J. Enzyme Inhibition, 1989, Vol. 3, pp. 1–11
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INHIBITORS OF FUNGAL STEROL SYNTHESIS: SQUALENE-EPOXIDATION AND C-14-DEMETHYLATION

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(Received 15 November, 1988)

KEY WORDS: C-14-Demethylation, Squalene-Epoxidation, Fungal Sterol Synthesis, Inhibitors

INHIBITORS AND TARGETS

In 1953 Langdon and Block[1] were able to demonstrate both the formation of squalene from acetate and the conversion of squalene to cholesterol in the liver of the intact rat. This was taken as a clear indication that squalene is an intermediate in the pathway from acetate to cholesterol. The following three decades provided a deep knowledge about sterol synthesis in mammals, plants and fungi (for reviews see references 2,3,4); nowadays fungal sterol synthesis, mainly that of ergosterol, has been and still is being studied intensively. This is particularly due to the fact that antifungal sterol biosynthesis inhibitors have become available as tools (and sometimes toys) for studies at the molecular level.

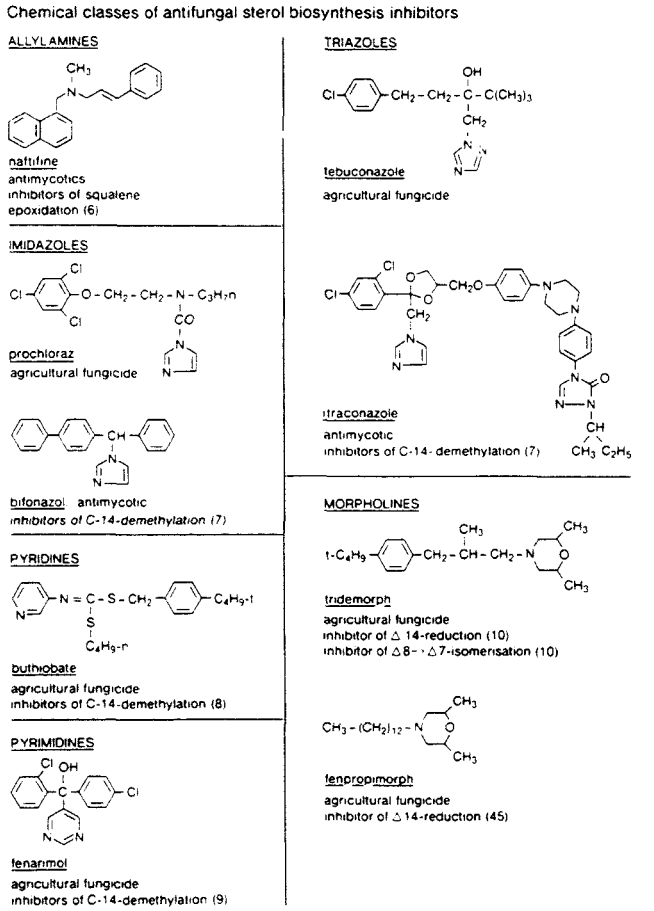
A selective and imcomplete list of sterol biosynthesis inhibitors (SBI's) is given in Table 1 to demonstrate the heterogenicity of the chemical classes that have been developed to date (for details see reference 5). In addition brief information on the various targets of these compounds is relevant and will be discussed following a short general description of fungal ergosterol biosynthesis (Figure 1).

Starting from 3-hydroxy-3-methyl-glutaryl-CoA, mevalonate is formed and subsequently isopentenylpyrophosphate, then geranylpyrophosphate and finally farnesylpyrophosphate are synthesized. A tail to tail reaction of farnesylpyrophosphate leads to squalene which, after epoxidation cyclises to lanosterol. The epoxidation is catalyzed by a microsomal NADPH-dependent monooxygenase, the squalene epoxidase.

