Biochemical Pharmacology, Vol. 44, No. 9, pp. 1701–1705, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

INHIBITION BY A NOVEL AZOLE ANTIFUNGAL AGENT WITH A GERANYL GROUP ON LANOSTEROL 14α-DEMETHYLASE OF YEAST

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(*Received* 2 *July* 1992; *accepted* 28 *August* 1992)

Abstract—AFK-108 (1-[2-(2,4-dichlorophenyl)-2-((2*E*)-3,7-dimethylocta-2,6-dienyloxy)ethyl]-1*H*-imidazole) is a new imidazole derivative characterized by a geranyl substituent showing strong antifungal activity. Azole antifungal agents are known to be potent inhibitors of lanosterol 14α-demethylase ($P450_{14DM}$) of fungi. The role of the geranyl group of AFK-108 on interaction of AFK-108 with the target was studied by using *Saccharomyces cerevisiae* $P450_{14DM}$ as the model enzyme. AFK-108 and some of its derivatives bound to oxidized $P450_{14DM}$ with one-to-one stoichiometry and inhibited the demethylase activity. AFK-108 derivatives having the longer farnesyl or the shorter prenyl group showed lower affinity than AFK-108 for the enzyme. AFK-108 caused 100% inhibition at the equivalent concentration to $P450_{14DM}$ in the reaction mixture (0.07 μM), while the farnesyl derivative inhibited the activity by 60% at the same concentration. AFK-108 interfered with the binding of CO to the ferrous $P450_{14DM}$. However, the interfering effect of the prenyl derivative was lower than that of AFK-108. Another AFK-108 derivative having the saturated 3,7-dimethyloctyl group was also a weaker inhibitor than AFK-108. These experiments suggest that the geranyl group of AFK-108 interacts with the substrate binding site of $P450_{14DM}$ that recognises the side chain of the substrate. AFK-108 is the first example of an azole derivative interacting with the side chain recognising region of the substrate binding site of $P450_{14DM}$.

Azole antifungal agents are known to inhibit ergosterol biosynthesis by fungi and to cause accumulation of 14α-methylsterols such as lanosterol and 24-methylene-24,25-dihydrolanosterol in the fungal cells [1]. This suggests that azole antifungal agents inhibit 14α-demethylation of ergosterol precursors. The 14α-demethylation of the precursor sterols is catalysed by the P450 monooxygenase, lanosterol 14α-demetylase ($P450_{14DM}$) [2], and azole antifungal agents act as potent inhibitors for this enzyme [1, 3, 4]. All azole antifungal agents so far tested interact with the heme iron of $P450_{14DM}$ via their azole nitrogen [1, 3–5]. However, their potency as $P450_{14DM}$ inhibitors depends on the hydrophobic substituents that may interact with the substrate binding site (or vicinity) of the enzyme [4, 5]. Moreover, stereoisomers of a chiral azole compound, such as triadimenol, showed different affinity for $P450_{14DM}$ [3, 6]. These facts suggest that an azole compound whose conformation is favorable for simultaneous interaction with the heme iron and the substrate binding site may act as a potent inhibitor.

Our recent studies indicated that three parts of the substrate, the 3-hydroxyl group [7], the body of sterol ring with 8-lanostene conformation [8] and the terminal part of the side chain [9], interacted with the active site of $P450_{14DM}$. Accordingly, the substrate binding site of the enzyme may consist of three regions interacting with each of these parts. Therefore, the azole derivatives that can interact simultaneously with at least one of these regions and the heme iron may act as potent $P450_{14DM}$ inhibitors [10]. AFK-108 (1-[2-(2,4-dichlorophenyl)-2-((2*E*)-3,7 - dimethylocta - 2,6 - dienyloxy)ethyl] - 1*H*-imidazole, compound **1** of Fig. 1) is a novel imidazole derivative having a geranyl group, which is closely similar to the sterol side chain, as a hydrophobic substituent [11]. In this study, we examined the interaction of AFK-108 and its derivatives (Fig. 1) with purified $P450_{14DM}$ of *Saccharomyces cerevisiae* focusing on the role of the geranyl group. Results indicate that the geranyl group occupied the substrate binding site of $P450_{14DM}$ that recognises the side chain of the substrate when the imidazole nitrogen bound to the heme iron. Consequently, AFK-108 acted as a potent inhibitor of $P450_{14DM}$.

