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INHIBITION OF STEROL $\Delta^8 \rightarrow \Delta^7$-ISOMERASE AND Δ^{14}-REDUCTASE BY FENPROPIMORPH, TRIDEMORPH AND FENPROPIDIN IN CELL-FREE ENZYME SYSTEMS FROM *SACCHAROMYCES CEREVISIAE*

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Key Word Index—*Saccharomyces cerevisiae*; Saccharomycetacae; sterol biosynthesis; sterol $\Delta^8 \rightarrow \Delta^7$-isomerase; sterol Δ^{14}-reductase; fungicides; morpholines.

Abstract—Enzymatic assay systems have been used to directly demonstrate the inhibition of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase and Δ^{14}-reductase during ergosterol biosynthesis in *Saccharomyces cerevisiae* by the structurally related fungicides, fenpropimorph, tridemorph and fenpropidin. Whilst tridemorph is shown to be a strong inhibitor of the $\Delta^8 \rightarrow \Delta^7$-isomerase, fenpropimorph and fenpropidin are found to be very potent inhibitors of both enzymic reactions. The dual site of action exhibited by these two fungicides predicts a lower risk of resistance development against this group of compounds.

INTRODUCTION

Fenpropimorph [1] (*N*-[3-(*p-tert*-butylphenyl)-2-methylpropyl]-*cis*-2,6-dimethylmorpholine) and tridemorph [2] (2,6-dimethyl-*N*-tridecylmorpholine) (Fig. 1) belong to an important group of ergosterol biosynthesis inhibiting fungicides (EBIs) known as the morpholines. Fenpropidin (*N*-[3-(*p-tert*-butylphenyl)-2-methylpropyl]-piperidine), though not technically a morpholine fungicide, is a related compound with similar activity [1] (Fig. 1). Concern over the reduced field performance of some sterol 14-demethylase inhibitors [3, 4], the other major group of EBIs, has increased the commercial importance of the morpholine group. For this reason interest in their mode of action has increased in recent years. At first there was some uncertainty as to the exact mode of action of this group of fungicides. Kato *et al.* [5] found that tridemorph led to the accumulation of sterols retaining the Δ^8-double bond in *Botrytis cinerea*, indicating inhibition of the sterol $\Delta^8 \rightarrow \Delta^7$-isomerase. Later Kerkenaar *et al.* [6] demonstrated that sterols possessing the $\Delta^{8,14}$-dienoid system accumulated in *Ustilago maydis* after tridemorph treatment. This indicated that tridemorph inhibited the sterol Δ^{14}-reductase.

More recent work on *Saccharomyces cerevisiae* and *Ustilago maydis* by Baloch *et al.* [7] showed that treatment with the above mentioned morpholines led to the accumulation of two main classes of sterols, those retaining the $\Delta^{8,14}$-dienoid system and those retaining a single nuclear Δ^8-double bond, suggesting that the activities of both of these enzymes are blocked. However, the three inhibitors did not block the two sites to the same extent. Fenpropimorph and fenpropidin caused a marked accumulation of $\Delta^{8,14}$-sterols and a much lesser one of Δ^8-sterols whilst the reverse was generally true of tridemorph. This showed that fenpropimorph and fenpropidin were better inhibitors of the Δ^{14}-reductase than tridemorph. It also suggested that tridemorph might be a better inhibitor of the $\Delta^8 \rightarrow \Delta^7$-isomerase than the other two fungicides. However, since the $\Delta^8 \rightarrow \Delta^7$-isomerase-catalysed reaction is preceded in the preferred ergosterol biosynthetic sequence by the Δ^{14}-reductase-catalysed reaction, this possibility could only be confirmed by testing the effect of the fungicides on the enzyme in isolation. The results of such an investigation are reported in this paper.

The relative inhibitory potencies of fenpropimorph, fenpropidin and tridemorph have been determined using two independent *in vitro* assays, one for the $\Delta^8 \rightarrow \Delta^7$-isomerase, the other for the Δ^{14}-reductase. In addition to these fungicides several isomers of fenpropimorph and fenpropidin have been assayed for their ability to inhibit the $\Delta^8 \rightarrow \Delta^7$-isomerase.

Not only are such investigations useful for determining the relative potencies of a number of inhibitors on individual enzymic steps, but they can also be used to elucidate those aspects of the fine structure of fungicidal compounds which are of greatest importance for optimal inhibitory activity.

