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# Anti-*Candida* Activity of YH-1715R, a New Triazole Derivative

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**Abstract** YH-1715R, (2R,3R)-2-(2,4-difluorophenyl)-3-(3-methoxy-1,2,4-isothiazol-3-yl-thio)-1-(1H-1,2,4-triazol-1-yl)-2-butanol, a new triazole derivative obtained by the structural modification of fluconazole, was found to exhibit potent anti-*Candida* activity against a wide variety of *Candida albicans* (*C. albicans*) (MIC: 0.4–12.5 mg/l). To investigate the mode of action of YH-1715R, its effect on ergosterol biosynthesis in cell-free extracts and whole cells of *C. albicans* was examined. The inhibitory activity of YH-1715R was approximately ten-fold higher than that of fluconazole. To determine the primary action mechanism of YH-1715R, its inhibitory activity against lanosterol 14α-demethylase (14α-DM), a major target for azole, was measured using gas-liquid chromatography. YH-1715R and fluconazole were found to inhibit 14α-DM with an $IC_{50}$ of 0.015 μM and 0.018 μM, respectively, plus the mode of inhibition of YH-1715R and fluconozole was noncompetitive with a $K_i$ of 0.0533 μM and 0.0975 μM.

**Key words:** Triazole, *Candida albicans*, 14α-demethylase, ergosterol biosynthesis, YH-1715R

Although the incidence of fungal infection has rapidly increased in the past two decades [1], the development of new antifungal drugs has been slow. Among the antifungal agents currently used in clinical therapy, azoles, such as fluconazole and itraconazole, are frequently used in the treatment of fungal infections caused by *Candida* spp. Azoles offer potential advantages over amphotericin B, with regard to reduced toxicity and the versatility of oral or intravenous administration. However, acquired or intrinsic resistance to these compounds is increasing, and failure of azole therapy has already been reported [10]. Therefore, the treatment of fungal infections with azoles needs to be improved until new drugs become available.

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Accordingly, in the current study, YH-1715R (2R, 3R)-2-(2,4-difluorophenyl)-3-(3-methoxy-1,2,4-isothiazol-3-yl-thio)-1-(1H-1,2,4-triazol-1-yl)-2-butanol [3], a derivative of fluconazole, was synthesized and subsequently selected as a potential new azole antifungal by Yuhan Pharmaceutical Co. (Seoul, Korea). The *in vitro* susceptibility testing method employed was a microdilution adaptation of the guidelines set forth by the National Committee for Clinical Laboratory Standards (NCCLS) [6]. In addition, cell-free extracts and whole cells were incubated with [$^{14}$C]mevalonic acid or [$^{14}$C]acetate and the ergosterol contents analyzed by thin layer chromatography (TLC). To elucidate the action mechanism of YH-1715R, an assay system for 14α-DM activity was employed, since the cytochrome P-450 lanosterol 14α-demethylase (14α-DM) of *C. albicans*, which is involved in an important step in the biosynthesis of ergosterol, is the major target of azole antifungal agents [2, 4, 11, 12]. When the *in vitro* activities of YH-1715R (Fig. 1) were evaluated against several strains of *C. albicans*, it was found that YH-1715R effectively inhibited azole-resistant strains, making it suitable for treating azole-resistant *Candida* spp.