MATERIALS AND METHODS

AFK-108 and its derivatives (Fig. 1) were synthesized according to the method described elsewhere [11]. $P450_{14DM}$ and NADPH-P450 reductase were purified from semi-anaerobically grown cells of *S. cerevisiae* according to the methods of Yoshida and Aoyama [12] and Aoyama *et al.* [9, 13], respectively. Lanosterol (97% pure) was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Other chemicals were of the purest reagents from commercial sources. $P450_{14DM}$ activity of the reconstituted system consisting of the purified $P450_{14DM}$ and NADPH-P450 reductase was assayed as follows. The basic reaction mixture consisted of 0.07 μM $P450_{14DM}$, 1 U NADPH-P450 reductase and

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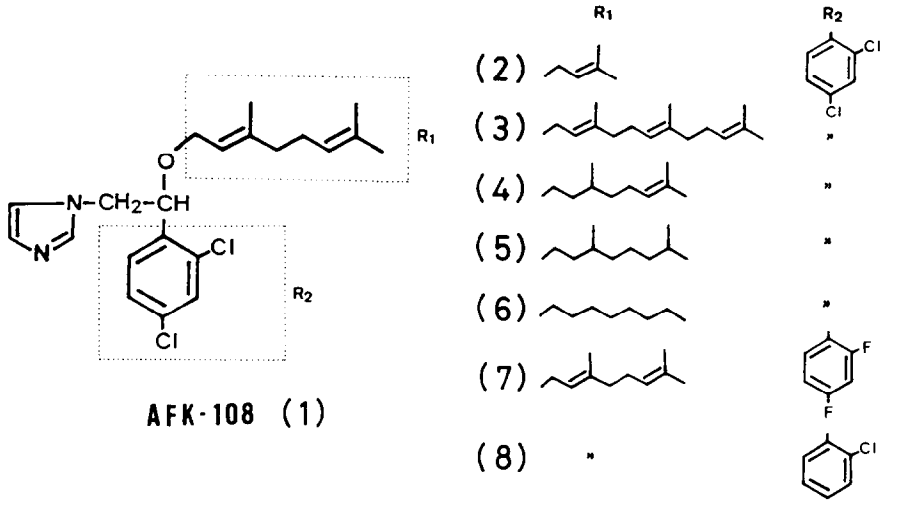

Fig. 1. Structural formula of AFK-108 and its derivatives used in this study. The numbers in parentheses are the numbers of the compounds cited in the text, **1** through **8**.

10 nmol lanosterol dispersed with dilauroylphosphatidylcholine and 0.1 M potassium phosphate, pH 7.5. AFK-108 (or its derivatives) was added to the reaction mixture as a dimethyl sulfoxide solution (5 μL) to give various final concentrations. The reaction was started by the addition of NADPH to give a final concentration of 0.15 mM and a final volume of 1.0 mL. The reaction was run for 5 min at 30° under aerobic conditions with constant shaking. The extracted lanosterol and its 14-demethylated product were analysed with GLC as described previously [2, 9]. Demethylase activity was calculated from the amount of the 14-demethylated product by using cholesterol as the internal standard for the GLC assay. Binding of azole compounds to $P450_{14DM}$ was detected spectrophotometrically. Detailed conditions for the spectrophotometric analysis are given in the figure legends.

RESULTS

Spectrophotometric analysis of the interaction of AFK-108 and its derivatives with $P450_{14DM}$

Binding of AFK-108 (**1**) and its derivatives (**2** and **3** of Fig. 1) to ferric $P450_{14DM}$ was analysed spectrophotometrically. AFK-108 (**1**) and its derivatives having a prenyl (**2**) or farnesyl (**3**) group caused the same spectral change of ferric $P450_{14DM}$. Figure 2 represents a representative spectral change caused by **1**. This spectral change is characteristic of the Type II spectral change indicating that the imidazole nitrogen of **1** (also **2** and **3**) bound to the heme iron of $P450_{14DM}$ as an external 6th ligand.

