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INHIBITION OF ERGOSTEROL BIOSYNTHESIS IN *SACCHAROMYCES CEREVISIAE* AND *USTILAGO MAYDIS* BY TRIDEMORPH, FENPROPIMORPH AND FENPROPIDIN

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Key Word Index—*Saccharomyces cerevisiae*, Saccharomycetacae, *Ustilago maydis*, Ustilaginaceae, sterol biosynthesis, sterol Δ^{14}-reductase, sterol $\Delta^{8} \rightarrow \Delta^{7}$-isomerase, fungicides, ergosterol

Abstract—The structurally related fungicides, tridemorph, fenpropimorph and fenpropidin have been shown to inhibit the sterol Δ^{14}-reductase and $\Delta^{8} \rightarrow \Delta^{7}$-isomerase during ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* However, although the three fungicides are able to inhibit both enzymes, tridemorph inhibits the Δ^{8}-Δ^{7}-isomerase better than the Δ^{14}-reductase whilst the reverse is true for fenpropidin and to a lesser extent for fenpropimorph

INTRODUCTION

Tridemorph [1] (2,6-dimethyl-*N*-tridecylmorpholine) and fenpropimorph [2] (*N*-[3-(*p-tert*-butylphenyl)-2-methylpropyl]-*cis*-2,6-dimethylmorpholine) (Fig 1) are important agricultural fungicides that are particularly useful against powdery mildews Fenpropidin (*N*-[3-(*p-tert*-butylphenyl)-2-methylpropyl]-piperidine) (Fig 1) is a related compound with similar activity [2] The prime cause of their fungitoxicity appears to be their inhibition of ergosterol biosynthesis which leads to the formation of abnormal mycelial membranes However, there is still a degree of uncertainty about the step in the ergosterol biosynthetic pathway that they inhibit Kato *et al* [3] showed that tridemorph at a concentration of 10 μM caused the accumulation of fecosterol and smaller amounts of two other Δ^{8}-sterols at the expense of ergosterol in *Botrytis cinerea* This suggested that the sterol $\Delta^{8} \rightarrow \Delta^{7}$-isomerase had been inhibited, thereby preventing the conversion of fecosterol into episterol Kerkenaar *et al* [4] then found that *Ustilago maydis* grown in the presence of tridemorph accumulated ignosterol and smaller amounts of three other sterols that also appeared to possess the $\Delta^{8,14}$-dienoid system This indicated that tridemorph inhibited the sterol Δ^{14}-reductase, the enzyme that catalyses the reduction of the Δ^{14}-double bond introduced during 14α-demethylation The picture was further complicated by the more recent observations of Leroux and Gredt [5] and Berg *et al* [6] The former workers found that tridemorph, fenpropimorph and fenpropidin caused the accumulation of fecosterol and ergosta-8,22,(24)28-trienol in *B cinerea* and *Penicillium expansum* and of fecosterol and ergost-8-enol in *S cerevisiae* and *U maydis*, indicating that they inhibit the $\Delta^{8} \rightarrow \Delta^{7}$-isomerase but not the Δ^{14}-reductase The latter group found that tridemorph caused the accumulation of zymosterol, fecosterol and stigmasta-8,24(28)-dienol in *Saprolegnia ferax* again indicating that it inhibits only the $\Delta^{8} \rightarrow \Delta^{7}$-isomerase

This paper reports the results of our investigations into the inhibition of ergosterol biosynthesis by tridemorph, fenpropimorph and fenpropidin in *S cerevisiae* and *U maydis* which we believe show that these fungicides inhibit both the $\Delta^{8} \rightarrow \Delta^{7}$-isomerase and the Δ^{14}-reductase, but to different degrees

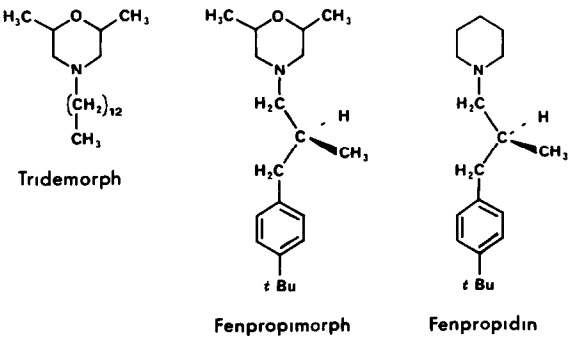

Fig. 1 Structures of the fungicides (fenpropimorph is shown as the more active *cis*-(−)-*S*-enantiomer although it is used as the (±)-*RS*-mixture, fenpropidin is drawn with the same stereochemistry by analogy)

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Abbreviations The trivial names of the sterols used in the text have the following systematic names ergosterol = ergosta-5,7,22-trien-3β-ol, fecosterol = 5α-ergosta-8,24(28)-dien-3β-ol, episterol = 5α-ergosta-7,24(28)-dien-3β-ol, ignosterol = 5α-ergosta-8,14-dien-3β-ol, zymosterol = 5α-cholesta-8,24-dien-3β-ol, lanosterol = 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol, 24,25-dihydrolanosterol = 4,4,14α-trimethyl-5α-cholesta-8-en-3β-ol

