BIOCHEMICAL MECHANISMS INVOLVED IN SELECTIVE FUNGITOXICITY OF FUNGICIDES WHICH INHIBIT STEROL 14α-DEMETHYLATION

Promotor : dr. ir. J. Dekker
Emiritus-hoogleraar in de fytopathologie

Co-promotor : dr. ir. M.A. de Waard
Universitair hoofddocent

J.C. Kapteyn

BIOCHEMICAL MECHANISMS INVOLVED IN SELECTIVE FUNGITOXICITY OF FUNGICIDES WHICH INHIBIT STEROL 14α-DEMETHYLATION

Proefschrift
ter verkrijgen van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op dinsdag 20 april 1993
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen.

CIP-gegevens Koninklijke Bibliotheek, Den Haag

ISBN 90-5485-103-1

VOORWOORD

Het onderzoek, dat heeft geleid tot dit proefschrift, is gefinancierd door Schering Agrochemicals Ltd. De publicatie van het proefschrift is mede mogelijk gemaakt door financiële bijdragen van Schering en het LEB-fonds.

Dit proefschrift had niet tot stand kunnen komen zonder de medewerking van een aantal medewerkers van de vakgroep Fytopathologie. Zondermeer de meeste dank ben ik verschuldigd aan Dr. Ir. Maarten de Waard. Maarten, ik dank je niet alleen voor het in mij gestelde vertrouwen, maar evenzeer voor de uitstekende begeleiding, je niet-aflatende betrokkenheid bij het project en je vele adviezen en bijdragen aan dit proefschrift. Ik ben Prof. Dr. Ir. J. Dekker dankbaar voor zijn bereidheid om als promoter op te treden, zijn adviezen voor dit proefschrift en het kritisch doornemen van de manuscripten.
Mijn dank gaat ook uit naar Dr. Ir. J. Guan en Christiane Stehmann voor de fijne samenwerking en het uitwisselen van ideeën in "vloeiend Nederlands" ("Wat zeg je ?"). Vermeldenswaard zijn ook de bijdragen van de studentes Eline Verschoor en Karin van Es aan het onderzoek. De isolatie van P450-enzymen uit *Ustilago maydis* en *Saccharomyces cerevisiae* was een "frustrerende" bezigheid, maar met jullie hulp is het toch gelukt!

I am very grateful to Drs. John B. Pillmoor and Richard J. Milling for their critical reading and editing of the huge amounts of interim reports, poster contributions and 'concept manuscripts'. I greatly appreciated their useful comments and the stimulatory discussions at Chesterford Park. I would also like to thank Dr. S. Wynne Ellis, not only for his outstanding performance at my wedding party, but also for his help and contineous interest in the project during his stay at Wageningen.

Ik wil mijn "mede-AIO-hokgenoten" en de andere labmedewerkers bedanken voor de gezelligheid en de getoonde belangstelling. De werksfeer was altijd prima. Hopelijk koopt mijn opvolger in "het hok" evenwel frequenter drop en zet deze regelmatig koffie!

Tenslotte wil ik mijn vrienden en familie, met name Willeke, bedanken voor hun betrokkenheid en steun gedurende de laatste 5 jaar.

STELLINGEN

1. Verschillen in toxiciteit tussen sterol 14α-demethyleringsremmers (DMIs) voor een bepaalde schimmel worden voor een gedeelte bepaald door verschillen in affiniteit van deze fungiciden voor het cytochroom P450-afhankelijke sterol 14α-demethylase van het desbetreffende organisme.

   Dit proefschrift.

2. Biochemische screeningstoetsen, waarmee structuur-activiteit-relaties van potentiële sterol biosynthese-remmers (SBIs) kunnen worden bestudeerd met als doel de remmende werking van deze middelen te optimaliseren, dienen bij voorkeur te worden uitgevoerd met cel-vrije extracten van het pathogeen, waartegen men een SBI-fungicide wil ontwikkelen.

   Dit proefschrift.

3. Spectrofotometrische studies naar de interactie van azool-fungiciden met het cytochroom P450-afhankelijke sterol 14α-demethylase zijn niet bruikbaar om de *in vitro* toxiciteit van deze middelen te evalueren, wanneer gebruik gemaakt wordt van microsomale preparaten van filamenteuze schimmels.

   Yoshida and Aoyama, In: *In vitro* and *in vivo* evaluation of antifungal agents, ed. H. Vanden Bossche, Elsevier Science Publishers BV., Amsterdam, 1986, pp. 123-134.
   Dit proefschrift

4. De algemeen gebezigde terminologie van "P450 isozymes" ter aanduiding van verschillende cytochroom P450-afhankelijke enzymen, die verschillende reacties katalyseren, is conform de wetenschappelijke definitie van het begrip "isozyme" onjuist.

   Lehninger, In: Biochemistry. Ed. Lehninger, Worth Publishers Inc., New York, 1979, pp. 244-245.

5. Chemische gewasbescherming zal de komende decennia onontbeerlijk blijven voor een doelmatige beheersing van ziekten en plagen in de land-en tuinbouw.

6. De voortgaande achteruitgang van vleermuispopulaties in Nederland is meer het gevolg van schaalvergroting en intensivering van het agrarisch landgebruik dan van de toepassing van pesticiden en is daardoor sterk regio-gebonden.

Limpens and Kapteyn. Myotis **29** (1991) 39-48.
Kapteyn. Zoogdier **3** (1993) in press.

7. De introductie van transgene ziekte-resistente gewassen in land-en tuinbouw zal niet realiseerbaar zijn, indien niet meer aandacht wordt besteed aan professionele publieksvoorlichting over de biologische veiligheid van genetisch gemodificeerde organismen.

Van Veen, In: De biologische veiligheid van genetisch gemodificeerde organismen in de natuur, een zwaartepunt in het DLO-onderzoek, Dienst Landbouwkundig Onderzoek, Wageningen, 1991.

8. Glucomannoproteïnen spelen een essentiële rol in de celwandsynthese van schimmels behorende tot de Ascomyceten en de Basidiomyceten.

Van Rinsum *et al.* Yeast **7** (1991) 717-726.

9. Korfbal zal publicitair de concurrentie met andere (zaal)sporten pas volledig aankunnen, wanneer de amusementswaarde van deze sport middels spelregelwijzigingen wordt verhoogd en het wordt toegelaten tot de Olympische spelen.

10. De door het Ministerie van Verkeer en Waterstaat gefinancierde televisiespotjes, waarin autogebruikers worden gewezen op gevaarlijk rijgedrag, is pure kapitaalvernietiging zolang bewindslieden zelf straffeloos het slechte voorbeeld blijven geven.

Stellingen behorend bij het proefschrift:

Biochemical mechanisms involved in selective fungitoxicity of fungicides which inhibit sterol 14α-demethylation.

Wageningen, 20 april 1993 Hans Kapteyn

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1. General aspects of fungicides

Plant pathogens cause considerable crop losses which are cautiously estimated at about 10 percent world-wide.[1] Several practices of modern agriculture have enhanced the destructive potential of diseases and made disease management with use of fungicides inevitable. Two of the most important practices are the use of genetically similar crop plants in continuous monoculture and the use of plants susceptible to pathogens. In addition, the increasing human population, especially in developing countries, also necessitates the use of fungicides in effective disease management.[1,2]

While in 1900 only sulphur and copper fungicides were applied in agriculture, nowadays approximately 100 different antifungal compounds are available, which differ markedly in their fungitoxic spectrum, field performance and biochemical mode of actions.[3]

With respect to their mode of action fungicides can be divided in two classes: compounds with non-specific and specific mode of actions. The non-specific fungicides are characterized as multi-site inhibitors. Their selective action between fungi and plants is based on differences in uptake by plants and fungi. Most of these fungicides are protective, lack eradicative or curative activity and therefore, have to be applied repeatedly to remain effective. Development of resistance to these multi-site inhibitors based on changes at the sites of action is generally of low probability in practice, since a fungus cannot escape from all inhibitory effects without loosing its fitness.[3,4] However, resistance to these inhibitors may occur due to detoxification or reduced accumulation.[4] Sulphur, copper, mercurials, dithiocarbamates (maneb, zineb, thiram), phthalimides (captan, folpet) and quinoxaline derivatives are well known examples of non-specific inhibitors.[3,5]

Compounds with a specific mode of action interfere with only one or very few sites of fungal metabolism. Other characteristics of this group are that most of these chemicals are systemic and have protective and curative activity. The specific mode of action and the selective fungitoxicity make those chemicals often suitable for application in modern integrated plant protection strategies with use of biological control measures, soil and hygiene techniques and cultivation of resistant plant cultivars. The disadvantage of specific site inhibitors is the relatively high risk of resistance development.[3,4,6] However, the speed of resistance development is different among the single-site inhibitors and depends on the fungal genetic control mechanisms. Fungal resistance to some single-site inhibitors such as benzimidazoles, phenylamides and carboxamides developed rapidly in

one step as a result of mutation in one gene, which has a major effect on the affinity of the target site for the fungicide. A more gradual, stepwise development of resistance is characteristic for the sterol 14α-demethylation inhibitors (DMIs). This feature of DMI fungicides may be explained by the fact that resistance to DMIs is under polygenetic control which implies that several mutant genes are required to achieve a resistance level which may lead to failure of disease control.[7]

1.2. Aimes and outlines of the present study

Relatively little is known about the mechanisms of natural insensitivity and acquired (field or laboratory) resistance of fungi to DMI fungicides. Therefore, the present study was undertaken to get a better fundamental understanding of the physiological/biochemical parameters which are involved in the selective fungitoxicity of DMIs. In most of these studies prochloraz was used as the test fungicide, since this compound possesses a different spectrum of activity from other DMIs, controlling *Pseudocercosporella herpotrichoides*, *Alternaria spp.*, *Septoria spp.*, *Sclerotinia spp.*, *Monilinia spp.* and *Botrytis spp.* as well as cereal powdery mildews.[8,9] Extension of knowledge on the basis of DMI-selectivity may eventually guide synthesis of entirely new fungicides or lead to improvement of the existing DMIs. In addition, it may help to design anti-resistance strategies and increase our knowledge on fungal biochemistry and physiology, which is still relatively limited. Before presenting the results obtained in this study a literature review on the mode of action and on the mechanisms of selective fungitoxicity of modern single-site inhibitors is given (Chapter 2). In this chapter ample attention is paid to sterol biosynthesis in fungi, its inhibition by DMI fungicides and the interaction of DMIs with their target site, cytochrome-P450 dependent sterol 14α-demethylase. It is assumed that the molecular mechanisms of natural insensitivity of fungi to DMIs may be closely related to those involved in acquired resistance such as differential accumulation, metabolism and affinity for the target enzyme.[10] Therefore, at first, differential accumulation and metabolism of DMIs by various fungal pathogens, including the laboratory-generated DMI-resistant isolates of *Penicillium italicum*, were studied (Chapter 3). Chapter 4 describes the energy-dependent DMI-efflux system, a proposed resistance mechanism in *P. italicum*, in more detail. Special attention was paid to the role of the membrane potential in mediating this efflux system. A major part of the study was spent on the isolation of microsomal P450 isozymes from *Ustilago maydis* and the spectrophotomtric analysis of their interaction with several DMIs (Chapter 5). The isolation of microsomal P450 isozymes from fungal plant pathogens has only recently been described for the first time.[11] Hence, further information on P450 isozymes from plant pathogens was highly desirable. Guan *et al.*[12] also developed a method to prepare cell-free extracts from *P. italicum*, in which activity of the target enzyme, $P450_{14DM}$, could be demonstrated. These developments made it possible to study the affinity of various compounds, including

prochloraz and its analogues, for $P450_{14DM}$ from this fungus according to enzymatic and spectrophotometric methods (Chapter 6). Chapter 7 covers the development of a cell-free sterol 14α-demethylase system from the economically important fungal pathogen *Botrytis cinerea*. The success of this assay opened the opportunity to investigate the affinity of prochloraz and its analogues for *B. cinerea* $P450_{14DM}$ and to undertake some structure-activity relationship studies (Chapter 8). Chapter 9 gives a final discussion of the results obtained.

References

1. Schwinn, F.J., Significance of fungal pathogens in crop protection. *Pestic. Outlook* 1 (1992) 18-25.
2. Fry, W.E., Principles of Plant Disease Management. Academic Press, Inc., London, UK., 1982, 378 pp.
3. Buchenauer, H., Physiological Reactions in the Inhibition of Plant Pathogenic Fungi. *In*: Haug, G. & Hoffmann, H. (Eds.), Chemistry of Plant Protection Vol. 6, Controlled Release, Biochemical Effects of Pesticides, Inhibition of Plant Pathogenic Fungi. Springer-Verlag, Berlin, Germany, 1990, p. 217-292.
4. Dekker, J., The development of resistance to fungicides. *Progr. Pest. Biochem. Toxicol.*. 4 (1985) 165-218.
5. Hassall, K.A., The Biochemistry and Uses of Pesticides, Structure, Metabolism, Mode of Action and Uses in Crop Protection. VCH Verlag, Weinheim, Germany, 1990, 536 pp.
6. Dekker, J., Development of resistance to modern fungicides and strategies for its avoidance. *In*: Lyr, H.(Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 39-52.
7. Georgopoulos, S.P., The genetics of fungicide resistance. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 53-62.
8. Birchmore, R.J., Brookes, R.F., Copping, L.G. & Wells, W.H., BTS 40542 - A new broad spectrum fungicide. *Proc. Br. Crop Protect. Conf. Pest. Dis.* 2 (1977) 593-598.
9. Copping, L.G., Birchmore, R.J., Wright, K. & Godson, D.H., Structure-activity relationships in a group of imidazole-1-carboxamides. *Pestic. Sci.* 15 (1984) 280-284.
10. Lyr, H., Selectivity in modern fungicides and its basis. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 31-38.
11. Guan, J., Braks, H.M.J., Kerkenaar, A. & De Waard, M.A., Interaction of microsomal cytochrome P450 isozymes isolated from *Penicillium italicum* with DMI fungicides. *Pestic. Biochem. Physiol.* 42 (1992) 24-34.
12. Guan, J., Stehmann, C., Ellis, S.W., Kerkenaar, A. & De Waard, M.A., Ergosterol biosynthesis in a cell-free preparation of *Penicillium italicum* and its sensitivity to DMI fungicides. *Pestic. Biochem. Physiol.* 42: 262-270.

CHAPTER 2

SELECTIVE FUNGICIDES WITH A SPECIFIC MODE OF ACTION.

2.1. Review of fungicides with a specific mode of action.

Benzimidazoles and benzimidazole generating compounds. The benzimidazoles (benomyl) and benzimidazole-generating compounds (thiophanate-methyl) were introduced in the late 1960's. They are effective inhibitors of a broad range of fungi consisting of Ascomycetes, Basidiomycetes and Deuteromycetes, but none of the Oomycetes.[1] Their primary mode of action is inhibition of microtubule assembly due to their interaction with the protein ß-tubulin, an important constituent of microtubules. Therefore, mitosis, cell-division and other microtubule-dependent processes are impaired.[2] The biochemical basis for the selective fungitoxic action of benzimidazoles is due to their differential affinity for the tubulin from the different fungal species. Acquired fungal resistance to benzimidazoles is caused by a single gene mutation resulting in slightly changed tubulin with reduced affinity for these compounds.[2]

2-Aminopyrimidines. 2-Aminopyrimidines (ethirimol) are characterized by a selective and systemic activity against powdery mildews. Their mode of action is inhibition of adenosine deaminase catalysing the deamination of adenosine. As a consequence, fungal purine metabolism is disturbed leading to prevention of appressoria and haustoria formation.[3] Adenosine deaminase is present in many fungi, but only the enzyme from powdery mildews is sensitive. This may account for the high selective toxicity towards powdery mildews.[3] The mechanism of acquired field resistance in powdery mildews is not known.

Phenylamides. The group of phenylamides (metalaxyl, furaxyl) consists of highly active, systemic fungicides against fungi of the order of *Peronosporales*. They inhibit r-RNA synthesis of Oomycetes by specific interference with a chromatin bound RNA-polymerase complex.[4] Fungal resistance to phenylamides is based on reduced affinity of the target enzyme complex for these fungicides due to a single gene mutation.[4] It is not known why phenylamides act selectively against Oomycetes.

Carboxamides. The carboxamides (carboxin) represent systemic fungicides controlling mainly Basidiomycetes, like smuts, bunts and rusts.[5] They specifically interfere with the succinate-ubiquinone oxidoreductase system of the fungal mitochondrial electron transfer chain.[6] The selectivity of carboxamides between different fungal species is primarily due to differences in sensitivity of the target site for the chemicals. Acquired carboximide

resistance was attributed to a single gene mutation resulting in an altered structure of the succinate-ubiquinone oxido-reductase complex.[7,8]

Aromatic hydrocarbon fungicides (AHFs). AHFs like quintozene and chloroneb are an heterogenous group of compounds which are highly active against some soil-borne fungi and used against postharvest diseases.[9] Their primary toxic effect is probably an induced lipid peroxidation of membranes caused by inhibition of electron transport from flavin enzymes such as cytochrome c-reductase to substrates. The basis of the selective fungitoxicity has not yet been elucidated.[10]

Dicarboximides. Iprodionine and vinclozolin are well known examples of this type of fungicides which are mainly used against *B. cinerea*, *Monilinia* and *Sclerotinia* spp.[11] Dicarboximides are supposed to have a similar mode of action as the AHFs. Like for the AHFs, the reason for the selectivity of dicarboximides is not yet clear.[10] Strains of several pathogens, for example *B. cinerea*, which are cross-resistant to dicarboximides and AHFs, can easily be isolated in the laboratory, but have also been observed in practice.[12]

Organophosphorous fungicides. Organophosphorous fungicides are classified in three chemical classes: phosphoric acid amides (triamiphos), phosphorothionates (pyrazophos) and phosphothiolates (Kitazin-P). The first two groups are primarily active against powdery mildews, whereas the last one predominantly acts against rice diseases e.g. *Pyricularia oryzae*.[13] The primary targets of the phosphoric acid amides and phosphorothionates are not yet understood. The phosphothiolates interfere in phospholipid synthesis by inhibition of the conversion of phosphatidyl ethanolamine to phosphatidyl choline by transmethylation of S-adenosylmethionine. This leads to drastic alterations of membrane structure and function.[14] Studies on the mechanisms of Kitazin-P resistance of *P. oryzae* showed that besides fungal detoxification of the fungicide by cleavage of its S-C bond, other resistance mechanisms must be involved.[15] The mechanism of resistance to pyrazophos was most probably related to the inability of resistant strains to activate pyrazophos to its hydrolysis product, PP-pyrazophos.[16]

Polyene macrolide antibiotics. The polyene macrolide antibiotics (nystatin, pimaricin and amphotericin A or B) are produced by *Streptomyces* spp. and used as antimycotics in medicine. They are selective toxicants of cell membranes and preferably bind with the fungal sterols of membranes. This results in increase of membrane permeability, leakage of cellular constituents and altered transport of nutrients.[17] The mechanism of resistance is still unknown, but resistance might be ascribed to changes in lipid and sterol compositions.[18,19]

Polyoxins and nikkomycin. The polyoxins and nikkomycin are closely related antibiotics, synthesized by *Streptomyces* spp. The polyoxins are specific inhibitors of chitin synthase. Therefore, these compounds are highly selective inhibitors of fungi containing chitin in their cell walls.[20,21] Oomycetes which contain cellulose as cell wall constituent are insensitive. The natural sensitivity of fungal species was correlated to the extent of penetration of polyoxins into the fungal cells,[22] whereas acquired fungal resistance could be related to reduced polyoxin accumulation.[23] The differences in sensitivity among fungi and resistance mechanisms may be attributed to structural changes of peptides involved in polyoxin uptake mechanisms.[24]

Melanin synthesis inhibitors (MSIs). The MSIs (tricyclazole) display their mode of action in pigmented Ascomycetes and Deuteromycetes. Rice blast caused by *P. oryzae* is excellently controlled by these chemicals.[25] Inhibition of melanin synthesis is regarded as the primary mode of action of MSIs, which causes a lack of appressoria melanization leading to a loss of pathogenicity.[26-28]

Sterol biosynthesis inhibitors (SBIs). The first representatives of this type of fungicides were introduced 25 years ago. Nowadays, they are the most widely used antifungal compounds in agriculture and in medicine. SBIs are highly active at low doses against fungi belonging to Ascomycetes, Basidiomycetes and Deuteromycetes. SBIs can be subdivided into several classes of structurally unrelated compounds which specifically inhibit sterol biosynthesis at different steps in the pathway.[29,30]

2.2. Ergosterol biosynthesis in fungi and its inhibition.

Sterol composition. Most species of Ascomycetes, Basidiomycetes and Deuteromycetes produce ergosterol as the abundant sterol. However, in rust fungi and powdery mildews other C_{14}-desmethyl sterols are synthesized. Most Oomycetes such as the Pythiaceous fungi *Pythium* and *Phytophthora* do not synthesize sterols or do not require them for vegetative growth.[31,32] The role of sterols is twofold; first, they are involved in maintaining proper membrane fluidity and integrity. For this 'bulk' membrane function large quantities of sterols are needed, but structural requirements are in this case less specific. Second, they have a regulatory or 'sparking' function, for which small amounts are needed and structural requirements are more demanding. C_{14}-desmethyl sterols like ergosterol appear to satisfy both functions in fungi.[31,32]

Ergosterol biosynthesis. Many aspects of fungal ergosterol biosynthesis were reviewed by Mercer,[33] Kato,[34] and summarized by Vanden Bossche[35] and Weete.[32] The first sterol structure occurring in the sterol pathway, lanosterol, is formed by cyclization of 2,3-

epoxysqualene, which originates from mevalonic acid via the isoprenoid pathway (Fig. 1).

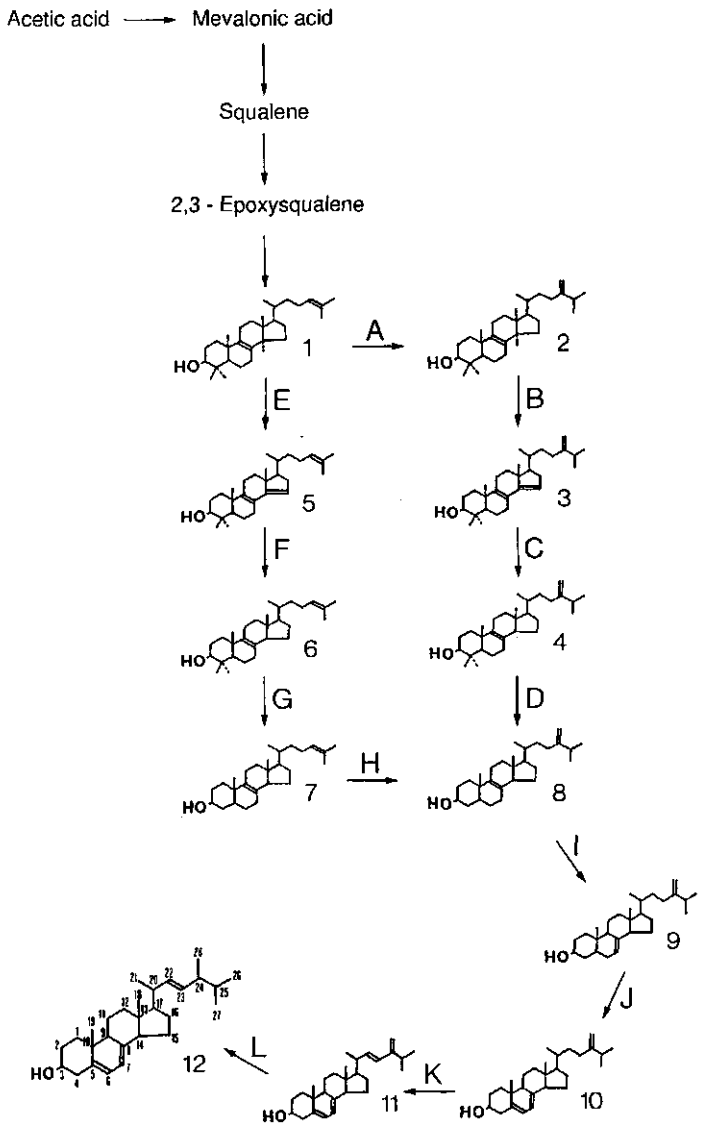


Fig. 1. Ergosterol biosynthesis pathway in fungi and its inhibition steps by various compounds. 1, Lanosterol; 2, 24-Methylenedihydrolanosterol (Eburicol); 3, 4,4-Dimethyl-ergosta-8,14,24(28)-trienol; 4, 4,4-Dimethyl-ergosta-8,24(28)-dienol; 5, 4,4-Dimethyl-cholesta-8,14,24-trienol; 6, 4,4-Dimethyl-cholesta-8,24-dienol; 7, Cholesta-8,24-dienol (Zymosterol); 8, Ergosta-8,24(28)-dienol (Fecosterol); 9, Episterol; 10, Ergosta-5,7,24(28)-trienol; 11, Ergosta-5,7,22,24(28)-tetraenol; 12, Ergosterol (From Kato, 1986).

However, an important difference between yeasts and filamentous fungi arises in the pathway following lanosterol metabolism. In filamentous fungi alkylation at the C24-position is the first step (A) leading to the production of 24-methylenedihydrolanosterol (eburicol). Eburicol is subsequently demethylated at C_{14} (B) which is mediated by cytochrome P450-dependent sterol 14α-demethylase. Then the $\Delta^{14(15)}$ double bond of the demethylation product, $C_{4,4}$-dimethylergosta-8,14,24(28)-trienol, is reduced (C), followed by demethylation of C_4 (D) resulting in the synthesis of ergosta-8,24(28)-dienol (fecosterol). In yeasts, however, the alkylation at the C_{24}-position occurs after C_{14}-demethylation (E), reduction of the $\Delta^{14(15)}$ double bond (F) and C_4-demethylation (G). The fecosterol conversion to ergosterol is similar for yeasts and filamentous fungi. The $\Delta^{8(9)}$ double bond of fecosterol is isomerized to a Δ^7 double bond (I), followed by an introduction of a Δ^5 double bond (J), a Δ^{22} double bond (K) and the introduction of a $\Delta^{24(28)}$ double bond (L).

Inhibition of ergosterol biosynthesis. Ergosterol biosynthesis may be impaired at different steps. Representative inhibitors are categorized as follows:[25,30,34]

a) squalene epoxidase inhibitors: allylamines, terbinafine and naftifine.[36]
b) 2-3-oxidosqualene cyclase inhibitors: 2-aza-2,3 dihydrosqualene and 2,3 imino-squalene.[31,32]
c) C_{24}-sterolmethyltransferase inhibitors (step A and H): 25-azacholesterol and 24-epiiminolanosterol.[31,32]
d) sterol 14α-demethylase inhibitors (step B and E): imidazole, triazole, pyrimidine and pyridine derivatives.[35]
e) $\Delta^{14(15)}$-reductase inhibitors (step C and F): morpholine derivatives.[37,38]
f) Δ^7-Δ^8-isomerase inhibitors (step I): morpholine derivatives.[37,38]
g) Δ^{22}-desaturase inhibitors (step K): triarimol.[32,34]
h) $\Delta^{24(28)}$-reductase inhibitors (step L): 25-azacholesterol.[32,34]

In economical respect, the sterol 14α-demethylation inhibitors (DMIs), especially imidazoles and triazoles, are the most important group of sterol biosynthesis inhibitors applied in agriculture and medicine. Examples of widely used agricultural fungicides are triadimenol, propiconazole, prochloraz and imazalil. Itraconazole, ketoconazole and fluconazole are well known antimycotics used in clinical and veterinary medicine.[29] Only a limited number of derivatives of piperazine, pyrimidine and pyridine are developed as DMIs.

Inhibition of sterol biosynthesis by DMIs results in direct depletion of ergosterol and accumulation of abnormal sterol intermediates. These changes in sterol composition alter membrane fluidity, permeability and activities of membrane-bound enzymes, since the fluidity of membranes is an important regulatory factor for these kind of enzymes.[32]

Examples of membrane-bound enzyme activities which are disturbed by DMIs are microsomal ATPases, mitochondrial cytochrome oxidase, cytochrome *c* peroxidase and fatty acid coenzyme A desaturase.[35] A major consequence of ergo-sterol depletion and/or accumulation of 14α-methylsterols is the increased and uncoordinated activation of chitin synthase. The increased and irregular disposition of chitin disturbes the processes of cell-division and -separation in yeasts leading to chains and clusters of cells. In filamentous fungi it stimulates branching and causes misformed, swollen hyphae due to an uncoordinated formation of septa and cell walls.[35,39]

Development of resistance to DMIs. Like for all site-specific fungicides, DMIs are more prone to resistance than conventional multisite inhibitors. Under laboratory conditions DMI-resistant mutants of fungi can easily be obtained, but most of these strains show reduced fitness with regard to spore germination, mycelial growth and pathogenicity.[40-42] However, experiments with *Sphaerotheca fuliginea* demonstrated that DMI-resistance is not always associated with reduced fitness.[43] It is now generally accepted that the polygenic nature of DMI-resistance may explain the still limited number of DMI-resistant plant pathogens observed in the field, rather than reduced fitness of DMI-resistant strains. In the polygenic control of DMI-resistance the effect of individual genes is generally small. Therefore, a highly resistant strain may only be obtained by crossing different first step mutants or by stepwise selection of several mutant genes interacting with each other in the same nucleus.[44,45]

DMI-resistance may in theory be based on a single gene mutation leading to a non-functional cytochrome P450 sterol 14α-demethylase.[46] This phenomenon has been described for nystatine-resistant mutants of *U. maydis*, *Candida albicans* and *Saccharomyces cerevisiae* which are cross-resistant to DMIs.[47-50] These mutants were relatively tolerant to the accumulated 14α-methylsterols, but their growth rate as well as their morphology were greatly impaired. Therefore, this type of resistance is unlikely to occur in the field. Only a gene mutation resulting in unimpaired binding of the substrate lanosterol, but a decreased affinity for DMIs might lead to resistant strains with unimpaired sterol composition and competitive fitness.[46,51] In this respect, Vanden Bossche *et al.*,[52,53] demonstrated that azole-resistant isolates of *C. albicans* did contain P450 isozymes with less affinity to various DMIs than the azole-sensitive ones. These resistant isolates were less pathogenic than the sensitive strains. Target site mutations, however, have not yet been observed with filamentous fungi. In filamentous fungi, like *Aspergillus nidulans*, *P. italicum*, *Monilinia fructicola* and *Nectria haematococca* var. *cucurbitae*, DMI-resistance is probably based on an increased energy-dependent efflux of the fungicides, by which accumulation is reduced.[42,54-57] Other potential mechanisms like tolerance to toxic sterols, failure to toxify accumulated 14α-methylsterols,[58] disability to activate the fungicides,[59,60] deposition of fungicides in lipid droplets or vacuoles[61] and an

induced resistance response related to a transient accumulation of sterol precursors[62] seem to have limited relevance.

Selectivity of DMIs. Numerous species of P450 enzymes exist in mammals and higher plants. Therefore, azole antifungals are required to have a high selective affinity for fungal cytochrome P450-dependent sterol 14α-demethylase to avoid inhibitory effects on host P450 isozymes.[63] The observed inter-species selectivity of DMIs is most probably due to significantly lower binding affinities to related cytochromes P450 operative in mammals and plants.[35,46,64] However, despite their high target specificity, DMIs also show a remarkable selective toxicity to fungi, reflected by the spectrum of diseases controlled by a particular DMI-fungicide.[29] The reason for the selective action of particular DMIs is still not well understood, but could be due to differences in the sterol 14α-demethylase resulting in differential binding affinity for the various inhibitors.[65] This supposition is supported by studies which demonstrated that the sterol 14α-demethylase from *S. cerevisiae* showed differences in affinity for various DMIs.[66-68] Similar results were found with the enzyme from *C. albicans*, *Aspergillus fumigatus* and *P. italicum*.[69-71] Pye & Marriott[72] showed that drastic differences with regard to the binding affinities of DMIs to $P450s_{14DM}$ from three *Candida* species could partly explain their selective activities. However, in the case of *Mucor rouxii*, lack of sensitivity to DMIs cannot be ascribed to a low affinity of the DMIs for the target enzyme because abnormal sterols readily accumulated at concentrations well below those needed to inhibit growth.[73] This capacity to tolerate 14α-methylsterols is also a plausible mechanism which can account for the selective fungitoxicity of DMIs.[73] The natural insensitivity of several fungi to triadimefon was attributed to the extent of its metabolic activation to triadimenol and the fungal sensitivity to the individual enantiomers of triadimenol produced.[74] In case of triforine, the selective fungitoxic action is based on either differences in fungal accumulation or metabolism.[75]

2.3. Cytochrome P450

To comprehend the biochemical basis which determines the affinity of DMIs for fungal sterol 14α-demethylase an overview of cytochrome P450 is required. This is detailed in the following sections.

Definition, function and distribution of P450. Cytochrome P450 (P450) is the collective name given for a special class of haem containing proteins which show after reduction with dithionite and in the presence of carbon monoxide (CO) a maximal absorption at 450 nm.[39,76,77] This unique spectrophotometric feature of P450 is due to

binding of a thiolate anion (S^-) from a cysteine residue in the apoprotein and of CO to the reduced haem iron to its fifth and sixth ligand, respectively (Fig. 2).

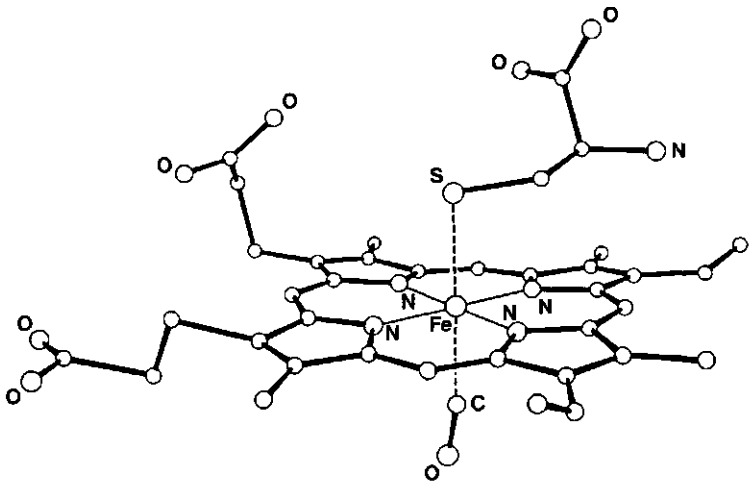


Fig. 2. Proposed perspective drawing of the iron protoporphyrin in interaction with carbon monoxide (CO). The fifth ligand is a thiolate anion of cysteine. The sixth coordination position of the haem is CO (From Vanden Bossche, 1985).

P450s are present in most eukaryotes and in a few bacteria. Molecular weights range between 45 and 60 Kd. In animals and plants the enzyme system has been detected in almost all tissues examined. The eukaryotic P450s are mainly located in the endoplasmatic reticulum and mitochondria. Bacterial P450s have no apparent membrane association.[77] P450s can be divided in two classes. The first group consists of P450s involved in regiospecific monooxygenation of many physiologically important endogenous lipophilic substrates like steroids, fatty acids, prostanoids, and secondary metabolites of plants and microbial species. An example is lanosterol 14α-demethylase in sterolbiosynthesis. Characteristic of this class of P450s is that these enzymes are generally substrate-specific and not greatly affected by foreign chemicals, although they might be able to metabolize these chemicals at a low rate. The second class is represented by P450s which are induced to detectable levels after chronic exposure to xenobiotics such as drugs, pesticides, food-additives ect.[39,76] However, besides substrate monooxygenations many other reactions are catalyzed by P450s like for example epoxidation, *N*-, *S*-, and *O*-dealkylation, desulfurization, deamination, dehalogenation and isomerization.[77] To metabolize such diverse substrates P450 shows considerable molecular multiplicity. It is not known how many forms of P450 exist, but the figure might be between 30 and 200.

The catalytic mechanism of P450. The monooxygenase reaction catalyzed by P450 requires NADPH and molecular oxygen. In general, the reaction equation is as follows:

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$$

The essential components of the cytochrome P450-dependent monooxygenase in microsomal membranes are: the NADPH-cytochrome P450-reductase with FAD and FMN as flavin prosthetic groups, P450 and phospholipids. Cytochrome b_5 may also be involved. The function of the P450-reductase is to transfer electrons from NADPH to P450. P450 provides the oxygen- and the substrate-binding site. Phospholipids mediate the interactions between P450 and the reductase. They also facilitate electron transfer and stabilize and/or induce functionally active conformation of P450.[39] The catalytic sequence can be summarized as follows: 1) binding of a substrate (RH) to P450, 2) reduction of the two flavin prosthetic groups of P450-reductase by NADPH, 3) transfer of one of the two electrons to P450 and consequent reduction of the haem iron, 4) binding of molecular oxygen to form a Fe^{2+}-cytochrome-P450 dioxygen complex, 5) transfer of the 2nd electron from P450-reductase or from cytochrome b_5 to the latter complex, 6) Splitting of the oxygen-oxygen bond with the incorporation of the distal oxygen into a molecule of H_2O, 7) incorporation of the 2nd oxygen atom into the substrate, 8) dissociation of the product (ROH).[39,64,76]

Substrate-induced spectral changes of P450. In the oxidized state, P450s exist in either a low-spin hexacoordinate form or a high-spin pentacoordinate form. In the low-spin hexacoordinate form of P450 the sixth haem ligand is most probably the oxygen atom from water, whereas such a ligand is absent in the pentacoordinate form (Fig. 3). The equilibrium between the low- and high-spin configuration is affected by the accessibility of water to the haem iron which is determined by the hydrophobicity and steric hinderance of the haem pocket. Most P450s have been found to be extensively low-spin in their resting form.[77] Binding of a substrate to oxidized P450 causes certain spectral change of the cytochrome. The substrate-induced spectral change is caused by removal or exchange of the sixth ligand of oxidized P450 concomitant with the binding of a substrate. Two different types of spectral changes can be distinguished. If a lipophilic substrate (AH) binds to the substrate site near the sixth coordination position, the accessibility of water to the haem is reduced. In consequence, the cytochrome moves from the hexacoordinated low-spin to the pentacoordinated high-spin configuration showing an absorbance maximum at approximately 385 nm. This spectral change is shown by the type-I difference spectrum (Fig. 3). In contrast, if a substrate which is bound to the substrate site also acts as an external sixth haem ligand (AXH) instead of water, the cytochrome P450 is converted to an artificial low-spin complex. The type II spectral change is induced by this kind of substrate binding (Fig. 3). The wavelengths of maximal and minimal absorption of a low-spin complex of P450 not only depends on the nature of the sixth ligand, but also on the spin state of the cytochrome. Since microsomal P450

consists of a mixture of low- and high-spin forms, the absorbance maxima and minima in type II difference spectra may vary from 425 to 430 nm and 390 to 410 nm, respectively.[76] The type II spectral change can be induced by all kinds of amines, such as pyridines, imidazoles, triazoles, isonitriles, amides and anilines.[39,78-79]

Sterol 14α-demethylase ($P450_{14DM}$) in fungi. Studies on fungal sterol 14α-demethylase ($P450_{14DM}$) are generally not very extensive. Only $P450_{14DM}$ from the yeasts *S. cerevisiae* and *C. albicans* is studied in great detail. Spectral characteristics indicated that the oxidized form of $P450_{14DM}$ from these yeasts is in a low-spin state and its reduced CO

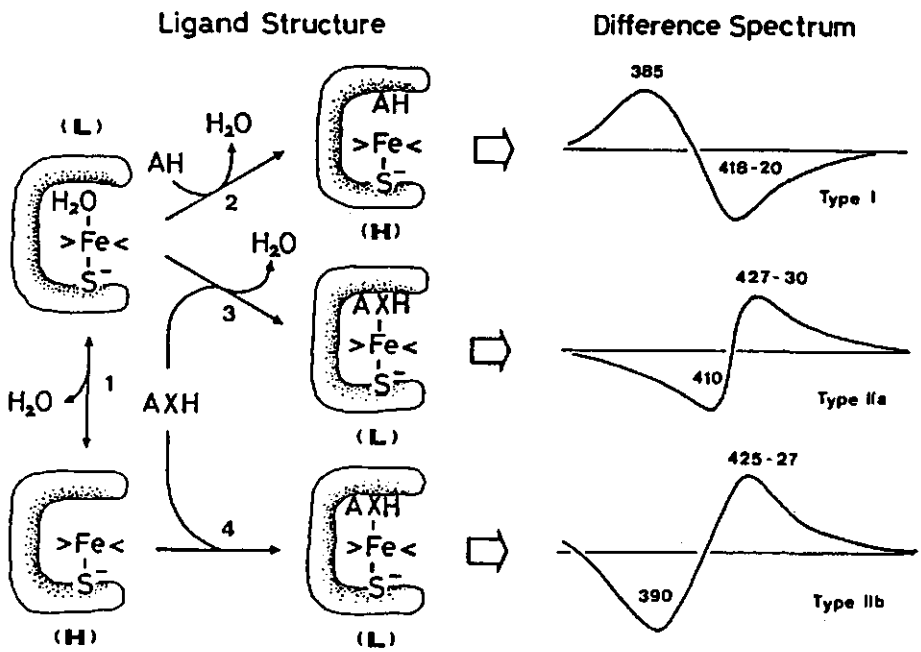


Fig. 3. Spin-state equilibrium, substrate binding, and the substrate-induced spectral change of cytochrome P450 in relation to ligand structure of the cytochrome. S^- represents the thiolate fifth ligand characterizing the cytochrome. Spin state of each structure is represented by H (high-spin) or L (low-spin). The substrate-free resting form is in equilibrium between the high- spin (penta-coordinate) and the low-spin (water-ligated) forms (reaction 1). Reaction 2 indicates binding of a substrate that has no coordinating group (AH) to the low-spin form. This interaction converts the water-ligated low-spin form to the penta-coordinate high-spin form and induces the type I spectral change. Reactions 3 and 4 represent binding of another type of substrate (AXH), which contains a coordinating atom (X), to the low-spin and high-spin forms, respectively. In these cases, X coordinates to the haem-iron, an artificial low-spin complex is formed, and the type II spectral change is induced. The shape of the type II difference spectrum is affected by the spin state of the substrate-free form, and they are discriminated as type IIa and type IIb in this figure (From Yoshida, 1988).

complex has an absorption peak at 447 nm. Molecular weights were estimated to be 58 and 51 Kd, respectively. Studies using reconstituted model membrane systems with $P450_{14DM}$ and NADPH-cytochrome P450-reductase from both yeast species indicated that three NAD(P)H-dependent oxygenase reactions are involved in the removal of the 14α-methylgroup from lanosterol.[64,76,80] It is noted that the *Candida* enzyme prefers NADH rather than NADPH, as co-factor.[81] In the first step the 14α-methylgroup (CH_3) is oxidized to a 14α-hydroxymethyl group (CH_2OH), in the second step the hydroxymethyl-group is oxidized to a formaldehyd group (CHO) and in the third step the latter is oxidized and lost as formate (HCOOH). The cytochromes $P450_{14DM}$ from both yeasts catalyzed the entire process of demethylation in which the initial hydroxylation of the 14α-methylgroup is the rate-limiting step.[39,64,76,80] The reconstituted system of *S. cerevisiae* was also used to unravel the mechanisms which determine the substrate-specificity of $P450_{14DM}$. It was demonstrated that the 3-hydroxy group, the $\Delta^{8(9)}$ double bond and the terminal part of the side chain of lanosterol are essential for orienting the substrate in the active site of $P450_{14DM}$.[63,82-84] There are hardly any data available on $P450_{14DM}$ from filamentous fungi. This is most probably due to the unstable nature and relatively low content of this enzyme in these organisms.[71,85,86] However, a few papers on the isolation of microsomal P450 from the filamentous fungi *A. fumigatus* and *P. italicum* have recently been published.[85-87] The complexes of CO with reduced microsomal P450 isozymes from these fungi were characterized by absorption maxima at 451 and 449 nm, respectively.[85-87] In addition, several reports appeared on the preparation of cell-free extracts from the same two filamentous fungi and from *U. maydis* which are able to synthesize ergosterol from [^{14}C]mevalonate.[69,71,88] To date, no reports have appeared on the purification of $P450_{14DM}$ or on reconstituted systems with this enzym from filamentous fungi. This is a prerequisitive for further characterization of the enzyme from these fungi. It is known that sterol 14α-demethylation in filamentous fungi occurs after methylation at C_{24} of lanosterol,[33] which takes place in mitochondria.[89] This may have been the reason why so far, one has failed to develop reconstituted membrane systems from filamentous fungi, which are able to synthesize desmethylsterols.

