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PF1163A, a Novel Antifungal Agent, Inhibit Ergosterol Biosynthesis at C-4 Sterol Methyl Oxidase

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PF1163A and B are a pair of antifungal agents isolated from a fermentation broth of *Penicillium* sp. PF1163A inhibited ergosterol synthesis in *Saccharomyces cerevisiae*, resulting in an accumulation of 4,4-dimethylzymosterol and a decrease of ergosterol. The ERG25 strain overexpressing the *ERG25* gene was resistant to PF1163A. ERG25p is a C-4 sterol methyl oxidase known to be essential for the viability of yeast and fungi because of the known role of *ERG25* gene disruption in *S. cerevisiae*-led lethality. ERG25p is the enzyme responsible for the first step in the removal of the two methyl groups at the C-4 position of sterol. From the results obtained here, we conclude that PF1163A is a novel natural antifungal that inhibits C-4 sterol methyl oxidase.

The incidence of life-threatening systemic fungal infections has increased in the last decade due to a rise in immunocompromised patients infected by immunodeficiency virus (HIV) and receiving cancer chemotherapy and immunosuppressants. Only a small number of compounds are used in therapies for systemic fungal diseases, and all of them have drawbacks. As in the case of amphotericin B, the polyenes have serious side effects[1]. Fluconazole has been widely used to treat fungal infections caused by *Candida albicans*, but now we are witnessing the emergence of intrinsically resistant species, such as *Candida glabrata*, *Candida krusei* and a variety of *C. albicans* acquired resistance[2]. Thus, new drugs and therapies are urgently needed.

The ergosterol biosynthesis pathway has been recognized as a good target for antifungal therapy and has been well characterized in *Saccharomyces cerevisiae*[3]. The sterols are major membrane lipids of yeast and fungi and play a crucial role in their viability, membrane fluidity, and permeability. *S. cerevisiae* mutants that lack sterols are not viable, and inhibitors of sterol biosynthesis kill the pathogenic yeast and fungi.

A number of agents that inhibit the ergosterol biosynthesis have been discovered and developed, including several natural products. The morpholines inhibit two different steps in ergosterol biosynthesis, namely, the Δ^8-Δ^7-isomerase and the Δ^{14}-reductase, and this has prompted their use as agricultural fungicides[4,5]. The allylamines inhibit the squalene epoxidase in the ergosterol pathway, and one allylamine compound, terbinafine, is used as a topical agent for mycoses of the skin[6,7]. The azoles inhibit ergosterol biosynthesis at lanosterol 14α-demethylase. Many azole compounds are used both topically and systemically[8], although those more recently developed azoles have undesirable side effects resulting from host steroid biosynthesis sensitivity[9]. Another series of compounds from natural products has been reported to inhibit lanosterol 14α-demethylase, but these compounds do not show *in vivo* efficacy[10,11].

In the course of screening for antifungal agents, we have discovered PF1163A and B, a pair of novel compounds isolated from microbial broths of *Penicillium* sp.[12]. In our experiments, we found that PF1163A was as potent as fluconazole in inhibiting the synthesis of ergosterol *in vitro*. In this paper, we report treatment with PF1163A caused an accumulation of 4,4-dimethylzymosterol, and that the strain overexpressing the *ERG25* gene was resistant to PF1163A. Thus, PF1163A is suspected to inhibit C-4 sterol methyl oxidase.

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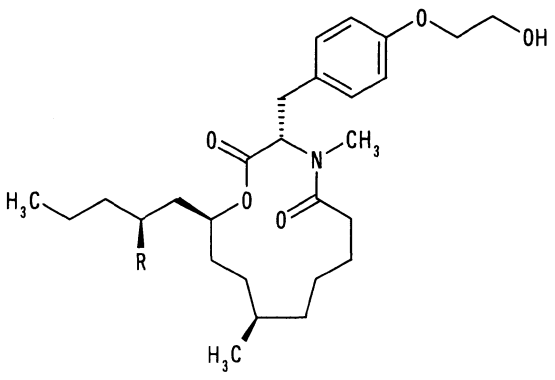
Materials and Methods

Strains, Inhibitors and Culture Conditions

Table 1 lists the *S. cerevisiae* strains used in this work. All yeast strains were derivatives of YPH499[13]. They were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) or SD (0.67% Yeast Nitrogen Base without amino acids, 2% glucose) supplemented with appropriate amino acids and 0.004% adenine. For solid media, 2% Bacto-agar was added. Ergosterol and lanosterol were purchased from Sigma. PF1163A was purified by the method reported previously[12].