RESULTS

Effect of the fungicides on S cerevisiae (*N C Y C* 739)

Batches of yeast were grown for 18 hr in the presence of 10 μM tridemorph, 1 μM fenpropimorph or 10 μM fenpropidin or in the absence of fungicide (control) These fungicide concentrations were chosen because previous experimentation had shown that they would cause an approximately 50% inhibition of growth, based upon cell dry wt measurements, relative to the control over the culture period The sterols were then extracted and separated by TLC into 4,4-dimethyl-, 4α-methyl- and 4-demethyl sterol classes The 4α-methyl sterol class was relatively minor and was not examined further The 4,4-dimethyl- and 4-demethyl sterols were then analysed by UV spectroscopy, GC and GC/MS Table 1 gives the relative proportions of the sterols identified expressed as percentages of the total sterol and the sterol class

The 4,4-dimethylsterols of the control yeast cells were made up of lanosterol, a small amount of 24,25-dihydrolanosterol and a larger amount of 4,4-dimethylcholesta-8,24-dienol However in the yeast cells grown in the presence of all three fungicides lanosterol and 24,25-dihydrolanosterol were not detected and were replaced by the $\Delta^{8\,14}$-sterols, 4,4-dimethylcholesta-8,14-dienol and 4,4-dimethylcholesta-8,14,24-trienol This qualitative change was accompanied by a marked quantitative change in the fenpropimorph and fenpropidin-treated yeast where the 4,4-dimethylsterols increased from 23% of the total sterol (control) to 31 2% and 44 9% respectively, this change was not seen in the tridemorph-treated yeast where the 4,4-dimethylsterols constituted 19% of the total

The 4-demethylsterols of the control yeast cells were made up of ergosterol, zymosterol, ergosta-5,7,22,24(28)-tetraenol, episterol and a sterol that was identified as fecosterol from its RR_t, its mass spectrum (particularly the presence of a fragment ion at m/z 314 $[M-84]^+$ which indicates the presence of a 24-methylene side chain) and the absence of UV absorption in the 245–250 nm region The 4-demethylsterols of yeast grown in the presence of tridemorph and fenpropimorph were made up of very small amounts of ergosterol and zymosterol along with one major component with a RR_t identical to that of fecosterol The fenpropidin 4-demethylsterols showed a slight variation from this in that this major component was accompanied by significant quantities of a sterol with a RR_t of 1 14 The major component of the fungicide-treated yeast 4-demethylsterols was identified as being a mixture of fecosterol and ignosterol for the following reasons (i) both sterols have an RR_t of 1 15 on the GC column used—moreover it is known that fecosterol and ignosterol do not separate from one another either as the free sterols, their acetates or their TMSi-derivatives on most GC liquid phases, (ii) GC/MS of the 4-demethyl sterol fractions gives a mass spectrum of the RR_t 1 15 peak that has a molecular ion at m/z 398 and a fragment ion at m/z 314 which indicates the presence of fecosterol but does not rule out the presence of ignosterol whose mass spectrum contains only ions that are also seen in the mass spectrum of fecosterol, (iii) the UV spectrum of the 4-demethylsterol fraction has a pronounced peak at 250 nm with a shoulder at 245 nm which is characteristic of $\Delta^{8\,14}$-sterols [7] and which is therefore exhibited by ignosterol [8], (iv) the 250 nm peak in the UV spectrum of the 4-demethyl sterol fraction must be solely due to the RR_t 1 15 peak in the case of the tridemorph and fenpropimorph-treated yeast because the only other sterols present are ergosterol and zymosterol which are not $\Delta^{8,14}$-sterols (in the 4-demethylsterol fraction from fenpropidin-treated yeast the sterol with the RR_t of 1 14, identified by its mass spectrum as ergosta-8,14,22-trienol, also contributes to the 250 nm peak in the UV spectrum) Table 2 gives the results of calculations aimed at determining what proportions of the GC RR_t 1 15 peak are constituted by fecosterol and ignosterol respectively in each of the three fungicide treatments These calculations are based on (i) knowledge of the quantity of sterol represented by each RR_t 1 15 peak, (ii) estimation of the quantity of $\Delta^{8\,14}$-sterol in each 4-demethylsterol fraction from its UV spectrum and (iii) knowledge that all the $\Delta^{8,14}$-sterols are in the GC RR_t 1 15 peak in the case of the tridemorph and fenpropimorph treatments and in the GC RR_t 1 14 and 1 15 peaks in the fenpropidin treatments They show that the GC RR_t 1 15 peak from the tridemorph treatment is an approximately 1 1 mixture of fecosterol and ignosterol whilst those from the fenpropimorph and fenpropidin treatments are roughly 19 1 and 49 1 respectively in favour of ignosterol

Effect of the fungicides on U maydis

Control and fungicide-treated batches of *U maydis* were cultured and analysed similarly Table 3 gives the relative proportions of the 4-demethylsterols identified Those from the control consisted of ergosterol and slightly smaller amounts of ergost-7-enol and ergosta-7,22-dienol, neither fecosterol nor any other Δ^8-sterol was detected