Interaction of fungal $P450_{14DM}$ with DMIs. Azole antifungal agents induced type II spectral changes of purified $P450_{14DM}$ from *S. cerevisiae* and *C. albicans*.[76,80] Type II spectra were also obtained with microsomal preparations of *C. albicans*, *A. fumigatus* and *P. italicum* upon addition of various DMIs.[52,85,86] This means that an azole nitrogen atom (N_3 of imidazole; N_4 of triazole) coordinates with the haem iron of ferric P450 to form an artificial low-spin complex. The spectral studies with purified yeast $P450_{14DM}$ further demonstrated that the DMIs formed one-to-one complexes with the ferric cytochrome.[76,80] This conclusion was supported by findings that several azole antifungals completely inhibited reconstituted sterol 14α-demethylase activity when added at a concentration equal to that of $P450_{14DM}$.[66-68,80] However, $P450_{14DM}$ also showed type II spectral changes

with simple, non-toxic pyridines and imidazoles, but not at equimolar concentrations. Consequently, affinity of these ligands is far less than reported for the azole antifungal agents suggesting that the azole fungicides or antimycotics interact not only with the haem iron, but also at some region of the apoprotein.[76] In addition, reconstituted demethylase activity of *S. cerevisiae* and binding of CO to reduced $P450_{14DM}$ was differentially inhibited by various DMIs, including stereoisomers of diniconazole and triadimenol. This inhibition correlated with their inhibitory effects on growth.[63,66-68,90] Similar results were also found when the inhibitory effects of various DMIs on CO binding were studied with microsomal cytochrome P450 isozymes from *C. albicans*.[35,52,64,91] Based on these results it was deduced that the affinity of DMIs for $P450_{14DM}$ is predominantly determined by their N_1 substituent regulating their mobility in the haem crevice and most probably their interaction with the substrate binding site.[35,63,64,67,68] Consequently, inhibition of sterol 14α-demethylase activity by DMIs is supposed to result from interaction at two sites of the enzyme: first, the DMIs inhibit substrate binding by occupying the substrate binding site at the apoprotein located near the haem. Second, the azole moiety of the DMIs coordinates with the ferric haem, containing the cytochrome in its low spin-state, thereby preventing the transition to the high spin-state, which is required for enzyme activity.[80]

Methods for determining the potency of DMIs against $P450_{14DM}$. The most common method to evaluate the potency of azoles to inhibit activity of $P450_{14DM}$ is by measuring the incorporation of [^{14}C]mevalonic acid into desmethylsterols of azole-treated cell-free extracts or reconstituted sterol 14α-demethylase systems.[80] Reconstituted systems have been described for *S. cerevisiae* and *C. albicans*, whereas cell-free bioassays have been reported for *U. maydis*, *A. fumigatus* and *P. italicum*. As already mentioned before, both enzyme assays revealed that the differential inhibition of $P450_{14DM}$ activity by a number of DMIs correlated with their fungicidal activity or *in vitro* sterol biosynthesis inhibition in intact cells.[63,66-71,88]

The affinity of DMIs for $P450_{14DM}$ can also be examined spectrophotometrically by measuring the ability of DMIs to induce type II spectral changes of the cytochrome. According to Wiggins & Baldwin[92] the increase in value of ΔA (maximuum absorption - minimum absorption) in type II spectra after successive additions of various isomers of diclobutrazol to partly purified yeast P450, as well as the final saturation value (ΔA_{max}) reflected their fungicidal activity. Yoshida & co-workers also demonstrated that the differences in affinity of various stereoisomers of diniconazole and triadimenol for yeast $P450_{14DM}$ determined by type II spectral analysis correlated with their inhibitory effects on $P450_{14DM}$ activity.[66,68] In principle, from the results of the spectral titration of the cytochrome with DMIs the affinity of these compounds for the enzyme can be calculated using Lineweaver-Burke double reciprocal plots and expressed in binding constants (K_d values). However, determination of K_d values of DMIs is often hampered by technical

difficulties due to their extremely high affinity for $P450_{14DM}$ and to the fact that accurate spectrophotometric analysis can not be done with P450 concentrations below 0.1 μM.[66-68]

In contrast, Vanden Bossche *et al.*[91] suggested that the ability of an azole derivative to induce a type II spectrum is not necessarily indicative for a possible inhibitory effect on a P450-dependent reaction. This view was supported by the finding that various DMIs induced similar type II spectral changes of purified yeast $P450_{14DM}$, although they differed in their fungitoxicity.[67] Guan *et al.*[86] also concluded that the induction of type II spectral changes of microsomal P450 isozymes from *P. italicum* by several DMIs did not give a clue on their fungitoxicity.

Spectrophotometric studies in which the displacement of DMIs from reduced P450-DMI complexes by CO is examined, were suggested to give a better indication of the interaction between $P450_{14DM}$ and DMIs.[52,76] This suggestion was based on the fact that inhibition of CO binding to reduced $P450_{14DM}$ from *S. cerevisiae* and reduced microsomal P450 isozymes from *C. albicans* by DMIs correlated with their inhibitory effects on *in vitro* and *in vivo* sterol 14α-demethylation.[52,66-68,90,91] However, such a correlation could not be found when CO displacement experiments were conducted with purified $P450_{14DM}$ from *C. albicans* and microsomal preparations of *A. fumigatus* and *P. italicum*.[70,80,85-87]

References

1. Delp, C.J., Benzimidazole and related fungicides. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanism of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 233-244.
2. Davidse, L.C., Biochemical aspects of benzimidazole fungicides, action and resistance. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 245-258.
3. Hollomon, D.W., Target Sites of Hydroxypyrimidine Fungicides. *In*: Köller, W. (Ed.), Target Sites of Fungicide Action, CRC Press Inc., Boca Raton Ann Harbor, London, 1992, p. 31-42.
4. Davidse, L.C., Biochemical aspects of phenylamide fungicides, action and resistance. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 275-282.
5. Schewe, T. & Lyr, H., Mechanism of action of carboxin fungicides and related compounds. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 133-142.
6. White, G.A., A potent effect of 1,4-oxathiin systemic fungicides on succinate oxidation by a particulate preparation from *Ustilago maydis*. *Biochem. Biophys. Res. Commun.* **44** (1971) 1212-1219.
7. Georgopoulos, S.P., Chrysayi, M. & White, Carboxin resistance in the haploid, the heterozygous diploid, and the plant parasitic dicaryotic phase of *Ustilago maydis*. *Pestic. Biochem. Physiol.* **5** (1975) 543-551.
8. White, G.A. & Georgopoulos, S.G., Target Sites of Carboxamides. *In*: Köller, W. (Ed.), Target Sites of Fungicide Action, CRC Press Inc., Boca Raton Ann Harbor, London, 1992, p. 1-30.
9. Lyr, H., Aromatic hydrocarbon fungicides. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 63-74.
10. Edlich, W. & Lyr, H., Target Sites of Fungicides with Primary Effects on Lipid Peroxidation. *In*: Köller, W. (Ed.), Target Sites of Fungicide Action, CRC Press Inc., Boca Raton Ann Harbor, London, 1992, p. 53-68.

11. Pommer, E.-H. & Lorenz, G., Dicarboximides. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 91-106.
12. Leroux, P. & Fritz, R., Antifungal activities of dicarboximides and aromatic hydrocarbons and resistance to these fungicides. *In*: Trinci, A.P.J. & Ryley, J.F. (Eds.). Mode of Action of Antifungal Agents. Cambridge, 1984, p. 207-237.
13. Schreiber, B., Organophosphorous fungicides. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 299-308.
14. Kodama, O., Yamashita, K. & Akatsuka, T., Edifenphos, inhibitor of phosphatidylcholine biosynthesis in *Pyricularia oryzae*. *Biol. Chem.* **44** (1980) 1015-1021.
15. Uesugi, Y., Kodama, O. & Akutsaka, T., Inhibition of fungal metabolism of some organophosphorous compounds by pieronyl butoxide. *Agric. Biol. Chem.* **42** (1978) 2181-2183.
16. De Waard, M.A. & Van Nistelrooy, J.G.M., Mechanism of resistance to pyrazophos in *Pyricularia oryzae*. *Neth. J. Pl. Path.* **86** (1980) 251-258.
17. Hamilton-Miller, J.M.T., Fungal sterols and the mode of action of the polyene antibiotics. *Adv. Appl. Microbiol.* **17** (1974) 109-134.
18. Fryberg, M., Oehlschlager, A.C. & Unrau, A.M., Sterol biosynthesis in antibiotic sensitive and resistant *Candida*. *Arch. Biochem. Biophys.* **173** (1976) 171-177.
19. Dekker, J., The development of resistance to fungicides. *Progr. Pest. Biochem. Toxicol.* **4** (1985) 165-218.
20. Endo, A. & Misato, T., Polyoxin D, a competitive inhibitor of UDP-N-acetylglucosamine: chitin N-acetylglucosaminyltransferase in *Neurospora crassa*. *Biochem. Biophys. Res. Commun.* **37** (1969) 718-722.
21. Bowers, B., Levin, G. & Cabib, E., Effect of polyoxin D on chitin synthesis and septum formation in *Saccharomyces cerevisiae*. *J. Bacteriol.* **119** (1974) 564-575.
22. Cabib, E. & Bowers, B., Timing and function of chitin synthesis in yeast. *J. Bacteriol.* **124** (1975) 1586-1593.
23. Misato, T., Kakiki, K. & Hori, M., Mechanism of polyoxin resistance. *Neth. J. Pl. Path.* **83** (Suppl. 1) (1977) 253-260.
24. Gooday, G.W., *In*: Lyr, H. & Polter, C. (Eds.). Systemic fungicides. Abhandl. der Akad. Wissensch. DDR No.2, Akademie, Berlin, 1979, p.159-168.
25. Buchenauer, H., Physiological Reactions in the Inhibition of Plant Pathogenic Fungi. *In*: Haug, G. & Hoffmann, H. (Eds.), Chemistry of Plant Protection, Vol. 6, Controlled Release, Biochemical Effects of Pesticides, Inhibition of Plant Pathogenic Fungi. Springer-Verlag, Berlin, Germany, 1990, p. 217-292.
26. Woloshuk, C.P., Sisler, H.D., Chrysayi Tokousbalides, M. & Dutky, S.R., Melanin biosynthesis in *Pyricularia oryzae*: site of tricyclazole inhibition and pathogenicity of melanin-deficient mutants. *Pestic. Biochem. Physiol.* **14** (1980) 256-264.
27. Kubo, Y., Suzuki, K., Furusawa, I., Ishida, N. & Yamamoto, M., Melanin synthesis as a prerequisite for penetration by appressoria of *Colletotrichum lagenarium*: Site of inhibition by melanin-inhibiting fungicides and their action on appressoria. *Pestic. Biochem. Physiol.* **23** (1985) 47-55.
28. Yamaguchi, I. & Kubo, Y., Target Sites of Melanin Biosynthesis Inhibitors. *In*: Target Sites of Fungicide Action, CRC Press Inc., Boca Raton Ann Harbor, London, 1992, p. 101-118.
29. Scheinpflug, H. & Kuck, K.H., Sterol biosynthesis inhibiting piperazine, pyridine, pyrimidine and azole fungicides. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 173-204.
30. Köller, W., Antifungal Agents with Target Sites in Sterol Functions and Biosynthesis. *In*: Köller, W. (Ed.), Target Sites of Fungicide Action, CRC Press Inc., Boca Raton Ann Harbor, London, 1992, p. 119-206.
31. Burden, R.S., Cooke, D.T. & Carter, G.A., Inhibitors of sterol biosynthesis and growth in plants and fungi. *Phytochem.* **28** (1989) 1791-1804.
32. Weete, J.D., Structure and function of sterols in fungi. *Adv. Lipid Res.* **23** (1989) 115-167.
33. Mercer, E.I., The biosynthesis of ergosterol. *Pestic. Sci.* **15** (1984) 133-155.

34. Kato, T., Sterol-Biosynthesis in Fungi, a Target for Broad Spectrum Fungicides. *In*: Haug, G. & Hoffmann, H. (Eds.), Chemistry of Plant Protection, Vol. 1, Sterol Biosynthesis, Inhibitors and Anti-Feeding Compounds. Springer-Verlag, Berlin, 1986, p. 1-24.
35. Vanden Bossche, H., Mode of action of pyridine, pyrimidine and azole antifungals. *In*: Berg, D. & Plempel, M. (Eds.), Sterol Biosynthesis Inhibitors, Pharmaceutical and Agrochemical Aspects. Ellis Horwood Ltd., Chichester, UK, 1988, p. 79-119.
36. Ryder, N.S., Squalene epoxidase as a target for the allylamines. *Biochem. Soc. Trans.* **19** (1991) 774-777.
37. Kerkenaar, A., Mechanism of action of morpholine fungicides. *In*: Lyr, H. (Ed.), Modern Selective Fungicides, Properties, Applications, Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 159-172.
38. Mercer, E.I., The mode of action of morpholines. *In*: Berg, D. & Plempel, M. (Eds.), Sterol Biosynthesis Inhibitors, Pharmaceutical and Agrochemical Aspects. Ellis Horwood Ltd., Chichester, UK, 1988, p.120-150.
39. Janssen. P.A.J. & Vanden Bossche, H., Mode of action of cytochrome P-450 monooxygenase inhibitors. Focus on azole derivatives. *Arch. Pharm. Chem. Sci. Ed.* **15** (1987) 24-40.
40. De Waard, M.A. & Fuchs, A., Resistance to ergosterol-biosynthesis inhibitors II. genetic and physiological aspects. *In*: Dekker, J. & Georgopoulos, S.G. (Eds.), Fungicide resistance in crop protection. Pudoc, Wageningen, The Netherlands, 1982, p. 87-100.
41. Hollomon, D.W., Butters, J.A. & Hargreaves, J.A., Resistance to Sterol Biosynthesis-Inhibiting Fungicides. *In*: Green, M.B., Le Baron, H.M. & Moberg, W.K. (Eds.), Managing Resistance to Agrochemicals, from fundamental Research to practical Strategies. ACS Symposium Series **421** (1990) 199-214
42. Kalamarakis, A.E., De Waard, M.A., Ziogas, B.N. & Georgopoulos, S.G., Resistance to fenarimol in *Nectria haematococca* var. *cucurbitae*. *Pestic. Biochem. Physiol.* **40** (1991) 212-220.
43. Schepers, H.T.A.M., Fitness of isolates of *Sphaerotheca fuliginea* resistant or sensitive to fungicides which inhibit ergosterol biosynthesis. *Neth. J. Pl. Path.* **91** (1985) 65-76.
44. Georgopoulos, S.P., Genetics and population dynamics. *In*: Delp, C.J. (Ed.), Fungicide Resistance in North America, APS Press, St. Paul, Minnesota USA, 1988, p. 12-13.
45. De Waard, M.A. & Van Nistelrooy, J.G.M., Stepwise development of laboratory resistance to DMI fungicides in *Penicilium italicum*. *Neth. J. Pl. Path.* **96** (1990) 321-329.
46. Köller, W., Sterol demethylation inhibitors: mechanism of action and resistance. *In*: Delp, C.J. (Ed.), Fungicide Resistance in North America, APS Press, St. Paul, Minnesota, USA, 1988, p. 79-88.
47. Walsh, R.C. & Sisler, H.D., A mutant of *Ustilago maydis* deficient in sterol C-14 demethylation, characterization and sensitivity to inhibitors of ergosterol biosynthesis. *Pestic. Biochem. Physiol.* **18** (1982) 122-131.
48. Aoyama, Y., Yoshida,Y., Nishino, T., Katsuki, H., Maitra, U.S., Mohan, V.P. & Sprinson, D.B., Isolation and characterization of an altered cytochrome P-450 from a yeast mutant deficient in lanosterol 14α-demethylation. *J. Biol. Chem.* **262** (1987) 14260-14264.
49. King, D.J., Wiseman, A., Kelly, D.E. & Kelly, S.L., Differences in the cytochrome P-450 enzymes of sterol C-14 demethylase mutants of *Saccharomyces cerevisiae*. *Curr. Genet.* **10** (1985) 261-267.
50. Bard, M., Lees, N.D., Barbuch, R.J. & Sanglard, D., Characterization of a cytochrome P450 deficient mutant of *Candida albicans*. *Biochem. Biophys. Res. Commun.* **147** (1987) 794-800.
51. Sisler, H.D. & Ragsdale, N.N., Molecular design and target site analysis in fungicide development. *In*: Magee, P.S., Henry, D.R. & Block, J.H. (Eds.), Probing bioactive mechanisms. American Chemical Society, Washington, DC, 1989, p. 198-213.
52. Vanden Bossche, H., Marichal, P., Gorrens, J., Bellens, D., Verhoeven, H., Coene, M-C., Lauwers, W. & Janssen, P.A.J., Interaction of azole derivatives with cytochrome P-450 isozymes in yeast, fungi, plants and mammalian cells. *Pestic. Sci.* **21** (1987) 1-13.
53. Vanden Bossche, H., Marichal, P., Gorrens, J., Bellens, D., Moereels, H. & Janssen, P.A.J., Mutation in cytochrome P-450-dependent 14α-demethylase results in decreased affinity for azole antifungals. *Biochem. Soc. Trans.* **18** (1990) 56-59.
54. De Waard, M.A. & Van Nistelrooy, J.G.M., Differential accumulation of fenarimol by a wild type and fenarimol-resistant isolates of *Penicillium italicum*. *Neth. J. Pl. Path.* **90** (1984) 143-153.

55. De Waard, M.A. & Van Nistelrooy, J.G.M., Inhibitors of energy-dependent efflux of the fungicide fenarimol by *Aspergillus nidulans*. *Exp. Mycol.* **11** (1987) 1-10.
56. De Waard, M.A. & Van Nistelrooy, J.G.M., Accumulation of SBI fungicides in wild-type and fenarimol-resistant isolates of *Penicillium italicum*. *Pestic. Sci.* **22** (1988) 371-382.
57. Ney, C., Untersuchungen zur Resistenz von *Monilinia fructicola* (Wint) Honey gegenüber Ergosterol-Biosynthesehemmern. Thesis, University of Basel, Switzerland, 1988, 92 pp.
58. Watson, P.F., Rose, M.E., Ellis, S.W., England, H. & Kelly, S.L., Defective C5-6 desaturation and azole resistance: a new hypothesis for the mode of action of azole antifungals. *Biochem. Biophys. Res. Commun.* **164** (1989) 1170-1175.
59. Fuchs, A. & De Vries, F.W., Diastereomer-selective resistance in *Cladosporium cucumerinum* to triazole-type fungicides. *Pestic. Sci.* **15** (1984) 90-96.
60. Kalamarakis, A.E., Ziogas, B.N. & Georgopoulos, S.G., Resistance to ergosterol biosynthesis inhibitors in *Nectria haematococca*. *In*: Abstracts of 6th Int. Congr. Pest. Chem., IUPAC, Ottawa, Canada, 1986, 3E-06.
61. Hippe, S., Combined application of low temperature preparation and electronic microscopic autoradiography for the localization of systemic fungicides. *Histochemistry* **87** (1987) 309-315.
62. Smith, F.D. & Köller, W., The expression of resistance of *Ustilago avenae* to the sterol demethylation inhibitor triadimenol is an induced response. *Phytopathol.* **80** (1990) 584-590.
63. Yoshida, Y. & Aoyama, Y., Sterol 14α-demethylase and its inhibition: structural considerations on interaction of azole antifungal agents with lanosterol 14α-demethylase ($P\text{-}450_{14DM}$) of yeast. *Biochem. Soc. Trans.* **19** (1991) 778-800.
64. Vanden Bosche, H., Marichal, P., Gorrens, J., Coene, M-C., Willemsens, G., Bellens, D., Roels, I., Moereels, H. & Janssen, P.A.J., *Mycoses* **32** (Suppl.1) (1989) 35-52.
65. Burden, R.S., Cooke, D.T. & Carter, G.A., Review - Mechanism of action of herbicidal and fungicidal compounds on cell membranes. *Pestic. Sci.* **30** (1990) 125-140.
66. Yoshida, Y., Aoyama, Y., Takano, H. & Kato, T., Stereo-selective interaction of enantiomers of diniconazole, a fungicide, with purified P450/14DM from yeast. *Biochem. Biophys. Res. Commun.* **137** (1986) 513-519.
67. Yoshida, Y. & Aoyama, Y., Interaction of azole antifungal agents with cytochrome $P\text{-}450_{14DM}$ purified from *Saccharomyces cerevisiae* microsomes. *Biochem. Pharmacol.* **36** (1987) 229-235.
68. Yoshida, Y. & Aoyama, Y., Stereoselective interaction of azole antifungal agent with its target, lanosterol 14α-demethylase (cytochrome $P\text{-}450_{14DM}$): a model study with stereoisomers of triadimenol and purified cytochrome $P\text{-}450_{14DM}$ from yeast. *Chirality* **2** (1990) 10-15.
69. Ballard, S.A., Ellis, S.W., Kelly, S.L. & Troke, P.F., A novel method for studying ergosterol biosynthesis by a cell-free preparation of *Aspergillus fumigatus* and its inhibition by azole antifungal agents. *J. Med. Vet. Mycol.* **28** (1990) 335-344.
70. Hitchcock, C.A., Dickinson, K., Brown, S.B., Evans, E.G.V. & Adams, D.J., Interaction of azole antifungal antibiotics with cytochrome *P*-450-dependent 14α-sterol demethylase purified from *Candida albicans*. *Biochem. J.* **266** (1990) 475-480.
71. Guan, J., Stehmann, C., Ellis, S.W., Kerkenaar, A. & De Waard, M.A., Ergosterol biosynthesis in a cell-free preparation of *Penicillium italicum* and its sensitivity to DMI fungicides. *Pestic. Biochem. Physiol.* **42** (1992) 262-270.
72. Pye, G.W. & Marriott, M.S., Inhibition of sterol C14 demethylation by imidazole-containing antifungals. *Sabouraudia* **20** (1982) 325-329.
73. Weete, J.D. & Wise, M.L., Effects of triazoles on fungi. V. Response by a naturally tolerant species, *Mucor rouxii*. *Exp. Mycol.* **11** (1987) 214-222.
74. Deas, A.H.B., Carter, G.A., Clark, T., Clifford, D.R. & James, C.S., The enantiomeric composition of triadimenol produced during metabolism of triadimefon by fungi. III. Relationship with sensitivity to triadimefon. *Pestic. Biochem. Physiol.* **26** (1986) 10-21.
75. Gasztonyi, M. & Josepovits, G., Biochemical and chemical factors of the selective antifungal effect of triforine. I. The causes of selectivity of the contact fungicidal action. *Acta Phytopath. Acad. Sci. Hung.*, **10** (1975) 437-446.
76. Yoshida, Y., Cytochrome P450 of Fungi, Primary Target for Azole Antifungal Agents. *In*: McGinnis, M.R. (Ed.), Current Topics in Medical Mycology. Springer-Verlag, Berlin, Germany, 1988, p. 388-418.

77. Black, S. D. & Coon, M.J., *P*-450 Cytochromes: Structure and Function. *Adv. Enzymol.* **60** (1988) 35-87.
78. Schenkman, J.B., Sligar, S.G. & Cinti, D.L., Substrate interaction with cytochrome P-450. *Pharmacol. Ther.* **12** (1981) 43-71.
79. Testa, B. & Jenner, P., Inhibitors of cytochrome P-450s and their mechanism of action. *Drug Metab. Rev.* **12** (1981) 1-117.
80. Hitchcock, C.A., Cytochrome P-450-dependent 14α-sterol demethylase of *Candida albicans* and its interaction with azole antifungals. *Biochem. Soc. Trans.* **19** (1991) 782-789.
81. Goosey, M.W. & Moore, D.J., Sterol biochemistry in filamentous fungi: a coming of age. *Biochem. Soc. Trans.* **19** (1991) 769-799.
82. Aoyama, Y., Yoshida, Y., Sonoda, Y. & Sato, Y., Role of the 8-double bond of lanosterol in the enzyme-substrate interaction of cytochrome $P\text{-}450_{14DM}$ (Lanosterol 14α-demethylase). *Biochim. Biophys. Acta* **1001** (1989) 196-200.
83. Aoyama, Y., Yoshida, Y., Sonoda, Y. & Sato, Y., The 3-hydroxy group of lanosterol is essential f o r orienting the substrate in the substrate site of cytochrome $P\text{-}450_{14DM}$ (Lanosterol 14α-demethylase). *Biochim. Biophys. Acta* **1006** (1989) 209-213.
84. Aoyama, Y., Yoshida, Y., Sonoda, Y. & Sato, Y., Role of the side chain of lanosterol in substrate recognition and catalytic activity of lanosterol 14α-demethylase (cytochrome $P\text{-}450_{14DM}$) of yeast. *Biochim. Biophys. Acta* **1081** (1991) 262-266.
85. Ballard, S.A., Kelly, S.L., Ellis, S.W. & Troke, P.F., Interaction of microsomal P-450 isolated from *Aspergillus fumigatus* with fluconazole and itraconazole. *J. Med. Vet. Mycol.* **28** (1990) 327-334.
86. Guan, J., Braks, H.M.J., Kerkenaar, A. & De Waard, M.A., Interaction of microsomal cytochrome P450 isozymes isolated from *Penicillium italicum* with DMI fungicides. *Pestic. Biochem. Physiol.* **42** (1992) 24-34.
87. Marichal, P., Vanden Bossche, H., Gorrens, J., Bellens, D. & Janssen, P.A.J., Cytochrome P-450 of *Aspergillus fumigatus* - Effects of itraconazole and ketoconazole. *In*: Schuster, I. (Ed.), Cytochrome P-450: biochemistry and biophysics. Taylor & Francis, London, UK, 1988, p. 177-180.
88. Peacock, G.A. & Goosey, M. W., Separation of fungal sterols by normal-phase high-performance liquid chromatography. Application to the evaluation of ergosterol biosynthesis inhibitors. *J. Chromatogr.* **469** (1989) 293-304.
89. Vanden Bossche, H., Importance and role of sterols in fungal membranes. *In*: Kuhn, P.J., Trinci, A.P.J., Jung, M.J., Goosey, M.W & Copping, L.G. (Eds.), Biochemistry of cell walls and membranes in fungi. Springer-Verlag, Berlin Heidelberg, 1990, p. 135-157.
90. Yoshida, Y. & Aoyama, Y., Interaction of azole fungicides with yeast cytochrome P-450 which catalyzes lanosterol 14α-demethylation. *In*: Iwata, K., Vanden Bossche, H. (Eds.), In *In vitro* and *In vivo* Evaluation of Antifungal Agents. Elsevier Science Publishers B.V., Amsterdam, The Netherlands, 1986, p. 123-134.
91. Vanden Bossche, H., Bellens, D., Cools, W., Gorrens, J., Marichal, P., Verhoeven, H., Willemsens, G., De Coster, R., Beerens, D., Haelterman, C., Coene, M-C., Lauwers, W. & Le Jeune, L., Cytochrome P-450: target for itraconazole. *Drug Dev. Res.* **8** (1986) 287-298.
92. Wiggins, T.E. & Baldwin, B.C., Binding of azole fungicides related to diclobutrazol to cytochrome P450. *Pestic. Sci.* **15** (1984) 206-209.
93. Vanden Bossche, Biochemical targets for antifungal azole derivatives: hypothesis on the mode of action. *In*: McGinnis, M.R. (Ed.), Current topics in medical mycology, vol.1. Springer-Verlag, New York Berlin Heidelberg Tokyo, 1985, p. 313-350.

CHAPTER 3

BIOCHEMICAL MECHANISMS INVOLVED IN SELECTIVE FUNGITOXICITY OF TWO STEROL 14α-DEMETHYLATION INHIBITORS, PROCHLORAZ AND AN EXPERIMENTAL TRIAZOLE.

ACCUMULATION AND METABOLISM STUDIES.

Johan C. Kapteyn[a], John B. Pillmoor[b] and Maarten A. De Waard[a]

[a] Department of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, 6709 PD Wageningen, The Netherlands.

[b] Schering Agrochemicals Ltd., Chesterford Park Research Station, Saffron Walden, Essex CB10 1XL, UK.

(Accepted by Pesticide Science)

Abstract

The selective fungitoxic actions of prochloraz (an imidazole) and an experimental triazole fungicide 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4 triazol-1yl)-4(3*H*)-quinazolinone (**II**) were studied with selected phytopathogenic fungi. With the exception of *Ustilago maydis*, all the fungi tested were more sensitive to prochloraz than to **II**. A number of DMI-resistant mutants of *Penicillium digitatum* and *P. italicum* showed positive cross-resistance to both DMIs, but except for *P. italicum* isolate H17, the levels of resistance to **II** were much higher than to prochloraz.

The generally higher toxicity of prochloraz to the fungi investigated, as compared to **II**, could not be ascribed to the slightly higher accumulation of prochloraz. With regard to prochloraz, there was no general correlation between the sensitivity of the fungi tested and the amount of fungicide accumulated. A similar situation was evident for **II**. However, the DMI-resistant mutants of *P. italicum* did show a reduced accumulation of both azoles, which may account for a low level of acquired DMI-resistance in this fungus. However, since accumulation levels of the test compounds in the isolates with different

degrees of resistance were the same, additional mechanisms of resistance may be involved in isolates with relatively high degrees of DMI-resistance.

No detectable amounts of fungicide metabolites were found in most fungi tested over a 16 hour incubation period. Therefore, fungal metabolism is not generally responsible for the differences in sensitivity between fungi to each azole tested. It also does not generally explain the differential toxicities of prochloraz and II to each individual species. The exception to this was *Rhizoctonia solani* which metabolized prochloraz to a non-fungitoxic compound. This correlated with its low prochloraz sensitivity.

Introduction

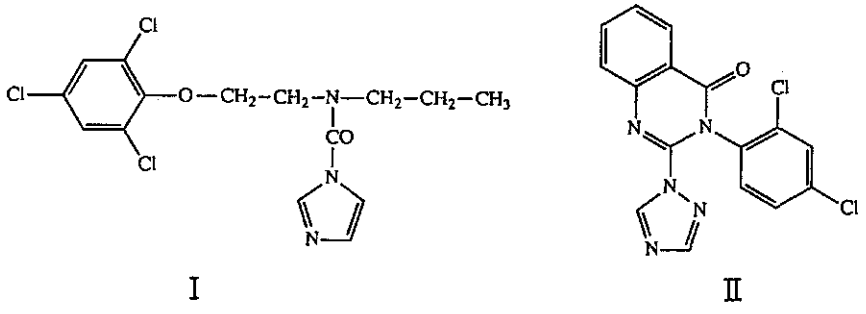
Sterol biosynthesis inhibitors (SBIs) are widely used in agriculture as fungicides combating plant diseases like powdery mildews, rusts and scabs. The primary mode of action of most SBIs is inhibition of sterol biosynthesis caused by binding of the fungicides to cytochrome P-450 dependent sterol 14α-demethylase.[1] Therefore these SBIs are called demethylation inhibitors (DMIs). The other major group of SBIs are the morpholine and piperidine derivatives which inhibit the Δ^{14}-reductase and Δ^{8}-Δ^{7}-isomerase in sterol biosynthesis.[2,3]

Relatively little is known about the mechanisms of natural insenstivity and acquired (field or laboratory) resistance of fungi to DMIs. A high degree of natural DMI insenstivity is shown by *Peronosporales* like *Pythium* and *Phytophthora* because these fungi do not synthesize sterols or do not require them for vegetative growth.[4] Growth of *Mucor* species, which do contain ergosterol as the main desmethylsterol, is also relatively insensitive to DMIs. This may be ascribed to the qualitative and quantitative requirements for sterols being less in *Mucor* than in more sensitive fungi.[5] Natural insensitivity of fungi to triforine appears to be based on either differences in fungal accumulation or metabolism of the compound.[6,7] Deas *et al.*[8] demonstrated that natural insensitivity of a number of fungi to triadimefon depends on the extent of metabolic activation of triadimefon to triadimenol and the sensitivity to the individual enantiomers of triadimenol produced.

The mechanisms of natural fungal insensitivity to DMIs may be closely related to those which account for acquired resistance. To date, decreased sensitivity to DMIs in practice has been observed in pathogens like *Erysiphe graminis* f.sp. *hordei*, *E. graminis* f.sp.*tritici*, *Sphaerotheca fuliginea*, *P. digitatum*, *Venturia inaequalis*, *Pseudocercosporella herpotrichoides* and *Rhynchosporium secalis*.[9-12] Most of these pathogens are either difficult to cultivate *in vitro* or are obligate pathogens. Therefore, studies on mechanisms of acquired resistance to DMIs have mainly been carried out with model fungi which are relatively easy to cultivate *in vitro*, like *Aspergillus nidulans*, *P. italicum*, *Monilinia fructicola* and *Nectria haematococca*.[13-17] In laboratory-generated mutants of these fungi, DMI-resistance is often based on an increased energy-dependent

efflux of the fungicide, by which accumulation is decreased.[13-17] A second proposed resistance mechanism involves an altered sterol composition, as has been found for a morpholine-resistant laboratory mutant of *Ustilago maydis* producing a yet unknown desmethylsterol instead of ergosterol.[18] Altered sterol composition has also been observed in DMI- and nystatine-resistant, sterol 14α-demethylation deficient mutants of *U. maydis*, *Candida albicans* and *Saccharomyces cerevisiae*.[19-23] Watson *et al.*[24] concluded that resistance of *S. cerevisiae* might be based on defective sterol C_{5-6} desaturation, as a result of which resistant isolates accumulated 14α-methylfecosterol, and, in contrast to sensitive strains, lacked the ability to synthesize toxic diols of 14α-methylsterols. Another possible mechanism of DMI-resistance may be reduced affinity of the target enzyme cytochrome-P450 for DMIs. In this respect, Vanden Bossche *et al.*[25-27] demonstrated that azole-resistant isolates of *C. albicans* contained cytochrome-P450 isozymes with less affinity for various azole DMIs than the azole-sensitive strains.

The present study describes the selective toxicity of two DMI-fungicides, prochloraz and the experimental compound 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4 triazol-1yl)-4(3*H*)-quinazolinone (Fig.1), to selected phytopathogenic fungi and attempts to determine whether the variation in biological activity of these azoles results from differences in accumulation and metabolism.



I II

Fig. 1. Structural formulae of prochloraz (**I**) and 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1yl)-4(3*H*)-quinazolinone (**II**).

Materials and Methods

Fungal isolates. The fungi used were *Botrytis cinerea*, *Cladosporium cucumerinum*, *Fusarium culmorum*, *Fusarium oxysporum* f.sp. *lycopersici*, *Glomerella cingulata*, *Rhizoctonia solani* and *U. maydis*. *P. italicum* W5 (wild-type) and the laboratory isolates A10-9 (low degree of DMI-resistance), E300-3 (medium degree of DMI-resistance) and H17 (high level of DMI-resistance) have been described before.[28,29] *P. herpotrichoides*

wheat- and rye-type (field isolates) were provided by Schering Agrochemicals Ltd. *Penicillium digitatum* W (wild-type) and the imazalil-resistant packing house isolates 5 and FMC originated from Janssen Pharmaceutica (Beerse, Belgium).

Chemicals. Technical prochloraz (I) and 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4 triazol-1yl)-4(3*H*)-quinazolinone (II) were provided by Schering Agrochemicals Ltd. along with U-[^{14}C] phenyl labelled prochloraz and U-[^{14}C] benzyl labelled compound II. Methanol, ethylacetate and toluene were purchased from Janssen Chimica (Beerse, Belgium); glucose and n-hexane from Lamers & Pleuger BV. (s'Hertogenbosch, The Netherlands); rifampicine from Sigma (Deisenhofen, Germany); dichloromethane, acetic acid and all other chemicals from Merck (Darmstadt, Germany).

Culture methods and preparation of mycelial suspensions. *B. cinerea*, *C. cucumerinum*, *G. cingulata*, *P. herpotrichoides* and *R. solani* were maintained on potato dextrose agar (PDA). *F. culmorum*, *F. oxysporum* f.sp. *lycopersici*, *U. maydis*, *P. italicum* and *P. digitatum* were maintained on malt extract agar (MA).

Mycelium of all filamentous fungi tested was grown in liquid medium containing 2% malt extract (Difco) and 1% mycological peptone (Oxoid L40). Flasks (300 ml) with medium (100 ml) were inoculated with 1 ml of a conidial suspension (final numbers of conidia ml^{-1} for *P. italicum* and *C. cucumerinum* 10^7 and for *F. culmorum* $2x10^5$) and incubated in an orbital shaker at 25°C and 180 rpm for 10.5, 12 and 10.5 h, respectively. For *R. solani*, 50 to 100 mycelial plugs (0.25 cm^2) were incubated at 25°C and 180 rpm for 60 h. Sporidia of *U. maydis* ($8x10^5$ ml^{-1}) were grown at 30°C and 180 rpm in a liquid medium described previously.[30] *P. herpotrichoides* ($5x10^5$ conidia ml^{-1}) was grown at 22°C and 100 rpm for 64 h. Conidia of *P. herpotrichoides* were obtained by growing mycelial plugs (5 plugs per plate) on water agar amended with rifampicine (3 μg ml^{-1}) for 12 days at 16°C under constant near UV light. The plates were flooded with 1.5 ml sterile distilled water and the conidia were removed by scraping the agar surface with a glass rod. Conidial suspension (1 ml) was spread onto PDA and incubated under near UV light for 7 days at 16°C. This produces a mass of conidia (R.J. Birchmore, personal communication).

Standard mycelial suspensions of all filamentous fungi were made by passing the suspension through a 0.21 mm pore sieve to remove clusters of mycelium and then collecting mycelium on a 0.02 mm pore sieve. Mycelium collected was washed once with tap water and twice with potassium phosphate buffer pH 6, containing 0.1 mM $CaCl_2$ and 50 mM glucose. Before sieving, mycelium of *R. solani* was partially fragmented for 10 s in a Polytron homogenizer (Kinematica, Kriens-Luzern, Switzerland). Sporidia of *U. maydis* were harvested by centrifugation at 2000*g* for 5 min and washed twice with potassium phosphate buffer. Standard biomass suspensions (average dry weight of about 3 mg ml^{-1}) of most test fungi were made by resuspending washed mycelium or sporidia (1 g

wet weight) in 50 ml of potassium phosphate buffer pH 6 as described previously.[13,14] Standard biomass suspensions of *P. herpotrichoides* were made by resuspending 1 g wet weight of mycelium in 100 ml buffer.

Toxicity assay. Toxicities of prochloraz and II to radial fungal growth were determined in Petri dishes containing PDA amended with these fungicides at various concentrations (from 1000-fold stock solutions in methanol). For *U. maydis*, MA was used. Inverted 5-mm agar discs with young mycelium or sporidia were placed on the agar surface (in duplicate, 3 discs per plate). All fungi but *P. herpotrichoides* (20°C) and *U. maydis* (30°C) were incubated at 25°C.

Accumulation studies. The amount of radioactive fungicides associated with the mycelium was determined as described below. Standard biomass suspensions (50-80 ml) of *F. culmorum*, *P. herpotrichoides*, *P. italicum* and *R. solani* were preincubated in a reciprocal water bath shaker at 25°C for 30 min. Standard biomass suspensions of *C. cucumerinum* and *U. maydis* were incubated under similar conditions at 22 and 30°C, respectively. Experiments were started by adding [^{14}C]prochloraz (spec. activity 1.3 MBq mmol^{-1}) and [^{14}C]compound II (spec. activity 1.2 MBq mmol^{-1}) from 100-fold stock solutions in methanol to final concentrations of 90 μM. At time intervals samples (5 ml) were taken, processed and radio-assayed as described previously.[13,14]

Metabolism studies. Metabolism of prochloraz and II was studied with standard biomass suspensions (20 ml) made from 16 h old liquid cultures by resuspending 1 g wet weight of biomass in 50 ml of fresh media. The metabolism experiments were initiated by adding [^{14}C]prochloraz (spec. activity 1.3 MBq mmol^{-1}) and [^{14}C]compound II (1.2 MBq mmol^{-1}) to final concentrations of 0.3 and 90 μM, respectively. Samples (10 ml) were taken after an additional 16 h incubation period and filtered on Whatman glass filter papers using a Millipore sampling manifold apparatus. [^{14}C]Label was extracted from mycelium or sporidia with dichloromethane (20 ml) by grinding in a Potter-type homogenizer. Label in culture filtrates was extracted twice with dichloromethane (10 ml). Extracts were dried under reduced pressure, resuspended in dichloromethane (0.5 ml) and spotted on silica gel TLC-plates (Merck, 60 F_{254}). The plates were developed immediately with ethylacetate/toluene (3:2) or ethylacetate/hexane/acetic acid (8:2:0.1) as solvent systems. Radioactive metabolites and fungicides were located by autoradiography using diagnostic films (Kodak, X-OMAT™ AR). The TLC plates were also bioassayed for fungicidal activity by spraying with a conidial suspension of *P. italicum* W5 in 1% KH_2PO_4, 0.43% $Na_2HPO_4.2H_2O$, 0.57% KNO_3, 0.14% MgSO4.7H_2O and 4.3% glucose. Growth was assessed after 3 days of incubation in glass trays at 100% R.H. and 25°C. Radioactivity in mycelial or sporidial extract, culture filtrate extract, mycelial or sporidial

(solid) residue and extracted culture filtrate (aqueous phase) was determined in a liquid scintillation system as described previously.[13,14]

Results

Toxicity assay. EC_{50} values of prochloraz to radial growth of the fungi tested ranged from 0.01 to 4.0 μM. *C. cucumerinum*, *F. culmorum* and *P. italicum* W5 were the most sensitive species, while *R. solani* and *U. maydis* were the least sensitive ones (Table 1).

Table 1: Toxicity of prochloraz and **II** to various fungal pathogens in radial growth assays on agar.

Fungus	Incubation period	EC_{50} (μM)	
	(days)	Prochloraz	**II**
B. cinerea	2	0.07	0.61
C. cucumerinum	7	0.01	0.09
F. culmorum	2	0.01	40.5
F. oxysporum f.sp. *lycopersici*	2	0.03	4.5
G. cingulata	3	0.05	0.59
P. digitatum W	3	0.02 (1)[1]	0.67 (1)[1]
P. digitatum 5	3	0.10 (5)	517 (772)
P. digitatum FMC	3	0.12 (6)	461 (688)
P. italicum	3	0.01 (1)	7.8 (1)
P. italicum A10-9	3	0.06 (6)	154 (20)
P. italicum E300-3	3	0.13 (13)	501 (64)
P. italicum H17	3	1.12 (112)	531 (68)
P. herpotrichoides wh	10	0.09	0.33
P. herpotrichoides ry	14	0.09	19.6
P. oryzae	4	0.03	5.0
R. solani	2	3.7	5.0
U. maydis	9	4.0	1.2

[1] ( ): resistance factor defined as ratio between EC_{50} of prochloraz or **II** for resistant and wild-type isolate.

Except for *U. maydis* all fungi tested were much less sensitive to **II** than to prochloraz (Table 1). EC_{50} values of **II** varied from 0.09 to 531 μM. *C. cucumerinum* appeared to be

the most sensitive fungus, while *P. italicum* isolate H17 had the lowest sensitivity. The DMI-resistant *P. italicum* isolates A10-9, E300-3 and H17 exhibited positively correlated cross-resistance to prochloraz and **II** (Table 1). The levels of resistance in isolates A 10-9 and E300-3 to prochloraz were lower than to **II**. In contrast, isolate H17 was more resistant to prochloraz than to **II**. Positively correlated cross-resistance to both azoles was also observed in imazalil-resistant isolates of *P. digitatum*. Both isolates showed a similar level of resistance, but were much more resistant to **II** than to prochloraz (Table 1).

Since *C. cucumerinum*, *F. culmorum*, *P. herpotrichoides* wheat- and rye-type, *P. italicum* isolates W5, E300-3 and H17, *R. solani* and *U. maydis* exhibited marked differences in sensitivity to prochloraz and **II**, these fungi were selected for accumulation and (except for *F. culmorum* and *P. italicum* E300-3) metabolism studies. The mechanism of DMI-resistance in *P. digitatum* is studied by Janssen Pharmaceutica (Vanden Bossche, pers. commun.)