Fig. 1. The structures of PF1163A and B.



PF1163A, R=OH; PF1163B, R=H.

Cloning and Construction of Plasmids

The plasmid pYD1 was constructed in our laboratory from pYES2. The pYD1 is a high copy expression vector that contains the *S. cerevisiae GPD1* promoter region[14], multiple cloning site, the *S. cerevisiae CYC1* transcription termination signal, and the *URA3* gene as a selectable marker. A 1.6-kb fragment containing the coding sequence for *ERG11* was amplified by PCR using strain *S. cerevisiae* FL100 genomic DNA as a template and the primers ERG11-3 and ERG11-4 shown in Table 2. The ERG11-3 and ERG11-4 primers were designed to contain the recognition site for *Kpn*I and *Eco*RI, respectively (italic letters in Table 2). The PCR product was digested with *Kpn*I and *Eco*RI and ligated into plasmid pYD1, resulting in the formation of pYD1-*ERG11*. pYD1-*ERG24*, pYD1-*ERG25* and pYD1-*ERG26* were constructed by basically the same method, with the only exception of that the PCR product of *ERG25* gene was digested with *Hin*dIII and *Xho*I.

Sterol Analysis

The *S. cerevisiae* PDR5 strain was inoculated at 1×10^7 cells/ml in 100 ml of SD medium with or without PF1163A. After incubating the culture at 28°C for 9 hours with shaking, the yeast cells were collected. Sterols were isolated as nonsaponifiables, as described previously[15]. GC/MS analyses of individual sterols were performed on a HP5890 series II GC running on HP Chemstation software. The capillary column (DB-17) has a film thickness of 30 m×0.25 mm×0.25 μm and the column temperature was

Table 1. Genotypes and origin of yeast strains used in these studies.

Strain	Genotype	Ref.
YPH499	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1*	13
PDR5	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1*	this work
ERG11	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1 pYD1-ERG11*	this work
ERG24	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1 pYD1-ERG24*	this work
ERG25	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1 pYD1-ERG25*	this work
ERG26	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-△63 his3-△200 leu2-△1 pdr5::TRP1 pYD1-ERG26*	this work

Table 2. PCR primers used in this work.

Gene	Oligonucleotide designation and sequence	
ERG11	ERG11-3;	5'-AAACGAG*GGTACC*AAGGATGTCTGCTACCA-3'
	ERG11-4;	5'-TTTCTC*GAATTC*AAACCTTAGATCTTTTGT-3'
ERG24	ERG24-3;	5'-GTGTAGA*GGTACC*AAGGATGGTATCAGCTT-3'
	ERG24-4;	5'-AGCAGT*GAATTC*AAAACTTAATAAACATAT-3'
ERG25	ERG25-3;	5'-CATC*AAGCTT*GTAGTACAGCCATAAAAAAA-3'
	ERG25-4;	5'-GTAC*CTCGAG*TTTGAAGTATGTTTCTTCTC-3'
ERG26	ERG26-3;	5'-GGCAAAC*GGTACC*AAATATGTCAAAGATAG-3'
	ERG26-4;	5'-GCTCCT*GAATTC*TGCCATTACAAACCTTCG-3'

programmed to run from 80 to 300°C (1 minute at 80°C, followed by an increase of 1°C/minute until the final temperature of 300°C, followed by 4 minutes at 300°C). Helium, the carrier gas, moved with a linear velocity of 50 cm/second in the splitless mode. The mass spectrometer ran in the electron impact ionization mode and was set to scan from 50 to 600 atomic mass units at 0.5-seconds intervals, at an ion source temperature of 200°C, with an electron energy of 70 ev.

