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SELECTIVITY OF ISOPRENOID-CONTAINING IMIDAZOLE ANTIFUNGAL COMPOUNDS FOR STEROL 14-DEMETHYLASE P450 ($P450_{14DM}$) AND 7-ETHOXYCOUMARIN *O*-DEETHYLASE P450 OF RAT LIVER MICROSOMES

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Abstract—The imidazole antifungal compound AFK-108 (1-[2-(2,4-dichlorophenyl)-2-((2*E*)-3,7-dimethylocta-2,6-dienyloxy)ethyl]-1*H*-imidazole) has been shown to be a potent inhibitor for yeast lanosterol 14α-demethylase ($P450_{14DM}$), interacting specifically with the sterol side-chain recognition part of the substrate site through its geranyl moiety. AFK-108 acted as a potent inhibitor for rat liver $P450_{14DM}$, while its farnesyl (AFK-110) and prenyl (AFK-122) homologues were weak inhibitors. This indicates that AFK-108 interacts with rat liver $P450_{14DM}$ in the same manner as with the yeast enzyme. However, the difference between the potency of AFK-108 and the homologues was greater in rat $P450_{14DM}$ than in the yeast enzyme. AFK-108 and its homologues partially inhibited 7-ethoxycoumarin *O*-deethylase activity of rat liver microsomes. The order of potency was AFK-122 > AFK-108 > AFK-110, indicating that some steric hindrance of the isoprenoid moiety might affect their potency. The inhibitory effect of AFK-108 for $P450_{14DM}$ was considerably higher than for 7-ethoxycoumarin *O*-deethylase P450, while the inhibition of AFK-110 and AFK-122 on these enzymes was of the same order of magnitude. These results suggest that azole compounds interacting with the side-chain recognition site of $P450_{14DM}$ may be good candidates as antifungal agents selective for fungal $P450_{14DM}$.

Key words: cytochrome P450; antifungal agent; sterol demethylase inhibitor; target selectivity; sterol demethylase; rat liver

Azole compounds acting as potent inhibitors of fungal sterol 14α-demethylase P450 ($P450_{14DM}$§ [1]) are used as antifungal agents [2]. Sterol 14α-demethylation is a key reaction in cholesterol biosynthesis by mammals which is also catalysed by $P450_{14DM}$ [3]. Accordingly, systemically applied azole antifungal agents may possibly inhibit mammalian $P450_{14DM}$. Furthermore, if these agents act as inhibitors for other P450s, many adverse reactions, such as the enhancement of pharmacologic activity and toxicity of co-administered drugs and perturbation of steroid hormone levels may occur. Therefore, azole antifungal agents are a pre-requisite for high selectivity for fungal $P450_{14DM}$.

Azole antifungal agents bind to the heme iron of $P450_{14DM}$ by their azole nitrogen [4, 5]. These compounds also interact with the substrate binding site or its vicinity with their hydrophobic substituents [6–8]. The affinity of azole antifungal agents for $P450_{14DM}$ is thus determined by how the hydrophobic substituents fit the substrate-binding site when they are anchored at the heme iron by the azole nitrogen [6]. In other words, the affinity of azole compounds for P450 may be modified by altering the structure of the hydrophobic substituents that may interact with the substrate binding site and by their geometry in relation to the azole nitrogen.

In previous work, Aoyama and Yoshida [9]

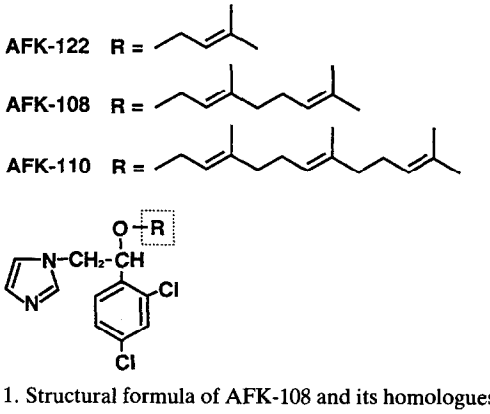

Fig. 1. Structural formula of AFK-108 and its homologues.