In contrast to cholesterol synthesis in mammals, ergosterol synthesis in fungi requires a side chain methylation at C-24, which is commonly performed at the lanosterol stage. 24-Methylenedihydranolanosterol has been found in many fungi such as various Candida spp.[12,13], *Ustilago maydis*[14], and numerous filamentous fungi[4,15,16,17]. However, 24-methylenedihydrolanosterol has not been found in *S. cerevisiae*[4] or *Saccharomycopsis lipolytica*[18] because side-chain alkylation proceeds with $\Delta^{8,24}$-cholesterol as substrate[19,20,21]. The side-chain alkylation enzymes require *S*-adenosylmethionine (SAM) as the donor of the 24-methyl group[22].

* Correspondence

TABLE I
Chemical classes of antifungal sterol biosynthesis inhibitors.



Independent of whether side-chain methylation proceeds first or later, either lanosterol or 24-methylenedihydrolanosterol is oxidatively demethylated at C-14 in the next step (Figure 2).

In the liver a single cytochrome P-450 is responsible for the oxidation sequence[22,24] as well as the lyase activity. Its apparent molecular weight after SDS-polyacrylamide gel-electrophoresis[25] is 51000 D. In the case of the yeast *S. cerevisiae*, which also uses lanosterol as substrate, it has also been found that one isozyme is responsible for the whole oxidation sequence[26]. The yeast cytochrome $P\text{-}450_{14DM}$ has an apparent molecular weight of 58000 D. As the last step in C-14-demethylation the reduction of the Δ^{14}-intermediate is performed by one $NADPH/H^{\oplus}$-dependent reductase[27].

The subsequent C-4-demethylations remove the methyl groups[4] as CO_2, and are followed by $\Delta^8 \rightarrow \Delta^7$-isomerisation that starts with α-protonation of the Δ^8 double bond and ends with elimination of a C-7 proton[28]. The $\Delta^8 \rightarrow \Delta^7$-isomerase in *S. cerevisiae* is localized in the microsomal fraction[27,29]. Δ^5-Desaturation leads to the

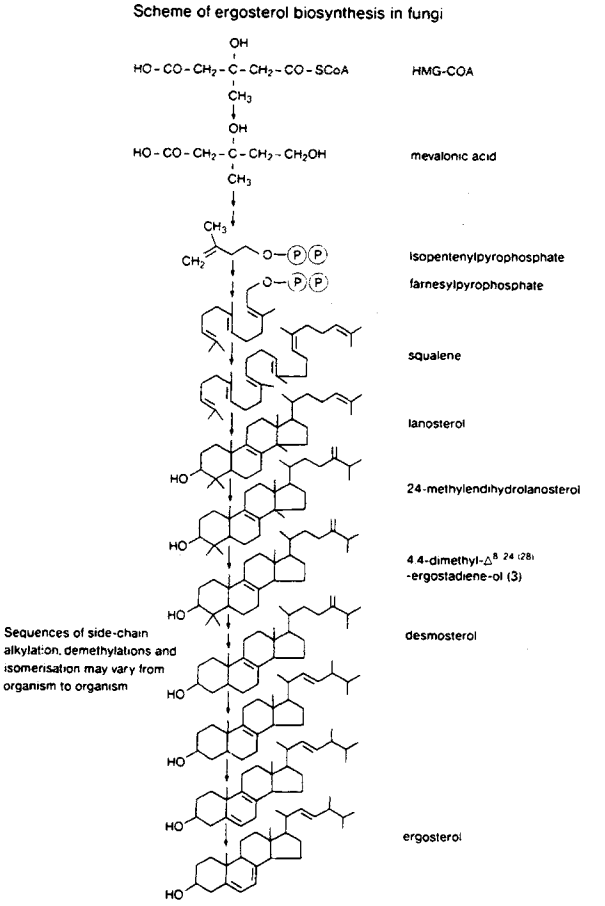


FIGURE 1 Scheme of ergosterol biosynthesis in fungi

$\Delta^{5,7}$-diene in ring B, and saturation of the $\Delta^{24(28)}$-double bond and formation of the Δ^{22}-double bond concludes ergosterol synthesis. The latter step (Δ^{22}-desaturation) could again involve a cytochrome P-450 dependent enzyme.