The extent of the Type II spectral change is dependent on the amount of P450–ligand complex. Thus, binding of **1**, **2** and **3** to $P450_{14DM}$ was estimated by spectrophotometric titration (Fig. 3). The result shown in Fig. 3 indicates that **1**, **2** and **3** formed one-to-one complexes with $P450_{14DM}$. The titration curve of **1** was linear up to the saturation point and the

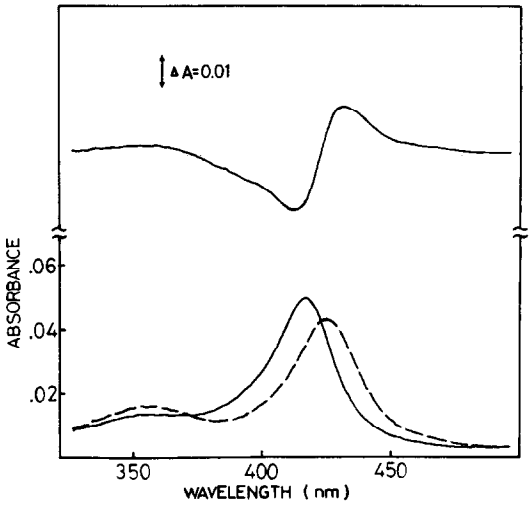

Fig. 2. Spectral change of ferric $P450_{14DM}$ caused by binding with AFK-108. $P450_{14DM}$ was diluted with 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol to give a final concentration of 0.45 μM. Then, the equivalent amount of AFK-108 to $P450_{14DM}$ was added. Spectra (——) and (— —) represent the free and AFK-108-bound form, respectively. The spectrum shown in the upper half is the difference spectrum (bound *minus* free).

concentration of **1** at the saturation point was equal to the $P450_{14DM}$ concentration (0.45 μM). This fact indicates that the dissociation constant (K_d) of **1** was considerably lower than the concentration of $P450_{14DM}$ used in the titration. However, titration at a lower concentration of $P450_{14DM}$ than 0.1 μM was inadequate for determining a reliable K_d because of the increasing noise of the spectrophotometer. Therefore, the exact K_d of **1** could not be determined.

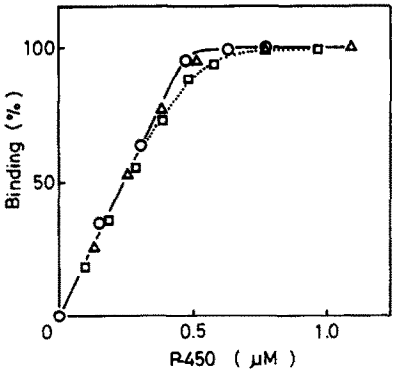

Fig. 3. Spectrophotometric titration of ferric $P450_{14DM}$ by AFK-108 and its derivatives. $P450_{14DM}$ (0.45 μM) in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol was titrated with the indicated amounts of AFK-108 (**1**) and its derivatives (**2** and **3**). Binding of these compounds to $P450_{14DM}$ was determined by the extent of difference spectra (see Fig. 2). (○) **1**, (△) **2** and (□) **3**.

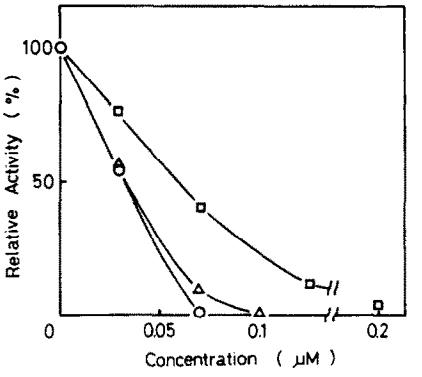

Fig. 5. Inhibition of $P450_{14DM}$ activity by AFK-108 and its derivatives having isoprenoid substituents of different sizes. $P450_{14DM}$ activity of the reconstituted system was assayed as described in Materials and Methods in the presence of the indicated amounts of AFK-108 (**1**) and its derivatives (**2** and **3**). (○) **1**, (△) **2** and (□) **3**.