The 4-demethylsterols from the tridemorph-treated culture consisted mainly of ergost-8-enol, ergosta-8,22-dienol and a sterol which, from its RR_t of 1 15 and its M^+ at m/z 398 could have been fecosterol, ignosterol or a mixture of both However, since the UV spectrum of these sterols showed the presence of trace amounts of $\Delta^{5\,7}$-sterol (λ_{max} 293, 282 and 272 nm) and the complete absence of $\Delta^{8,14}$-sterol (no λ_{max} 250 nm), this peak was identified as fecosterol Its mass spectrum confirmed this, with a fragment ion at m/z 314 ($[M-84]^+$) caused by cleavage allylic to the 24-methylene group

The 4-demethylsterols of the fenpropimorph and fenpropidin-treated cultures also included a sterol with an RR_t of 1 15 This sterol was however identified, in both cases, as being ignosterol rather than fecosterol because, (i) the UV spectrum showed a very pronounced absorption peak at 250 nm (as well as minor absorption maxima at 293, 282 and 272 nm due to trace amounts of $\Delta^{5\,7}$-sterol) and (ii) the mass spectrum was characteristic of ignosterol and devoid of any of the fragmentations peculiar to fecosterol, in particular that at m/z 314 In addition to ignosterol the fenpropimorph-treated culture contained a considerable quantity of ergost-8-enol, relatively small amounts of ergosta-8,22-dienol and ergost-7-enol and traces of ergosterol whilst the fenpropidin-treated culture contained only small amounts of ergost-8-enol and ergost-7-enol and traces of ergosterol

DISCUSSION

All three of the fungicides markedly block the formation of ergosterol in yeast and *U maydis* and cause the

Table 1 Relative amounts of identified sterols in control and fungicide inhibited yeast cultures

Sterol class	Sterol	RR_t^1	$[M]^+$	% of total sterol				% of sterol class			
				Con[2]	TM[3]	FM[4]	FP[5]	Con[2]	TM[3]	FM[4]	FP[5]
4,4-Dimethyl	24,25-Dihydrolanosterol	1 21	428	1 6	n d	n d	n d	6 6	n d	n d	n d
	4,4-Dimethylcholesta-8,14-dienol	1 22	412	n d	4 1	6 5	3 3	n d	18 1	15 8	6 7
	Lanosterol	1 25	426	3 8	n d	n d	n d	15 9	n d	n d	n d
	4,4-Dimethylcholesta-8,14,24-trienol	1 26	410	n d	5 8	11 4	26 6	n d	25 4	32 2	54 2
	4,4-Dimethylcholesta-8,24-dienol	1 27	412	17 6	10 1	14 2	15 0	75 5	44 5	40 4	30 6
4-Demethyl	Zymosterol	1 06	384	19 0	4 6	4 9	4 3	24 9	5 9	7 5	8 5
	Ergosterol	1 10	396	23 8	1 5	1 7	2 4	31 3	2 0	2 6	4 7
	Ergosta-5,7,22,24(28)-tetraenol	1 12	394	13 0	n d	n d	n d	17 1	n d	n d	n d
	Ergosta-8,14,22-trienol	1 14	396	n d	n d	n d	16 4	n d	n d	n d	32 1
	Fecosterol and/or Ignosterol	1 15	398	12 0[6]	60 5[7]	46 3[7]	20 8[8]	15 8[6]	78 4[7]	71 4[7]	40 3[8]
	Episterol	1 18	398	4 6	n d	n d	tr	6 1	n d	n d	tr

1, Retention time relative to cholesterol, 2, control, 3, tridemorph, 4, fenpropimorph, 5, fenpropidin, 6, fecosterol only because the UV spectrum showed no evidence of absorption at 250 nm characteristic of ignosterol, 7, a mixture of ignosterol and fecosterol because the UV spectrum had a strong absorption peak at 250 nm and the mass spectrum had a fragment ion at m/z 314 typical of fecosterol caused by cleavage allylic to the 24-methylene group, 8, largely ignosterol because the UV spectrum had a strong absorption peak at 250 nm whilst the mass spectrum had only a barely discernible fragment ion at m/z 314 (see Table 2), n d = not detected, tr = trace

Table 2 Estimated quantities and proportions of ignosterol and fecosterol in the GLC peak (RR_t = 1 15) from control and inhibited yeast cultures

	Control	TM[1]	FM[2]	FP[3]
μg of sterol in the GLC peak RR_t = 1 15[4,5]	86	52 1	62 6	51 4
μg of $\Delta^{8,14}$-sterols in 4-demethylsterols[6,7]	0	25 2	59 7	50 4
μg of fecosterol[8]	86	26 9	2 9	1 0
% of ignosterol in GLC peak RR_t = 1 15	0	48 4	95 4	98 1
% of fecosterol in GLC peak RR_t = 1 15	100	51 6	4 6	1 9