The function of sterols in fungal membranes has often been described as a contribution to membrane topology. Nowadays at least four functions have to be discussed (for review see reference 7). The contribution to membrane topology by interaction with phospholipids is called a "bulk function" requiring a concentration of about 15 μg/ml. Studies on sterol auxotrophic yeasts with cholestanol showed that "sparking" by minute amounts of ergosterol (1–10 ng/ml) is necessary to induce growth. A concentration of 100 ng/ml is required for certain limited areas ("critical domain function"), and at concentrations of 0.5–1 μg/ml ergosterol prevents detectable changes in properties of lipids possessing a so called "domain function"[31,32].

Application of antifungal sterol biosynthesis inhibitors leads to various typical physiological abnormalities which can be explained by lack of the end-product of

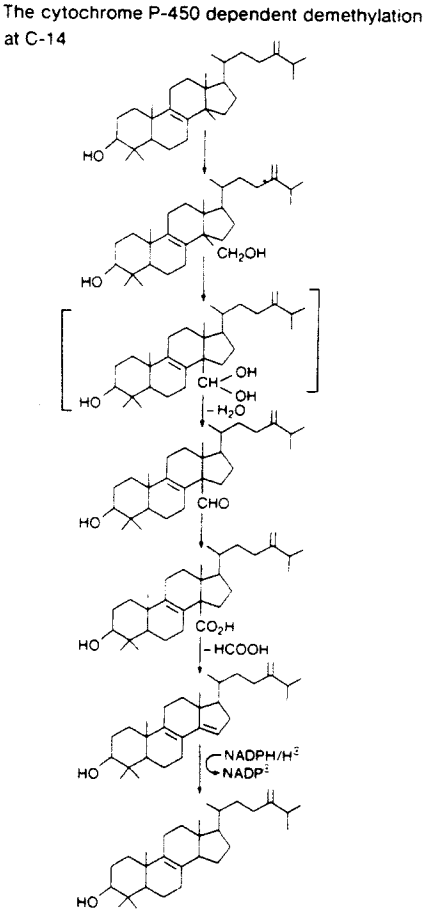


FIGURE 2 The cytochrome P-450 dependent demethylation at C-14

fungal sterol synthesis, including irregularities of the cell membrane, disappearance or deformation of membrane invaginations, accumulation of lomasome-like vesicles, deformation of buds, abberrant thickening of cell walls, and incomplete septa formation leading to impaired separation of budding cells resulting in chains of interconnecting cells[38].

Inhibition of Squalene Epoxidation

The inhibitors of squalene epoxidase are represented by the allylamines e.g. naftifine (Table 1). A large number of chemical variations have been made to optimize efficacy[34] but we may restrict ourselves here to naftifine and terbinafine ($-C=C(CH_3)_3$ instead of phenyl in naftifine).

Cell-free studies have been performed as squalene epoxidase can be assayed in microsomal fractions of yeasts[35,36]. The enzyme requires molecular oxygen, NAD(P)H

TABLE II
Inhibitory concentrations (μM) of naftifine and terbinafine against microsomal squalene eqoxidase from various sources.

Enzyme source	Naftifine		Terbinafine	
	K_i	IC_{50}[a] (μm)	K_i	IC_{50}[a] (μm)
C. albicans	1.1	1.1	0.03	0.03
C. parapsilosis	–	0.34	0.04	0.04
Rat liver	–	144	77	93
Guinea pig liver	–	> 100	–	4.0

[a] Concentration causing 50% inhibition of enzyme activity under standard assay conditions (36)

and FAD for activity. Attempts to investigate the enzyme system from filamentous fungi, however have been unsuccessful to date.

Naftifine and terbinafine are reversible inhibitors of Candida squalene epoxidation with high specificity[6]. In the Dixon plot they show non-competitive kinetics with respect to squalene (see Figure 3) but also to NADH and FAD[36]. The K_i-values for naftifine and terbinafine are shown in Table 2, reflecting not only their relative activity as antifungals but also their range of potency towards enzymes from different sources.