Short term accumulation studies. Time course studies of the accumulation of the two compounds by the fungi investigated are shown in Figures 2-4 and the maximum levels accumulated are given in Table 2. All the fungi investigated accumulated prochloraz to a higher, maximum level than they accumulated **II** (Table 2). Maximum accumulation of prochloraz and **II** by the fungi tested ranged from 2.3 to 12.9 and 0.4 to 8.6 nmol mg^{-1} dry weight of biomass, respectively. Both types of *P. herpotrichoides* accumulated

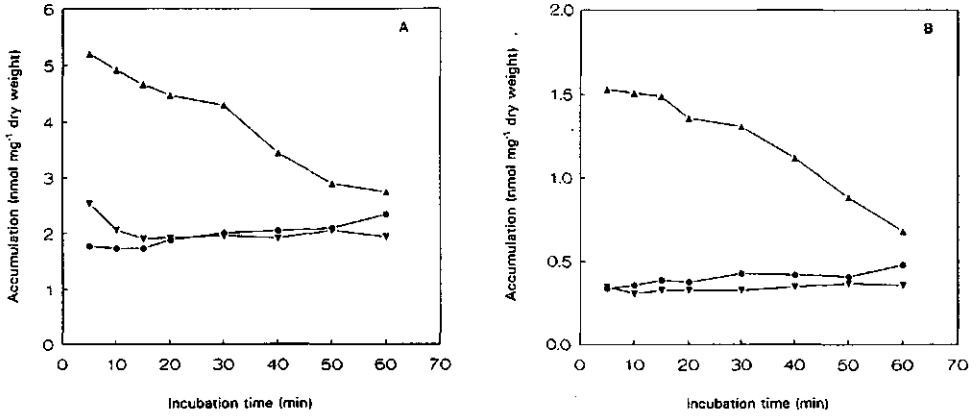


Fig. 2. Accumulation of (A) [^{14}C]prochloraz and (B) [^{14}C]compound **II** by wild-type W5 (▲) and DMI-resistant isolates E300-3 (●) and H17 (▼) of *Penicillium italicum*. Initial external DMI concentration 90 μM.

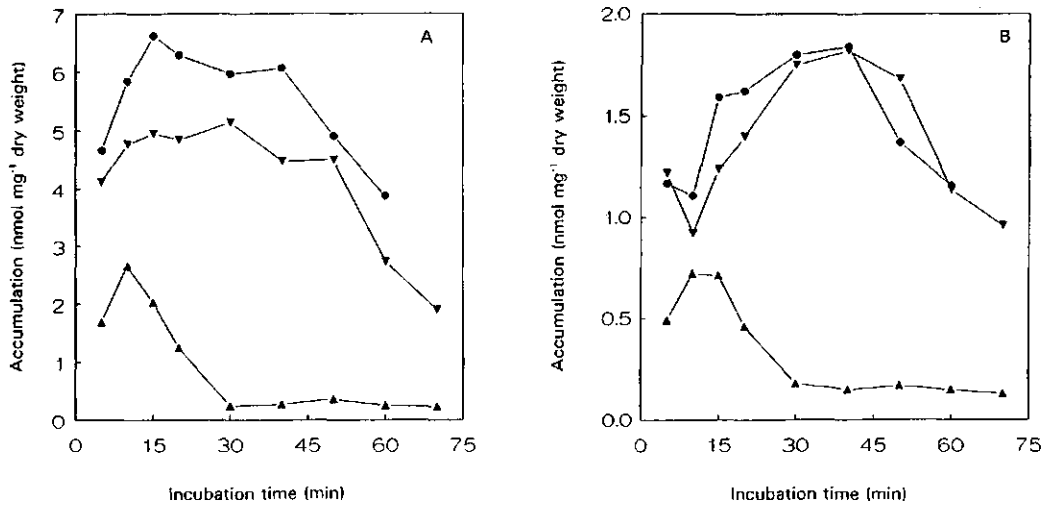


Fig. 3. Accumulation of (A) [^{14}C]prochloraz and (B) [^{14}C]compound **II** by *Cladosporium cucumerinum* (•), *Rhizoctonia solani* (▼) and *Ustilago maydis* (▲). Initial external DMI concentration 90 μM.

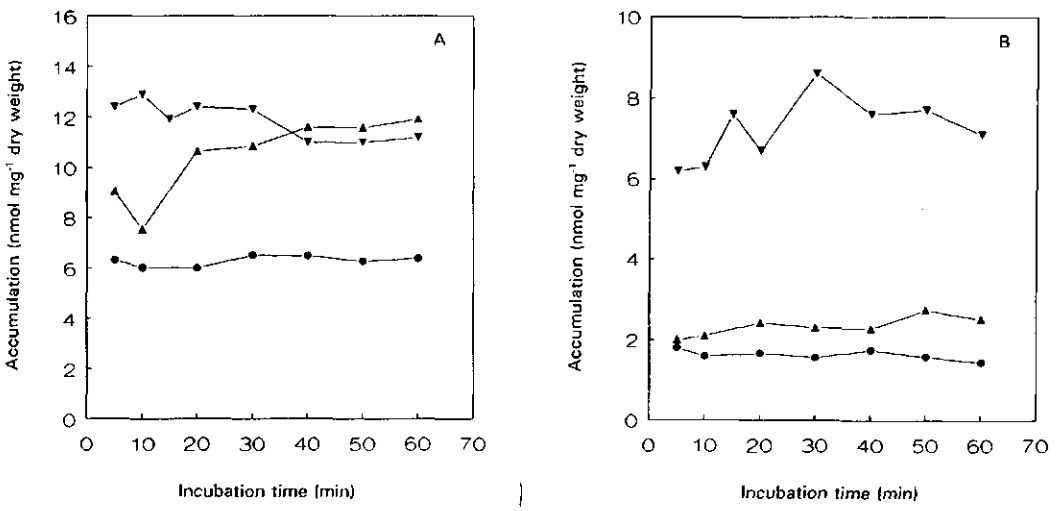


Fig. 4. Accumulation of (A) [^{14}C]prochloraz and (B) [^{14}C]compound **II** by *Fusarium culmorum*, (•), *Pseudocercosporella herpotrichoides* wheat- (▲) and rye-type (▼). Initial external DMI concentration 90 μM.

Table 2. Maximum accumulation of [^{14}C]prochloraz and [^{14}C]compound **II** by various fungal species. Initial external DMI concentration was 90 μM.

Fungus	Maximum[1] accumulation (nmol mg^{-1} dry weight)	
	Prochloraz	**II**
C. cucumerinum	6.6	1.8
F. culmorum	6.5	1.8
P. herpotrichoides wheat	12.0	2.7
P. herpotrichoides rye	12.9	8.6
P. italicum W5	5.2	1.5
P. italicum E300-3	2.3	0.5
P. italicum H17	2.5	0.4
R. solani	5.1	1.8
U. maydis	2.7	0.7

[1] Maximum levels of compound associated with the mycelium, taken from Figs. 2-4.

prochloraz to the greatest level, as compared with the other fungi. *P. herpotrichoides* rye-type also accumulated **II** to a higher level, as compared with the other fungal species. In contrast to the situation for prochloraz, where both types of *P. herpotrichoides* accumulated similar levels, the wheat-type accumulated much less **II** than did the rye-type.
U. maydis and the two resistant isolates of *P. italicum* accumulated the lowest amounts of both compounds, although 3-4 times more prochloraz than **II** was accumulated in all three cases.

P. italicum W5 showed a transient accumulation of prochloraz and **II**, while uptake by resistant isolates E300-3 and H17 was constantly low during the time course of the experiment (Fig. 2). Accumulation of prochloraz by both resistant isolates was similar. The same was true for **II**. *U. maydis* also showed transient accumulation of both compounds, but the accumulation levels were about 2 times lower than observed for *P. italicum* W5 (Fig. 3). *R. solani* and *C. cucumerinum* showed less pronounced transient accumulation curves (Fig. 3). Azole accumulation by *F. culmorum* and *P. herpotrichoides* (wheat- and rye-type) was relatively constant with time (Fig. 4).

Metabolism studies. Total recoveries of radioactivity from the fungi incubated with labelled prochloraz and **II** varied from 64 to 89% and 77 to 100%, respectively (Table 3 and 4). Total recovery of label in *P. herpotrichoides* wheat- and rye-type incubated with [^{14}C]prochloraz was low compared with the values obtained from other fungi, but this was not investigated further. After 16 h incubation with prochloraz radioactivity in mycelium of *R. solani* was relatively low compared with the other fungi tested (Table 3).

Table 3: Recovery (%) of added radioactivity from fungal cultures incubated with [^{14}C]prochloraz (0.3 μM) for 16 h.

Fungus	Replicate	Mycelium		Culture filtrate		Total
		Extract	residue	Organic phase	Aqueous phase	
C. cucumerinum	1	50	16	17	1	84
P. italicum W_5	3	44	3	41	1	89
P. italicum $E_{300\text{-}3}$	3	37	4	40	1	82
P. herpotrichoides wheat	2	34	9	20	1	64
P. herpotrichoides rye	2	26	8	32	1	67
R. solani	2	20	12	38	14	84
U. maydis	2	35	10	35	1	81

Table 4: recovery (%) of added radioactivity from fungal cultures incubated with **II** (90 μM) for 16 h.

Fungus	Replicate	Mycelium		Culture filtrate		Total
		Extract	residue	Organic phase	Aqueous phase	
C. cucumerinum	1	34	6	40	1	81
P. italicum W_5	3	14	1	75	2	92
P. italicum $E_{300\text{-}3}$	3	12	1	73	3	89
P. herpotrichoides wheat	2	14	6	60	1	81
P. herpotrichoides rye	2	16	4	56	1	77
R. solani	2	16	4	78	1	100
U. maydis	2	54	8	31	1	94

TLC of mycelial and culture filtrate extracts of all fungi incubated with [^{14}C]compound **II** for 16 h and developed with two different solvent systems did not demonstrate any metabolite of this triazole (data not shown). Similar results were obtained with prochloraz (data not shown), with the exception of *R. solani*. TLC of mycelial and culture filtrate extracts of this fungus indicated the production of one metabolite after 16 h incubation with prochloraz (Fig. 5). Bioassays of the TLC-plates by incubating them with a conidial suspension of *P. italicum* W5 demonstrated that the metabolite was non-fungitoxic (data not shown). It was also tested whether extracellular enzymes were responsible for the

metabolic degradation of prochloraz. For this purpose culture filtrate of *R. solani* was incubated for another 16 h with [^{14}C]prochloraz and extracted. TLC of this extract did not reveal any metabolites (data not shown).

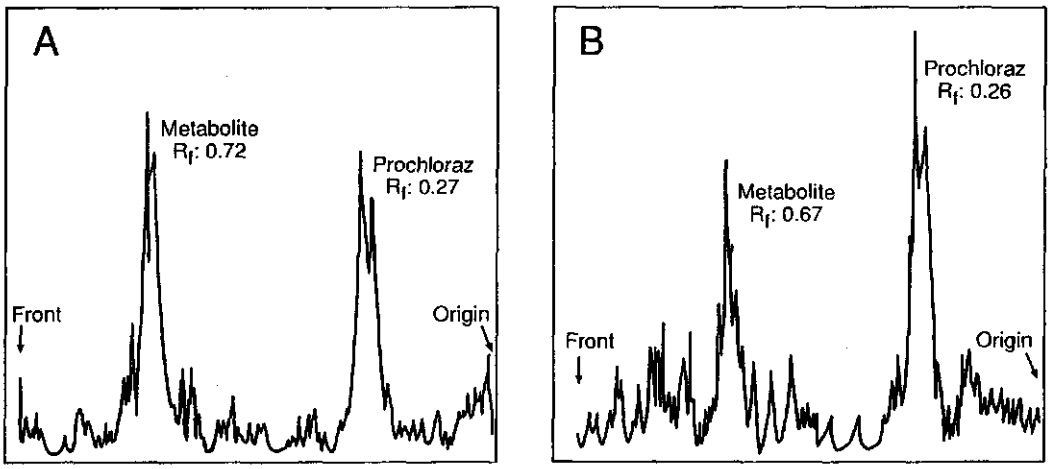


Fig. 5: Radioactivity TLC patterns from (A) an extract of the culture filtrate and (B) a mycelial extract of *Rhizoctonia solani* incubated with ^{14}C-prochloraz for 16 h at 25°C. Solvent system was ethylacetate:toluene, 3:2.

Discussion

Investigation of the fungitoxicity of prochloraz and II to a number of fungi in *in vitro* radial growth assays showed a range of sensitivities. Prochloraz was extremely toxic to all the fungi tested, except for *U. maydis* and *R. solani* (Table 1). In contrast, II showed much more variation in toxicity and was less active than prochloraz in all cases except against *U. maydis*. The difference in senstivity between both types of *P. herpotrichoides* to II is in line with data obtained with other triazoles.[12] As was expected, the DMI-resistant isolates of *P. italicum* and *P. digitatum* were less sensitive to the two compounds as compared with the wild type strains. However, the resistance factors of individual strains to the two compounds did differ markedly in most cases, indicating that the resistance mechanisms were not always equally effective in preventing the toxicity of the compounds.

From the initial toxicity studies, 9 fungi that exhibited marked differences in their responses to the two compounds were selected for further study to determine whether differences in uptake or metabolism could account for the observed differences in sensitivity.

The accumulation studies showed that there was no overall correlation between senstivity and the maximum amount of compound accumulated by the wild type fungi investigated for either prochloraz or II (Table 2). For example II was accumulated to a similar level by sensitive (*C. cucumerinum* and *P. herpotrichoides* wheat-type) and insensitive (*F. culmorum*, *P. italicum* and *R. solani*) species. *U. maydis* accumulated the lowest levels of II despite being of intermediate sensitivity. The highest levels of II were accumulated by the relatively insensitive *P. herpotrichoides* rye-type. Hence, a negative correlation exists between sensitivity and accumulation of II in wheat- and rye-types of *P. herpotrichoides* (Table 1 and 2). No obvious explanation for this can be given, but it might be that in the rye-type the availability of II at the target site is reduced by deposition in other cell compartments. For prochloraz, the three most senstitive fungi (*P. italicum*, *C. cucumerinum* and *F. culmorum*) accumulated a similar level of prochloraz as did the relatively insensitive *R. solani*. The two types of *P. herpotrichoides*, which were about 9 times more insensitive than the three most sensitive species, accumulated some 2 times more prochloraz than did the sensitive fungi. However, it is noted that the accumulation studies with *P. herpotrichoides* were undertaken with approximately half the biomass density than used for the other fungi and this may have resulted in a relatively higher level of accumulation.

Despite the lack of any overall correlation between sensitivity and maximum accumulation, it is noted that *U. maydis*, which was the most prochloraz insensitive species, did show a rapid transient and low accumulation of prochloraz (Fig. 3A). The low accumulation in this fungus may contribute to, but cannot fully explain, its more than 300-fold lower prochloraz sensitivity as compared with the other fungi.

Investigation of the accumulation of the two compounds by laboratory-generated mutants of *P. italicum* also showed reduced maximum accumulation of both compounds in the resistant isolates as compared with the wild-type isolate (Fig. 2). As has been described for other DMIs[15], accumulation of the compounds by the wild-type isolate showed an initial high accumulation followed by efflux. In contrast, the resistant isolates showed consistently low accumulation during the course of the experiment. Reduced initial accumulation may therefore be a major reason for the resistance of isolates E300-3 to both compounds and of H17 to II. However, isolate H17 showed similar accumulation of prochloraz to that of isolate E300-3, despite being some 10 times more resistant. In this case, the resistance of isolate H17 to prochloraz must involve an additional, as yet unknown, mechanism. These findings agree with those of Ney[16], who demonstrated that reduced accumulation alone could not account for the differential, laboratory-generated triazole-resistance in *M. fructicola*.

Similar, transient accumulation curves to that of wild-type *P. italicum* were also evident for *C. cucumerinum*, *R. solani* and *U. maydis* (Fig. 3). This might indicate that these fungi also possess an inducible, energy-dependent efflux mechanism, but this still remains to be confirmed. Transient accumulation did not, however, correlate with sensitivity as

both the sensitive *C. cucumerinum* and the relatively insensitive *R. solani* exhibited transient accumulation curves. *F. culmorum* and *P. herpotrichoides* wheat- and rye-type (Fig. 4) did not show transient accumulation which might indicate that these fungi possess constitutive efflux systems similar to those of *P. italicum* isolates E300-3 and H17. However, appearance of transient accumulation also depends on the initial external concentration and the nature of the DMI added. With respect to the latter aspect it is interesting to note that *F. culmorum* did, but *U. maydis* did not exhibit transient accumulation when incubated with 30 μM fenarimol (De Waard, unpublished results) in contrast to the present results. Furthermore, membrane or cell wall characteristics of these fungi might also account for the absence of transient accumulations. In this respect, it is noted that *P. herpotrichoides* produces a layer of mucilage around the hyphae.[31] This might have hindered or masked azole efflux by high non-specific binding of the compounds.

Comparison of the selective fungitoxicity of prochloraz and **II** showed that prochloraz was accumulated to a greater maximum level than was **II** in all cases (Table 2). However, the greater accumulation of prochloraz is not considered to provide a general explanation for the greater toxicity of this compound as compared with **II**. While the greater accumulation of prochloraz by *C. cucumerinum*, *P. herpotrichoides* wheat-type and *R. solani* might partially explain the observed 3 to 5 fold differences in sensitivity, it does not seem plausible that the same differences in uptake for *P. italicum* wild-type and *F. culmorum* can account for the up to 3000-fold differences in toxicity of the compounds. It is also noted that *U. maydis* was less sensitive to prochloraz than to **II**, despite accumulating more prochloraz.

Investigation of the metabolism of the two compounds demonstrated no evidence of metabolism of either compound over a 16 hour incubation period for most of the fungi tested. Therefore, differential sensitivity of the fungi to the two compounds is not generally due to metabolism. The only exception to this was for *R. solani* which did significantly metabolise prochloraz, but not compound **II**, to a non-fungitoxic compound (unpublished result). In this case, metabolism may account for the low sensitivity of this species to prochloraz. The metabolite was detected in both the mycelial and culture filtrate extracts but was not formed by incubation of prochloraz with the isolated culture filtrate for 16 hours (unpublished result). The metabolite is therefore probably produced in the mycelium and excreted into the medium. This assumption is supported by the fact that, after 16 h incubation with prochloraz, the amount of radiolabel in mycelium of *R. solani* was lower than in the other fungi tested (Table 2).

The present work has demonstrated that the selective fungitoxicity of prochloraz and **II** to a number of fungal species is not generally due to differential accumulation or metabolism. Consequently, other factors must also be involved in the observed selective fungitoxicity, for example differential affinity of the cytochrome-P450 to these DMIs,

altered sterol composition, removal of the toxic sterol precursors etc. Some of these mechanisms have been investigated in other studies.[32]

Acknowledgements:

The authors would like to thank Prof. Dr. J. Dekker and Drs. R.J. Milling, K. Wright and P. Russell for their critical comments. J.C. Kapteyn thanks Dr. R.J. Birchmore for providing cultures of *P. herpotrichoides* and acknowledges the financial support of Schering Agrochemicals Ltd., UK.

References

1. Kato, T., In *Chemistry of Plant Protection, Vol. 1*, ed. G. Haug & H. Hoffmann. Springer-Verlag, Berlin, 1986, p. 1-24.
2. Baloch, R.I. & Mercer, E.I., *Phytochemistry* **26** (1986) 663-668.
3. Kerkenaar, A., In *Modern Selective Fungicides, Properties, Applications, Mechanism of Action*, Lyr H.(Ed.), VEB Gustav Fischer Verlag, Jena, 1987, p. 159-172.
4. Weete, J.D., *Phytochemistry* **12** (1973) 1843-1864.
5. Weete, J.D. & Wise, M.L., *Exp. Mycol.* **11** (1987) 214-222.
6. Gasztonyi, M. & Josepovits, G., *Acta Phytopathol. Sci. Hung.* **10** (1975) 437-446.
7. Gasztonyi, M. & Josepovits, G., *Pestic. Sci.* **15** (1984) 48-55.
8. Deas, A.H.B., Carter, G.A., Clark, T., Clifford, D.R. & James, C.S., *Pestic. Biochem. Physiol.* **26** (1986) 10-21.
9. Köller, W., In *Fungicide Resistance in North America*, ed. C.J. Delp. APS Press, St. Paul, Minnesota USA, 1988, p. 79-88.
10. Brent, K.J. & Hollomon, D.W., In *Sterol Biosynthesis Inhibitors, Pharmaceutical and Agrochemical Aspects*, ed. D. Berg & M. Plempel. Ellis Horwood Ltd., Chichester, 1988, p. 332-346.
11. Hildebrand, P.D., Lockhart, C.L., Newbury, R.J. & Ross, R.G., *Can. J. Pl. Pathol.* **10** (1988) 311-316
12. Leroux, P., Gredt, M. & Boeda, P., *Pestic. Sci.* **23** (1988) 119-129.
13. De Waard, M.A. & Van Nistelrooy, J.G.M., *Neth. J. Pl. Pathol.* **90** (1984) 143-153.
14. De Waard, M.A. & Van Nistelrooy, J.G.M., *Exp. Mycol.* **11** (1987) 1-10.
15. De Waard, M.A. & Van Nistelrooy, J.G.M., *Pestic. Sci.* **22** (1988) 371-382.
16. Ney, C., Thesis, University Basel (1988).
17. Kalamarakis, A.E., De Waard, M.A., Ziogas, B.N. & Georgopoulos, S.G., *Pestic. Biochem. Physiol.* **40** (1991) 212-220.
18. Leroux, P. & Gredt, M., *Pestic. Sci.* **15** (1984) 85-89.
19. Walsh, R.C. & Sisler, H.D., *Pestic. Biochem. Physiol.* **18** (1982) 122-131.
20. Aoyama, Y., Yoshida, Y., Hata, S., Nishino, T., Katsuki, H., Maitra, U.S., Mohan, V.P. & Sprinson, D.B., *J. Biol. Chem.* **258** (1983) 9040-9042.
21. Aoyama, Y., Yoshida, Y., Nishino, T., Katsuki, H., Maitra, U.S., Mohan, V.P. & Sprinson, D.B., *J. Biol. Chem.* **262** (1987) 14260-14262.
22. King, D.J., Wiseman, A., Kelly, D.E. & Kelly, S.L., *Current Gen.* **10** (1985) 261-267.

23. Bard, M., Lees, N.D., Barbuch, R.J. & Sanglard, D., *Biochem. Biophys. Res. Commun.* **147** (1987) 794-800.
24. Watson, P.F., Rose, M.E., Ellis, S.W., England, H. & Kelly, S.L., *Biochem. Biophys. Res. Commun.* **164** (1989) 1170-1175.
25. Vanden Bossche, H., Marichal, P., Gorrens, J., Bellens, D., Verhoeven, H., Coene, M-C., Lauwers, W. & Janssen, P.A.J., *Pestic. Sci.* **21** (1987) 1-18.
26. Vanden Bossche, H., Marichal, P., Gorrens, J., Bellens, D., Moereels, H. & Janssen, P.A.J., *Biochem. Soc. Trans.* **18** (1990) 56-59.
27. Vanden Bossche, H., Willemsens, G. & Marichal, P., *CRC Crit. Rev. Microbiol.* **15** (1987) 57-72.
28. De Waard, M.A., Groeneweg, H. & Van Nistelrooy, J.G.M., *Neth. J. Pl. Pathol.* **88** (1982) 99-112.
29. De Waard, M.A. & Van Nistelrooy, J.G.M., *Neth. J. Pl. Pathol.* **96** (1990) 321-29
30. Coursen, C. & Sisler, H., *Am. J. Botany* **47** (1964) 541-549.
31. Reiss, J., *Trans. Brit. Mycol. Soc.* **56** (1971) 481-482.
32. Kapteyn, J.C., Pillmoor, J.B. & De Waard, M.A., *Pestic. Sci.* **34** (1992) 37-43.

CHAPTER 4

CHARACTERISATION OF ENERGY-DEPENDENT EFFLUX OF IMAZALIL AND FENARIMOL IN ISOLATES OF *PENICILLIUM ITALICUM* WITH A LOW, MEDIUM AND HIGH DEGREE OF RESISTANCE TO DMI-FUNGICIDES

J. Guan[1], J.C. Kapteyn[1], A. Kerkenaar[2] and M. A. De Waard[1]

[1] Department of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, 6709 PD Wageningen, the Netherlands.

[2] Denka International B.V., Hanzeweg 1, 3771 NG Barneveld, the Netherlands

(Netherlands Journal of Plant Pathology, 1992, **98**, 313-324)

Abstract

Differential accumulation of [^{14}C]imazalil and [^{14}C]fenarimol by germlings of wild-type and DMI-resistant isolates of *Penicillium italicum* was studied at various pH values. At pH 7 and 8 the low resistant isolate $E_{300\text{-}3}$ accumulated 22% and 35% less imazalil than the wild-type isolate W_5, respectively. Imazalil accumulation at pH 5 and 6 was similar. Isolate $E_{300\text{-}3}$ also accumulated less fenarimol as compared with the wild-type isolate. This difference was much more obvious than for imazalil and was observed at all pH values tested. Differences in accumulation of both imazalil and fenarimol between low ($E_{300\text{-}3}$), medium (H_{17}) and high resistant (I_{33}) isolates were not observed. These results suggest that decreased accumulation of DMIs is responsible for a low level of resistance only and that additional mechanisms of resistance might operate in isolates with a medium and high degree of resistance. With all isolates fenarimol accumulation was energy-dependent. This was not obvious for imazalil.

The wild-type and DMI-resistant isolates had a similar plasma membrane potential as determined with the probe [^{14}C]tetraphenylphosphonium bromide ([^{14}C]TPP$^+$). Various test compounds among which ATPase inhibitors, ionophoric antibiotics, calmodulin antagonists affected the accumulation of [^{14}C]TPP$^+$, [^{14}C]imazalil and [^{14}C]fenarimol. No obvious correlation between the effects of the test compounds on accumulation levels of

the fungicides and [^{14}C]TPP$^+$ could be observed. These results indicate that the plasma membrane potential does not mediate the efflux of DMI fungicides by *P. italicum*.

Introduction

Sterol biosynthesis inhibitors (SBIs) are systemic fungicides used in agriculture for the control of plant diseases. The primary mode of action of most SBIs (e.g. derivatives of imidazoles, triazoles and pyrimidines) is based on inhibition of cytochrome P450-dependent sterol 14α-demethylation.[1] Therefore, these SBIs are referred to as demethylation inhibitors (DMIs).

Resistance to DMIs has been reported for a number of fungi in both laboratory and field.[2,3] Studies with various fungi suggested that a large number of potential mechanisms of resistance can be involved.[4-12] A well documented mechanism is energy-dependent efflux of DMI-fungicides from mycelium of *Aspergillus nidulans* and *Penicillium italicum*. Increased efflux from fenarimol-resistant mutants of these fungi leads to secretion of DMIs into the external medium and hence prevents intracellular accumulation. Consequently, the target enzyme becomes less readily inhibited and the mutants less sensitive.[13-16] The ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD) and the plasma membrane ATPase inhibitor sodium orthovanadate were found to inhibit efflux causing accumulation of all DMIs tested.[15] This might be a consequence of dissepation of the electrochemical proton gradient ($\Delta\tilde{\mu}_H+$) maintained by plasma membrane ATPase across plasma membranes.[15] The $\Delta\tilde{\mu}_H+$ is composed of a proton gradient (Δ_H+) and a plasma membrane potential ($\Delta\psi$) and is known to drive transport processes of various xenobiotics such as benzoic and sorbic acid[17] and aminoglycoside antibiotics,[18] respectively. Accumulation of the aminoglycoside antibiotic gentamycin by *Staphylococcus aureus* was also found to be enhanced by DCCD.[19] DMI-resistant isolates of *A. nidulans* showed cross-resistance to the aminoglycoside antibiotic neomycin.[20] These results suggest that a similar mechanism may mediate the accumulation of DMIs and aminoglycosides. In addition, resistance to aminoglycoside antibiotics in *Saccharomyces cerevisiae* and *Escherichia coli* was found to correlate with decreased intracellular accumulation and was ascribed to a defect $\Delta\psi$.[21,22] Therefore, we investigated the $\Delta\psi$ of wild-type and DMI-resistant isolates of *P. italicum* using [^{14}C]tetraphenylphosphonium bromide ([^{14}C]TPP$^+$), a lipophilic cation, as a probe.

Accumulation of fenarimol and other DMIs, and of imazalil by a wild-type and a fenarimol-resistant isolate of *P. italicum* with a low degree of resistance to imazalil, differed in various aspects.[16] The main difference was that the differential accumulation by low-resistant isolates was obvious for fenarimol but absent for imazalil. Therefore, in the present study fenarimol and imazalil were selected as test compounds. The study compares the accumulation of these fungicides in isolates of *P. italicum* with a low, medium and high degree of resistance at different pH values. In addition, the effect of

compounds with known membrane-interfering properties on accumulation of [^{14}C]TPP$^+$ was compared with their effect on accumulation of both fungicides.

Materials and methods

Fungal isolates. *P. italicum* wild-type isolate W_5 and DMI-resistant isolates $E_{300\text{-}3}$, H_{17} and I_{33} with a low, medium and high degree of resistance, respectively, were used. The isolates have been described previously.[23,24] The fungus was maintained on malt-extract agar medium.

Culture methods and preparation of germling suspensions. Germlings were grown in liquid malt-extract medium.[14] Flasks (300 ml) with medium (100 ml) were inoculated with 1 ml of spore suspension (10^9 conidia per ml) collected from 7 to 10 days old agar plates. The flasks were incubated at 26°C on an orbital shaker at 200 rpm for 11 h. Germlings were harvested on a sieve (0.21 mm pores) and collected on a second sieve (0.02 mm pores) by intensive washing with tap water. Germlings were washed once again with 25 mM potassium phosphate buffer pH 7.0, containing 0.1 mM calcium chloride and 1% (w/v) glucose. Standard suspensions of germlings with an average dry weight of about 2 mg ml^{-1} were prepared by resuspending 1 g wet weight of germlings in 50 ml of the same buffer.

Fungicides and chemicals. [^{14}C]imazalil and imazalil sulphate (imazalil) were gifts from Janssen Pharmaceutica (Beerse, Belgium); [^{14}C]fenarimol from Lilly Research Center Ltd. (Erl Wood Manor, England). [^{14}C]tetraphenylphosphonium bromide ([^{14}C]TPP$^+$) was purchased from Amersham, (UK), TPP$^+$ from ICN Pharmaceuticals Inc. (New York, USA); calmidazolium, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), chlorpromazine, diethylstilbestrol (DES), gramicidine-S, nigericine and triflupromazine (TFP) from Sigma (Deisenhofen, Germany). Sodium orthovanadate (vanadate) and valinomycine and from Janssen Chimica (Beerse, Belgium). Fungicides and chemicals were prepared as 100-fold concentrated solution in methanol.

Determination of plasma membrane potential. Standard germling susensions (70 ml) in flasks (300 ml) were incubated on a reciprocal shaker at 26°C for 30 min. [^{14}C]TPP$^+$ (sp. act. 17.3 MBq mmol^{-1}), a commonly used probe for assessing the $\Delta\psi$,[25] was added to a final concentration of 10 μM. At time intervals, samples (5 ml) were taken and filtered on a Whatman GF/A glass filter using a Millipore sampling manifold apparatus. Pellets of germlings were washed 3 times with 1 mM $MgSO_4$ (5 ml) in 10 sec. This washing step reduces aspecific binding of [^{14}C]TPP$^+$ to anionic groups at the cell wall surface of the germlings.[25] A correction of [^{14}C]TPP$^+$ accumulation for internal, aspecific probe binding was made by subtracting the amount of probe bound under deenergized conditions from

the total amount of accumulated probe under energized conditions.[25-27] In preliminary experiments, remaining background adsorption of [^{14}C]TPP$^+$ by deenergized germlings was studied after various treatments: incubation with 0.2 M KCl for 50 min, incubation without carbon source in the presence of 0.2 or 1.0 M KCl for 6 h, heating at 100°C for 5 min, and incubation with 1% (v/v) toluene at 37°C or 50°C for 1 h according to Lolkema *et al.*[27] Germling-associated radioactivity was extracted with scintillation liquid (Aqua luma plus) overnight and counted in a liquid scintillation spectrometer. After correction of [^{14}C]TPP$^+$ accumulation for aspecific probe binding the $\Delta\psi$ was estimated with the Nernst equation.[25] The intracellular volume of *P. italicum* was taken to be 2.3 μl mg^{-1} dry weight of germlings.[16] Effects of test compounds on accumulation of [^{14}C]TPP$^+$ were studied by addition from 500-fold concentrated stock solutions in methanol, 50 min after the addition of [^{14}C]TPP$^+$. Accumulation of [^{14}C]TPP$^+$ was determined in samples (5 ml) taken 5 and 15 min after addition of the test chemicals. The correspondent amount of methanol was added to the controls. In these experiments [^{14}C]TPP$^+$ accumulation in the presence of test compounds was expressed as nmol mg^{-1} dry weight. The corresponding $\Delta\psi$ values were not calculated since the effect of the test compounds on aspecific probe binding was not determined.

Accumulation of [^{14}C]imazalil and [^{14}C]fenarimol. Experiments were carried out according to the method previously described.[14] Standard germling suspensions were shaken on a reciprocal shaker at 26°C for 30 min. Concentrations of [^{14}C]imazalil (sp. act. 11.1 MBq mmol^{-1}) and [^{14}C]fenarimol (sp. act. 5.6 MBq mmol^{-1}) in the germling suspensions were 10 and 90 μM, respectively. The final methanol concentration in the suspension was below 1%. Accumulation of DMIs was determined in germlings collected from 5-ml samples and washed 5 times with buffer (5ml) in 30 s. Further reduction of background adsorption of the DMIs failed since experimental conditions or chemicals tested which led to deenergization of the germlings gave a significant increase in accumulation of the DMIs. Test chemicals were added to the germling suspension after 40 min of incubation with [^{14}C]imazalil and [^{14}C]fenarimol. Effects of the test compounds on fungicide accumulation were determined 10 min after their addition to the suspension.

Results

Determination of plasma membrane potential. Accumulation of [^{14}C]TPP$^+$ by germlings was relatively fast in the initial 10 min and reached a stationary phase in about 50 min. The total amount of [^{14}C]TPP$^+$ accumulated in the stationary phase was similar for isolates W_5, $E_{300\text{-}3}$ and H_{17} (Fig. 1). In preliminary experiments background adsorption of [^{14}C]TPP$^+$ to germlings was determined according to various methods. Heat treatment of germlings slightly increased [^{14}C]TPP$^+$ accumulation (Table 1). Germlings treated with toluene at 37°C accumulated approximately 4-fold more [^{14}C]TPP$^+$ than untreated

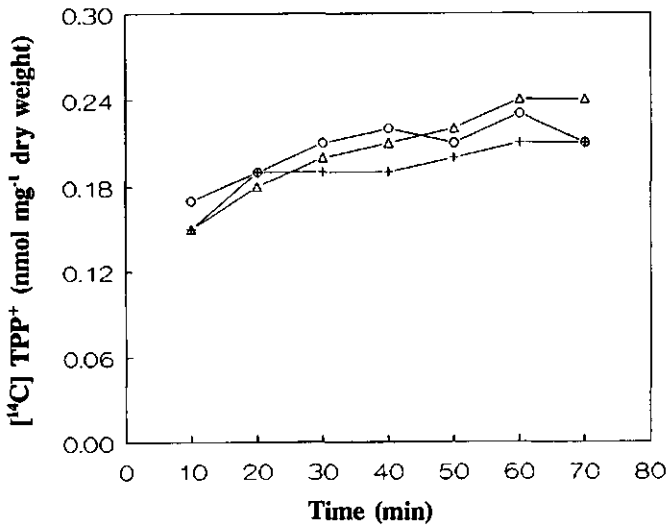


Fig. 1. Accumulation of [^{14}C]TPP$^+$ (10 μM) by wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△) and H_{17} (○) of *Penicillium italicum* in 23.4 mM potassium phosphate buffer pH 7, containing 0.1 mM calcium chloride and 1% (w/v) glucose.

Table 1. Relative effect of various treatments of germlings on accumulation of [^{14}C]TPP$^+$ (10 μM) by *Penicillium italicum* isolates W_5, $E_{300\text{-}3}$ and H_{17}

Treatment	Relative effect of treatment on accumulation level[1,2]		
	W_5	$E_{300\text{-}3}$	H_{17}
Heating (100°C, 5 min)	1.0	1.3	1.3
Toluene (1% (v/v), 37°C, 1 h)	4.4	4.2	-[3]
Toluene (1% (v/v), 50°C, 1 h)	0.7	0.7	0.8
KCl (0.2 M, 50 min)	0.8	0.8	0.8
Starvation[4] and KCl (0.2 M)	0.6	0.6	0.6
Starvation and KCl (1.0 M)	0.3	0.3	0.3

[1] Average accumulation levels of [^{14}]TPP$^+$ in untreated germlings of isolates W_5, $E_{300\text{-}3}$ and H_{17} were 0.25±0.04, 0.27±0.04 and 0.29±0.06 nmol mg^{-1} dry weight, respectively (n=6).
[2] Ratio between accumulation level in treated and untreated germlings.
[3] Not tested.
[4] Incubation without glucose in orbital shaker at 180 rpm for 6 h.

germlings. In contrast, treatment of germlings with toluene at 50°C slightly reduced accumulation of the probe, as did the incubation of germlings with 0.2 M KCl for 50 min (Table 1). Germlings which were deenergized by starvation for 6 h in the presence of 1.0 M KCl showed the lowest level of [^{14}C]TPP$^+$ accumulation suggesting a background adsorption of about 30% (Table 1). This percentage was used to correct $\Delta\psi$ for internal, aspecific probe binding. The $\Delta\psi$ values under equilibrium conditions were calculated to be 55.9±4.3, 58.1±4.3 and 59.8±6.7 mV (n=6) for isolates W_5, $E_{300\text{-}3}$ and H_{17}, respectively.

Accumulation of [^{14}C]imazalil and [^{14}C]fenarimol. Accumulation of [^{14}C]imazalil by germlings of the wild-type isolate W_5 was pH-dependent, gradually reached a stable level in about 30 min and increased with pH, except for pH 8 (Fig. 2). At pH 5 and 6 [^{14}C]imazalil accumulation by W_5 and the low-resistant isolate $E_{300\text{-}3}$ was similar, but at pH 7 and 8 accumulation by isolate $E_{300\text{-}3}$ was 22 and 35% less compared to the wild-type isolate (Fig. 2). No differential accumulation of [^{14}C]imazalil among isolates $E_{300\text{-}3}$, H_{17} and I_{33} was found (Fig. 3).

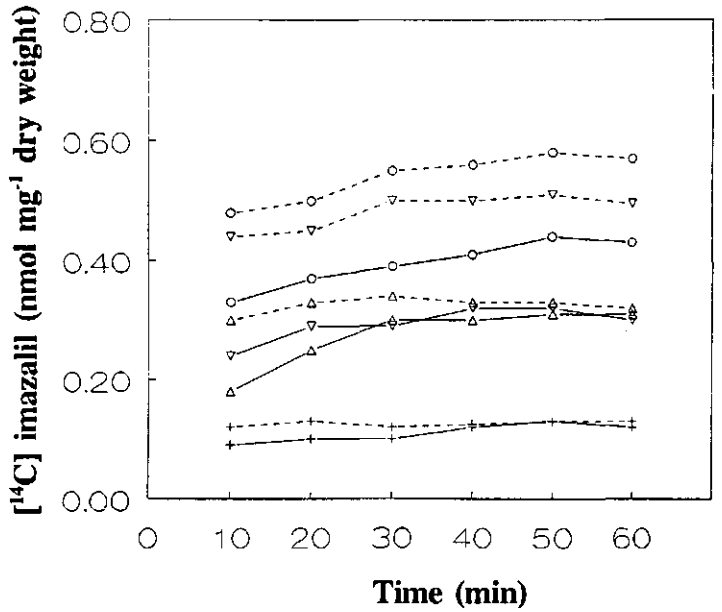


Fig. 2. Effect of external pH on accumulation level of [^{14}C]imazalil (10μM) by wild-type isolate W_5 (----) and DMI-resistant isolate $E_{300\text{-}3}$ (—) of *Penicillium italicum* in 23.4 mM potassium phosphate, containing 0.1 mM calcium chloride and 1% (w/v) glucose at pH 5 (+), 6 (△), 7 (○) and 8 (▽).

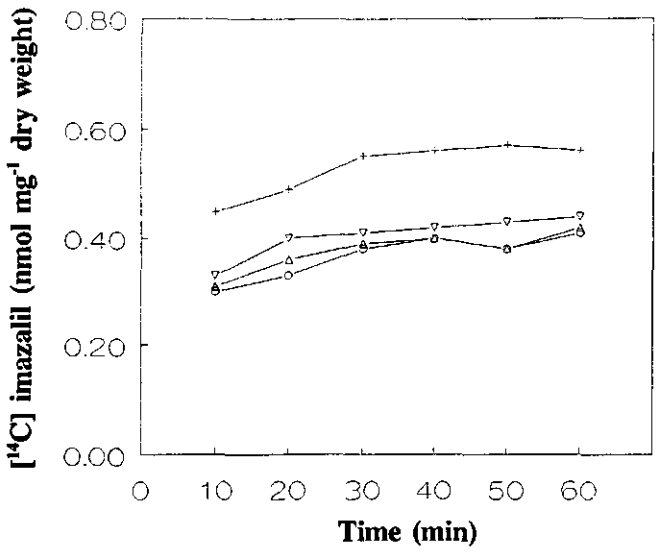


Fig. 3. Accumulation of [^{14}C]imazalil (10 μM) by wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△), H_{17} (○)and I_{33} (▽) of *Penicillium italicum* in 23.4 mM potassium phosphate buffer pH 7, containing 0.1 mM calcium chloride and 1% (w/v) glucose.

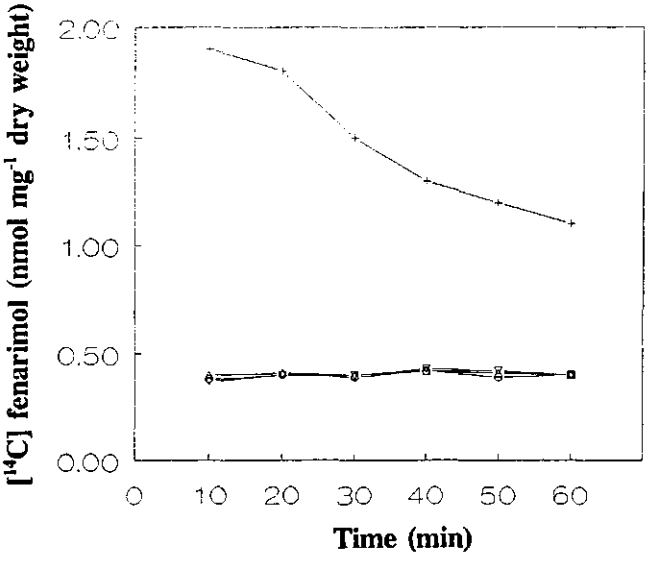


Fig. 4. Accumulation of [^{14}C]fenarimol (90 μM) by wild-type isolate W_5 (+) and DMI-resistant isolates $E_{300\text{-}3}$ (△), H_{17} (○) and I_{33} (▽) of *Penicillium italicum* in 23.4 mM potassium phosphate buffer pH 7, containing 0.1 mM calcium chloride and 1% (w/v) glucose.

The accumulation pattern of [^{14}C]fenarimol by wild-type isolate W_5 at pH 7 (Fig. 4) was similar to the typical transient accumulation curve of [^{14}C]fenarimol demonstrated previously by De Waard and Van Nistelrooy[14] who used 16-h-old mycelium. A minor difference was that in the present experiments equilibrium in accumulation was obtained less readily. Accumulation of [^{14}C]fenarimol was not significantly affected by changes of extracellular pH (results not shown). The accumulation of [^{14}C]fenarimol by resistant isolate $E_{300\text{-}3}$ was much lower than for wild-type isolate W_5 (Fig. 4). No differential accumulation of [^{14}C]fenarimol among isolates $E_{300\text{-}3}$, H_{17} and I_{33} was perceived. For all following experiments pH 7 was selected as a standard pH.

Effects of chemicals on accumulation of [^{14}C]TPP$^+$, [^{14}C]imazalil and [^{14}C]fenarimol. Differential accumulation of [^{14}C]imazalil and [^{14}C]fenarimol was observed between wild-type W_5 and low-resistant isolate $E_{300\text{-}3}$ only. Therefore, these two isolates were selected for experiments in which the effects of chemicals with known membrane-interfering properties on accumulation of [^{14}C]TPP$^+$ and both [^{14}C]DMIs were studied. The chemicals selected were the protonophoric uncoupler CCCP, the membrane ATPase inhibitors DES and vanadate, the calmodulin anatagonists chlorpromazine and TFP, and the ionophoric antibiotics gramicidine-S, nigericine and valinomycine. CCCP, DES, gramicidine-S and vanadate caused a slight increase in accumulation of [^{14}C]TPP$^+$ in both isolates (Table 2 and 3). The effect of TFP was more pronounced, but became weaker in time. Chlorpromazine slightly inhibited accumulation of [^{14}C]TPP$^+$ while nigericine and valinomycine hardly showed any effect.

On the other hand, all test compounds used strongly enhanced accumulation of [^{14}C]fenarimol in both isolates (Table 2 and 3). The relative effect of most compounds on accumulation by isolate $E_{300\text{-}3}$ was higher than by isolate W_5. The effect of the test compounds on accumulation of [^{14}C]imazalil was generally much weaker and not comparable with that of [^{14}C]fenarimol. Only CCCP and DES caused a small increase in accumulation whereas all other compounds tested led to a slightly lower accumulation level (Table 2 and 3).