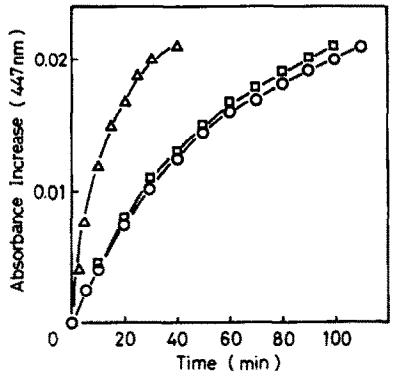

Fig. 4. Replacement with CO of AFK-108 and its derivatives bound to ferrous $P450_{14DM}$. AFK-108 (**1**) or derivative (**2** or **3**) was added to ferric $P450_{14DM}$ (0.45 μM) in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol to give a final concentration of 1.0 μM. Under these conditions, ferric $P450_{14DM}$ was saturated with these azoles (see Fig. 3). Then, the azole complexes of ferric $P450_{14DM}$ were reduced by sodium dithionite. After the reduction of the complexes was completed (30 min), CO was added to the reduced complexes, and appearance of the 447 nm absorption band due to the ferrous–CO complex of $P450_{14DM}$ was followed spectrophotometrically. (○) **1**, (△) **2** and (□) **3**.

Titration curves of **2** and **3** indicated some dissociation of these compounds from $P450_{14DM}$ under the experimental conditions. The deviation of the titration curve of **2** from that of **1** was slight but was highly reproducible, and the deviation of the titration curve of **3** from that of **1** was larger. Thus, the order of affinity for $P450_{14DM}$ was assumed to be **1** > **2** > **3**.

Compounds **1**, **2** and **3** interfered with the binding of CO to ferrous $P450_{14DM}$ (Fig. 4). When CO was added to ferrous $P450_{14DM}$ without azole compound, the 447-nm peak of the reduced CO complex appeared promptly (data not shown). In contrast, binding of CO to the ferrous $P450_{14DM}$ bound with azole compound took place over a considerably long time: the time necessary for full replacement with CO was about 110, 40 and 100 min for the complexes with **1**, **2** and **3**, respectively (Fig. 4). Absorption spectra of the ferrous $P450_{14DM}$ bound with **1**, **2** or **3** indicate the interaction of an imidazole nitrogen with the heme iron (data not shown). Therefore, conversion of the azole complexes of ferrous $P450_{14DM}$ to the CO complex is considered to be replacement of the azole nitrogen with CO at the heme iron. Thus, the results shown in Fig. 4 suggest that CO replaced **2** having a smaller prenyl group more readily than **1** or **3** having a larger geranyl or farnesyl group.

Effect of the size of the isoprenoid chain on the inhibitory effects of AFK-108 and its derivatives on $P450_{14DM}$ activity

AFK-108 (**1**) and its derivatives (**2** and **3**) inhibited lanosterol 14α-demethylation by the reconstituted system consisting of purified $P450_{14DM}$ and NADPH-P450 reductase (Fig. 5). Inhibition by **1** was quantitative and the activity was completely inhibited by 0.07 μM of **1** equivalent to the concentration of $P450_{14DM}$ in the reaction mixture. As discussed in the above section, such linear titration indicated that **1** showed no dissociation under the experimental conditions, and the K_d or IC_{50} of **1** was too low to estimate from the experiment shown in Fig. 5. To obtain some information about the IC_{50} of **1**, the demethylation reaction was assayed by using 0.007 μM of $P450_{14DM}$, but the amount of demethylated product was too low to estimate exact activity. However, it was confirmed that the activity was completely inhibited by 0.007 μM of **1** under these conditions (data not shown). Thus, it can be concluded that the K_d or IC_{50} of **1** is less than 0.007 μM. The inhibitory effect of **3** having the

