Sterol-Inhibiting Fungicides: Effects on Sterol Biosynthesis and Sites of Action

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ABSTRACT

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A group of fungicides, diverse with respect to chemical structure, spectrum of biological activity, and degree of systemicity, has recently been introduced for plant disease control. Fungal mutants selected for resistance to one member of this group of fungicides usually display resistance to other members as well. This phenomenon of cross-resistance indicates a common mode of action. Biochemical analysis of the toxic action of these compounds in fungal cells revealed that ergosterol biosynthesis is rapidly inhibited. This inhibition eventually curtails membrane synthesis and fungal growth. The details of the toxic action, effects on sterol biosynthesis, and sites of action of the sterol-inhibiting fungicides are discussed.

The introduction of compounds that inhibit ergosterol biosynthesis and membrane function in fungi is a recent innovation in the uses of chemicals to control plant pathogens. Almost all of these chemicals exhibit varying degrees of systemicity, and they control a wide range of diseases caused by Ascomycetes, Basidomycetes, and Deuteromycetes. The sterol-inhibiting fungicides appear to have several unique characteristics that differentiate them from other systemic agents. They are the largest group of compounds with the same specific mode of action; they are a diverse group of compounds with respect to chemical structures; and, most important, no field resistance to these chemicals has been reported.

STEROL BIOSYNTHESIS

Sterols, which are required for growth and reproduction by eucaryotic organisms, probably function primarily in a nonmetabolic manner. They serve as architectural components of membranes. Sterol biosynthesis is one aspect of general lipid metabolism in which acetate, the basic starting chemical unit, is converted into mevalonate. The condensation of five-carbon unit isoprenoids (formed by the loss of one carbon from mevalonic acid) eventually leads to the formation of squalene. The cyclization of squalene to the first sterol intermediate, lanosterol, is the first step in a series of complex reactions leading to the synthesis of ergosterol, the major sterol in higher fungi.

Figure 1 illustrates the general pathway of ergosterol biosynthesis. This synthesis involves the following reactions (23): (i) The introduction of a methyl group into lanosterol at C-24, accompanied by a double-bond shift from C-24(25) to C-24(28); (ii) removal of three methyl groups on the steroid nucleus (two at C-4 and one at C-14); (iii) double-bond shift from C-8(9) to C-7; (iv) introduction of a double bond at C-5(6) and C-22; and (v) reduction of the C-24(28) double bond. Many of the reactions have not been completely elucidated, and the precise order of the steps in the pathway may vary in different species of fungi.

The site of sterol biosynthesis is the smooth portion of the endoplasm reticulum; in part, this involves the microsomal, mixed function, oxidase system. Sterol carrier proteins, which are involved in all steps of sterol synthesis, allow the interaction of the sterol and enzymes necessary for various sterol conversions.

INHIBITORS OF ERGOSTEROL BIOSYNTHESIS

Compounds and antifungal activity. Two groups of compounds are listed in Table 1. The compounds in the first group have all been reported to inhibit the biosynthesis of ergosterol, as determined by biochemical analysis of their toxic action in fungal cells (see reference numbers in Table 1). The compounds in the second group (see footnote b in Table 1) are presumed to be inhibitors of ergosterol biosynthesis primarily on the basis of the similarity of their structures to those of known sterol-inhibitors.

Close structural similarities exist among a number of the compounds, for example, the substituted pyrimidines (triarimol, fenarimol, and nuarimol), triazoles (triadimefon, triadimenol, and bitertanol), and imidazoles (miconazole and imazalil).

Not all of the listed compounds have agricultural use. Miconazole and clotrimazole are antifungal agents used in medicine (26), and triarimol, azasterol, and dodecylimidazole have only experimental, nonagricultural use. It is difficult to determine from the literature the degree of systemicity of the agricultural fungicides. Although none has been reported to move in the symplast, local penetration or movement in the apoplast appears to be common.