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§ Abbreviations: $P450_{14DM}$, sterol 14α-demethylase P450; DTT, dithiothreitol; DLPC, dilauroylphosphatidylcholine.

showed that $P450_{14DM}$s of yeast and rat liver recognized the structure of the C_{17} side-chain of the sterol substrates such as lanosterol, 24,25-dihydrolanosterol and 24-methylene-24,25-dihydrolanosterol differently. It was also reported that yeast and rat liver $P450_{14DM}$s showed different recognition for the length of the C_{17} side-chain of 24,25-dihydrolanosterol [10, 11]. A novel azole antifungal compound AFK-108 (1-[2-(2,4-dichlorophenyl)-2-((2*E*)-3,7-dimethylocta-2,6-dienyloxy)-ethyl]-1*H*-imidazole, Fig. 1 and Ref. 12) interacted with the side-chain recognition site of yeast $P450_{14DM}$ with its geranyl group, and the AFK-108 homologues having a prenyl group (AFK-122) or a farnesyl group (AFK-110) showed lower affinity than AFK-108 for the P450 [13]. Consequently, it is interesting to compare the inhibitory effects of AFK-108 and its homologues (Fig. 1) for mammalian $P450_{14DM}$ with those for the yeast enzyme. This paper describes the inhibitory effects of AFK-108 and its homologues on partially purified rat liver lanosterol 14α-demethylase and on 7-ethoxycoumarin *O*-deethylase activity of rat liver microsomes.

MATERIALS AND METHODS

Partially purified $P450_{14DM}$ from rat liver microsomes. Liver microsomes (1000 mg protein) prepared from male untreated Wistar rats (8 weeks) were suspended in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol, 1 mM DTT, 0.1 mM EDTA and 0.25 mM phenylmethylsulfonyl fluoride to give a protein concentration of 7.0 mg/mL, and were solubilized with 0.6% sodium cholate for 60 min. The mixture was centrifuged at 102,000 *g* for 90 min. The resulting supernatant (412 mg protein) was applied to an Aminohexyl-Sepharose 4B column (1.5 × 16 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol, 0.6% sodium cholate, 1 mM DTT and 0.1 mM EDTA. The column was washed with 10 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol, 0.6% sodium cholate, 1 mM DTT and 0.1 mM EDTA. The adsorbed P450 was then eluted with 0.2% Emulgen 913 in the same buffer. The P450-containing eluate was dialysed overnight against 5 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol, 0.2% Emulgen 913, 1 mM DTT and 0.1 mM EDTA. The dialysate was applied to a column (1.5 × 7.0 cm) of DE-52 equilibrated with the dialysis buffer and the column was washed with the same buffer. The lanosterol 14α-demethylase activity was eluted from the column under these conditions, although most of the P450 remained in the column. The fractions rich in lanosterol 14α-demethylase activity were applied to a column (0.7 × 3.0 cm) of hydroxylapatite equilibrated with 5 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol, 0.05% Emulgen 913 and 1 mM DTT. The column was washed with 1.5 volumes of the equilibration buffer and $P450_{14DM}$ was eluted from the column with 150 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol, 0.05% Emulgen 913 and 1 mM DTT. The fractions rich in the lanosterol 14α-demethylase activity were collected as the partially purified $P450_{14DM}$ preparation.

Other materials. NADPH-P450 reductase was purified from phenobarbital-induced rat liver microsomes according to the method of Yasukochi and Masters [14]. Liver microsomes used as the enzyme preparation for the 7-ethoxycoumarin *O*-deethylase assay were prepared from untreated male Wistar rats according to the conventional method. AFK-108 and its derivatives were synthesized as described elsewhere [15]. Lanosterol (97% pure), chemically synthesized DLPC, 7-ethoxycoumarin and ketoconazole were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Aminohexyl-Sepharose 4B (EAH-Sepharose 4B), DE-52 and hydroxylapatite (Bio-Gel HT) were the products of Pharmacia Co. (Uppsala, Sweden), Whatman Co. (Maidstone, U.K.) and Bio-Rad Laboratories (Richmond, CA, U.S.A.), respectively. Other chemicals and biochemicals were the purest reagents obtainable commercially.