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-β-ol; episterol = 5α-ergosta-7,24(28)-dien-3β-ol; ignosterol = 5α-ergosta-8,14-dien-3β-ol.

RESULTS

Properties of the $\Delta^8 \rightarrow \Delta^7$-*isomerase enzyme preparation*

The acetone powder extract contained $\Delta^8 \rightarrow \Delta^7$-isomerase activity. As this enzyme preparation contained

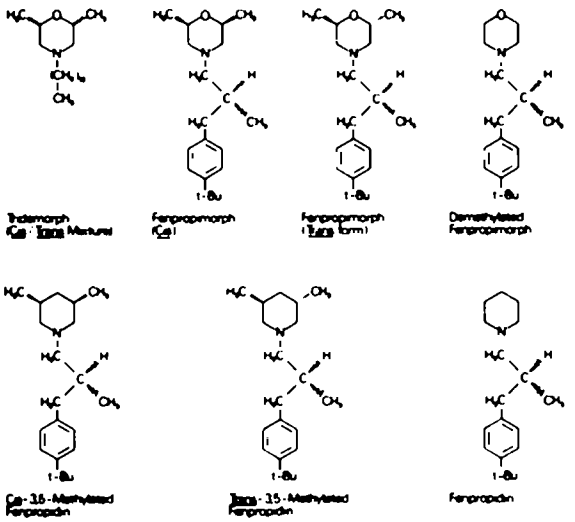

Fig. 1. Structure of fungicides and fungicide analogues.

Table 1. Inhibition of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase and sterol Δ^{14}-reductase by the fungicides fenpropimorph, fenpropidin and tridemorph

Fungicide	Sterol $\Delta^8 \rightarrow \Delta^7$-isomerase I_{50}* (μM)	Sterol Δ^{14}-reductase I_{50}* (μM)
Fenpropimorph	0.013	2.3
Fenpropidin	0.300	1.8
Tridemorph	0.033	98.0

Sterol $\Delta^8 \rightarrow \Delta^7$-isomerase: the enzyme preparation (1.985 ml) was incubated with fecosterol (20 nmol) and a range of fungicide concentrations (1 nM to 10 μM) at 30° for 3 hr.

Sterol Δ^{14}-reductase: the enzyme preparation (4.6 ml) was incubated with NADPH (1 mM), 5α-ergosta-8,14,24(28)-trien-3β-ol (0.25 μmol) and a range of fungicide concentrations (1–300 μM) at 30° for 3 hr.

*I_{50}, fungicide concentration required to reduce the percentage conversion value (of substrate to product) to half that of the control value.

very little endogenous sterol it allowed the study of $\Delta^8 \rightarrow \Delta^7$-isomerase using non-radiolabelled substrate. The reaction proceeded in a linear fashion for at least 3 hr and Michaelis Menten kinetics were followed, the apparent K_m with respect to fecosterol being 9.1 μM under the experimental conditions used.

The I_{50} values for tridemorph, fenpropimorph and fenpropidin for $\Delta^8 \rightarrow \Delta^7$-isomerase

Fecosterol (10 μM) was incubated with cell-free extract and a range of concentrations of the inhibitor under study for 3 hr at 30°. The sterols were extracted after this incubation period and analysed by GC using ergosterol as an internal standard. Both fecosterol (RR_t 1.039) and episterol (RR_t 1.073) were identified by GC/MS as detailed in the Experimental. To avoid errors due to small variations in the recovery of the sterols during the extraction procedures and variations in the volumes injected on to the GC, the percentage conversion of fecosterol to episterol were calculated rather than determining the absolute quantities of episterol produced. The percentage inhibition values were calculated as described in the Experimental.

The percentage inhibition values were plotted against fungicide concentration and the dose–response curves so obtained were used to determine the concentration of fungicide required to reduce the percentage conversion of fecosterol to episterol to half that of the control value (I_{50} value) for tridemorph, fenpropimorph and fenpropidin (Table 1). All three compounds are potent inhibitors of this enzymic conversion, fenpropidin (I_{50} 0.30 μM) was the least potent, tridemorph (I_{50} 0.033 μM) and fenpropimorph (I_{50} 0.013 μM) being 10 and almost 25 times more effective, respectively. Considering that the fecosterol concentration used in this assay (10 μM) was close to the apparent K_m (9.1 μM), the I_{50} values obtained are in the order of the inhibition constants (K_i values) [8]. This indicates that the $\Delta^8 \rightarrow \Delta^7$-isomerase has a much higher affinity (2–3 orders of magnitude) for tridemorph, fenpropimorph and fenpropidin than for its preferred substrate, fecosterol, and highlights the potency of these fungicides as inhibitors of this enzymic step.