1, Tridemorph-treated, 2, fenpropimorph-treated, 3, fenpropidin-treated, 4, calculated from its percentage in the 4-demethylsterols, 5, in the case of fenpropidin-treated cells the GLC peak RR_t = 1 14 is included because it is a $\Delta^{8,14}$-sterol, 6, calculated from the absorption spectra of the 4-demethylsterol fractions on the assumption that the molar absorption coefficient of $\Delta^{8,14}$-sterols is 18000 at 250 nm (see refs [12, 13]) and that the MW of the 4-demethyl-$\Delta^{8,14}$-sterols is 398, 7, this must be the wt of ignosterol (plus ergosta-8,14,22-trienol in the case of FP-treated yeast) because ignosterol is the only $\Delta^{8,14}$-sterol present in the 4-demethyl sterols, 8, obtained by subtraction of the wt of $\Delta^{8\ 14}$-sterol, estimated by the UV spectroscopy, from the wt of sterol corresponding to GLC peak RR_t = 1 15

Table 3 Relative amounts of identified 4-demethylsterols in control and fungicide-treated cultures of *U maydis*

			% of sterol			
Sterol	RR_t	$[M]^+$	Con[1]	TM[2]	FM[3]	FP[4]
Ergosta-8,22-dienol	1 07	398	n d	21 4	9 8	n d
Ergosterol	1 10	396	39 4	tr	tr	tr
Ergosta-7,22-dienol	1 11	398	28 0	n d	n d	n d
Fecosterol and/or Ignosterol	1 15	398	n d	26 9[5]	29 2[6]	85 4[6]
Ergost-8-enol	1 16	400	n d	51 7	41 8	8 5
Ergost-7-enol	1 19	400	30 2	n d	6 2	6 1

1, Control, 2, tridemorph-treated, 3, fenpropimorph-treated, 4, fenpropidin-treated, 5, fecosterol only because the UV spectrum shows no evidence of absorption at 250 nm characteristic of ignosterol, 6, ignosterol only because the UV spectrum has a strong absorption peak at 250 nm whilst the mass spectrum has no fragment ion at m/z 314, n d = not detected, tr = trace

accumulation of sterols that fall into two main categories, those with a $\Delta^{8,14}$-dienoid system and those with a single nuclear double bond This indicates that the sterol Δ^{14}-reductase and the sterol $\Delta^8 \rightarrow \Delta^7$-isomerase are being inhibited

The steps catalysed by these two enzymes are shown in Fig 2 which depicts the preferred pathways between lanosterol and ergosterol in yeast (thick, open arrows) and *U maydis* (thick, black arrows) Thus inhibition of the Δ^{14}-reductase would block the steps marked a in Fig 2 whilst inhibition of the $\Delta^8 \rightarrow \Delta^7$-isomerase would block that marked b Because of the lack of absolute substrate specificity of many of the enzymes involved in the lanosterol to ergosterol pathway the blocking of individual steps such as a and/or b causes the accumulation, not only of the sterol immediately preceding the blockage, but also of sterols derived from it that are not on the normal biosynthetic route, such sterols are termed abnormal sterols in Fig 2

Inhibition of the Δ^{14}-reductase by the three fungicides in yeast is indicated by the accumulation of 4,4-dimethylcholesta-8,14,24-trienol (**3**) along with 4,4-dimethylcholesta-8,14-dienol (**16**), ergosta-8,14,22-trienol (**17**) (fenpropidin treatment only) and ignosterol (**18**) The 4,4-dimethylcholesta-8,14-dienol is presumably formed from 4,4-dimethylcholesta-8,14,24-trienol by saturation of the Δ^{24}-double bond in a manner analogous to the formation of 24,24-dihydrolanosterol from lanosterol as occurs in the control The ergosta-8,14,22-trienol and ignosterol are presumably formed from 4,4-dimethylcholesta-8,14,24-trienol as a result of the lack of absolute substrate specificity of the enzymes catalysing the demethylations at C-4, 24-methylation, 22,23-dehydrogenation and 24(28)-reduction It is evident from the different proportions of total $\Delta^{8,14}$-sterol accumulating that the three fungicides do not inhibit the Δ^{14}-reductase in yeast equally well Tridemorph is clearly the weakest of the three since only about 40% of the total sterol present is $\Delta^{8,14}$-sterol in contrast to the > 60% in the fenpropimorph and fenpropidin-treated cultures

Inhibition of the $\Delta^8 \rightarrow \Delta^7$-isomerase by the three fungicides in yeast is indicated by the accumulation of fecosterol (**10**, Fig 2) This is only marked in the case of tridemorph where fecosterol accounts for about 30% of the total sterol present in contrast to the 2% or less found in the fenpropimorph and fenpropidin-treated yeast However, the low levels of fecosterol accumulated in the latter does not necessarily indicate that fenpropimorph

