Interaction of azole antifungal antibiotics with cytochrome *P*-450-dependent 14 α -sterol demethylase purified from *Candida albicans*

Christopher A. HITCHCOCK,*† Keith DICKINSON,* Stanley B. BROWN,† E. Glyn V. EVANS* and David J. ADAMS*§

Departments of *Microbiology and †Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

The interaction of azole antifungal antibiotics with purified *Candida albicans* cytochrome *P*-450-dependent 14 α -sterol demethylase (*P*-450_{DM}) was measured spectrophotometrically and by inhibition of enzyme activity. Ketoconazole and ICI 153066 (a triazole derivative) formed low-spin complexes with the ferric cytochrome and induced type II difference spectra. These spectra are indicative of an interaction between the azole moiety and the sixth co-ordination position of *P*-450_{DM} haem. Both azoles inhibited the binding of CO to the sodium dithionite-reduced ferrous cytochrome, and inhibited reconstituted *P*-450_{DM} activity by binding to the cytochrome with a one-to-one stoichiometry. Similarly, total inhibition of enzyme activity occurred when equimolar amounts of clotrimazole, miconazole or fluconazole were added to reconstituted *P*-450_{DM}. These results correlated with the inhibition of *P*-450_{DM} in broken cell preparations, confirming that all five azoles are potent inhibitors of ergosterol biosynthesis in *C. albicans*.

INTRODUCTION

Candida albicans is a widespread opportunistic fungal pathogen that causes superficial and systemic candidoses in human and animal hosts (Odds, 1988). The azole (*N*-substituted imidazole or triazole) antifungal antibiotics are prescribed commonly for the treatment of mycoses, including candidoses (Fromtling, 1988). Azoles probably work by inhibiting cytochrome *P*-450-dependent 14 α -sterol demethylase (*P*-450_{DM}; Vanden Bossche, 1985), an important enzyme in ergosterol (ergosta-5,7,22-trien-3 β -ol) biosynthesis in fungi and in cholesterol (cholest-5-en-3 β -ol) biosynthesis in mammals. The accumulation of 14 α -methylated sterols in azole-treated cells disrupts membrane structure and function (Yeagle *et al.*, 1977), resulting in an inhibition of growth (Vanden Bossche, 1985). The selectivity of azoles for fungal cells *in vivo* is believed to reside in their greater affinity for fungal rather than mammalian *P*-450_{DM} (e.g. see Marriott *et al.*, 1986; Vanden Bossche *et al.*, 1986).

P-450_{DM} purified from rat liver (Trzaskos *et al.*, 1986) and from the yeast, *Saccharomyces cerevisiae* (Aoyama *et al.*, 1987), catalyses the complete oxidative demethylation of 24,25-dihydrolanosterol to 4,4-dimethyl-5 α -cholesta-8,14-dien-3 β -ol and formic acid, via three mono-oxygenation steps (i.e. CH₃ → CH₂OH → CHO → HCO₂H). The reaction requires NADPH and molecular O₂, and is inhibited by CO. The interaction of azoles with the yeast cytochrome has been studied in detail by Yoshida and co-workers (Aoyama *et al.*, 1983; Yoshida *et al.*, 1986; Yoshida & Aoyama, 1987). All of the azoles tested induced type II difference spectra when bound to ferric *P*-450_{DM}, and some of them inhibited CO binding to the ferrous cytochrome. This activity is attributed to co-ordination of the azole moiety with *P*-450_{DM} haem,

while hydrophobic *N*-substituent groups of the azole molecule interact with the apoprotein of the cytochrome. In contrast, studies of azole binding to *P*-450_{DM} from pathogenic fungi, such as *C. albicans*, have been confined to broken cell preparations. For example, azole derivatives do induce type II difference spectra and inhibit CO binding to total cytochrome *P*-450 in microsomal preparations from *C. albicans* (Vanden Bossche *et al.*, 1986, 1987). However, the commonest method of assessing the potency of azoles against *C. albicans* *P*-450_{DM} is by measuring the incorporation of [¹⁴C]mevalonic acid into the 14 α -methylated sterols of cell lysates (e.g. see Pye & Marriott, 1982; Marriott *et al.*, 1986; Vanden Bossche *et al.*, 1986; Hitchcock *et al.*, 1987).

