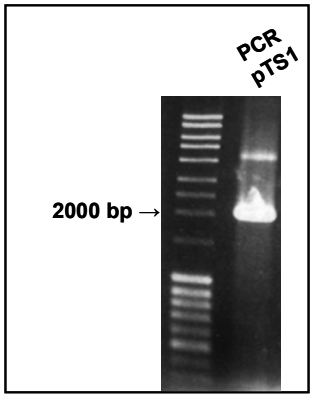


Figure 14: DNA-gel electrophoresis (1% agarose) of the PCR product of *Candida tropicalis* Oxidoreductase DNA (2077 bp).

4.2.5.4 Topo Cloning of the *Candida tropicalis* Oxidoreductase

As DNA amplification by PCR can produce incorrect DNA, the determination of the correct nucleotide sequence was inevitable, to avoid mutation, deletion or insertions of the constructs. Therefore the amplified PCR product was cloned into the pCR®-Blunt II-TOPO® vector according to manufacturers' protocol Zero Blunt® TOPO® PCR Cloning Kit. This vector has the distinct advantage that it incorporates PCR products easily without further modification and purification and also provides several sites for sequencing primers flanking the insert.

Competent bacteria Escherichia coli strain DH 10 B were transformed with the pCR®-Blunt II-TOPO® vector including the *Candida tropicalis* oxidoreductase insert, with transformants selected on LB plates containing 100 µg/mL ampicillin. Chosen transformants were grown over night at 37°C and 220 rpm in 2 mL cultures of LB medium containing 100 µg/mL ampicillin. Vector DNA was extracted following the protocol *small-scale preparations of plasmid DNA/Lysis by alkali* as described above. Restriction analysis with EcoR I followed by DNA gel electrophoresis confirmed the

successful insertion of the PCR amplificate. One transformant was selected and amplified at 37°C and 220 rpm in a 100 mL culture of LB media containing 100 µg/mL ampicillin. Bacteria were centrifuged and plasmid DNA was isolated as described above. DNA concentration was determined spectrally at λ = 260 nm to be 0.15 µg/µL.

4.2.5.5 Sequencing

The pCR®-Blunt II-TOPO® vector including the *Candida tropicalis* oxidoreductase insert was sequenced by GATC (Konstanz, Germany) utilising the two pCR®-Blunt II-TOPO® primers: M13 forward and M13 reverse flanking the insert. A second sequencing was performed with the specific primer chosen from the middle of the coding part of the DNA.

M13 forward Primer
5'-TGT AAA ACG ACG GCC AGT-3'
M13 reverse Primer
5'-CAG GAA ACA GCT ATG ACC-3'
Sequencing Primer (MWG)
5'-GCT GAA TAC GGT GAA GGT GAC G-3'

Sequencing results were compared on the Laser Gene/MegAlign software (DNA-Star) to the DNA sequence of *Candida tropicalis* oxidoreductase reported by Sutter et al. [91] (EMBL-EBI: M35199), to confirm the correct sequence.

4.2.5.6 Preparation of DNA Fragments for Ligation

The 2069 bp DNA fragment encoding the *Candida tropicalis oxidoreductase* was excised from the pCR®-Blunt II-TOPO® vector with BspH I, Pvu II and Xho I. The pFastBac™ Dual *Candida albicans* CYP51 vector (6770 bp) was linearised with Pvu II and Xho I. After dephosphorylation of the pFastBac™ Dual *Candida albicans* CYP51 vector the DNA fragments were separated by gel electrophoresis on a 1.2% agarose gel and visualised using ethidiumbromide staining (Figure 15 A). The bands corresponding to the expected 2069 bp and 6770 bp fragments were excised from the gel and the DNA was purified. The DNA fragments were controlled by gel electrophoresis (Figure 15 B)

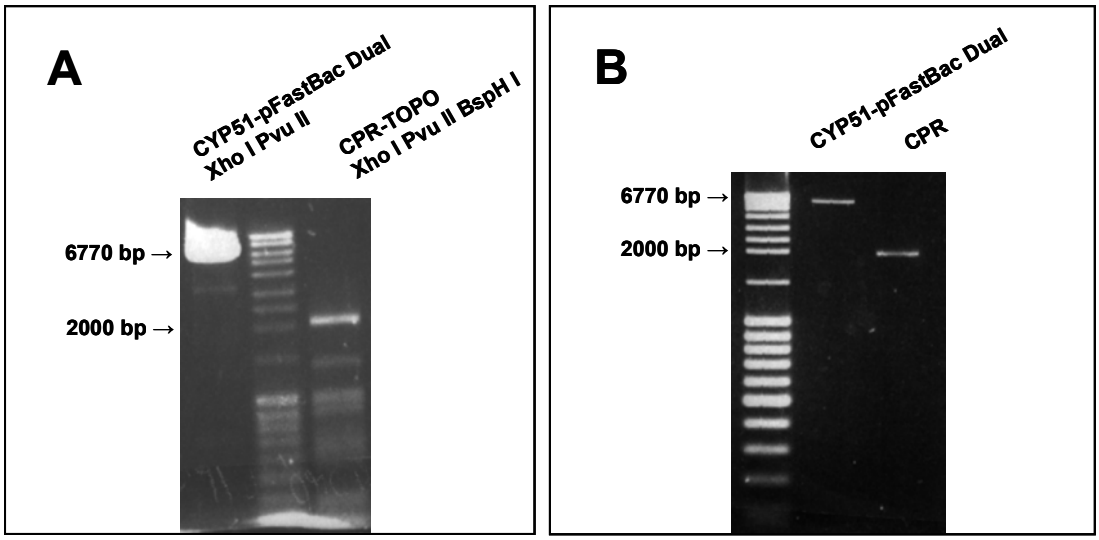


Figure 15: A DNA-gel electrophoresis on 1.2% agarose of pFastBac Dual-*Candida albicans* CYP51 - vector linearised with Xho I and Pvu II and *Candida tropicalis* Oxidoreductase DNA excised from CPR-TOPO by Xho I, Pvu II and BspH I.

B Purified DNA-fragments (1% agarose).

4.2.5.7 Ligation of pFastBac™ Dual-*Candida albicans* CYP51 Vector and *Candida tropicalis* Oxidoreductase

The ligation of *Candida tropicalis* Oxidoreductase into the pFastBac™ Dual *Candida albicans* CYP51 vector and amplification was performed as described above. The correct insertion of the DNA fragment was controlled and could be confirmed by digests with the following restriction enzymes: Afl III, BamH I, Bgl II, BspH I, EcoR I, Hinc II, Nco I, Sma I. DNA fragments were separated by gel electrophoresis by a 1.5% agarose gel and ethidiumbromide staining (Figure 16 A-F).

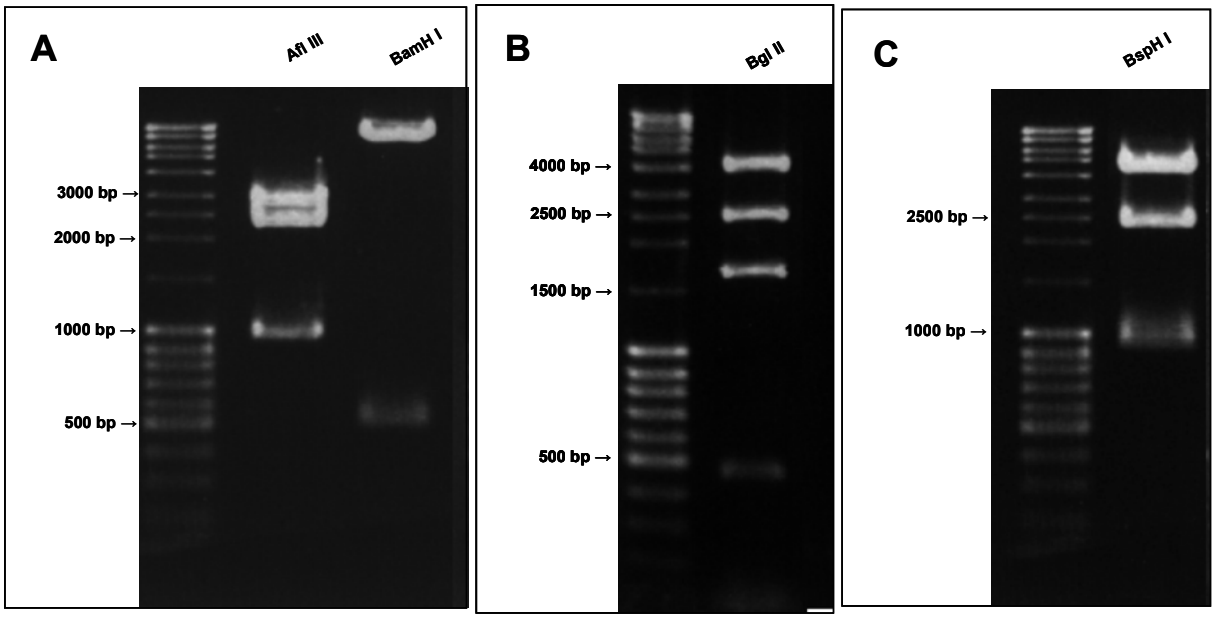


Figure 16: DNA-gel electrophoresis on 1.5% agarose of restriction analysis of pFastBac Dual-*Candida albicans* CYP51-*Candida tropicalis* Oxidoreductase construct with

A Afl III 982 bp, 2398 bp, 5459 bp and BamH I 495 bp, 8344 bp

B Bgl II 470 bp, 1683 bp, 2505 bp, 4181 bp

C BspH I 1008 bp, 2474 bp, 5357 bp.

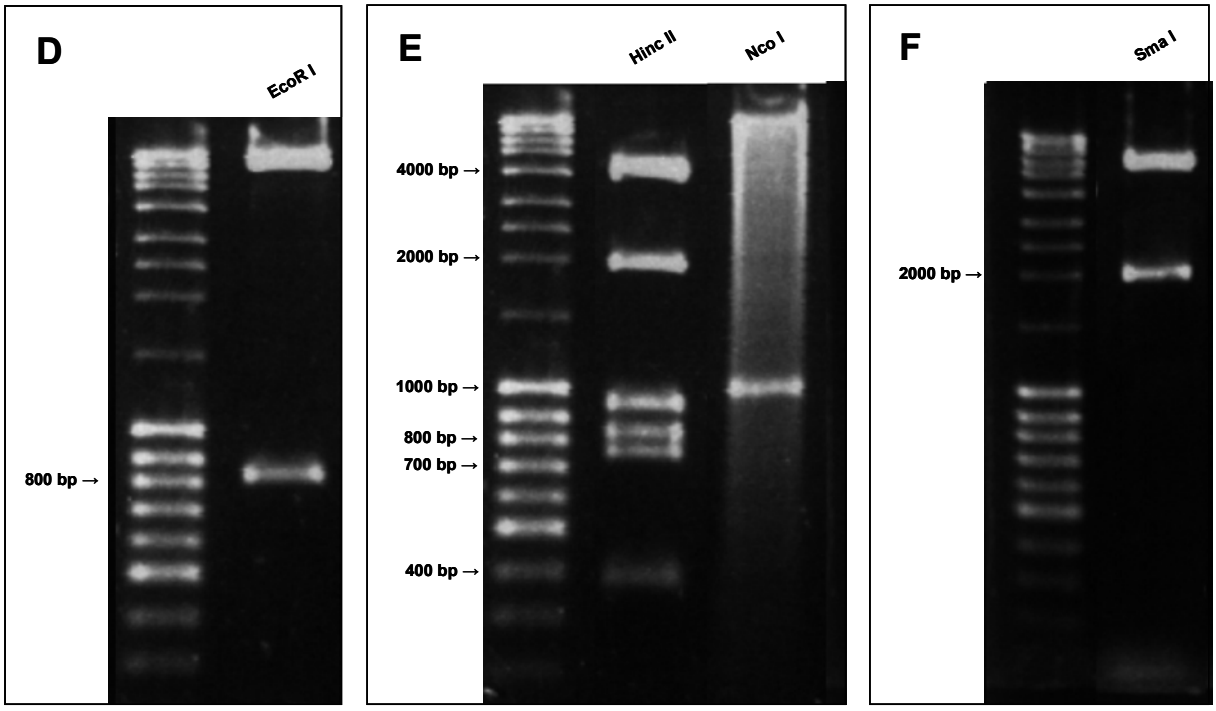


Figure 16: DNA-gel electrophoresis on 1.5% agarose of restriction analysis of pFastBac Dual-*Candida albicans* CYP51-*Candida tropicalis* Oxidoreductase construct with

D EcoR I 807 bp, 8032 bp

E Hinc II 358 bp, 729 bp, 802 bp, 948 bp, 1941 bp, 4061 bp and Nco I 989 bp, 7850 bp

F Sma I 2000 bp, 6839 bp.

4.2.6 Bacmid-DNA Production

The pFastBac™ Dual *Candida albicans* CYP51 *Candida tropicalis* oxidoreductase DNA construct was transformed into MAX Efficiency® DH10Bac™ competent *E.coli.* by a modified heat shock method according to the Bac-to-Bac® Baculovirus Expression System instruction manual (Invitrogen, Karlsruhe, Germany). Three different 5-fold serial dilutions (1:5 ; 1:25 ; 1:625) of the *E.coli* cells in SOC medium were prepared and 100 µL of each dilution were plated on LB agar plates containing 100 µg/mL ampicillin, 50 µg/mL kanamycin, 10 µg/mL gentamycin, 7 µg/mL tetracyclin, 80 µg/mL X-Gal and 40 µg/mL IPTG. The plates were incubated for 48 h at 37°C.

Insertion of the DNA construct by transposition into the mini-*att* Tn7 attachment site on the bacmid disrupts the expression of the LacZα peptide. Colonies containing the recombinant bacmid are white in a background of blue colonies that harbour the unaltered bacmid.

Selected white colonies were amplified over night at 37°C and 220 rpm in 2 mL cultures of LB medium containing 100 µg/mL ampicillin, 50 µg/mL kanamycin, 10 µg/mL gentamycin and 7 µg/mL tetracyclin. The bacteria were pelleted at 13 900 rpm ≡ 20 000 × g for one minute and vector DNA was extracted as described above for 2 mL cultures. Recombinant bacmid DNA is bigger than 135 kb in size making restriction analysis difficult. Thus a PCR, utilising specific primers flanking the region of insertion, was performed to confirm the correct insertion of the DNA construct into the bacmid DNA. According to the instructions manual a ~ 6285 bp fragment comprising of the 2069 bp oxidoreductase fragment, the 1656 bp CYP51 fragment and ~2560 bp fragment from the bacmid, should result. If the transposition had not occured a 300 bp PCR product would result. A second PCR using the primers of the Candida tropicalis oxidoreductase was also performed. PCR products confirming the successful transposition were separated by gel electrophoresis on a 1.2% (A) and 1% (B) agarose gel and visualised with ethidiumbromide (Figure 17 A+B).