Preparation of 4,4-Dimethylzymosterol

The *S. cerevisiae* PDR5 strain was inoculated in 100 ml of YPD medium at 28°C with shaking overnight. The yeast cells were collected by centrifugation and re-inoculated at 1×10^7 cells/ml in 10 liters of YPD medium containing PF1163A. The culture was incubated at 28°C for 9 hours with shaking. Next, the yeast cells were collected and sterols were saponified in 10% (w/v) KOH in 90% (v/v) ethanol at 80°C for 1 hour. Nonsaponifiable sterols were extracted twice with *n*-hexane and dried in a vacuum evaporator. The free sterols were resolved by TLC in $CHCl_3$-methanol (100 : 1), and 4,4-dimethylzymosterol was separated.

Overexpression Assay

S. cerevisiae strains ERG11, ERG24, ERG25 and ERG26 were used in the overexpression assay. A 96-well microtitre MIC format was used. Serial 2-fold dilutions of inhibitors were prepared, starting from a concentration of 100 μg/ml. An overnight culture grown in YPD medium was diluted in fresh SD medium without uracil to achieve a cell density of 1×10^5 cells/ml. An aliquot (100 μl) of the cell suspension was added to the serially diluted inhibitors. After incubation at 28°C for 24 hours, the absorbance was read and the minimum inhibitory concentration value was determined.

Results

Sterol Analysis and Identification of Sterols

As previously reported[16], azoles inhibited 14-demethylase of yeasts and fungi, resulting in an accumulation of lanosterol (molecular mass, 426). As shown in Fig. 2, two major peaks appeared in the strains treated with PF1163A, whereas the major peak was ergosterol in the absence of the compound. The compound corresponding to peak A (*m/z* 396) was identified as an ergosterol by mass-spectrum fragmentation analysis. The molecular ion of peak C was observed at 412 Da and the fragmentation pattern (Fig. 3) was identical to the spectrum of 4,4-dimethylzymosterol observed by BYSKOV *et al.*[17]. The compound of peak C was separated and confirmed by ^{13}C NMR analysis (data not shown). The minor peak B (*m/z* 412) was not identified.

Antifugal Activity

Our sterol analysis confirmed that treatment with PF1163A elicited the accumulation of 4,4-dimethylzymosterol, and it furthermore suggested that PF1163A inhibits ERG25p aside from ERG11p. The overexpression system is known to be a valid and selective method for examining the drug's mode of action[18]. Therefore, we genetically characterized the mode of action of PF1163A by measuring the growth inhibition of PF1163A for *S. cerevisiae* strains overexpressing the *ERG11*, *ERG24*,

Fig. 2. GC analysis of sterols accumulating in *S. cerevisiae* PDR5 strain treated with 20 μg/ml of PF1163A.