In order to probe further the interaction of azoles with fungal *P*-450_{DM}, we have purified the cytochrome from microsomal preparations of *C. albicans* (Hitchcock *et al.*, 1989b). The cytochrome has spectral and enzymic properties like those of *P*-450_{DM} purified from rat liver (Trzaskos *et al.*, 1986) and *S. cerevisiae* (Yoshida & Aoyama, 1984; Aoyama *et al.*, 1987) and is able to C-14-demethylate lanosterol (4,4,14 α -trimethyl-5 α -cholesta-8,24-dien-3 β -ol) to 4,4-dimethyl-5 α -cholesta-8,14,24-trien-3 β -ol *in vitro*. We report now a detailed investigation of the interaction of some azole antifungals (Fig. 1) with *C. albicans* *P*-450_{DM}, and show that the azole-cytochrome complex has properties very similar to those of the *S. cerevisiae* cytochrome complex (Yoshida & Aoyama, 1987).

MATERIALS AND METHODS

Materials

Biochemicals were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Solvents and other chemicals

Abbreviations used: *P*-450_{DM}, cytochrome *P*-450-dependent 14 α -sterol demethylase; Me₂SO, dimethyl sulphoxide; NSF, non-saponifiable lipid fraction; IC₅₀, concn. causing 50% of maximum inhibition.

† Present address: Pfizer Central Research, Sandwich, Kent CT13 9NJ, U.K.

§ To whom correspondence should be addressed.

Fluconazole

ICI 153066

Ketoconazole

Miconazole

Clotrimazole

Fig. 1. Structural formulae of azole antifungal antibiotics used in the present study

were of analytical grade and purchased from BDH, Liverpool, U.K. DL-[2-¹⁴C]Mevalonic acid (dibenzyl-diethyldiamine salt; sp. radioactivity 1.89 GBq/mmol) was purchased from Amersham International, Amersham, Bucks., U.K. Azole antifungals were obtained from the following sources: clotrimazole (1-[*o*-chloro- $\alpha\alpha$ -diphenylbenzyl]imidazole), miconazole {1-[2,4-dichloro- β -(2,4-dichlorobenzyl)oxy]phenethyl]imidazole} and ketoconazole, {*cis*-1-acetyl-4-[4-[(2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)methyl)-1,3-dioxolan-4-yl]-methoxy]phenyl]piperazine} from Sigma Chemical Co.; ICI 153066 [1-(2,4-dichlorophenyl)-1(4-fluorophenyl)-2-(1,2,4-triazol-1-yl)ethanol], from ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, U.K.; and fluconazole [2-(2,4-difluorophenyl)-1,3-bis(1*H*-1,2,4-triazol-1-yl)propan-2-ol]] from Pfizer Central Research, Sandwich, Kent, U.K.

Purification of *P*-450_{DM} and cytochrome *P*-450 reductase

P-450_{DM} was purified from *C. albicans* (serotype A; NCPF 3153) microsomes as described by Hitchcock *et al.* (1989b). Stock solutions of *P*-450_{DM} (1–2 nmol/ml) in 100 mM-potassium phosphate (pH 7.4) containing

30 mM-nicotinamide, 4 mM-*N*-acetylcysteine and 20% (v/v) glycerol (storage buffer) were stored at –70 °C. NADPH:cytochrome *c* (cytochrome *P*-450) reductase was purified from rat liver microsomes using the affinity chromatography method described by Ardies *et al.* (1987).

Analytical methods

The interaction of azole antifungals with *P*-450_{DM} was analysed spectrophotometrically using a Shimadzu recording spectrophotometer, essentially as described by Yoshida & Aoyama (1987). Assays were done using stock solutions of *P*-450_{DM} diluted to 0.1–0.2 nmol/ml with storage buffer. Azoles were dissolved in dimethyl sulphoxide (Me₂SO) and added to the cytochrome to give a final Me₂SO concentration of 1% (v/v). *P*-450_{DM} was quantified from its sodium dithionite-reduced CO difference spectrum, essentially as described by Omura & Sato (1964).