The fungitoxic spectrum of these compounds varies. None is used to control diseases caused by the Phycomycetes. Most of the compounds control various powdery mildew, rust, and smut pathogens. In addition, individual compounds may also control diseases

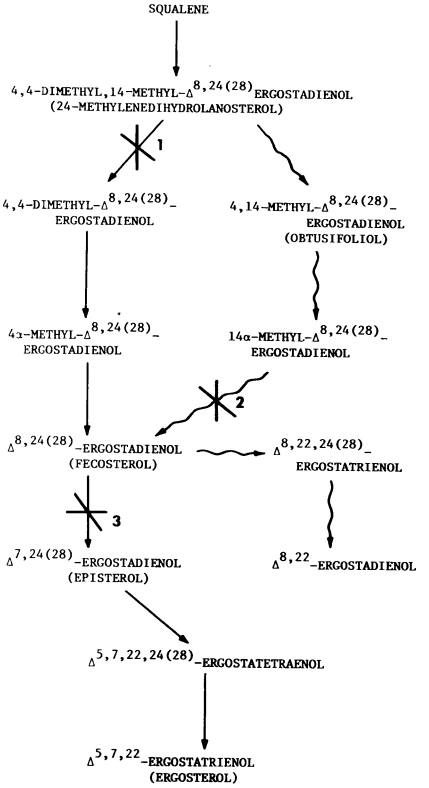

Fig. 1. Scheme of sterol biosynthesis (adapted from references 17 and 23). Numbers and X's indicate major points of inhibition by ergosterol-inhibiting fungicides (Table 2). Straight arrows represent normal sterol biosynthetic pathway; wavy arrows represent alternate or abnormal pathways induced by the fungicides.

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caused by species in the genera of *Monilinia*, *Septoria*, *Venturia*, *Colletotrichum*, *Fusarium*, *Aspergillus*, *Penicillium*, *Verticillium*, *Cercospora*, *Thielaviopsis*, *Helminthosporium*, *Rhizoctonia*, and *Botrytis* (4).

General effects. Based on various assays and analyses of the toxic action of sterol-inhibiting fungicides, these compounds can be considered to have the following general characteristics: They fail to inhibit spore germination or initial cell growth and dry weight increase; they alter cell morphology, causing abnormal growth patterns, swollen hyphae, and/or excessive hyphal branching; they have no immediate effects on respiratory metabolism or macromolecule synthesis; and they cause the accumulation of free fatty acids and sterol intermediates in cells. Fungal mutants selected in the laboratory for resistance to one member of this group of fungicides usually display resistance to other members as well.

These inhibitors rapidly curtail the biosynthesis of ergosterol. This effect has been demonstrated in experiments in which neutral lipids in treated mycelium were labeled using [^{14}C]acetate. Growth-inhibiting concentrations of imazalil and concentrations 10 times below that necessary to reduce growth completely inhibited incorporation of [^{14}C]acetate into the desmethyl sterols (ergosterol) in mycelium of *Aspergillus nidulans* Eidam (Wint.) within 30 min after the addition of the fungicide (30).

Even though ergosterol biosynthesis is quite sensitive to inhibition by these toxicants, mycelial growth and various aspects of metabolism (respiration, protein and nucleic acid synthesis) are only mildly affected for a period of time after the curtailment of the synthesis of the sterol. The levels of ergosterol in treated mycelium do not decline rapidly, which indicates that the rate of use of the sterol in membrane synthesis is slower than is its biosynthesis. However, once the level of ergosterol becomes depleted, an interference in membrane synthesis occurs; growth inhibition and changes in morphology and metabolism are then noted. Labeling of the neutral lipids in treated mycelium indicates that certain sterol intermediates start to accumulate immediately upon inhibition of ergosterol biosynthesis. On the other hand, free fatty acids accumulate in treated mycelium only after longer incubation periods with the inhibitors and after cessation of membrane synthesis. This accumulation results from continued de novo synthesis of free fatty acids, a decline in the utilization of triglycerides and polar lipids, and from the degradation of the existing phospholipids in membranes (23,31).

Although chemical analysis of the toxic action of an inhibitor on cellular metabolism ultimately determines mode of action, other assays can often be used to characterize toxicity further. Perhaps one of the best supplemental assay methods uses fungicide resistant mutants