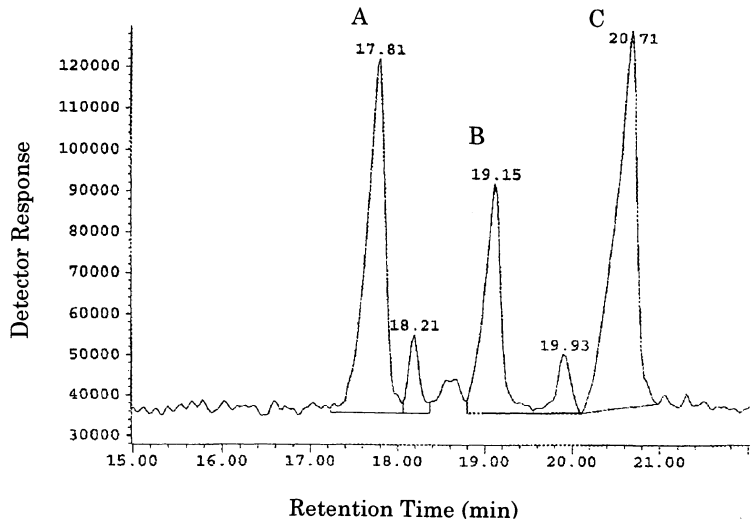
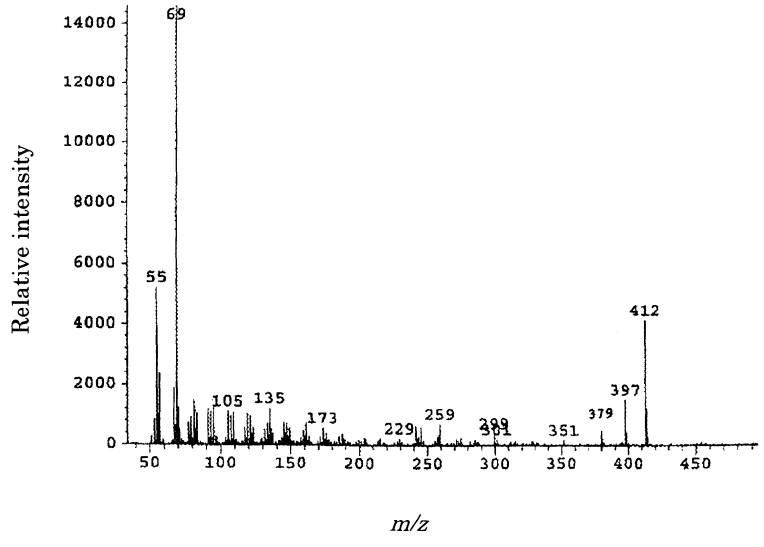


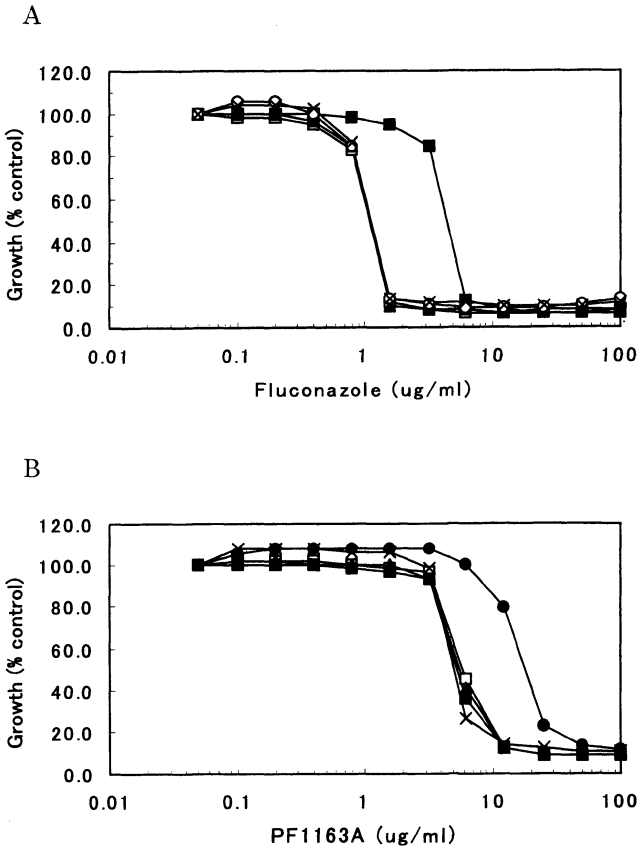
Fig. 3. Mass spectrum of GC/MS peak C identified as 4,4-dimethylzymosterol.



ERG25, and *ERG26* genes. The *S. cerevisiae* FL100 *ERG11*, *ERG24*, *ERG25*, and *ERG26* genes were amplified by the PCR method and cloned on the multicopy pYD1 plasmid. pYD1 contained the *S. cerevisiae GPD1* promoter region induced by glucose in the medium. Each plasmid was transformed into the *S. cerevisiae* PDR5 strain, resulting in the construction of the ERG11, ERG24, ERG25, and ERG26 strains. Figure 4 shows the effects of PF1163A on the growth of the recombinant strains.