Lanosterol 14 α -demethylase activity was assayed in a reconstituted system containing *P*-450_{DM}, *P*-450 reductase and NADPH (Hitchcock *et al.*, 1989b). The conversion of lanosterol to 4,4-dimethyl-5 α -cholesta-8,14,24-trien-3 β -ol was measured by g.l.c.; sterols were quantified using cholestane as internal standard with peak areas calculated by electronic integration. The details of all of these methods are described by Hitchcock *et al.* (1989b).

Measurement of sterol biosynthesis in cell lysates with [2-¹⁴C]mevalonic acid

Sterol biosynthesis was assayed by the incorporation of DL-[2-¹⁴C]mevalonic acid into the non-saponifiable lipid fraction (NSF) of cell lysates, as described by Barrett-Bee *et al.* (1986). Azoles were added to the lysates in Me₂SO as described above; control assays received Me₂SO only. Further details are given in the Figure legends.

RESULTS

Interaction of azoles with ferric *P*-450_{DM}

The absolute absorption spectrum of *P*-450_{DM} is shown in Fig. 2. The oxidized cytochrome absorbed at 552 and 528 nm with a Soret maximum at 417 nm, indicating that it was in the low-spin state. The Soret peak shifted by approx. 7 nm towards the red end of the spectrum when a saturating amount of ketoconazole was added to the cytochrome (Fig. 2a). This spectral change is characteristic of the interaction of a nitrogen atom with the sixth co-ordination position of haem iron (White & Coon, 1982; Yoshida & Aoyama, 1987). Addition of a saturating amount of ICI 153066 to the cytochrome also induced a red shift (Fig. 2b) indicating that the triazole moiety interacted with the haem iron of ferric *P*-450_{DM}. However, the spectral change was smaller (3 nm) than that induced by ketoconazole, giving a Soret maximum at 420 nm. Ketoconazole or ICI 153066 complexes with ferric *P*-450_{DM} gave rise to typical type II difference spectra with absorption maxima and minima at 428 and 410 nm respectively (Fig. 3). Fig. 3 shows clearly that the magnitude of the spectral change was greater with ketoconazole in an experiment where 0.1 nmol of either azole was added to 0.2 nmol of the cytochrome. The increase in value of absorbance (peak maximum minus peak minimum) measured after successive additions of azole gave the saturation curves shown in Fig. 4. The

Fig. 2. Spectral change of ferric *P*-450_{DM} induced by (a) ketoconazole and (b) ICI 153066

P-450_{DM} was diluted with storage buffer to a concentration of 0.1 nmol/ml and absolute spectra were recorded: —, *P*-450_{DM}; ●—●, *P*-450_{DM} after the addition of azole to a concentration of 0.2 nmol/ml.

Fig. 3. Difference spectra of ferric *P*-450_{DM} induced by (a) ketoconazole and (b) ICI 153066

P-450_{DM} was diluted with storage buffer to a concentration of 0.2 nmol/ml and spectra were recorded in the presence of 0.1 nmol of azole/ml.

spectral change was linearly dependent on the concentration of azole and was saturated when equimolar amounts of azole were added to *P*-450_{DM}. These data indicate that the antifungals formed a one-to-one complex with the cytochrome, precluding a meaningful kinetic analysis of the data, and consequently, calculation of a dissociation constant of the azole-*P*-450_{DM} complex. The difference between the plateau levels of the titration curves reflects the difference in magnitude of the spectral interaction of the azoles shown in Fig. 3.