PCR conditions

Primer forward Reductase (CPR-Xho If) (MWG, Ebersberg, Germany) c = 0.1 µg/µL
5'-ATC GCT CGA GTC ATG GCA TTA GAT AAG-3'
Primer reverse Reductase (CPR-Pvu IIr) (MWG, Ebersberg, Germany) c = 0.1 µg/µL
5'-TAA TCA GCT GTT CAT ATT TTC CTT ACC AG-3'

H_2O sterile	36.5 µL	Melting Temperature	94°C / 30 sec
Bacmid DNA c = 0.1 µg/µL	1 µL	Annealing Temperature	60°C / 30 sec
dNTP's	5 µL	Elongation Temperature	72°C / 1 min
Reductase Primer each	1 µL	→ 30 Cycles	
Taq Buffer	5 µL	ultimate Elongationstep	72°C / 10 min
Taq Polymerase	0.5 µL		
∑	50 µL		

Primer Bacmid M13 Forward (Invitrogen)
5'-GTT TTC CCA GTC ACG AC -3'
Primer Bacmid M13 Reverse (Invitrogen)
5'-CAG GAA ACA GCT ATG AC-3'

H_2O sterile	36.5 µL	Melting Temperature	94°C / 45 sec
Bacmid DNA c = 0.1 µg/µL	1 µL	Annealing Temperature	55°C / 45 sec
dNTP's	5 µL	Elongation Temperature	72°C / 3 min
Bacmid Primer each	1 µL	→ 30 Cycles	
Taq Buffer	5 µL	ultimate Elongationstep	72°C / 7 min
Taq Polymerase	0.5 µL		
∑	50 µL		

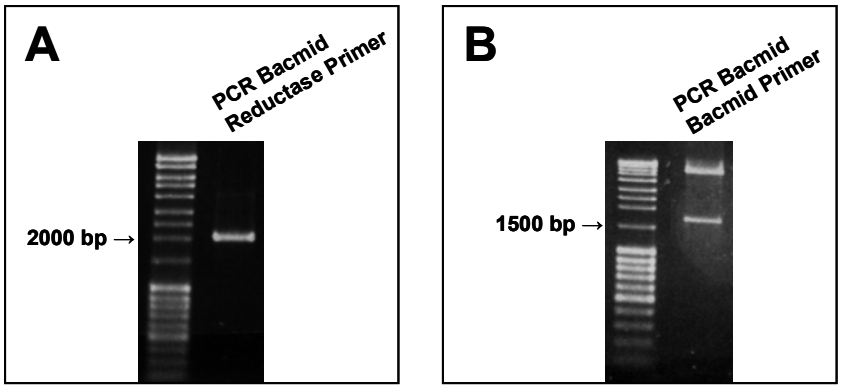


Figure 17: A 1.2% agarose gel of PCR product of Bacmid utilising Reductase Primers rendering 2069 bp Reductase fragment DNA-gel electrophoresis (1.2% agarose) of the PCR amplification product of C.trp. Reductase (2069 bp) using recombinant bacmid DNA as template.
B 1% agarose gel of PCR product of Bacmid utilising Bacmid specific Primers flanking the insert ~ 6284 bp.

One transformant was selected and amplified over night in a 100 mL culture of LB media containing 100 µg/mL ampicillin, kanamycin 50 µg/mL, gentamycin 10 µg/mL and tetracyclin 7 µg/mL at 37°C and 220 rpm. Bacteria were centrifuged for 10 min at 4°C at 4 000 rpm ≡ 500 × g and bacmid DNA was isolated with a Plasmid Midi Kit from Quiagen (Hilden, Germany). The DNA concentration was determined to be 0.1 µg/µL.

4.2.7 Cell culture procedures

A Sf9 insect cell line was used for all experiments. Aliquots of 4 - 5 × 10^6 cells per mL in SF 3 Baculo Express medium supplemented with 10% DMSO were kept in liquid nitrogen in special cryovials. Cryopreservation was performed by freezing cells for 24 h at -20°C, then for 48 h at -70°C and finally in liquid nitrogen.

One aliquot of Sf9 insect cells was quickly thawed at 37°C, diluted with 10 mL SF 3 Baculo Express medium and incubated in a 10 mL cell culture flask at 28°C. Cells were left for 2 h to attach and then the cell culture medium was changed to remove DMSO. Optimal growth conditions in cell culture flasks were reached from 50% confluence.

Cells were cultured in cell culture flasks, 6-well plates at 27°C or at room temperature in 100 mL or 250 mL Erlenmeyer flasks or in 1 800 mL Fernbach flasks on an orbital shaking platform at 100 rpm. Optimal growth conditions needed a minimum of 0.5 x 10^6 cells/mL, maximal cell density was reached between 6 and 10 x 10^6 cells/mL. Cell numbers were counted by the trypanblue exclusion test.

4.2.8 Baculovirus Production/Transfection of Sf9 cells with Bacmid DNA

Cells were transfected with a mixture of Cellfectin® and bacmid DNA according to the Invitrogen Bac-to-Bac® Baculovirus Expression System protocol.

In a 6-well tissue culture plate 1 × 10^6 cells per well were seeded in 2 mL fetal calf serum (FCS) free SF 3 Baculo Express medium and kept at 27°C for one hour. Bacmid DNA and Cellfectin® were diluted with FCS-free SF 3 Baculo Express medium, mixed and incubated for 30 minutes at room temperature. Each mixture was diluted with additional medium and the cells were transfected with these mixtures. The medium was changed after 5 h. A Viral P1 stock could be attained from the supernatant of the cells after 72 h. Cells showed clear signs of late infection: increase in cell diameter and size of cell nuclei as well as detachment from the plate. The cell supernatant was collected and centrifuged for 5 min at 500 rpm ≡ 50 × g. The resulting supernatant was transferred to sterile tubes, supplemented with 10% FCS for protease protection and stored protected from light at 4°C and -70°C, respectively. According to the manufacturer this viral stock P1 should have a viral titer of 1 x 10^6 to 1 x 10^7 plaque performing units per millilitre (pfu/mL).

For virus amplification 8 x 10^6 cells were seeded on a 10 cm cell culture plate and infected with a multiplicity of infection (MOI) of 0.05. The MOI is defined as the

number of virus particles per cell and is calculated as described below. The titer of P1 was assumed to be 1 x 10^6 pfu.

$$\text{Inoculum required [mL]} = \frac{\text{MOI [pfu / cell] x number of cells}}{\text{Titer of viral stock [pfu / mL]}}$$

Cells were incubated for 96 h at 27°C and from the cell supernatant viral stock P2 was collected as described above. Virus titer of P2 was determined performing a viral plaque assay following the manufacturers' manual. Briefly 0.5 x 10^6 cells per well were seeded on 6 well tissue culture plates in TNM-FH medium and allowed to attach and incubated over night. Ten serial 10-fold dilutions of the virus were prepared in 1.5 mL of TNM-FH medium. From each well medium was removed, discarded and replaced by a virus dilution. Cells were incubated for 4 h at 27°C. A 0.8% sterile, low melting agarose solution in TNM-FH medium was prepared. Virus containing medium was removed from the cells and replaced by a 2 mL overlay of agarose suspension. After hardening of the agarose at room temperature for 1 h, the cells were incubated for 7-10 days at 27°C until plaques became visible. Plaques in each dilution were counted and the titer was calculated to be 2 x 10^7 pfu/mL as shown below.

$$\text{Titer [pfu/mL]} = \text{number of plaques x dilution factor x } \frac{1}{\text{mL of inoculum / well}}$$

4.2.9 Protein Expression in Sf 9 Insect-cells

Sf9 insect cells were cultured in 1800 mL Fernbach flasks in 500 mL SF 3 Baculo Express medium, supplemented with 10% FCS. Cells were cultured at room temperature at 100 rpm on an orbital shaking platform. At a cell density between 3.4 x 10^6 and 4 x 10^6 cells/mL cells were infected using a MOI of 0.01 pfu/mL. To achieve a higher yield of functional cytochrome P450 enzyme the medium was supplemented with 4 µg/mL hemin from a 2 mg/mL hemin stock solution prepared in 0.4 M NaOH/ethanol (1:1).

Cell growth was monitored daily. The infection was allowed to proceed until 30 - 50% of the cells stained with the trypan blue dye exclusion test and cells showed signs of late to very late infection. This stage was reached 3 - 4 days post infection. Cells were then harvested by centrifugation at 500 rpm ≡ 50 × g (Megafuge 1.0 R Heraeus) in 50 mL Sarstedt tubes, the supernatant was discarded, cell pellets were

resuspended in 3 mL of 0.05 M potassium phosphate buffer pH 7.4 containing 0.5 M sucrose and the suspensions were stored at -70°C until further use.

4.2.10 Preparation of Microsomes

Microsomes were prepared according to the standard operating procedure "SUPERSOME™ Preparation" from BD-Gentest Corporation with small modifications. Shortly: Cell suspensions stored at -70°C were thawed on ice and weighed. Weights of all cell suspensions were determined and the 4-fold amount of suspension weight of deionised water was added to the suspensions. The suspension was transferred to a 60 mL homogeniser vessel and homogenised with 20 strokes of a teflon pestle at 4°C on the Potter S Homogeniser apparatus (Sartorius). The suspension was then transferred to a fresh cooled glass homogeniser and homogenised with 10 strokes of a glass pestle. The resulting suspension was transferred to 32 mL polycarbonate ultra centrifuge tubes (Beckmann Instruments Inc. Palo Alto, CA, USA), adjusted in weight with deionised water and centrifuged on a Beckmann L8-M ultracentrifuge in a Ti 70 rotor at 10 000 rpm ≡ 10 000 × g at 4°C for 10 min. Supernatants were transferred to fresh ultra centrifuge tubes, adjusted in weight and spun for 30 min at 30 000 rpm ≡ 92 000 × g at 4°C. Pellets containing microsomes were weighed. The amount of potassium phosphate buffer needed to resuspend the pellets was calculated as described below:

Volume to resuspend pellets = 0.35 x weight of pellets + 1 mL

Pellets were gently resuspended using a glass stirring rod. The resulting suspension was transferred to a small volume glass homogeniser and homogenised with a minimum of 10 strokes. The microsomal suspension was aliquoted and stored at -70°C until further use. Microsomal preparations of 4 insect cell preparations were thawed on ice, pooled, homogenised with a glass homogeniser, aliquoted and stored at -70°C. These pooled suspensions were used for incubation procedures described in the following Chapter 4.3 of *Candida albicans* CYP51. Short centrifugation times were chosen to minimize the inactivation of cytochrome P450 enzymes observed when applying long centrifugation times (personal communication Christopher Patten / BD Gentest Cooperation).

4.2.11 Characterisation of Microsomal Preparations

Protein concentration of the microsomal preparations was determined to be 50 µg/µL, following the method of Bradford utilising bovine serum albumin as reference. The cytochrome P450 content of the microsomal preparations was determined to be 1 µM by CO difference absorbance spectra according to Omura and Sato [92,93]. To assess if the expressed enzyme was functional or not, microsomes were incubated and analysed according to the *Candida albicans* CYP51 lanosterol assay described below.

Kinetic characterisations of the microsomal preparations are described in the following chapter.

4.2.12 Conclusions

The two cDNA sequences encoding the *Candida albicans* CYP51 and *Candida tropicalis* oxidoreductase were successfully cloned into the pFastDual vector. The following transposition experiment rendered a bacmid DNA construct harbouring both sequences and with this baculovirus particles could be gained by transfecting Sf9 cells. Having amplified and titrated the virus particles large scale cell suspensions could be infected with the recombinant virus to produce *Candida albicans* CYP51 and *Candida tropicalis* oxidoreductase in one cell. Microsomal preparation of the infected cells produced membranes harboring functional enzymes that were catalytically active and produced FF-MAs from lanosterol. Sufficiently high amounts of membranes could be produced to conduct kinetic and inhibition studies described in the following chapter.

4.2.13 Outlook

The single virus approach chosen for this project had the distinct advantages of being time-and labour-efficient, cell lysates can be used without supplementation of the respective reductase since it is ensured that cells express both enzymes parallel [88] [94]. Drawbacks are the relatively lower yields of the single enzymes compared to expression experiments where only one of the two enzymes is expressed [94] [87]. Furthermore the ratio of expression of CYP to oxidoreductase cannot be influenced using a single virus. A further optimisation of the expression system could be to add hemin to the incubations in form of a hemin albumin complex [95] [87], thereby reducing the toxicity of hemin for insect cells and gaining higher expression yields.

4.3 Inhibition Studies on human CYP51 and Candida albicans CYP51

4.3.1 Introduction

The aim of part of this work was to assess the specificity of azole antifungal compounds for their therapeutic target enzyme fungal CYP51 compared to the human analogue. Analytical procedures for the quantitation of the substrate and the product of human and fungal lanosterol-14α-demethylase activity have been described in Chapter 4.1 [96]. Human CYP51 co-expressed in the baculovirus system with the respective human oxidoreductase was available from BD Gentest. The successful co-expression of *Candida albicans* CYP51 and *Candida tropicalis* cytochrome P450 oxidoreductase was described in the previous chapter.

To assess the inhibitory potency of azoles on human and fungal CYP51, IC_{50} values of 13 fungicides used agriculturally, 7 antifungal agents used in human medicine and two cytostatic drugs were determined by monitoring formation of FF-MAS by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the presence of different azole concentrations.

4.3.2 Materials and Methods

4.3.2.1 Chemicals and Reagents

Human CYP51 was a kind gift from Gentest [96]. Engineering of *Candida albicans* CYP51 was performed as described in chapter 4.2. Membranes obtained from the 10 000 × g pellet were used for the incubation procedure described below. Bitertanol, cyproconazole, flusilazole, hexaconazole, imazalil, myclobutanil, penconazole, prochloraz, propiconazole, tebuconazole, triadimefon and triadimenol were from Dr. Ehrenstorfer (Augsburg, Germany). Epoxiconazole was a kind gift from the Swiss Federal Research Station (Wädenswil, Switzerland). Fluconazole was kindly provided by Pfizer GmbH (Karlsruhe, Germany). Clotrimazole was from Bufa (Uitgeest, The Netherlands). Fadrozole and letrozole were kind gifts from Novartis Pharma AG (Basel, Switzerland). Bifonazole, ketoconazole, itraconazole, miconazole and all other chemicals were of the highest grade available from Sigma/Fluka (Taufkirchen, Germany). The inhibitory potency of voriconazole was determined with a "*solutio ad infusionem*".

4.3.2.2 Analysis of Microsomes

Protein concentration of CYP51 and cytochrome P450 content were determined as described in Chapter 4.2.

4.3.2.3 *Candida albicans* CYP51 Inhibition Assay

Stock solutions of lanosterol (1 mM) and Triton X 100 (16 mg/mL) were prepared in isopropanol. Stock solutions and dilution series of azoles were prepared in ethanol, except for ketoconazole, itraconazole and miconazole where dimethylsulfoxide was used. A master mix containing 975 µL of potassium phosphate buffer (0.1 M; pH 7.4), 40 µL of a $NADP^+$ solution (50 mM), 75 µL of a magnesium chloride solution (66 mM), 50 µL of a glucose-6-phosphate solution (100 mM) and 7 µL of a glucose-6-phosphate dehydrogenase solution (0.1 U/µL) was prepared. Of this solution 76 µL were mixed with 4 µL of a 1:1 mix of lanosterol and Triton X 100 solution and 1 µL of azole dilution. The reaction mixture was preincubated for 5 min at 37°C and the reaction was started by addition of 20 µL of *Candida albicans* CYP51. After 40 min incubation 100 µL of isopropanol containing the internal standard d_6-cholesterol (5 µM) were added to stop the reaction. Protein was removed by centrifugation at 15 000 × g for 10 min. The supernatant was transferred to low volume insert sample-vials and 10 µL were injected onto the HPLC column and analysed by LC-MS/MS as described previously in Chapter 4.1 [96]. In a dose-finding study, dilution series based on concentration steps of factors of ten were used to define the concentration range to be tested in the main experiment.

The 10 000 × g membranes used contain cell nuclei and DNA of the insect cells. A second dose-finding study was performed to assess if the presence of high DNA concentrations has an influence on the free azole concentration, and thus on the IC_{50} value. Both studies based on the identical dilution series and concentration steps. Additionally 1 µg of calf thymus DNA were added to the incubations.

For all 21 azole compounds no differences were detected between the two dose-finding studies as seen in Figure 18 showing exemplary the two dose-finding studies of itraconazole. Since the presence of DNA in the incubations had no influence the main experiments were performed without additional DNA.

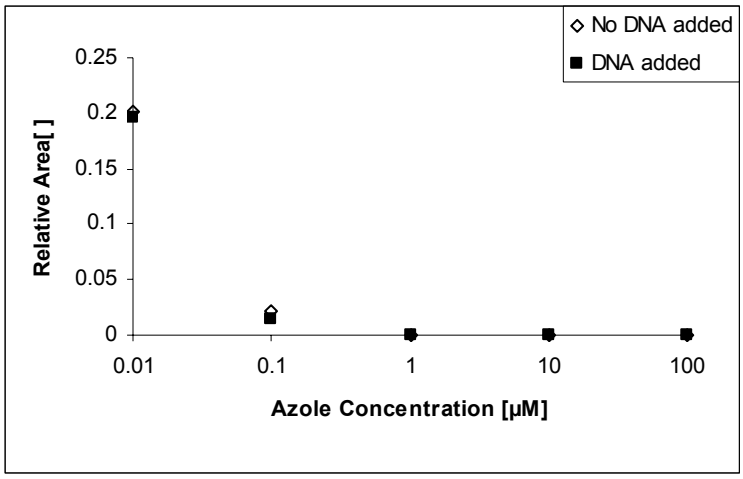


Figure 18: Dose finding studies of itraconazole with and without additional DNA.