Table 1. Inhibitors of ergosterol biosynthesis

Chemical class	Name used in text	Chemical name	Other names	Reference[a]
Triazole	Triadimefon	1-(4-Chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanone	Bay MEB 6447; Bayleton	(3)
	Triadimenol	β-(4-Chlorophenoxy)-α-(1,1,dimethylethyl)-1*H*-1,2,4-triazole-1-ethanol	Bay KWG 0519; Baytan	(3,14)
	Bitertanol	β-([1,1-Biphenyl]-4-yloxy)-α-(1,1-dimethylethyl)-1*H*-1,2,4-triazole-1-ethanol	Bay KWG 0599; Baycor	(19)
	Fluotrimazole	1-(3-Trifluoromethyltriphenyl)1,2,4-triazole	Bay 6683; Persulon	(6)
	Diclobutrazol[b]	1-(2,4-Dichlorophenyl)-4-4-dimethyl-2-(1,2,4 triazol-1-yl) pentan-3-ol	ICI 296; Vigil	(6)
	Propiconazol[b]	1-[[2-(2,4-Dichlorophenyl)-4-propyl-1-3-dioxolan-2-yl]methyl]-1*H*-1,2,4 triazole	Tilt; CGA 64250	
	CGA 64251[b]	1-[[2-(2,4-Dichlorophenyl)-4-ethyl-1,3-dioxolan-2-yl]methyl]-1*H*-1,2,4-triazole	Vangard	
Imidazole	Imazalil	1-[2-(2,4-Dichlorophenyl)-2-(2-propenyloxy) ethyl]-1*H*-imidazole	Fungaflor	(5,20,31)
	Miconazole[c]	1-[2-(2,4-Dichlorophenyl)-2-[2,4-dichlorophenyl)methoxy]] ethyl-1*H*-imidazole		(15,33)
	Clotrimazole[c]	Bisphenyl(2-chlorophenyl)-1-imidazolyl methane	Bay L 5097; Canestan	(6)
	Prochloraz	N-Propyl-*N*[2-(2,4,6-trichlorophenoxy) ethyl] imidazole-1-carboxamide	BTZ 40502	(20,21)
	Fenapanil[b]	2-(imidazol-1-ylmethyl)-2-phenyl-hexanentrile	RH 2161; Sisthane	
	XE 326[b]	1-[2-(2,4-Dichlorophenyl)-1-3 dixolan-2-yl methyl] imidazole		
	Dodecylimidazole[c]	1-Dodecylimidazole		(15)
Pyrimidine	Triarimol[c]	α(2,4-Dichlorophenyl)-α-phenyl-5-pyrimidinemethanol	EL-273	(22,25,27)
	Fenarimol	α-(2-Chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidinemethanol	EL-222; Rubigan; Bloc	(2,10)
	Nuarimol	α-2(Chlorophenyl)-α-(4-fluorophenyl)-5-pyrimidinemethanol	EL-228; Trimidal	(2)
Morpholine	Tridemorph	2,6-Dimethyl-*N*-tridecylmorpholine	Calixin	(17,18)
	Fenpropemorph[b]	4-[3-(4-(1,1-dimethyl)phenyl)-2-methyl] propyl-2,6-dimethylmorpholine	BAS 42100; Corbel	
Piperazine	Triforine	*N,N'*-Bis-(1-formanido-2,2,2,-trichloro-ethyl) piperazine	Cela W-524; Funginex; Saprol	(27,28)
Pyridyl	Buthiobate	N-3-Pyridyl-*s*-*n*-butyl-*s*-*p*-*t*-butyl-benzylimidodithiocarbonate	S-1358; Denmert	(16,17)
Miscellaneous	Azasterol[c]	15-aza-24-methylene-D-homo cholesta-8,14-diene-3β-ol	A25822B	(35)

[a] Reference for mode of action.
[b] Suspected inhibitors of sterol biosynthesis based on structural similarities with known inhibitors.
[c] Nonagricultural use.

and measures patterns of cross-resistance. The term "cross-resistance" is used when a change in one genetic factor in the fungal strain results in the development of resistance to different fungicides (8). Cross-resistance usually occurs only between compounds that have similar modes of action.

Patterns of cross-resistance with fungicide-resistant mutants of *Ustilago maydis* (DC) Corda to clotrimazole, imazalil, miconazole, fenarimol, nuarimol, triadimefon, and tridemorph were studied by Barug and Kerkenaar (1). Cross-resistance was reciprocal in all cases except for triadimefon and imazalil and for triadimefon and tridemorph. Cross-resistance between the sterol-inhibitors and prochloraz was also established, indicating a similar mode of action for this compound. This was later confirmed by biochemical analysis of the antifungal activity of prochloraz (21).

Fluotrimazole, buthiobate, and triforine were not included in the study by Barug and Kerkenaar because these compounds do not inhibit growth of *U. maydis*. However, cross-resistance for these, as well as other inhibitors of ergosterol biosynthesis, has been demonstrated (13,27,28).

Specific effects. The specific mechanisms of inhibition of sterol biosynthesis by the group of compounds under discussion have been reviewed recently (23,31). In addition, a detailed list of references dealing with the mode of action of each compound is given in Table 1. The primary site of interference by these inhibitors occurs in the sterol biosynthetic pathway and results in the inhibition of ergosterol synthesis and the accumulation of sterol intermediates (Table 2). The most significant interference within this pathway, for all compounds except tridemorph and azasterol, is the selective inhibition of sterol C-14α demethylation (Fig. 1, reactions 1 and 2). Reactions involving squalene cyclization, C-24 methylation, and C-4 demethylation are generally unaffected.