Enzyme assay. The lanosterol 14α-demethylase activity of the reconstituted system consisting of the partially purified $P450_{14DM}$ and NADPH-P450 reductase was assayed according to the method developed for the assay of the yeast counterpart [16] with the following modifications. The partially purified $P450_{14DM}$ (0.12 nmol, 20 μL), NADPH-P450 reductase (1.2 U, 30 μL) and lanosterol micelles in DLPC (10 nmol lanosterol in 50 μg DLPC, 50 μL) were well mixed in a test tube. The mixture was diluted with 1.0 mL of 0.2 M potassium phosphate buffer, pH 7.5, and EDTA (0.5 mM), glucose-6-phosphate (10 mM) and glucose-6-phosphate dehydrogenase (0.25 U) were added to the solution to give the indicated concentrations. Various concentrations of azole compounds were added to the mixture as 10 μL of DMSO solutions. The reaction was started by the addition of 10 μL of 30 mM NADPH. The final volume of the reaction mixture was 2.0 mL. The reaction was run at 37° for 20 min and terminated by the addition of 5.0 mL of 20% KOH in methanol. The mixture was spiked with 16.5 nmol of cholesterol as the internal standard and saponified at 80° for 60 min. Sterols were extracted from the saponified mixture and analysed by GLC as described previously [16]. The amount of the demethylated product, 4,4-dimethylcholesta-8,14,24-trienol, was determined in relation to the cholesterol internal standard and the activity was expressed as the amount of product formed per min. 7-Ethoxycoumarin *O*-deethylase activity of rat liver microsomes was assayed by the method of Greenlee and Poland [17]. 7-Ethoxycoumarin *O*-deethylase and aminopyrine *N*-demethylase activities of the partially purified $P450_{14DM}$ were assayed in the same reaction mixture as that of the lanosterol demethylase assay except that lanosterol was omitted from the DLPC micelles and 2.0 mM 7-ethoxycoumarin and 1.0 mM aminopyrine, respectively, were added as the substrates. 7-Hydroxycoumarin was determined according to the method of Greenlee and Poland [17] and formaldehyde was determined by the method of Nash [18]. Azole compounds were added to the reaction medium as 10 μL of DMSO solution.

Other methods. P450 content of the rat liver

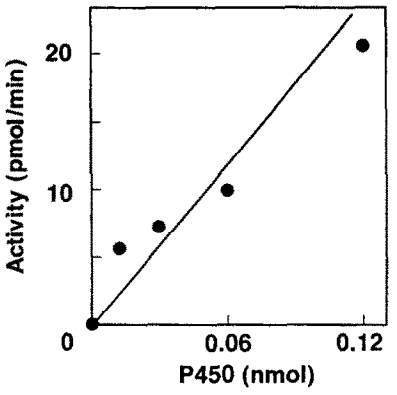

Fig. 2. Dependency on $P450_{14DM}$ concentration of lanosterol 14α-demethylase activity. Activity of the reconstituted system containing various amounts of the partially purified $P450_{14DM}$ was assayed as described in Materials and Methods.

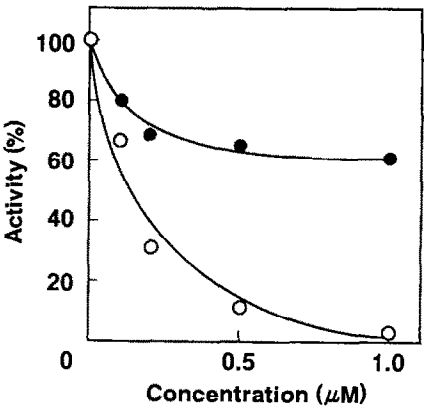

Fig. 3. Inhibition by ketoconazole of lanosterol 14α-demethylase activity of the reconstituted system and 7-ethoxycoumarin *O*-deethylase activity of the liver microsomes from untreated rats. Activities of lanosterol 14α-demethylase and 7-ethoxycoumarin *O*-deethylase were assayed as described in Materials and Methods in the presence of indicated concentrations of ketoconazole. Ketoconazole was added to the reaction mixture as 10 μL of DMSO solution and the same volume of the solvent was added to the control. (○) Lanosterol 14α-demethylase and (●) 7-ethoxycoumarin *O*-deethylase.

microsomes was determined from the carbon monoxide-difference spectrum of sodium dithionite-reduced microsomes by using $\Delta\ \varepsilon\ 450\text{–}490 = 91.1\ mM^{-1}\,cm^{-1}$ [19]. The P450 content of the partially purified preparation was calculated from the absolute spectrum of the oxidized form by using $\varepsilon_{418} = 107\ mM^{-1}\,cm^{-1}$ [20]. Protein was assayed by the method of Lowry *et al.* [21] using bovine serum albumin as standard.