For the main experiments, the concentration range spanned at least three decades with 7 logarithmically spaced values and the expected IC_{50} at the geometric mean. All experiments were run in duplicate.

4.3.2.4 Human CYP51 Inhibition Assay

Incubations with human CYP51 were performed similar to the *Candida albicans* CYP51 assay, as described in Chapter 4.1 [96]. Dose-finding studies for the main experiments were performed as described for *Candida albicans* CYP51, except that no second experiment with additional calf thymus DNA was performed. Human CYP51 supersomes from BD Gentest Cooperation had been prepared from insect cells following a very similar procedure to the one described in Chapter 4.1 (personal communication Chris Patten/BD Gentest). In contrast to the source of fungal CYP51, only the membranes of the 100 000 × g pellet were used as supersomes. DNA present in the insect cells had been removed by centrifugation at 10 000 × g thus intercalation of azole compounds into the DNA and the loss of inhibitory compounds does not have to be controlled for.

4.3.2.5 Determination of K_m and V_{max}

Determination of K_m and V_{max} requires incubation procedures using different substrate concentrations. The described incubation assay for *Candida albicans* was performed with dilution series of lanosterol resulting in an inter assay substrate concentration ranging from 1 to 50 µM. The amount of isopropanol in the incubation assay was kept constant. For human CYP51 only turn over rates could be determined due to limited availability of enzyme.

4.3.2.6 Data Analysis (IC_{50} Values, K_m, V_{max})

For both assays the area of FF-MAS corrected by the area of the internal standard (relative area) was plotted against the log (base 10) of the concentration of the azole. Four parameters were estimated by fitting the two replicate area data sets to a probit curve: the FF-MAS relative area at azole concentration 0 and ∞, the relative area at half maximum inhibition and the slope of the probit curve. Statistical analysis was performed by a nonlinear mixed-effects model fit by maximum likelihood run on the statistics software "R". A random error was attributed to the FF-MAS relative area at azole concentration 0, to account for differences between replicates for the extrapolated null measurement.

When solubility limits did not allow experimental determination of a second zero product data point, one theoretical high dose data point was added with full inhibition (zero product formation) in order to generate a complete lognormal inhibition curve for the statistical analysis. In the human CYP51 assay for fluconazole, itraconazole, fadrozole and letrozole no IC_{50} values could be calculated due to the low inhibitory potency and the limited solubility.

Kinetic parameters K_m and V_{max} were determined by non-linear regression analysis according to Kakkar et al., equation 2 [97].

4.3.3 Results

4.3.3.1 Analytical Procedures

Formation of FF-MAS, the product of the reaction catalysed by human and *Candida albicans* CYP51 with lanosterol, could be unequivocally detected by LC-MS/MS. Atmospheric pressure photoionisation or atmospheric pressure chemical ionisation had to be used instead of electrospray ionisation (ESI) for the analysis of lanosterol, FF-MAS and the internal standard d_6-cholestrol since steroids are too lipophilic and exhibit no moieties for protonation or deprotonation processes typically used for ESI. In comparison to the established methods for the quantitation of product formation of CYP51, like gas chromatography mass spectrometry or release of radio-labelled formic acid, the established method has the distinct advantages of minimal sample preparation and short HPLC run times. No extraction or derivatisation steps are necessary for the analytical LC-MS/MS method, thus artefact formation is negligible.

4.3.3.2 *Candida albicans* CYP51

More than 80% of the total enzyme activity was localised in the 10 000 × g membrane fraction, 10% in the 100 000 × g fraction and less than 10% in the supernatant of the microsomal preparations described in Chapter 4.1. Thus the 10 000 × g fractions were used for inhibition studies. Figure 19 displays the activity distribution in graphical form. The cytochrome P450 content of the Sf9 membranes was determined to be 1 µM, resulting in a cytochrome P450 enzyme concentration in the assay of 0.2 µM. The absorption maxima of the carbon monoxide-bound form were located at 450 nm with no peak at 420 nm, indicating a stable production (data not shown).

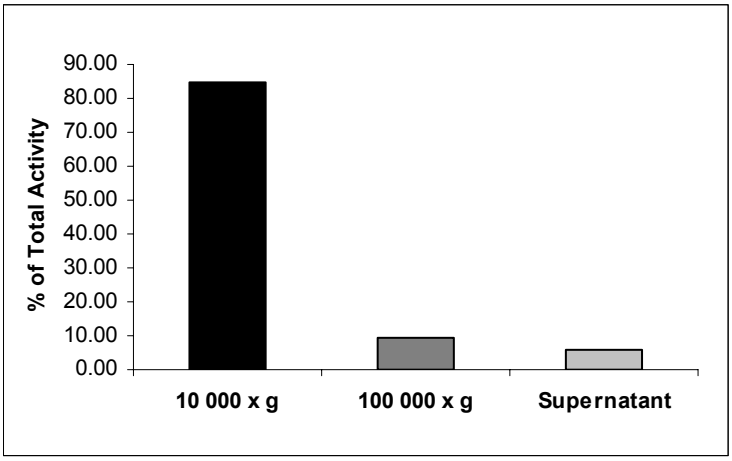


Figure 19: Activities of the 10 000 × g, 100 000 × g fractions and the supernatant of the microsomal preparations of the Sf9 cells.

Protein concentration of the membranes was determined to be 50 µg/µL, resulting in an assay-protein concentration of 10 µg/µL. The concentration of organic solvents in the assay was 5%.

Product formation was linear from 5 to 40 µL incubated microsomes as shown in Figure 20.

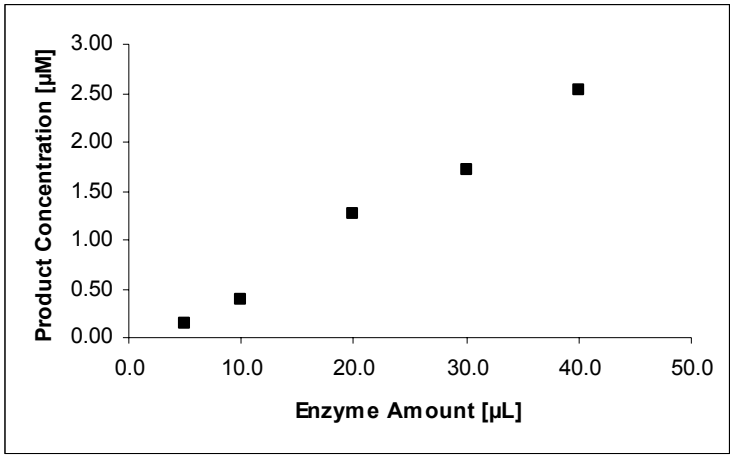


Figure 20: Linear product formation by addition of varying amounts of *Candida albicans* CYP51 (lanosterol-14α-demethylase) membranes to the incubation assay.

Product formation velocity was not linear over time. As shown in Figure 21 the velocity is highest in the first 10 minutes and then slows down. Between 40 and 60 minutes virtually no substrate turn-over takes place. A possible explanation could be the deterioration of the enzyme over time. The product formation was sufficiently constant from 10 to 40 minutes incubation time to determine IC_{50} values.

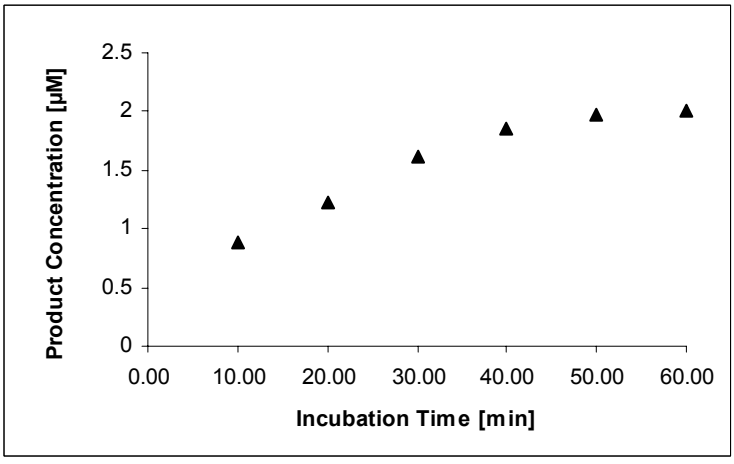


Figure 21: Time course from 10 to 60 minutes of product formation by *Candida albicans* CYP51 (lanosterol-14α-demethylase) membranes.

Determination of V_{max} was performed with 40 minutes incubation time and thus the V_{max} value does not represent the true maximum velocity achieved in the first 10 minutes of the incubation. A compromise between sufficient product formation to obtain reproducible analytical signals and the maximum velocity had to be found.

K_m and V_{max} of *Candida albicans* CYP51 were determined to be 7.4 µM and 0.46 pmol / min × pmol P450, respectively. Substrate concentration for determination of IC_{50} values was chosen to be 20 µM, equivalent to approximately 2.7 times K_m. The turn-over of the enzyme was constant, allowing for the determination of IC_{50} values under constant conditions. The rate of lanosterol-14α-demethylation using 20 µM lanosterol was determined to be 0.5 pmol product formed / min × pmol *Candida albicans* CYP51. Using the described incubation procedure and the lanosterol concentration could be monitored parallel to product formation. In the absence of inhibitors, approximately 20% of the substrate added was metabolised to FF-MAS within 40 minutes.

4.3.3.3 Human CYP51

The P450 contents of human CYP51 supersomes had been determined to be 20 µM, resulting in an assay concentration of 0.1 µM. Protein concentration of the membranes was determined to be 50 µg/µL, resulting in an assay protein concentration of 0.3 µg/µL. The concentration of organic solvents in the assay was 5%. Product formation was linear from 10 to 40 minutes and from 0.05 to 0.2 µM enzyme concentration (data not shown). The turn-over number using 15 µM lanosterol was 0.1 pmol / min × pmol P450, thus only 1% of the total substrate amount is metabolised. No substrate depletion was observed visually using the described analytical and incubation procedure. Substrate concentration for determination of IC_{50} values was chosen to be 15 µM.

4.3.3.4 Inhibitory Potencies Determined for Azole Compounds on Human and Fungal CYP51

Table 9 displays the IC_{50} values and respective geometric standard errors determined for human CYP51 and *Candida albicans* CYP51. The inhibitory potencies of azole fungicides on recombinant human CYP51 ranged approximately 30-fold, from 1.30 to 37.2 µM. The most potent compounds are bitertanol and epoxiconazole; the least potent are imazalil and triadimenol. The IC_{50} values of the antifungal agents showed a wider span (500-fold) from 0.057 (miconazole) to >30 µM (fluconazole).

Miconazole was the most potent of all azoles tested. Four of the 7 antifungal agents (miconazole, bifonazole, clotrimazole and ketoconazole) were more potent than the most potent fungicide (bitertanol). These are all imidazole compounds and the older compounds on the marked compared to the two newer triazole compounds, fluconazole and itraconazole. The two cytostatic drugs tested display only low inhibitory potency (≥ 100 µM).

Table 9: Data of the inhibition of human lanosterol-14α-demethylase (hCYP51) and *Candida albicans* lanosterol-14α-demethylase (cCYP51) by azoles. IC_{50} values are given in [µM] concentrations. The standard error (x/:) is given as a factor because estimates of IC_{50} values and standard errors were estimated on a log(concentration)-probit model.

Azole	IC_{50} hCYP51	x/:	IC_{50} cCYP51	x/:	Ratio
Fungicides					
Bitertanol	1.30	1.29	0.059	1.22	22
Cyproconazole	22.8[a]	1.62	0.10	1.16	228
Epoxiconazole	1.95	1.51	0.22	1.13	9
Flusilazole	3.36	1.58	0.085	1.22	40
Hexaconazole	15.6[a]	1.5	0.066	1.28	236
Imazalil	36.1	1.95	0.082	1.10	440
Myclobutanil	29.0[a]	1.57	0.14	1.16	207
Penconazole	19.3	1.40	0.076	1.15	254
Prochloraz	5.00	1.23	0.098	1.25	51
Propiconazole	8.25	1.41	0.15	1.14	55
Tebuconazole	3.61	2.08	0.35	1.40	10
Triadimefon	9.95	1.62	0.13	1.30	77
Triadimenol	37.2[a]	1.36	0.33	1.27	113
Antifungal agents					
Bifonazole	0.80	1.80	0.30	1.13	3
Clotrimazole	0.85	1.47	0.091	1.12	9
Fluconazole	>30 (23% inhibition)[b]		0.051	1.09	588
Itraconazole	≈ 30 (53% inhibition)[b]		0.039	1.13	769
Ketoconazole	0.43	1.24	0.064	1.08	7
Miconazole	0.057	1.24	0.072	1.09	0.8
Voriconazole	≈ 30 (63% inhibition)[c]				
Cytostatic drugs					
Fadrozole	≈ 100 (54% inhibition)[b]		32.2	1.41	3
Letrozole	>100 (11% inhibition)[b]		13.3	1.26	8

[a] One theoretical high-dose/zero-product point added

[b] No full sigmoid curve available due to limited solubility

[c] No full data set (solutio ad infusionem; chemical instability)

The inhibitory potency of the fungicides on *Candida albicans* CYP51 ranged only 6-fold, from 0.059 to 0.35 µM. The most potent compounds were bitertanol and hexaconazole; the less potent compounds were tebuconazole and triadimenol. The IC_{50} values of the antifungal agents also showed a small range of 8 from 0.039 (itraconazole) to 0.3 µM (bifonazole). Itraconazole was the most potent of all azoles tested. The two cytostatic drugs displayed a 30- to 100-fold lower inhibitory potency against *Candida albicans* CYP51.

The IC_{50} values determined for the human enzyme and the *Candida albicans* variant do not correlate with each other (Figure 22 shows a graphical plot). Further they do not correlate with log p values of the azole compounds as determined by Spearman rank correlation (data not shown). The log p (or partition coefficient) describes the differential solubility of a compound in octanol and water and is a measure of the hydrophobicity and hydrophilicity of a substance.

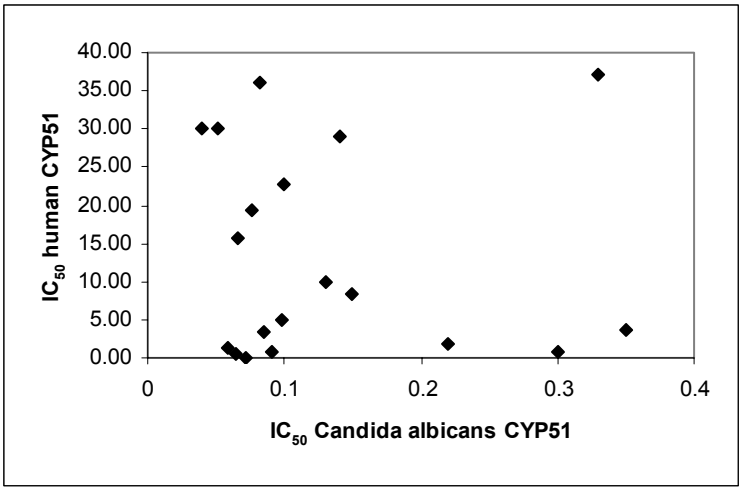


Figure 22: IC_{50} values determined for antifungal azole compounds used agriculturally and in human medicine and human CYP51 (y-axis) are plotted against IC_{50} values for the same compounds and *Candida albicans* CYP51 (lanosterol-14α-demethylase) (x-axis).

A ratio of the IC_{50} values determined for the human enzyme divided by the value determined for the *Candida albicans* enzyme are given in Table 9. The values for the ratio range from 0.8 (miconazole) to 769 (itraconazole). A small ratio indicates an unfavourable, small difference between the inhibitory potency for the fungal CYP51, compared to the human enzyme, i.e. low selectivity. This is the case for the agriculturally used fungicides epoxiconazole and tebuconazole and for bifonazole,

clotrimazole, ketoconazole and miconazole, compounds used as antifungal drugs in human medicine.

The larger the ratio is, the better the selectivity for the fungal variant of the enzyme. Such a favourable ratio was found for the agricultural compounds cyproconazole, hexaconazole, imazalil, myclobutanil and penconazole. Of the medicinal agents fluconazole and itraconazole showed such a desirable high ratio.