The I_{50} values obtained (Table 1) also show that the 2,6-dimethyl morpholine moiety, which is common to both fenpropimorph and tridemorph, is more effective for the inhibition of the $\Delta^8 \rightarrow \Delta^7$-isomerase enzyme than the piperidine moiety of fenpropidin. To investigate this further and to gain more information about the structural features of the *N*-containing ring required to inhibit this enzyme, the effects of a number of fenpropimorph and fenpropidin analogues on $\Delta^8 \rightarrow \Delta^7$-isomerase activity were investigated. Analogues of fenpropimorph and fenpropidin were chosen as they have a common *N*-substituent (Fig. 1), thus any differences observed in inhibitory activity are likely to be due only to changes in the *N*-heterocyclic ring.

Structure–activity relationship of $\Delta^8 \rightarrow \Delta^7$-isomerase inhibition by fenpropimorph, fenpropidin and their analogues

The structural features considered were: (i) the importance of the presence of methyl groups at the two positions *meta* to the nitrogen of the *N*-heterocyclic rings, (ii) the configuration of these methyl groups, i.e. whether they were *cis* or *trans*, and (iii) the importance of the oxygen in the morpholine ring. The inhibitory activity of these analogues was tested at a single inhibitor concentration (0.0125 μM) close to the I_{50} value of fenpropimorph. The results obtained (Table 2) show that the methyl groups of fenpropimorph have an important influence on its inhibitory activity, as (*N*-[3-(*p*-*tert*-butylphenyl)-2-methylpropyl]-*cis*-3,5-dimethylpiperidine) (hereafter referred to as *cis*-3,5-methylated fenpropidin) gave an inhibitory effect equivalent to that of fenpropimorph itself (48.4 and 48.5%, respectively). However, the methyl groups must be in the *cis*-configuration. The *trans*-isomers of fenpropimorph (25.6% inhibition) and 3,5-methylated fenpropidin (12.1% inhibition) display inhibitory activities below those of their non-methylated analogues (36.7 and 17.6%, respectively). The role of oxygen is secondary, only becoming apparent when the

Table 2. Relative inhibition of sterol $\Delta^8 \rightarrow \Delta^7$-isomerase activity by fenpropimorph and fenpropidin analogues

Inhibitors	Percentage inhibition*
Cis-fenpropimorph	45.5
Trans-form of fenpropimorph	26.6
Demethylated fenpropimorph	36.1
Fenpropidin	17.6
Cis-3,5-methylated fenpropidin	48.4
Trans-3,5-methylated fenpropidin	12.1

The enzyme preparation (1.985 ml) was incubated with fecosterol (20 nmol) and inhibitor at a final concentration of 0.0125 nM at 30° for 3 hr.

*Percentage inhibition = percentage conversion (of fecosterol to episterol) in the control − percentage conversion in the test/percentage conversion in the control × 100.

methyl groups are either *trans* to each other or absent (see above) when the morpholine ring shows approximately double the activity of the piperidine ring in both cases.

The relative potencies of tridemorph, fenpropimorph and fenpropidin as inhibitors of the sterol Δ^{14}-reductase

A cell-free enzyme preparation from semi-anaerobically grown yeast cells was incubated with a range of inhibitor concentrations (1–300 μM) for 3 hr using [^{14}C]5α-ergosta-8,14,24(28)-trienol as substrate. The sterols from each sample were then extracted, acetylated and chromatographed on $AgNO_3$-impregnated TLC plates. Radioautography of these plates showed the radiolabel to be distributed between two zones in the samples from the fenpropidin experiment and four zones in those from the tridemorph and fenpropimorph experiments. Zone 1 (R_f 0.16) was found to co-chromatograph with prepared 5α-ergosta-8,14,24(28)-trienol acetate, thus zone 2 (R_f 0.65) was the product no longer containing the $\Delta^{8,14}$-heteroannular double bond system. This zone was identified as fecosterol by GC-MS in an analogous non-radioactive experiment. The two minor zones observed in the tridemorph and fenpropimorph experiments were identified as ignosterol (R_f 0.3) and 5α-ergosta-8-enol (R_f 0.7). It would appear that the sterol $\Delta^{24(28)}$-reductase was also active in these enzyme preparations giving small amounts of ignosterol from 5α-ergosta-8,14,24(28)-trienol and 5α-ergosta-8-enol from fecosterol. However, in terms of Δ^{14}-reductase activity, both the $\Delta^{8,14}$-sterols were considered as the substrates and both the Δ^8-sterols as the products. The radiolabel in each zone was quantified. As the recovery of total sterol may vary slightly from incubation to incubation and thereby introduce errors into the calculation, percentage conversion values were then determined as described in the Experimental. The percentage inhibition values were plotted against inhibitor concentration and the I_{50} values for tridemorph, fenpropimorph and fenpropidin determined (Table 1). The results obtained clearly show that tridemorph is a weak inhibitor of this enzyme whilst fenpropimorph and fenpropidin are almost equally strong inhibitors. This suggests that differences in the *N*-substituent (*n*-tridecyl in tridemorph; 3(*p*-*tert*-butylphenyl)-2-methylpropane in fenpropimorph and fenpropidin) play a more significant role in the inhibition of the Δ^{14}-reductase than differences in the *N*-heterocycles (2,6-dimethylmorpholine in tridemorph and fenpropimorph; piperidine in fenpropidin).