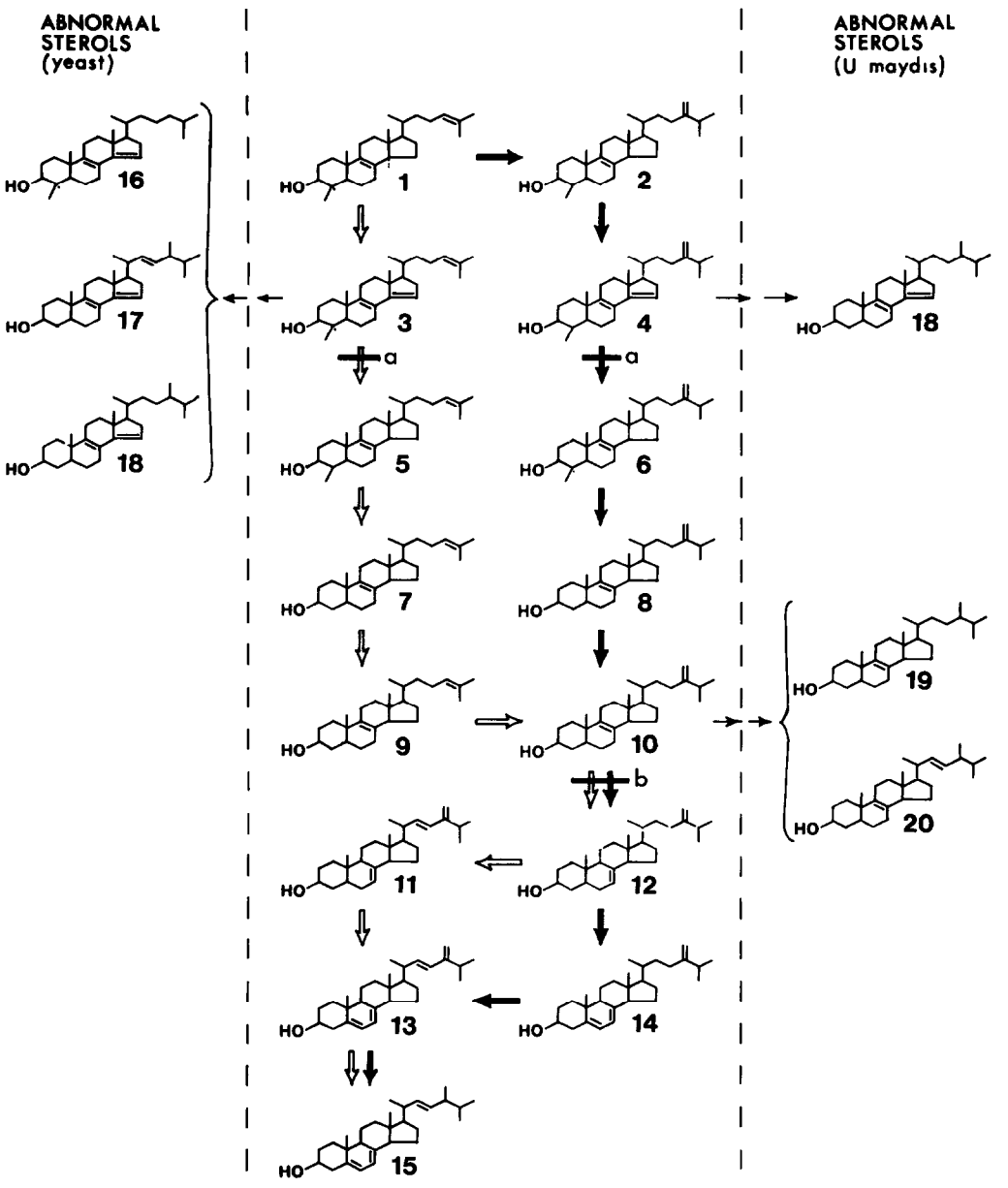

Fig 2 Probable preferred reaction sequences involved in the conversion of lanosterol into ergosterol in *Saccharomyces cerevisiae* and *Ustilago maydis* (**1** = lanosterol, **2** = 24-methylene-24,25-dihydrolanosterol, **3** = 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, **4** = 4,4-dimethyl-5α-ergosta-8,14,24(28)-trien-3β-ol, **5** = 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol, **6** = 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol, **7** = 4α-methyl-5α-cholesta-8,24-dien-3β-ol, **8** = 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol, **9** = zymosterol, **10** = fecosterol, **11** = 5α-ergosta-7,22,24(28)-trien-3β-ol, **12** = episterol, **13** = ergosta-5,7,22,24(28)-tetraen-3β-ol, **14** = ergosta-5,7,24(28)-trien-3β-ol, **15** = ergosterol, **16** = 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol, **17** = 5α-ergosta-8,14,22-trien-3β-ol, **18** = ignosterol, **19** = 5α-ergosta-8-en-3β-ol, **20** = 5α-ergosta-8,22-dien-3β-ol) ⇨, reactions involved in the probable preferred pathway in *Saccharomyces cerevisiae*, ➡, reactions involved in the probable preferred pathway in *Ustilago maydis*, → ,reactions resulting from blockage of the preferred pathways by the fungicides

and fenpropidin are poor inhibitors of the $\Delta^8 \rightarrow \Delta^7$-isomerase They could result from an over-efficient blockage of the Δ^{14}-reductase by the particular concentration of fungicide used allowing relatively little of the normal biosynthetic intermediates to proceed through to fecosterol