It should be noted however, that for the interpretation of these ratios the absolute IC_{50} values have to be taken into account. Both fadrozole and letrozole only show a relatively low ratio of 3 and 8, respectively. In contrast to the four antifungal agents used in human medicine displaying a low ratio, the IC_{50} values of the cytostatic compounds determined for both enzymes are very high, thus no problems arise from the low ratio.

4.3.4 Discussion

4.3.4.1 Comparison of Enzyme Kinetics

Candida albicans CYP51 has been expressed before in *Saccharomyces cerevisiae,* purified on chromatographic columns and reconstituted by Lamb and co-workers [14] [98] [99]. The K_m values and respective V_{max} values are given in Table 10 and are compared to the values determined for the *Candida albicans* CYP51-*Candida tropicalis* oxidoreductase construct. The K_m values reported are 3 to 4 times higher compared to the value determined for the construct described above. K_m is a constant for the affinity of a substrate to an expressed enzyme and is independent of the enzyme amount used.

The difference between the literature value and the one described above can be in part explained by the fact, that a different expression system was used by Lamb et al., the *Candida albicans* CYP51 was expressed alone and was later reconstituted with an oxidoreductase.

Additionally the non-purified enzyme shows a higher affinity to its substrate, than the purified ones described in literature. In the living organism cytochrome P450 enzymes and the respective oxidoreductase form a complex anchored in the cell membrane. By purifying the enzyme it is removed from its “natural” environment, the cell membrane. This could have an influence on the enzyme conformation and thus on its substrate affinity indicated by the higher K_m values.

Table 10: Kinetic parameters K_m and V_{max} of heterologously expressed and purified *Candida albicans* lanosterol-14α-demethylase (cCYP51) reported in literature compared to our value.

	K_m	V_{max}
This manuscript	7.4 µM	0.46 pmol product / min × pmol P450
Lamb et al.[14] [a]	20.8 µM	0.47 nmol product / min × nmol P450
Lamb et al. [98] [b]	21 µM	0.24 ±0.04 nmol product / min × nmol P450
Lamb et al. [99] [a]	29.4 µM	0.15 nmol substrate converted / min × nmol P450

[a] To achieve catalytic activity enzymes were reconstituted with rabbit NADPH oxidoreductase

[b] To achieve catalytic activity enzymes were reconstituted with yeast cytochrome b_5 and NADH cytochrome b_5 reductase or with yeast oxidoreductase

The V_{max} value determined for the described non-purified enzyme is only comparable to one of those reported in literature. The other two values are 2 to 3 times lower than our value. Differences could be explained by the fact that different analytical methods were used to monitor product formation and that for the lowest V_{max} value reported substrate turn-over was determined rather than product formation.

Further explanations for the differences found both for K_m and V_{max} could be the different analytical procedures used to calculate both constants (non-inverse vs. double inverse Lineweaver-Burk plots) and that only one standard error is given making comparison difficult.

Human CYP51 has also been heterologously expressed in yeast and purified [14]. Catalytic activity was reconstituted by addition of rabbit NADPH oxidoreductase and K_m and V_{max} were determined to be 29.4 µM and 0.474 pmol product / minute × pmol P450.

4.3.4.2 Comparison with Literature IC_{50} Values

Lamb and co-workers determined IC_{50} values for ketoconazole and itraconazole with a formic release assay [14]. They found a less than 10-fold difference between the fungal and the human enzyme (Table 11 and 12). The values for ketoconazole on both enzymes and for itraconazole on *Candida albicans* could be reproduced here. For the human variant of the enzyme and itraconazole however an IC_{50} value close to 30 µM was determined in contrast to the finding of Lamb et al (0.61 µM). At our hands no explanation can be given for this finding.

Table 11: Comparison of literature IC_{50} values for *Candida albicans* lanosterol-14α-demethylase (cCYP51) with our reported IC_{50} values.

Substance	This Manuscript	Lamb et al. 1999[a]	Lamb et al. 2000[b]	Lamb et al. 1997[b]	Van den Bosche 1987/1998[c]
Imazalil	0.082				0.05
Penconazole	0.076				0.019
Propiconazole	0.15				0.061
Bifonazole	0.30				1.170
Clotrimazole	0.091				0.036
Fluconazole	0.051			0.053	0.249
Itraconazole	0.039	0.06	0.0076		0.031
Ketoconazole	0.064	0.08	0.008	0.006	0.03
Miconazole	0.072				0.076

[a] Purified *Candida albicans* CYP51, formic release assay [14]

[b] *Candida albicans* CYP51, cell free biosynthesis of ergosterol from [2-^{14}C] mevalonate [15] [100]

[c] *Candida albicans* CYP51, inhibition of CO-binding to central heme iron [101,102]

Table 12: Comparison of literature IC_{50} values for human lanosterol-14α-demethylase (hCYP51) with our reported IC_{50} values.

Substance	This Manuscript	[14][a]
Itraconazole	≈ 30 (53% inhibition)	0.58
Ketoconazole	0.43	0.61

[a] Purified human CYP51, formic release assay [14]

Literature describes a [2-^{14}C] mevalonate assay using mevalonate as substrate, a compound present in the cholesterol biosynthesis a few steps upstream of lanosterol. The actual substrate of CYP51, lanosterol, first has to be synthesised in the assay. Utilising such a [2-^{14}C] mevalonate ergosterol inhibition assay Lamb et al. determined IC_{50} values of 0.053 for fluconazole, 0.0076 for itraconazole and 0.008 - 0.004 for ketoconazole [15] [100]. The values for itraconazole and ketoconazole are a factor of 5 - 10 below those reported by the same group using a different assay and to the values in this manuscript (Table 12). The IC_{50} values determined with a formic acid release assay are close to the ones determined here. An explanation could be that the formic acid release assay measures production of FF-MAS from the direct

substrate of CYP51-lanosterol. IC_{50} values are dependent on the substrate concentration used. For the [2-^{14}C] mevalonate assay a substrate concentration cannot be determined. Another interpretation of the difference in IC_{50} values could be that the azole compounds added inhibited not only CYP51 but also enzymes necessary for the production of lanosterol.

Vanden Bosche et al reported IC_{50} values for *Candida albicans* and a variety of azole compounds, including fungicides used for agricultural purposes given in Table11 [101,102]. Numerical IC_{50} values fit well to the ones determined here for some examples, e.g. itraconazole, ketoconazole and miconazole. For all other compounds however the values do not compare well to the ones described in Table 9 nor were they applicable to those of Lamb. The inhibitory potency is assessed by the inhibition of CO-binding to the central heme iron of CYP51. Questionable is whether this assay can fully assess the inhibitory potency of mixed type inhibitors or non-competitive inhibitors, this could explain the differences observed.

Lamb et al. postulate that in crude membrane fractions of rat or human liver azole compounds are scavenged by other microsomal enzymes present. Thus higher concentrations of inhibitor are necessary and higher IC_{50} values result. Trzaskos and Henry already reported in 1989 that the sensitivity of rat CYP51 towards flusilazole is enhanced by partial purification of the enzyme from rat hepatic microsomes [103]. A protective effect by other susceptible P450 isoenzymes present in crude microsomal fractions of mammalian preparations is postulated.

By expressing both human CYP51 and *Candida albicans* CYP51 heterologously in the same system a similar protein background is present in both incubation processes. The problem of other cytochrome P450 enzymes scavenging part of the azole added to the incubation has been avoided, since insect cell lines used do not express cytochrome P450 enzymes endogenously in such a high level as are present in liver microsomes.

A good test system to assess the different inhibitory potency of azole compounds towards human and fungal CYP51 could be established. For a variety of compounds, e.g. epoxiconazole, tebuconazole, bifonazole, clotrimazole, miconazole, the difference between the inhibition of human CYP51 and fungal CYP51 is smaller than by a factor of 10. For a number of other compounds however, e.g. imazalil, fluconazole, itraconazole, a major difference between the inhibition of fungal and

human enzyme was observed. Specificity for the fungal enzyme compared to the human analogue is favourable for all antifungal azole compounds with view to the likely roles of FF-MAS and the resulting T-MAS in the endocrine system. The two cytostatic drugs tested should and do have a low inhibitory potency on both enzymes. A complete evaluation of the azole compounds, including a comparison of the IC_{50} values of this chapter to plasma concentrations reached, is given in the discussion, Chapter 6.

5 Azole Residues in Wine Samples

5.1.1 Introduction

Azoles (imidazoles and triazoles) are used as fungicides on a wide variety of crops, e.g. bananas, wheat, barley and grapes. Residue levels determined by authorities are not generally published. To gain insight into selected levels of residues of azole fungicides wine samples were analysed. Grapes are especially vulnerable to moulds, which leads to a widespread use of antifungal agents. The physicochemical and biological processes of wine-making do not eliminate fungicides, which leads to observable amounts in wine samples [104]. The determination of residue levels is necessary for food safety monitoring and regulatory purposes.

Up to now azole levels in wine samples were mainly analysed by gas chromatography/electron ionisation mass spectrometry (GC-EI/MS) [105,106]. This technique involves many preceding sample work-up steps like extraction, clean up or interference removal and derivatisation. Sample preparation could be simplified by automated solid phase micro-extraction techniques as described for triadimefon, propiconazole, myclobutanil and penconazole [107]. Methods based on liquid chromatography/tandem mass spectrometry (LC-MS/MS) are described for the quantitation of several azoles used in human medicine in body fluids of humans and rats [108,109]. A single analytical procedure is desirable to screen beverage samples for all azoles applied as fungicides. In complex matrix this is only achievable with single MS or more conveniently with MS-MS based methods and has been described previously for 8 and more analytes within a single LC-run [96,110] . The method presented here allows the quantitation of 13 azoles in wine samples without any sample preparation other than centrifugation, within one LC-run using a tandem mass spectrometer as detector. Analytes can be distinctly detected by GC-MS [104] [106] and LC-MS [111,112] with similar sensitivity to the method described but tandem mass spectrometry has the advantage of unequivocal analyte identification due to the ion selection of two quadrupole mass analysers.

5.1.2 Materials and Methods

5.1.2.1 Chemicals and reagents

Epoxiconazole was a kind gift from the Swiss Federal Research Station, Wädenswil, Switzerland. All other azole compounds were from Dr. Ehrenstorfer (Augsburg, Germany). LC grade water was from Roth (Karlsruhe, Germany). All other chemicals were from Sigma/Fluka (Taufkirchen, Germany). All solvents used were LC grade or better. Wine samples were chosen randomly out of private donations from members of the department.

5.1.2.2 Sample preparation

Wine samples were centrifuged at 15 000 x g for 5 minutes to remove particles. The supernatant was analysed by LC-MS/MS without further sample work-up.

5.1.2.3 Optimisation of mass spectrometry methods

The LC-ESI-MS/MS method was optimised with respect to sensitivity, analyte identification and quantitative measurement. Infusion experiments were carried out to examine ionisation and fragmentation patterns of the analytes. A syringe pump (Single Syringe Pump 11, Harvard Apparatus Inc., Holliston, USA) was used to provide a constant analyte infusion (300 µL/min) into the LC eluent via a T-connection. Analyte concentrations were chosen in a range of 5 - 100 ng/µL to obtain a constant signal in the Q1 scan mode. Basic source and MS parameters such as declustering potential (DP), focussing potential (FP), collision energy (CE) and exit potential (CXP) were optimised using the “quantitative optimisation” function of “Analyst 1.3.1” (Applied Biosystems, Darmstadt, Germany).

5.1.2.4 Liquid chromatography-tandem mass spectrometry

To determine the 13 different azole compounds qualitatively or quantitatively in wine samples, 10 µL of supernatant were injected on a Synergi Hydro RP LC column (80 A, 4 µm, 150 x 2 mm Phenomenex, Aschaffenburg, Germany) with a C18 ODS guard column (4 x 3 mm, Phenomenex, Aschaffenburg, Germany) using an Agilent 1100 autosampler and an Agilent 1100 LC-pump (Agilent Waldbronn, Germany). The samples were separated by gradient elution with water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B) using the following conditions: 50% A isocratic for 1 min, linear to 0% A within 5 min and isocratic for 5 min at 0% A with a flow rate of 250 µL/min. The LC system was coupled directly to a triple stage quadrupole mass spectrometer (API 3000, Applied Biosystems,

Darmstadt, Germany) equipped with a turbo ion spray source. Analytes were detected in the positive ion mode at a vaporizer temperature of 400°C. An ion spray voltage of 2 000 V was applied. Spectral data were recorded with N_2 as collision gas (CAD = 4) in the multiple reaction monitoring (MRM) mode with a dwell time of 50 ms for each transition monitoring the following MS/MS ion-transitions (Table 13).

Table 13: MS parameters for the detection of 13 different azoles by HPLC-MS/MS with electrospray ionisation. DP: Declustering Potential, CE: Collision Energy, CXP: collision cell exit potential.

Compound		Transition [*m/z*]	Ret. time [min]	DP [V]	CE [V]	CXP [V]
Penconazole	Quantifier	284.0 → 158.9	8.76	56	45	4
	Qualifier	284.0 → 70.0		56	27	4
Myclobutanil	Quantifier	289 → 70.1	7.31	76	31	4
	Qualifier	289 → 125.1		76	41	4
Cyproconazole	Quantifier	292.0 → 70.1	6.65	66	31	4
	Qualifier	292.0 → 125.1		66	49	4
Triadimefon	Quantifier	294.0 → 57.1	7.67	71	45	2
	Qualifier	294.0 → 69.1		71	31	4
Triadimenol	Quantifier	296.0 → 70.1	5.83 6.24*	41	17	2
Imazalil	Quantifier	297.1 → 159	2.25	86	27	6
	Qualifier	297.1 → 200.9		86	21	4
Tebuconazole	Quantifier	308.1 → 70.0	7.88	76	53	2
	Qualifier	308.1 → 124.8		76	51	4
Hexaconazole	Quantifier	314.0 → 70.1	8.40	71	33	4
	Qualifier	314.0 → 159.1		71	41	6
Flusilazole	Quantifier	316.1 → 165.3	8.06	66	39	4
	Qualifier	316.1 → 109		66	79	4
Epoxiconazole	Quantifier	330.0 → 121.2	7.57	46	29	4
	Qualifier	330.0 → 129.1		46	61	4
Bitertanol	Quantifier	338.1 → 70.0	8.13	41	23	2
	Qualifier	338.1 → 99.0		41	21	4
Propiconazole	Quantifier	342 → 159.0	8.97	71	47	4
	Qualifier	342 → 69.2		71	33	4
Prochloraz	Quantifier	377.9 → 309.7	6.99	41	19	14
	Qualifier	377.9 → 70		41	33	4

* Two isomers are separated on the column. For quantitation purposes the second peak was used.

Quantitation of azoles was based on calibration curves obtained after addition of known amounts of azoles (0.1 – 50 ng/mL) to two red wine and one white wine

sample containing no azoles. Calibration curves were calculated from 11 duplicate data points using “Analyst 1.3.1”. Limits of detection (LODs) and limits of quantitation (LOQs) were measured in three independently spiked and analysed samples. Signal to noise (s/n) ratios where determined with Analyst 1.3.1 software applying a standard deviation of three. A peak with s/n ≥ 3 was judged as above the LOD and a peak with s/n ≥ 9 as above the LOQ. Precision values at the 1 ng/mL and at the 10 ng/mL level where each calculated from 20 consecutive injections of the same sample.

5.1.3 Results and Discussion

5.1.3.1 Sample Work-Up

Centrifugation was the only sample preparation step used to eliminate particles, such as crystals of tartar and yeast of very young wines. The same is true for beer samples (data not shown).

5.1.3.2 High performance liquid chromatography-mass spectrometry

5.1.3.3 Ionisation and fragmentation

Ionisation

Table 13 summarises precursor ions of all azoles of which analytical standards were available. In the positive ion mode, protonation was the major ionisation mechanism for all azoles. Triadimefon was the only compound that could be sufficiently ionised in the negative ion mode to produce detectable product-ions in the third quadrupole. Due to adequate sensitivity in the electrospray mode, other ionisation techniques like APCI (atmospheric pressure chemical ionisation) or APPI (atmospheric pressure photoionisation) were not tested. For different azoles literature describes APCI, in positive and negative ion mode [111,113] and APPI [114] as ionisation techniques. However references are not consistent in the evaluation of the different ionisation techniques: for imazalil APCI and ESI gave similar results in terms of sensitivity [112]; for bitertanol, tebuconazole and triadimefon APCI in positive mode was considered superior to ESI in positive and negative mode and to APCI in negative mode [115]. The declustering potential was optimised for each compound in the positive ion mode to achieve highest sensitivity.