Inhibition of CO binding to ferrous *P*-450_{DM}

In contrast to ferric *P*-450_{DM}, addition of azoles to

Fig. 4. Spectral titration of ferric *P*-450_{DM} with ketoconazole (●) and ICI 153066 (■)

P-450_{DM} was diluted with storage buffer to a concentration of 0.2 nmol/ml and titrated with increasing amounts of azole. The magnitude of the resulting difference spectrum was plotted as a function of azole concentration.

ferrous *P*-450_{DM} (produced by reduction of ferric cytochrome with sodium dithionite) did not induce a spectral change in the cytochrome. However, the affinity of the antifungals for ferrous *P*-450_{DM} could be assessed by measuring their ability to inhibit the binding of CO to the azole-ferrous-*P*-450_{DM} complex. In these experiments, azoles were added to ferric *P*-450_{DM} before its reduction to the ferrous form with sodium dithionite and gassing with CO. The resulting reduced CO difference spectrum was used to quantify the cytochrome. Fig. 5 shows the results of an experiment where 0.2 nmol of *P*-450_{DM} was titrated with increasing amounts of ketoconazole or ICI 153066. Both azoles inhibited the binding of CO to the ferrous *P*-450_{DM}-azole complex in a dose-dependent way. However, in contrast to ferric *P*-450_{DM}, there was a striking difference between the azoles in their affinity for ferrous *P*-450_{DM}, as judged by their ability to inhibit CO binding. The concentration of ketoconazole required to inhibit CO binding to 50% of the cytochrome (IC_{50}) was 0.1 nmol, indicating that the azole bound to ferrous *P*-450_{DM} with one-to-one stoichiometry. The larger IC_{50} value of ICI 153066 (0.7 nmol) suggests that the triazole was less able than ketoconazole to inhibit the formation of a CO-ferrous-*P*-450_{DM} complex.

Inhibition of reconstituted *P*-450_{DM} activity by azoles

The catalytic activity of *P*-450_{DM} in a reconstituted system was assayed by measuring the conversion of lanosterol to its C-14-demethylated product, 4,4-dimethyl-5 α -cholesta-8,14,24-trien-3 β -ol. Fig. 6 shows the results of an experiment in which 0.1 nmol of *P*-450_{DM} was titrated with increasing amounts of ketoconazole or ICI 153066. The inhibition of enzyme activity was linearly dependent on azole concentration, and total inhibition of enzyme activity occurred at a concentration equal to that of *P*-450_{DM}, indicating that the antifungals bound to *P*-450_{DM} with one-to-one stoichiometry. Essentially the same results were obtained with the imid-

Fig. 5. Inhibition of CO binding to ferrous $P-450_{DM}$ complexes of ketoconazole (●) and ICI 153066 (■)

$P-450_{DM}$ was diluted with storage buffer to a concentration of 0.2 nmol/ml and different amounts of azole in Me_2SO were added to the cytochrome; control assays received Me_2SO only. Each sample was reduced with sodium dithionite and gassed with CO, and the resulting ferrous $P-450_{DM}$ was measured as a difference spectrum.

Fig. 6. Inhibition of reconstituted $P-450_{DM}$ activity by ketoconazole (●) and ICI 153066 (■)

Lanosterol (23 nmol) was metabolized aerobically by $P-450_{DM}$ in the following reconstituted system in 1 ml of storage buffer: $P-450_{DM}$ (0.1 nmol), dilauroyl phosphatidylcholine (240 nmol), NADPH (1 μ mol), glucose 6-phosphate (20 μ mol) and glucose-6-phosphate dehydrogenase (1 unit). Different amounts of azole were added to the reconstituted system in Me_2SO ; control assays received Me_2SO only. Lanosterol and the C-14 demethylated metabolite were analysed by g.l.c., as described in the Materials and methods section. 100% activity corresponds to control $P-450_{DM}$ activity in the range 0.03–0.04 nmol of lanosterol metabolized/min. Values are the means of triplicate determinations which varied by < 15%.

azoles clotrimazole and miconazole and the triazole fluconazole: total inhibition of enzyme activity occurred when equimolar amounts of azole were added to $P-450_{DM}$.