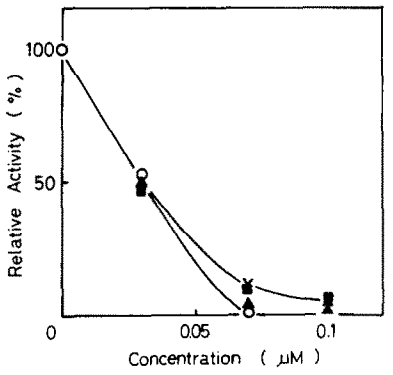

Fig. 6. Effects of saturation of the double bond and removal of the methyl groups of the geranyl moiety on the inhibitory effect of AFK-108. $P450_{14DM}$ activity was assayed under the same conditions as in Fig. 5 in the presence of AFK-108 (**1**) and its derivatives (**4**, **5** and **6**). (○) **1**, (▲) **4**, (■) **5** and (×) **6**.

longer farnesyl group on the demethylase activity was considerably lower than that of **1**. As shown in Fig. 5, **3** inhibited the activity by 60% at 0.07 μM and the IC_{50} of **3** estimated from Fig. 5 was 0.06 μM. The result shown in Fig. 5 seems to suggest that the inhibitory effect of **2** having the shorter prenyl group was comparable to that of **1**. However, **2** did not cause complete inhibition at the concentration equivalent to that of $P450_{14DM}$ (0.07 μM, Fig. 5), and this result was highly reproducible. Therefore, **2** is considered to be a weaker inhibitor than **1**.

Role of the double bonds of the geranyl moiety in the inhibitory effect of AFK-108

Figure 6 summarizes the results of the inhibition experiment with the AFK-108 derivatives regarding the double bond and the branched methyl groups of the geranyl moiety (**4**, **5** and **6** of Fig. 1). The AFK-108 derivatives having the saturated 3,7-dimethyloctyl (**5**) or octyl (**6**) group instead of the geranyl group were significantly weaker inhibitors than **1**. In contrast, **4** having the 6-double bond was a potent inhibitor comparable to **1**. These findings suggest the important role of the double bond ($^6\triangle$) at the terminal part of the geranyl moiety in the inhibitory effect of AFK-108 on $P450_{14DM}$.

Role of the dichlorophenyl group in the inhibitory effect of AFK-108

Substitution of the dichlorophenyl moiety with another halophenyl group modified the inhibitory effect of AFK-108. The AFK-108 derivatives used in this experiment were **7** and **8** having 2,4-difluorophenyl and 2-chlorophenyl groups, respectively, instead of the 2,4-dichlorophenyl group of AFK-108 (Fig. 1). Substitution of chloride with fluoride (**7**) did not alter the inhibitory effect but removal of the chloride at 4-position (**8**) reduced the inhibitory effect of AFK-108. This finding suggested the 2,4-dichlorophenyl group plays an important role in the interaction of AFK-108 with $P450_{14DM}$.

DISCUSSION

AFK-108 (**1**) is a novel imidazole-containing antifungal compound [11, 14] having a unique geranyl group as a hydrophobic substituent. As in the case of other azole antifungal agents [1, 3–5], the imidazole nitrogen of **1** interacted with the heme iron of $P450_{14DM}$ and formed a one-to-one complex (Figs 2 and 3). AFK-108 (**1**) acted as a potent inhibitor of $P450_{14DM}$ causing complete inhibition at an equivalent concentration to that of the enzyme, even of 0.07 μM or less (Fig. 5). The inhibitory effect of **1** was reduced when the geranyl group was replaced by a longer farnesyl group (**3**) (Fig. 5). Substitution of the geranyl group with a shorter prenyl group (**2**) also reduced the inhibitory effect but to a lesser extent (Fig. 5). This order of potency of **1**, **2** and **3** as $P450_{14DM}$ inhibitor was consistent with their spectrophotometrically determined affinity for $P450_{14DM}$ (Fig. 3). Compounds **1**, **2** and **3** interfered with the CO binding to ferrous $P450_{14DM}$, and the interfering effects of **1** and **3** were stronger than that of **2**. In a previous paper [4], we reported that azole antifungal agents having large hydrophobic substituents, such as ketoconazole and itraconazole, strongly interfered with the binding of CO to ferrous $P450_{14DM}$, while small azoles such as triadimefon were readily replaced by CO. This phenomenon was interpreted by limited mobility of the large compounds in the heme pocket of ferrous $P450_{14DM}$ that might be due to some interaction between the hydrophobic substituents and the apoprotein [4]. The present result described above is consistent with this interpretation, and suggests that the large geranyl and farnesyl groups of **1** and **3** might interact considerably with the apoprotein of ferrous $P450_{14DM}$. Thus, **1** was the most potent inhibitor according to all criteria, indicating that $P450_{14DM}$ interacts most favorably with **1** having a geranyl group.