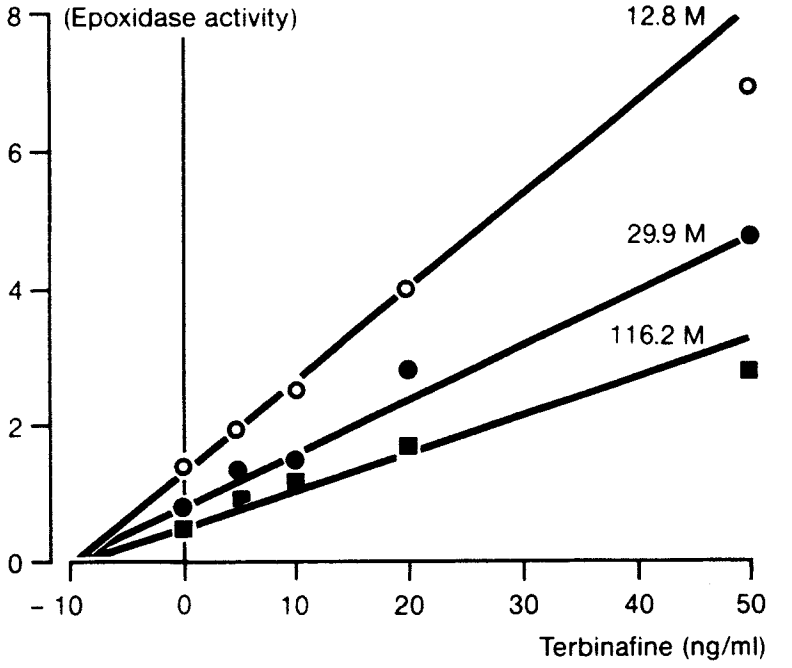
Surprisingly in contrast to the MIC values established for the two Candida species[6], there is only a small difference in sensitivity of the epoxidase system *in vitro*. Obviously there are other physiological factors involved in determining susceptibility to growth inhibition.

The molecular mechanism of epoxidase inhibition is not yet understood, but the discovery of the allylamines has induced work on fungal squalene epoxidase although still only little is known. The enzyme complex from rat liver has been purified[38]. The reducing equivalents are transferred from NADPH via a protein with NADPH-cytochrome-C-reductase activity to the terminal oxidase. This terminal enzyme is not of the cytochrome P-450 type, contains no haem, and is obviously a flavoprotein[38]. The Candida enzyme exhibits no differences with respect to cofactor requirements[35]. Studies by Ryder[6] have shown that the allylamines most likely act on the terminal oxidase, probably by allosteric interactions. A possible interaction would be attachment to a lipid binding site on the enzyme because a specific interaction of the allylamines with certain anionic phospholipids has been observed[6].

Inhibition of Cytochrome P-450$_{14DM}$

Studies on the mode of action of sterol biosynthesis inhibitors are normally performed by GC/MS-analysis of sterol patterns after treatment as compared to untreated controls. A typical example is given in Table 3 using HWG 1608 (common name tebuconazole, for structure see Table 1) as a representative of triazoles[18].

A dose dependent accumulation of lanosterol is observed in *S. lipolytica*, but in



Reproduced from Science G. Petranyi, N.S. Ryder and A. Stuetz. *Science*, **224**, 1239–1241 (1984).

FIGURE 3 Inhibition of Candida albicans squalene epoxidase by terbafine (Dixon analysis) for various squalene concentrations. (copyright 1984 by the AAAs)

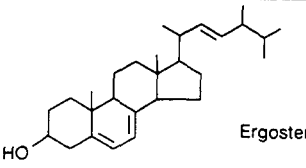
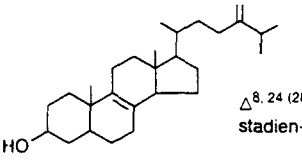
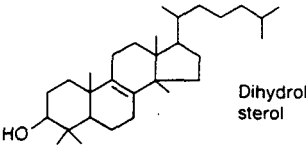
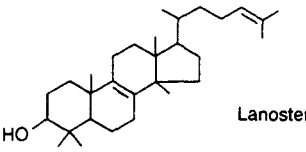
filamentous fungi side-chain alkylation proceeds prior to C-14-demethylation so that 24-methylenedihydrolanosterol and often other 14-α-methylsterols such as obtusifoliol are detected[7,18]. This clearly indicates that the cytochrome P-450$_{14DM}$ involved in the oxidative removal of the C-14-methyl groups is inhibited.