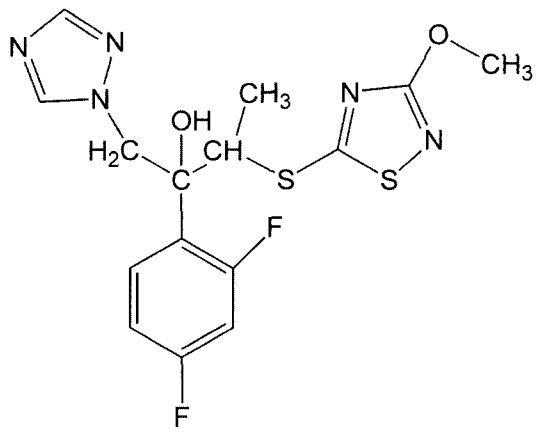


**Fig. 1.** Structure of YH-1715R.

## MATERIALS AND METHODS

### Chemicals and Reagents

The YH-1715R [2R,3R)-2-(2,4-difluorophenyl)-3-(3-methoxy-1,2,4-isothiazol-3-yl-thio)-1-(1H-1,2,4-triazol-1-yl)-2-butanol] and fluconazole were provided by the chemical synthesis team at Yuhan Pharmaceutical Co. (Seoul, Korea) [3]. The miconazole, ketoconazole, cofactors, and other biochemicals were purchased from Sigma (St. Louis, MO, U.S.A.). The following isotopes (specific activity) were purchased from Amersham (Buckinghamshire, U.K.): [$^{14}$C]acetate (56 mCi/mmol) and [2-$^{14}$C]mevalonic acid (60 mCi/mmol). The silica gel plates (Kiesel gel $60F_{254}$), toluene, diethyl ether, and chloroform were purchased from Merck Co. (Darmstadt, Germany). Sabouraud's dextrose media were obtained from Difco Co. (Detroit, MI, U.S.A.).

### Organism and Culture Conditions

The fungal strains used were *C. albicans* (ATCC10231, ATCC28838, ATCC11651, ATCC64550, ATCC64124, ATCC62342, IFO1385) and clinical isolates of *C. albicans* (YH-019, Yuhan Pharmaceutical Co., Seoul, Korea). Cells were maintained on slants of Sabouraud's dextrose agar at 4°C. To culture the yeast growth form, cells were inoculated and grown at 30°C for 18 h in Sabouraud's dextrose broth, pH 5.6. Late exponential phase cells (18 h) were harvested and washed by centrifugation, and used to prepare microsomes.

### Broth Microdilution Susceptibility Test

The tests were based on those described previously by Park *et al.* [8, 9] and the NCCLS (document M27-A) [6]. Fluconazole, miconazole, and ketoconazole were dissolved in DMSO. The stock solutions were diluted with RPMI 1640 medium supplemented with glutamine (Sigma Chemical Co., St Louis, MO, U.S.A.) and 2% glucose, buffered to pH 7.0 with 0.156 M 3-*N*-morpholinopropane-sulphonic acid (MOPS; Sigma). The final antifungal concentrations were 0.0975- 100 mg/l and the inoculum size was $10^4$ cells/ml. The tests were performed in 96-well microtitre plates that were incubated at 35°C for 24 h. The MICs for YH-1715R, fluconazole, and ketoconazole were read as the lowest concentration of the agent that inhibited growth by 80%.

### Preparation of *C. albicans* Microsomes

The *C. albicans* microsome preparation was based on the method of Park *et al.* [8, 9]. Harvested cells were suspended in 2.5 volumes of 0.1 M potassium phosphate (pH 7.4, 1 mM EDTA) and centrifuged at 5,000 ×*g* for 5 min. The washed cells were then resuspended in 0.1 M potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM DTT, and 0.25 mM phenylmethylsulfonylfluoride [PMSF] at $2\times10^9$ cells/ml and broken by shaking with glass beads (0.45- 0.5 mm) in a B. Braun disintegrator (MSK, B. Braun Biotech. GMBH, Schwarzenberger-WEG, Germany) for a total of 3 min interspersed with periods of cooling. The cell debris was removed by centrifugation at 11,000 ×*g* for 20 min, then the supernatant was centrifuged at 110,000 ×*g* for 1 h. The resulting microsomal pellets were resuspended in 0.1 M potassium phosphate (pH 7.4), 1 mM EDTA, and 20% glycerol, divided into aliquots, and stored at - 70°C.