RESULTS

Lanosterol 14α-demethylase activity of the partially purified rat liver $P450_{14DM}$

Upon reconstitution with 1.2 U of NADPH-P450 reductase in the presence of DLPC, the partially purified $P450_{14DM}$ from rat liver microsomes catalysed the conversion of lanosterol to the 14-demethylated product, 4,4-dimethylcholesta-8,14,24-trienol. The reaction proceeded linearly with time up to 30 min and the activity was dependent on the amount of P450 (Fig. 2). On the basis of these fundamental observations, the standard assay conditions for the lanosterol 14α-demethylase activity with the partially purified rat liver $P450_{14DM}$ were set up as described in the Materials and Methods. A representative value of the demethylase activity under these conditions was 0.2 nmol/min/nmol P450 or 0.9 nmol/min/mg protein. These values indicated that the $P450_{14DM}$ preparation was still contaminated with other proteins and P450s. However, the P450 preparation showed neither 7-ethoxycoumarin *O*-deethylase nor aminopyrine *N*-demethylase activity.

Inhibitory effect of ketoconazole on the lanosterol 14α-demethylase activity of the partially purified rat liver $P450_{14DM}$ and 7-ethoxycoumarin O-deethylase activity of the rat liver microsomes

Before evaluating the effects of AFK-108 and its homologues, the inhibitory effects of ketoconazole, a typical azole antifungal agent, on lanosterol 14α-demethylase activity of the partially purified rat liver $P450_{14DM}$ and 7-ethoxycoumarin *O*-deethylase activities of the rat liver microsomes were studied as a reference. Ketoconazole inhibited the lanosterol 14α-demethylase activity with an apparent IC_{50} of 0.12 μM (Fig. 3). This IC_{50} value was twice as high as the $P450_{14DM}$ concentration in the reaction medium (0.06 μM). For comparison, ketoconazole showed complete inhibition of the yeast lanosterol 14α-demethylase below 0.1 μM and the apparent IC_{50} corresponded to half of the $P450_{14DM}$ concentration in the medium [4]. These facts clearly indicate that the susceptibility of the rat liver $P450_{14DM}$ to ketoconazole inhibition is less than that of the yeast enzyme.

Ketoconazole caused about 40% inhibition of the 7-ethoxycoumarin *O*-deethylase activity of the rat liver microsomes at 1.0 μM (Fig. 3). However, little further inhibition was observed when the ketoconazole concentration was elevated up to 10 μM. It can therefore be concluded that more than one P450 species is responsible for 7-ethoxycoumarin *O*-deethylase activity, only part of which is inhibited by ketoconazole. The dose–response profile of ketoconazole inhibition on the ketoconazole-sensitive fraction of the ethoxycoumarin deethylase activity was close to that on the lanosterol demethylase activity. However, the partially purified $P450_{14DM}$ preparation used in this study showed no 7-ethoxycoumarin *O*-deethylase activity, as mentioned above, and inhibitory effects of AFK homologues on this activity were different from those on lanosterol 14α-demethylase (see below). Therefore, the ketoconazole-sensitive 7-ethoxycoumarin *O*-deethylase activity was not due to $P450_{14DM}$.

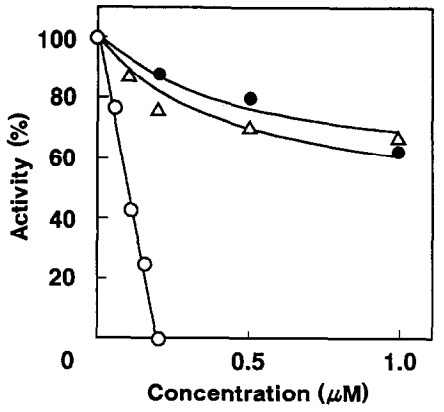

Fig. 4. Inhibition of lanosterol 14α-demethylase activity of the reconstituted system by AFK-108 and its homologues. Lanosterol 14α-demethylase activity of the reconstituted system was assayed as described in Materials and Methods in the presence of indicated concentrations of the AFK homologues. The AFK homologues were added to the reaction mixture as 10 μL of DMSO solution and the same volume of the solvent was added to the control. (○) AFK-108, (●) AFK-110 and (△) AFK-122.