DISCUSSION

Fenpropimorph, tridemorph and fenpropidin have been shown to inhibit both the sterol Δ^{14}-reductase and the $\Delta^8 \rightarrow \Delta^7$-isomerase using *in vitro* assays from *S. cerevisiae*. The reaction mechanisms of the two enzyme-catalysed steps are shown in Fig. 2. The mechanism of isomerization is likely to involve protonation at C-9 on the α-face of the sterol nucleus, resulting in a high energy

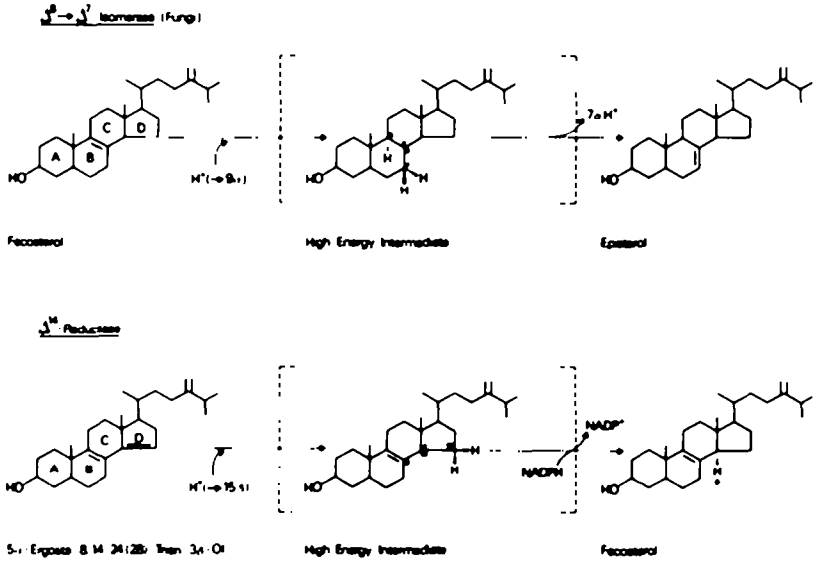

Fig. 2. Reaction mechanisms.

intermediate (HEI) with a carbonium ion at C-8 [9]. This carbonium ion is then stabilized by the loss of a proton from C-7 [10]. The mechanism of the Δ^{14}-double bond reduction most likely involves the donation of a proton at C-15 on the β-face, again resulting in the formation of a HEI, but with a carbonium ion at C-14. NADPH then donates a hydride ion which becomes the 14α-hydrogen atom, thereby stabilizing the molecule [11].

Fenpropimorph (I_{50} 0.013 μM) and tridemorph (I_{50} 0.033 μM) were both found to be extremely strong inhibitors of the isomerization step, but fenpropidin, whilst still very potent, is a full order of magnitude less active, suggesting that the *N*-substituents play a relatively minor role and that other features of the *N*-heterocyclic ring are more important for optimal inhibitory activity. This was confirmed by the changes in potencies observed with the fenpropimorph and fenpropidin analogues (Table 2). *Cis*-3,5-methylated fenpropidin had a much higher inhibitory activity than fenpropidin itself and roughly equal to that of fenpropimorph. The non-methylated analogues, 2,6-demethylated fenpropimorph and fenpropidin, were markedly less active than the *cis*-methylated compounds. Introduction of the *trans*-methyl groups reduced inhibitory activity still further. In the presence of the *cis*-methyl groups the role of the heterocyclic oxygen is not apparent and only appears to be significant when the methyl groups are either absent or in the *trans*-configuration when it apparently doubles the inhibitory activity. These findings clearly demonstrate the importance of the methyl groups and their correct configuration.