Discussion

Energy-dependent efflux of a number of DMIs from DMI-resistant isolates of *A. nidulans* and *P. italicum* has been well documented.[13-16] The increased efflux of DMIs in resistant isolate $E_{300\text{-}3}$, which is high-resistant to fenarimol and low-resistant to imazalil, is probably responsible for a lower level of accumulation at which the DMIs do not readily saturate the target enzyme in sterol biosynthesis. Imazalil was the only exception among DMIs since no differential accumulation between 16-h-old mycelium of sensitive and resistant isolates could be observed.[16] However, the present study indicates that a significant difference in accumulation could be observed if 11-h-old germlings were used

and the pH of the external medium was 7.0 or higher (Fig. 1). Mycelium (16-h-old) probably accumulates more aspecifically bound imazalil than germlings and may, therefore, mask small differences in accumulation. In this respect, it is noted that the toxicity of imazalil to 11-h-old germlings of *P. italicum* in liquid medium is relatively

Table 2. Effect of various test compounds on accumulation of [^{14}C]imazalil (10 μM), [^{14}C]fenarimol (90 μM) and [^{14}C]TPP$^+$ (10 μM) by germlings of *Penicillium italicum* isolate W_5 at pH 7.0.

Compound	Concentration (μM)	Relative effect of chemicals on on accumulation[1]			
		Imazalil	Fenarimol	TPP[3] t=5	TPP[3] t=15
Calmidazolium	10	0.8[2]	2.7[2]	-[4]	-
,,	25	0.8	3.2	-	-
CCCP	50	1.8	4.5	1.2	1.5
,,	100	1.2	5.2	1.6	1.7
Chlorpromazine	10	0.5	1.4	-	-
,,	50	0.7	3.9	-	-
,,	100	0.6	2.9	0.9	0.9
DES	10	0.9	2.2	-	-
,,	50	1.3	4.7	1.0	1.3
,,	100	1.4	3.0	1.3	1.7
TFP	10	-	1.8	-	-
,,	50	0.7	5.0	1.7	1.4
,,	100	0.8	2.9	2.2	1.9
Gramicidine-S	50	-	-	1.5	1.2
,,	100	-	1.3	1.6	1.5
Nigericine	100	-	2.7	-	1.0
Valinomycine	100	-	2.0	-	1.0
Vanadate	100	-	2.0	1.1	1.2

[1] Average accumulation levels of [^{14}C]imazalil, [^{14}C]fenarimol and [^{14}C]TPP$^+$ in untreated mycelium 50 min after adding the radiolabeled compounds were 0.58 ± 0.05, 1.10 ± 0.07 and 0.26 ± 0.04 nmol mg^{-1} dry weight of germlings, respectively.

[2] Ratio between accumulation level in treatments and controls determined 10 min after addition of the test compounds.

[3] Relative effect measured 5 and 15 min after addition of the test compounds.

[4] Not tested.

[5] Abbreviations used: DES (diethylstilbestrol), CCCP (carbonyl cyanide 3-chlorophenylhydrazone), TFP (triflupromazine), TPP$^+$ (tetraphenylphosphonium bromide).

high as compared to its toxicity to 16-h-old mycelium in liquid cultures, but comparable to its toxicity determined in radial growth experiments.[28] In addition, imazalil has a pK_a value of 6.5. Hence, at pH 7.0 it is mainly in a neutral form and accumulation may not be affected by the presence of its protonated form as compared with pH 6.0 used in previous studies.[16]

Table 3. Effect of various test compounds on accumulation of [^{14}C]imazalil (10 μM), [^{14}C]fenarimol (90 μM) and [^{14}C]TPP$^+$ (10 μM) by germlings of *Penicillium italicum* isolate $E_{300\text{-}3}$ at pH 7.0.

Compound	Concentration (μM)	Relative effect of chemicals on accumulation[1,2]			
		Imazalil	Fenarimol	TPP[3]	
				t=5	t=15
Calmidazolium	10	0.8	4.6	-	-
,,	25	0.8	6.5	-	-
CCCP	50	1.7	7.9	1.2	1.5
,,	100	1.5	-	1.5	1.7
Chlorpromazine	10	0.7	3.0	-	-
,,	50	0.9	7.8	-	-
,,	100	0.5	1.1	0.6	0.6
DES	10	1.0	1.1	-	-
,,	50	1.2	7.7	1.2	1.5
,,	100	1.3	7.1	1.3	1.8
TFP	10	-	4.8	-	-
,,	50	0.7	12.7	1.0	0.8
,,	100	0.6	9.2	1.5	1.1
Gramicidine-S	50	-	-	1.3	1.3
,,	100	-	3.8	1.4	1.3
Nigericine	100	-	14.6	-	0.9
Valinomycine	100	-	5.7	-	0.9
Vanadate	100	-	1.6	1.2	1.2

[1] Average accumulation levels of [^{14}C]imazalil, [^{14}C]fenarimol and [^{14}C]TPP$^+$ in untreated mycelium 50 min after adding the radiolabeled compounds were 0.40 ± 0.05, 0.42 ± 0.04 and 0.28 ± 0.05 nmol mg^{-1} dry weight of germlings, respectively.

[2] Ratio between accumulation level in treatments and controls determined 10 min after additionof the test compounds.

[3] Relative effect measured 5 and 15 min after addition of the test compounds.

[4] Not tested.

[5] Abbreviations used: DES (diethylstilbestrol), CCCP (carbonyl cyanide 3-chlorophenylhydrazone), TFP (trifluprazine), TPP$^+$ (tetraphenylphosphonium bromide).

Fenarimol is in a neutral form at all pH values tested. This probably explains why the external pH does not affect accumulation of this fungicide.

Increase in [^{14}C]fenarimol accumulation by the protonophoric uncoupler CCCP, the membrane ATPase inhibitors DES and vanadate, the calmodulin antagonists chlorpromazine and TFP, and the ionophoric antibiotics gramicidine-S, nigericine and valinomycine (Table 2), confirms previous results that a mechanism of energy-dependent efflux is probably involved in accumulation of fenarimol and other DMIs.[15,16] In contrast to fenarimol, accumulation of imazalil only slightly increased upon incubation with CCCP and DES while all other test chemicals decreased imazalil uptake (Table 2). *P. italicum* wild-type W_5 also did not exhibit transient accumulation of imazalil in time (Fig. 2) as demonstrated for fenarimol and other DMIs.[16,29] These results suggest that imazalil accumulation is not or only slightly mediated by a mechanism of energy-dependent efflux. It might be that the energy-dependent efflux of imazalil is hampered by intracellular protonation of the fungicide.

Isolates $E_{300\text{-}3}$, H_{17} and I_{33} with a low, medium and high degree of resistance to DMIs, respectively, did not show differential accumulation of fenarimol and imazalil (Fig. 2 and 3). An explanation is that increased resistance in isolates H_{17} and I_{33} is based on other mechanisms such as decreased affinity of the target site for the toxicant. Alternatively, it might also be that the relatively high background adsorption of the fungicides masks any further decrease in accumulation in medium- and high-resistant isolates.

The lipophilic cation TPP^+ is a commonly used probe for measuring $\Delta\psi$.[25] Preliminary experiments showed that various methods to determine the background adsorption of the probe by deenergized germlings did not provide comparable results (Table 1). This finding is in agreement with data from Lolkema *et al.*[27] It is noted that toluene treatment of the germlings at 37°C, a method used by them to determine the background adsorption of [^{14}C]TPP^+ to bacterial cells, was not useful (Table 1). Apparently, different methods have to be used for different organisms. In our tests starvation of germlings in the presence of 1 M KCl for 6 h led to the lowest accumulation levels of the probe. With this method about 30% of the accumulated probe remained associated with the deenergized germlings (Table 1). This value was arbitrarily chosen to correct the amount of [^{14}C]TPP^+ accumulated for aspecific probe binding. The $\Delta\psi$ values were calculated to be 55.9 ± 4.3, 58.1 ± 4.3 and 59.8 ± 6.7 mV for isolates W_5, $E_{300\text{-}3}$ and H_{17}, respectively. These results indicate that the isolates do not differ in $\Delta\psi$.

The relative effects of various compounds on the accumulation of [^{14}C]TPP^+ by *P. italicum* isolates W_5 and $E_{300\text{-}3}$ were also studied. The effects observed were marginal (Table 2 and 3). For example, CCCP, DES, gramicidine-S and vanadate only enhanced accumulation of [^{14}C]TPP^+ with a factor below 2.0, while nigericine and valinomycine hardly had any effect (Table 2 and 3). There is no obvious explanation for the fact that addition of CCCP and DES resulted in an increased accumulation of [^{14}C]TPP^+ which was unexpected from their known effects on membrane processes. No correlation between

the effects of compounds on accumulation of [^{14}C]TPP^{+} and both fungicides could be observed. For example, accumulation of [^{14}C]fenarimol could be significantly enhanced by test compounds, such as nigericine and valinomycine, which hardly influenced accumulation of [^{14}C]TPP^{+} (Table 2 and 3). Therefore, results suggest that the $\Delta\psi$ is not involved in accumulation of fenarimol and imazalil by germlings of wild-type and DMI-resistant isolates of *P. italicum*.

Energy-dependent efflux of DMI fungicides (except for imazalil) in fungi resembles in several aspects multi-drug resistance (MDR) in mammalian cancer cells.[30] Firstly, these mammalian MDR cells are cross resistant to structurally non-related drugs. DMI-resistant isolates of *A. nidulans*, *Botrytis cinerea* and *Ustilago avenae* also show cross-resistance to unrelated fungitoxicants and antibiotics such as cycloheximide, acriflavine, neomycin, 8-azaguanidine, thiourea and nikkomycin.[20,31-33] In this respect, it is worthwile to investigate whether the DMI-resistant isolates of *P. italicum* are also cross-resistant to unrelated fungitoxic xenobiotics. Secondly, in both type of organisms resistance is based on reduced accumulation of compounds mediated by energy-dependent efflux. Thirdly, the accumulation of both anticancer drugs and DMI fungicides can be reversed by calmodulin antagonists such as TFP, chlorpromazine and calmidazolium (Table 2 and 3).[34] In addition, cycloheximide which reversed drug accumulation in MDR cells[35] also increased accumulation of fenarimol in *A. nidulans* (De Waard, unpublished results). These lines of similarity suggest that DMI-resistance in fungi may be based on a similar mechanism as present in MDR mammalian cancer cells. This topic will be the subject of future studies.

Acknowledgement.

The critical reading of the manuscript by Prof. Dr. J. Dekker is acknowledged.

References

1. Kato, T., In *Chemistry of Plant Protection, Vol. 1*, ed. G. Haug & H. Hoffmann, Springer-Verlag, Berlin, 1986, p. 1-24.
2. Köller, W. & Scheinpflug, H., *Pl. Disease* **171** (1987) 1066-1074.
3. Brent, K.J. & Hollomon, D.W., In *Sterol Biosynthesis Inhibitors, Pharmaceutical and Agrochemical Aspects*, ed. D. Berg & M. Plempel. Ellis Horwood Ltd., Chichester, 1988, p.332-346.
4. Deas, A.H.B., *Pestic. Sci.* **17** (1986) 69-70.
5. Hitchcock, C.A., Barrett-Bee, K.J. & Russell, N.J., *J. Med. Vet. Mycol.* **25** (1986) 29-37.
6. Portillo, F. & Gancedo, C., *Mol. Gen. Genet.* **199** (1985) 493-499.
7. Smith, F.D. & Köller, W., *Phytopathol.* **80** (1990) 584-590.
8. Taylor, F.R., Rodrigues, R.J. & Parks, L.W., *J. Bacteriol.* **155** (1983) 64-68.
9. Vanden Bossche, H., Marichal, P., Correas, J., Bellens, D., Moereels, H. & Janssen, P.A.J., *Biochem. Soc. Trans.* **1818** (1990) 56-59.

10. Walsh, R.L. & Sisler, H.D., *Pestic. Biochem. Physiol.* **18** (1982) 122-131.
11. Watson, P.F., Rose. M.E. & Kelly, S.L., *J. Med. Vet. Mycol.* **26** (1988) 153-162.
12. Weete, J.D., *Abstracts 6th Internat. Congr. on Pestic. Chem., IUPAC*, Ottawa, Canada, 1986, 3C-06.
13. De Waard, M.A. & Van Nistelrooy, J.G.M., *Pestic. Biochem. Physiol.* **10** (1979) 210-229.
14. De Waard, M.A. & Van Nistelrooy, J.G.M., *Neth. J. Pl. Pathol.* **90** (1984) 143-153.
15. De Waard, M.A. & Van Nistelrooy, J.G.M., *Exp. Mycol.* **11** (1987) 1-10.
16. De Waard, M.A. & Van Nistelrooy, J.G.M., *Pestic. Sci.* **22** (1988) 371-382.
17. Warth, A.D., *J. Appl. Bacteriol.* **43** (1977) 215-230.
18. Eisenberg, E.S., Mandel, L.J., Kaback, H.R. & Miller, M.H., *J. Bacteriol.* **157** (1984) 863-867.
19. Gilman, S. & Saunders, V.A., *J. Antimicrob. Chemother.* **17** (1986) 37-44.
20. Van Tuyl, J.M., *Mededelingen Landbouwhogeschool, Wageningen, The Netherlands*, (1977) 77-2.
21. Perlin, D.S., Brown, G.L. and Hafer, J.E., *J. Biol. Chem.* **263** (1988) 18118-18122.
22. Damper, P.D. & Epstein, W., *Antimicrob. Agents Chemother.* **20** (1981) 803-808.
23. De Waard, M.A., Groeneweg, H. & Van Nistelrooy, J.G.M., *Neth. J. Pl. Pathol.* **88** (1982) 99-112.
24. De Waard, M.A. & Van Nistelrooy, J.G.M., *Neth. J. Pl. Pathol.* **96** (1990) 321-329.
25. Kashket, E.R., *Ann. Rev. Microbiol.* **39** (1985) 219-242.
26. Lolkema, J.S., Hellingwerf, K.J. & Konings, W.N., *Biochim. Biophys. Acta* **681** (1982) 85-94.
27. Lolkema, J.S., Abbing, A., Hellingwerf, K.J. & Konings, W.N., *Eur. J. Biochem* **130** (1983) 287-292.
28. Guan, J. Kerkenaar, A. & De Waard, M.A., *Neth. J. Pl. Pathol.* **95** (Supplement 1) (1989) 73-86.
29. Kapteyn, J.C., Pillmoor, J.B. & De Waard, M.A. *Pestic. Sci.* (in press).
30. Bradley, G., Juranka, P.F. & Ling, V., *Biochim. Biophys. Acta* **948** (1988) 87-128.
31. Leroux, P., Gredt M. & Fritz, R., *Phytiatrie-Phytopharmacie* **25** (1976) 317-334.
32. De Waard, M.A. & Gieskes, S.A., *Neth. J. Pl. Pathol.* **83** (1977) 177-188.
33. Krämer, W., Berg, D. & Köller, W., In *Combating Resistance to Xenobiotics - Biological and Chemical Approaches*, ed. M.G. Ford, D.W. Hollomon, B.P.S. Khambay & R.M. Sawicki. Ellis Horwood Ltd., Chichester, 1987, p. 291-305.
34. Endicott, J.A. & Ling, L., *Ann. Rev. Biochem.* **58** (1989) 137-171.
35. Gottesman, M. & Pastan, I., *J. Biol. Chem.* **263** (1988) 12163-12166.

CHAPTER 5

ISOLATION OF MICROSOMAL CYTOCHROME-P450 ISOZYMES FROM *USTILAGO MAYDIS* AND THEIR INTERACTION WITH STEROL DEMETHYLATION INHIBITORS.

Johan C. Kapteyn,[a] John B. Pillmoor[b] and Maarten A. de Waard[a]

[a] Department of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, 6709 PD Wageningen, The Netherlands.

[b] Schering Agrochemicals Ltd., Chesterford Park Research Station, Saffron Walden, Essex CB10 1XL, UK.

(Pesticide Science 1992, **34**, 37-43)

Abstract

A procedure for the isolation of microsomes containing cytochrome- P450 isozymes from *Ustilago maydis* is described. Yields of P450 amount to approximately 19±6 pmol mg^{-1} of microsomal protein. The wavelength of maximum absorbance of the reduced carbon monoxide difference spectrum is 448-449 nm. The azole fungicides prochloraz, etaconazole, imazalil, triadimefon and 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4 triazol-1-yl)-4(3*H*)-quinazolinone, which differ markedly in toxicity to *U. maydis*, all induce type II binding difference spectra at extremely low concentrations (10^{-9}-10^{-8} M). The DMI concentrations which cause half saturation of type II binding difference spectra (IC_{50}) do not correlate with the fungicidal activities of the azoles. Binding of carbon monoxide to ferrous cytochrome-P450 was only slightly inhibited to different degrees by the DMIs tested. However, the inhibition of CO binding also does not correlate with fungitoxicity of the DMIs. The results of this paper suggest that the spectrophotometric studies with this preparation are not useful for evaluating selective toxicity of DMIs to intact sporidia of *U. maydis*.

Introduction

Sterol demethylation inhibitors (DMIs) are antifungal agents which inhibit the biosynthesis of ergosterol by binding to cytochrome-P450 dependent sterol 14α-demethylase ($P450_{DM}$).[1] Derivatives of imidazole, triazole, pyrimidine and pyridine belong to this group of fungicides.

Relatively little is known about the mechanisms of DMI selectivity. A possible mechanism for selective toxicity may be differential affinity of fungal $P450_{DM}$ for the DMIs. In this respect, Vanden Bossche *et al.*[2,3] demonstrated that microsomal P450 isozymes isolated from *Candida albicans* and *Saccharomyces cerevisiae* show much greater affinity to azole fungicides than those from plants or mammals. This finding correlated with the observation that the azoles inhibited sterol biosynthesis to a greater extent in yeasts than in plant or mammalian systems. In addition, azole-resistant isolates of *C. albicans* contained P450 isozymes with lower affinity to various DMIs than azole-sensitive strains.[3,4] Yoshida and Aoyama[5-7] and Yoshida *et al.*[8] studied the interaction of DMIs, including a number of stereo-isomers, with $P450_{DM}$ purified from *S. cerevisiae* microsomes. They suggested that the inhibitory effect of azole antifungals on $P450_{DM}$ was predominantly determined by the N_1 substituent of the azole moieties regulating the mobility of the molecules in the haem crevice of $P450_{DM}$. Similar results were obtained using microsomal preparations of *C. albicans*.[9-11]

The isolation of $P450_{DM}$ is a prerequisite to studying the differential affinity of the target site to various DMIs. In contrast to the isolation of yeast P450 isozymes, it is difficult to isolate these enzymes from filamentous fungi. This is probably because of the instability and low content of the enzymes. Therefore, few papers deal with P450 in these organisms, but two papers on the isolation of P450 from filamentous fungi have recently been published. Marichal *et al.*[12] developed a method to isolate microsomes from the filamentous fungus *Aspergillus fumigatus* which contained relatively stable P450 isozymes. With this method, microsomal preparations with a P450 content of 45 to 50 pmol mg^{-1} protein were obtained. Ballard *et al.*[13] also succeeded in isolating P450-containing microsomes from *A. fumigatus*. They used a slightly different method which resulted in a P450 content of about 19 pmol mg^{-1} protein.

In this paper a novel procedure is described for isolating P450 from the phytopathogenic fungus *Ustilago maydis*. The sporidia of this fungus multiply in a yeast-like fashion by budding, but the fungus has no physiological or systematic relation to yeasts. The interactions of the DMIs prochloraz, etaconazole, imazalil, triadimefon and experimental fungicide 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4 triazol-1-yl)-4(3*H*)-quinazolinone (Fig. 1) with the P450 isozymes of this fungus are discussed.

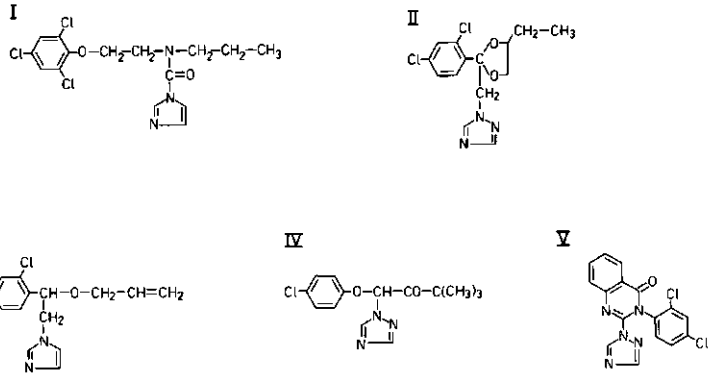


Fig. 1. Structural formulae of (**I**) prochloraz, (**II**) etaconazole, (**III**) imazalil, (**IV**) triadimefon and (**V**) 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4 triazol-1-yl)-4(3*H*)-quinazolinone

Materials and Methods

Chemicals. 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4 triazol-1-yl)-4(3*H*)-quinazolinone (Compound V) and prochloraz were obtained from Schering Agrochemicals Ltd. (Saffron Walden, UK). Imazalil sulphate (imazalil) was a gift from Janssen Pharmaceutica (Beerse, Belgium). Etaconazole and triadimefon were acquired from Ciba-Geigy (Basel, Switzerland) and Bayer (Leverkusen, Germany), respectively. Novozym™234 was purchased from Novo Bio Labs (Bagsvaerd, Denmark); ethylenediaminetetraacetic acid (EDTA), ß-mercaptoethanol (MCE), sodium dithionite and dithiothreitol (DTT) from Sigma (Deisenhofen, Germany); toluenesulfonyl fluoride (TSF) from Janssen Chimica (Beerse, Belgium). TSF was used as solution in acetone (2 M).

Culture methods. Sporidia of *U. maydis* were grown in liquid medium described by Coursen and Sisler[14] at 30°C for 20 or 38 h in an orbital shaker (180 rpm). Inoculum was 10^6 sporidia ml^{-1} medium.

Toxicity assay. Sensitivity of *U. maydis* to prochloraz, etaconazole, imazalil, triadimefon and compound V was assessed in liquid medium. Sporidial suspensions (10 ml; 10^4 sporidia ml^{-1}) in conical flasks (25 ml) were incubated with different fungicide concentrations (from 100-fold stock solutions in DMSO) at 30°C for 38 h in an orbital shaker (180 rpm). After incubation, dry weight of sporidia was determined by harvesting on a Büchner funnel and drying in an oven at 50°C for 20 h.

Isolation procedure for P450 isozymes. Sporidia grown for 20 h (9 to 12 g wet weight of sporidia) were harvested by centrifugation at 2000*g* for 10 min and washed twice with

incubation medium consisting of 100 mM potassium phosphate buffer pH 5.8 and 0.5 M ammonium sulphate; the second washing also contained 14 mM MCE. Standard sporidial suspensions were made by resuspending 1 g wet weight of sporidia into 25 ml incubation medium containing Novozym™234 (1 mg ml^{-1}) and 1 mM TSF. After incubating the sporidial suspension (200-300 ml) at 28°C for 15 min, sphaeroplasts and/or 'plasticised' cells were harvested by centrifugation (10 min; 1000*g*) and washed with cold incubation medium (100 ml). The pellets were carefully resuspended with a teflon pestle in cold 10 mM potassium phosphate buffer pH 7.0 containing 2.0 M sorbitol (150 ml) and recentrifuged at 1000*g* for 15 min. All subsequent procedures were carried out at 4°C and the buffers used were modified media described previously by Marichal *et al.*[12] Sphaeroplasts were resuspended in 10 mM potassium phosphate buffer pH 7.0 containing 14 mM MCE, 0.25 M sorbitol, 1.0 mM TSF, 0.1 mM DTT and 0.1 mM EDTA (75 ml). The sphaeroplasts were collapsed by gentle disruption in a Braun Potter-type homogenizer (Salm and Kipp, Breukelen, The Netherlands) using a loosely-fitting Potter-tube to avoid generating a vacuum. Homogenization was achieved by moving the rotating pestle (100 rpm) slowly 10 times up and down. Glycerol (12% (v/v)) was added and the suspension was homogenized by another 10 strokes. The concentration of glycerol was increased to 22% (v/v) and a further 10 strokes were applied. Cell wall fragments and uncollapsed cells were removed by centrifugation at 2500*g* for 10 min. The resulting supernatant was centrifuged for 20 min at 25000*g* to sediment mitochondria, filtered through glass wool to remove the floating lipid layer and centrifuged at 120000*g* for 80 min. After removing the supernatant by aspiration, the solid microsomal pellet was carefully separated from a putative glycogen pellet with a spatula and transferred to a washing medium (15 ml) consisting of 100 mM potassium phosphate buffer pH 7.4, 14 mM MCE, 10 mM EDTA, 0.1 mM DTT and 20% (v/v) glycerol. The microsomal pellets were recentrifuged at 120000*g* for 1 h and resuspended in 100 mM potassium phosphate buffer pH 7.4, containing 20% (v/v) glycerol (5ml). Microsomal samples (0.7 ml) were stored in Eppendorf centrifuge tubes at -70°C until use.

Spectrophotometric analysis. Spectrophotometric analysis of the microsomal samples was conducted with an Aminco DW-2C UV-VIS spectrophotometer. The P450 isozyme content of the microsomal fractions was determined according to Omura and Sato[15] by measuring the reduced carbon monoxide difference spectrum. A microsomal sample (0.7 ml) was thawed, diluted with 100 mM potassium phosphate buffer pH 7.4 containing 20% (v/v) glycerol (0.7 ml) and centrifuged at 2000*g* for 1 min to remove denatured protein. The microsomal sample (1.4 ml) was reduced with sodium dithionite (17 mg ml^{-1}) and divided between the reference and sample cuvettes. The cuvette-holder was kept at 20°C. After establishing the baseline manually the sample cuvette was saturated with carbon monoxide (approx. 20 bubbles; 30 s) and tightly closed. The reduced CO difference

spectrum was recorded 1 min later and at various time intervals up to 15 min. The absorbance difference between 448 and 490 nm ($OD_{(448\text{-}490\ nm)}$) after 15 min incubation with CO was used for calculation of the P450 isozyme content.

The interaction of DMIs with P450 isozymes was studied by measuring type II binding difference spectra with standard microsomal preparations of 100 nM P450. The microsomal preparation (1.4 ml) was divided between both cuvettes, and after establishing the baseline manually, the DMIs were added to the sample cuvette with increments of 0.7 μl from 1000x concentrated stock solutions in DMSO. The maximum amount of DMSO (15 μl) added to the sample cuvette caused no change in the spectrum over the region scanned.

Effects of DMIs on formation of the CO-complex ($OD_{(448\text{-}490\ nm)}$) were studied with standard microsomal samples of 100 nM P450. In these studies a microsomal sample (1.4 ml) was pre-incubated with DMIs (1.4 μl from 1000x concentrated stock solutions in DMSO) for 2 min prior to reduction. After bubbling the sample cuvette with CO (approx. 20 bubbles; 30 s) the reduced CO difference spectrum was recorded at various time intervals.

Protein content. Protein content of microsomal preparations was determined by the Bio-Rad protein assay (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands).

Results

Toxicity assay. The EC_{50} values of various DMIs to growth of *U. maydis* in liquid culture ranged from 0.01 to 35 μM (Table 1). Imazalil had the highest inhibitory effect on growth of *U. maydis*, followed by etaconazole, prochloraz, Compound V and triadimefon.

Isolation procedures for P450 isozymes. The standard isolation procedure described under 'Materials and Methods' yielded microsomal preparations which showed a reduced CO difference spectrum (Fig. 2). The wavelength of maximum absorbance varied from 448 to 449 nm between experiments. The small shoulder at approximately 424 nm indicates that denaturation of P450 isozymes to P420 hardly occurred during isolation of microsomes. However, after 9 min the peak at 424 nm did increase a little suggesting that a small amount of P450 isozymes was converted to P420 during this time. The specific content of P450 isozymes isolated in the standard procedure amounted to 19 ± 6 pmol mg^{-1} protein and 106 ± 29 pmol g^{-1} wet weight of sporidia (mean $\pm$ SEM of 13 isolations). Microsomal preparations which had been stored at -70°C in 100 mM phosphate buffer pH 7.4 containing 20% (v/v) glycerol for one month did not show any decline in P450 isozyme content.

Table 1. Toxicity of DMIs to growth of *Ustilago maydis* in synthetic liquid medium according to Coursen and Sisler[14].

DMI	EC_{50} (M)[a,b]
Imazalil	$1.1x10^{-8}$
Etaconazole	$1.7x10^{-7}$
Prochloraz	$8.5x10^{-6}$
V	$1.6x10^{-5}$
Triadimefon	$3.5x10^{-5}$

[a] Concentration of DMI which caused 50% growth inhibition.

[b] EC_{50} values are based on duplicate experiments.

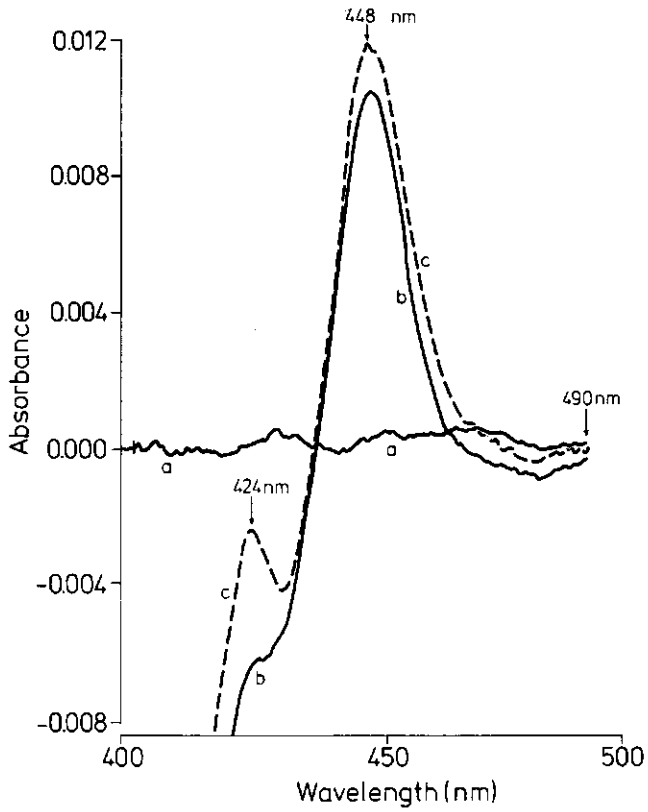


Fig. 2. Reduced carbon monoxide difference spectrum of P450 isozymes in microsomal preparation from *Ustilago maydis* obtained after homogenizing sporidial sphaeroplasts with a Potter-type homogenizer. Baseline (a); Spectra recorded 1 (b) and 9 (c) minutes after addition of CO.

It was observed that several factors were important for successful isolation of microsomal P450 isozymes. Preliminary experiments showed that the age of sporidial cultures of *U. maydis* was a critical factor. Yield of P450 isozymes was maximal when 20 h old sporidial cultures of *U. maydis* were used for isolation (data not shown). At this stage cultures of *U. maydis* were in the late log phase. The method of disrupting the sporidia was also crucial. Mechanical breakage using a French press or Braun homogenizer led to the isolation of P420 isozymes and to contamination of the samples by cytochrome oxidase (data not shown). Disruption of sporidial sphaero-plasts by sonication (20 KHz, 10 s) in 10 mM potassium phosphate buffer pH 7.0, consisting of 14 mM MCE, 0.1 mM DTT, 0.1 mM EDTA, 0.25 M sorbitol and 1 mM TSF before addition of 20% (v/v) glycerol also was not as successful as the standard disruption method. Sonication led to very dense proteinaceous microsomal preparations (2.5 to 4 mg protein ml^{-1}) containing P450 isozymes, but also small amounts of P420 and traces of cytochrome oxidase (data not shown).

Reduction of the microsomal samples with sodium dithionite was a critical step in the assay procedure to determine the content of P450 isozymes accurately. Optimal reducing effect was attained at a concentration of about 17 mg dithionite ml^{-1} microsomal suspension (data not shown). This concentration of dithionite was used in all the following experiments.

Type II difference spectra. Type II difference spectra of microsomal P450 isozymes (100 nM) from *U. maydis* were recorded after stepwise addition of various DMIs. Typical examples for prochloraz and compound V are shown in Fig. 3. The type II spectra were characterized by an absorbance maximum at approximately 428 nm and a minimum at approximately 410 nm. The increase in $OD_{(428\text{-}410\ nm)}$ in relation to azole concentration yielded saturation curves (Fig. 4). Saturation of type II spectral response was attained at concentrations ranging from 10^{-5} and $5x10^{-4}$ M for most fungicides, but was not fully achieved for triadimefon and compound V. Therefore the concentrations of triadimefon and compound V which caused 50% of maximum type II spectral response (IC_{50}) should be regarded as minimum values and amounted approximately 10^{-5} and 10^{-6} M (Table 2). The other azoles had lower IC_{50} values in the order of 10^{-7} M.

CO displacement tests. The relative affinities of prochloraz, imazalil, etaconazole and compound V for *U. maydis* microsomal P450 isozymes were also studied by determining their inhibitory effects on binding of CO to the P450 isozymes. It appeared to be a consistent trend that imazalil, prochloraz and compound V (10^{-6} M) inhibited binding of CO to P450 (10^{-7} M) to about a similar extent (Table 3). Etaconazole was the most effective compound of all DMIs tested, but still only caused 25% inhibition of CO binding. Except for imazalil, the extent of inhibition of CO/P450 complex formation by the DMIs tested varied significantly between experiments.

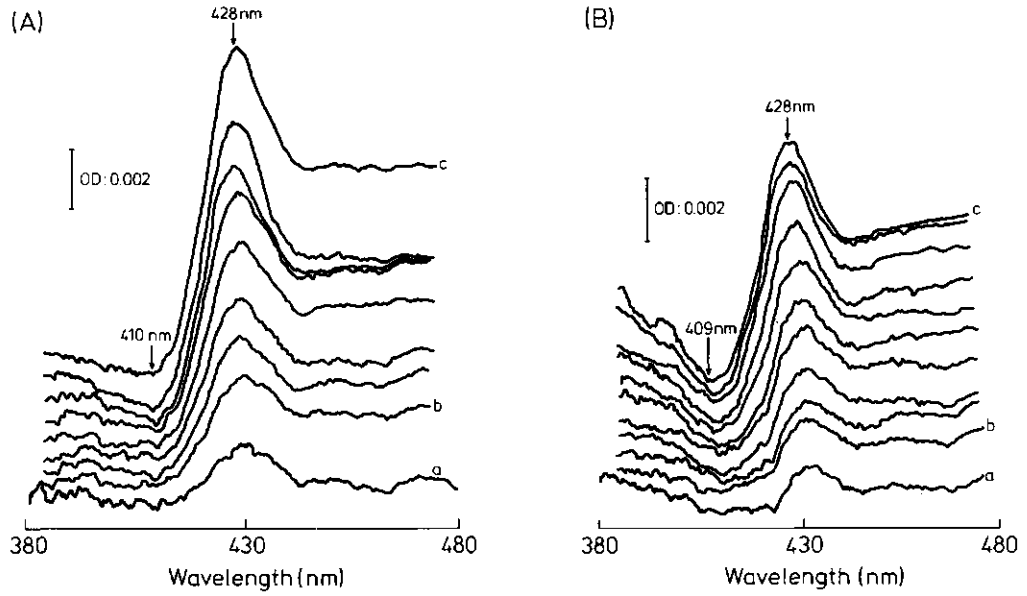


Fig. 3. Type II binding difference spectra of (A) prochloraz and (B) compound V obtained after stepwise addition of DMIs to microsomal preparations of *Ustilago maydis* containing P450 isozymes (100 nM). Baseline (a); Final DMI concentration in sample cuvette ranged from 10^{-9} (b) to 10^{-5}M (c).

Table 2. Maximum absorbance difference of type II spectra ($OD_{(428\text{-}410\ nm)max}$) upon saturation with DMIs and concentrations of DMIs which induce half saturation response in type II difference spectra of microsomal P450 isozymes obtained from *Ustilago maydis* (IC_{50}).

DMIs	$OD_{(428\text{-}410\ nm)max}$ (10^{-3})	IC_{50} (M)
Imazalil	7.9±3.3 (4)[a]	$6.3\pm4.1\times10^{-7}$
Etaconazole	6.5±1.7 (4)	$4.4\pm1.4\times10^{-7}$
Prochloraz	5.4±1.6 (3)	$7.0\pm4.1\times10^{-8}$
Compound V	4.3±1.6 (3)	$1.3\pm0.6\times10^{-6}$
Triadimefon	8.8±2.5 (3)	$2.9\pm2.1\times10^{-5}$

[a] ( ): number of replicates

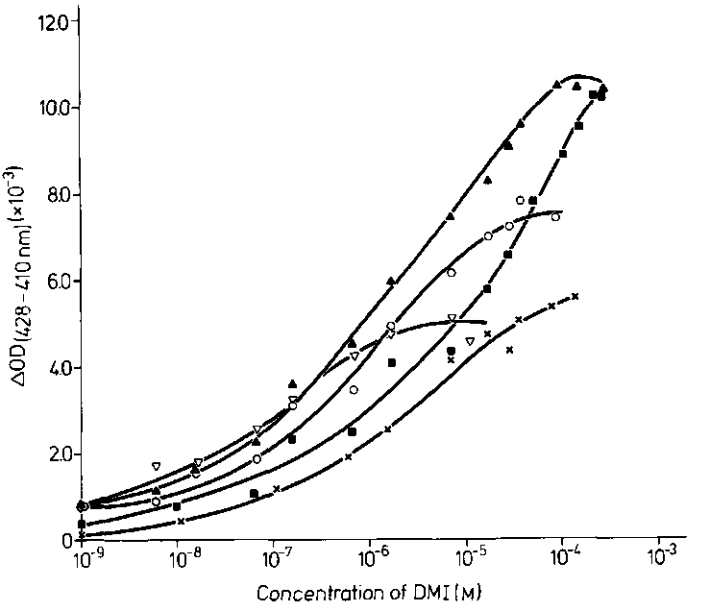


Fig.4. Type II spectral response ($OD_{(428\text{-}410\ nm)}$) of microsomal P450 isozymes (100 nM) from *Ustilago maydis* induced by stepwise addition of prochloraz (▽), etaconazole (○), imazalil (▲), triadimefon (■) and compound V (×).

Table 3. Inhibitory effects of various DMIs on binding of carbon monoxide to microsomal P450 isozymes from *Ustilago maydis*. Concentration of P450 isozymes and DMIs were 10^{-7} and 10^{-6} M, respectively. At each time of spectrophotometric measurement binding of CO in the control treatment (DMSO) was taken as 100%.

Time after CO-treatment (min)	Binding of CO in treated samples as percentage of control of the specific treatments			
	Imazalil (3)[b]	Prochloraz	Compound V	Etaconazole
1	81.9±3.8[c]	88.0±7.6	87.8±10.5	72.4±10.9
3	84.5±1.3	92.1±5.8	82.2±17.7	73.8±09.3
6	87.1±0.8	95.8±6.5	86.4±11.9	75.3±08.8
10	89.2±0.2	96.1±6.5	84.8±13.5	74.7±08.0
15	80.0±0.9	100.9±7.0	92.3±16.3	77.1±11.2

Average $OD_{(448\text{-}490\ nm)}$ ($x10^{-3}$) values of all controls at t=1, 3, 6, 10 and 15 min after CO addition were 8.3±0.9, 9.1±1.2, 9.5±1.2, 9.7±1.4 and 9.4±1.0, respectively.
( ): number of replicates of the specific treatments.
Percentage values correspond to the controls of the specific treatments.

Discussion

This study demonstrates that microsomes containing stable P450 isozymes can be isolated from *U. maydis*. Yields amounted to approximately 19 pmol mg^{-1} of microsomal protein, which corresponds well with results of Hitchcock *et al.*[16] and Ballard *et al.*[13], who isolated comparable quantities of microsomal P450 isozymes from *C. albicans* and *A. fumigatus*, respectively. The maximum absorbance of the reduced CO difference spectrum was at 448-449 nm. All the azole fungicides investigated induced type II binding difference spectra at extremely low concentrations (10^{-9}-10^{-8} M) (Fig. 3). This indicates that the nitrogen atom at position three and four of the imidazole and triazole rings, respectively, interacted with the ferric iron at the haem centre of P450. The artefactual peaks at approximately 430 nm recorded in the base lines (Fig. 3) were due to the high protein content of the microsomal preparations which exceeded the protein concentration limit that can be tolerated by the optical system.[17]

The DMI-concentrations which induced half saturation of the response in type II spectra (IC_{50}) demonstrated that the microsomal P450 preparation from *U. maydis* had different affinities for the antifungal azoles tested (Table 2). The apparent IC_{50} values, however, did not correlate with the fungicidal activity of the DMIs tested. For example, prochloraz, poorly active against *U. maydis*, exhibited an IC_{50} value of similar order (10^{-7} M) as etaconazole and imazalil, which were more toxic by several orders of magnitude (Table 1 and 2). The IC_{50} values may also be over-estimated since, due to the high protein content of the microsomal preparations, maximal type II difference response may not be proportional to protein concentration.[17] It was not possible to work with less microsomal protein, because then the concentration of P450 isozymes would have been lower than 100 nM, which was regarded as the minimal concentration for accurate spectrophotometric analysis.

The maximum type II spectral response also did not correlate with the toxicity of DMIs to *U. maydis*. However, these values changed considerably between experiments for reasons unknown (Table 2). Wiggins and Baldwin[18] used microsomes of *S. cerevisiae* and partially purified yeast P450 preparations. They concluded that in both cases the final saturation value did relate to fungicidal activity. Hence, in order to demonstrate such a correlation with *U. maydis* it may also be necessary to purify the $P450_{DM}$ from the microsomal preparations. The concentrations at which type II spectral responses were saturated were similar for all DMIs tested and ranged from 10^{-5} to $5x10^{-4}$ M (Fig. 4), which is far higher than the actual concentration of P450 used (10^{-7} M). It is known that azole fungicides bind with $P450_{DM}$ with one-to-one stoichiometry.[6,7,10,19] The fact that stoichiometric interaction was not observed in the present study might be explained by partitioning of the lipophilic azoles in the microsomal membranes, thus making the DMIs unavailable to the $P450_{DM}$ at low concentrations. In addition, the microsomes isolated from *U. maydis* almost certainly contain a variety of P450 isozymes which could differ in

affinity for DMIs and mask the specific $P450_{DM}$. This view is supported by work of Henry and Sisler[20] who demonstrated that *U. maydis*, grown under identical conditions to those used in the present experiments, was able to demethylate p-chloro-N-methylaniline (PMCA), a classical model P450 type substrate. The N-demethylation of PCMA by intact sporidia appeared to be constitutive and far less sensitive to DMIs than sterol biosynthesis or growth of *U. maydis*.

It has been suggested that the CO-displacement studies may be useful to evaluate DMI fungicides.[5-11,21] This suggestion was based on studies with purified $P450_{DM}$ from *S. cerevisiae* and microsomal P450 isozymes from *C. albicans*. These studies have shown that the differences in inhibition of sterol 14α-demethylation by a number of DMIs, including stereoisomers of triadimenol and diniconazole, correlated with their ability to impair binding of CO to the ferrous cytochrome(s).[5-11] The present study revealed that the inhibitory effects of prochloraz, imazalil, compound V and etaconazole on CO/P450 complex formation were small and, except for imazalil, variable (Table 3). This might be due to fact that $P450_{DM}$, highly sensitive to DMIs, forms only a minor and variable proportion of the total amount of microsomal P450 isozymes from *U. maydis*. The relatively low variability observed with imazalil may relate to its higher water solubility as compared with the other azoles tested. Despite this low level of inhibition and high variability, the CO displacement studies demonstrated that the azoles did differ in their affinity for the P450 isozymes (Table 3). However, the affinity did not correlate with fungicidal activity. For example, imazalil was more readily displaced by CO than etaconazole despite the fact that etaconazole was about 10 times less toxic to *U. maydis* than imazalil. This lack of correlation implies that the CO displacement studies with microsomal preparations are not useful for evaluating selective toxicity of DMIs to whole cells of *U. maydis*. These conclusions are in line with results from Marichal *et al.*[12] and Ballard *et al.*[13], who studied the interaction of microsomal P450 isozymes obtained from *A. fumigatus* with fluconazole, ketoconazole and itraconazole. Fungitoxicity of DMIs could also, therefore, be determined by other parameters like accumulation, metabolism of the fungicide or disposition in lipid compartments in the cells. However, it may also be that the current CO displacement tests with microsomal preparations are not suitable for determination of the specific inhibition of $P450_{DM}$ by azoles. Firstly, this may be due to the crude nature of these preparations containing multiple forms of P450, which may mask the specific interaction of the DMIs with $P450_{DM}$. Secondly, the CO concentration in the sample cuvette is far above the DMI concentration thus preventing the DMIs from effectively competing for the P450 isozymes. Thirdly, as suggested by Yoshida[21], interference of the azoles with CO binding or oxygen activation may not be essential for their inhibition of $P450_{DM}$ activity. This view is mainly based on the observation that buthiobate, triadimefon and triadimenol, which have relatively small substituents at N_1 of the azole group, exhibit practically no interference with CO, while these azole fungicides inhibit reconstituted lanosterol 14α-demethylase activity from *S.cerevisiae*.[5,6,21,22] Furthermore,

Hitchcock *et al.*[19] found that the ability of the triazole-related compound ICI 153066 to inhibit reconstituted $P450_{DM}$ activity from *C. albicans* also did not relate to its ability to interfere with the binding of CO to the ferrous $P450_{DM}$. Consequently, interaction of the azole moiety of DMIs with the haem iron may be of minor importance with respect to their ability to inhibit the biochemical activity of the $P450_{DM}$. Therefore, affinity to $P450_{DM}$ may be mainly determined by binding of the N_1 substituent of DMIs, possibly to the substrate binding site of the cytochrome. This is especially relevant for antimycotics with large N_1 substituents like ketoconazole and itraconazole which may tightly bind to the substrate binding site in the haem crevice of $P450_{DM}$ and in consequence impair binding of CO to the haem iron. These compounds thus inhibit both substrate binding and oxygen activation.[5,6,21]

Results of the present study demonstrate that spectroscopic analysis of *U. maydis* microsomes does not give clear information about the affinity of the azole fungicides to $P450_{DM}$ from this fungus. Purification of $P450_{DM}$ and/or using reconstituted $P450_{DM}$ assay systems may resolve this question. To test the intrinsic toxicity of DMIs to $P450_{DM}$ activity it may be easier and more valuable to test the sensitivity of enzyme activity in cell-free systems.