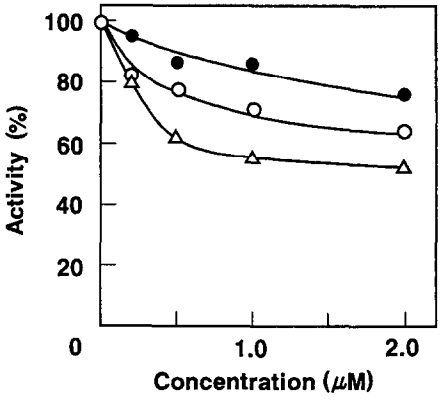

Fig. 5. Inhibition of 7-ethoxycoumarin *O*-deethylase activity by AFK-108 and its homologues. 7-Ethoxycoumarin *O*-deethylase activity of the liver microsomes from the untreated rats was assayed as described in Materials and Methods in the presence of indicated concentrations of the AFK homologues. The AFK homologues were added to the reaction mixture as 10 μL of DMSO solution and the same volume of the solvent was added to the control. (○) AFK-108, (●) AFK-110 and (△) AFK-122.

Inhibitory effects of AFK-108 and its homologues on lanosterol 14α-demethylase activity of the partially purified rat liver P450$_{14DM}$

AFK-108 is one of the most potent inhibitors for yeast P450$_{14DM}$ and causes complete inhibition at 0.07 μM or less with one to one stoichiometry to the enzyme [13]. AFK-108 inhibited the lanosterol 14α-demethylase activity of rat liver P450$_{14DM}$ (Fig. 4). The inhibitory effect of AFK-108 was stronger than that of ketoconazole (Fig. 3), and complete inhibition was observed at 0.2 μM. However, nearly 50% of the activity remained at 0.06 μM corresponding to the concentration of P450 in the reaction medium. This fact indicates that the affinity of AFK-108 for the rat liver P450$_{14DM}$ is lower than that for the yeast enzyme. The AFK-108 homologues having the shorter prenyl (AFK-122) and the longer farnesyl (AFK-110) groups also inhibited the rat liver P450$_{14DM}$. However, their inhibitory effects were weaker than AFK-108 and the apparent IC_{50} of these compounds was assumed to be 40–50 times higher than that of AFK-108 by extrapolating the data shown in Fig. 4. AFK-110 and AFK-122 were also weaker inhibitors than AFK-108 for yeast P450$_{14DM}$ [13]. However, AFK-110, the weakest one, completely inhibited the yeast enzyme at 0.2 μM [13] and the dose–response profile of AFK-110 inhibition of the yeast P450$_{14DM}$ [13] was almost the same as that of AFK-108 inhibition of the rat enzyme (Fig. 4). The difference between the inhibitory effects of AFK-122 and AFK-108 on the yeast enzyme was very small and AFK-122 completely inhibited the yeast enzyme at 0.1 μM [13]. These observations indicate that rat liver P450$_{14DM}$ is less sensitive than the yeast counterpart for AFK-108 and its homologues. The difference in sensitivity for AFK-108 and the other two compounds is considerably larger in the rat liver enzyme compared with the yeast enzyme [13]. This indicates that the rat liver P450$_{14DM}$ discriminates the length of the isoprenoid chains of the AFK homologues more strictly than the yeast enzyme.

Inhibitory effects of AFK-108 and its homologues on 7-ethoxycoumarin O-deethylase activity of the rat liver microsomes

Figure 5 summarizes the effects of AFK-108 and its homologues on the 7-ethoxycoumarin *O*-deethylase activity of rat liver microsomes. AFK-122 was as potent an inhibitor as ketoconazole and was the strongest of the homologues. The order of potency was AFK-122 > AFK-108 > AFK-110 and was in the reverse order of the size of their isoprenoid moieties. The inhibition by these compounds was incomplete as in the case of ketoconazole (Fig. 3), and about 50% of the activity remained, even in the presence of 10 μM of AFK-122. This suggests that the AFK homologues might inhibit the same P450 species as that which is sensitive to ketoconazole.

DISCUSSION

In the preceding paper, Aoyama *et al.* [13] reported that the potent inhibitory effect of AFK-108 on yeast P450$_{14DM}$ was due to the simultaneous interaction of its geranyl moiety and imidazole nitrogen with the substrate-binding site and the heme iron, respectively. Affinities of the AFK-108 derivatives having other isoprenoid chains or modified geranyl groups were lower than that of AFK-108, indicating that yeast P450$_{14DM}$ favorably interacted with the geranyl group that was structurally similar to the C_{17} side-chain of lanosterol [13]. On the basis of these facts, Aoyama *et al.* [13] concluded that the isoprenoid moiety of AFK homologues

interacted with the side-chain recognition part of the substrate-binding site of yeast $P450_{14DM}$.