Cytochrome P-450 has been isolated from various sources such as mammalian livers[39] and spectroscopic studies with respect to azole interactions have been performed. Recently Vanden Bossche *et al.*[40] compared interactions of azole derivatives with cytochrome P-450 isozymes from yeast, plant and mammalian cells.

The principle of interaction is always the same. With the free electron pair of the heterocyclic nitrogen (N-3 in imidazoles or N-4 in 1,2,4-triazoles) the azole binds directly to the iron atom of the prosthetic Fe-porphyrin complex of the cytochrome system (schematic drawing of the active site shown in Figure 4).

The interaction yields a type II difference binding spectrum (Figure 5). The azoles compete with, for example, carbon monoxide for binding to the sixth coordination position of the reduced haem iron in the prosthetic group. Even stereochemical implications for the fungicidal efficacy of fungicide isomers could be evaluated on the basis of binding capacity[41]. Titration of cytochrome P-450$_{14DM}$ with azoles indicated stoichiometric binding of the fungicide to the cytochrome[42]. With enzyme systems from different sources[40] variations with respect to binding capacities have been found,

TABLE III
Distribution of sterols in Saccharomycopsis lipolytica after treatment with HWG 1608.

Sterol (%)	Untreated	HWG 1608 (ppm) 0.5	1	5	10
 Ergosterol	100	63.4	59.2	14.3	10.4
 $\Delta^{8,24(28)}$-Ergostadien-ol (3)	< 0.02	5.8	6.3	15.3	15.1
 Dihydrolanosterol	< 0.02	10.8	10.3	11.2	7.4
 Lanosterol	< 0.02	20.0	24.3	59.2	67.0

but in explaining the *in vivo* situation of anti-fungal azoles additional aspects causing selectivity apart from the P-450-system also have to be considered[7] i.e. differences in uptake, translocation, metabolism etc.

The whole C-14-demethylation sequence involves as a final step a NADPH/$H^{\oplus}$-dependent reduction of the Δ^{14}-double bond, which is formed simultaneously with the loss of formic acid (Figure 2). Fortunately the morpholines can be used as tools for studying this reaction.

Inhibition of Sterol- Δ^{14}-Reductase

Although Kato *et al.*[43] in 1980 had described an inhibition of Δ^{8} -> Δ^{7}-isomerase in *Botrytis cinerea* by the morpholine derivative tridemorph, only a year later Kerkenaar *et al.*[10] came to a different conclusion with studies on sterol synthesis in *Ustilago maydis*. They showed convincing data — additionally supported by ^{1}H-NMR and UV-spectroscopy — that in *U. maydis* $\Delta^{8,14}$-ergostadienol (ignosterol) accumulated after tridemorph administration, together with other $\Delta^{8,14}$-sterols.

In 1984 Baloch *et al.*[44]. demonstrated that these obvious discrepancies in fact are simply explicable by comparing the effects of tridemorph and fenpropimorph (Table 1) on sterol synthesis in *S. cerevisiae* and *U. maydis*. The compounds inhibited both the sterol Δ^{8} -> Δ^{7}-isomerase and the sterol Δ^{14}-reductase but to different degrees.