Using the techniques described it would be interesting to compare the P450 content of the wild-type strain used in the present study to that of the mutant deficient in 14α-sterol demethylation described by Walsh and Sisler.[23]

Acknowledgement

The authors would like to thank Dr. R.J. Milling and Dr. S.W. Ellis for their critical comments. J.C. Kapteyn also acknowledges the financial support of Schering Agrochemicals Ltd., UK.

References

1. Kato, T., *Chemistry of plant protection*, ed. G. Haug & H. Hoffmann. Springer-Verlag, Berlin, 1986, pp.1-24.
2. Vanden Bossche, H., Willemsens, G., Marichal, P., Cools, W. & Lauwers, W., *Mode of action of antifungal agents*, ed. A.P.J. Trinci & J.F. Ryley. Cambridge Univ. Press, 1984, pp. 321-341.
3. Vanden Bossche, H., Marichal, P., Gorrens, J., Bellens, D., Verhoeve, H., Coene, M-C., Lauwers, W. & Janssen, P.A.J., *Pestic. Sci.*, **21** (1987) 1-18.
4. Vanden Bossche, H., Marichal, P., Gorrens, J., Bellens, D., Moereels, H. & Janssen, P.A.J., *Biochem. Soc. trans.*, **18** (1990) 56-59.
5. Yoshida, Y. & Aoyama, Y., *In vitro and In vivo evaluation of antifungal agents*, ed. K. Iwata & H. Vanden Bossche. Elsevier Science Publishers B.V., Amsterdam, 1986, pp.123-134.
6. Yoshida, Y. & Aoyama, Y., *Biochem. Pharmacol.*, **36** (1987) 229-235.
7. Yoshida, Y. & Aoyama, Y., *Chirality*, **2** (1990) 10-15.

8. Yoshida, Y., Aoyama, Y., Takano, H. & Kato, T., *Biochem. Biophys. Res. Commun.*, **137** (1986) 513-519.
9. Vanden Bossche, H., Bellens, D., Cools, W., Gorrens, J., Marichal, P., Verhoeven, H., Willemsens, G., De Coster, R., Beerens, D., Haelterman, C., Coene, M-C., Lauwers, W. & Le Jeune, L., *Drug Dev. Res.*, **8** (1986) 287-298.
10. Vanden Bossche, H., *Sterol biosynthesis inhibitors, pharmaceutical and agrochemical aspects*, ed. D. Berg & M. Plempel. Ellis Horwood, Chichester, 1988, pp. 79-119.
11. Vanden Bossche, H., *Recent Trends in the Discovery, Development and Evaluation of antifungal Agents*, ed. R.A. Fromtling. J.R. Proust Science Publishers S.A., Barcelona, 1987, pp. 207-221.
12. Marichal, P., Vanden Bossche, H., Gorrens, J., Bellens, D. & Janssen, P.A.J., *Cytochrome P-450: biochemistry and biophysics, Proc. 6th Int. Conf. Biochemistry and Biophysics of Cytochrome P-450 in Vienna, 1988*, ed. I. Schuster. Taylor & Francis, London-New York-Philadelphia, 1989, pp. 177-180.
13. Ballard, S.A., Kelly, S.L., Ellis, S.W. & Troke, P.F., *J. Med. Vet. Mycol.*, **28** (1990) 327-334
14. Coursen, C. & Sisler, H:, *Am. J. Bot.*, **47** (1964) 541-549.
15. Omura, T. & Sato, R., *J. Biol. Chem.*, **239** (1964) 2370-2385.
16. Hitchcock, C.A., Dickinson, K., Brown, S.B., Evans, E.G. & Adams, D.J., *Biochem. J.*, **263** (1989) 573-579.
17. Jefcoate, C.R., *Methods in Enzymology*, ed. S. Fleischer & L. Packer. Academic Press, New york, 1978, vol.52, part C, pp. 258-279.
18. Wiggins, T.E. & Baldwin, B.C., *Pestic. Sci.*, **15** (1984) 206-209.
19. Hitchcock, C.A., Dickinson, K., Brown, S.B., Evans, E.G. & Adams, D.J., *Biochem. J.*, **266** (1990) 475-480.
20. Henry, M.J. & Sisler, H., *Pestic. Biochem. Physiol.*, **22** (1984) 262-275.
21. Yoshida, Y., *Current topics in medical mycology*, ed. M.R. McGinnis. Springer-Verlag, New York, 1988, vol 2, pp. 388-418.
22. Ayoama, Y., Yoshida, Y., Hata S., Ninshino, T. & Katsuki, H., *Biochem. Biophys. Res. Commun.*, **115** (1983) 642-647.
23. Walsh, R.C. & Sisler, H., *Pestic. Biochem. Physiol.*, **18** (1982) 122-131.

CHAPTER 6

INTERACTION OF AZOLE FUNGICIDES AND RELATED COMPOUNDS WITH CYTOCHROME-P450 ISOZYMES FROM *PENICILLIUM ITALICUM* IN *IN VITRO* ASSAYS

Johan C. Kapteyn,[a] Richard J. Milling,[b] Donald J. Simpson[b] and Maarten A. De Waard[a]

[a] Department of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, 6707 PD Wageningen, The Netherlands.

[b] Schering Agrochemicals Ltd., Chesterford Park Research Station, Saffron Walden, Essex CB10 1XL, UK.

(Accepted by Pesticide Science)

Abstract

The interaction of various sterol demethylation inhibitors (DMIs) and experimental compounds with cytochrome-P450 dependent sterol 14α-demethylase ($P450_{14DM}$) from *Penicillium italicum* was studied by difference spectroscopy using a preparation of microsomal P450 isozymes and assays with cell-free extracts capable of synthesizing ergosterol from [^{14}C]mevalonate. The EC_{50} values (concentrations of compounds which inhibit radial growth of *P. italicum* by 50%) of the compounds ranged from $3x10^{-8}$ M to levels higher than 10^{-3} M. All the compounds investigated gave type II difference spectra and interfered with the binding of carbon monoxide (CO) to microsomal P450 isozymes. However, the differences in the IC_{50} values (concentrations of compounds which cause 50% of maximal type II spectral change) between compounds and their inhibition of CO binding did not correlate with the fungicidal activity of the compounds. Hence, neither type II difference spectra nor CO-displacement tests with microsomal preparations from this fungus can be used for studying the selective fungitoxicity of azoles. Data obtained using the cell-free sterol 14α-demethylase assay revealed larger differences between the compounds in their inhibition of sterol 14α-demethylation. The I_{50} values (concentrations of compounds which inhibit cell-free sterol biosynthesis by 50%) varied from $4.3x10^{-9}$ to $4.4x10^{-5}$ M. This assay was able to rank the compounds in order of fungitoxicity, but the I_{50} values did not quantitatively reflect the differences in toxicity. Consequently, the observed differential inhibition of $P450_{14DM}$ activity between the compounds can not fully explain their selective fungitoxicity. Additional mechanisms must be involved. The

present study supports the general opinion that the affinity of azoles for $P450_{14DM}$ depends on the nature of their N_1 substituent. However, it was demonstrated that the nature of the azole moiety was also of importance in determining the affinity of DMIs for $P450_{14DM}$.

Introduction

Sterol demethylation inhibitors (DMIs) are antifungal agents used in agriculture and medicine. Their primary mode of action is inhibition of sterol biosynthesis due to binding to cytochrome-P450 dependent sterol 14α-demethylase ($P450_{14DM}$).[1] Derivatives of imidazole and triazole constitute the most important group of DMIs.

Great differences in both potency and selectivity have been found for various DMIs.[2] The biochemical basis of selective fungitoxicity of DMIs, however, has not yet been elucidated. Previous work has demonstrated that the selective fungitoxicity of prochloraz and an experimental triazole fungicide to a number of fungi is not generally due to differential fungal accumulation or metabolism.[3] Another possible mechanism for selectivity may be differential affinity of DMIs for the $P450_{14DM}$. The binding affinity of DMIs for $P450_{14DM}$ can be studied in several ways: For example spectrophotometrically by determining the ability of carbon monoxide (CO) to displace DMIs from reduced $P450_{14DM}$, or by measuring the ability of DMIs to induce type II difference spectra.[4-18] It can also be studied enzymatically by measuring the inhibitory effect of DMIs on ergosterol biosynthesis in fungal cell-free extracts[6,9,11,19-23] or reconstituted systems.[4-7,9,11,23] Such studies with $P450_{14DM}$ purified from *Saccharomyces cerevisiae* and with microsomal P450 isozymes from *Candida albicans* indicated that the interaction of azole DMIs is twofold: first, the N_3 and N_4 of the imidazole and triazole rings, respectively, bind to the ferric iron at the haem centre of $P450_{14DM}$ and second, the N_1 substituent of the azoles interacts with the apoprotein of the enzyme, presumably within the substrate binding site. The degree of inhibitory action of DMIs on $P450_{14DM}$ activity has been suggested to be predominantly determined by the second interaction.[4-12] This view was mainly based on CO-displacement studies which showed that replacement of the imidazole ring of ketoconazole by a triazole moiety did not affect the affinity to microsomal P450 from *C. albicans*.[11,12] Similar results were obtained when the triazole ring of itraconazole was replaced by an imidazole ring.[11,12] Comparison of ketoconazole with deacylated ketoconazole (nor-ketoconazole) showed that minor structural changes in the N_1 substituent did affect the interaction with P450 isozymes from *C. albicans*.[11] Further evidence for the importance of the N_1 substituent of azoles in fungicidal activity was also deduced from the differences in affinity of yeast $P450_{14DM}$ for the different isomers of triadimenol, diniconazole, diclobutrazol and paclobutrazol.[6,7,13,19,20] Moreover, the two isomers of diclobutrazol differentially inhibited CO binding to microsomal P450 isozymes from *Ustilago maydis* which correlated with their differences in fungitoxicity.[14] Since the inhibition of *in vitro* and *in vivo* sterol 14α-demethylation by various DMIs correlated

with their ability to impair the formation of CO-$P450_{14DM}$ complexes, the spectrophotometric analysis of the interaction of DMIs with $P450_{14DM}$ has been proposed to be an effective method for evaluating their *in vitro* fungicidal action.[4-12,14] However, more recently, spectrophotometric studies with microsomal P450 isozymes from *Aspergillus fumigatus*, *Penicillium italicum* and *U. maydis* demonstrated that the ability of CO to displace DMIs from such complexes did not always correlate with their *in vitro* toxicity.[15-18] In contrast, the inhibitory effects of DMIs on ergosterol biosynthesis by cell-free preparations of *A. fumigatus* and *P. italicum* did reflect to some degree their *in vitro* fungitoxic action.[21,22] Furthermore, the potency of the triazole, ICI 153066, in inhibiting cell-free and reconstituted $P450_{14DM}$ activity from *C. albicans* also did not relate to its ability to impair binding of CO to purified $P450_{14DM}$.[23]

In the present paper the interaction of prochloraz (compound **I**), prochloraz analogues (compounds **II** to **IX**), the structurally unrelated compound **X**, and racemic mixtures of etaconazole, imazalil, triadimenol and triadimefon (compounds **XI** to **XIV**) (Fig. 1) with microsomal P450 isozymes from *P. italicum* was measured spectrophotometrically. The interaction was also studied by measuring the inhibitory effect of the compounds on ergosterol biosynthesis in cell-free extracts of *P. italicum*. The results of both analytical methods were compared and discussed in relation to the toxicity of the compounds to *P. italicum*.

Materials & methods

Chemicals. Imazalil sulphate (imazalil) was a gift from Janssen Pharmaceutica (Beerse, Belgium). Etaconazole was obtained from Ciba-Geigy (Basel, Switzerland). Triadimefon and triadimenol were provided by Bayer (Leverkusen, Germany). Prochloraz and experimental compounds (II-X) were from Schering Agrochemicals Ltd. Dimethyl sulfoxide (DMSO) and sodium dithionite were purchased from Merck (Darmstadt, Germany). DL[2-^{14}C]mevalonate was from Du Pont De Nemours (s'Hertogenbosch, The Netherlands). All test compounds were dissolved in DMSO.

Culture methods, toxicity assays. *P. italicum* wild-type was maintained on malt extract agar. Liquid mycelial cultures were prepared as described previously.[18,22] Toxicity of DMIs and related compounds to radial growth of *P. italicum* on potato dextrose agar (PDA) was determined according to Kapteyn *et al.*[3]

Isolation and spectrophotometric analysis of microsomes. Microsomes containing stable P450 isozymes were isolated and analysed spectrophotometrically as described previously.[17,18]

Preparation of cell-free extracts and sterol biosynthesis assays. A cell-free extract of *P. italicum* was prepared by mechanical disruption of 32 ml mycelial paste (80 mg fresh weight of mycelium in 1 ml 100 mM potassium phosphate buffer pH 7.5) using a Bead-Beater

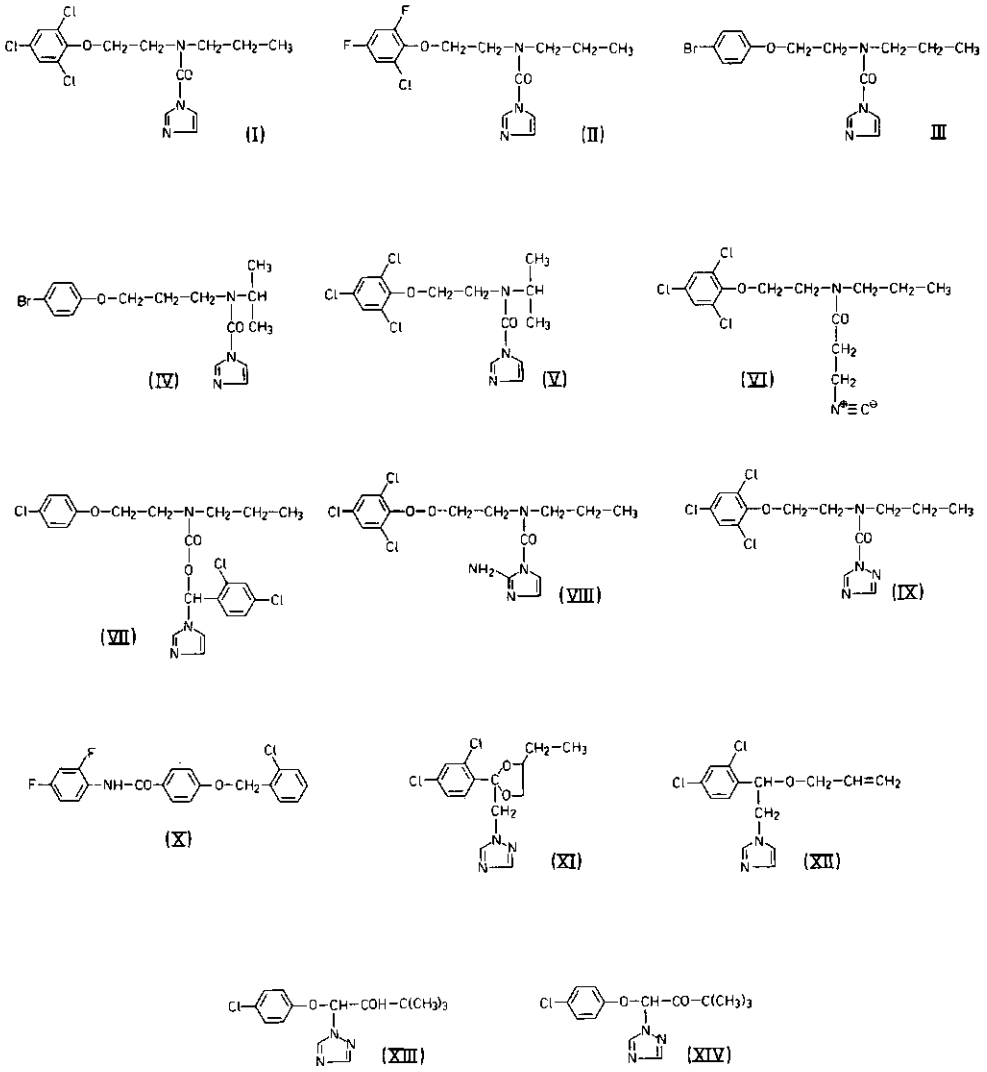


Fig. 1 : Structural formulae of prochloraz (**I**), prochloraz analogues (**II-IX**), the unrelated compound **X** and reference DMIs etaconazole (**XI**), imazalil (**XII**), triadimenol (**XIII**) and triadimefon (**XIV**). Reference DMIs were used as racemic mixtures.

(Biospec Products, Bartlesville, Oklahoma, USA) as described previously.[22] The sterol biosynthesis assay was also carried out according to Guan *et al.*[22] The test compounds were added from 1000-fold stock concentrations in DMSO. Radiolabeled non-saponifiable $C_{4,4}$-dimethylated, C_4-monomethylated and C_4-desmethylated sterols produced in the assay were separated by thin layer chromatography (TLC). Radioactive zones were located by autoradiography, cut from the plate and the radioactivity present was determined by liquid scintillation counting.

Results

Toxicity assay. Prochloraz had the lowest EC_{50} value ($3.1x10^{-8}$ M) of all the compounds tested. EC_{50}s of the prochloraz analogues ranged from $4.2x10^{-8}$ M to levels higher than 10^{-3} M (Table 1). EC_{50}s for analogues **VIII**, **IX** and compound **X** could not be determined accurately, since they did not show any inhibitory effect at a concentration of 10^{-3} M. The EC_{50} values of the reference DMIs tested ranged from $2.4x10^{-7}$ to $2.1x10^{-3}$ M (Table 1).

Table 1: Toxicity of prochloraz, experimental compounds and various reference DMIs to radial growth of *Penicillium italicum* on PDA.

Compounds	EC_{50} (M)[1,2]
Prochloraz series:	
I (Prochloraz)	$3.1x10^{-8}$
II	$4.2x10^{-8}$
III	$4.0x10^{-7}$
IV	$4.9x10^{-7}$
V	$6.9x10^{-6}$
VI	$3.8x10^{-5}$
VII	$7.6x10^{-4}$
VIII	$>10^{-3}$
IX	$>10^{-3}$
X	$>10^{-3}$
Reference DMIs:	
XI (Etaconazole)	$2.4x10^{-7}$
XII (Imazalil)	$3.3x10^{-7}$
XIII (Triadimenol)	$1.6x10^{-4}$
XIV (Triadimefon)	$2.1x10^{-3}$

[1] Concentration of compounds which caused 50% inhibition of radial growth of *P. italicum* on PDA.

[2] EC_{50} values based on two replicate experiments.

Due to changes in experimental conditions imazalil had an approximately 20 times lower EC_{50} value than reported before.[22]

Type II binding difference spectra. Type II difference spectra of microsomal P450 isozymes (100 nM) from *P. italicum* could be recorded for all compounds tested including the non-azoles VI and X, the latter of which is unrelated to prochloraz (data not shown). Compounds III, V and IX were not tested. A typical type II spectrum of prochloraz is presented in Fig. 2. The type II spectral changes induced by all azole agents were characterized by an absorbance maximum at approximately 428-429 nm and minima varying from 387 to 403 nm (Table 2). In contrast, the non-azole VI and the unrelated compound X gave rise to absorbance maxima at about 434 nm. For most compounds

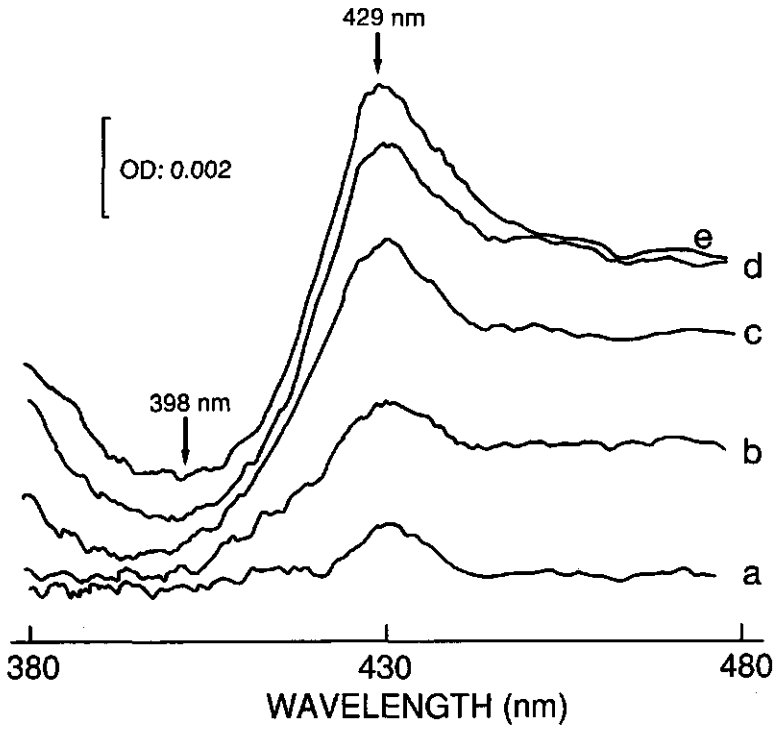


Fig. 2 : Type II difference spectra of prochloraz recorded after stepwise addition to microsomal preparations of *Penicillium italicum* containing P450 isozymes (10^{-7} M). Final prochloraz concentrations in sample cuvette: 0 (a), $5x10^{-9}$ M (b), $5x10^{-8}$ M (c), $6.2x10^{-6}$ M (d) and $1.6x10^{-5}$ M (e).

Table 2: Characteristics of type II difference spectra of microsomal P450 isozymes (10^{-7} M) induced by prochloraz, experimental compounds and reference DMIs and their inhibition of CO binding to these enzymes.

Compounds	Type II spectrum (nm) minimum	maximum	IC_{50} (M)[1]	CO-binding (%)[2]
Prochloraz series:				
I (Prochloraz)	390-392	428-429	$3.2\pm3.9\times10^{-8}$ (5)[3]	49.1±10.3 (5)
II	390-392	428-429	$4.1\pm1.8\times10^{-8}$ (3)	47.8±04.5 (3)
IV	390-392	428-429	$1.0\pm0.5\times10^{-8}$ (3)	93.1±13.6 (3)
VI	396-399	434-435	$5.4\pm7.3\times10^{-8}$ (3)	17.6±04.4 (4)
VII	398-402	429-430	9.5×10^{-8} (2)	81.6±04.8 (3)
VIII	398-403	429-430	$1.5\pm2.5\times10^{-6}$ (3)	98.1±04.9 (3)
X	390-398	432-434	3.3×10^{-9} (2)	84.3±13.4 (4)
Reference DMIs:				
XI (Etaconazole)	390-394	428-429	$1.3\pm0.7\times10^{-8}$ (5)	72.2±0.00 (2)
XII (Imazalil)	390-394	428-429	$2.0\pm1.0\times10^{-9}$ (5)	43.5±0.00 (2)
XIII (Triadimenol)	388-390	428-429	$5.4\pm1.4\times10^{-8}$ (5)	73.5±13.7 (3)
XIV (Triadimefon)	387-390	427-429	$4.3\pm4.1\times10^{-8}$ (6)	100.8±13.3 (3)

[1] Concentration of compounds which induced half saturation response in type II difference spectra.

[2] Binding of CO in DMI-treated samples (10^{-6} M) as percentage of the control treatments measured 6 min after CO addition. Figures calculated from values presented in Fig. 4. Average $OD_{(448-490nm)}$ values of all controls at t=6 min after CO addition was $8.4\pm0.9\times10^{-3}$ (n=11).

[3] ( ): number of replicates.

tested satuaration response in the type II spectra was attained at almost equimolar concentrations (10^{-7} M) of P450 isozymes and antifungal compounds (data not shown). A satuaration response for compounds **VI, VII, VIII** and **X** was found at concentrations of about 10^{-6}, 10^{-6}, 10^{-5} and 2×10^{-8} M, respectively. Most compounds examined showed IC_{50} values in the order of 10^{-8} M (Table 2). Imazalil and **X** exhibited relatively low IC_{50} values of 2.0×10^{-9} and 3.3×10^{-9} M, respectively, whereas **VIII** showed an exceptionally high IC_{50} of 1.5×10^{-6} M. Dissociation constants (K_d values) of the P450 isozymes and test compounds were not determined due to technical difficulties.

Spearman's ranking correlation coefficients (r_s) between the EC_{50} (Table 1) and IC_{50} values for the prochloraz analogues, for the reference DMIs and for all the compounds tested amounted to 0.70, 0.80 and 0.53, respectively. In all three cases there was no correlation between the EC_{50} and IC_{50} values (P=0.05).

CO displacement tests. Reduced CO difference spectra with a maximum absorbance at 449 nm recorded at various time intervals after addition of CO are shown in Fig. 3A. The relative affinity of DMIs and related chemicals for the ferrous P450 isozymes was studied by determining their inhibitory effects on CO-P450 complex formation. Except for triadimefon and **VIII**, all chemicals tested were able to interfere with the binding of CO, but to significantly different degrees (Fig. 4 and Table 2). The isonitrile analogue (**VI**) was the most potent inhibitor of CO-P450 complex formation, while **IV**, **VIII** and triadimefon (**XIV**) hardly interfered with CO binding (Fig. 4). **VI** was also the only compound which induced the formation of a maximum at 420 nm in addition to the one at 449 nm (Fig. 3B), indicating that it caused denaturation of the P450 isozymes. Spearman's r_s coefficients between the EC_{50}s and the inhibitory effects of the prochloraz analogues, of the reference DMIs and of all compounds tested on CO binding to the microsomal P450 isozymes were 0.10, 0.70 and 0.45, respectively. In all three cases there was no correlation (P=0.05).

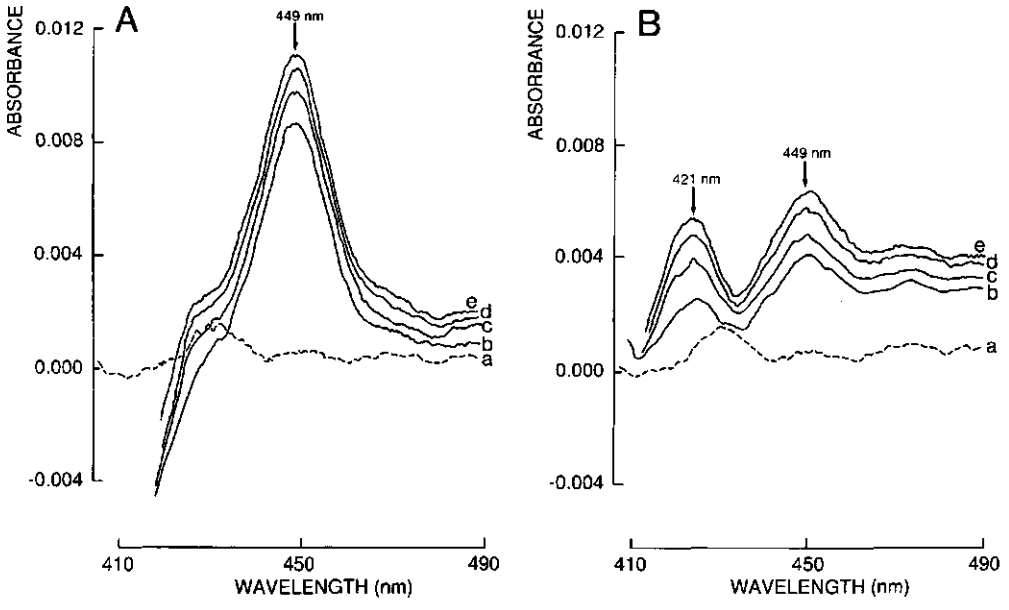


Fig. 3 : Reduced CO difference spectra of microsomal P450 isozymes (10^{-7} M) from *Penicillium italicum* preincubated with 0.1% DMSO (A) and 10^{-6} M **VI** (B) recorded before (a) and 1 (b), 3 (c), 10 (d) and 15 min (e) after CO-treatment.

Cell-free sterol biosynthesis assay. Total incorporation of label into non-saponifiable lipids varied between experiments from 18 to 24% of the total radioactivity added. On average the percentage of radioactivity incorporated into ergosterol amounted to 16% of the non-saponifiable lipids. Fig. 5 (control treatment) shows an autoradiogram of a TLC separation of non-saponifiable lipids produced from [2-^{14}C]mevalonate by a cell-free extract of *P. italicum*. Based on TLC and radio-HPLC analysis by Guan *et al.*[22] bands 1

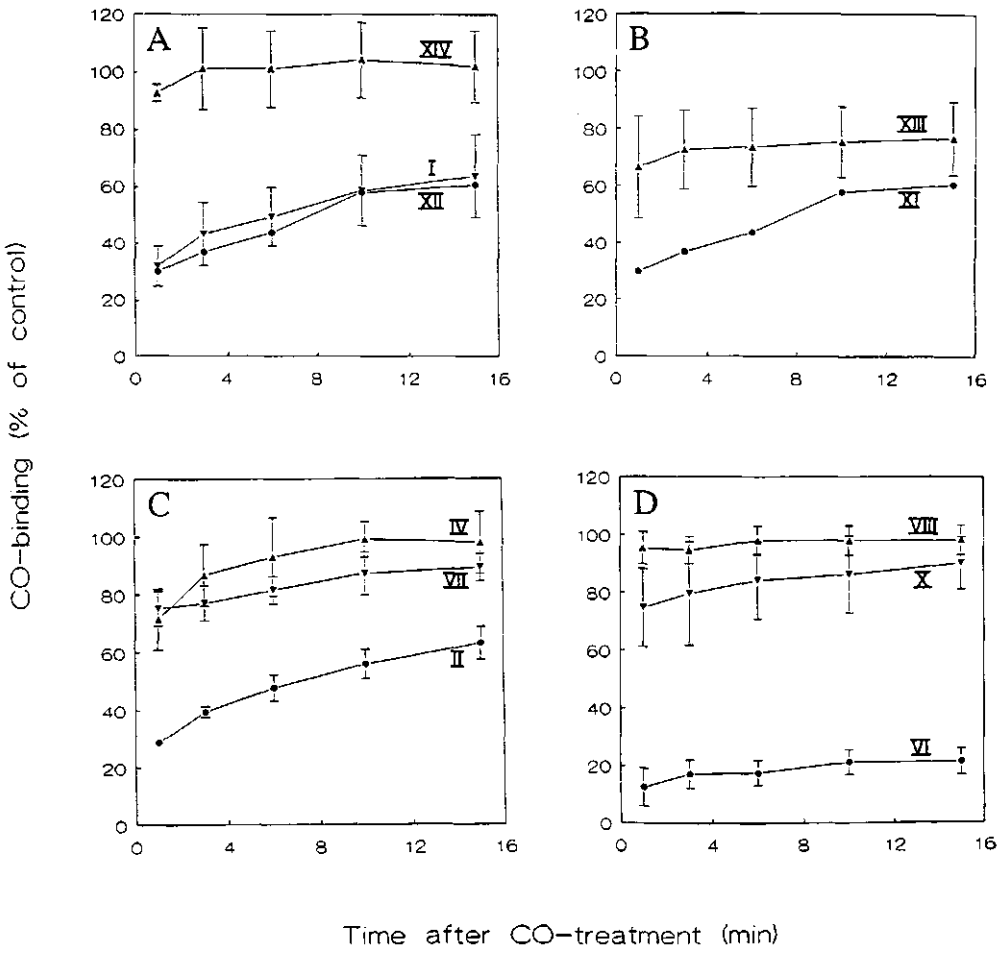


Fig. 4 : Displacement of (A) prochloraz (**I**; ▾), imazalil (**XII**; •) and triadimefon (**XIV**; ▴); (B) etaconazole (**XI**; •) and triadimenol (**XIII**; ▴); (C) **II** (▪), **IV** (▴) and **VII** (▾); (D) **VI** (•), **VIII** (▴) and **X** (▾) from microsomal P450 isozymes (10^{-7} M) of *Penicillium italicum* after reduction with dithionite and CO treatment. Binding of CO as percentage of control (DMSO) plotted against time after CO treatment. Concentration of all test compounds 10^{-6} M.

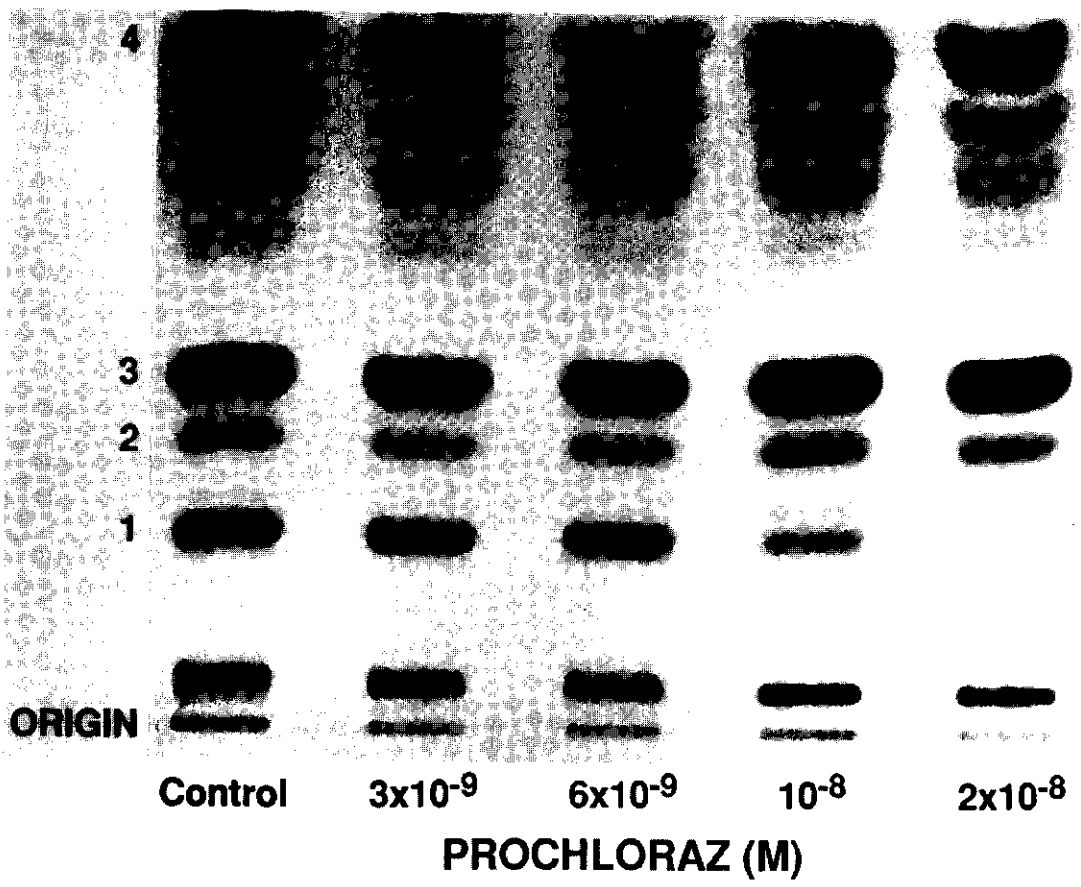


Fig. 5 : Autoradiogram of a TLC separation of non-saponifiable lipids synthesized from [2-^{14}C]mevalonate by a cell-free preparation of *Penicillium italicum* in the presence of increasing concentrations of prochloraz. Bands 1, 2, 3 and 4 contained C_4-desmethyl sterols, C_4-monomethyl sterols, $C_{4,4}$-dimethyl sterols and squalene, respectively. All other bands were not identified.

and 2 are assigned to ergosterol and C_4-monomethyl sterols, respectively. Band 3 contains the $C_{4,4}$-dimethyl sterols, lanosterol and 24-methylenedihydrolanosterol (eburicol). Radioactivity in band 4 is most probably squalene while the other bands are not yet identified. Prochloraz inhibited incorporation of radiolabel into C_4-desmethyl sterols at very low concentrations (Fig. 5); it had an I_{50} value (concentration which inhibits incorporation of label into C_4-desmethylsterols by 50%) of 8.0×10^{-9} M (Table 3). All other compounds tested also had inhibitory effects, but over a wide range of I_{50} values (Table 3). Prochloraz analogue **II** was the most potent inhibitor tested (I_{50} 4.3×10^{-9} M).

Table 3: Inhibitory effects of prochloraz, experimental compounds and reference DMIs on cell-free 14α-demethylase activity from *Penicillium italicum*.

Compounds	I_{50} (M)[1]
Prochloraz series:	
I (Prochloraz)	$8.0\pm1.0\times10^{-9}$ (3)[2]
II	$4.3\pm1.0\times10^{-9}$ (4)
III	$1.4\pm0.2\times10^{-8}$ (3)
IV	$1.8\pm0.1\times10^{-8}$ (3)
V	$1.0\pm0.2\times10^{-7}$ (3)
VI	$9.2\pm2.7\times10^{-7}$ (3)
VII	$3.5\pm1.1\times10^{-7}$ (3)
VIII	$9.0\pm0.5\times10^{-7}$ (3)
IX	$6.5\pm1.1\times10^{-7}$ (3)
X	$4.4\pm1.0\times10^{-5}$ (3)
Reference DMIs:	
XI (Etaconazole)	$8.7\pm1.1\times10^{-9}$ (4)
XII (Imazalil)	$9.0\pm1.0\times10^{-9}$ (4)
XIII (Triadimenol)	$4.2\pm1.8\times10^{-8}$ (3)
XIV (Triadimefon)	$7.2\pm1.0\times10^{-8}$ (3)

[1] Concentration which inhibits incorporation of [2-^{14}C]mevalonate into C4-desmethyl sterols by 50%.

[2] ( ): number of replicates.

Prochloraz, etaconazole, imazalil, **III** and **IV** all had comparable I_{50}s of approximately 10^{-8} M. Triadimenol and triadimefon were slightly less effective, but still exhibited I_{50} values at 4.2×10^{-8} and 7.2×10^{-8} M, respectively. All other chemicals showed higher I_{50} values ranging from approximately 10^{-7} to 10^{-6} M. Compound **X** was the weakest inhibitor of cell-free sterol 14α-demethylation found (I_{50} 4.4×10^{-5} M). It is noted that DMSO (0.1% (v/v)) did not have any inhibitory effect on cell-free sterol 14α-demethylation. With respect to prochloraz and experimental compounds **II-X** Spearman's r_s coefficient was 0.96. This value decreased to 0.86 when the reference DMIs were included, while r_s was 1.0 for the reference DMIs alone (Table 3). In all three cases there was a ranking correlation between the EC_{50} and I_{50} values ($P=0.05$).

Discussion

The present study demonstrates that a number of related azoles is capable of inducing type II difference spectra with microsomal preparations of P450 isozymes from *P.*

italicum (Table 2). This indicated that the ferric haem iron of P450 was occupied by the N_3 and N_4 of the imidazole and triazole rings, respectively. However, the non-azole **VI** and the unrelated compound **X** also induced type II difference spectra, suggesting that the isonitrile carbon of analogue **VI** must also interact with the haem iron. It is unknown which functional group of compound **X** is responsible for the induction of the type II spectrum. These findings confirm literature data that type II spectra are not only induced by compounds with nitrogenous heterocyclic rings, but generally by all kinds of amines and isonitriles.[24,25] Comparison of the spectral characteristics showed differences in the absorbance maxima in type II spectra of azole and non-azole compounds. Maxima of type II spectra induced by the azoles ranged from 427 to 430 nm, whereas those of the non-azole compounds **VI** and **X** were at approximately 434 nm. Apparently, the type II spectral characteristics induced are determined by the chemical nature of the interacting moiety. This observation corresponds to data obtained by others.[24] Irrespective of their toxicity to *P. italicum*, the concentration of most DMIs which induced a half saturation response of type II spectra (IC_{50}) was in the order of 10^{-8} M (Table 2). For reasons unknown imazalil had an exceptionally low IC_{50}, as did the unrelated compound **X**. The relatively high IC_{50} of compound **VIII** can be explained by the fact that the amino group at C_2 of the heterocyclic ring hampers the interaction of N_3 with the sixth ligand of the cytochrome ferric haem iron. The observed absence of a correlation between the ability of azoles to induce type II spectra and their fungitoxicity is in line with most recent literature data.[8,12,17,18]

The CO-displacement test has previously been reported as being suitable for assessing the *in vitro* fungicidal activity of DMIs,[4-12] but more recently, this has been contradicted.[15-18] In the present paper the compounds interfered differentially with the binding of CO to the ferrous cytochrome(s) (Fig. 4; Table 2), but the differential inhibition of CO-P450 complex formation did not correlate with their toxicity to *P. italicum* (Table 1 and 2). For instance, compound **IV**, which is almost as toxic as etaconazole, was readily displaced by CO, while the isonitrile **VI**, which is relatively non-toxic, was the most potent inhibitor of CO-P450 complex formation. This may relate to the fact that isonitriles have a very high affinity for (haem) iron atoms.[26] However, the denaturing effect of this compound on P450 (Fig. 3B) may also explain the relatively strong inhibition of CO binding. The low biological activity of **VI** could be due to the fact that this compound is likely to be susceptible to metabolic degradation. It was therefore concluded that CO-displacement tests could not be used here for structure-activity correlation studies. The lack of correlation between fungitoxicity and inhibition of CO-P450 complex formation is in agreement with results from comparable studies with *A. fumigatus*, *P. italicum* and *U. maydis*.[15-18] Most probably this is due to the crude nature of the microsomal preparations consisting of multiple P450 isozymes, which may mask the specific interaction of the compounds with $P450_{14DM}$. Another explanation is, as Yoshida suggested, that impairment of CO binding or oxygen activation by azoles may not be

essential for their ability to inhibit sterol 14α-demethylase activity.[27] This view was based on the finding that various DMIs, that hardly interfered with CO binding, did inhibit reconstituted lanosterol 14α-demethylase activity from *S. cerevisiae*. Hitchcock *et al.*[23] also demonstrated in a comparison of two azoles that the inhibition of cell-free sterol biosynthesis did not relate to their inhibitory effect on CO binding to the purified ferrous $P450_{14DM}$ from *C. albicans*. The present study is also supportive of this view.

The correlation between fungitoxicity of the compounds examined and their ability to inhibit cell-free sterol 14α-demethylation was much better, especially within the group of prochloraz analogues (Table 1 and 3). It is emphasized, however, that although the cell-free bioassay was suitable for ranking the analogues in order of fungitoxicity, the I_{50} values obtained did not quantitatively reflect the differences in fungitoxicity. For example, the relatively large differences in toxicity between compounds **V** and **VII** could not be explained by their small differences in inhibitory effects on cell-free sterol 14α-demethylation. The same tendency was found for the racemic mixtures of the reference DMIs (Table 1 and 3). For instance, triadimenol and triadimefon, which were relatively inactive against *P. italicum*, still showed relatively low I_{50} values. Consequently, the differential toxicity of prochloraz, the related structures and reference DMIs is not determined only by differential affinity for the $P450_{14DM}$. Additional mechanisms, such as metabolism, uptake and disposition, will also determine their fungitoxicity. Similar results were found by Guan *et al.*[22] Considering the fact that triadimefon needs to be converted to triadimenol to exert its fungitoxicity,[28] it was surprising to notice that triadimefon was only a slightly weaker inhibitor of cell-free $P450_{14DM}$ activity than triadimenol (Table 3). This might be explained by the fact that the cell-free extract of *P. italicum* is able to reduce triadimefon to triadimenol. This phenomenon has also been observed with cell-free extracts of *Cladosporium cucumerinum*.[29] The interpretation of these results is hampered by the possibility that the composition of the racemic mixture of triadimenol is different from the stereoisomer composition present after the putative conversion of triadimefon by the cell-free extract. It is well documented that the four triadimenol stereoisomers differ in their ability to bind with $P450_{14DM}$.[6] The inhibitory potency of the single stereoisomers of the reference DMIs on cell-free $P450_{14DM}$ activity will be studied in future research. The isonitrile compound **VI** and unrelated compound **X**, to which *P. italicum* was relatively insensitive, belonged to the least effective inhibitors of sterol 14α-demethylase activity having I_{50}s in the order of 10^{-6} and 10^{-5} M, respectively. Interestingly, compound **VI** denatured P450 to P420 (Fig. 3B), but was still a weak inhibitor of cell-free sterol biosynthesis. The low activity of **VI** in the cell-free assay might, however, be related to its relatively high susceptibility to metabolic degradation.