pK_a values of the morpholines are difficult to determine accurately due to the poor solubility of the neutral species in aqueous solution. However, all three fungicides are believed to have pK_a values falling within the range 7-10. Therefore, at physiological pH the fungicides are likely to be present largely in their protonated forms with a positive charge on the tertiary nitrogen atom [12]. It is likely that the protonated species of the fungicides are responsible for the observed inhibition by mimicking the HEI formed during the isomerase reaction in a manner similar to that described by Rahier *et al.* [12] where tridemorph, fenpropimorph and fenpropidin were demonstrated to inhibit the sterol cycloeucalenol–obtusifoliol isomerase in higher plants. It can be envisaged that the *N*-heterocycle of the fungicide positions itself on the active site of the isomerase normally occupied by ring B of fecosterol with the positively charged nitrogen at the point normally taken up by C-8 (Fig. 2). Any variation in the inhibitory activity observed with different inhibitor molecules, all of which will possess a positively charged nitrogen at physiological pH, must then be due to differences in structure at other parts of the inhibitor molecule. A possible interpretation of the data is that (i) the *trans*-configuration of the methyl groups always presents at least one of the methyl groups in such a way that it prevents a 'perfect fit', (ii) the absence of methyl groups passively allows a better fit, though the precise positioning is not obtained and (iii) in the *cis*-configuration, one or both of the methyl groups positively interacts with the sterol binding site, ensuring the best 'fit' and therefore the most complete inhibition. Moreover since the mechanism of the isomerase reaction (Fig. 2) involves the addition of a proton from a sub-site of the enzyme to C-9 [9] it is also possible that the *cis* methyl groups, or at least one of them, interact with this 'sub-site' thereby more effectively blocking the enzyme activity.

Taken overall, the binding of the fungicides to the $\Delta^8 \rightarrow \Delta^7$-isomerase active site could be regarded as consisting of two elements, the most important of which is the binding to the site normally occupied by the B ring of fecosterol. The second element of the binding could be a property of the *N*-substituent. The *N*-*tert*-butylphenyl-2-methylpropyl group gives better inhibitory activity than the tridecyl group since fenpropimorph is a stronger inhibitor than tridemorph, but this effect can be almost completely counteracted by more efficient binding at the primary site since tridemorph is a more effective inhibitor than fenpropidin.

Fenpropimorph and fenpropidin (I_{50} values 2.3 and 1.8 μM, respectively) were both found to be strong inhibitors of the Δ^{14}-reductase while tridemorph is relatively weak with an I_{50} value about 50 times less than the 3-phenyl propyl amines. This result is consistent with previous findings [7] in whole yeast cells where fenpropimorph and fenpropidin caused a far greater accumulation of $\Delta^{8,14}$-sterols than tridemorph. Fenpropimorph and fenpropidin are related by having the same *N*-substituent, i.e. the 3-(*p*-*tert*-butylphenyl)-2-methylpropyl residue, but they show almost identical inhibitory activity. However, fenpropimorph and tridemorph, which are structurally identical in the *N*-heterocyclic ring, have different *N*-substituents and they show very different degrees of inhibitory activity. Although these findings differ from those obtained for the inhibition of the $\Delta^8 \rightarrow \Delta^7$-isomerase they may be explained using arguments similar to those already used. The positively charged nitrogen atom of the inhibitors can again be envisaged to be located at the position normally occupied by C-8 of the preferred substrate, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol in yeast, with the *N*-substituent distributing itself along the region normally occupied by rings C and D and the side chain. The proposed reaction mechanism (Fig. 2) involves the formation of a HEI carbonium ion with the positive charge formally located at C-14. The charge on the heterocyclic nitrogen of the inhibitors can be considered to be delocalized [13, 14] with the charge being distributed between the N-atom and the adjoining C-atoms. Thus, it is possible that the fungicides can mimick the HEI, albeit not so accurately as on the $\Delta^8 \rightarrow \Delta^7$-isomerase. The less intensive binding and a less 'perfect fit' with that part of the active site normally occupied by ring B of the substrate would then allow the correct binding of other structural features of the inhibitors to assume a proportionally greater importance in determining overall inhibitory activity. In addition, the reaction mechanism of the Δ^{14}-reductase involves the donation of a proton to C-15 of the preferred substrate, presumably from a sub-site on the enzyme. This emphasizes the importance of that region of the active site normally occupied by rings C and D of the sterol molecule for Δ^{14}-reductase activity. Bearing in mind that fenpropimorph and fenpropidin showed much stronger inhibitory activity against the Δ^{14}-reductase than tridemorph, this indicates that the 3-(*p*-*tert*-butylphenyl)-2-methylpropane *N*-substituent binds far more efficiently and thereby interferes more effectively with this region of the active site than the long floppy tridecyl *N*-substituent of tridemorph.