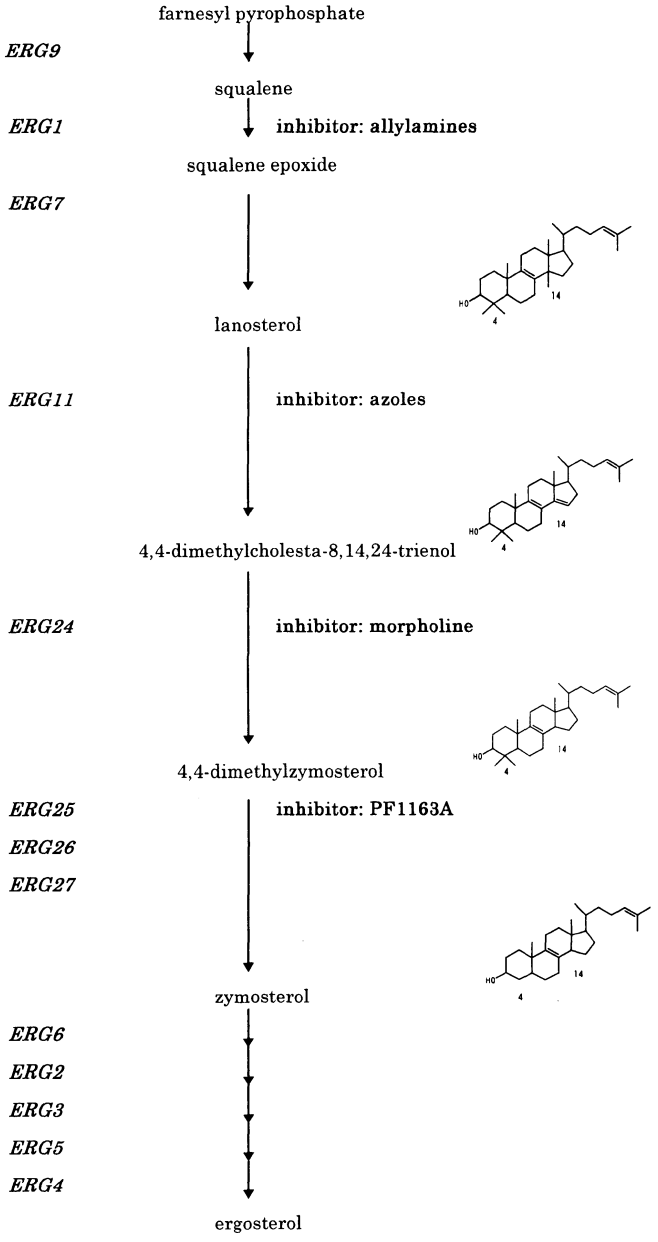
The ERG11 strain overexpressing *ERG11* gene was more resistant to fluconazole than other strains, to the extent that we had expected (Fig. 4A). PF1163A had an MIC of 12.5 μg/ml for PDR5, ERG11, ERG24, and ERG26 strains, in contrast to a markedly higher MIC of 50 μg/ml for the ERG25 strain with an elevated copy number (Fig. 4B).

Fig. 4. Growth inhibition of *S. cerevisiae* strains by PF1163A.



PDR5 (□), ERG11 (■), ERG24 (▲), ERG25 (●) and ERG26 (×) were grown in medium containing fluconazole (A) or PF1163A (B). Growth was plotted as a percent of control in the absence of inhibitor.

Fig. 5. PF1163A involvement in the ergosterol biosynthetic pathway from farnesyl pyrophosphate to ergosterol in *S. cerevisiae*.