The present study supports the general view that the affinity of azole antifungals for $P450_{14DM}$ is influenced by the N_1 substituent of the azole moiety,[4-12] since structural changes in the N_1 substituent of prochloraz affected the inhibition of $P450_{14DM}$ activity from *P. italium* (Table 3). For instance, replacement of the propyl chain in the N_1

substituent of prochloraz by an isopropyl group (**V**) caused an approximately 12-fold loss of activity in the cell-free assay. This finding agrees with that described by Baillie.[30]

However, the affinity of azoles to $P450_{14DM}$ is not exclusively determined by their N_1 substituents, since replacing the imidazole ring of prochloraz by a triazole moiety (**IX**) abolished its toxicity to *P. italicum* and decreased its inhibitory effect on cell-free ergosterol synthesis nearly 100-fold (Table 1 and 3). Baillie[30] also found that prochloraz analogues in which the imidazole group was replaced by a triazole ring were much less active against for example *Erysiphe graminis*. It is possible that the triazole group of compound **IX**, due to the electron-withdrawing effect of the carbonyl group, may not be a good enough ligand to bind to the haem iron, or the compound may be too hydrolytically unstable (C.G. Earnshaw, pers. commun.). This implicates that the N_1 substituent of prochloraz also has an effect on the interaction of the azole moiety with the haem iron. The antifungal activity of imidazole-1-carboxylates, to which prochloraz is related in terms of structure and biological activity, was also lost when the imidazole ring was replaced by a triazole moiety.[31] The same phenomenon was observed for ketoconazole and its triazole analogue interacting with microsomal P450 from piglet testis.[11] Addition of an amino group at C_2 of the imidazole ring (**VIII**) led to a 100-fold reduction of the inhibitory effect on cell-free sterol biosynthesis. These observations agree with findings that substitutions in the 2, 4, and 5 positions of the imidazole, or 1, and 3 positions of the triazole diminish the potency of azoles against yeast $P450_{14DM}$ activity.[25,32] Consequently, the affinity of this group of compounds for $P450_{14DM}$ is dependent on both the azole part and the N_1 substituent of the molecule.

Acknowledgements

The authors would like to thank Drs. J.B. Pillmoor and C.G. Earnshaw for their critical comments. J.C. Kapteyn acknowledges the financial support of Schering Agrochemicals Ltd, Saffron Walden, UK.

References

1. Kato, T., In *Chemistry of Plant Protection*, ed. G. Haug and H. Hoffmann. Springer Verlag, Berlin, 1986, pp. 1-24.
2. Scheinpflug, H. & Kuck, K.H., In *Modern Selective Fungicides - Properties, Applications, Mechanisms of Action*, ed. H. Lyr. VEB Gustav Fischer Verlag, Jena, 1987, pp. 173-204.
3. Kapteyn, J.C., Pillmoor, J.B. & De Waard, M.A., *Pestic. Sci.*, (in press).
4. Yoshida, Y. & Aoyama, Y., In *In-vitro and In-vivo Evaluation of Antifungal Agents*, ed. K. Iwata & H. Vanden Bossche. Elsevier Science Publishers BV., Amsterdam, 1986, pp. 123-134.
5. Yoshida, Y. & Aoyama, Y., *Biochem. Pharmacol.*, **36** (1987) 229-235.
6. Yoshida, Y. & Aoyama, Y., *Chirality*, **2** (1990) 10-15.

7. Yoshida, Y., Aoyama, Y., Takano, H. & Kato, T., *Biochem. Biophys. Res. Commun.*, **137** (1986) 513-519.
8. Vanden Bossche, H., Bellens, D., Cools, W., Gorrens, J., Marichal, P., Verhoeven, H., Willemsens, G., De Coster, R., Beerens, D., Haelterman, C., Coene, M-C., Lauwers, W. & Le Jeune, L., *Drug Dev.*, **8** (1986) 287-298.
9. Vanden Bossche, H., In *Sterol Biosynthesis Inhibitors, Pharmaceutical and Agrochemical Aspects*, ed. D. Berg & M. Plempel. Ellis Horwood, Chichester, 1988, pp. 79-119.
10. Vanden Bossche, H., In *Recent Trends in the Discovery, Development and Evaluation of Antifungal Agents*, ed. R.A. Fromtling. J.R. Proust Science Publishers S.A., Barcelona, 1987, pp. 207-21.
11. Vanden Bossche, H., Marichal, P., Gorrens, J., Coene, M-C., Willemsens, G., Bellens, D., Roels, I., Moereels, H. & Janssen, P.A.J., *Mycoses*, **32** (Suppl. 1) (1989) 35-52.
12. Vanden Bossche, H., Marichal, P., Gorrens, J., Geerts, H. & Janssen, P.A.J., *Ann. N.Y. Acad. Sci.*, **544** (1988) 191-207.
13. Wiggins, T.E. & Baldwin, B.C., *Pestic. Sci.*, **15** (1984) 206-209.
14. Carelli, A., Farina, G., Gozzo, F., Merlini, L. & Kelly, S.L., *Pestic. Sci.*, **35** (1992) 167-170.
15. Marichal, P., Vanden Bossche, H., Gorrens, J., Bellens, D. & Janssen, P.A.J., In *Cytochrome P-450: Biochemistry and Biophysics, Proc. 6th Int. Conf. Biochemistry and Biophysics of Cytochrome P-450, Vienna, 1988*, ed. I. Schuster. Taylor & Francis, London-New York-Philadelphia, 1989, pp. 177-180.
16. Ballard, S.A., Kelly, S.L., Ellis, S.W. & Troke, P.F., *J. Med. Vet. Mycol.*, **28** (1990) 327-334.
17. Kapteyn, J.C., Pillmoor, J.B. & De Waard, M.A., *Pestic. Sci.*, **34** (1992) 37-43.
18. Guan, J., Braks, H.M.J., Kerkenaar, A. & De Waard, M.A., *Pestic. Biochem. Physiol.*, **42** (1992) 24-32.
19. Baldwin, B.C. & Wiggins, T.E., *Pestic. Sci.*, **15** (1984) 156-166.
20. Gadher, P., Mercer, E.I., Baldwin, B.C. & Wiggins, T.E., *Pestic. Biochem. Physiol.*, **19** (1983) 1-10.
21. Ballard, S.A., Ellis, S.W., Kelly, S.L. & Troke, P.F., *J. Med. Vet. Mycol.*, **28** (1990) 335-344.
22. Guan, J., Stehmann, C., Ellis, S.W., Kerkenaar, A. & De Waard, M.A., *Pestic. Biochem. Physiol.*, **42** (1992) 262-270.
23. Hitchcock, C.A., Dickinson, K., Brown, S.B., Evans, E.G.V. & Adams, D.J., *Biochem. J.*, **266** (1990) 475-480.
24. Schenkman, J.B., Sligar, S.G. & Cinti, D.L., *Pharmacol. Ther.*, **12** (1981) 43-71.
25. Janssen, P.A.J. & Vanden Bossche, H., *Arch. Pharm. Chem. Sci. Ed.*, **15** (1987) 24-40.
26. Malatesta, L., In *Progress in Inorganic Chemistry*, ed. F.A. Codon. Interscience, New York, 1959, pp. 233-379.
27. Yoshida, Y., In *Current Topics in Medical Mycology*, ed M.R. McGinnis, Springer-Verlag, New York, 1988, vol.2, pp. 388-418.
28. Deas, A.H.B., Carter, G.A., Clark, T., Clifford, D.R. & James, C.S., *Pestic. Biochem. Physiol.*, **26** (1986) 10-21.
29. Gasztonyi, M. & Josepovits, G., *Pestic. Sci.* **15** (1984) 48-55.
30. Baillie, A.C., In *Synthesis and Chemistry of Agrochemicals*, ed. D.R. Baker, J.G. Fenyes, W.K. Moberg & B. Cross. ACS Symposium Series **355** (1987) 328-339.
31. Imai, T., Takao, H., Yamaguchi, K., Uchida, T., Goto, T. & Umetsu, N., *Pestic. Sci.*, **34** (1992) 119-125.
32. Hitchcock, C.A., *Biochem. Soc. Trans.*, **19** (1991) 782-787.

CHAPTER 7

DEVELOPMENT OF A CELL-FREE ASSAY OF *BOTRYTIS CINEREA* TO BIOCHEMICALLY SCREEN FOR STEROL BIOSYNTHESIS INHIBITORS

Christiane Stehmann, Johan C. Kapteyn and Maarten A. De Waard

Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, the Netherlands.

(To be submitted to Pesticide Science)

Abstract

An assay for measuring ergosterol biosynthesis in cell-free extracts of the filamentous plant pathogen *Botrytis cinerea* is described. The extracts capable of synthesizing C4-desmethyl sterols from [2-^{14}C]mevalonate were prepared by mechanical disruption of young conidial germlings in a Bead-Beater apparatus. The C_4-desmethyl sterol fraction consisted of three distinct compounds and totalled 39% of the non-saponifiable lipids formed. Ergosterol accounted for 63% of the C_4-desmethyl sterols. Only little amounts of C_4-monomethyl sterols were synthesized, while $C_{4,4}$-dimethyl sterols made up 29% of the non-saponifiable lipids. The latter fraction mainly consisted of lanosterol (54%) and eburicol (28%). The cell-free system had a narrow pH optimum (pH 7.3-7.4) for synthesis of C_4-desmethyl sterols. Cell-free synthesis of C_4-desmethyl sterols was inhibited by the imidazole fungicide imazalil, concomitant with an accumulation of eburicol. The IC_{50} value (concentration of fungicide which inhibits cell-free synthesis of C_4-desmethyl sterols by 50%) was 9.1×10^{-9} M. This indicates that imazalil is a potent inhibitor of the cytochrome-P450 dependent sterol 14α-demethylase of *B. cinerea*. The method described may be used to biochemically screen compounds for inhibition of sterol biosynthesis in an agriculturally important plant pathogen.

Introduction

In the last two decades the development of resistance to benzimidazole and dicarboximide fungicides has severely hampered chemical control of *Botrytis cinerea*. During this period, fungicides which inhibit sterol biosynthesis (SBIs) were developed. Sterol demethylation inhibitors (DMIs) constitute the largest group of SBIs. The primary

target of these antifungals is the cytochrome-P450 dependent sterol 14α-demethylase ($P450_{14DM}$).[1,2] Although DMIs have a broad antifungal spectrum only few of them show substantial biological activity against the economically important plant pathogen *B. cinerea*.[3-6] Registration of more DMIs for the control of *B. cinerea* is therefore desirable. A cell-free sterol biosynthesis assay from this filamentous fungus would be useful to study the quantitative structure activity relationships of potential DMI-fungicides so that the toxicity of DMIs to *B. cinerea* could be optimized. The information would aid in the understanding of the selectivity of DMIs for different pathogens and could be used to screen for SBIs which inhibit sterol synthesis at sites other than the $P450_{14DM}$.

Cell-free extracts capable of synthesizing ergosterol from the sterol precursor [2-^{14}C]mevalonate have been largely confined to *Saccharomyces cerevisiae*,[7,8] and the yeast form of *Candida albicans*.[9-11] These assays have often been used to optimize the fungitoxicity of candidate fungicides. This is not an ideal situation, since the sensitivities of $P450_{14DM}$ activity of different fungi to a specific DMI are not necessarily the same. For a long time attempts to develop comparable assays from plant pathogenic filamentous fungi have been unsuccessful.[12] This might have been due to instability of the membrane-bound $P450_{14DM}$ during preparation of the cell-free extract or to a relatively low concentration of the enzyme in filamentous fungi. A difference in the sterol biosynthetic pathway (transmethylation of lanosterol instead of zymosterol, probably in mitochondria) might be an additional explanation.[13,14] However, the development of sterol 14α-demethylase assays has recently been reported for the filamentous fungi *Aspergillus fumigatus*[15] and *Penicillium italicum*.[16] Nevertheless, a similar assay for an important plant pathogen is still not available.

In this paper a method is described to obtain a cell-free extract of the filamentous plant pathogen *B. cinerea* capable of synthesizing C_4-desmethyl sterols. To evaluate the validity of this assay, the inhibitory action of the imidazole DMI-fungicide imazalil on $P450_{14DM}$ activity was studied.

Materials & methods

Chemicals. [2-^{14}C]mevalonate, dibenzethylenediamine salt in ethanol (specific activity 1.9 GBq mmol^{-1}), was purchased from Amersham International plc (Amersham, UK). NAD^+, $NADP^+$, NADPH, ATP, glucose-6-phosphate, L-methionine, dimethyl sulfoxide (DMSO), oxytetracycline, dithiothreitol (DTT) and mercaptoethanol (MCE) were purchased from Sigma (St. Louis, Mo., USA). Reduced glutathione and N-acetyl cysteine were from Boehringer (Mannheim, Germany). Nicotinic acid amide was from Hoffmann-La Roche (Basle, Switzerland). Imazalil (sulphate salt) was kindly supplied by Janssen Pharmaceutica (Beerse, Belgium).

Fungus and culture conditions. The monoascospore isolate of *B. cinerea* SAS-56,[17] a gift from Dr. F. Faretra (Bari, Italy), was maintained on PDA slants. Conidia of *B. cinerea* were obtained from PDA cultures (30 ml) in Petri dishes (diameter 9 cm) incubated at 20°C for 24 hours in the dark and for 14 to 20 days under near UV light. Subcultures were made every three to four days by transferring agar plugs with young sporulating mycelium to the centre of agar plates. Flasks (2 l) with liquid synthetic media (1 l) prepared according to Fritz *et al.*[19] were inoculated with washed conidia collected from these plates (initial density $2x10^6$ conidia ml^{-1}). The cultures were incubated in a rotary shaker (200 rpm) in the dark at 20°C for 14 h. By then, germination percentages of conidia exceeded 90%. Germinated conidia had one to three (usually two) germ tubes. The average germ tube length of the longest germ tube was 1.40 ± 0.06 μm.

Preparation of cell-free extract. Cell-free extracts were prepared according to a modified method described by Guan *et al.*[16] A standard germling suspension of *B. cinerea* was made by passing 14-hour-old cultures (harvest amounted to about 3 g wet weight l^{-1} medium) through a 0.2 mm pore sieve to remove clusters of mycelium and collecting the germlings on a 0.05 mm pore stainless steel sieve. Germlings collected were washed extensively with running cold tap water and twice with 100 mM ice-cold potassium phosphate buffer, pH 7.5 (250 ml). Washed germlings were resuspended in the buffer to give a ratio of 100 mg wet weight (27 mg dry weight) ml^{-1} buffer. Subsequent steps were carried out at 0-4°C. A 32 ml Bead-Beater vessel (Biospec Products, Bartlesville, Okl., USA) containing 15 g glass beads (0.5 mm diameter) was completely filled with germling suspension. Remaining air was removed by evacuation at -1 bar for 5 min. The vessel was again completely filled with germling suspension. Fungal cells were disrupted four times for 30 sec with 30 sec intervals, while the outer jacket of the vessel was filled with ice-water. The disruptor was driven at 100 V. The resulting homogenate was filtered through two layers of gauze (Klinion, Medical Care, Untermöhlen, Utrecht, The Netherlands) presoaked in buffer. Glycerol was immediately added to the filtrate (22% (v/v) final concentration), gently mixed and centrifuged twice at 3000*g* for 10 min to sediment cell debris. The resulting supernatant was used immediately for sterol biosynthesis assays. The protein concentration in cell-free preparations was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Veenendaal, The Netherlands) with bovine-γ-globulin as a standard. The absence of any intact cells was verified by microscopic observation and streak tests on PDA.

Sterol biosynthesis assay. Standard sterol synthesis assays were carried out according to a modified method of Ballard *et al.*[15] Incubation mixtures (1 ml) consisted of cell-free extract (924 μl), cofactor solution (50 μl; containing 1 μmol NADPH, 1 μmol $NADP^+$, 1 μmol NAD^+, 3 μmol glucose-6-phosphate, 5 μmol ATP, 1 μmol reduced glutathione in distilled water adjusted to pH 7.2 with 1 M KOH), L-methionine (5 μl; 2 μmol) and

divalent cation solutions (10 μl of 0.5 M $MgCl_2$ and 5 μl of 0.4 M $MnCl_2$, both adjusted to pH 7.0 with 1 M KOH). The combined incubation mixtures (20 ml) were adjusted to pH 7.4 with 5 M K_2HPO_4 (which never exceeded 1% of the total volume) and samples of 994 μl were divided into screw-capped tubes. DMSO (control treatments) or DMSO-solutions of imazalil (1 μl) were added to the incubation mixtures. Sterol biosynthesis was started by adding [2-^{14}C]mevalonate (5 μl; 18.5 kBq). Mixtures were incubated in a reciprocal water bath shaker (80 strokes min^{-1}) at 20°C in the dark for 3 h. Reaction was stopped by adding 1.5 ml of freshly prepared 20% KOH (w/v) in ethanol (90%, v/v). Effects of pH on cell-free sterol biosynthesis were studied by adjusting incubation mixtures with KH_2PO_4 (3 M) or K_2HPO_4 (5 M) to the pH values tested. The pH of mixtures was measured again after incubation.

Saponification, sterol extraction and analysis. Saponification of incubation mixtures, extraction and analysis of the non-saponifiable lipids (NSLs) by thin layer chromatography (TLC) and radio-HPLC were carried out as described previously.[17] In order to identify components of the C_4-desmethyl, C_4-monomethyl and $C_{4,4}$-dimethyl sterol fractions separated on TLC plates, zones containing sterol fractions were cut out of the plates, extracted with chloroform and re-chromatographed on TLC-plates pretreated with $AgNO_3$ as described by Kerkenaar *et al.*[19] Ergosterol, lanosterol and eburicol (24-methylene dihydrolanosterol) were identified by co-chromatography with authentic standards.

Inhibitory effects of imazalil on sterol biosynthesis. Effects of imazalil on sterol biosynthesis were investigated by incubating cell-free extracts with the inhibitor at various concentrations. Incorporation of radiolabel into C_4-desmethyl sterols was calculated as a percentage of total incorporation into NSLs.[8] Subsequently, corresponding incorporation rates of fungicide treatments were calculated as percentages of control treatments (100%). Using the soft-ware program Lotus 1-2-3 percentages were plotted against fungicide concentration on a logarithmic scale, regression analysis of these inhibitor-response data was performed and the imazalil concentration which inhibited synthesis of C_4-desmethyl sterols by 50% (IC_{50}) was calculated. The experiment was repeated four times.

Results

Characterization of cell-free sterol biosynthesis. Incorporation of [2-^{14}C]mevalonate into NSLs accounted for 27.0$\pm$4.6% (n=10) of the total radiolabel added. Separation of these NSLs by TLC resulted in a typical separation pattern as illustrated by the autoradiogram presented in Fig. 1 (control lane). Upon co-chromatography with authentic samples of ergosterol and lanosterol and by comparison with literature data[8,11,15] bands 2, 3 and 4 were tentatively identified as C_4-desmethyl sterols, C_4-monomethyl sterols and

$C_{4,4}$-dimethyl sterols, respectively. Similarly, band 7 was identified as squalene. Literature data suggest that band 6 is probably 2,3-oxido-squalene.[8,20] The identities of bands 1 and 5, composed of several zones, are unknown. C_4-desmethyl sterols represented the major component of the sterols formed (39.0±4.4% of the NSLs); $C_{4,4}$-dimethyl sterols accounted for 28.0±2.9%, while only minor amounts (3.2±1.1%) of C_4-monomethyl sterols were synthesized (n=10).

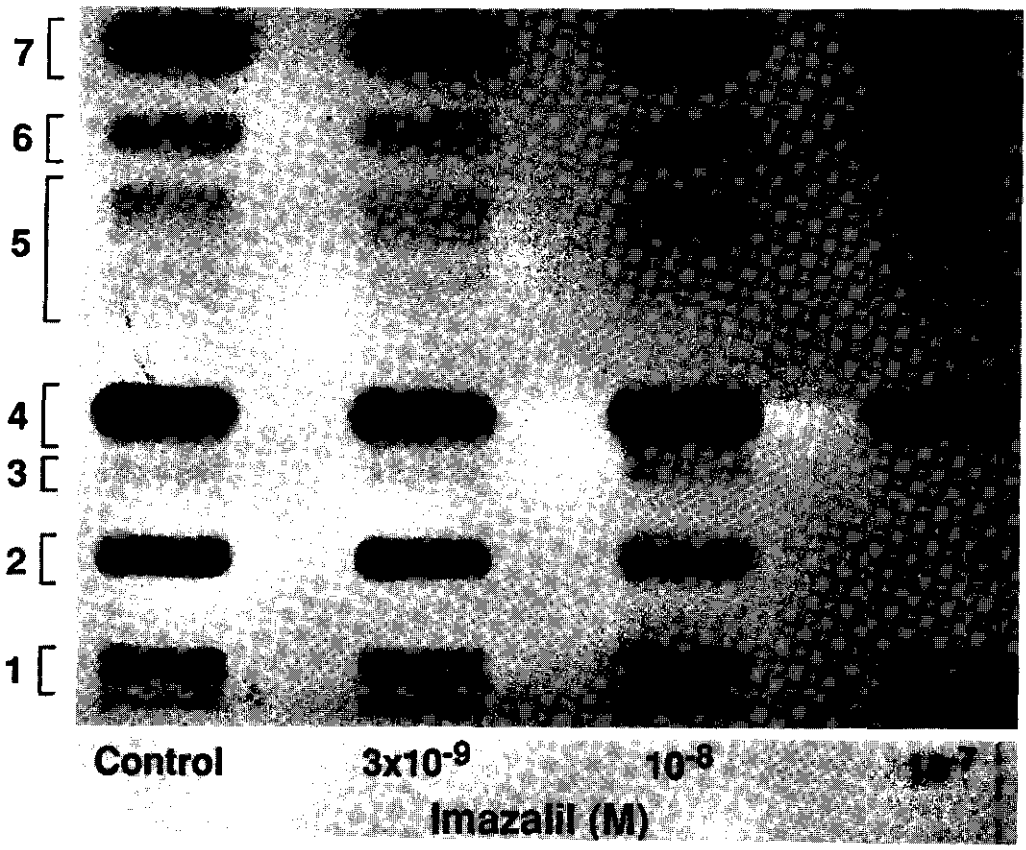


Fig. 1: Autoradiogram of a TLC separation of non-saponifiable lipids synthesized from [2-^{14}C]mevalonate in cell-free assays of *Botrytis cinerea* in the absence and presence of imazalil at pH 7.4. Bands 2, 3, 4, 6 and 7 contained C_4-desmethyl sterols, C_4-monomethyl sterols, $C_{4,4}$-dimethyl sterols, 2,3-oxidosqualene (tentative) and squalene, respectively. The identities of bands 1 and 5, which contained several sterols, are unknown.

Results of a typical time-course experiment (Fig. 2) show that incorporation of [2-^{14}C]mevalonate into NSLs was approximately linear with time up to 2 h. This was also true for synthesis of C_4-desmethyl sterols. Formation of $C_{4,4}$-dimethyl sterols decreased after 1 h. The amount of radiolabel incorporated into C_4-monomethyl sterols was low and decreased after 1 h.

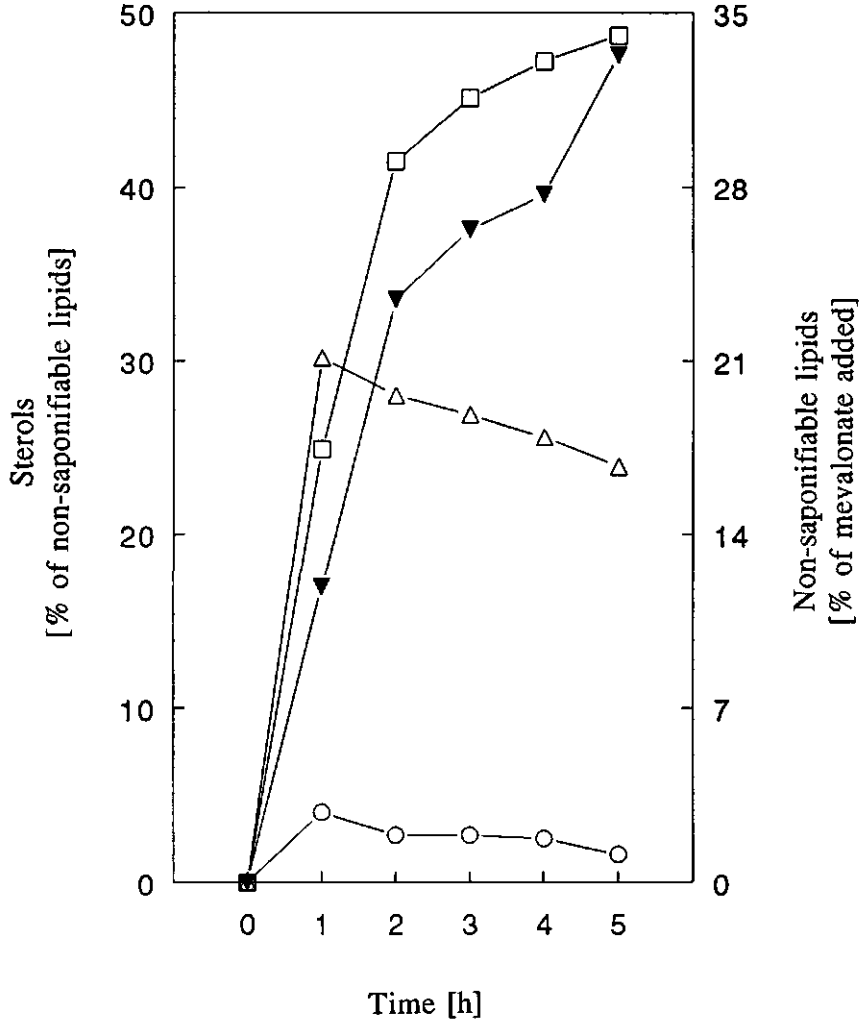


Fig. 2: Time course of synthesis of non-saponifiable lipids (▾), $C_{4,4}$-dimethyl sterols (Δ), C_4-monomethyl sterols (o) and C_4-desmethyl sterols (□) in a cell-free preparation of *Botrytis cinerea*. Left ordinate: radioactivity in sterol fractions as percentage of radioactivity in non-saponifiable lipids. Right ordinate: radioactivity in non-saponifiable lipids as percentage of total radioactivity added.

Half or double the standard [2-^{14}C]mevalonate concentration (8.5 nmol ml^{-1} incubation mixture) resulted in similar incorporation percentages of the radiolabel into NSLs and C_4-desmethyl sterol fractions (results not shown), indicating that sterol biosynthesis was linear with the substrate concentration. The intermediate concentration was chosen in all other studies. In a further experiment cell-free sterol biosynthesis was investigated in extracts diluted with potassium phosphate buffer to cover a range of protein concentrations from 1.1 to 9.1 mg ml^{-1}. Synthesis of NSLs and C_4-desmethyl sterols proved to be more or less proportional to the protein content and hence the amount of enzyme present in cell extracts, except for the lowest concentration tested (Fig. 3). The

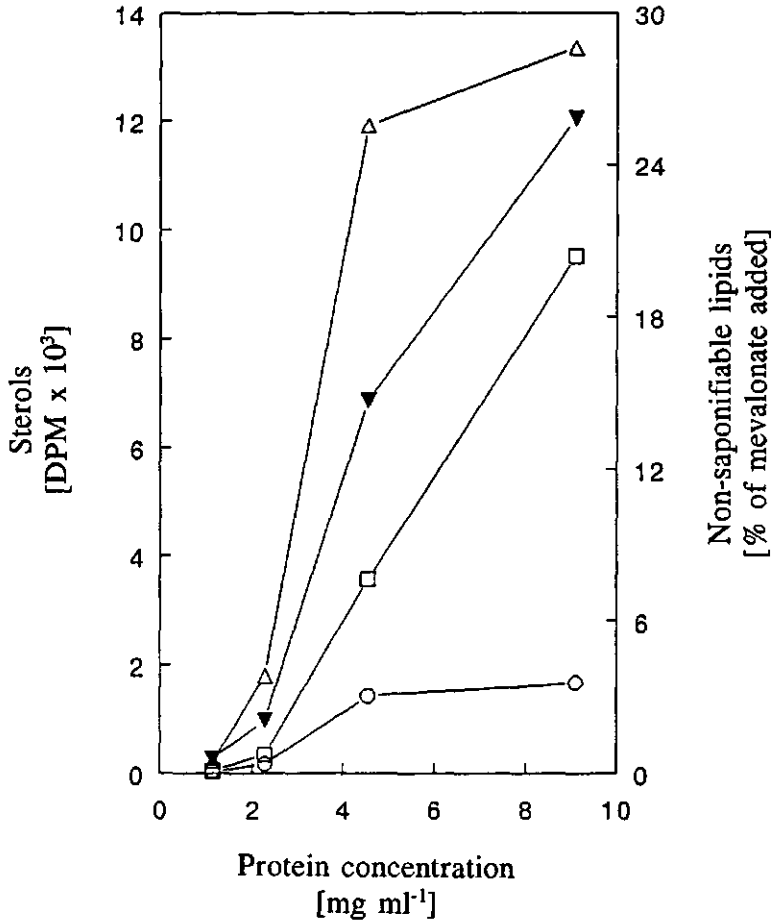


Fig. 3: Effect of protein concentration of cell-free extracts on synthesis of non-saponifiable lipids (▾), C_4-desmethyl sterols (□), C_4-monomethyl sterols (o), $C_{4,4}$-dimethyl sterols (Δ) and squalene (○). Left ordinate: radioactivity in sterol fractions as desintegrations min^{-1}. Right ordinate : radioactivity in non-saponifiable lipids as percentage of total radioactivity added.

highest protein concentration investigated led to the highest amount of C_4-desmethyl sterols and the highest ratio between C_4-desmethyl sterols and total NSLs synthesized. Therefore, this concentration was used throughout the experiments.

Stability of sterol biosynthetic activity did not siginificantly decrease upon storage at -18°C for 8 days (data not shown). Cell-free extracts were not contaminated with mycelial fragments, since no cells could be detected microscopically after the second centrifugation of the cell-free extract and PDA-plates inoculated with droplets of extract did not show any fungal growth, although some bacterial colonies were noticed. Addition of oxytetracycline (10 mg ml^{-1}) to incubation mixtures had no significant effect on cell-free sterol biosynthesis but did inhibit bacterial growth on PDA plates. Cell-free extracts heated at 80°C for 1 min incorporated only 0.2% of [2-^{14}C]mevalonate added into NSLs. Omission of either the cofactor solution or the divalent cations in the incubation mixture yielded only 0.2 and 1.0% of incorporation of the radiolabel into NSLs, respectively.

Essential factors for biosynthesis of C_4-desmethyl sterols. The intensity of disruption of the germlings was a crucial point. Microscopical examination showed that the standard procedure disrupted more than 90% of the cells (Fig. 4a and b). Protein concentrations of the resulting cell-free extracts were in the range of 9.0±1.1 mg ml^{-1} (n=10). Lower disruption speeds of the Bead-Beater, shorter disruption periods or the use of smaller glass beads resulted in less severe disruption of conidial germlings and yielded cell-free

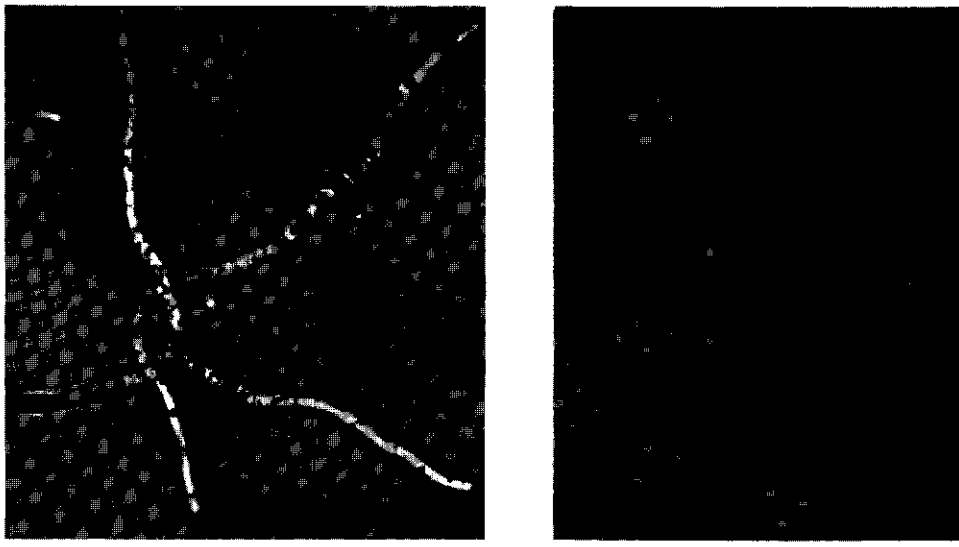


Fig. 4: Conidial germlings before **(A)** and after **(B)** the standard disruption procedure. Magnification: 200x.

extracts with lower protein concentrations. Similarly, higher disruption speeds, longer disruption periods and the use of larger glass beads increased protein concentrations of cell-free extracts and then germlings were more severely disrupted than in the standard assay. Incorporation of [2-^{14}C]mevalonate into C_4-desmethyl sterols by these extracts was poor, although $C_{4,4}$-dimethyl sterols were formed (results not shown). Similar results were obtained when glycerol was not added to the cell-free extract immediately after filtration of the germling homogenate (Table 1). Evacuation of the disruption vessel was also essential for synthesis of C_4-desmethyl sterols (data not shown).

Activity of the cell-free assay was strongly affected by the pH of the incubation mixture, which in itself did not change significantly during incubation. Incorporation of radiolabel into NSLs and C_4-desmethyl sterols had narrow pH optima between pH 7.4-7.6 and pH 7.3-7.4, respectively. Incorporation into C_4-desmethyl sterols at pH 7.1 and 7.7

Table 1: Effect of composition of the disruption buffer on sterol biosynthesis in cell-free extracts of *Botrytis cinerea*

Standard composition[1]	NSLs[2]	Sterol ratio[3]		
		Eburicol	lanosterol	C_4-desmethyl sterols
None	18.5	1.0	1.2	2.0
+ MCE[5] (14 mM) + DTT (10 mM)	09.3	1.0	8.7	n.d.[4]
+ MCE (14 mM) + DTT (10 mM) + EDTA (10 mM)	10.2	1.0	8.7	n.d.
+ N-Ac (10 mM) + NAA (10 mM)	01.4	n.d.	n.sd.	n.d.
- glycerol (22%)	18.9	1.0	5.9	n.d.

[1] K_2HPO_4 - KH_2PO_4 buffer pH 7.5 supplied with 22% glycerol

[2] NSLs synthesized as percentage of [2-^{14}C]mevalonate added

[3] Ratio relative to eburicol; based on HPLC analysis

[4] Not detected

[5] MCE: mercaptoethanol; DTT: dithiothreitol; EDTA: ethylene diamine tetraacetic acid; N-AC: N-acetyl cysteine: NAA: nicotinic acid amide

was 39 and 45% lower than at pH 7.4, respectively (Fig. 5). Formation of $C_{4,4}$-dimethyl and C_4-monomethyl sterols did not show an obvious pH optimum.

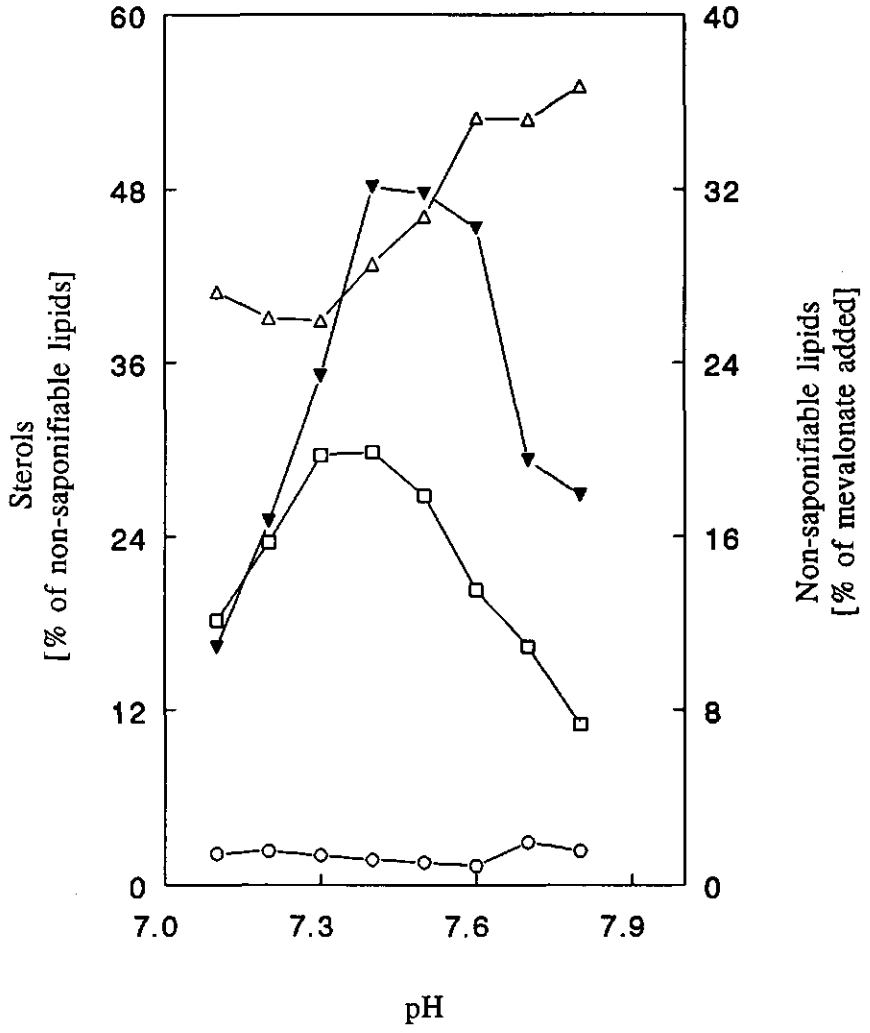


Fig. 5: Effect of pH on incorporation of [2-^{14}C]mevalonate into non-saponifiable lipids (▾), $C_{4,4}$-dimethyl sterols (Δ), C_4-monomethyl sterols (o) and C_4-desmethyl sterols (□) in cell-free preparations of *Botrytis cinerea*. Left ordinate: radioactivity in sterol fractions as percentage of radioactivity in non-saponifiable lipids. Right ordinate: radioactivity in non-saponifiable lipids as percentage of total radioactivity added.

Addition of L-methionine to the cofactor solution enhanced *in vitro* production of C_4-desmethyl sterols. Samples supplied with L-methionine incorporated on average comparable amounts of radiolabel into NSLs as those incubated without L-methionine, but the presence of the compound caused an increase in C_4-desmethyl sterols of about 30 to 40% of total NSLs. When L-methionine was replaced by the same molar amount of S-adenosyl methionine, C_4-desmethyl sterols accounted for 52 to 59% of NSLs, but total incorporation of [2-^{14}C]mevalonate into the sterol fraction was very low (only 5 to 6% of the radiolabel added). The addition of glucose-6-phosphate dehydrogenase did not improve cell-free synthesis of C_4-desmethyl sterols.

In preliminary experiments activity of cell-free extracts using different disruption buffers was investigated. Interestingly, replacement of the standard potassium buffer by a sodium potassium phosphate buffer significantly reduced synthesis of C_4-desmethyl sterols and also resulted in accumulation of C_4-monomethyl sterols (Table 2). Disruption of germlings in Tris-HCl-buffer, commonly used for Δ^{24} sterol:S-adenosyl methionine transferase assays,[21-23] reduced synthesis of NSLs by 50% and resulted in a relatively high amount of eburicol (Table 2), indicating negative effects on $P450_{14DM}$ activity. In further experiments the effect of different supplements, often used in $P450_{14DM}$ assays of yeasts,[7,11,24] were tested. MCE, DTT and EDTA reduced incorporation of [^{14}C]mevalonate into NSLs by about 50% (Table 1). More interestingly, they blocked formation of C_4-desmethyl sterols concomitant with an accumulation of lanosterol, which indicates an inhibitory effect of these compounds on side chain alkylation of lanosterol.

Table 2: Effect of different disruption buffers (100 mM) on sterol synthesis in cell-free extracts of *Botrytis cinerea*.

Buffer pH 7.5	NSLs[1]	Sterol ratio[2]			
		Eburicol	lanosterol	C_4-monomethyl sterols	C_4-desmethyl sterols
K_2HPO_4 - KH_2PO_4	21.3	1.0	3.3	n.d.[3]	4.1
Na_2HPO_4 - KH_2PO_4	18.3	1.0	1.7	2.5	2.6
Tris-HCl	11.0	1.0	0.1	n.d.	0.3

[1] NSLs as percentage of [^{14}C]mevalonate added

[2] Relative to eburicol; based on HPLC analysis

[3] Not detected

Addition of N-acetyl cysteine and nicotinic acid amide to the disruption buffer severely impaired cell-free sterol synthesis (Table 1). Hence, the 100 mM potassium phosphate buffer (pH 7.5) was chosen in the standard assay.

Inhibition studies. The imidazole fungicide imazalil inhibited radial growth of *B. cinerea* by 50% at $1.5x10^{-6}$ M. Imazalil inhibited cell-free incorporation of [2-^{14}C]mevalonate into C_4-desmethyl sterols (Fig. 1). Inhibition coincided with an increase of radiolabelled $C_{4,4}$-dimethyl sterols. Effects of imazalil on the formation of C_4-monomethyl sterols could not be observed. Fig. 6 shows a dose-response curve of imazalil for inhibition of C_4-desmethyl sterol biosynthesis obtained from four replicate experiments. The IC_{50} value was calculated to be $9.1\pm1.5x10^{-9}$ M. DMSO (0.1%) in control treatments did not have any significant effects on sterol synthesis.

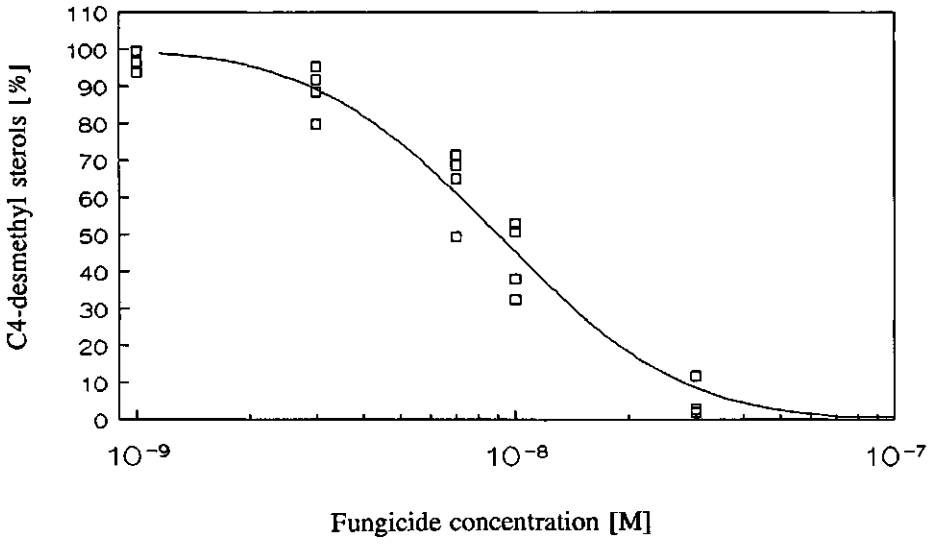


Fig. 6: Inhibition of incorporation of [2-^{14}C]mevalonate into C_4-desmethyl sterols in cell-free assays (n=4) of *Botrytis cinerea* at pH 7.4 by imazalil. Abscis: concentration of test compound. Ordinate: radioactivity in C_4-desmethyl sterols as percentages of control treatment.