In *U maydis* inhibition of the Δ^{14}-reductase is indicated by the accumulation of ignosterol (**18**) which is only seen in the fenpropimorph and fenpropidin-treated cultures This presumably arises from 4,4-dimethylergosta-8,14,24(28)-trienol (**4**) as a result of the lack of absolute substrate specificity of the enzymes catalysing the 4-demethylation and $\Delta^{24(28)}$ reduction steps Indeed evidence from GC/MS and UV absorption at 250 nm has indicated the presence of small amounts of 4,4-dimethylergosta-8,14,24(28)-trienol in the sterols of

fenpropimorph-treated *U maydis* Fenpropidin appears to be a far more potent inhibitor of the Δ^{14}-reductase than fenpropimorph since ignosterol constituted over 80% of the sterols in *U maydis* treated with it in contrast to about 30% in the sterols of *U maydis* treated with fenpropimorph

Inhibition of the $\Delta^8 \rightarrow \Delta^7$-isomerase in *U maydis* by the three fungicides is indicated by the accumulation of fecosterol (**10**), ergost-8-enol (**19**) and ergosta-8,22-dienol (**20**) in tridemorph-treated cultures, of ergost-8-enol and ergosta-8,22-dienol in fenpropimorph-treated cultures and of ergost-8-enol and in fenpropidin-treated cultures It would appear that tridemorph is the most potent and fenpropidin the least potent of the three fungicides as inhibitors of this enzyme in *U maydis* as judged by the percentage of Δ^8-sterols in the 4-demethylsterols of the three treatments (i e tridemorph, 100%, fenpropimorph, 51 6%, fenpropidin, 8 5%) However, too much weight should not be put upon this interpretation for the reason mentioned earlier, namely that the efficiency of inhibition of the Δ^{14}-reductase will have a bearing on the apparent efficiency of inhibition of the $\Delta^8 \rightarrow \Delta^7$-isomerase since the former precedes it in the biosynthetic sequence

The following conclusions can be drawn from the foregoing observations, (i) tridemorph inhibits the $\Delta^8 \rightarrow \Delta^7$-isomerase well in both fungi and inhibits the Δ^{14}-reductase in yeast, (ii) fenpropimorph inhibits the Δ^{14}-reductase well in both fungi and inhibits the $\Delta^8 \rightarrow \Delta^7$-isomerase in *U maydis* and probably in yeast and (iii) fenpropidin inhibits the Δ^{14}-reductase well in both fungi and inhibits the $\Delta^8 \rightarrow \Delta^7$-isomerase in *U maydis* and possibly in yeast

The question now arises as to why this should be so A possible clue to the answer to this question lies in the structures of the fungicides They fall into two overlapping pairs, namely (i) tridemorph and fenpropimorph, which have 2,6-dimethylmorpholine rings but different *N*-substituents on them and (ii) fenpropimorph and fenpropidin, which have different *N*-containing ring systems but identical *N*-substituents (i e the 3-(*p-tert*-butylphenyl)-2-methylpropane residue) The fact that the fungicides of pair (ii) inhibit the Δ^{14}-reductase better than the $\Delta^8 \rightarrow \Delta^7$-isomerase suggests that Δ^{14}-reductase inhibitory activity resides mainly in the *N*-substituent rather than the *N*-ring system Conversely the fact that tridemorph inhibits the $\Delta^8 \rightarrow \Delta^7$-isomerase better than the Δ^{14}-reductase suggests that the $\Delta^8 \rightarrow \Delta^7$-isomerase activity resides in the 2,6-dimethylmorpholine ring rather than the tridecyl *N*-substituent This is supported by the experimental observations that are consistent with, though not proof of, fenpropimorph being a better inhibitor of the $\Delta^8 \rightarrow \Delta^7$-isomerase than fenpropidin These suggestions being correct, one can surmise that the *N*-heterocycle of the fungicides positions itself on the active site of the $\Delta^8 \rightarrow \Delta^7$-isomerase on the place that is normally taken up by ring B of fecosterol and thereby blocks the isomerization reaction The difference in inhibitory potency between tridemorph and fenpropimorph on the one hand and fenpropidin on the other would then be due to the more efficient binding of the 2,6-dimethylmorpholine ring system than the piperidine ring system to the sterol ring B enzyme site One can further surmise that the *N*-heterocycle of the fungicides also positions itself on the active site of the Δ^{14}-reductase on the place normally taken up by ring B of the $\Delta^{8,14}$-sterol (normally 4,4-dimethylcholesta-8,14,24-trienol in yeast and 4,4-dimethylergosta-8,14,24(28)-trienol in *U maydis*) and that the *N*-substituent distributes itself along the region normally occupied by rings C and D and the side chain thereby blocking reduction of the Δ^{14}-double bond The difference in inhibitory potency between fenpropimorph and fenpropidin on the one hand and tridemorph on the other would then be due to the better fit and more efficient binding of the *N*-substituent to the sterol 'rings C and D plus side chain' enzyme site