### Sterol Biosynthesis Assay

Washed *C. albicans* (ATCC 10231) cells were incubated with radiolabelled substrates, as previously described [8, 9]. Briefly, for the whole-cell sterol biosynthesis, the washed cells were resuspended in a 0.1 mM potassium phosphate buffer containing 1% (w/v) glucose. The test compounds (YH-1715R or fluconazole) were then added to the cell suspension ($2\times10^9$ cells/ml) and preincubated for 10 min. The reaction was initiated by the addition of 10 µl of [$^{14}$C]acetate (1 µCi, specific activity 56 µCi/mmol). For the cell-free sterol biosynthesis, the washed cells were broken as described above, then the cell lysates were centrifuged at 5,000 ×*g* for 20 min. The supernatant was incubated with a solution containing 0.1 M potassium phosphate (pH 7.4), 0.1 µM NADP, 1 µM NAD, 5 µM ATP, 3 µM glucose-6-phosphate, 3 µM $MgCl_2$, 2 µM $MnCl_2$, 0.7 unit glucose-6-phosphate dehydrogenase, 3 µM reduced glutathione, and 0.25 µCi [2-$^{14}$C] mevalonic acid at 37°C for 2 h. The reaction was stopped by the addition of 1 ml of 15% (w/v) KOH and 90% ethanol, and the samples saponified at 80°C for 1 h. The nonsaponifiable lipids were then extracted with petroleum ether and the samples evaporated to dryness. The quantitation of the sterol biosynthesis was as previously described [9].

### Sterol 14α-Demethylase (14α-DM) Assay

The sterol 14α-demethylase assay was conducted using a slight modification of the method described previously [5]. Lanosterol (dissolved in Tween 80 at a weight ratio of 70:1 detergent:sterol) was added to a final concentration of 100 µM to 1 ml of 20% (w/v) glycerol, 0.1 M phosphate, pH 7.4, 1 mM KCN, 1 mM NADPH, 10 mM glucose-6-phosphate, and 0.2 units glucose-6-phosphate dehydrogenase that had been preincubated at 37°C for 4 min. The incubation was carried out in the presence of 2 mg of microsomes at 37°C for 10 min. The reaction was stopped by the addition of 1 ml of 15% (w/v) KOH and 90% ethanol, and the samples saponified at 80°C for 1 h. The sterols were extracted with petroleum ether, dried under $N_2$ gas, and analyzed by GLC. The enzymatic activity was calculated from the relative amounts of substrate and product in the incubated samples compared with the unincubated controls. GLC analysis was conducted on a HewlettPackard 5890 GLC system under the following conditions: oven temperature, 260°C; injector, 300°C; detector,

300°C; detection Ultra-1 column (25 m×0.2 mm×0.33 μm); carrier gas, nitrogen; flow rate, 2 ml/min.

## RESULTS AND DISCUSSION

During the course of the chemical synthesis of fluconazole derivatives, YH-1715R was selected for the current study based on primary screening systems that included disk diffusion susceptibility and broth microdilution susceptibility tests against *C. albicans* (data not shown). To examine whether YH-1715R inhibited the growth of *Candida* species and to determine its anti-*Candida* activity, cells were grown in the presence of the test compounds (YH-1715R or fluconazole) and MIC values measured. As summarized in Table 1, for *C. albicans*, YH-1715R produced *in vitro* MIC values ranging from 0.4 to 12.5 mg/ml, depending on the strain tested. The MIC values (mg/l) for YH-1715R were much lower than those for fluconazole. Furthermore, YH-1715R significantly inhibited azole-resistant strains, such as ATCC64550 (1.56 mg/l), ATCC64124 (12.5 mg/l), ATCC62342 (0.78 mg/l), and IFO 1385 (1.56 mg/l). As such, the MICs for fluconazole in relation to these azole-resistant strains were approximately 16- to 64-fold higher than those for YH-1715R, suggesting that YH-1715R may be more suitable for treating azole-resistant *Candida* spp.