Identification of radiolabelled sterols. NSLs were analyzed by radio-HPLC. In control samples, six major peaks with retention times of 4.9 (peak 1), 10.0 (peak 2), 12.8 (peak 3), 16.6 (peak 4, consisting of two peaks), 22.8 (peak 5) and 25.1 min (peak 6) were observed (Fig. 7A). Peaks 4, 5 and 6 had retention times identical to those of authentic

ergosterol, lanosterol and eburicol, respectively, as demonstrated by UV-detection at 210 and 280 nm. The identities of other sterols were not studied any further.

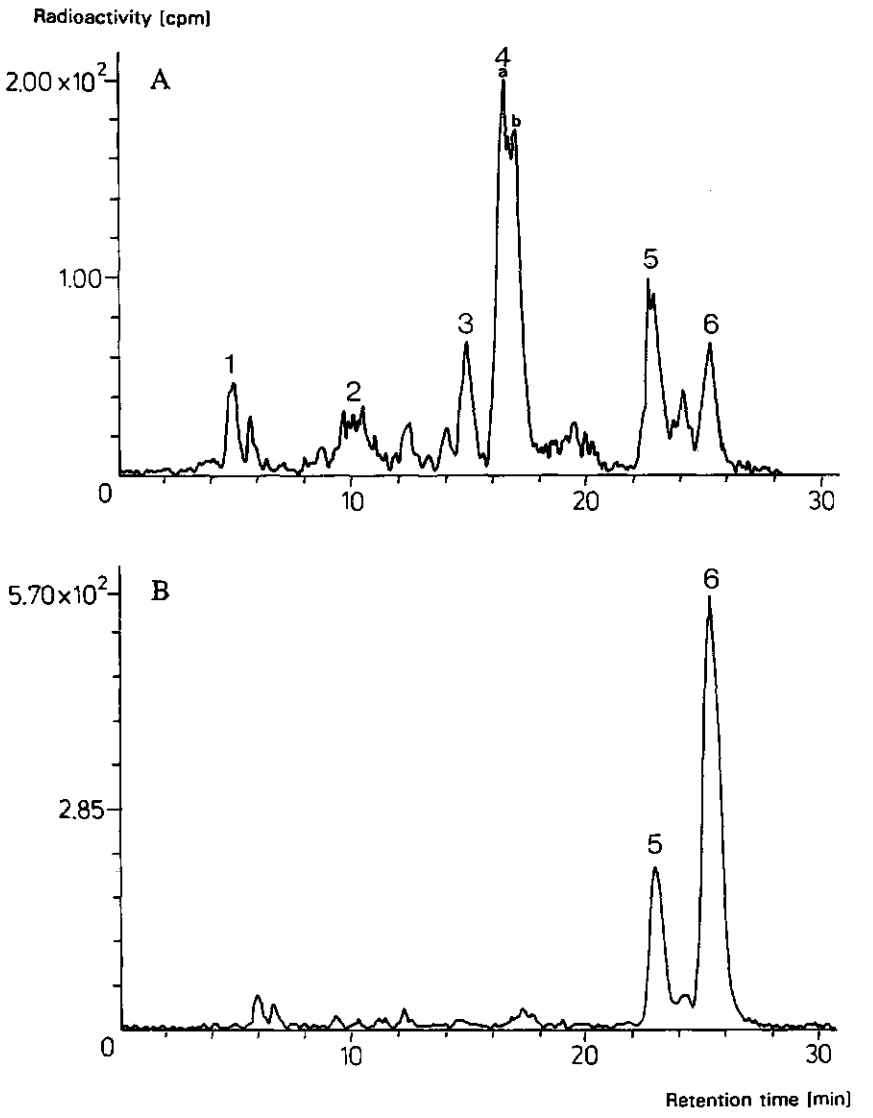


Fig. 7: Radio-HPLC separation of radiolabelled non-saponifiable lipids extracted from control **(A)** and imazalil-treated ($3x10^{-8}$ M) **(B)** cell-free sterol biosynthesis assays of *Botrytis cinerea* at pH 7.4. Peak 4 is composed of a mixture of ergosterol (4a) and another non-identified C_4-desmethyl sterol (4b). The identities of peak 5 and 6 are lanosterol and eburicol, respectively.

In imazalil-treated samples ($3x10^{-8}$ M) only two major peaks were observed (Fig. 7B): peak 5 (lanosterol) and 6 (eburicol). The size of the latter increased significantly. Sterols were further analyzed by separation on TLC plates pretreated with $AgNO_3$. The C4-desmethyl sterol fraction proved to consist of three sterols with Rf-values of 0.02, 0.07 and 0.21 which accounted for 12.6, 63.1 and 24.3% of the C_4-desmethyl sterol fraction, respectively. The compound with a Rf-value of 0.07 cochromatographed with authentic ergosterol. The other two C_4-desmethyl sterols were not identified. $C_{4,4}$-dimethyl sterols separated into four distinct bands with Rf-values of 0.04, 0.31, 0.36 and 0.42. Compounds with Rf-values of 0.36 and 0.42 co-chromatographed with authentic eburicol and lanosterol and accounted for 53.6 and 28.2% of the $C_{4,4}$-dimethyl sterol fraction, respectively. No attempts were undertaken to identify the other sterols.

Discussion

Cell-free extracts of *B. cinerea* actively synthesized C_4-desmethyl sterols and other sterols from [2-^{14}C]mevalonate. The disruption procedure was relatively vigorous compared with those described for other filamentous pathogens,[15,16] but this proved to be essential. The protein concentration of cell-free extracts in standard assays was 9.0 ± 1.1 mg ml^{-1}, which is comparable to those described for assays developed for yeasts[7,8,11] and ten times higher than reported for cell-free systems of *A. fumigatus*[15] and *P. italicum*.[16] The necessity of a relatively strong method to disrupt the germlings may be explained by the fact that cells of *B. cinerea* are surrounded by a layer of mucilage. However, it may also be due to differences in quality and quantity of $P450_{14DM}$ in different fungi and $P450_{14DM}$ stability. Additional crucial points for the preparation of active cell-free extracts were the addition of glycerol to filtrates of cell-homogenates before centrifugation (Table 1) and the pH of the incubation mixture (Fig. 5). The cell-free system had a narrow pH optimum for synthesis of C_4-desmethyl sterols between pH 7.3 and 7.4. This result is different from that reported for the cell-free assay of *P. italicum*,[16] but resembles data obtained for *A. fumigatus*.[15]

Under optimal conditions the average amount of radioactivity incorporated into C_4-desmethyl sterols was about 39.0% of total NSLs. In intact cells incorporation of radiolabel into C_4-desmethyl sterols accounts for more than 90% of the NSLs formed.[25] The relatively low incorporation of label into C_4-desmethyl sterols under cell-free conditions is probably not due to a feed-back inhibition by ergosterol, since addition to the incubation mixture of the polyene antibiotic nystatin (10^{-5} M), which forms complexes with C_4-desmethyl sterols, did not influence cell-free synthesis of C_4-desmethyl sterols (results not shown). However, increased synthesis of NSLs in extracts in the presence of imazalil (results not shown) is supportive of feed-back inhibition.

Incorporation of [2-^{14}C]mevalonate by cell-free extracts of *B. cinerea* into C_4-desmethyl sterols is higher than reported for cell-free preparations derived from other filamentous fungi[15,16] and *C. albicans*.[9] *B. cinerea* assays yielded relatively low amounts of lanosterol as compared with cell-free assays of *A. fumigatus*[15] and *P. italicum*.[16] This is probably due to the addition of L-methionine to the incubation mixture, since its biologically activated form S-adenosyl methionine is used for the C_{24} side chain alkylation of lanosterol.[21,26] Interestingly, the addition of S-adenosyl methionine itself resulted in a very high conversion of [2-^{14}C]mevalonate to C_4-desmethyl sterols, but in an extremely low formation of total sterols. We have no explanation for this phenomenon.

Analysis of the C_4-desmethyl sterol fraction with argentation TLC showed three distinct C_4-desmethyl sterols. The C_4-desmethyl sterol which co-chromatographed with authentic ergosterol accounted for 63.1% of the C_4-desmethyl sterols synthesized. Radio-HPLC analysis of NSLs revealed three C_4-desmethyl sterol peaks (peaks 3, 4a and 4b), which were absent upon imazalil-treatment of cell-free extracts (Fig 7). The identities of peak 1 and 2 are most probably non-sterols and C_4-monomethyl sterols, respectively. Peak 4a had a retention time identical to that of standard ergosterol. This indicates that ergosterol is the major C_4-desmethyl sterol synthesized in cell-free extracts. The other two C_4-desmethyl sterols are not yet identified. According to literature data on sterol composition of intact cells of *B. cinerea* these sterols might be $\Delta^{5,8,22}$-ergostatrienol and episterol.[27-29] Only a minor amount (3.2$\pm$ 1.1%) of C_4-monomethyl sterols was synthesized under cell-free conditions, which is in agreement with literature data on the sterol content of intact *B. cinerea* cells.[25] The other sterols in the NSLs identified by co-chromatography on radio-HPLC and argentation TLC were lanosterol and eburicol. These data suggest that the cell-free sterol synthesis follows the same pathway as described for intact mycelium.[28,29]

Treatment of cell-free extracts with imazalil led to accumulation of eburicol and depletion of C_4-desmethyl sterols (Fig. 1 and 7). This suggests that sterol 14α-demethylation is blocked by inhibition of $P450_{14DM}$ activity and that eburicol is the substrate for the $P450_{14DM}$. The IC_{50} value of imazalil was extremely low ($9.1\pm1.5\times10^{-9}$ M) indicating a potent inhibition of $P450_{14DM}$ activity of this fungus. The EC_{50} value of imazalil for radial growth is 1.5×10^{-6} M. This difference may be explained by the accumulation, distribution and metabolism of the compound in fungal cells, the intracellular pH or some other unknown factors. These factors may be of relatively great importance for the fungicidal activity of DMIs against *B. cinerea* and may explain the present low number of DMIs which are used for control of this fungus

The cell-free assay provides a simple method to screen biochemically for compounds which potentially inhibit sterol biosynthesis at C_{14}-demethylation or, alternatively, at any other site in the sterol biosynthetic pathway between the precursor squalene and the end product ergosterol.

Acknowledgements

The authors are grateful to Prof.Dr.J. Dekker, Prof.Dr.P.J.G.M. De Wit and Dr.R.J. Milling for critical reading of the manuscript. J.C. Kapteyn and C. Stehmann acknowledge the financial support of Schering Agrochemicals Ltd and the Deutsche Akademische Austauschdienst.

References

1. Kuck, K.-H. & Scheinpflug, H. In *Chemistry of Plant Protection*, **Vol.1**, ed. G. Haug & H. Hoffman. Springer, Berlin, 1986, pp. 65-96.
2. Vanden Bossche, H. & Janssen, P.A.J. In *Target Sites of Fungicide Action*, ed.W. Köller. CRC Press, London, 1992, pp. 227-254.
3. Kaspers, H., Brandes, W. & Scheinpflug, H., *Pflanzenschutz Nachr. Bayer* **40** (1987) 1-110.
4. Birchmore, R.J., Brookes, R.F., Copping, L.G. & Wells, W.H. In *Proc. Br. Crop Protect. Conf. - Pests and Diseases*, 1977, pp. 593-598.
5. Copping, L.G., Birchmore, R.J., Wright, K. & Godson, D.H., *Pest. Sci.*, **15** (1984) 280-284.
6. Shirane, N., Murabayashi, A., Masuko, M., Uomori, A., Yoshimura, Y., Seo, S., Uchida, K. & Takeda, K., *Phytochemistry*, **29** (1990) 2513-2520.
7. Gadher, P., Mercer, E.I., Baldwin, B.C. & Wiggins, T.E., *Pestic. Biochem. Physiol.*, **19** (1983) 1-10.
8. Mercer, E.I., *Tagungsber. Akad. Landwirtschaftswiss. DDR*, **253** (1987) 115-120.
9. Marriott, M.S., *J. Gen. Microbiol.*, **117** (1980) 235-255.
10. Barrett-Bee, K.J., Lane, A.C. & Turner, R.W., *J. Med. Vet. Mycol.*, **24** (1986) 155-160.
11. Hitchcock, C.A., Brown, S.B., Evans, E.G.V. & Adams, D.J., *Biochem. J.*, **260** (1989) 549-556.
12. Vanden Bossche, H. In *Sterol Biosynthesis Inhibitors*, ed. D. Berg & M. Plempel. Ellis Horwood Ltd., Chichester, 1988, pp. 79-119.
13. Mercer, E.I., *Pestic. Sci.*, **15** (1984) 133-155.
14. Vanden Bossche, H. In *Biochemistry of Cell Walls and Membranes in Fungi*, ed. P.J. Kuhn, A.P.J. Trinci, M.J. Jung, M.W. Goosey & L.G. Copping. Springer Verlag, Berlin, 1990, pp.135-155.
15. Ballard, S.A., Ellis, S.W., Kelly, S.L. & Troke, P.F., *J. Med. Vet. Mycol.*, **28** (1990) 335-344.
16. Guan, J., Stehmann, C., Ellis, S.W., Kerkenaar, A. & de Waard, M.A., *Pestic. Biochem. Physiol.*, **42** (1992) 262-270.
17. Faretra, F., Antonacci, E. & Pollastro, S., *J. Gen. Microbiol.*, **134** (1988) 2543-2550.
18. Fritz, R., Leroux, P. & Gredt, M., *Phytopath. Z.*, **90** (1977) 152-163.
19. Kerkenaar, A., Uchiyama, M. & Versluis, G.G., *Pestic. Biochem. Physiol.*, **16** (1981) 97-104.
20. Ryder, N.S., Seidl, G. & Troke, P.F., *Antimicrobiol. Agents Chemother.*, **254** (1984) 483-487.
21. Bailey, R.B., Thompson, E.D. & Parks, L.W., *Biochem. Biophys. Acta*, **334** (1974) 127-136.
22. Thompson, E.D., Bailey, R.B. & Parks, L.W., *Biochem. Biophys. Acta*, **334** (1974) 116-126.
23. Moore, J.T. & Gaylor, J.L., *J. Biolog. Chem.*, **215, 18** (1970) 4684-4688.
24. Hitchcock, C.A., Dickinson, K., Brown,S.B., Evans, E.G.V. & Adams, D.J., *Biochem. J.*, **263** (1989) 573-579.
25. Pontzen, R. & Scheinpflug, H., *Neth. J. Pl. Path.*, **95 Supplement 1** (1989) 151-160.
26. Ator, M.A., Schmidt, S.J., Adams, J.L. & Dolle, R.E., *Biochem.*, **28** (1989) 9633-9640.
27. Berg, D., Born, L., Büchel, K.H., Holmwood, G. & Kaulen, J., *Pflanzenschutz Nachr. Bayer*, **40,2** (1987) 111-132.
28. Loeffler, R.S.T. & Hayes, A.L., *Phytochemistry*, **29 (11)** (1990) 3423-3425.

29. Steel, C.C., Baloch, R.I., Mercer, E.I. & Baldwin, B.C., *Pestic. Biochem. Physiol.*, **33** (1989) 101-111.

CHAPTER 8

INHIBITION OF STEROL SYNTHESIS IN CELL-FREE EXTRACTS OF *BOTRYTIS CINEREA* BY PROCHLORAZ AND PROCHLORAZ ANALOGUES.

Johan C. Kapteyn[a], Richard J. Milling[b], Donald J. Simpson[b] and Maarten A. De Waard[a]

[a] Department of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, 6709 PD Wageningen, The Netherlands.

[b] Schering Agrochemicals Ltd., Chesterford Park Research Station, Saffron Walden, Essex CB10 1XL, UK.

(To be submitted to Pesticide Science)

Abstract

The sensitivity of cytochrome-P450 dependent sterol 14α-demethylase ($P450_{14DM}$) to prochloraz and several prochloraz analogues was studied in a cell-free assay of *Botrytis cinerea*. The EC_{50} values (concentrations which inhibited radial growth of *B. cinerea* by 50%) of the compounds tested ranged from $3.3x10^{-8}$ to $1.7x10^{-5}$ M. The IC_{50} values (concentrations which inhibited cell-free sterol synthesis by 50%) in cell-free assays of *B. cinerea* ranged from $2.6x10^{-9}$ to $4.4x10^{-7}$ M. The assay ranked the compounds in order of fungitoxicity, but the IC_{50} values did not quantitatively reflect the differences in toxicity. Therefore, the differential inhibition of cell-free $P450_{14DM}$ activity by these compounds can not fully account for their differences in toxicity to *B. cinerea*. Additional mechanisms must be involved.

The compounds tested were generally more potent in the *Botrytis* assay than in similar assays developed for *Penicillium italicum* and, in particular, *Saccharomyces cerevisiae*. This correlated with the relatively higher toxicity of most test compounds to *B. cinerea*. Results suggest that the cell-free assay of *B. cinerea* is more useful to biochemically screen candidate fungicides as inhibitors of sterol 14α-demethylase activity than similar assays from model organisms.

The present study confirms that the affinity of prochloraz analogues for $P450_{14DM}$ depends on the nature of their N_1 substituent and their azole moiety. It was also found that addition of an amino group at C_2 of the imidazole moiety of prochloraz (VI) resulted in inhibition of C_4-desmethyl sterol biosynthesis at a different site to $P450_{14DM}$. This was

confirmed by the observation that laboratory-generated triadimenol-resistant isolates of *B. cinerea* did show reduced sensitivity to triadimenol and prochloraz, but not to compound **VI**.

Introduction

Control of grey mould caused by *Botrytis cinerea*, an economically important plant pathogen, is highly dependent on the application of fungicides. However, the number of fungicides which are available is limited due to resistance development to benzimidazoles and dicarboximides. Therefore, attention is focussed on other types of fungicides such as the sterol 14α-demethylation inhibitors (DMIs). However, only a few DMIs have been found to be effective,[1-5] and other commercial DMIs possess only low or moderate efficacy.[3] Availability of more DMIs for *Botrytis* control would be desirable. Sufficient knowledge on the biochemical mechanisms which are responsible for the observed selective toxicity of DMIs to *B. cinerea* is lacking. DMI fungicides inhibit sterol biosynthesis by binding to cytochrome-P450 dependent sterol 14α-demethylase ($P450_{14DM}$).[6] The differences in toxicity of DMIs to *Aspergillus fumigatus* and *Penicillium italicum* were shown to be, at least in part, due to differential affinities for $P450_{14DM}$ in these fungi.[7-9] The recent development of a cell-free sterol biosynthesis assay from *B. cinerea*[10] makes it possible to investigate whether the observed differences in toxicity of DMIs to this fungus also result from different potencies of $P450_{14DM}$ inhibition. In addition, the cell-free assay opens the opportunity to optimize the inhibitory action of DMIs against fungal $P450_{14DM}$ activity and to search for other potential target sites in fungal sterol biosynthesis. These studies may eventually lead to the development of improved DMIs and of new types of sterol biosynthesis inhibitors (SBIs) active against *B. cinerea*.

In this paper the inhibitory potencies of prochloraz, several prochloraz analogues and the experimental triazole fungicide 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)-4(3*H*)-quinazolinone (Fig. 1) on the activity of $P450_{14DM}$ in cell-free extracts of *B. cinerea* are described. The results are discussed in relation to the fungitoxicities of the compounds to *B. cinerea*. The data from the assays are compared with those obtained with similar cell-free assays developed for *P. italicum*[7] and *Saccharomyces cerevisiae*.

Materials & Methods

Chemicals. Prochloraz (**I**), experimental triazole fungicide 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)-4(3*H*)-quinazolinone (**II**), and prochloraz analogues (**III-VIII**) were donated by Schering Agrochemicals Ltd. Dimethyl sulfoxide (DMSO) was

purchased from Merck (Darmstadt, Germany). DL[2-^{14}C]mevalonate (spec. activity 1.9 Gbq mmol^{-1}) was from Du Pont De Nemours (s'Hertogenbosch, The Netherlands). All test compounds were dissolved in DMSO.

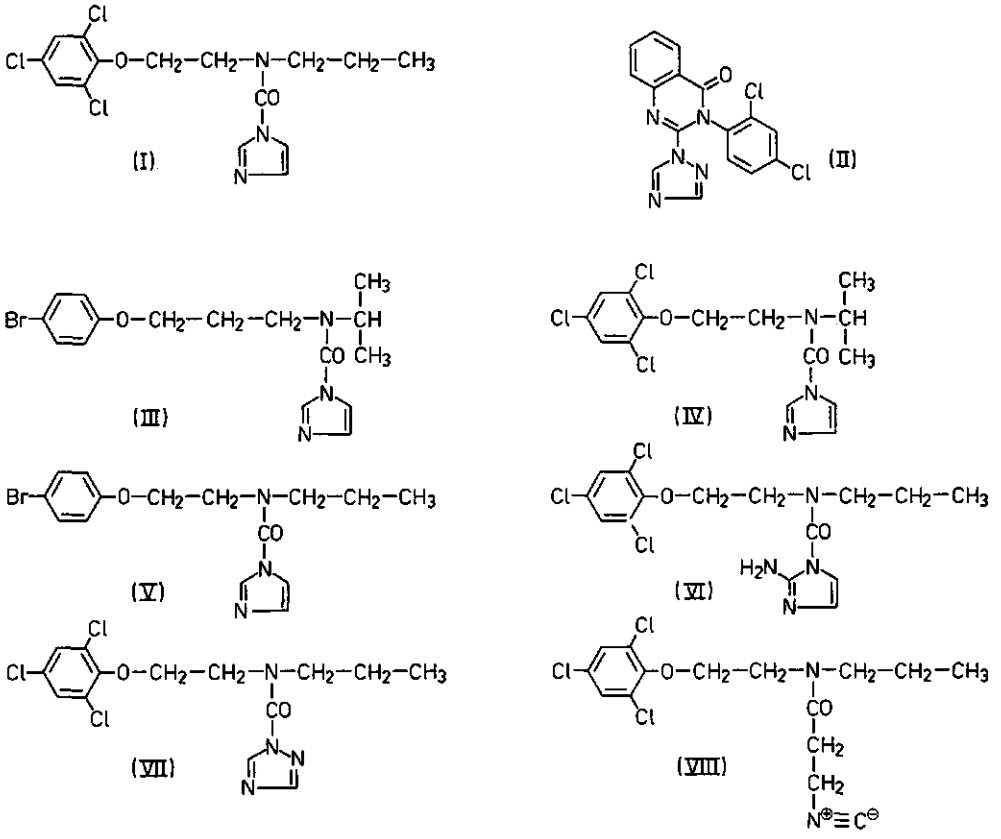


Fig. 1: Structural formulae of prochloraz (**I**), experimental triazole fungicide 3-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)-4(3*H*)-quinazolinone (**II**) and various prochloraz analogues (**III-VIII**)

Culture methods, radial growth inhibition tests. The monoascospore isolate of *B. cinerea* SAS-56[11] was donated by Dr. F. Faretra (Bari, Italy). The laboratory-generated triadimenol-resistant isolates T_N-150/6-6 and T_N-25 of *B. cinerea* were obtained from Dr. B.N. Ziogas (Athens, Greece). Isolate SAS-56 was maintained on potato dextrose agar (PDA); the resistant isolates on PDA amended with triadimenol (10 μg ml^{-1}). Liquid germling cultures of SAS-56 were prepared as described by Stehmann *et al.*[10] Toxicity of compounds to radial growth on PDA was determined according to Kapteyn *et al.*[12]

Preparation of cell-free extracts and sterol biosynthesis assays. Cell-free extracts of *B. cinerea* isolate SAS-56 were obtained by mechanical homogenization of germling suspension (100 mg fresh weight of germlings in 1 ml 100 mM potassium phosphate buffer pH 7.5) using a Bead-Beater (Biospec. Products, Bartlesville, Okl., USA).[10] The average protein content was 9.0 ± 1.0 mg ml^{-1} (n=10). Sterol synthesis in cell-free extracts of this fungus was assayed as described previously.[10] The test compounds were added from 1000-fold concentrated stock solutions in DMSO. Radiolabelled $C_{4,4}$-dimethyl, C_4-monomethyl and C_4-desmethyl sterols synthesized in the bioassay were separated by thin layer chromatography (TLC). Radioactive zones were located by autoradiography, cut from the plate and the radioactivity present was determined by liquid scintillation counting. The amount of radioactivity incorporated into different sterol fractions was expressed as a percentage of the radioactivity in total non-saponifiable lipids (NSLs) recovered from the bands on the TLC plates.[7-9] NSLs were further analysed with radio-HPLC as described previously.[8-10]

With regard to *S. cerevisiae*, the preparation of cell-free extracts and the sterol biosynthesis assays were carried out according to Kato and Kawase.[11] The average protein concentration used in the yeast cell-free assay was 29.5 ± 2.0 mg ml^{-1} (n=4). Analysis of NSLs was performed by radio-HPLC using a 5 μm Hypersil silica column (25 cm x 4.6 mm) (Hichrom, Reading, U.K.). Mobile phase was heptane/isopropanol (98:2) at 1 ml min^{-1} flow rate. Cell-free sterol 14α-demethylation was calculated as radioactivity incorporated into C_4-desmethylsterol sterols as a proportion of radioactivity in lanosterol and the C_4-desmethyl sterols.

Results

Radial growth inhibition tests. Among the compounds tested prochloraz was the strongest inhibitor of radial growth of *B. cinerea* isolate SAS-56 with an EC_{50} of $3.3x10^{-8}$ M (Table 1). Experimental triazole fungicide **II** exhibited an EC_{50} of $2.0x10^{-7}$ M. EC_{50}s of the prochloraz analogues ranged from $1.4x10^{-6}$ to $1.7x10^{-5}$ M (Table 1). Compound **VIII** had the weakest fungitoxicity of all the compounds tested. Except for prochloraz, compounds **III** and **V**, all test compounds were relatively more fungitoxic to *Botrytis* than to *Penicillium*. Prochloraz was equally toxic to both fungi (Table 1). Spearman's ranking correlation coefficient (r_s) between the EC_{50}s for *B. cinerea* and those for *P. italicum* was 0.26 indicating that these fungi differed markedly in sensitivity to most of the compounds tested (Table 2).

As expected, isolates T_N-150/6-6 and T_N-25 were resistant to triadimenol exhibiting resistance levels of 9 and 10, respectively (Table 3). The triadimenol-resistant isolates were also resistant to prochloraz but as sensitive to **VI** as the wild-type isolate SAS-56 (Table 3).

Table 1: Toxicity of prochloraz and related compounds to radial growth of *Botrytis cinerea* and *Penicillium italicum* on PDA (EC_{50}) and inhibitory effects of these compounds on cell-free C_4-desmethyl sterol synthesis of *B. cinerea*, *P. italicum* and *Saccharomyces cerevisiae* (IC_{50}).

Compound	*B. cinerea*		*P. italicum*		*S. cerevisae*
	EC_{50} [M][1,2]	IC_{50} [M][2,3]	EC_{50} [M][1,4,5]	IC_{50} [M][2,3,4]	IC_{50} [M][3,5]
I	$3.3\pm3.2\times10^{-8}$ (1)[6]	$2.6\pm0.1\times10^{-9}$ (1)	3.1×10^{-8} (1)	$8.0\pm1.0\times10^{-9}$ (1)	2.2×10^{-7} (1)
II	$2.0\pm0.9\times10^{-7}$ (6)	$1.2\pm0.1\times10^{-8}$ (5)	7.1×10^{-5} (2290)	$1.9\pm0.5\times10^{-8}$ (2)	2.9×10^{-7} (1)
III	$1.4\pm0.9\times10^{-6}$ (42)	$1.1\pm0.4\times10^{-8}$ (4)	4.9×10^{-7} (16)	$1.8\pm0.1\times10^{-8}$ (2)	1.2×10^{-7} (0.5)
IV	$1.8\pm1.1\times10^{-6}$ (55)	$3.1\pm1.5\times10^{-8}$ (12)	6.9×10^{-6} (222)	$1.0\pm0.2\times10^{-7}$ (12)	8.8×10^{-7} (4)
V	$3.2\pm2.0\times10^{-6}$ (97)	$9.2\pm0.6\times10^{-9}$ (3)	4.0×10^{-7} (13)	$1.4\pm0.2\times10^{-8}$ (2)	-
VI	$8.3\pm0.8\times10^{-6}$ (252)	$1.5\pm0.4\times10^{-8}$ (6)	$>10^{-3}$ (>32260)	$9.0\pm0.5\times10^{-7}$ (112)	$>10^{-6}$ (>5)
VII	$1.1\pm0.4\times10^{-5}$ (333)	$4.4\pm1.7\times10^{-7}$ (169)	$>10^{-3}$ (>32260)	$6.5\pm1.1\times10^{-7}$ (81)	1.1×10^{-5} (50)
VIII	$1.7\pm0.6\times10^{-5}$ (515)	$1.0\pm0.5\times10^{-7}$ (38)	3.8×10^{-5} (1226)	$9.2\pm2.7\times10^{-7}$ (115)	9.7×10^{-7} (4)

[1] Concentration of compound which inhibited radial fungal growth on PDA by 50%.

[2] Figures based on three replicates.

[3] Concentration of compound which inhibited incorporation of [2-^{14}C]mevalonate into C_4-desmethyl sterols by 50%.

[4] Data from Kapteyn *et al.*[7]

[5] Figures based on two replicates.

[6] Between brackets: ratio between the inhibitory effects of compounds and prochloraz.

[7] Not tested.

Table 2: Ranking correlation between the fungitoxic activities of various compounds to *Botrytis cinerea* and *Penicillium italicum* (EC_{50}) and their inhibitory effects on sterol 14α-demethylation in cell-free extracts of *B. cinerea* and *P. italicum* (IC_{50} values).

EC_{50}			IC_{50}	
	B. cinerea	*P. italicum*	*B. cinerea*	*P. italicum*
EC_{50} (*B. cinerea*)	x	0.26*	0.76	0.81
EC_{50} (*P. italicum*)		x	0.83	0.83
IC_{50} (*B. cinerea*)			x	0.88
IC_{50} (*P. italicum*)				x

* Spearman's correlation coefficient (r_s) followed by an asterisk indicates the absence of a ranking correlation.

Table 3: Toxicity of triadimenol, prochloraz and compound **VI** to radial growth of *Botrytis cinerea* wild-type isolate SAS-56 and triadimenol-resistant isolates T_N-25 and T_N-150/6-6.

Isolate	Triadimenol		Prochloraz		Compound **VI**	
	EC_{50} [M][1]	Q[2]	EC_{50} [M]	Q	EC_{50} [M]	Q
SAS-56	1.8×10^{-6}	-	2.0×10^{-8}	-	6.6×10^{-6}	-
T_N-150/6-6	1.6×10^{-5}	9	1.3×10^{-7}	7	3.8×10^{-6}	0.7
T_N-25	1.8×10^{-5}	10	1.1×10^{-7}	6	7.4×10^{-6}	1.1

[1] Figures based on two replicates.

[2] Q (resistance level): ratio between EC_{50} of resistant isolate and isolate SAS-56.

Inhibition of cell-free sterol 14α-demethylation in B. cinerea SAS-56. The total incorporation of radioactivity into NSLs was on average 17.9±3.7% of radiolabel added (n=10). Fig. 2A shows an autoradiogram of a TLC plate on which labelled $C_{4,4}$-dimethyl, C_4-monomethyl and C_4-desmethyl sterols, and other NSLs synthesized in control treatments were separated. TLC and radio-HPLC analyses by Stehmann *et al.*[10] indicated that band 1 contains ergosterol and another, yet unidentified C_4-desmethyl sterol. Band 2 represents C_4-monomethyl sterols, while band 3 contains the $C_{4,4}$-dimethyl sterols, lanosterol and 24-methylenedihydrolanosterol (eburicol). Band 5 was tentatively identified as 2,3-oxidosqualene[12,13] whilst band 6 was most probably squalene. All other bands were not identified. Percentage of radioactivity incorporated into C_4-desmethyl sterols was on average 38.3±8.6% of the NSLs recovered from TLC. Prochloraz inhibited incorporation

of radiolabel into C_4-desmethyl sterols at extremely low concentrations concomitant with an accumulation of eburicol (Figs. 2A, 2B, 3A and 3B). It was the most potent inhibitor tested and had an IC_{50} of $2.6x10^{-9}$ M (Table 1). All other compounds tested inhibited C_4-desmethyl sterol synthesis to lower degrees. The triazole analogue **VII** was the weakest inhibitor with an IC_{50} of $4.4x10^{-7}$ M (Table 1). Except for compound **VI** (see 3.4), all test compounds impaired C_4-desmethyl sterol synthesis due to inhibition of $P450_{14DM}$ activity. The Spearman's r_s value between the EC_{50} values for radial growth and the IC_{50} values was 0.76 indicating that a ranking correlation existed between these values (Table 2).

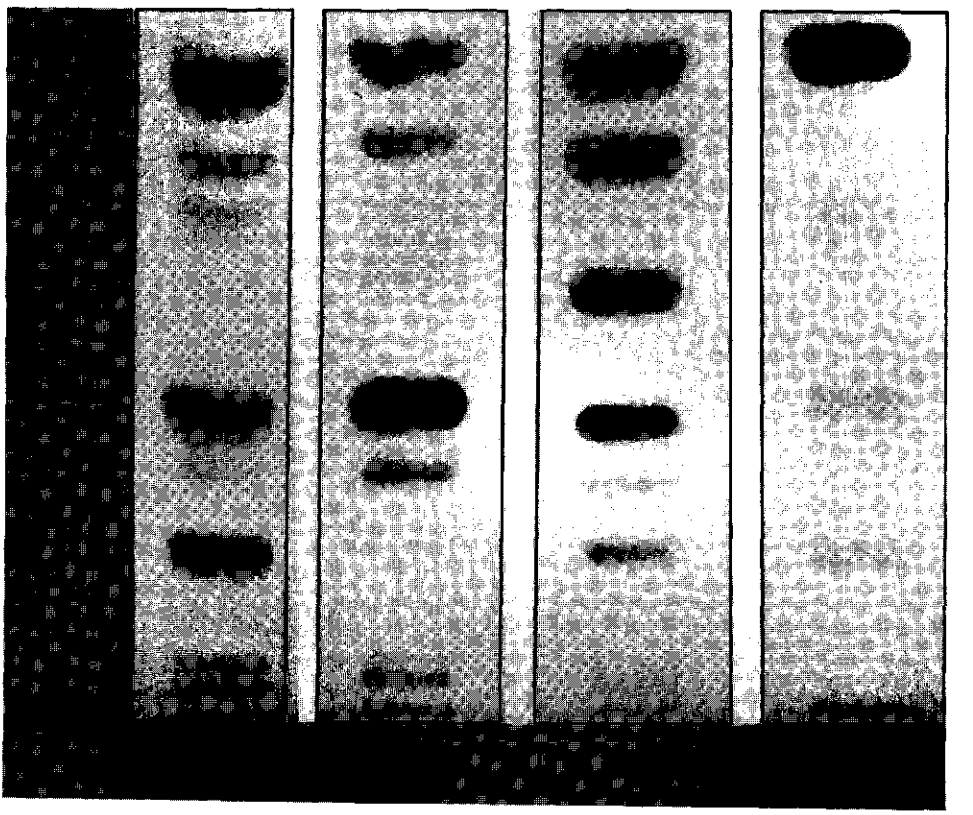


Fig. 2: Autoradiogram of a TLC separation of non-saponifiable lipids synthesized from [2-^{14}C] mevalonate by a cell-free preparation of *Botrytis cinerea* in the presence of (A) DMSO (control), (B) 10^{-8} M prochloraz, (C) $5x10^{-8}$ M compound **VI** and (D) 10^{-5} M terbinafine. Bands 1, 2, 3, 5 and 6 contained C_4-desmethyl sterols, C_4-monomethyl sterols, $C_{4,4}$-dimethyl sterols, 2,3-oxidosqualene (putative), and squalene, respectively. All other bands were not identified.

Inhibition of cell-free sterol 14α-demethylation in S. cerevisiae. The cell-free system of *S. cerevisiae* was used to make preliminary comparisons of the inhibitory potencies of test compounds in similar assays from filamentous and yeast-like fungi. The most potent inhibitors in the yeast assay were prochloraz, compounds **II** and **III** with IC_{50}s of $2.2x10^{-7}$, $2.9x10^{-7}$ and $1.2x10^{-7}$ M, respectively (Table 1). The weakest inhibitor was **VII** with an IC_{50} of $1.1x10^{-5}$ M (Table 1).

Inhibition of cell-free sterol synthesis at other sites. Incubation of the cell-free extract of *B. cinerea* with compound **VI** led to a depletion of $C_{4,4}$-dimethyl and C_4-desmethyl sterols, and an accumulation of bands 4 (putatively 2,3-oxidosqualene) and 5 (identity unknown) (Fig. 2C). HPLC-analysis of NSLs of compound **VI**-treated assays yielded two new peaks (6 and 7) concomitant with a reduction in synthesis of lanosterol (4), eburicol (5) and C_4-desmethyl sterols (2 and 3) (Fig. 3C). Squalene synthesis was not significantly affected. In contrast to prochloraz, compound **VI** ($5x10^{-8}$ M) did not significantly decrease the ratio between lanosterol and eburicol synthesized. (Fig. 3B, 3C). Terbinafine, a squalene epoxidase inhibitor, led to a decrease in synthesis of $C_{4,4}$-dimethyl, C_4-monomethyl and C_4-desmethyl sterols concomitant with an accumulation of squalene (Fig. 2D). Radio-HPLC analysis of the NSLs formed upon incubation of yeast cell-free extract with compound **VI** showed no detectable C_4-desmethyl sterols. Lanosterol synthesis was hardly influenced and no new peaks were observed (data not shown).

Discussion

The present study demonstrates that prochloraz and a number of prochloraz analogues inhibit cell-free $P450_{14DM}$ activity of *B. cinerea* to different degrees (Table 1). Prochloraz was the most active compound in the cell-free assay and also showed the highest toxicity to radial growth of this fungus. Although the most potent inhibitors in the cell-free assay tended to be the most toxic to radial growth of *Botrytis* (r_s=0.76), the differences in IC_{50} values did not fully reflect the observed differences in toxicity. For example, the relatively large difference in toxicity between prochloraz and **III-VIII** did not relate to the small difference in inhibition of $P450_{14DM}$ activity (Table 1). Consequently, this finding indicates that the toxicity of prochloraz and its analogues to *B. cinerea* is not only determined by their differential inhibition of $P450_{14DM}$ activity. Other mechanisms must also be involved, such as for instance differential accumulation or metabolism. The relatively low inhibition of radial growth and of $P450_{14DM}$ activity by compound **VIII** might be explained by its relatively high susceptibility to metabolic breakdown. These results are in agreement with those found for *P. italicum*.[7,9]

B. cinerea and *P. italicum* differed greatly in sensitivity to most of the test compounds (Table 1). This was also demonstrated by the absence of a ranking correlation between

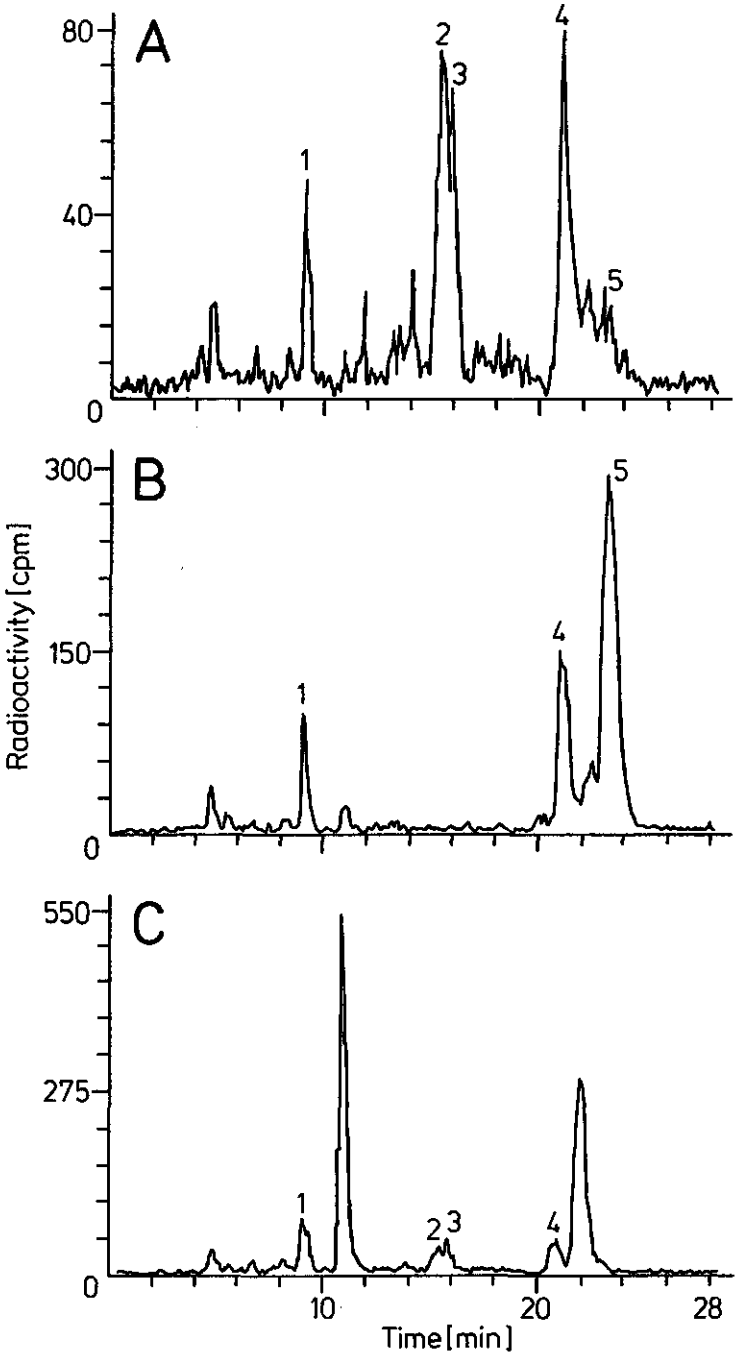


Fig. 3: Radio-HPLC separation of radiolabelled non-saponifiable lipids extracted from (A) control, (B) prochloraz-treated (10^{-8} M) and (C) compound VI-treated ($5x10^{-8}$ M) cell-free assays of *Botrytis cinerea*. The identities of peak 2, 4, and 5 are ergosterol, lanosterol and eburicol, respectively. All other peaks were not identified.

the EC_{50} values for both fungi (r_s 0.26; Table 2). The cell-free assay of *B. cinerea* ranked the compounds in almost the same order of intrinsic potency as the cell-free system of *P. italicum* (r_s=0.88) (Table 2). However, to some extent all the compounds tested were less potent in the cell-free assay from *P. italicum* than in the one from *B. cinerea* (Table 1) suggesting that the $P450_{14DM}$ from *B. cinerea* has a slightly higher affinity for the compounds tested than the demethylase from *P. italicum*. This was reflected in the generally higher toxicity of the test compounds to *Botrytis* (Table 1). However, for some test compounds this correlation was absent. For example, compound **V** was almost equally potent in both cell-free assays, but was less toxic to *B. cinerea* than to *P. italicum* (Table 1). An alternative explanation for the relatively high potency of test compounds in the cell-free assay of *B. cinerea* may be a relatively low $P450_{14DM}$ concentration in cell-free extracts from this fungus. The validity of this hypothesis could not be tested, since the actual concentration of $P450_{14DM}$ in both cell-free extracts was too low to be determined by CO difference spectroscopy.

Prochloraz and compounds **II** and **III** were relatively potent inhibitors of yeast $P450_{14DM}$ activity while compounds **VI** and **VII** were relatively weak inhibitors (Table 1). This agrees with data from the *Botrytis* and *Penicillium* assays. Prochloraz was far less potent in the yeast cell-free assay than in those from *B. cinerea* and *P. italicum* (Table 1). This also applies for the other test compounds, except for compound **VIII**. Consequently, the yeast cell-free assay is less suitable as a biochemical screening test of candidate fungicides than the assays of both filamentous fungi. This observation may relate to the fact that both *B. cinerea* and *P. italicum* belong to the *Euascomycetidae* while *S. cerevisiae* is a member of the *Hemiascomycetidae*.

Structural modifications in the N_1 substituent of prochloraz significantly affected its inhibition of $P450_{14DM}$ activity from *B. cinerea* (Table 1). For instance, when the propyl chain in the N_1 substituent of prochloraz was replaced by an isopropyl group (**IV**), the activity in the cell-free assay was reduced by a factor 12. This is in agreement with literature data indicating that the affinity of azole fungicides for $P450_{14DM}$ is influenced by the N_1 substituent of the azole moiety.[7,14-18]

Modifications in the azole moiety of prochloraz had relatively more impact on $P450_{14DM}$ activity, since substitution of the imidazole ring of prochloraz by a triazole moiety (**VII**) reduced its inhibitory effect on cell-free sterol synthesis and its fungicidal activity 169 and 333-fold, respectively (Table 1). These results are consistent with data obtained from cell-free assays of *S. cerevisiae* and *P. italicum* (Table 1) and data from other studies.[19-21] It has been postulated that the triazole group of **VII** might not be a good enough ligand, due to the electron-withdrawing effect of the carbonyl group.[7] Consequently, the present study confirms that the affinity of this group of compounds for $P450_{14DM}$ is dependent on both the azole part and the N_1 substituent of the molecule.