5.1.3.4 Fragmentation

Fragmentation was performed with nitrogen as collision gas and the important potentials like the collision energy were optimised for each azole and transition

(Table 13). The most sensitive fragment ion was used to generate a quantifier transition and the second most sensitive one to generate a qualifier transition. Triadimenol did not generate a second utilisable transition, for myclobutanil the qualifier transition was less sensitive than the quantifier transition by a factor of five. For all other compounds the qualifier transition was as sensitive as the quantifier transition. Bitertanol, cyproconazole, epoxiconazole, hexaconazole, myclobutanil, penconazole, prochloraz, tebuconazole and triadimenol all showed m/z = 70 as a prominent product-ion. With the exception of epoxiconazol the transition of $[M\text{-}H]^+ \rightarrow$ m/z = 70 was chosen as qualifier or quantifier transition. For bitertanol, penconazole and triadimenol this product-ion can be explained by a McLafferty rearrangement since these compounds have one γ-hydrogen available [116]. Figure 23 shows a product-ion spectrum of penconazole with the proposed McLafferty rearrangement.

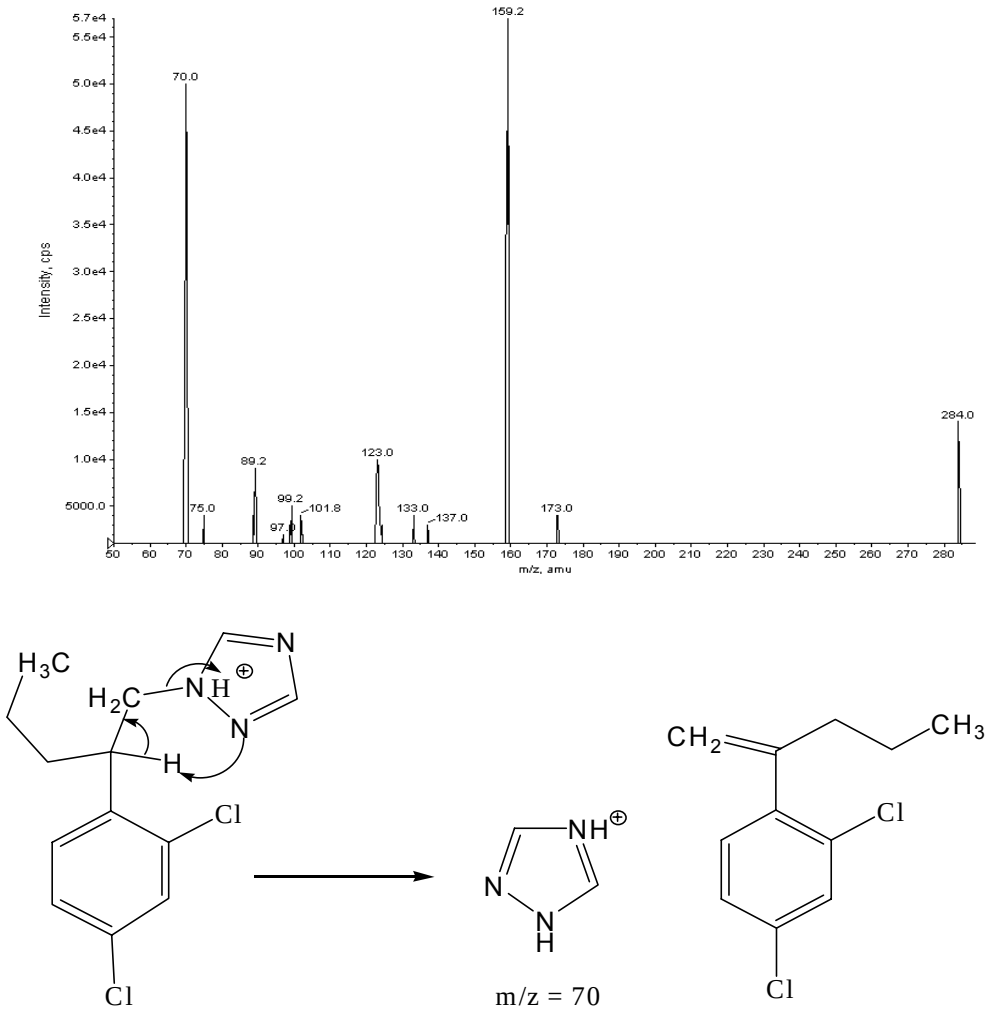


Figure 23: Product-ion spectrum of penconazole and proposed McLafferty rearrangement.

Cyproconazole, epoxiconazole, hexaconazole myclobutanil and tebuconazole do not have a γ-hydrogen. The product-ion of m/z = 70 could be explained by a three or four-membered transition state with the neighbouring methylene group, followed by a cleavage of the nitrogen-carbon-bond. However the product-ion m/z = 70 of prochloraz cannot be explained by this, since prochloraz does not have a neighboured methylene group.

Both propiconazole and triadimefon show m/z = 69 as product-ion corresponding to a simple cleavage of the triazole ring plus hydrogen. To a small extent they also show m/z = 70 as product-ion, which could be explained by the above mentioned three to four membered transition state.

Imazalil has m/z = 69 as characteristic product-ion which corresponds to a McLafferty rearrangement of an imidazole ring. Flusilazole shows a completely different fragmentation pattern compared to the other azoles with neither m/z = 70

nor m/z = 69 as product-ion, which could be attributed to the silicon atom in the structure.

Due to the availability of standards for all 13 azoles unequivocal analyte identification was possible using quantifier and qualifier transitions and comparing retention times with standards in matrix solutions

5.1.3.5 Liquid chromatography

The chromatographic conditions were optimised with respect to sensitivity. To achieve a very efficient ionisation the analytes should elute from the column with a relatively high percentage of organic solvent to ensure complete evaporation of the LC solvents. Further no analyte should elute with the solvent front of the column which would lead to ion suppression in the electrospray process. This was achieved for all azoles with a Synergy Hydro RP column, acidified water as solvent A and a gradient starting at 50% A. Figure 24 A shows an overlay of the 13 single traces obtained by measuring the 13 specific quantifier MS/MS transitions at LOQ level in a spiked wine sample. Figure 24 B shows the quantifier transitions of standards spiked in wine of triadimenol and tebuconazole. These two azoles where detected in the sample shown in Figure 24 C. The signals eluting at 1.8 minutes with the solvent front are no azole compounds. Apart from imazalil eluting at 2.3 min all azoles are well separated from the solvent front. The use of compound specific MS-MS transitions does not require a complete resolution between the analytes. Sample matrix (wine, vs. water) has no influence on the separation.

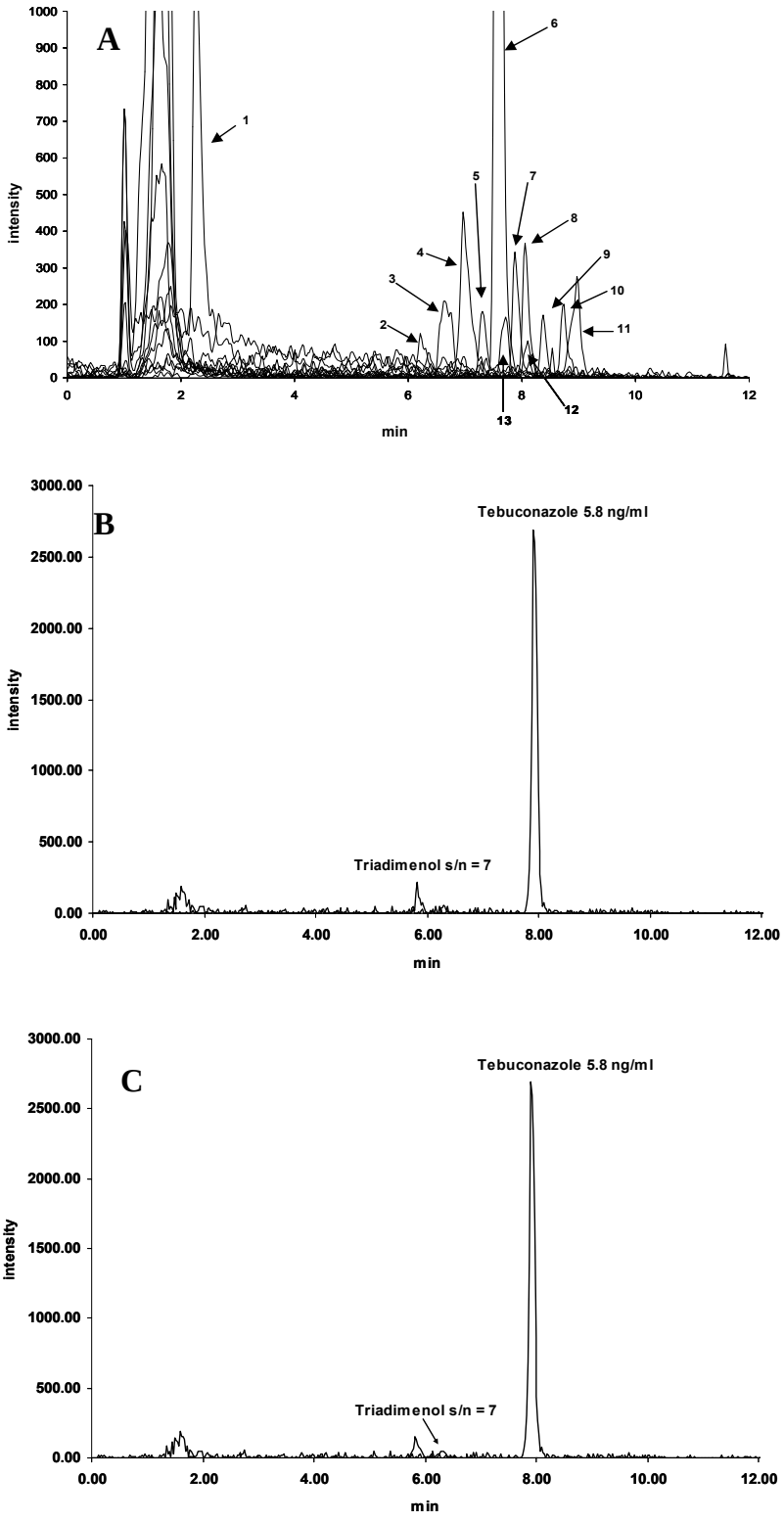


Figure 24: Chromatograms showing

A) Transitions of all 13 azoles of a standard in wine matrix spiked at LOQ level: imazalil (1), triadimenol (2), cyproconazole (3), prochloraz (4), myclobutanil (5), epoxiconazole (6), tebuconazole (7), flusilazole (8), hexaconazole (9), penconazole (10), propiconazole (11), bitertanol (12), triadimefon (13)

B) Transitions of tebuconazole and triadimenol from a 10 ng/mL standard

C) Transitions of tebuconazole (5.8 ng/mL) and triadimenol (<LOQ) of a real sample

5.1.3.6 Quantitation

The only work-up step centrifugation has no effect on the azole concentrations (data not shown). This is also demonstrated by identical accuracy, precision and slope values of calibration standards and curves acquired in water or wine matrix (Table 14 and 15). As it is not a problem to get robust quantitative results and satisfactory data, the use of stable isotope labelled internal standards is not necessary. Furthermore an isotope labelled standard is not available for many azoles. The limits of quantitation (LOQs) ranged between 0.25 and 7.5 ng/mL. For all azoles tested the LOQ was lower than the maximum residues levels (MRL) of the European Union by at least a factor of 4 (Table 14 and 15) enabling the use of the method for screening of beverages, especially wine, without any sample enrichment step.

Table 14: Method validation parameters II

Compound	Linearity	r^2	Precision [%] (1 ng/mL)	Precision [%] (10 ng/mL)
Penconazole	0.25 - 50	0.983	13.3	5.8
Myclobutanil	0.75 - 50	0.995	7.9	2.6
Cyproconazole	0.75 - 50	0.994	9.0	4.2
Triadimefon	7.5 - 50	0.990	n.d.	13.7
Triadimenol	0.75 - 50	0.992	12	5.3
Imazalil	5 - 50	0.987	n.d.	5.1
Tebuconazole	0.5 - 50	0.992	9.2	6.9
Hexaconazole	0.5 - 50	0.994	12.6	6.1
Flusilazole	0.75 - 50	0.994	9.1	10.7
Epoxiconazole	5 - 50	0.998	10.1	7
Bitertanol	1 - 50	0.977	12.1	8.6
Propiconazole	0.75 - 50	0.994	5.3	4.1
Prochloraz	0.75 - 50	0.993	8.4	3.5

Table 15: Method validation parameters

Compound	MRL [ng/g] *		LOD [ng/mL]	LOQ [ng/mL]	Accuracy [%]
Penconazole	200	Quantifier	0.25	0.25	80 - 120
		Qualifier	0.25		
Myclobutanil	1 000	Quantifier	0.5	0.75	80 - 116
		Qualifier	2.5		
Cyproconazole	---	Quantifier	0.5	0.75	80 - 117
		Qualifier	0.5		
Triadimefon	2 000	Quantifier	5	7.5	80 - 118
		Qualifier	0.75		
Triadimenol	2 000	Quantifier	0.5	0.75	80 - 116
Imazalil	20	Quantifier	2.5	5	80 - 117
		Qualifier	2.5		
Tebuconazole	---	Quantifier	0.25	0.5	80 - 118
		Qualifier	0.25		
Hexaconazole	100	Quantifier	0.25	0.5	80 - 116
		Qualifier	0.25		
Flusilazole	---	Quantifier	0.5	0.75	80 - 115
		Qualifier	0.5		
Epoxiconazole	50	Quantifier	n.d.**	n.d.**	80 - 119
		Qualifier	n.d.**		
Bitertanol	50	Quantifier	0.75	1	80 - 116
		Qualifier	0.5		
Propiconazole	500	Quantifier	0.5	0.75	80 - 117
		Qualifier	0.25		
Prochloraz	50	Quantifier	0.5	0.75	80 - 120
		Qualifier	0.25		

* Appendix II German Regulation for Maximum Residue Levels in Food (RHmV)

** Contamination in water and wine with a signal to noise of 8, thus LOD and LOQ could not be determined

5.1.3.7 Application to wine samples

103 randomly chosen wine samples from the following 11 different countries were analysed (in brackets the number of samples from the specific country): Germany (50), Italy (19), Spain (6), France (5), Chile (7), Czech Republic (1), Greece (2), Switzerland (1), South Africa (9), Australia (2), USA (1). The samples consisted of 49 red, 42 white and 12 sparkling wines. Among the grape varieties where e.g. Bacchus, Cabernet Sauvignon, Merlot, Riesling, Sauvignon Blanc as well as various blends. In more than 75% of the samples azoles were detected, but all concentrations were below the known MRL (Table 16 and Figure 25).

Table 16: Results of 103 wine samples tested

Azole detected	Occurrence	Range of levels determined [ng/mL]
Cyproconazole	2/103	< LOQ
Epoxiconazole	3/103	6.5 - 30
Flusilazole	9/103	0.5 - 0.75
Myclobutanil	25/103	0.5 - 35
Penconazole	26/103	0.25 - 1.2
Tebuconazole	55/103	0.25 - 33
Triadimenol	33/103	0.5 - 2.2

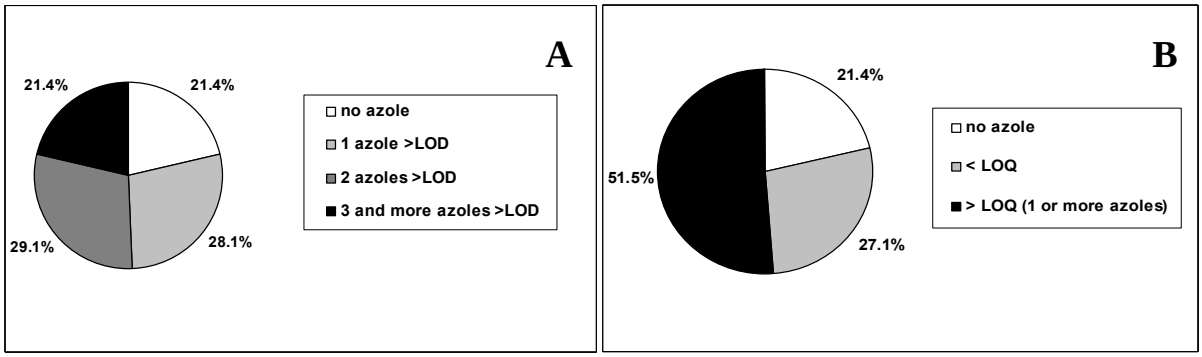


Figure 25: Pie charts showing results of residue determination in 103 wine samples

A) Number of different azole compounds found in one sample

B) Findings were sorted on the basis of the ability to quantify one compound found or not

Tebuconazole is the most commonly used fungicide and was detected in 53% of the samples. Azole fungicides residues were observed in wine samples of every country but a statistical analysis of the data is not possible since the samples for each country do not represent a valid cross section. In one wine sample produced under ecological control no azoles were detected. Interestingly, about 50% of the wine samples contained more than one respectively 21% more than two azole

fungicides. Applying more than two azole fungicides to one acre in one season is against “good agricultural practice”. In the winemaking progress, e.g. for Bordeaux blends, a variety of grapes from different vineyards, sometimes from more than one producer, are blended to one product. Thus the more than two azoles in one wine sample could be explained by the usage of different fungicides on different acres and a mixture of the grapes of different origin in the cellar. Nevertheless in the case of wine samples containing more than two azoles the levels of azoles are clearly below the MRL.