Discussion

The ergosterol biosynthesis pathway has been characterized in *S. cerevisiae*, and many inhibitors of ergosterol biosynthesis have been discovered and synthesized (Fig. 5). Fluconazole has been used widely to treat fungal infections caused by *C. albicans* and other *Candida* species, but the emergence of azole-resistant strains is now a problem. Thus, we face an urgent need to discover novel compounds and new antifungal target sites. As all of the genes prior to the sterol C-24 methyltransferase (*ERG6*) gene are essential for viability, other genes such as ERG25, ERG26, and ERG27 genes are candidates as targets for the discovery of novel antifungal drugs.

C-4 demethylation is separated into three reactions and the genes involved in each step were cloned[19~21]. ERG25p converts the 4α-methyl group to an alcohol, then to an aldehyde, and finally to a carboxylic acid. ERG26p removes the 3α-hydrogen and leads up to the decarboxylation of a 3-ketocarboxylic acid sterol intermediate. ERG27p converts the 3-keto to the β-hydroxy sterol. ERG25p contains three histidine clusters and a KKXX golgi-to-endoplasmic

reticulum retrieval signal[19]. The non-heme diiron enzymes such as hydroxylases and fatty acid desaturases contain histidine clusters[22].

Previously we discovered novel PF1163A and B, a pair of novel compounds isolated from the microbial broths of *Penicillium* sp. PF1163A was as potent as fluconazole in inhibiting the synthesis of ergosterol.

In this study, we elucidated the mode of action by sterol analysis and overexpression assay. Treatment with PF1163A resulted in the appearance of another sterol (*m/z* 412) that was different from lanosterol (*m/z* 396). This sterol was identified as 4,4-dimethylzymosterol by fragmentation analysis of the mass-spectrum. According to the overexpression assay, the ERG25 strain overexpressing the *ERG25* gene was resistant to PF1163A. On the other hand, the MICs of PF1163A for the ERG11, ERG24, and ERG26 strains was the same as that for the PDR5 strain expressing the wild-type levels of each gene. These results strongly suggest that PF1163A is an inhibitor of ERG25p, C-4 sterol methyl oxidase. As there are no previous reports of any other inhibitor of C-4 sterol methyl oxidase, PF1163A is a novel inhibitor of the enzyme. PF1163A may be a useful tool for developing a new antifungal drug and for studying the ergosterol biosynthesis of yeast and fungi.