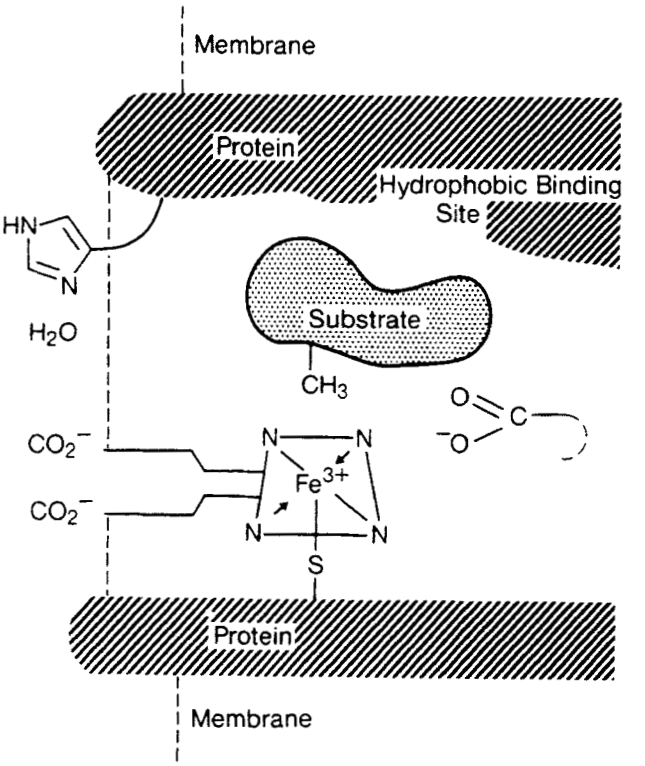


FIGURE 4 Cytochrome P_{450}. Model enzyme site

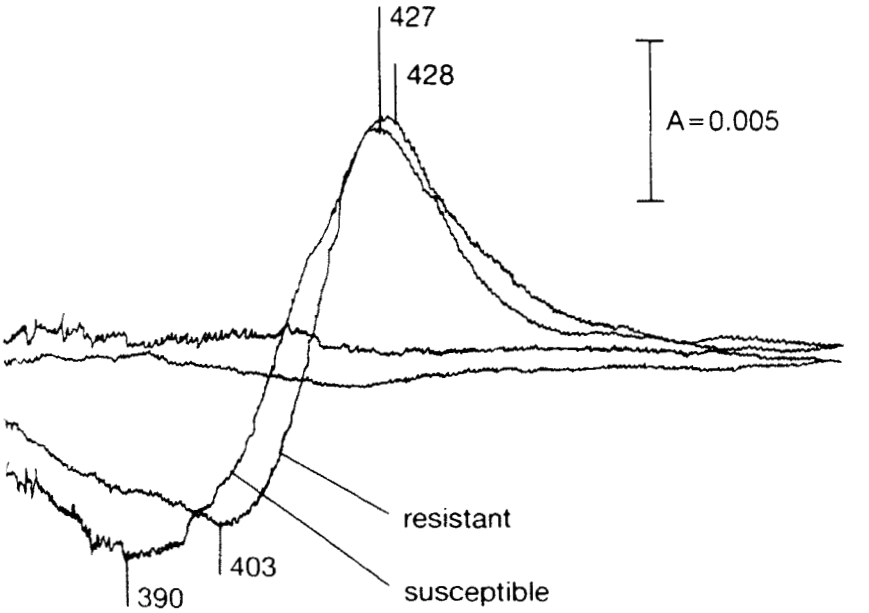


FIGURE 5 Triadimenol binding spectra of microsomal cytochrome P-450 from susceptible and resistant *S. lipolytica*