Inhibition of cell lysate $P-450_{DM}$ activity by azoles

The inhibition of cell lysate 14α -sterol demethylase activity by azoles was measured by the incorporation of $[2-^{14}C]$ mevalonic acid into the 14α -methylated sterols of lysates. Sterols were separated from the other NSF components and fractionated into desmethylated and 4,4-dimethylated classes by one-dimensional t.l.c., using published methods (Hitchcock *et al.*, 1989a). In control experiments without azole antifungals, approx. 60% of the total NSF radioactivity was incorporated into desmethylated sterols, the major component of which is ergosterol (Hitchcock *et al.*, 1989a). In experiments containing azoles to inhibit $P-450_{DM}$, radioactivity was incorporated into the 4,4-dimethylated sterol fraction which comprises mainly lanosterol and 24-methylene-24,25-dihydrolanosterol (Vanden Bossche, 1985; Hitchcock *et al.*, 1989a). Fig. 7 shows the results of a typical experiment where increasing amounts of ketoconazole were added to cell lysates incubated with $[2-^{14}C]$ -

Fig. 7. Effect of ketoconazole on incorporation of $[2-^{14}C]$ mevalonic acid into desmethylated (●) and 4,4-dimethylated (▲) sterols of a cell lysate

Cell lysates containing ketoconazole in Me_2SO were incubated with 0.25 μ Ci of $[2-^{14}C]$ mevalonic acid for 30 min. The reaction was stopped by saponifying lipids with 3 M-KOH in 90% (v/v) ethanol at 70 °C for 1 h, before NSF lipids were extracted with light petroleum (b.p. 60–80 °C). Approx. 40% of the total radioactivity was recovered in the NSF, which was fractionated into desmethylated and 4,4-dimethylated sterols by silica-gel H t.l.c., with toluene/diethyl ether (9:1, v/v) as developing solvent. The amounts of radioactivity associated with the desmethylated and 4,4-dimethylated sterol fractions are expressed as percentages of the total NSF radioactivity. Values are the means of triplicate determinations which varied by < 5%. The control values for desmethylated (○) and 4,4-dimethylated (△) sterols are also shown.

Table 1. Effect of azole antifungals on cell lysate 14 α -sterol demethylase activity

The IC₅₀ value is the concentration of azole giving 50% inhibition of incorporation of [2-¹⁴C]mevalonic acid into desmethylated sterols of cell lysate NSF lipids. Values are the means $\pm$ s.d. of experiments on three separate cell lysates (individual values varied by < 5%).

Azole antifungal	10 ⁷ $\times$ IC ₅₀ (M)
Ketoconazole	0.61 $\pm$ 0.06
ICI 153066	0.78 $\pm$ 0.09
Fluconazole	0.74 $\pm$ 0.02
Miconazole	1.10 $\pm$ 0.35
Clotrimazole	0.61 $\pm$ 0.01

mevalonic acid. There was a reciprocal relationship between the amount of radioactivity incorporated into 4,4-dimethylated sterols and that lost from desmethylated sterols, as a function of ketoconazole concentration. The concentration of ketoconazole required to give 50% inhibition of incorporation of radiolabel into desmethylated sterols is shown in Table 1, together with the IC₅₀ values of the other azoles used in this study. When the IC₅₀ values for the azoles were compared, there was less than a 2-fold variation between them, indicating that the antifungals had a similar potency against cell lysate *P*-450_{DM}.

DISCUSSION

The aim of the present study was to investigate the interaction of azole antifungals with *P*-450_{DM} purified from *C. albicans*, and to compare the properties of the azole-*P*-450_{DM} complex with those for the *S. cerevisiae* cytochrome complex (Aoyama *et al.*, 1983; Yoshida *et al.*, 1986; Yoshida & Aoyama, 1987).

In common with *S. cerevisiae* *P*-450_{DM}, the interaction of ketoconazole with *C. albicans* *P*-450_{DM} induced a red shift of the Soret peak from 417 to 424 nm. The smaller red shift induced by ICI 153066 relative to ketoconazole has been noted for another triazole antifungal, itraconazole, when bound to *S. cerevisiae* *P*-450_{DM} (Yoshida & Aoyama, 1987). Such spectral shifts are characteristic of an azole nitrogen atom (N-3, imidazole; N-4, triazole) co-ordinating with the haem iron of ferric *P*-450 to form an artificial low-spin complex (White & Coon, 1982; Yoshida & Aoyama, 1987). Comparison of the binding of a number of azole derivatives to *S. cerevisiae* *P*-450_{DM} has demonstrated that the spectral properties of the cytochrome-azole complex depend on the co-ordinating species (imidazole or triazole), rather than the N-1 substituent groups (Yoshida & Aoyama, 1987). The spectral titration of *C. albicans* *P*-450_{DM} with ketoconazole or ICI 153066 demonstrated that both azoles bound to the cytochrome with high affinity (one-to-one stoichiometry). The same was true for buthiobate (Aoyama *et al.*, 1983) diniconazole (Yoshida *et al.*, 1986), ketoconazole and itraconazole (Yoshida & Aoyama, 1987) complexes with the *S. cerevisiae* cytochrome.