The relationship of the *N*-heterocyclic ring of these fungicides to ring B of the sterol nucleus is further indicated by the finding that tridemorph [9, 10] and fenpropimorph [Wang, Z-X, Khalil, I A and Mercer, E I, unpublished observations, Benveniste, P, personal communication] inhibit the enzyme catalysing the opening of the 9β,19-cyclopropyl ring in higher plant sterol biosynthesis On the other hand the importance of a good structural fit between their *N*-substituents and the sterol rings C, D and side chain is highlighted by a number of observations of Himmele and Pommer [2] They reduced the phenyl group in fenpropimorph and fenpropidin and separated the *cis*- and *trans*-1,4-cyclohexyl isomers of each They found that the *trans*-1,4-isomers were much more potent fungicides, and presumably sterol Δ^{14}-reductase inhibitors, than the *cis*-1,4-isomers The *trans*-1,4-isomers have an essentially planar structure that matches the sterol rings C, D and side chain well in contrast to the bent structure of the *cis*-1,4-isomers which does not They also showed that the (−)-enantiomer of fenpropimorph, which has the *S*-configuration at C-2 of the *N*-substituent, is a far better fungicide than the (+)-*R*-enantiomer This implies that the C-2 methyl group is a recognition feature of the molecules, only matching features of the sterol C, D rings and side chain when it has the correct configuration

EXPERIMENTAL

Organisms and growth conditions *Saccharomyces cerevisiae* (NCYC 739, high sterol strain) was grown in 40 ml batches for 18 hr at 28–30° in an orbital shaker rotating at 160 rpm in a medium containing per litre, $(NH_4)_2SO_4$ (2 g), KH_2PO_4 (2 g), Na_2HPO_4 (0 5 g), $MgSO_4\ 7H_2O$ (0 25 g), $MnSO_4\ 4H_2O$ (0 025 g), D-glucose (20 g), yeast extract (Difco) (1 g) and the appropriate quantity of tridemorph, fenpropimorph or fenpropidin The fungicides were added in Me_2CO soln so as to give concentration ranges of 0 5–100 μM, the concentration of Me_2CO in the fungicide-inhibited and control media was 0 5% The cells were then harvested by centrifugation at 2000 *g*, washed twice with H_2O, freeze dried and the dry wt determined From the latter the % inhibition, relative to the control, was determined for each fungicide concn It was found that 1 μM fenpropimorph gave a 54% inhibition of growth whilst 10 μM tridemorph and fenpropidin gave 49% and 60% inhibitions, respectively These cells, inhibited *ca* 50%, were then analysed for their sterol content

Ustilago maydis (DC) Cda, ATCC 14826 was grown on Coursen and Sisler's nutrient medium [14] supplemented with yeast extract (Oxoid) in 40 ml batches for 18 hr at 25° in an orbital shaker rotating at 160 rpm The fungicides were added in the same way as for yeast It was found that 4 μM tridemorph gave 36% inhibition of growth whilst 0 8 μM fenpropidin and 0 08% fenpropimorph give 58% and 53% inhibition respectively These cells, inhibited *ca* 50%, were analysed for their sterol content

Sterol extraction and analysis The freeze-dried cells were saponified in ethanolic KOH containing 0 25% pyrogallol as an

Table 4 Ionic species in the mass spectra of sterols in control and treated *S cerevisiae* and *U maydis* cultures

	4-Demethyl sterols											4,4-Dimethyl sterols				
RR_t^1	1 06[2]	1 07[3]	1 10[2 3]	1 11[3]	1 12[2]	1 14[2]	1 15[4]	1 15[5]	1 16[3]	1 18[3]	1 19[3]	1 21[2]	1 22[2]	1 25[2]	1 26[2]	1 27[2]
Fragmentation[6]																
$[M]^+$	384 (18)[7]	398 (20)	396 (20)	398 (10)	394 (37)	396 (55)	398 (24)	398 (30)	400 (37)	398 (6)	400 (30)	428 (10)	412 (18)	426 (35)	410 (65)	412 (24)
$[M-Me]^+$	369 (15)	383 (10)		383 (6)	379 (2)	381 (28)	383 (15)	383 (18)	385 (12)	383 (4)	385 (9)	413 (37)	397 (9)	411 (49)	395 (20)	397 (10)
$[M-H_2O]^+$		380 (2)	378 (2)	380 (2)	376 (2)									408 (30)	392 (11)	
$[M-Me-H_2O]^+$	351 (4)	365 (2)	363 (28)	365 (2)	361 (36)	363 (7)	365 (4)	365 (5)	367 (3)		367 (3)	395 (18)	379 (3)	393 (42)	377 (18)	379 (4)
$[M-43]^+$		355 (3)				353 (17)										
$[M-59]^+$			337 (10)		335 (4)											
$[M-43-H_2O]^+$						335 (8)										
$[M-84]^+$							314 (4)			314 (17)						
$[M-84-Me]^+$							299 (5)									
$[M-SC]^+$		273 (28)	271 (7)	273 (14)	271 (28)	271 (27)	273 (10)	271 (32)	273 (14)		273 (8)	315 (4)	299 (4)			
$[M-SC-2H]^+$	271 (10)	271 (32)	269 (5)	271 (36)	269 (12)	269 (12)	271 (32)			271 (95)				313 (5)	297 (5)	299 (3)
$[M-SC-H_2O]^+$		255 (16)	253 (10)	255 (12)			255 (5)		255 (6)		255 (24)					
$[M-SC-2H-H_2O]^+$			251 (5)		251 (17)					253 (10)				295 (6)		
$[M-SC-27]^+$	246 (8)	246 (38)		246 (15)			246 (7)		246 (4)		246 (6)					274 (2)
$[M-SC-42]^+$	231 (7)	231 (14)		231 (5)			231 (12)		231 (10)		231 (12)	273 (5)		273 (22)		259 (8)
$[M-SC-27-H_2O]^+$	228 (8)						228 (17)									
$[M-SC-56]^+$												259 (6)		259 (25)		
$[M-SC-42-H_2O]^+$	213 (18)	213 (18)	211 (16)	213 (8)	211 (17)		213 (20)		213 (18)	213 (8)	213 (22)	255 (3)		255 (26)		241 (8)
$[M-SC-56-H_2O]^+$												241 (8)		241 (36)		
$[M-251]^+$					143 (30)											
$[M-253]^+$			143 (38)													