These findings confirm the value of *in vitro* assays of the inhibition of individual enzymic steps as compared with whole cell investigations. The ambiguity concerning the

relative potencies of these compounds as inhibitors of the $\Delta^8 \rightarrow \Delta^7$-isomerase, caused by this enzyme being later in the preferred biosynthetic sequence than the Δ^{14}-reductase, has been clarified, showing that fenpropimorph and not tridemorph is the most potent inhibitor of the isomerization step. The structural aspects of the inhibitor molecules contributing to their activity have been elucidated and quantified. The potency of fenpropimorph and to a lesser extent fenpropidin as inhibitors of both the Δ^{14}-reductase and the $\Delta^8 \rightarrow \Delta^7$-isomerase has been unambiguously demonstrated. Such a dual site of action markedly reduces the risk of resistance development and promises the continued success of this group of compounds as commercial fungicides.

EXPERIMENTAL

Organism. Saccharomyces cerevisiae (NCYC 739, high sterol strain) was used in the preparation of cell free systems; it was maintained on Sabouraud-dextrose agar (Oxoid).

Growth conditions and preparation of cell free extract for $\Delta^8 \rightarrow \Delta^7$*-isomerase assay.* This procedure was adapted from that of Yabusaki *et al.* [10]. *Saccharomyces cerevisiae* (NCYC 379) was grown as described by Katsuki and Bloch [15]. After harvesting and washing the cells by centrifugation they were resuspended in 0.1 M phosphate buffer, pH 6.8 containing 5 mM *N*-acetyl-L-cysteine at 0.5 g cells/ml buffer and homogenized using a Bronwill MSK cell mill with liquid CO_2 cooling. The resulting homogenate was centrifuged at 2500 *g* for 10 min at 4° and the supernatant used as the cell-free extract. An acetone powder of the cell-free extract was prepared as described by Moore and Gaylor [16]. This powder was suspended in the above buffer (10 mg/ml) and the resulting solution used as the enzyme preparation.

Growth conditions and preparation of cell free extract for the Δ^{14}*-reductase assay. Saccharomyces cereviciae* (NCYC 739, high sterol strain) was grown semi-anaerobically in 2 l fermentation bottles for 72 hr at 30° in a medium containing per litre; bacto tryptone (Difco) (10 g), yeast extract (Difco) (5 g) and glucose (20 g). The cells were harvested by centrifugation and washed 2 × with 0.1 M potassium phosphate buffer, pH 6.8. Semi-anaerobically grown and washed yeast cells were resuspended (at 0.75 g cells/ml) in 0.1 M phosphate buffer pH 6.2 and ruptured using a pre-cooled French pressure cell operating at 0.1379 GPa. The unbroken cells were removed by centrifugation at 1000 *g* for 10 min at 4°. The resulting supernatant was filtered through glass wool to remove the floating lipid layer and centrifuged at 8000 *g* for 20 min at 4°. The supernatant was again filtered though glass wool and used immediately as the enzyme preparation.

Substrate preparation. Fecosterol was prepared as follows: yeast cells, grown semi-anaerobically as described above, were harvested by centrifugation and then resuspended in 0.1 M phosphate buffer (pH 6.8) to the extent 0.5 g fr. wt cell/ml buffer. The cells were aerobically adapted overnight in 10% glucose and sufficient tridemorph to give a final concentration of 200 μM. After harvesting by centrifugation the cells were saponified with ethanolic KOH containing 0.5% pyrogallol as an antioxidant. The non-saponifiable lipid was extracted into $CHCl_3$ and separated on Whatmann LK6F silica gel TLC plates into three sterol classes (4,4-dimethyl-, 4α-methyl- and 4-demethyl-) using cyclohexane EtOAc (80 : 20) as the developing solvent. Ergosterol was used as a marker for the 4-demethylsterols. This class of sterols was eluted and acetylated. The acetylated sterols were separated by argentation chromatography on 6% $AgNO_3$-impregnated silica gel TLC plates developed twice in toluene. The major zone (R_f 0.65) was eluted and hydrolysed in ethanolic KOH. This zone was identified as fecosterol by GC/MS and found to be 97% pure by GC.