In the present study, the ability of the azole-ferrous-cytochrome complex to inhibit CO binding to haem was used as a guide to the affinity of ketoconazole and ICI 153066 for the ferrous cytochrome. Working with

S. cerevisiae *P*-450_{DM} under the same experimental conditions, Yoshida & Aoyama (1987) have reported that azoles with relatively large N-1 substituent groups (such as ketoconazole and itraconazole) bind more tightly to the ferrous cytochrome than do derivatives with smaller N-1 substituents. It is thought that large N-1 substituents lower the mobility of the azole molecule in the haem crevice, and therefore increase the affinity of the azole for the cytochrome. This argument may explain why ICI 153066, which has a relatively small N-1 substituent group, has a lower affinity for *C. albicans* ferrous *P*-450_{DM} than does ketoconazole.

All of the azole antifungals used in the present study inhibited reconstituted *P*-450_{DM} activity by forming an azole-cytochrome complex with a one-to-one stoichiometry. This fact indicates that the potency of ICI 153066 against enzyme activity does not relate to its ability to interfere with the binding of CO to the ferrous cytochrome. However, it is likely that the azoles work *in vivo* by binding to the active site of *P*-450_{DM} with high affinity and constraining the ferric cytochrome in its low-spin state. Besides inhibiting the binding of substrate molecules to the active site, this mode of action would prevent a low-spin to high-spin transition in the cytochrome and would therefore inhibit enzyme activity. The azoles used in the present study were roughly equipotent against *P*-450_{DM} in cell lysates, in agreement with their inhibition of reconstituted enzyme activity. This phenomenon applies also to the *S. cerevisiae* cytochrome (Yoshida & Aoyama, 1986).

C. albicans and *S. cerevisiae* cytochromes *P*-450_{DM} have similar spectral and enzymic properties *in vitro* (Aoyama *et al.*, 1984, 1987; Hitchcock *et al.*, 1989b). Moreover, comparison of the nucleotide sequences of the *P*-450_{DM} genes of both organisms has revealed that there is 62% amino acid identity between the proteins (Kalb *et al.*, 1987; Lai & Kirsch, 1989). The interaction of ketoconazole with *C. albicans* *P*-450_{DM} shows the same spectral and enzyme inhibitory characteristics as those of the *S. cerevisiae* cytochrome-ketoconazole complex. Taken together, the above data suggest strongly that *C. albicans* and *S. cerevisiae* cytochromes *P*-450_{DM} share the same structural features in the apoprotein of the haem crevice. Clearly, the efficacy of an azole as an inhibitor of *P*-450_{DM} depends on the geometric orientation of its azole moiety to the haem iron, which is determined by the binding of its N-1 substituents to the apoprotein of the cytochrome. All five azoles used in the present study have the appropriate structures for high-affinity binding to *P*-450_{DM}, making them potent inhibitors of ergosterol biosynthesis in *C. albicans*. Azoles inhibit a number of mammalian cytochrome *P*-450 isoenzymes, including *P*-450_{DM} (Ortiz de Montellano & Reich, 1986). Thus, future studies similar to those described here but with *P*-450s purified from mammalian cells will provide valuable background information for the development of new systemically active azoles with enhanced activity against fungal, rather than mammalian, cytochromes *P*-450_{DM}.

The financial support of the Medical Research Council and the Yorkshire Cancer Research Campaign is acknowledged.