1, Retention time relative to cholesterol, 2, *Saccharomyces cerevisiae*, 3, *Ustilago maydis*, 4, From control yeast and *U maydis*, tridemorph-treated yeast and *U maydis* and fenpropimorph-treated yeast, 5, From fenpropimorph-treated *U maydis* and fenpropidin-treated yeast and *U maydis*, 6, SC, sterol side chain, 27, C_2H_3 (loss of C-16 and C-17), 42, C_3H_6 (loss of C-15 to C-17), 43, C_3H_7 (loss of C-25 to C-27 in a Δ^{22}-sterol), 56, C_4H_8 (loss of C-15 to C-17 plus C-32 in a 14α-methyl sterol), 59, C_3H_7O (loss of C-1 to C-3 in a $\Delta^{5\ 7}$-sterol), 84, C_6H_{12} (loss of C-23 to C-28 in a 24-methylene sterol), $[M-251]^+$, $[M-253]^+$ give a fragment of m/z 143 which is characteristic of $\Delta^{5\ 7}$-sterols, 7, Figures in parentheses are the relative intensities of ions

antioxidant The unsaponifiable lipid from yeast cells was extracted with $CHCl_3$ and then subjected to TLC on Whatman LK6F silica gel plates using cyclohexane–EtOAc (85 15) for development Ergosterol and lanosterol were used as markers of the 4-demethylsterol zone ($R_f \sim 0.29$) and 4,4-dimethylsterol zone ($R_f \sim 0.4$) respectively These two zones were scraped off and extracted $\times 3$ with Et_2O The sterols from *U maydis* were separated similarly but using *n*-hexane–Et_2O–HOAc (8 2 1) as the developing solvent on Uniplate silica gel GF plates with a preadsorbent zone The combined sterol bands ($R_f \sim 0.3$–0.35) were eluted as described above The resulting sterols were then analysed by UV, GC and GC/MS Quantities of $\Delta^{5,7}$-sterols and $\Delta^{8\,14}$-sterols were calculated from their UV spectra in *n*-hexane on the assumption that their molar absorption coefficients were 11820 (282 nm) [11] and 18000 (250 nm) [12, 13] GC employed an SE-30, WCOT quartz capillary column 25 m × 0.2 mm, splitless injection, 280° injection port, sample vol 3 μl $CHCl_3$, N_2 1 ml/min, temp programmed from 50° (1 min) to 280° (12 5 min) to 290° (15 min) at 40°/min and 2°/min respectively, FID, 300° detector oven

GC/MS analysis by quadripole HP 5992B utilized the same GC column temperature programmed between 100° and 290° at 32°/min with He carrier gas and an ionizing potential of 70 eV The fragmentation patterns of the individual sterols are listed in Table 4 The MS of GC peak RR_t 1 15 varied with its origin as shown From the available data the following identifications were made RR_t 1 06, zymosterol, RR_t 1 07, ergosta-8,22-dienol, RR_t 1 10, ergosterol, RR_t 1 11, ergosta-7,22-dienol, RR_t 1 12, ergosta-5,7,22,24(28)-tetraenol, RR_t 1 14, ergosta-8,14,22-trienol, RR_t 1 15 from control and tridemorph-treated yeast and *U maydis* and from fenpropimorph-treated yeast, fecosterol, RR_t 1 15 from fenpropimorph-treated *U maydis* and fenpropidin-treated yeast and *U maydis*, ignosterol, RR_t 1 16, ergost-8-enol, RR_t 1 18, episterol, RR_t 1 19, ergost-7-enol, RR_t 1 21, 24,25-dihydrolanosterol, RR_t 1 22, 4,4-dimethylcholesta-8,14-dienol, RR_t 1 25, lanosterol, RR_t 1 26, 4,4-dimethylcholesta-8,14,24-trienol, RR_t 1 27, 4,4-dimethylcholesta-8,24-dienol

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