5.1.3.8 Conclusions

The method described shows good accuracy and precision and can be utilised as screening method for azoles in wine samples. With centrifugation as the only sample work-up step and a short LC-gradient as many as 100 samples can be measured in a day. Good chromatographic behaviour of the analytes on the column used, results in good peak shapes and LOQs well below the maximum residue levels.

Wines from 11 different countries were tested and at least one sample from every country tested positive for an azole fungicide. Tebuconazole was the most frequently determined azole. In 50.5% of the 103 samples 2 or more azole compounds could be detected.

6 Discussion

6.1 Analytical Procedures for Product Determination

The first inhibition studies on aromatase (CYP19) described in this manuscript utilise a fluorescent pseudo substrate, DBF. This system has the distinct advantages of a possible high throughput design, availability and cheap instrumentation. Disadvantages of the DBF system are the potential spectrometric interferences in the fluorescence detection, DBF not being specifically metabolised only by CYP19 and DBF being a pseudo substrate for CYP19. Potency ranking of compounds inhibiting CYP19 however is well possible with this setup.

This was shown in Chapter 3 Tables 4 and 6 upon comparison with data obtained with the CYP19 / testosterone assay. This assay utilizing estradiol product analysis was developed to evaluate the inhibitory potential of azole compounds on a CYP19 system with a natural substrate.

Further inhibition studies with azole compounds were conducted on human CYP51. Its inhibition, and thus the inhibition of FF-MAS production and the resulting T-MAS, is considered an unwanted side-effect for all compounds described. Incubation procedures evaluating the inhibitory potency of azoles have to be performed with one of the natural substrates. Out of the four natural substrates accepted by most lanosterol-14α-demethylases lanosterol was chosen.

Due to the very lipophilic characteristics of FF-MAS and estradiol, analytical methods based on LC-MS/MS technique with photospray ionisation were established for both CYP51 assays and for the CYP19 / testosterone assay. With this technique direct analysis of product formation is possible with protein precipitation as only sample work up step. In comparison to literature procedures utilising expensive radio labelled compounds or techniques measuring product formation indirectly or complicated and time consuming sample preparation steps, the methods presented here have the distinct advantages of quick sample preparation and fast, direct product measurement. Artefact formation is kept minimal, since no derivatisation or extraction procedures are necessary. The products of the enzymatic reactions of CYP19 and CYP51, estradiol and FF-MAS, can be quantified using standard calibration curves.

For determination of IC_{50} values, areas corrected by the area of the internal standard could be used. Drawback of the methods is the expensive equipment needed.

Pesticide residue levels determined by authorities are not generally published. To get a picture of residue levels of azoles in food in a model case an LC-ESI-MS/MS based method was developed for the determination of azole compounds in wine. Centrifugation was the only sample workup step. The limits of quantitation achieved were at least 4 times lower than the maximum residue levels for azole fungicides in wine prescribed, thus the method can be conveniently used as a screening assay for azole residues in wine samples. Of 103 wine samples tested 75% tested positive on at least one azole compound, all residues were below the maximum residue levels set by the authorities though.

The analytical methods presented here for the detection of estradiol with d_6-cholesterol as internal standard, and FF-MAS with the same internal standard can be combined into one method as subsequently described (Batchelor Thesis Fischer). This makes it possible to incubate aromatase and lanosterol-14α-demethylase in one analytical assay and analyse both products formed in one HPLC run. On the one hand this set up could mimic the physiological surroundings where more than one enzyme is present in the cell membrane. On the other hand a high throughput set up could be established and the inhibitory potency for one compound on both enzymes could be determined in one analytical and experimental set up. First double incubation experiments however showed that the activities of the CYP51 enzyme are influenced negatively by the presence of the additional enzyme. This cannot be overcome by supplementation of additional NADPH regenerating system. Which influence this decline in activity has on the inhibitory potencies and on the rank order of potencies of the different azole compounds has still to be assessed.

6.2 Enzyme Assays and IC_{50} values

One aim of this work was to assess the specificity of azole compounds for their therapeutic target enzyme fungal CYP51 compared to the human analogue. A functional enzyme complex comprising of the *Candida albicans* lanosterol-14α-demethylase and the *Candida tropicalis* oxidoreductase was expressed in the baculovirus system in sufficiently high yields. The microsomal fraction displayed constant turn-over rates and IC_{50} values showed only small standard errors. For the

following incubation procedures a similar protein background compared to the human CYP51 variant and human CYP19 could be set up.

Studies to assess the inhibitory potency of 21 azole compounds on both the human and fungal CYP51 variant were conducted.

The comparison of IC_{50} values determined for the same compounds with different enzyme sources and substrates is difficult. Even when the same assay is used in different labs a ten-fold difference in IC_{50} value for one compound can occur as seen with placental microsomes expressing aromatase and fadrozole as inhibitor [50,51]. IC_{50} values are dependent on the substrate concentration used. K_i values are independent of the substrate concentration used and are therefore more comparable with values determined by other laboratories. However K_i values bring with them the disadvantage that more samples are necessary to obtain a single value compared to a single IC_{50} value. For K_i values first a dose finding study has to be performed and a K_i value can only be derived from a second experiment, with additional samples. The first dose finding study however already brings with it an IC_{50} value. On top high-throughput design is difficult to set up.

As discussed in Chapter 3.2 for the two CYP19 assays, IC_{50} values can be influenced not only by the use of different substrate concentrations but by a variety of parameters e.g. substrate chosen, protein concentration and incubation time.

Although DBF is a pseudo substrate for aromatase, it gives a good estimate of the inhibitory potency of the substance tested. The values obtained are a mean factor of 58 lower than those obtained with the testosterone assay and the natural substrate, but ranking of inhibitors on the basis of DBF data is well possible. To eliminate the uncertainty factor of a pseudo substrate the other three assays were based on one of the natural substrates of the enzyme.

Another possibility to explain differences in IC_{50} values determined in different assays could be different protein concentrations used in the incubations. Using microsomal proteins from human livers, animal livers and Sf9 cells Obach et al. showed that protein concentrations substantially higher than 0.1 mg/mL where required to bind fractions of the test compound [60]. Protein concentrations in the utilised assays were 0.11 mg/mL for the CYP19 / testosterone assay, 0.022 mg/mL for the CYP19 / DBF assay, 0.3 mg/mL for the hCYP51 assay and can therefore be neglected as putative confounder. For the cCYP51 assay however, the protein concentration was 10 mg/mL, and thus rather high. If azole compounds bound

unspecifically to the microsomal protein, more substance would be needed to inhibit the enzyme, thus leading to higher IC_{50} values. The IC_{50} values determined with the assay were rather low and consistent with literature values. Therefore an influence of the protein concentration on the IC_{50} values is regarded negligible.

Incubation time is another confounding factor for the determination of IC_{50} values. For the three LC-MS/MS based assays the incubation time is 40 minutes. The CYP19 / DBF assay utilises an incubation time of 30 minutes only. For ketoconazole a time dependent up to 4-fold increase in IC_{50} values for CYP3A4 supersomes derived from insect cells and human liver microsomes has been reported [61]. The increase with time was consistent with the metabolic depletion of the inhibitor. Ketoconazole is a substrate for human liver microsomes and CYP3A4. If metabolites are less inhibitory than the parent compound IC_{50} values increase with incubation time, if metabolites are inhibitorier than the parent compound however a decrease in IC_{50} values with incubation time occurs.

Metabolism by aromatase has been reported for methadone, levo-alpha-acetylmethadol, 7-ethoxycoumarine, aflatoxin B1 and buprenorphine [48,62-65]. For azoles no such metabolic pathways have been reported and in the articles cited above azoles have been used as probes to inhibit the monitored reactions.

Up to know a very strict substrate specificity is postulated for CYP51 [117] and literature gives no evidences that CYP51 metabolises other substrates than lanosterol or respective analoges. Thus an effect on the inhibitor concentration through metabolic depletion and on IC_{50} values is thought to be of minor importance.

6.3 Overall Assessment of Azole Compounds

Table 17 gives an overview of all IC_{50} values determined with the four different assays for 21 azole compounds. Calculations of Spearman rank correlations among the IC_{50} values showed that only the IC_{50} values determined for CYP19 with DBF and testosterone correlate with each other. Further no correlation exists between any of the sets of IC_{50} values and the respective octanol water partition coefficient values of the azole compounds. Thus lipophilic properties of the compounds do not seem to be the dominating factor influencing inhibition of various enzymes.

Table 17: Summary of IC_{50} values determined with the four enzyme systems: human CYP19 (aromatase) with Dibenzylfluorescein (DBF) or testosterone as substrate and human CYP51 (lanosterol-14α-demethylase) and*Candida albicans* CYP51 (lanosterol-14α-demethylase) both with lanosterol as substrate, log p values of the respective compounds and overall evaluation of the inhibitory potencies.

Compound	log p values	CYP19 / DBF	CYP19 / Testosterone	hCYP51	cCYP51	overall assessment		
Fungicides						**CYP19**	**hCYP51**	**cCYP51**
Bitertanol	3.675	>20	>>300	1.30	0.059	++	o	++
Cyproconazole	2.538	8.5	≈ 100	22.8[a]	0.10	++	+	+
Epoxiconazole	2.873	1.44	≈ 100	1.95	0.22	++	o	+
Flusilazole	3.835	0.055	7.7	3.36	0.085	o	o	++
Hexaconazole	3.494	9.9*	96	15.6[a]	0.066	+	+	++
Imazalil (I)	4.062	0.072	3.6	36.1	0.082	o	+	++
Myclobutanil	3.301	0.47	47	29.0[a]	0.14	+	+	+
Penconazole	3.942	0.85	47	19.3	0.076	+	+	++
Prochloraz (I)	4.01	0.047	0.44	5.00	0.098	-	o	++
Propiconazole	3.207	3.2	199	8.25	0.15	++	o	+
Tebuconazole	3.192	5.8	609	3.61	0.35	++	o	+
Triadimefon	2.6	17.5	483	9.95	0.13	++	o	+
Triadimenol	2.704	12.6	972	37.2[a]	0.33	++	+	+
Antifungal drugs								
Bifonazole (I)	5.054	0.019	0.24	0.80	0.3	-	-	+
Clotrimazole (I)	5.761	0.11	1.2	0.85	0.091	o	-	++
Fluconazole	0.306	>140	>>300	>30 (23 % inhibition)[b]	0.051	++	++	++
Itraconazole	3.291	>70	≈ 100	≈ 30 (53 % inhibition)[b]	0.039	++	+	++
Ketoconazole (I)	2.879	5.6	281	0.43	0.064	++	-	++
Miconazole (I)	6.417	0.064	8.2	0.057	0.072	o	--	++
Aromatase inhibitors								
Fadrozole (I)	1.875	0.0076	0.66	≈ 100 (54 % inhibition)[b]	32.2	++	++	+
Letrozole	1.516	0.015	0.13	>100 (11 % inhibition)[b]	13.3	++	++	+

IC_{50} values are classified into 5 categories: ++ (very favourable), + (favourable), o (neutral), -(unfavourable), -- (very unfavourable)

(I) Imidazole compound

[a] One theoretical high-dose/zero-product point added

[b] No full sigmoid curve available due to limited solubility

[c] No full data set (solutio ad infusionem; chemical instability

The last three columns give an overview of the evaluation of the inhibitory potencies of the azole compounds towards the three substrate/enzyme systems utilising the natural substrates. Preferably the anti fungal compounds should not inhibit human CYP19 or human CYP51, but show strong inhibitory potency against fungal CYP51. The cytostatic compounds should strongly inhibit aromatase, but should not show any activity against the two lanosterol-14α-demethylases. The IC_{50} values are classified into 5 categories: ++ (very favourable), + (favourable), o (neutral), -(unfavourable), -- (very unfavourable).

Nine agents show desirable patterns of inhibition on the three enzymes, indicated by 3 ++ or 3 + ratings. These include the two cytostatic drugs, fadrozole and letrozole and fluconazole and itraconazole, the two antifungal agents used in human medicine, both being comparably new compounds on the market. Further the fungicides cyproconazole, hexaconazole, myclobutanil, penconazole and triadimenol show such desirable inhibition patterns.

Fadrozole and letrozole proved to be very strong inhibitors of their therapeutic target enzyme and only had a very small effect on the human variant of CYP51. They had a moderate effect on fungal CYP51 thus meeting all requirements for cytostatic aromatase inhibitors.

Fluconazole and itraconazole both have little to no effect on aromatase and human CYP51, but are among the most potent inhibitors of fungal CYP51. This profile is desirable for antifungal agents used in medicine, especially since these compounds are administered in high doses, orally and in the case of fluconazole even intravenously.

The agricultural fungicides mentioned above all show a little effect on aromatase and/or human CYP51, but strongly inhibit the fungal CYP51 which is favourable.

Five agents show undesirable patterns of inhibition, with either inhibition of human CYP19 or inhibition of human CYP51 or both. These include prochloraz, bifonazole, clotrimazole, ketoconazole and miconazole.

Bifonazole and prochloraz strongly inhibit aromatase, the potencies reaching those of the compounds designed for this purpose. Clotrimazole and miconazole moderately inhibit aromatase and only ketoconazole does not show inhibitory potency towards CYP19. The agents used in human medicine strongly inhibit human CYP51; the agriculturally used prochloraz inhibits human CYP51 only moderately.

The seven other compounds tested do not give such a clear picture. Imazalil shows an inhibitory effect on aromatase but inhibits human CYP51 only to a small extent. Imazalil is a strong inhibitor of fungal CYP51. Bitertanol, epoxiconazole, propiconazole, tebuconazole and triadimefon all show no effect on aromatase and inhibit human CYP51 only to a moderate extend. Bitertanol, epoxiconazole are slightly stronger inhibitors of a fungal enzyme than the other three compounds. Finally flusilazole shows moderate effects on both human CYP19 and human CYP51 and strongly inhibits fungal CYP51.

The conclusion can be drawn that the majority of the compounds, fourteen, can be directly assigned to a group with either favourable profiles or rather unfavourable profiles. Seven compounds show a more complex picture of inhibitory potencies. Up to now the roles of FF-MAS and the resulting T-MAS in the endocrine system and steroidogenesis are not completely clear, thus a weighting of the effects on human CYP19 and human CYP51, which is more severe, cannot yet be conducted.

6.4 Enzyme Inhibition and Exposure

All quantitative considerations made below have model character. Plasma concentrations cannot be equated with concentrations near the enzyme in the target cells. Pharmacokinetic considerations such as bioavailability and duration of exposure must also be considered case by case.

6.5 Inhibitory Potencies vs. Exposure to Fungicides

From an agricultural exposure study for epoxiconazole an exposure dose of ≤100 mg (0.3 µmol) per person and day was estimated [24]. From this a maximum plasma level of 0.004 µM was derived (please see Chapter 3.2) which is by a factor of 480 below the IC_{50} value of epoxiconazole and thus can be regarded as safe. Unfortunately exposure data of other fungicides are not published.