Based on previous reports on the action of azole-compounds, it was presumed that YH-1715R might have the same target, lanosterol 14α-DM, for its inhibitory action, like other known azole antifungals [2, 4, 11, 12]. Thus, to examine the inhibitory activity of YH-1715R in relation to ergosterol biosynthesis, the rate of sterol synthesis was measured in the presence and absence of the test compounds (YH-1715R or fluconazole). The total cellular sterols in *C. albicans* were synthesized using either [$^{14}$C]acetate in whole cells or [$^{14}$C]mevalonic acid in cell-free extracts as a precursor in the presence of the test compounds. The ergosterol biosynthesis was measured by the incorporation of [$^{14}$C]-labeled precursors, thereby enabling

**Table 1.** *In vitro* antifungal activity of YH-1715R and fluconazole against *C. albicans*.

| Organisms | MIC (mg/l) | |
|---|---|---|
| | YH-1715R | Fluconazole |
| *C. albicans* ATCC 10231 | 0.4 | 1.56 |
| *C. albicans* ATCC 11651 | 3.125 | 6.25 |
| *C. albicans* ATCC 28838 | 0.2 | 3.125 |
| *C. albicans* IFO 1385 | 1.56 | 100 |
| *C. albicans* YH 019[a] | 0.4 | 1.56 |
| *C. albicans* ATCC 64550 | 1.56 | 100 |
| *C. albicans* ATCC 64124 | 12.5 | >200 |
| *C. albicans* ATCC 62342 | 0.78 | 12.5 |

[a]Clinical isolate.