Inhibitors of C-14α demethylation typically cause marked accumulation in the following sterol intermediates: C-14 dimethyl (24-methylenedihydrolanosterol), C-4 methyl (obtusifoliol), and C-4 desmethyl (14α methyl $\Delta^{8,24(28)}$-ergostadienol) sterols. In the presence of these inhibitors, no sterols are synthesized that do not contain the C-14α methyl group. Although all compounds listed in Table 2 except tridemorph and azasterol cause the accumulation of C-4 methyl and dimethyl sterols, all have not been reported to cause accumulation of the C-14 methyl (C-4 desmethyl) sterol.

The accumulation of methyl and dimethyl sterols indicates that C-4 demethylations may be carried out along an alternate or abnormal pathway. It has been suggested that these reactions are carried out by C-4 demethylases of the normal pathway or by an alternate demethylase system, either of which may be less sensitive to inhibition and operate only at high substrate concentrations (31). Therefore, C-4 demethylases operating in the abnormal pathway can affect the conversion rate and quantities of the methyl and dimethyl sterols. Consequently, whether or not the C-14 methyl sterol is recovered from treated fungi may depend on the length of the incubation period and whether the organism has the specific C-4 demethylase to convert obtusifoliol to a C-4 desmethyl sterol.

The mixed function oxidases and their corresponding electron carriers are involved in the various sterol demethylation reactions. The sterol-inhibiting fungicides do not appear to affect general, mixed-function, oxidase activity. Instead, certain inhibitors are selective for a reaction and/or enzyme in the C-14 demethylation sequence (31).

A desmethyl sterol ($\Delta^{5,7}$-ergostadienol) was recovered in fungi treated with triarimol and the analogous compounds, nuarimol and fenarimol (2,10,21). Because all other desmethyl sterols were depleted by conversion to ergosterol, it would suggest that these compounds prevent the conversion of the $\Delta^{5,7}$ sterol to a sterol containing a C-22(23) double bond. Reduction of the C-24(28) double bond is linked to C-22(23) dehydrogenation; therefore, the former reaction also does not occur in fungi treated with the pyrimidine fungicides. Although the reductions of the C-22(24) double bond and of the 24-methylene group have been identified as reactions of high sensitivity, it is possible that the inhibition of these two reactions is a secondary effect of the inhibition of sterol C-14α demethylation (22).

Dodecylimidazole has also been reported to have more than one mechanism of action within the sterol biosynthetic pathway (15). The mechanisms of action depend on concentration. At 0.1–0.25 μg/ml, the toxicant inhibits C-14 demethylation in sporidia of *U. maydis*; at 1.0 μg and above, it inhibits 2,3 oxidosqualene cyclization and subsequent transmethylation. As a result of the latter mode of action, squalene, 2,3 oxidosqualene, and lanosterol accumulate in sporidia instead of C-4 methyl and dimethyl sterols.

Neither tridemorph nor azasterol inhibits C-14 demethylation. Fungi treated with azasterol accumulate the desmethyl sterol $\Delta^{8,14}$-ergostadienol (35). Demethylation of the C-14 group results in a sterol containing a C-14(15) double bond. C-4 demethylation can proceed even though the C-14(15) double bond is not reduced. Because the $\Delta^{8,14}$ desmethyl sterol accumulates, the azasterol apparently inhibits the C-14(15) reductase enzyme. No further reactions—such as insertion of a double bond at C-22(23), or a double-bond shift from C-8(9) to C-7(8) and insertion of a doublebond at C-5(6)—can take place unless the C-14(15) bond is reduced.

The desmethyl sterols recovered from fungi treated with tridemorph were fecosterol, $\Delta^{8,22,24(28)}$-ergostatrienol, and $\Delta^{8,22}$-ergostadienol (17). Because all the accumulated sterols contain the Δ^{8} double bond, tridemorph can be considered to block $\Delta^{8} \rightarrow \Delta^{7}$ isomerization between fecosterol and episterol (Fig. 1, reaction 3). The accumulation of the $\Delta^{8,22,24(28)}$ and $\Delta^{8,22}$ desmethyl sterols indicates that the alternate reactions take place in the side chain because of the inhibition of the $\Delta^{8} \rightarrow \Delta^{7}$ isomerase. Trifluperidol, a hypochloesteremic agent, also inhibits ergosterol biosynthesis in yeast, prevents $\Delta^{8} \rightarrow \Delta^{7}$ isomerization, and causes accumulation of desmethyl sterols similar to those reported for tridemorph (32).