6.6 Inhibitory Potencies vs. Exposure to Antifungal Drugs

Maximum serum concentrations of pharmaceutical antifungal compounds reported in literature were given in Chapters 1.2 and 3.2. In short (in brackets the application route): bifonazole 0.051 µM (dermal) [38], clotrimazole 0.029 µM (dermal)

(German Summary of Product Characteristics), itraconazole 2.8 µM (oral) (German Summary of Product Characteristics), ketoconazole 6.6 µM (oral) [38], miconazole 2.4 µM (oral) and 0.024 µM (vaginal), [38] letrozole 0.129 µM (oral) (German Summary of Product Characteristics).

In Chapter 3.2 these are compared to the IC_{50} values determined for CYP19 and testosterone as substrate. For letrozole the maximum plasma concentration corresponds to the IC_{50} value determined, which is favorable since CYP19 is the therapeutic target of azoles used as cytostatic drugs. The antifungal drugs bifonazole and miconazole were identified as critical since their maximum plasma concentrations are within a factor of five of the IC_{50} values and thus quite close to a concentration producing 50 percent enzyme inhibition. For clotrimazole, itraconazole, ketoconazole and miconazole (vaginal dosing) a factor of at least 35 was estimated for the difference between the IC_{50} values and maximum plasma concentrations.

Ratios between the maximum plasma concentrations and the IC_{50} values for human CYP51 were calculated on the basis of the values cited above and the values given in Table 17 to be 0.064, 0.034, 0.093, 15, 42 and 0.42 for bifonazole, clotrimazole, itraconazole, ketoconazole, miconazole (oral) and miconazole (vaginal), respectively. For the oral antifungal drug ketoconazole the maximum plasma concentration is a factor of 15 above the determined IC_{50} value, for the oral use of miconazole the factor is even larger. Even for the topical use of miconazole the maximum plasma concentration is within a factor of 3 of the IC_{50} value and thus quite close. Problems could arise especially from the usage of miconazole gels for infections of mucous membrane of the mouth of children, where parts of the dose could be ingested and become available systemically. For the orally applied ketoconazole and vaginal applications of miconazole treatment during pregnancy is contraindicated (German Summaries of Product Characteristics).

It is postulated that FF-MAS plays an important role in the control of oocyte meiosis in mammals. The meiotic division of female germ cells is initiated prior to ovulation and is terminated by the insemination. With the addition of FF-MAS to culture media effects like a resumption of meiosis and an increased rate of fertilisation could be shown [17]. Inhibition of human CYP51 resulting in a lack of FF-MAS might lead to a disturbed fertilisation process. A use of the above mentioned antifungal agents in women wishing to become pregnant has to be reconsidered. With view to a likely role of T-MAS in the testis and on meiosis during sperm

production, the use of these compounds in men could also lead to a disturbed fertility and thus to a disturbed fertilisation process.

Usage of bifonazole, clotrimazole and itraconazole is considered unproblematic with view of the inhibition of human CYP51 due to the larger factors between maximum plasma concentrations and the IC_{50} values determined.

6.7 Inhibitory Potencies vs. Consumer Exposure

Exposure to fungicides from residues in food items can show large margins of safety. In Chapter 5 residue levels of azole fungicides in wine were determined and tebuconazole showed the highest levels of 30 ng/mL [66]. Consumption of 500 mL wine would thus result in a dose of 15 µg, equivalent to 0.048 µmol. As described in Chapter 3.2 a plasma level of 0.0005 µM results, which is by factor of 7 000 below the IC_{50} value of tebuconazole. If the apparent volume of distribution is higher than 1 L/kg body weight and the compound does not accumulate at the enzymes, the safety factors are even larger.

The German Federal Institute for Risk Assessment (BfR) conducting residue analysis of pesticides in food follows the policy that the assessment results will, in principle, be made publicly accessible whilst maintaining the confidentiality of protected data. Thus residues below the limit values set by authorities are not generally made public. In an attempt to assess the risk of residues on foods for the consumer the following scenario was developed: as an exposure dose the maximum value for the acceptable daily intake of a compound was used.

Assuming 100% bioavailability, a body weight of 70 kg and an apparent volume of distribution of 1 liter per kg body weight, maximum plasma levels were derived. Volumes of distribution of azole compounds vary from 0.6 (fluconazole) to 20 L per kg body weight (miconazole) [3]. The value chosen ranges among the lower values reported, thus if the apparent volume of distribution is higher than 1 L/kg body weight and accumulation at the enzyme can be excluded, the safety factors are even larger. To determine safety factors these maximum plasma levels were divided by the respective IC_{50} values determined for human CYP19 and human CYP51. The results for compounds with low IC_{50} values for one of the two enzymes are given in Table 18.

Table 18: Summary of the maximum values for the acceptable daily intake set by authorities, the derived maximum plasma concentrations (Plasma Concentration) and the safety factors derived for human CYP19 (aromatase) and human CYP51 (lanosterol-14α-demethylase).

Compound	max. ADI [mg/kg bw]	Plasma Conc. [µM]	Safety Factor hCYP19	Safety Factor hCYP51
Bitertanol	0.01	0.03	0.0001	0.02
Flusilazole	0.001	0.003	0.0004	0.0009
Imazalil	0.03	0.1	0.03	0.003
Prochloraz	0.01	0.03	0.07	0.006
Propiconazole	0.04	0.12	0.0006	0.01
Triadimefon	0.03	0.1	0.0002	0.01

The results show that the smallest safety factors are derived for imazalil and prochloraz on human CYP19 and bitertanol, propiconazole and triadimefon on human CYP51. However even the lowest safety factor is a factor of 14 below the respective IC_{50} value. Thus even when the highest acceptable daily dose of a compound is taken in, the IC_{50} values are not reached under the assumed “worst case scenario”.

6.8 Conclusions

Based on the findings presented, the following conclusions can be drawn.

For agricultural workers the risk set by exposure to azole fungicides with respect to human CYP51 and CYP19 can be regarded as negligible when safety measures are adhered to.

With view to the pharmaceutical drugs, as a matter of principle, the usage of bifonazole, miconazole and ketoconazole has to be viewed with caution in respect to the high level of inhibition of human CYP51 and/or CYP19.

On consumer safety authorities follow the principle that residue levels should be kept as low as possible, not more than necessary for the given task and never higher than hygienically justifiable. Under the precaution that the acceptable daily intake amounts set by authorities for azole compounds are not exceeded, the residues do not pose a threat to consumer safety regarding the mentioned enzymes.

Inhibition of CYP19 with the consequence of a reduction of estradiol levels has to be regarded as a possible disrupting effect of the hormone balance. The relevance of FF-MAS and T-MAS in the endocrine system however still has to be evaluated completely bringing with it the question of how much importance has to be attached to the inhibition of human CYP51.

7 Summary

Azoles are important chemicals used as antifungal agents in agriculture and human medicine, but also as cytostatic drugs in tumour chemotherapy.

Antifungal activities are based on inhibition of lanosterol-14α-demethylase (CYP51), a phylogenetically widely distributed and highly conserved cytochrome P450 monooxygenase. In mammals and yeasts CYP51 catalyses the three-step oxidative removal of the methyl group # 32 of lanosterol to produce follicular fluid meiosis activating steroid (FF-MAS), an important step in sterol biosynthesis. For fungi the later resulting ergosterol is an essential compound of the cell membrane. Exposed fungi lack ergosterol, which leads to a collapse of the cell membrane.

In animals and humans cholesterol, the downstream product of lanosterol-14α-demethylation necessary for the synthesis of bile acids, mineral corticoids, glucocorticoids and sex steroids, can be supplemented with food intake. However FF-MAS and the resulting T-MAS (testis meiosis activating steroids), the direct products of the CYP51 reaction, act as meiosis-activating steroids on ovaries and testes and are not supplemented with food intake. Inhibition of CYP51 in humans may therefore affect the endocrine system and is an unwanted side effect of azoles.

Aromatase (CYP19) catalyses, among other reactions, the demethylation of testosterone to estradiol. CYP19 is another cytochrome P450 monooxygenase inhibited by azoles. Reduction of estrogen levels by CYP19 inhibition is the working principle of cytostatic drugs used in breast cancer therapy but is considered an unwanted side effect for azoles used to treat fungal infections.

A favourable fungicide or antifungal drug should be a strong inhibitor of fungal CYP51. In contrast human CYP51 and human CYP19 should not be inhibited by an azole fungicide or antifungal agent. The favourable cytostatic drug should show a high potency towards human CYP19. Neither human CYP51 nor fungal CYP51 should be inhibited by a cytostatic drug. The aim of this work was to assess: are fungicides and antifungal drugs strong inhibitors of fungal CYP51? In return do they not inhibit human CYP51 and human CYP19? Do cytostatic drugs strongly inhibit human CYP19? And in return do they not inhibit human CYP51 or fungal CYP51?

In a first step inhibitory potencies of 22 azole compounds used for the three purposes were tested in an inhibition assay on commercially available human CYP19 utilising a fluorescent pseudo substrate dibenzylfluorescein (DBF). IC_{50} values of 13

fungicides used in agriculture ranged more than 750-fold from 0.047 to 35 µM. The potency of seven antifungals used as human drugs spanned more than 7 000-fold, from 0.019 to >138 µM.

The two tumour therapeutic agents were the most potent inhibitors, with IC_{50} values of 0.0076 and 0.015 µM. The data indicate that the top-ranking azoles used as antifungal agents or antifungal drugs are almost as potent inhibitors of aromatase *in vitro* as the drugs used in tumour therapy.

Drawback of this first assay was the use of a fluorescent pseudo substrate instead of the natural substrate testosterone. Therefore a test system utilising testosterone was developed and estradiol product formation was measured by a fully validated analytical method based on liquid chromatography – tandem mass spectrometry utilising photospray ionisation (APPI). All 22 azole compounds were tested on this new system.

Again the two cytostatic drugs were the most potent inhibitors. However, azoles used as fungicides, e.g. prochloraz, or as antifungal drugs, e.g. bifonazole, were almost as potent inhibitors of aromatase as the drugs used in tumour therapy. Comparison of plasma concentrations that may be reached in antifungal therapy do not allow for large safety factors for e.g. bifonazole and miconazole. The IC_{50} values were compared to the data obtained with the fluorescent pseudo substrate. A high correlation was found, indicating that the fluorescence assay with DBF can well be used for potency ranking and screening of chemicals for aromatase inhibition.

The specificity of azole compounds for their therapeutic target enzyme fungal CYP51 compared to the human analogue was to be evaluated. A functional human CYP51 was available from BD Gentest Cooperation. A functional enzyme complex comprising of the *Candida albicans* lanosterol-14α-demethylase and the *Candida tropicalis* oxidoreductase was expressed in the baculovirus system in sufficiently high yields. Thus for the following incubation procedures a similar protein background compared to the human CYP51 variant and human CYP19 could be set up.

Incubation procedures based on the natural substrate lanosterol were developed and product formation was measured by liquid chromatography -APPI- tandem mass spectrometry. Inhibitory potencies of 21 azole compounds on both the human and fungal CYP51 variant were thereby determined.

The specificity for the therapeutic target enzyme compared to the human enzyme varied from little to high. Little specificity holds true for agriculturally used fungicides, e.g. epoxiconazole and tebuconazole and azole compounds used in human medicine, e.g. bifonazole, clotrimazole, ketoconazole and miconazole. Compounds showing high specificity for their therapeutic target enzyme includes agricultural fungicides such as imazalil or penconazole and compounds used in human medicine such as fluconazole and itraconazole.

It should be noted however, that for the interpretation of these ratios the levels of the original IC_{50} values have to be taken into account. This is seen for bifonazole and fadrozole, since both have a ratio of 3, but IC_{50} values determined for bifonazole were much lower.

Preferably the antifungal compounds should not inhibit human CYP19 or human CYP51, but show strong inhibitory potency against fungal CYP51. The cytostatic compounds should strongly inhibit aromatase, but should not show any activity against the two lanosterol-14α-demethylases. When comparing inhibitory potencies on CYP19, human CYP51 and *Candida albicans* CYP51 a number of agents show desirable patterns of inhibition on the three enzymes. These include e.g. the two cytostatic drugs, the two antifungal agents used in human medicine, fluconazole and itraconazole, and a wide variety of the fungicides, e.g. cyproconazole and hexaconazole. Undesirable patterns of inhibition were exhibited by a number of compounds, e.g. prochloraz, bifonazole, ketoconazole and miconazole. Seven compounds show a more complex picture of inhibitory potencies though.

Up to now the role of FF-MAS and T-MAS in the endocrine system and steroidogenesis is not completely clear, thus a weighting of the effects on human CYP19 and human CYP51, which is more severe, cannot yet be conducted.

To get a picture of residue levels of azoles in food in a model case an LC-ESI-MS/MS based method was developed for the determination of azole compounds in wine. All residues found were below the maximum residue levels set by authorities.

To classify the inhibitory potencies on the different enzyme systems IC_{50} values obtained were compared to exposure levels measured in farmers, maximum plasma concentrations in humans reported after exposure to antifungal drugs and to acceptable daily intake levels set by authorities.

Based on the findings presented, the following conclusions can be drawn. The risk for agricultural workers set by exposure to azole fungicides with respect to human CYP51 and CYP19 can be regarded as negligible when safety measures are adhered to. As a matter of principle however, the usage of bifonazole, miconazole and ketoconazole has to be viewed with caution in respect to the high level of inhibition of human CYP51 and/or CYP19.

On consumer safety authorities follow the principle that residue levels should be kept as low as possible, not more than necessary for the given task and never higher than hygienically justifiable. Under the assumption that the acceptable daily intake amounts set by authorities for azole compounds are not exceeded the residues do not pose a threat to consumer safety judged by our findings.

Inhibition of CYP19 with the consequence of a reduction of estradiol levels has to be regarded as a possible disrupting effect of the hormone balance. The relevance of FF-MAS and T-MAS in the endocrine system however still has to be evaluated completely bringing with it the question of how much importance has to be attached to the inhibition of human CYP51.

8 Zusammenfassung

Die Azole stellen eine wichtige Gruppe von Chemikalien dar, die als Fungizide sowohl in der Landwirtschaft also auch in der Medizin eingesetzt werden. Ein weiteres Einsatzgebiet in der Humanmedizin ist die Tumortherapie.

Die fungizide Wirkung beruht auf der Hemmung des Enzyms Lanosterol-14α-Demethylase (CYP51), einem phylogenetisch weit verbreiteten und hochkonserviertem Cytochrom P450 Isoenzym. In Säugetieren und Hefen katalysiert CYP51 die drei-stufige oxidative Demethylierung der Methylgruppe am C14 des Lanosterols zum „Follicular Fluid Meiosis Activating Steroid (FF-MAS)“, einen wichtigen Schritt der Steroidbiosynthese. Für Pilze ist das später resultierende Ergosterol ein essentieller Bestandteil der Zellmembran. Gegenüber Azolen exponierten Pilzen fehlt Ergosterol was zu einem Zusammenbruch der Zellmembran führt.

Säugetiere können Cholesterol, das später entstehende Produkt der Lanosterol-14α-Demethylierung, das zur Synthese von Gallensäuren, Mineralcorticosteroiden, Glucocorticoiden und Sexualhormonen nötig ist, mit der Nahrung aufnehmen. FF-MAS und das resultierende T-MAS (Testis Meiosis Activating Steroids), die direkten Produkte der CYP51 katalysierten Reaktion, wirken als Meiose-aktivierende Steroide auf Ovarien und Hoden und werden nicht mit der Nahrung aufgenommen. Eine Hemmung der CYP51 Aktivität könnte das endokrine System beeinflussen und wird als unerwünschte Nebenwirkung der Azole betrachtet.

Aromatase (CYP19) katalysiert unter anderem die oxidative Demethylierung von Testosteron zu Östradiol. CYP19 ist ein weiteres Cytochrom P450 Isoenzym, das durch Azole gehemmt wird. Die Verringerung der Östrogenspiegel durch Inhibition von CYP19 ist das Wirkprinzip der als Zytostatika genutzten Azole, bei den Fungiziden wird es als unerwünschte Nebenwirkung angesehen.