Radiolabelled 5α-ergosta-8,14,24(28)-trien-3β-ol was prepared by a method adapted from that of Bottema and Parks [17] as follows: *Saccharomyces cerevisiae* was grown semi-anaerobically for 72 hr at 30° in a medium containing 1% tryptone, 0.5% yeast extract and 2% glucose. After harvesting by centrifugation the cells were resuspended in 0.1 M phosphate buffer (pH 6.2) to the extent of 0.5 g fr. wt cells/ml buffer. To 50 ml of cell suspension was added [U-^{14}C]acetate (100 μCi), [methyl-^{14}C]methionine (50 μCi) and sufficient fenpropidin to give a final concentration of 200 μM. The cells were then aerobically adapted overnight at 30°. The 5α-ergosta-8,14,24(28)-trien-3β-ol was extracted and purified using the same procedure as used for the preparation of fecosterol. The major zone on the argentation TLC plate (R_f 0.18) was identified as a $\Delta^{8,14}$-sterol by UV spectroscopy as it gave the characteristic peak at 250 nm with a shoulder at 245 nm [18] and was found to be 98% pure 5α-ergosta-8,14,24(28)-trien-3β-ol by GC. Its positive identification was made by GC/MS from an analogous non-radiolabelled preparation. The [^{14}C]5α-ergosta-8,14,24(28)-trien-3β-ol was quantified from its UV absorption spectrum on the assumption that the molar absorption coefficient of $\Delta^{8,14}$-sterols is 18 000 at 250 nm [19, 20] and that the M_r is 396. The radioactivity of a known aliquot was determined and the specific activity of the [^{14}C]5α-ergosta-8,14,24(28)-trien-3β-ol was calculated to be 5×10^5 dpm/μmol.

Assay of $\Delta^8 \rightarrow \Delta^7$*isomerase.* The standard assay mixture contained the following constituents in a final volume of 2 ml: fecosterol (20 nmol) added in 5 μl EtOH, 1.985 ml enzyme preparation (protein content, 5 mg/ml, determined using the method of Bradford *et al.* [21]) and fungicide dissolved in EtOH (10 μl) to give the required concn. A no-fungicide control contained 10 μl EtOH. The mixtures were incubated for 3 hr at 30° with continuous shaking. The reaction rate during the incubation period was linear. The reaction was terminated by the addition of 4 ml EtOH. The sterols were extracted with *n*-hexane and analysed by GC. The activity of the $\Delta^8 \rightarrow \Delta^7$-isomerase was determined by measuring the percentage conversion of fecosterol (*RR*, 1.039) to episterol (*RR*, 1.073) by GC using ergosterol as internal standard both to calculate the *RR*,s and to determine the relative quantities of fecosterol and episterol. The percentage conversion values were calculated as follows: area counts in episterol peak/area counts in both the episterol and fecosterol peaks, × 100. Assuming the percentage conversion values in the control to be 100%, the percentage inhibition values were calculated using the following relationship: percentage conversion in control − percentage conversion in test/percentage conversion in the control × 100. The percentage inhibition values so obtained were then plotted against fungicide concn. and from the resulting curves the concn. of fungicides giving 50% inhibition relative to the control (I_{50}) were determined.

To determine the relative potencies of fenpropimorph and fenpropidin analogues the incubations were prepared as described above but the inhibitor concn. used in each case was 0.0125 μM (close to the I_{50} value of fenpropimorph). The percentage inhibition values were then calculated as shown above.

Δ^{14}*-Reductase assay.* The standard assay mixture contained the following in a final volume of 5 ml; enzyme preparation (4.6 ml) (protein content 30 mg/ml), 5α-ergosta-8,14,24(28)-trien-3β-ol (0.25 μmol) added in 200 μl EtOH, NADPH (5 μmol) added in 100 μl 0.1 μM phosphate buffer pH 6.2 and 125 μl of an ethanolic soln of fungicide to give the required concentrations. The mixtures were incubated for 3 hr at 30° with gentle shaking. The reaction rate was found to be linear during this period. Ethanolic KOH was used to terminate the reaction. The mixture