A geranyl group contains two double bonds and two branched methyl groups. Comparison of the inhibitory effects of the AFK-108 derivatives (**4**, **5** and **6**) with that of AFK-108 (**1**) suggest the importance of the double bond at the terminal part ($^6\triangle$) (Fig. 6). The structures of the geranyl group and the side chain of lanosterol are fundamentally the same. In the previous papers [9, 15], we suggested that lanosterol was the best substrate for *S. cerevisiae* $P450_{14DM}$, while 24,25-dihydrolanosterol was a poor one with low affinity, i.e. saturation of the 24-double bond of lanosterol reduced the affinity for the enzyme. The double bond at the terminal part of the geranyl group ($^6\triangle$) corresponds structurally to the 24-double bond of lanosterol. Accordingly, the lower inhibitory effect of **5** corresponds to the low efficiency of 24,25-dihydrolanosterol as the substrate, suggesting that the geranyl group of AFK-108 is likely to interact with the substrate binding site recognising the side chain of the substrate (side chain binding site).

As indicated by the spectral change shown in Fig. 2, the imidazole nitrogen (N_3) of AFK-108 bound to the heme iron of $P450_{14DM}$. Accordingly, if the geranyl group of AFK-108 interacts with the side chain binding site of the enzyme, as discussed above, this compound must cause simultaneous interaction

of the heme iron and the side chain binding site with its imidazole and geranyl groups, respectively. The 14α-methyl group of lanosterol is thought to be located near the heme iron when it binds to the substrate binding site [10]. Therefore, the distance between the heme iron and the side chain binding site is assumed to be 10–12 Å. Although the exact conformation of AFK-108 in the active site of $P450_{14DM}$ is not known, some conformations give a similar distance as above between N_3 of imidazole and the terminal part of the geranyl group. Consequently, simultaneous interaction of the imidazole and the geranyl group of AFK-108 with the heme iron and the side chain binding site, respectively, may be possible. Some azole compounds such as ketoconazole and itraconazole are large enough to interact simultaneously with the side chain binding site and the heme iron. However, the structures of the large substituents of ketoconazole and itraconazole are considerably different to that of the sterol side chain, and there is no evidence suggesting their interaction with the side chain binding site. Thus, AFK-108 is the first example of an azole compound that interacts with the side chain binding site of $P450_{14DM}$.

The dichlorophenyl group of AFK-108 was also important for the inhibitory effect. The chloro- or fluoro-phenyl group forms part of many known azole antifungal agents. However, it has not yet been determined whether this group interacts with a specific region of the substrate binding site of $P450_{14DM}$ or not. In the case of AFK-108, the dichlorophenyl group may interact with the substrate binding site recognising the body of the sterol ring, because the geranyl group is considered to interact with the side chain binding site as discussed above.

We conclude that AFK-108 is a unique $P450_{14DM}$ inhibitor interacting with the side chain recognising region of the substrate binding site of $P450_{14DM}$. As described in previous papers [9, 15, 16], $P450_{14DM}$s of different origins recognised differently the structure of the sterol side chain. It is therefore expected that a selective inhibitor for fungal $P450_{14DM}$ could be designed by modification of AFK-108.

Acknowledgement—This work was supported by a Grant-in-Aid for Scientific Research (No. 03671075) from the Ministry of Education, Science and Culture of Japan to Y.Y.

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