References

1) PERFECT, J. R.; M. H. LINDSAY & R. H. DREW: Adverse drug reactions to systemic antifungals. Prevention and management. Drug Saf. 7: 323~363, 1992
2) WHITE, T. C.: Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. 41: 1482~1487, 1997
3) LEES, N. D.; M. BARD & D. R. KIRSCH: Biochemistry and molecular biology of sterol synthesis in *Saccharomyces cerevisiae*. Crit. Rev. Biochem. Mol. Biol. 34: 33~47, 1999
4) BALOCH, R. I. & E. I. MERCER: Inhibition of sterol Δ^8-Δ^7-isomerase and 14-reductase by fenpropimorph tridemorph and fenpropidin in cell-free enzyme systems from *Saccharomyces cerevisiae*. Phytochemistry 26: 663~668, 1987
5) BALOCH, R. I.; E. I. MERCER, T. E. WIGGINS & B. C. BALDWIN: Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropimorph and fenpropidin. Phytochemistry 23: 2219~2226, 1984
6) RYDER, N. S.: Squalene epoxidase as a target for the allylamines. Biochem. Soc. Trans. 19: 774~777, 1991
7) RYDER, N. S.: Terbinafine: mode of action and properties of the squalene epoxidase inhibition. Br. J. Dermatol. 126: 2~7, 1992
8) ISAACSON, D. M.; E. L. TOLMAN, A. J. TOBIA, M. E. ROSENTHALE, J. L. MCGUIRE, H. VANDEN BOSSCHE & P. A. JANSSEN: Selective inhibition of 14 alpha-desmethyl sterol synthesis in *Candida albicans* by terconazole, a new triazole antimycotic. J. Antimicrob. Chemother. 21: 333~343, 1988
9) GEORGOPAPADAKOU, N. H. & T. J. WALSH: Human mycoses: Drugs and targets for Emerging Pathogens. Science 264: 371~373, 1994
10) MATSUKUMA, S.; T. OHTSUKA, H. KOTAKI, H. SHIRAI, T. SANO, K. WATANABE, N. NAKAYAMA, Y. ITEZONO, M. FUJIU & N. SHIMANO: A new series of natural antifungals that inhibit P450 lanosterol C-14 demethylase. I. Taxonomy, fermentation, isolation and structural elucidation. J. Antibiotics 45: 151~159, 1992
11) O'SULLIVAN, J.; D. W. PHILLIPSON, D. R. KIRSCH, S. M. FISHER, M. H. LAI & W. H. TREJO: Lanomycin and glucolanomycin, antifungal agents produced by *Pycnidiophora dispersa*. I. Discovery, isolation and biological activity. J. Antibiotics 45: 306~312, 1992
12) NOSE, H.; A. SEKI, T. YAGUCHI, A. HOSOYA, T. SASAKI, S. HOSHIKO & T. SHOMURA: PF1163A and B, new antifungal antibiotics produced by *Penicillium* sp. I. Taxonomy of producing strain, fermentation, isolation and biological activities. J. Antibiotics 53: 33~37, 2000
13) SIKORSKI, R. S. & P. HIETER: A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19~27, 1989
14) BITTER, G. A. & K. M. EGAN: Expression of heterologous genes in *Saccharomyces cerevisiae* from vectors utilizing the glyceraldehyde-3-phosphate dehydrogenase gene promoter. Gene 32: 263~274, 1984
15) MOLZAHN, S. W. & R. A. WOODS: Polyene resistance and the isolation of sterol mutants in *Saccharomyces cerevisiae*. J. Gen. Microbiol. 72: 339~348, 1972
16) MARRIOTT, M. S.: Inhibition of sterol biosynthesis in *Candida albicans* by imidazole-containing antifungals. J. Gen. Microbiol. 117: 253~255, 1980
17) BYSKOV, A. G.; C. Y. ANDERSON, L. NORDOHOLM, H. THOGERSEN, X. GUOLIANG, O. WASSERMAN, J. V. ANDERSEN, E. GUDDAL & T. ROED: Chemical structure of sterols that activate oocyte meiosis. Nature (London) 374: 559~562, 1995
18) SLATER-RADOSTI, C.; G. VAN ALLER, R. GREENWOOD, R. NICHOLAS, P. M. KELLER, W. E. DEWOLF, Jr., F. FAN, D. J. PAYNE & D. D. JAWORSKI: Biochemical and genetic characterization of the action of triclosan on *Staphylococcus aureus*. J. Antimicrob. Chemother. 48: 1~6, 2001
19) BARD, M.; D. A. BRUNER, C. A. PIERSON, N. D. LEES, B. BIERMANN, L. FRYE, C. KOEGEL & R. BARBUCH: Cloning and characterization of *ERG25*, the *Saccharomyces cerevisiae* gene encoding C-4 sterol methyl oxidase. Proc. Natl. Acad. Sci. USA 93: 186~190, 1996
20) GACHOTTE, D.; R. BARBUCH, J. GAYLOR, E. NICKEL & M. BARD: Characterization of the *Saccharomyces cerevisiae ERG26* gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) involved in sterol biosynthesis. Proc. Natl. Acad. Sci. USA 95: 13794~13799, 1999
21) GACHOTTE, D.; S. E. SEN, J. ECKSTEIN, R. BARBUCH, M. KRIEGER, B. D. RAY & M. BARD: Characterization of the *Saccharomyces cerevisiae ERG27* gene encoding the 3-keto reductase involved in C-4 sterol demethylation. Proc. Natl. Acad. Sci. USA 96: 12655~12660, 1999
22) SHANKLIN, J.; E. WHITTLE & B. G. FOX: Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. Biochemistry 33: 12784~12794, 1994