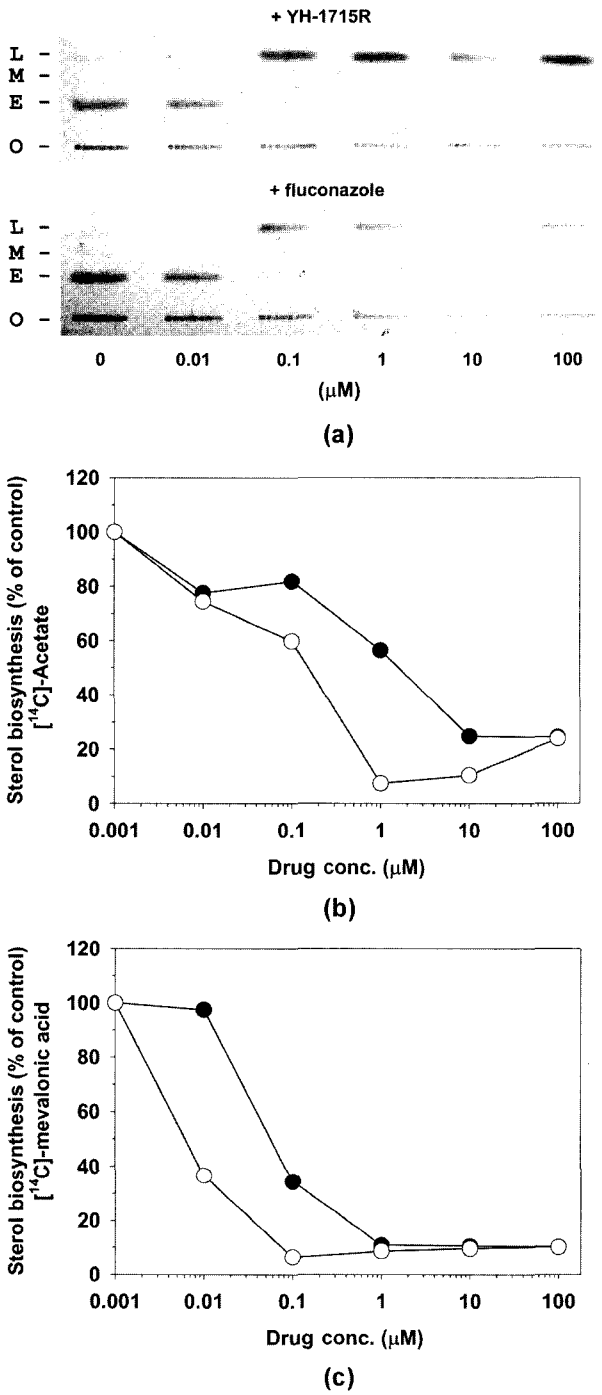


**Fig. 2.** Inhibition of ergosterol biosynthesis in *C. albicans*.
(a) Autoradiograph of TLC showing separation of labelled nonsaponifiable lipids from *C. albicans* cells incubated with [$^{14}$C]acetate in the presence of YH-1715R and fluconazole. The cells were incubated with [$^{14}$C]acetate in a 0.1 M potassium phosphate buffer (pH 6.5) containing 1% (w/v) glucose at 30°C for 2 h. Abbreviations: L, 4,4-dimethyl sterols (lanosterol); M, 4-methylsterols; E, ergosterol; O, origin. (b) Effects of YH-1715R (○) and fluconazole (●) on sterol biosynthesis (whole cells). The whole cells were incubated with [$^{14}$C]acetate, as described for (a). (c) Effects of YH-1715R (○) and fluconazole (●) on sterol biosynthesis (cell-free extracts). The cell-free extracts were incubated with [$^{14}$C]mevalonic acid, as described in Materials and Methods.

the inhibitory effects of YH-1715R or fluconazole to be examined. As shown in Fig. 2, the incorporation of either [$^{14}$C]acetate or [$^{14}$C]mevalonic acid into ergosterol was strongly inhibited by the presence of YH-1715R or fluconazole in a dose-dependent manner, with the former compound exhibiting a more potent inhibitory effect. As shown in Fig. 2A, the decrease in the incorporation of [$^{14}$C]acetate into ergosterol was a consequence of the inhibition of sterol 14α-DM that catalyzes the demethylation of C14-methyl group from lanosterol [2, 7]. In all cases, the cells incorporated [$^{14}$C]acetate or [$^{14}$C]mevalonic acid exclusively into the ergosterol fraction and the accumulated sterols were lanosterol or its derivatives, suggesting that the target was 14α-DM [2,7] (data not shown). That is, the $IC_{50}$s for these azoles as regards inhibiting ergosterol synthesis from [$^{14}$C]acetate were approximately 0.16 μM (YH-1715R) and 1.05 μM (fluconazole) (Fig. 2B). This trend was even maintained when [$^{14}$C]mevalonic acid was used as a precursor (0.005 μM for YH-1715R and 0.055 μM for fluconazole) (Fig. 2C). In addition, the inhibition of ergosterol biosynthesis by these azoles showed a similar potency pattern in both cell-free extracts and whole cells.

To determine whether YH-1715R specifically inhibited *C. albicans* 14α-DM activity *in vitro*, an enzyme assay was carried out using GLC [5]. Prior to this experiment, the apparent kinetic parameters for *C. albicans* microsomal 14α-DM were determined to be 61 μM (apparent $K_m$ for lanosterol) and 1.747 μM ($V_{max}$) (not shown). As shown in Fig. 3A, both YH-1715R and fluconazole were found to inhibit *C. albicans* 14α-DM activity in a dose-dependent manner. The mode of inhibition of 14α-DM by YH-1715R and fluconazole was found to be noncompetitive with a $K_i$ of 0.0533 μM and 0.0975 μM, respectively (Figs. 3B and 3C, respectively). Taken together, these results suggest that the anti-*Candida* activity of YH-1715R was due to the inhibition of 14α-DM activity. The potent *in vitro* antifungal activities of YH-1715R were presumed to arise from its potential ability to inhibit the fungal cytochrome P-450 14α-DM, which catalyzes the 14-demethylation of lanosterol in the biosynthesis of egosterol, an important element of the fungal membrane. As such, a structural similarity between YH-1715R and lanosterol was speculated to be an essential factor accounting for the antifungal potency of YH-1715R. Thus, YH-1715R was favorably overlaid to fit the lanosterol skeleton in such a manner that the aromatic ring and intramolecular hydrogen bonding ability to coordinate with the C(2)-hydroxy hydrogen and ionic functionality of the C(3)-sulfhydryl linkage of YH-1715R were located on the B and D rings of the steroid. Plus, the C(3)-methyl group and methylene carbon atom from the triazolylmethyl group of YH-1715R occupied the positions of the 13β-methyl and 14α-methyl groups of lanosterol, respectively. The 2R, 3R absolute configuration, as well as the location of the methyl substituent at the C(3)-position