was saponified and the non-saponifiable lipid extracted with *n*-hexane and subjected to TLC on silica gel plates using cyclohexane–EtOAc (80 : 20). Ergosterol was used as the marker to identify the 4-demethylsterol zone, which was eluted, acetylated and separated on $AgNO_3$-impregnated TLC plates as described above. The radiolabelled zones were located by radioautography. In an analogous non-radioactive experiment the observed zones were identified by GC-MS. The first major zone (R_f 0.16) was identified as 5α-ergosta-8,14,24(28)-trienol, the second (R_f 0.65) was identified as fecosterol. In the samples from the fenpropimorph and tridemorph experiments two additional minor zones were observed at R_f 0.3 and R_f 0.7 and were identified as ignosterol and 5α-ergosta-8-enol, respectively. Considering the $\Delta^{8,14}$-sterols as the substrates and the Δ^{8}-sterols as the products, the percentage conversion values of substrate(s) to product(s) can still be calculated as described for the $\Delta^{8} \rightarrow \Delta^{7}$-isomerase assay. Taking the percentage conversion in the control to be 100%, the degree of inhibition in the samples treated with fungicide were calculated. The percentage inhibition values were then plotted against fungicide concn. and the I_{50} values determined from the resulting curves.

GC analysis. GC analysis was carried out on SGE BP-5, WCOT quartz column 15 × 0.3 mm with splitless, on-column injection, sample volume 0.5 μl toluene, carrier gas helium 8 ml/min, column oven temp. programmed from 100° (1 min) to 260° (1 min) to 270° (5 min) at 40°/min and 2°/min, respectively. Detection was by FID, with a detector oven temp. of 300°.

GC-MS analysis was carried out using a HP 5890A GC connected to a VG 7070 MS and an ionization potential of 80 eV. The GC column used was a SE 54/80 and a temperature programme was from 220 to 350° at 6°/min with He as carrier gas.

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REFERENCES

1. Himmele, W. and Pommer, E.-H. (1980) *Angew. Chem. Int. Ed. Engl.* **19**, 194.
2. Konig, K. H., Pommer, E.-H. and Sanne, W. (1965) *Angew. Chem. Int. Ed. Engl.* **4**, 336.
3. Butters, J., Clark, J. and Hollowmon, D. W. (1983) *Proc. 10th. Int. Congress Plant Prot., Brighton* **2**, 644.
4. Buchenauer, H., Budde, K., Hellwald, K.-H., Taube, E. and Kirchner, R. (1984) *Br. Crop Prot. Conf.* **2**, 483.
5. Kato, T., Shoami, M. and Kawase, Y. (1980) *J. Pestic. Sci.* **5**, 69.
6. Kerkenaar, A., Uchiyama, M. and Versluis, G. G. (1981) *Pestic. Biochem. Physiol.* **16**, 97.
7. Baloch, R. I., Mercer, E. I., Wiggins, T. E. and Baldwin, B. C. (1984) *Phytochemistry* **23**, 2219.
8. Chou, T. (1974) *Molec. Pharmacol.* **10**, 235.
9. Wilton, D. C., Rahimtula, A. D. and Akhtar, M. (1969) *Biochem. J.* **114**, 71.
10. Yabusaki, Y., Nishima, T., Ariga, N. and Katsuki, H. (1979) *J. Biochem.* **85**, 1531.
11. Caspi, E., Moreau, J. P. and Ramm, P. J. (1974) *J. Am. Chem. Soc.* **96**, 8107.
12. Rahier, A., Schmitt, P., Huss, B., Benveniste, P. and Pommer, E. H. (1986) *Pestic. Biochem. Physiol.* **25**, 112.
13. Greenberg, A., Winkler, R., Smith, B. L. and Liebman, J. F. (1982) *J. Chem. Education* **59**, 397.
14. Port, G. N. J. and Pullmann, A. (1973) *Theoret. Chim. Acta* **31**, 231.
15. Katsuki, H. and Bloch, K. (1967) *J. Biol. Chem.* **242**, 222.
16. Moore, J. T., Jr. and Gaylor, J. L. (1969) *J. Biol. Chem.* **244**, 6334.
17. Bottema, C. K. and Parks, L. W. (1978) *Biochim. Biophys. Acta* **531**, 301.
18. Dorfman, L. (1953) *Chem. Rev.* **53**, 47.
19. Fieser, L. F. and Ourisson, G. (1953) *J. Am. Chem. Soc.* **75**, 4404.
20. Adams, W. J., Petrow, V. and Royer, R. (1951) *J. Chem. Soc.* 678.
21. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.