Sterol carrier proteins bind the water insoluble sterol intermediates, enabling enzymatic conversion; aid in activation of microsomal enzyme reactions of the sterol intermediates; and transfer the sterol to intercellular sites. It has been suggested that inhibitors of ergosterol biosynthesis bind to the carrier proteins and prevent microsomal-mediated C-14 demethylation and other enzyme interactions with the sterol carrier protein-sterol complex (15,23,31).

Table 2. Effect of inhibitors of ergosterol biosynthesis on the accumulation of sterol intermediates

	Sterol intermediates[a]			
Compound	C-4 Dimethyl	C-4 Methyl	C-4 Desmethyl (C-14 methyl)	Other desmethyl
Triforine	+	+	+	
Triarimol	+	+	+	+
Nuarimol	+	+	+	+
Fenarimol	+	+	+	+
Miconazole	+	+	+	
Imazalil	+	+	+	
Triadimefon	+	+		
Fluotrimazole	+	+		
Clotrimazole	+	+		
Dodecylimidazole	+	+		
Buthiobate	+	+		
Tridemorph				+
Azasterol				+

[a]Sterol intermediates named in text; + indicates accumulation.

Effect on gibberellin activity. Triarimol and the analogous compounds ancymidol (a growth retardant) (29), nuarimol and fenarimol (4), and triadimefon and imazalil (4) have been reported to retard plant growth. This growth effect is annulled by the addition of gibberellin. Because these sterol-inhibiting fungicides act systemically, antigibberellin activity in plants may be an important consideration in their use as fungicides. The initial stages of gibberellin synthesis, like sterol biosynthesis, involve a portion of the isoprenoid pathway. In addition, many of the reactions, particularly cyclizations and oxidations that occur in the latter stages of sterol synthesis, are similar to those stages of gibberellin biosynthesis (23,31).

Ancymidol has been shown to affect the latter stages of the gibberellin biosynthetic pathway inhibiting the conversion of kaurene to kaurenol (7). In this reaction sequence, the C-4α methyl group of kaurene undergoes oxidative demethylation in a manner similar to sterol demethylation. This inhibition of demethylation presumably would explain the similarities in the toxic action of certain sterol-inhibiting fungicides in ergosterol and gibberellin biosynthesis.

RESISTANCE

Although the development of mutants resistant to inhibitors of ergosterol biosynthesis is common in the laboratory, the emergence of mutants in the field has yet to be reported. Dekker (8) has described the possible mechanisms of resistance as decreased permeability, metabolism (increase of detoxification or decrease of conversion into a fungitoxic compound), decreased affinity at the site of action, circumvention of the site of action, and compensation. The resistance mechanism involving affinity for the site appears to be most common with the specific-site inhibitors (8). However, this mechanism has not as yet been demonstrated with the inhibitors of ergosterol biosynthesis. Decreased permeability (via increased efflux of the fungicide) and metabolism (via decreased conversion to the fungitoxic compound) have been reported for fenarimol (11) and triadimefon (14), respectively.

Resistance to the sterol-inhibitors appears to be multigenic because the levels of fungicide required to inhibit growth of the mutants vary appreciably (1). Genetic analysis showed that resistance to imazalil is multigenic (34). Multigenic resistance could imply either multiple sites of action within the ergosterol biosynthetic pathway (as in pyrimidine fungicides) and/or more than one mechanism of resistance.

In addition, multigenic resistance may affect cellular processes, thus reducing overall fitness of the mutants. This implies that the mutants, because of diminished growth and reproduction, are not as pathogenic as the wild type strain under field conditions. Reduced overall fitness of mutants has been suggested as a reason for the lack of field resistance to triforine and triarimol (13).

There appears to be a precise structural and configurational requirement for sterols to be effective in membranes. In higher fungi, ergosterol possesses these requirements, which allow for the optimum activity of membranes. Fungal mutants resistant to the polyene antibiotics exhibit altered sterol patterns, and these sterols are considered to be biogenetically more primitive than ergosterol (12). It was suggested that increased resistance to the polyenes, as related to altered sterol patterns, is linked to decreased fitness of the mutants (9). However, this mechanism may not apply to the sterol-inhibiting fungicides because mutants of species of *Aspergillus* resistant to the pyrimidine fungicides were shown not to have altered sterol patterns (24,28). It is evident that the biochemical mechanisms for increased resistance as well as reduced fitness of the mutants need further clarification.

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