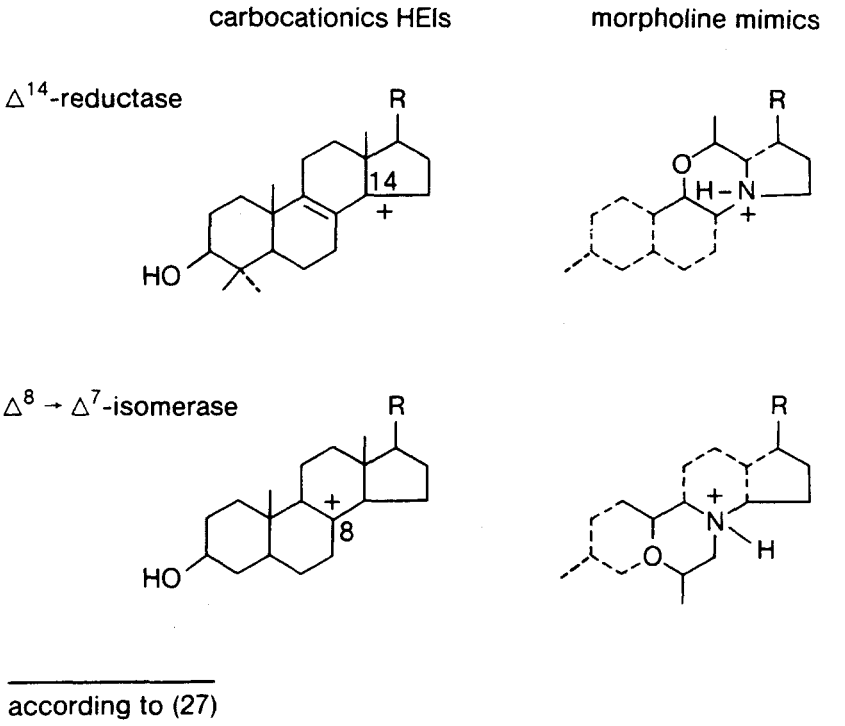


FIGURE 6 Morpholine fungicides as mimics of the HEPs of sterol Δ^{14}-reductase and sterol $\Delta^{18} \rightarrow \Delta^{7}$-isomerase

Again Kerkenaar *et al.*[45], in the same year, showed that fenpropimorph inhibited the sterol Δ^{14}-reductase in *P. italicum*, yielding an accumulation not of ignosterol ($\Delta^{8,14}$-ergstadienol) but of $\Delta^{8,14,24(28)}$-ergostatrienol.

Also in 1984 it was confirmed with the rice blast fungus *Pyricularia oryzae* that fenpropimorph led to ignosterol accumulation, and tridemorph was a more potent inhibitor of sterol $\Delta^{8} \rightarrow \Delta^{7}$-isomerase[46].

To explain the molecular inhibitory effect of the morpholines on the two enzymes, Benveniste *et al.*[47-50] proposed a structural similarity of the protonated morpholinium cations to the carbocationic high-energy-intermediates (HEIs) generated in the reactions (Figure 6). This fits with the concept of transition state analogues as potent enzyme inhibitors.

CONCLUSIONS

Sterol biosynthesis inhibitors besides fulfilling a useful function in studies on the mechanistic aspects of the respective enzyme action also have an economic impact and some concluding remarks will focus on their importance to modern developments in the life sciences industry (for review see reference 51).

Research on sterol biosynthesis inhibitors within the last 15 years has revolutionized the treatment of fungal diseases of crops as well as the therapy of human and

animal mycoses. The chemistry of inhibitors has become highly diversified and includes the chemical classes of piperazines, pyridines, pyrimidines, imidazoles, 1,2,4-triazoles, morpholines and piperidines. The modes of actions on these different classes are understood at the molecular level so that drug design can be used to develop new compounds rather than empirical variations.

In plant protection, sterol biosynthesis inhibitors represent almost traditional fungicides in cereals, fruits and grapes, applied as sprays or seed dressings. Compounds under recent development display an even broader spectrum of biological activity and can combat plant diseases on other crops (eg. peanuts, oilseed rape).

A separate development of 1,2,4-triazoles as plant growth regulators has also proved possible. These compounds show beneficial effects mainly in rice, rape, orchard fruits, ornamentals and turf. Generally azoles may positively influence plant physiology leading for example to improved temperature stress tolerance and drought resistance.

With respect to medical uses, dermal mycoses and vaginal infections are regarded as the classical indications for topically administered sterol biosynthesis inhibitors. Modern concepts are directed towards oral therapy of primarily pathogenic or opportunistic systemic mycoses. Of increasing importance is the treatment of immunocompromised or immunodeficient patients; AIDS victims, for example, often suffer from severe secondary fungal infections.

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