Ein ideales Azol, genutzt als Fungizid oder Antimykotikum, sollte Pilz-CYP51 stark inhibieren. Andererseits sollten sowohl humanes CYP19 wie auch humanes CYP51 durch ein solches Azol nicht inhibiert werden. Ein ideales Azol-Zytostatikum sollte eine starke inhibitorische Potenz gegenüber humanem CYP19 aufweisen. Hingegen sollten von diesem Azol humanes und Pilz-CYP51 nicht inhibiert werden. Ziel dieser Arbeit war es nun festzustellen: sind Fungizide und Antimykotika starke Inhibitoren von Pilz-CYP51? Zeigen Fungizide und Antimykotika keine Aktivität

gegenüber humanem CYP19 und humanem CYP51? Sind Zytostatika starke Inhibitoren von humanem CYP19? Zeigen Zytostatika keine Aktivität gegenüber humanem CYP51 und Pilz-CYP51?

In einem ersten Teil wurde die inhibitorische Potenz von 22 Azolen, die in den drei beschriebenen Gebieten eingesetzt werden, mittels eines Testsystems an humanem CYP19 und einem fluoreszierenden Pseudosubstrat getestet (Fluoreszenzassay). Die IC_{50} Werte von 13 landwirtschaftlich genutzten Fungiziden variierten um den Faktor 750 von 0.047 bis 35 µM. Die in der Humanmedizin genutzten Azole sogar um den Faktor 7 000 von 0.019 to >138 µM. Die beiden Tumortherapeutika waren die stärksten Inhibitoren mit IC_{50} Werten von 0.0076 und 0.015 µM. Die Daten zeigen aber auch, dass die stärksten Fungizide bzw. Antimykotika *in vitro* die Aromatase ähnlich stark hemmen wie die beiden Zytostatika.

Der Nachteil dieses ersten Testsystems lag in der Verwendung eines fluoreszierenden Pseudosubstrates anstelle des natürlichen Substrates Testosteron. Deswegen wurde ein System basierend auf dem natürlichen Substrat Testosteron etabliert. Die Produktbildung wurde mittels einer validierten, analytischen Methode, basierend auf Hochdruckflüssigkeitschromatographie gekoppelt mit Tandem Massenspektrometrie nach Photosprayionisation, gemessen. Alle 22 Azole wurden mit dem neuen Testsystem untersucht.

Die beiden Zytostatika waren erneut die potentesten Inhibitoren. Jedoch waren Azole, die als Fungizide oder Antimykotika eingesetzt werden, z.B. Prochloraz oder Bifonazol, gleich starke Inhibitoren der Aromatase wie die Zytostatika. Ein Vergleich mit Plasmakonzentrationen, die im Rahmen einer Fungizidtherapie erreicht werden, ergab keine großen Sicherheitsfaktoren z.B. für Bifonazol oder Miconazol. Ferner wurden die IC_{50} Werte mit Daten des Fluoreszenzassays verglichen. Hierbei wurde eine hohe Korrelation gefunden, was darauf hindeutet, dass der oben erwähnte Fluoreszenzassay für Rangbestimmungen und Screeningverfahren für Chemikalien und deren Inhibition von Aromatase genutzt werden kann.

Die Spezifität der Azole für das Pilz-CYP51 Enzym im Vergleich zu dem humanen Analogon sollte beurteilt werden. Das menschliche CYP51 wurde von „BD Gentest Cooperation“ zur Verfügung gestellt. Ein katalytisch aktiver Enzymkomplex, der sich aus der Lanosterol-14α-Demethylase von *Candida albicans* und der Oxidoreduktase von *Candida tropicalis* zusammensetzt, ließ sich im Baculovirussystem in ausreichend hoher Menge exprimieren. Im Vergleich zu

humanem CYP19 und CYP51 konnte somit für die anschließenden Inkubationsexperimente ein ähnlicher Proteinhintergrund geschaffen werden.

Inkubationsexperimente wurden mit dem natürlichen Substrat Lanosterol entwickelt und die Produktbildung wurde mittels Hochdruckflüssigkeits-chromatographie-Tandem Massenspektrometrie gemessen. Die inhibitorische Potenz von 21 verschiedenen Azolen auf menschliches und Pilz-CYP51 wurde bestimmt.

Die Spezifität für das therapeutische Zielenzym im Vergleich zu der humanen Variante reichte von gering bis hoch. Eine geringe Selektivität zeigen sowohl landwirtschaftlich genutzten Fungizide, z.B. Epoxiconazol und Tebuconazol, als auch Substanzen, die in der Humanmedizin als Antimykotika eingesetzt werden, z.B. Bifonazol, Clotrimazol, Ketoconazol und Miconazol. Substanzen die eine hohe Spezifität für das therapeutische Zielenzym aufweisen sind z.B. Imazalil und Penconazol, zwei landwirtschaftlich genutzte Fungizide, und Fluconazol und Itraconazol, zwei Antimykotika.

Angemerkt sei jedoch, dass bei der Interpretation des Verhältnisses zwischen den inhibitorischen Potenzen die absoluten IC_{50} Werte berücksichtigt werden müssen. Das zeigt sich am Beispiel von Bifonazol und Fadrozol. Für beide ergibt sich ein Verhältnis von 3 zwischen den beiden IC_{50} Werten, die Original IC_{50} Werte des Bifonazols sind aber sehr viel niedriger als die des Fadrozols.

Vorzugsweise sollten die als Fungizide bzw. Antimykotika eingesetzten Azole weder menschliches CYP19 noch menschliches CYP51 hemmen. Pilz-CYP51 sollte jedoch stark gehemmt werden. Die Zytostatika sollen die Aromatase stark inhibieren, aber keine Wirkung auf die beiden Lanosterol-14α-Demethylase Enzyme haben. Ein Vergleich der inhibitorischen Wirkstärke der Substanzen auf menschliches CYP19 und CYP51 und Pilz-CYP51 zeigt, dass einige Azole das erwünschte Bild zeigen. Dazu gehören die beiden Zytostatika, sowie Fluconazol und Itraconazol, zwei Antimykotika, die in der Humanmedizin eingesetzt werden und einige Fungizide, wie z.B. Cyproconazol und Hexaconazol. Ein unerwünschtes Bild zeigen eine Reihe von Azolen, z.B. Prochloraz, Bifonazol, Ketoconazol und Miconazol. Sieben Azole weisen ein komplexes Bild an inhibitorischen Wirkstärken auf.

Bis jetzt ist die Rolle des FF-MAS und T-MAS im endokrinen System und der Steroidbiosynthese nicht endgültig geklärt. Eine Gewichtung der Effekte auf

menschliches CYP19 und menschliches CYP51, welcher relevanter für das endokrine System ist, kann nicht abschließend bestimmt werden.

Um einen modellartigen Eindruck der Rückstände von Azolen in Lebensmitteln zu erhalten, wurde eine auf LC-ESI-MS/MS basierende Methode zur Rückstandsanalytik von Azolen im Wein entwickelt. Alle gefunden Rückstände aus 103 Weinproben aus aller Welt lagen unterhalb der von den Behörden festgelegten Rückstandshöchstmengen.

Um die inhibitorische Wirkung der Azole auf die verschiedenen Enzymsysteme in einem größeren Zusammenhang zu bringen, wurden die IC_{50} Werte mit Expositionsdaten von Bauern, maximalen Plasmaspiegeln in Patienten nach der Einnahme von Antimykotika und mit Expositionsgrenzwerten für die Langzeitaufnahme von Pflanzenschutzmittelrückständen („Acceptable Daily Intake Levels“, ADI) verglichen.

Basierend auf den dargestellten Ergebnissen können folgende Schlussfolgerungen gezogen werden. Das Risiko, dem landwirtschaftliche Arbeiter durch die Exposition gegenüber Azolfungiziden ausgesetzt sind, kann im Bezug auf menschliches CYP19 und CYP51 als vernachlässigbar eingestuft werden, wenn die entsprechenden Sicherheitsvorkehrungen getroffen werden. Im medizinischen Bereich muss grundsätzlich der Einsatz von Bifonazol, Miconazol und Ketoconazol mit Blick auf die hohe inhibitorische Potenz gegenüber menschlichem CYP19 und 51 vorsichtig betrachtet werden.

In Bezug auf die Lebensmittelsicherheit verfolgen die Behörden das Ziel, die Rückstandsmengen so niedrig wie möglich zu halten. Pflanzenschutzmittel sollten nur in den Mengen verwendet werden, die dem Zweck dienlich sind, aber niemals höher als hygienisch vertretbar. Unter der Annahme, dass die ADI Werte eingehalten werden, stellen die Rückstände auf Lebensmitteln in Bezug auf die oben genannten Enzymsysteme keine Bedrohung für den Verbraucher da.

Die Inhibition von CYP19, mit dem Ergebnis der Reduktion der Östrogenspiegel, muss als Störung des Hormonsystems angesehen werden Die Bedeutung von FF-MAS und T-MAS im endokrinen System muss noch abschließend geklärt werden und damit auch die Frage, wie viel Bedeutung der Inhibition von menschlichem CYP51 beigemessen werden muss.

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10 Appendix

In vivo studies of some azole fungicides are summarized and quoted from Zarn et al. [2] with special focus on effects putatively connected to disturbed steroidogenesis.

Bitertanol was evaluated by the JMPR in 1998 (FAO/WHO 1999). An ADI of 0-0.01 mg/kg body weight (bw) was allocated based on a NOEL of 1 mg/kg bw in a three-generation rat study and reduced pup survival rates at 5 mg/kg bw. Histopathologic changes in the adrenal glands of dogs and rats were seen at 1.2 and 81 mg/kg bw, respectively. In dogs, at 5 mg/kg bw, cataracts and reduced prostate weight with histopathologic changes were seen. In male rats, at 300 mg/kg bw, the relative testis weights were increased, and in females, the absolute ovary and adrenal weights were decreased with histopathologic changes. At the maternotoxic level of 100 mg/kg bw, rat fetuses showed effects such as cleft palate and hydrocephalus.

Cyproconazole induced cleft palate, hydrocephalus and hydronephroses in rat embryos after treatment of the dams with 20 mg/kg bw (the lowest dose tested); 50 mg/kg bw increased incidences of resorptions and dead fetuses were observed (Machera 1995).

Flusilazole was evaluated by the JMPR in 1995 (FAO/WHO 1996). An ADI of 0-0.001 mg/kg bw was allocated based on a NOEL of 0.14 mg/kg bw in a 1-year dog study. At 0.7 mg/kg bw, effects on the liver of the dogs were observed. In a 14-day rat study, the levels of testosterone and estradiol were reduced at 20 mg/kg bw. In isolated Leydig cells, the IC_{50} for testosterone production was 3.5 µM for flusilazole and 1 µM for ketoconazole. In a 2-year rat study, 31 mg/kg bw flusilazole induced Leydig cell tumours of the testis; flusilazole was fetotoxic and embryotoxic at 9 mg/kg bw in a developmental study. Toxicity to the dams was observed at 27 mg/kg bw. In in vitro teratogenicity assays with rat embryos, changes at the branchial apparatus were seen at 6.25 µM (Menegola et al. 2001).

Hexaconazole was evaluated by the JMPR in 1990 (FAO/WHO 1991). An ADI of 0-0.005 mg/kg bw was allocated based on a NOEL of 0.47 mg/kg bw in a 2-year rat study. At 4.7 mg/kg bw an increased incidence of Leydig cell tumours in testis was

observed. In a 90-day rat study, histopathologic changes were observed in the adrenal glands at 2.5 mg/kg bw and reduced testis and adrenal weights were observed at 250 mg/kg bw. In rats, hexaconazole was fetotoxic at 25 mg/kg bw. Isolated rat Leydig cells showed reduced testosterone and increased progesterone production on exposure to hexaconazole (0.1-30 µM). In a 29-day mouse study, lack of corpora lutea and smaller ovaries were observed in females and histopathologic changes in the testis and epididymis and enlarged adrenals were observed in males at 14 mg/kg bw. In a 90-day dog study, weights of ovaries and testes were reduced at 125 mg/kg bw.

Imazalil was evaluated by the JMPR in 2000 (FAO/WHO 2001). In a 1-year dog study, an ADI of 0-0.03 mg/kg bw was allocated based on a NOEL of 2.5 mg/kg bw and effects on body weight, the liver and clinical symptoms at 20 mg/kg bw. At a dose range of 5-20 mg/kg bw imazalil was fetotoxic in rats, mice and rabbits.

Myclobutanil was evaluated by the JMPR in 1992 (FAO/WHO 1993a). An ADI of 0-0.03 mg/kg bw was allocated based on a NOEL of 2.5 mg/kg bw in a 2-year rat study and many effects on the male reproductive system at 10 mg/kg bw. Reduced testis weight, testis atrophy, reduced or absent spermatid production, necrotic epididymis and atrophy of the prostate were observed with increasing doses.

Penconazole was evaluated by the JMPR in 1992 (FAO/WHO 1993b). An ADI of 0-0.03 mg/kg bw was allocated based on a NOEL of 3 mg/kg bw and reduced testis weight with atrophic changes at 17 mg/kg bw in a 1-year dog study. In long-term studies, increased prostate weights were observed in mice at 9.8 mg/kg bw and in rat testis at 202 mg/kg bw. In a two-generation rat study, relative ovary weights were increased at 125 mg/kg bw. At the maternotoxic level of 500 mg/kg bw, penconazole was also fetotoxic.

Prochloraz was evaluated by the JMPR in 2001 (FAO/WHO 2002). An ADI of 0-0.01 mg/kg bw was allocated based on a NOEL of 1.3 mg/kg bw and liver effects at 5 mg/kg bw in a 2-year rat study. In a 90-day rat study, ovary and thyroid weights were increased in females at 6 mg/kg bw. In a 90-day dog study, reduced testis and prostate weights were observed at 7 mg/kg bw. In a rat reproduction study, at 27

mg/kg bw a tendency to prolonged gestation, increased total litter losses, smaller litter sizes and increased pup mortality was observed. In trout, 25 nM (9.4 µg/L) prochloraz impaired the spermatogenesis (Le Gac et al. 2001).

Propiconazole was evaluated by the JMPR in 1987 (FAO/WHO 1988). An ADI of 0-0.04 mg/kg bw was allocated based on a NOEL of 4 mg/kg bw slight effects on the liver and hematology parameters at 20 mg/kg bw in a 2-year rat study. In a rat reproduction study, reduced testis and epididymis weights in pups were observed at 21 mg/kg bw. However, testis weights of rats were increased at 256 mg/kg bw in a short-term study.

Tebuconazole was evaluated by the JMPR in 1994 (FAO/WHO 1995). An ADI of 0-0.03 mg/kg bw was allocated based on a NOEL of 3 mg/kg bw and histopathologic changes in the adrenal glands and questionable cataracts at 4.5 mg/kg bw in a 1-year dog study. In a 90-day rat study, histopathologic changes in the adrenals were found at 36 mg/kg bw. In rat reproduction studies, reduced litter sizes and reduced survival indices were observed at 73 mg/kg bw. In a mouse teratogenicity study, increased incidences of runts and malformations such as cleft palate were observed at 30 mg/kg bw. In rats and rabbits, malformations and embryotoxicity occurred at 100 mg/kg bw.

Triadimefon was evaluated by the JMPR in 1981/1985 (FAO/WHO 1982, 1986). An ADI of 0-0.03 mg/kg bw was allocated based on a NOEL of 2.5 mg/kg bw and body weight effects and reduced hematopoesis at 25 mg/kg bw in a 2-year rat study. In a rat reproduction study, the male/female sex ratio in F2 was reduced at 77 mg/kg bw and the female fertility index was one-third of control. In a supplementing study, treated males (77 mg/kg bw) were mated with untreated females. The pregnancy rate was significantly lower, but the ratio of pregnant to inseminated females was not affected. It was concluded that triadimefon impaired the sexual behavior of the males. The testosterone level of these male rats was doubled. In in vitro teratogenicity studies with rat embryos, changes at the branchial apparatus were seen at 25 µM for both triadimefon and triadimenol (Menegola et al. 2000).

Triadimenol was evaluated by the JMPR in 1989 (FAO/WHO 1990). An ADI of 0-0.05 mg/kg bw was allocated based on a NOEL of 5 mg/kg bw in a rat two-generation study and on retarded development of pups at 20 mg/kg bw. In a mouse short-term study and a dog long-term study, changes in the cholesterol levels were seen at 170 mg/kg bw and 45 mg/kg bw, respectively and in a 2-year rat study, ovary weights were reduced at 144 mg/kg bw. In rat and rabbit teratogenicity studies, increased incidences of resorptions were observed at 120 mg/kg bw.

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Publications

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