The reconstituted rat liver lanosterol 14α-demethylase system showed the greatest sensitivity to AFK-108 (Fig. 4), indicating that the mode of interaction of rat liver $P450_{14DM}$ with these compounds was essentially the same as that of the yeast enzyme [13]. However, the inhibitory effects of the AFK homologues on the rat liver enzyme (Fig. 4) were lower than those on the yeast enzyme. AFK-108 caused about 40% inhibition of the reconstituted rat liver demethylase system at 0.06 μM that was equivalent to the P450 concentration in the reaction medium (Fig. 4), while it inhibited the yeast enzyme stoichiometrically below 0.07 μM [13]. AFK-110 caused only 40% inhibition on the rat liver enzyme even at 1.0 μM (Fig. 4), although it completely inhibited the yeast enzyme at 0.2 μM [13]. The inhibitory effect of AFK-122 on the rat liver enzyme was as low as AFK-110 (Fig. 4), while this compound was only a slightly weaker inhibitor than AFK-108 of the yeast enzyme [13]. In addition, the sensitivity for ketoconazole of rat liver $P450_{14DM}$ (Fig. 3) was also lower than that of the yeast enzyme [4]. This suggests that rat liver $P450_{14DM}$ is less sensitive for azole compounds than the yeast counterpart.

The difference between the inhibitory effects of AFK-108 and the other two homologues on rat liver $P450_{14DM}$ was remarkable (Fig. 4). Although qualitatively the same difference was observed on yeast $P450_{14DM}$ the difference was not so large [13]. It was reported by Aoyama and Yoshida [9] that yeast and rat liver $P450_{14DM}$s recognized the structure of the C_{17} side-chain of the sterol substrates differently. Aoyama *et al.* [10] also demonstrated that the trimming of the C_{17} side-chain of lanosterol up to and including C_{25} did not reduce the affinity for yeast $P450_{14DM}$. On the other hand, Sonoda *et al.* [11] reported that rat liver $P450_{14DM}$ showed lower affinity for the lanosterol derivatives having the trimmed C_{17} side-chains than for the parent. Consequently, it can be concluded that rat liver $P450_{14DM}$ has stricter selectivity for the size of the C_{17} side-chain of the sterol substrate than the yeast enzyme and this may reflect on their selectivity and sensitivity for the AFK homologues.

The AFK homologues partially inhibited the 7-ethoxycoumarin *O*-deethylase activity of the rat liver microsomes (Fig. 5). Partial inhibition of this activity was also observed with ketoconazole (Fig. 3), buthiobate [22], a pyridine-containing $P450_{14DM}$ inhibitor [23], and triadimenol (Yoshida *et al.*, unpublished). Therefore, at least one species of P450 responsible for 7-ethoxycoumarin O-deethylation may be sensitive to a wide variety of $P450_{14DM}$ inhibitors. The order of potency of the AFK homologues as inhibitors of this P450 was inversely correlated with the length of their isoprenoid moieties, suggesting some steric hindrance with the isoprenoid moiety. The inhibitory effect of AFK-108 for this activity was weaker than that for lanosterol 14α-demethylase activity, while AFK-122 inhibited this activity more strongly than the lanosterol demethylase (Figs 4 and 5). On the basis of these observations, it is concluded that AFK-108 shows high selectivity for $P450_{14DM}$ because of the structure-specific interaction of its geranyl moiety with the substrate-binding site of the enzyme, while AFK-122, the prenyl homologue of AFK-108, does not show marked selectivity for those P450s susceptible to azole compounds.

It is apparent from the results described in this paper that the geranyl moiety of AFK-108 favorably interacts with the substrate-binding site of $P450_{14DM}$ that recognizes the C_{17} side-chain of the substrate. The substrate-binding site of rat liver $P450_{14DM}$ strictly discriminates the size of isoprenoid chain both for the sterol substrates and the AFK homologues. In contrast, yeast $P450_{14DM}$ showed rather low selectivity for the isoprenoid chains of the substrates and the AFK homologues. As discussed in previous papers [9, 24, 25], $P450_{14DM}$s from different origins showed different selectivity for the side-chain of sterols and this difference may explain the different substrates for sterol 14-demethylation in the sterol biosynthetic pathways. It can thus be concluded that the azole compounds interacting with the side-chain recognition part of the active site of $P450_{14DM}$ are good candidates for selective inhibition of fungal $P450_{14DM}$.

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