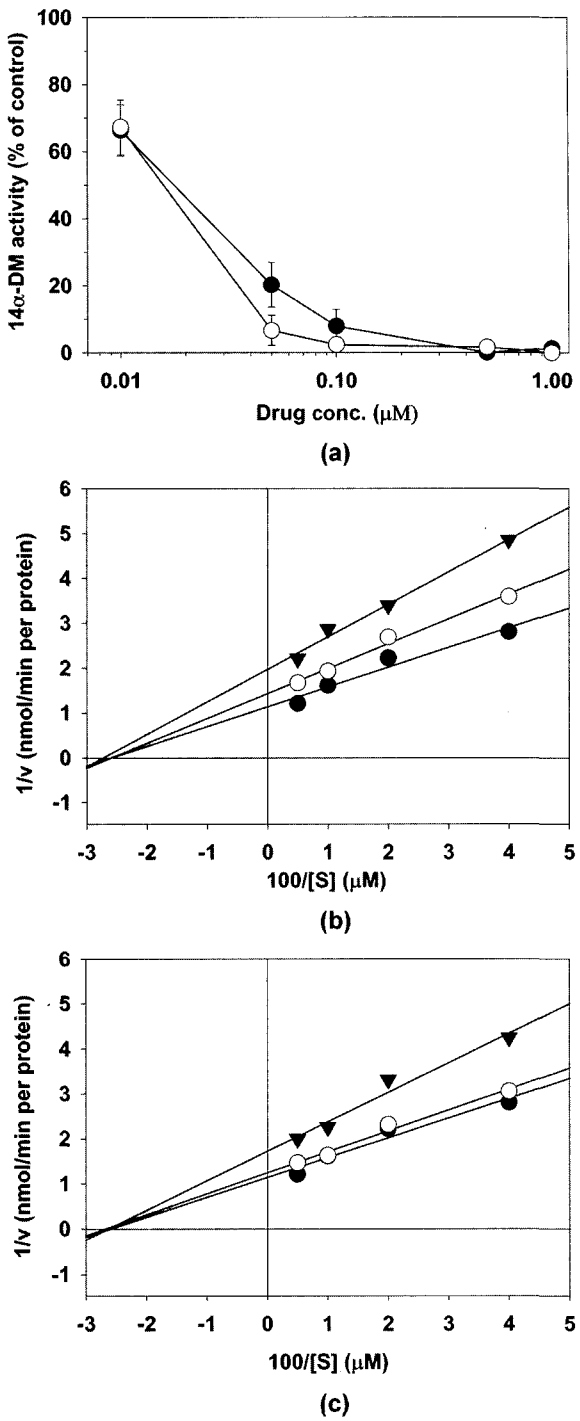


**Fig. 3.** Effect of YH-1715R and fluconazole on *C. albicans* 14α-DM activity.

(a) Effect of YH-1715R (○) and fluconazole (●) on *C. albicans* microsomal 14α-DM activity. The enzyme assays were carried out using lanosterol in 0.1 M Tris buffer (pH 7.5) at 37°C for 10 min. (b) Lineweaver-Burk plot of inhibition of *C. albicans* 14α-DM by YH-1715R. The reaction mixtures contained varying amounts of lanosterol (25, 50, 100, and 200 μM). The reciprocals of the velocity (1/*V*) and substrate concentration (1/[S]) are shown on the ordinate and abscissa, respectively. (c) Lineweaver-Burk plot of inhibition of *C. albicans* 14α-DM activity by fluconazole. The reaction mixtures were the same as those described for (b).

of YH-1715R, were also identified as key structural elements of the antifungal potency.

In summary, when comparing the *in vitro* activities of the triazole derivatives YH-1715R and fluconazole against *Candida* spp., YH-1715R exhibited lower MICs compared to fluconazole. In addition, the anti-*Candida* activity of YH-1715R was found to be based on inhibiting *C. albicans* 14α-DM. Therefore, these results suggest the possibility of designing and synthesizing a new family of triazole derivatives that are more effective than those currently available from the azole family. YH-1715R was also found to inhibit azole-resistant strains and 14α-DM in a noncompetitive manner, making it suitable for treating azole-resistant *Candida* spp. However, further *in vivo* studies with experimental animal models are needed to confirm this activity.

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