REFERENCES

- Aoyama, Y., Yoshida, Y., Hata, S., Nishino, T. & Katsuki, H. (1983) *Biochem. Biophys. Res. Commun.* **115**, 642-647

- Aoyama, Y., Yoshida, Y. & Sato, R. (1984) *J. Biol. Chem.* **259**, 1661–1666
- Aoyama, Y., Yoshida, Y., Sonada, Y. & Sato, R. (1987) *J. Biol. Chem.* **262**, 1239–1243
- Ardies, C. M., Lasker, J. M., Blosswick, B. P. & Lieber, C. S. (1987) *Anal. Biochem.* **162**, 39–46
- Barrett-Bee, K. J., Lane, A. C. & Turner, R. W. (1986) *J. Med. Vet. Mycol.* **24**, 155–160
- Fromtling, R. A. (1988) *Clin. Microbiol. Rev.* **1**, 187–217
- Hitchcock, C. A., Barrett-Bee, K. J. & Russell, N. J. (1987) *J. Med. Vet. Mycol.* **25**, 329–333
- Hitchcock, C. A., Brown, S. B., Evans, E. G. V. & Adams, D. J. (1989a) *Biochem. J.* **260**, 549–556
- Hitchcock, C. A., Dickinson, K., Brown, S. B., Evans, E. G. V. & Adams, D. J. (1989b) *Biochem. J.* **263**, 573–579
- Kalb, V. F., Woods, C. W., Turi, T. G., Dey, C. R., Sutter, T. R. & Loper, J. C. (1987) *DNA* **6**, 529–537
- Lai, M. H. & Kirsch, D. R. (1989) *Nucleic Acids Res.* **17**, 804
- Marriott, M. S., Pye, G. W., Richardson, K. & Troke, P. F. (1986) in *In Vitro and In Vivo Evaluation of Antifungal Agents* (Iwata, K. & Vanden Bossche, H., eds.), pp. 143–146, Elsevier, Amsterdam
- Odds, F. C. (1988) *Candida and Candidosis*, 2nd edn., Bailliere Tindall, London
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378
- Ortiz de Montellano, P. R. & Reich, N. O. (1986) in *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., ed.), pp. 273–314, Plenum Press, New York
- Pye, G. W. & Marriott, M. S. (1982) *Sabouraudia* **20**, 325–329
- Trzaskos, J., Kawata, S. & Gaylor, J. L. (1986) *J. Biol. Chem.* **261**, 14651–14657
- Vanden Bossche, H. (1985) in *Current Topics in Medical Mycology* (McGinnis, M. R., ed.), pp. 313–351, Springer-Verlag, New York
- Vanden Bossche, H., Bellens, D., Cools, W., Gorrens, P. M., Verhoeven, H., Willemsens, G., De Coster, R., Beerens, D., Haelterman, C., Coene, M. C., Lauwers, W. & Le Jeune, L. (1986) *Drug Dev. Res.* **8**, 287–298
- Vanden Bossche, H., Marichal, P., Gorrens, J., Bellens, D., Verhoeven, H., Coene, M., Lauwers, W. & Janssen, P. A. J. (1987) *Pestic. Sci.* **21**, 289–306
- White, R. E. & Coon, M. J. (1982) *J. Biol. Chem.* **257**, 3073–3083
- Yeagle, P. L., Martin, R. B., Lala, A. K., Lin, H. K. & Block, K. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4924–4926
- Yoshida, Y. & Aoyama, Y. (1984) *J. Biol. Chem.* **259**, 1655–1660
- Yoshida, Y. & Aoyama, Y. (1986) in *In Vitro and In Vivo Evaluation of Antifungal Agents* (Iwata, K. & Vanden Bossche, H. eds.), pp. 123–134, Elsevier, Amsterdam
- Yoshida, Y. & Aoyama, Y. (1987) *Biochem. Pharmacol.* **36**, 229–235
- Yoshida, Y., Aoyama, Y., Takano, H. & Kato, T. (1986) *Biochem. Biophys. Res. Commun.* **137**, 513–519

Received 1 September 1989/17 October 1989; accepted 3 November 1989