Addition of an amino group at C_2 of the imidazole ring of prochloraz (**VI**) resulted in inhibition of C_4-desmethyl sterol biosynthesis at a different site. Compound **VI** inhibited

both $C_{4,4}$-dimethyl and C_4-desmethyl sterol synthesis concomitant with the accumulation of 2,3-oxidosqualene (putative) and another, as yet unidentified NSL (Figs. 2C and 3C). Similar inhibition patterns by **VI** were observed in cell-free assays of *P. italicum* (unpublished results). Since the ratio between the levels of lanosterol and eburicol synthesized in the *B. cinerea* assays did not increase upon treatment with compound **VI** ($5x10^{-8}$ M) (Fig. 3C), it was concluded that compound **VI** is not a potent inhibitor of $P450_{14DM}$ activity of *B. cinerea*. This is in agreement with previous observations that substitutions at C_2 of the imidazole moiety decrease inhibition of $P450_{14DM}$ activity by imidazole compounds.[22,23] Compound **VI** did not inhibit squalene epoxidase, since TLC patterns of NSLs synthesized upon incubation with **VI** did not resemble those obtained upon incubation with the squalene epoxidase inhibitor terbinafine (Fig. 2D). The results presented indicate that the primary site of action of compound **VI** in *B. cinerea* is located between 2,3-oxidosqualene and lanosterol, possibly 2,3-oxidosqualene lanosterol-cyclase. The unidentified NSL (Fig. 2C, band 4) might be 2,3;22,23-dioxidosqualene, a product which is not a normal intermediate in sterol synthesis but may accumulate upon incubation with inhibitors of 2,3-oxidosqualene lanosterol-cyclase.[24-26] To date, most inhibitors of 2,3-oxidosqualene lanosterol-cyclase have been designed as amine analogues of the transient carbocationic high energy intermediate, occurring in the epoxide ring opening during the cyclization of 2,3-oxidosqualene.[6,27,28] Amines such as *N*-(1-*n*-dodecyl)-heterocycles, which are protonated at physiological pH, have been suggested to inhibit the cyclase due to binding of their positively charged nitrogen atom to the catalytic site of the enzyme.[29] Therefore, it might be that protonation of the amino group of compound **VI** is responsible for inhibition of 2,3-oxidosqualene lanosterol-cyclase activity.

Further evidence that prochloraz and compound **VI** have different modes of action was obtained from studies with the laboratory-generated triadimenol-resistant isolates of *B. cinerea* (T_N-150/6-6 and T_N-25). These isolates showed cross resistance to triadimenol and prochloraz but not to **VI** (Table 3). The mechanism of DMI-resistance in these isolates of *B. cinerea* is not known, but will be subject of further study. Like most compounds tested, compound **VI** was less potent in the assays from *P. italicum* and *S. cerevisiae* than in the one from *B. cinerea* (Table 1). Radio-HPLC analysis of the NSLs formed upon incubation of yeast cell-free extracts with compound **VI** (unpublished results) suggests that in yeast compound **VI** is an inhibitor of $P450_{14DM}$ and not earlier in the pathway.

Results described in this paper underline the validity of biochemical screening tests and emphasize that such tests should preferentially be carried out with cell-free assays from the target pathogen of interest.

Acknowledgements

The authors are grateful to Professor Dr. J. Dekker and Dr. J.B. Pillmoor for their critical reading of the manuscript. J.C. Kapteyn acknowledges the financial support of Schering Agrochemicals Ltd.

References

1. Kaspers, H., Brandes, C. & Scheinpflug, H., *Pflanzenschutz-Nachrichten Bayer*, **40** (1987) 81-110.
2. Murabayashi, A., Musuko, M., Shirane, N., Hayashi, Y. & Makisumi, Y., *Proc. 1990 Br. Crop Prot. Conf. - Pests and Diseases* (Brighton, UK), pp.423-430.
3. Elad, Y., *Plant Pathol.*, **41** (1992) 47-54.
4. Copping, L.G., Birchmore, R.J., Wright, K. & Godson, D.H., *Pestic. Sci.*, **15** (1984) 280-284.
5. K., Uchida, T., Goto, T. & Umetsu, N., *Pestic. Sci.*, **34** (1992) 119-125.
6. Köller, W., In *Target Sites of Fungicide Action*, ed. W. Köller, CRC Press, Boca Raton Ann Arbor, London, 1992, pp. 255-310.
7. Kapteyn, J.C., Milling, R.J., Simpson, D.J. & De Waard, M.A., *Pestic. Sci.*, (1993) in press.
8. Ballard, S.A., Ellis, S.W., Kelly, S.L. & Troke, P.F., *J. Med. Vet. Mycol.*, **28** (1990) 335-344.
9. Guan, J., Stehmann, C., Ellis, S.W., Kerkenaar, A. & De Waard, M.A., *Pestic. Biochem. Physiol.*, **42** (1992) 260-262.
10. Stehmann, C., Kapteyn, J.C. & De Waard, M.A., *Pestic. Sci.*, (submitted).
11. Kato, T. & Kawase, Y., *Agr. Biol. Chem.*, **40** (1976) 2379-2388.
12. Faretra, F., Antonacci, E. & Pollastro, S., *J. Gen. Microbiol.* **134** (1988) 2543-2550.
13. Kapteyn, J.C., Pillmoor, J.B. & De Waard, M.A., *Pestic. Sci.* (1992) in press.
14. Yoshida, Y., & Aoyama, Y., *In vitro and In vivo Evaluation of Antifungal Agents*, ed. K. Iwata & H. Vanden Bossche. Elsevier Science Publishers BV, Amsterdam, 1986, pp.123-134.
15. Yoshida, Y. & Aoyama, Y., *Chirality*, **2** (1990) 10-15
16. Vanden Bossche, H., *Recent Trends in the Discovery, Development and Evaluation of Antifungal Agents*, ed. Fromtling, R.A. J.R. Proust Science Publishers S.A., Barcelona, 1987, pp.207-221.
17. Vanden Bossche, H., *Sterol Biosynthesis Inhibitors, Pharmaceutical and Agrochemical Aspects*, ed. Berg, D. & Plempel, M. Ellis Horwood, Chichester, 1988, pp. 79-119.
18. Vanden Bossche, H., Marichal, P., Gorrens, J., Coene, M-C., Willemsens, G., Bellens, D., Roels, I., Moereels, H. & Janssen, P.A.J., *Mycoses*, **32** (Suppl. 1) (1989) 35-52.
19. Baillie, A.C., *Synthesis and Chemistry of Agrochemicals*, ed. D.R. Baker, Fenyes, J.G., Moberg, W.K. & Cross, B. ACS Symposium Series **355** (1987) 328-339.
20. Hitchcock, C.A., *Biochem. Soc. Trans.*, **19** (1991) 782-787.
21. Imai, T., Takao, H., Yamaguchi, K., Uchida, T., Goto, T. and Umetsu, N., *Pestic. Sci.*, **34** (1992) 119-125.
22. Janssen, P.A.J. & Vanden Bossche, H., *Arch. Pharm. Chem. Sci. Ed.*, **15** (1987) 24-40.
23. Hitchcock, C.A., *Biochem. Soc. Trans.*, **19** (1991) 782-787.
24. Orth, A.B., Henry, M.J. & Sisler, H.D., *Pestic. Biochem. Physiol.*, **37** (1990) 182-191.
25. Field, R.B. & Holmlund, C.E., *Arch. Biochem. Biophys.* **180** (1977) 465-471.
26. Schmitt, P., Gonzales, R., Benveniste, P., Ceruti, M. & Cattel, L., *Phytochemistry*, **26** (1987) 2709-2714.
27. Duriatti, A., Bouvier-Nave, P., Benveniste, P., Schuber, F., Delprino, L., Balliano, G. & Cattel, L., *Biochem. Pharmacol.* **34** (1985) 2765-2777.

28. Cattel, L., Ceruti, M., Viola, F., Delprino, L., Balliano, G., Duriatti, A. & Bouvier-Nave, P., *Lipids* **21** (1986) 31-38.
29. Mercer, E.I., Morris, P.K. & Baldwin, B.C., *Comp. Biochem. Physiol.*, **80B** (1985) 341-346.

CHAPTER 9

GENERAL DISCUSSION

The term 'selective fungitoxicity' of fungicides comprises the phenomena of natural insensitivity and acquired (field or laboratory) resistance of fungi to these compounds. The molecular mechanisms of natural insensitivity of fungi to fungicides can be closely related to those responsible for acquired resistance. This also holds true for DMI-fungicides.[1] These compounds differ greatly in both potency and selectivity.[2] However, the biochemical mechanisms which account for their selective fungitoxicity are not well documented.

A potential mechanism of selective fungitoxicity is metabolism of these antifungal compounds. Fungal metabolism may either be a detoxification or an activation process.[3] So far, natural insensitivity to DMIs based on detoxification has only been found for *Aspergillus niger*, *Colletotrichum atramentarium* and *Stemphylium radicinum*.[4] These fungi were able to detoxify triforine. Fungitoxicity of triadimefon depends on fungal/plant activation of this compound to triadimenol and the fungal sensitivity to the triadimenol enantiomers produced.[5] To date, acquired resistance based on metabolism has only been reported for triadimefon-resistant isolates of *Cladosporium cucumerinum*[6] and *Nectria haematococca*.[7] These resistant isolates failed to convert triadimefon to triadimenol. In the present study metabolism of prochloraz and an experimental triazole in selected plant pathogenic fungi which markedly differed in sensitivity to these DMIs was investigated (Chapter 3). The selective toxicity of these azoles did not obviously relate to metabolism. Only *Rhizoctonia solani* metabolised prochloraz to a non-fungitoxic compound. This might contribute to its low prochloraz sensitivity. This metabolite was most probably formed in the mycelium and excreted into the medium.

Another potential mechanism of selective fungitoxicity studied was differential accumulation of DMIs (Chapter 3). To date, natural DMI-insensitivity based on low accumulation has only been described for *Trichothecium roseum* and *R. solani*.[4] These insensitive fungi accumulated triforine to a relatively low level as compared with triforine-sensitive fungi. The present accumulation studies demonstrated no general correlation between fungal sensitivity to prochloraz and an experimental triazole, and accumulation of these fungicides (Chapter 3). However, DMI-resistant isolates of *Penicilium italicum* did show reduced accumulation of prochloraz, the experimental triazole, fenarimol and imazalil (Chapters 3 & 4). Reduced accumulation of prochloraz, the experimental triazole and fenarimol might be due to an increased energy-dependent efflux mechanism which has been described before for other DMIs.[8] Imazalil accumulation, however, was not obviously mediated by an energy-dependent efflux (Chapter 4). An energy-dependent efflux mechanism has also been proposed to be

responsible for acquired DMI-resistance in *Aspergillus nidulans*, *Monilinia fructicola* and *N. haematococca* var. *cucurbitae*.[9-11] Accumulation levels of DMIs tested by isolates of *P. italicum* with different degrees of resistance were similar. Hence, reduced accumulation is responsible for a low degree of resistance only. Additional mechanisms of resistance may be involved in isolates with relatively high levels of DMI-resistance (Chapters 3 & 4). This supposition is supported by results of Ney who found that the observed differential DMI-resistance in *M. fructicola* could also not be attributed to reduced accumulation only.[10] However, slight differences in accumulation between the low- and high-resistant strains of *P. italicum* and *M. fructicola* might be masked by aspecific binding of DMIs to mycelium and may, therefore, not be detectable with the methods applied. Therefore, the possibility that the high degree of resistance to DMIs results from a further increase in efflux capacity can not fully be excluded. The observation that different mutations in *N. haematococca* var. *cucurbitae* show additive effects in decreasing fenarimol accumulation by mycelium is in support of this possibility.[11] This is probably also true for *A. nidulans* since all laboratory-generated DMI-resistant mutants of this fungus exhibited reduced accumulation of fenarimol.[12]

Since plasma membrane ATPase inhibitors and ionophoric antibiotics were found to inhibit efflux and to increase accumulation of DMIs, efflux has been suggested to be mediated by the electrochemical proton gradient maintained across the plasma membranes by plasma membrane ATPase.[8,9] The electrochemical proton gradient consists of a proton gradient and a plasma membrane potential. However, no correlation between the accumulation of DMIs and TPP^+, a probe commonly used for measuring plasma membrane potential, could be found (Chapter 4). Consequently, the plasma membrane potential does not play a role in mediating the energy-dependent efflux of DMIs by *P. italicum*. Hence, the mechanism beyond this efflux of fungicides remains unclear. However, there are some similarities between DMI-resistance in *P. italicum* and multidrug resistance (MDR) of mammalian cancer cells (Chapter 4). For instance, both phenotypes are under polygenetic control and show cross-resistance to chemically unrelated compounds (e.g. antibiotics). In addition, DMI and multidrug resistance are both ascribed to reduced accumulation caused by an ATP-dependent efflux of the compounds concerned. In mammalian cancer cells MDR is often related to overexpression of membrane-associated P-glycoproteins which act as relatively aspecific transport proteins for drugs.[13] It might be that similar transport proteins are operative in *P. italicum* and account for the reduced accumulation of DMIs by the resistant isolates of *P. italicum*. The genetic background of pleiotropic drug resistance (PDR) in yeast, which is comparable with MDR in mammalian cells, has also been subject of extensive studies.[14] From these studies it has been suggested that a PDR1 encoded protein transcription regulator controls the expression of various target genes mediating PDR in yeast like for instance the genes which encode for plasma membrane ATPases and other putative transport proteins.[14] Future research should elucidate whether similar genes are present in

the DMI-resistant isolates of *P. italicum* and/or *A. nidulans*. In this respect, DNA fragments from yeast which encode for these genes may be useful heterologous probes to detect whether similar genes are involved in filamentous fungi.

The selective fungitoxicity of DMIs may also be explained by differences in affinity of these antifungals for their target enzyme, cytochrome-P450 dependent sterol 14α-demethylase ($P450_{14DM}$). Therefore, microsomal P450 isozymes were isolated from *Ustilago maydis* to study the affinity of several DMIs for the $P450_{14DM}$ from this fungus (Chapter 5). Gentle mechanical homogenization of sphaeroplasts and differential centrifugation of homogenates yielded microsomal preparations containing P450 isozymes with good quality and stability. Yields of P450 isozymes amounted to approximately 19 ± 6 pmol mg^{-1} of microsomal protein. Next to *P. italicum*,[15] this is the second report of a successful isolation of microsomal P450 isozymes from a phytopathogenic fungus. A similar procedure for the isolation of these enzymes from *U. maydis* has recently been published by Carelli *et al.*[16] The successful isolation procedures described made it possible to study spectrophotometrically the affinity of various DMIs, including prochloraz and prochloraz analogues, for the P450 isozymes from *U. maydis* and *P. italicum* (Chapters 5 and 6). All compounds tested induced type II binding difference spectra when added at extremely low concentrations (10^{-9}-10^{-8} M) to the oxidized forms of the P450 isozymes from both fungi. This indicated that the heterocyclic nitrogen atom of the DMIs tested interacted with the oxidized haem iron atom of the cytochromes. The IC_{50} values (concentration of DMIs which causes a half saturation response in type II binding spectra) did not correlate with the fungitoxicity of the compounds. This finding agrees with data from Guan *et al.*[15] and supports the general assumption that the affinity of DMIs for $P450_{14DM}$ is not determined by their interaction with the haem iron. It is generally accepted that the affinity is largely dependent on the interaction of the N_1 substituent of the antifungals with the apoprotein of the enzyme (Chapter 2). It has been proposed that this interaction can be studied by measuring the ability of carbon monoxide (CO) to displace DMIs from the isozymes (Chapter 2). The present study showed that CO differentially displaced the DMIs tested, including prochloraz and its analogues, from the microsomal P450 isozymes of *U. maydis* and *P. italicum* (Chapters 5 & 6). However, the differences in inhibition of CO-P450 complex formation by DMIs tested did not correlate with their toxicity to *U. maydis* and *P. italicum*. It was concluded that the CO-displacement tests with microsomal preparations from these fungal species do not give relevant information about the selective fungitoxic action of DMIs (Chapters 5 & 6). This conclusion agrees with recent literature data.[15,17-19]

Another method for studying the affinity of DMIs for $P450_{14DM}$ is by measuring the inhibitory effects on ergosterol biosynthesis in cell-free extracts or reconstituted systems (Chapter 2). Such cell-free sterol synthesis assays have been reported for *Saccharomyces cerevisiae*, *Candida* spp., *U. maydis*, *Aspergillus fumigatus* and recently for *P. italicum*.[20-26] Using the assay of *P. italicum* it was demonstrated that the DMIs tested differentially

inhibited cell-free sterol 14α-demethylase activity (Chapter 6). The cell-free assay was able to rank the compounds tested in order of fungitoxicity, but I_{50} values did not quantitatively reflect differences in toxicity. This implicates that differential toxicity of DMIs to *P. italicum* is partly determined by their differences in affinity for the $P450_{14DM}$. Obviously, additional mechanisms which influence toxicity are involved. Similar results were obtained by Guan *et al.*[26]

Results in Chapter 6 indicate that, in contrast to the spectrophotometric studies, the cell-free sterol biosynthesis assay of *P. italicum* is far more useful for evaluating the differences in inhibitory potency of DMIs on $P450_{14DM}$ activity of this fungus. Therefore, it was decided to develop a similar cell-free assay for *Botrytis cinerea*. Chapter 7 describes the successful development of the assay, an improved version of the ones described for *A. fumigatus* and *P. italicum*.[25,26] It is the first time that this is achieved for an economically important plant pathogenic fungus. Cell-free preparations of *B. cinerea* were capable of synthesizing a relatively high percentage of C_4-desmethylsterols (39% of total non-saponifiable lipids). $P450_{14DM}$ activity was very sensitive to the azole fungicides imazalil (Chapter 7), prochloraz and several prochloraz analogues (Chapter 8). The potency of the compounds tested in inhibiting $P450_{14DM}$ activity of *B. cinerea* (IC_{50}) ranked with the fungicidal activity to this fungus (EC_{50}). However, as for *P. italicum*, I_{50} values did not fully reflect differences in toxicity, suggesting that differential affinity of the DMIs for the enzyme is not the only factor accounting for their differences in toxicity. Comparison of the cell-free assays of *B. cinerea* and *P. italicum* showed that these systems ranked prochloraz and its analogues nearly in the same order of intrinsic potency (Chapter 8). However, all compounds tested were less potent in the cell-free assay from *P. italicum* than in the one from *B. cinerea*, suggesting that the $P450_{14DM}$ from *B. cinerea* has a generally higher affinity for the compounds tested. This suggestion could not be confirmed. Most compounds tested were far less potent in the cell-free assay from *S. cerevisiae* than in those from *B. cinerea* and *P. italicum* indicating that the cell-free sterol synthesis assay of *S. cerevisiae* is less suitable as a model system to biochemically screen azole compounds as potential DMIs with fungitoxicity to *B. cinerea* than the assays from both filamentous fungi (Chapter 8). This finding may be related to the fact that both filamentous fungi are members of the *Euascomycetidae* while *S. cerevisiae* belongs to the order of the *Hemiascomycetidae*.

The present study confirms the general view that the affinity of azole antifungals for $P450_{14DM}$ is dependent on the N_1 substituent of the azole ring since modifications in the structure of the N_1 substituent of prochloraz affected the inhibition of $P450_{14DM}$ activity of *P. italicum*, *B. cinerea* and *S. cerevisiae* (Chapters 6 & 8). However, the nature of the azole moiety is also significant since substituting the imidazole ring of prochloraz by a triazole moiety reduced inhibition of $P450_{14DM}$ activity considerably (Chapter 6 & 8). It has been postulated that the triazole moiety in triazole analogues of the imidazole carboxylates is presumably not a good enough ligand to interact with the haem iron, due

to the electron-withdrawing effect of the carbonyl group. It might also be that the compound is too hydrolytically unstable (Chapter 6 & 8).

Addition of an amino group at C_2 of the imidazole ring of prochloraz reduced fungitoxicity and inhibition of cell-free $P450_{14DM}$ activity significantly (Chapters 6 & 8). Most probably, the amino substituent sterically hinders the interaction with the haem iron of the $P450_{14DM}$. However, concomitant with the reduced potency to inhibit $P450_{14DM}$ activity this modification also led to a change in its mode of action. It was demonstrated that in cell-free extracts of *B. cinerea* and *P. italicum* the analogue did not induce an accumulation of $C_{4,4}$-dimethyl sterols but of two other, non-identified non-saponifiable lipids. One of these is probably 2,3-oxidosqualene. Therefore, the compound might be an inhibitor of 2,3-oxidosqualene lanosterol-cyclase (Chapter 8). This hypothesis is supported by the fact that laboratory-generated triadimenol-resistant isolates of *B. cinerea* which were also resistant to prochloraz did not show resistance to the C_2-amino-substituted analogue (Chapter 8). Like most compounds tested the analogue was less potent in the assays from *P. italicum* and *S. cerevisiae* than in the one from *Botrytis*. In yeast, the analogue did not inhibit 2,3-oxidosqualene lanosterol-cyclase activity, but only $P450_{14DM}$ activity.

The results described (Chapter 8) suggest that biochemical screening tests should preferentially be carried out with cell-free assays from the target pathogen of interest.

References

1. Lyr, H., Selectivity in modern fungicides and its basis. *In*: Lyr, H. (ed.), Modern Selective Fungicides, Properties, Applications and Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p. 31-38.
2. Scheinpflug, H. & Kuck, K.H., Sterol biosynthesis inhibiting piperazine, pyridine, pyrimidine and azole fungicides. *In*: Selective Fungicides, Properties, Applications and Mechanisms of Action. VEB Gustav Fischer Verlag, Jena, Germany, 1987, p.31-38.
3. Dekker, J., The development of resistance to fungicides. *Progr. Pest. Biochem. Toxicol.* **4** (1985) 165-218.
4. Gasztonyi, M. & Josepovits, G., Biochemical and chemical factors of the selective antifungal effect of triforine. I. The causes of selectivity of the contact fungicidal action. *Acta Phytopath. Acad. Sci. Hung.*, **10** (1975) 437-446.
5. Deas, A.H.B., Carter, G.A., Clark, T., Clifford, D.R. & James, C.S., The enantiomeric composition of triadimenol produced during metabolism of triadimefon by fungi. III. Relationship with sensitivity to triadimefon. *Pestic. Biochem. Physiol.* **26** (1986) 10-21.
6. Fuchs, A. & De Vries, F.W., Diastereomer-selective resistance in *Cladosporium cucmerinum* to triazole-type fungicides. *Pestic. Sci.* **15** (1984) 90-96.
7. Kalamarakis, A.E., Ziogas, B.N. & Georgopoulos, S.G., Resistance to ergosterol biosynthesis inhibitors in *Nectria haematococca*. *In*: Abstracts of 6th Int. Congr. Pest. Chem., IUPAC, Ottawa, Canada, 1986, 3E-06.
8. De Waard. M.A. & Van Nistelrooy, J.G.M., Accumulation of SBI fungicides in wild-type and fenarimol-resistant isolates of *Penicillium italicum*. *Pestic. Sci.* **22** (1988) 371-382.

9. De Waard, M.A. & Van Nistelrooy, J.G.M., Inhibitors of energy-dependent efflux of the fungicide fenarimol by *Aspergillus nidulans*. *Exp. Mycol.* **11** (1987) 1-10.
10. Ney, C., Untersuchungen zur Resistenz von *Monilinia fructicola* (Wint) Honey gegenüber Ergosterol-Biosynthesehemmern. Thesis, University of Basel, Switzerland, 1988, 92 p.
11. Kalamarakis, A.E., De Waard, M.A., Ziogas, B.N. & Georgopoulos, S.G., Resistance to fenarimol in *Nectria haematococca* var. *cucurbitae*. *Pestic. Sci.* **40** (1991) 212-220.
12. De Waard, M.A. & Van Nistelrooy, J.G.M., Mechanism of resistance to fenarimol in *Aspergillus nidulans*. *Pestic. Biochem. Physiol.* **10** (1979) 219-229.
13. Endicott, J.A. & Ling, V., The biochemistry of P-glycoprotein-mediated multidrug resistance. *Ann. Rev. Biochem.* **58** (1989) 137-171.
14. Balzi, E. & Goffeau, A., Review: Multiple or pleiotropic drug resistance in yeast. *Biochim. Biophys. Acta.* **1073** (1991) 241-252.
15. Guan, J., Braks, H.M.J., Kerkenaar, A. & De Waard, M.A., Interaction of microsomal cytochrome P450 isozymes isolated from *Penicillium italicum* with DMI fungicides. *Pestic. Biochem. Physiol.* **42** (1992) 24-34.
16. Carelli, A., Farina, G., Gozzo, F., Merlini, L. & Kelly, S.L., Interaction of tetraconazole and its enantiomers with cytochrome P450 from *Ustilago maydis*. *Pestic. Sci.* **35** (1992) 167-170.
17. Hitchcock, C.A., Cytochrome P-450-dependent 14α-sterol demethylase of *Candida albicans* and its interaction with azole antifungals. *Biochem. Soc. Trans.* **19** (1991) 782-789.
18. Marichal, P., Vanden Bossche, H., Gorrens, J., Bellens, D. & Janssen, P.A.J., Cytochrome P-450 of *Aspergillus fumigatus* - Effects of itraconazole and ketoconazole. *In*: Schuster, I. (Ed.), Cytochrome P-450: biochemistry and biophysics. Taylor & francis, London, UK, 1988, p. 177-180.
19. Ballard, S.A., Kelly, S.L., Ellis, S.W. & Troke, P.F., Interaction of microsomal P450 isolated from *Aspergillus fumigatus* with fluconazole and itraconazole. *J. Med. Vet. Mycol.* **28** (1990) 327-334.
20. Yoshida, Y. & Aoyama, Y., Interaction of azole antifungal agents with cytochrome P-450_{14DM} purified from *Saccharomyces cerevisiae* microsomes. *Biochem. Pharmacol.* **36** (1987) 229-235.
21. Yoshida, Y & Aoyama, Y., Stereoselective interaction of azole antifungal agent with its target, lanosterol 14α-demethylase (cytochrome *P*-450_{14DM}): a model study with stereoisomers of triadimenol and purified cytochrome *P*-450_{14DM} from yeast. *Chirality* **2** (1990) 10-15.
22. Pye, G.W. & Marriott, M.S., Inhibition of sterol C14 demethylation by imidazole-containing antifungals. *Sabouraudia* **20** (1982) 325-329.
23. Hitchcock, C.A., Dickinson, K., Brown, S.B., Evans, E.G.V. & Adams, D.J., Interaction of azole antifungal antibiotics with cytochrome *P*-450-dependent 14α-sterol demethylase purified from *Candida albicans*. *Biochem. J.* **266** (1990) 475-480.
24. Peacock, G.A. & Goosey, M.W., Separation of fungal sterols by normal-phase high-performance liquid chromatography. Application to the evaluation of ergosterol biosynthesis inhibitors. *J. Chromatogr.* **469** (1989) 292-304.
25. Ballard, S.A., Ellis, S.W., Kelly, S.L. & Troke, P.F., A novel method for studying ergosterol biosynthesis by a cell-free preparation of *Aspergillus fumigatus* and its inhibition by azole antifungal agents. *J. Med. Vet. Mycol.* **28** (1990) 335-344.
26. Guan, J., Stehmann, C., Kerkenaar, A. & De Waard, M.A., Ergosterol biosynthesis in a cell-free preparation of *Penicillium italicum* and its sensitivity to DMI fungicides. *Pestic. Biochem. Physiol.* **42** (1992) 262-270.

SUMMARY

Sterol demethylation inhibitors (DMIs) are antifungal agents which inhibit the biosynthesis of ergosterol by binding to cytochrome-P450-dependent sterol 14α-demethylase ($P450_{14DM}$). These compounds significantly differ in both toxicity and selectivity. This thesis presents results of studies on several potential mechanisms of selective fungitoxicity of DMIs.

Chapter 2 gives a literature review on the mode of action of DMIs, their specific interaction with $P450_{14DM}$ and selective fungitoxicity.

Chapter 3 describes the selective fungitoxic actions of two DMIs, prochloraz and an experimental triazole fungicide, to selected plant pathogenic fungi showing significant differences in sensitivity to these compounds. Results indicate that the relatively high toxicity of prochloraz to the fungi tested could not be explained by the slightly higher acccumulation of prochloraz. As for prochloraz, there was no general correlation between sensitivity of the fungi tested and fungicide accumulation. The same situation held true for the triazole compound. However, the low-DMI-resistant isolate of *P. italicum*, $E_{300\text{-}3}$, did exhibit decreased accumulation of both DMIs, which may be responsible for the low level of acquired DMI-resistance in this isolate. Since the high-resistant isolate H_{17} did not show a further decrease in accumulation of both DMIs, additional mechanisms for resistance in this particular isolate may be involved. Results also show that under the experimental conditions used most fungi tested did not metabolise both DMIs. This implicates that fungal metabolism also does not play a major role in selective fungitoxicity of DMIs. Metabolism also does not generally explain the differences in toxicities of prochloraz and the triazole compound to each individual species. In this respect, *Rhizoctonia solani* behaved exceptionally, since this fungus metabolised prochloraz to a non-fungitoxic compound. This correlates with its low prochloraz sensitivity.

In Chapter 4 the role of plasma membrane potential in mediating the observed reduced accumulation of two other DMIs, fenarimol and imazalil, by resistant isolates of *P. italicum* was studied. As found for prochloraz and the experimental triazole compound (Chapter 3), the results confirm that decreased accumulation of these DMIs is responsible for a low level of resistance only and that additional mechanisms may operate in isolates with a medium and high degree of resistance. With all isolates of *P. italicum* fenarimol accumulation was mediated by an energy-dependent efflux. This was not obviously the case for imazalil. Since no correlation between the accumulation of DMIs and tetraphenyl phosphonium bromide, a probe for measuring plasma membrane potential, could be found, it was concluded that the plasma membrane potential is not involved in accumulation of fenarimol and imazalil by *P. italicum*. Hence, the mechanism involved in the reduced fungicide accumulation remains unsolved.

Chapter 5 describes a procedure to isolate microsomal cytochrome P450 isozymes from *Ustilago maydis*. As demonstrated by difference spectroscopy (type II binding spectra) the the DMIs investigated bind with their heterocyclic nitrogen atom to the oxidized haem iron atom in the protoporphyrinic moiety of the isozymes. However, the DMI concentrations which cause half saturation of type II binding spectra (IC_{50}-type II) did not correlate with the fungicidal activities of the azoles. In the carbon monoxide(CO)-displacement tests binding of CO to ferrous cytochrome-P450 was only slightly inhibited to different degrees by the DMIs tested. The inhibition of CO binding did not correlate with fungitoxicity of these DMIs. It was, therefore, concluded that the spectrophotometric studies are not useful for evaluating toxicity of DMIs to *U. maydis*.

In Chapter 6 the interaction of various DMIs and experimental compounds with $P450_{14DM}$ from *P. italicum* was studied by difference spectroscopy using microsomal P450 isozymes and assays with cell-free extracts capable of synthesizing ergosterol from [^{14}C]mevalonate. Similar to the results obtained with microsomal preparations of *U. maydis* (Chapter 5), neither type II binding spectra nor CO-displacement tests were useful to assess fungitoxicity of the test compounds. In contrast, the cell-free sterol 14α-demethylase assay gave valuable information. I_{50} values (concentrations of compounds which inhibit cell-free sterol synthesis by 50%) varied from $4.3x10^{-9}$ to $4.4x10^{-5}$ M. The assay ranked the compounds tested in order of fungitoxicity. Results presented in Chapter 6 support the general view that the inhibitory potency of azole fungicides against $P450_{14DM}$ activity is influenced by the N_1 substituent of the azole moiety, since structural changes in the N_1 substituent of prochloraz clearly affected the inhibition of cell-free $P450_{14DM}$ activity. However, the intrinsic inhibitory potency of azoles is not exclusively affected by their N_1 substituent. It was demonstrated that the nature of the azole moiety is also important.

Chapter 7 presents the development of a sterol 14α-demethylase assay with cell-free extracts of the filamentous plant pathogen *Botrytis cinerea*. Extracts were obtained by mechanical disruption of young germlings in a Bead-Beater apparatus. The C_4-desmethyl sterol fraction accounted for 39% of the non-saponifiable lipids formed and consisted of three distinct compounds. Ergosterol was the major one. The cell-free system had a pH optimum for synthesis of C_4-desmethyl sterols at pH 7.3-7.4. Cell-free synthesis of C_4-desmethyl sterols was inhibited by the imidazole fungicide imazalil (IC_{50} $9.1x10^{-9}$ M).

The *Botrytis* assay was used to screen prochloraz and several prochloraz analogues for their intrinsic inhibitory potency (Chapter 8). Their IC_{50} values ranged from $2.6x10^{-9}$ to $4.4x10^{-7}$ M. The compounds were also tested in cell-free assays of *Saccharomyces cerevisiae*. The test compounds were less potent in the yeast assay than in the one of *Botrytis*. Therefore, it was concluded that the *Botrytis* assay is more suitable for screening compounds biochemically for their potency to inhibit $P450_{14DM}$ activity than the yeast assay. Results confirm that the inhibitory activity of prochloraz analogues on $P450_{14DM}$ activity depends on the nature of their N_1 substituent and their azole moiety.

Unexpectedly, addition of an amino group at C_2 in the imidazole moiety of prochloraz caused a change in its mode of action in sterol synthesis of *P. italicum* and *B. cinerea* (Chapter 8). This was supported by the fact that laboratory-generated triadimenol-resistant strains of *B. cinerea* exhibited cross resistance to triadimenol and prochloraz, but not to its amino-substituted analogue. Like most compounds, this analogue was less potent in the assays from *P. italicum* and *S. cerevisiae* than in the one from *B. cinerea*. In yeast, this compound did not inhibit sterol biosynthesis at another step in the pathway, but only $P450_{14DM}$ activity. These findings indicate that biochemical screening tests should preferentially be carried out with cell-free assays from the target pathogen of interest.

It is concluded that the selective fungitoxicity of DMIs is in part determined by their potency to inhibit $P450_{14DM}$ activity. Further studies should elucidate which other mechanisms are involved in selective fungitoxicity.

SAMENVATTING

Steroldemethyleringsremmers (DMIs) zijn fungitoxische middelen, die de biosynthese van ergosterol remmen door interactie met cytochroom P450-afhankelijk sterol 14α-demethylase ($P450_{14DM}$). Deze stoffen verschillen aanzienlijk in zowel toxiciteit als selectiviteit. Dit proefschrift geeft de resultaten weer van studies naar enkele potentiële mechanismen, die betrokken zijn bij de selectieve fungitoxiciteit van DMIs.

Hoofdstuk 2 bevat een literatuuroverzicht over het werkingsmechanisme van DMIs, de specifieke interactie van deze middelen met $P450_{14DM}$ en hun selectieve fungitoxiciteit.

Hoofdstuk 3 beschrijft de selectieve fungitoxische werking van twee DMIs, prochloraz en een experimenteel triazool-fungicide, tegen geselecteerde plant-pathogene schimmels, die significant verschillen in gevoeligheid voor deze stoffen. De resultaten tonen aan, dat de relatief hoge toxiciteit van prochloraz voor de schimmels niet verklaard kon worden door de iets hogere accumulatie van prochloraz. Er was geen correlatie tussen de gevoeligheid van de onderzochte schimmels voor prochloraz en accumulatie van dit fungicide. Hetzelfde gold voor het experimentele triazool-fungicide. Het *P. italicum* isolaat $E_{300\text{-}3}$, dat een lage graad van DMI-resistentie bezit, vertoonde echter wel een lagere accumulatie van beide DMIs, hetgeen de lage graad van verworven DMI-resistentie kan verklaren. Het feit, dat het hoog-resistente isolaat H_{17} geen verdere verlaging in accumulatie van beide DMIs te zien gaf, kan betekenen dat er additionele resistentie-mechanismen werkzaam zijn. De resultaten tonen ook aan, dat onder de toegepaste experimentele condities de meeste schimmels beide DMIs niet omzetten. Dit impliceert dat metabolisme door schimmels ook geen grote rol speelt in de selectieve fungitoxiciteit van DMIs. Metabolisme kan over het algemeen ook niet de verklaring vormen voor de verschillen in toxiciteit van prochloraz en het triazool-fungicide voor de verschillende schimmelsoorten. Alleen *Rhizoctonia solani* was in dit opzicht een uitzondering. Deze schimmel was namelijk in staat om prochloraz om te zetten tot een niet-fungitoxische stof. Dit correleert met de lage gevoeligheid van *R. solani* voor prochloraz.

In hoofdstuk 4 wordt de rol bestudeerd van de plasmamembraanpotentiaal bij de verminderde accumulatie van twee andere DMIs, fenarimol en imazalil, door resistente isolaten van *P. italicum*. De verkregen resultaten bevestigen de bevindingen met prochloraz en het experimentele triazool-fungicide (Hoofdstuk 3), dat verminderde accumulatie verantwoordelijk is voor een lage graad van resistentie en dat additionele mechanismen wellicht werkzaam zijn in isolaten met hogere resistentie-niveau's. In alle isolaten van *P. italicum* werd de fenarimol-accumulatie bepaald door een energie-afhankelijke efflux. Dit was niet duidelijk het geval voor imazalil. Er kon geen correlatie worden aangetoond tussen de accumulatie van de onderzochte DMIs en van tetrafenyl-fosfonium bromide, een stof, die voor metingen van de plasmamembraanpotentiaal gebruikt wordt. Op grond van dit resulaat wordt geconcludeerd dat de plasmamembraan-

potentiaal niet betrokken is bij accumulatie van fenarimol en imazalil. Vooralsnog blijft het mechanisme, dat verantwoordelijk is voor de verminderde accumulatie van de fungiciden, onopgehelderd.

Hoofdstuk 5 geeft een beschrijving van een procedure om microsomale cytochroom P-450 iso-enzymen uit *Ustilago maydis* te isoleren. Met behulp van verschilspectrofotometrie (type II spectra) werd gedemonstreerd dat de onderzochte DMIs met hun heterocyclische stikstof atoom een binding aangaan met het geoxideerde haem ijzer atoom in de protoporfyrine ring van de iso-enzymen. De DMI-concentraties, waarbij 50% verzadiging van de geïnduceerde type II spectra optreedt (IC_{50}-type II), correleerden echter niet met de fungitoxiteit van de azolen. De binding van koolmonoxide (CO) aan het gereduceerde cytochroom P450 werd slechts in geringe mate geremd door de onderzochte DMIs. De remming van de CO-binding correleerde echter niet met de fungitoxiciteit van de DMIs. Daarom werd geconcludeerd, dat de spectrofotometrische studies niet geschikt zijn voor de evaluatie van de toxiciteit van DMIs voor *U. maydis*.

In hoofdstuk 6 wordt de interactie van verscheidene DMIs en experimentele stoffen met $P450_{14DM}$ van *P. italicum* bestudeerd met behulp van microsomale P450 iso-enzymen en celvrije extracten, die in staat zijn om vanuit [^{14}C]mevalonaat ergosterol te synthetiseren (sterol 14α-demethylase toets). Overeenkomstig de resultaten, die verkregen waren met microsomale preparaten van *U. maydis* (Hoofdstuk 5), bleken noch de type II bindingsspectra noch het vermogen van CO om DMIs van microsomale P450 iso-enzymen te verdrijven bruikbaar om inzicht te krijgen in de fungitoxiciteit van de teststoffen. In tegenstelling hiermee gaf de sterol 14α-demethylase toets wel waardevolle informatie. De I_{50}-waarden (concentraties van stoffen, waarbij de celvrije sterol synthese voor 50% geremd wordt) varieerden van $4{,}3 \times 10^{-9}$ tot $4{,}4 \times 10^{-5}$ M. De toets rangschikte de middelen in volgorde van fungitoxiciteit. De resultaten in hoofdstuk 6 ondersteunen de algemene mening dat de remmingscapaciteit van azool fungiciden beinvloed wordt door de N_1 substituent van de azoolring. Structurele veranderingen in de N_1 substituent van prochloraz hadden namelijk een duidelijk effect op de remming van de celvrije $P450_{14DM}$-activiteit. De remmingscapaciteit van de azolen wordt echter niet uitsluitend beïnvloed door hun N_1 substituent. Er werd namelijk ook aangetoond dat de aard van de azoolring eveneens effect heeft op de remmingscapaciteit.

Hoofdstuk 7 beschrijft de ontwikkeling van een sterol 14α-demethylase toets met met celvrije extracten van de filamenteuze plant-pathogene schimmel *Botrytis cinerea*. De extracten werden verkregen door homogenisatie van jonge kiemlingen in een Bead-Beater apparaat. De C_4-desmethylsterol-fractie vormde 39% van de totale hoeveelheid geproduceerde, niet-verzeepbare vetten en bestond uit drie verschillende sterolen, waarvan ergosterol het hoofdbestanddeel vormde. Het celvrije systeem had een pH-optimum voor synthese van C_4-desmethylsterolen bij pH 7,3-7,4. De synthese van C_4-desmethylsterolen werd sterk geremd door het imidazool-fungicide imazalil (IC_{50}-waarde $9{,}1 \times 10^{-9}$ M).

De *Botrytis* toets werd vervolgens gebruikt om prochloraz en enkele prochloraz-analogen te onderzoeken op intrinsieke remmingscapaciteit van sterol 14α-demethylase activiteit (Hoofdstuk 8). De IC_{50}-waarden varieerden van $2{,}6x10^{-9}$ tot $4{,}4x10^{-7}$ M. De toetsstoffen werden ook onderzocht in celvrije extracten van *Saccharomyces cerevisiae*. De toetsstoffen waren in de gist-toets minder actief dan in die van *Botrytis*. De *Botrytis*-toets is daarom geschikter om stoffen biochemisch te screenen op hun remmingscapaciteit voor $P450_{14DM}$-activiteit dan de gist-toets. De resultaten bevestigen dat de remmende werking van prochloraz-analogen op $P450_{14DM}$ activiteit afhangt van de aard van de N_1 substituent en de azoolring. Een onverwacht resultaat was, dat aanwezigheid van een amino-groep op de C_2-plaats van de imidazool-ring van prochloraz een wijziging te weeg bracht in het werkingsmechanisme. De amino-gesubstitueerde analoog van prochloraz bleek een andere stap in de sterolbiosynthese te remmen. Dit werd ondersteund door het feit, dat laboratorium-gegenereerde triadimenol-resistente stammen van *B. cinerea* kruisresistentie vertoonden tegen triadimenol en prochloraz, maar niet tegen de amino-gesubstitueerde prochloraz-analoog. Deze analoog was minder actief in de celvrije toetsen van *P. italicum* en *S. cerevisiae*. In gist remde deze stof de sterol biosynthese niet op een andere plaats in de synthese-route, maar alleen de $P450_{14DM}$ activiteit. Deze resultaten geven aan, dat biochemische screeningstoetsen bij voorkeur dienen te worden uitgevoerd met celvrije extracten van het pathogeen, waartegen men fungiciden wil ontwikkelen.

Geconcludeerd wordt, dat de selectieve fungitoxiciteit van DMIs voor een deel bepaald wordt door hun intrinsieke remmingscapaciteit van de $P450_{14DM}$-activiteit. Vervolgstudies moeten uitwijzen welke andere mechanismen bij de selectieve fungitoxiciteit betrokken zijn.

CURRICULUM VITAE

Hans Kapteyn werd geboren op 30 november 1962 te Alkmaar. Na het behalen van het VWO-diploma aan de Chr. Scholengemeenschap "Jan Arentsz" te Alkmaar werd in 1981 aangevangen met de studie Planteziektenkunde aan de toenmalige Landbouwhogeschool te Wageningen. De doctoraalstudie omvatte de hoofdvakken Fytopathologie en Plantenfysiologie, en het bijvak Nematologie. Gedurende deze fase van zijn studie verrichtte hij biochemisch onderzoek naar schimmelresistentie tegen sterolbiosynthese-remmers en naar de nematicide werking van thiofeenverbindingen. Verder hield hij zich bezig met de zuivering en gedeeltelijke karakterisering van proteolytische enzymen uit haver. Vervolgens werd op de vakgroep Fytopathologie aan de Landbouwuniversiteit te Wageningen het onderzoek verricht, dat geleid heeft tot dit proefschrift. Vanaf 1 januari 1993 is hij als postdoctoraal onderzoeker verbonden aan de afdeling Moleculaire Celbiologie van de Faculteit Biologie van de Universiteit van Amsterdam.

Correspondentie adres: Akeleistraat 32, 6707 BZ Wageningen.