A NEW SERIES OF NATURAL ANTIFUNGALS THAT INHIBIT P450 LANOSTEROL C-14 DEMETHYLASE

II. MODE OF ACTION

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From a *Penicillium* sp. we identified a new series of antifungals having a tetrahydropyran skeleton with an alkenyl side chain. We elucidated the mode of action of Ro 09-1470, the most active compound of the series. Treatment of *Candida albicans* with the compound caused an accumulation of C-14 methyl intermediates of ergosterol at concentrations of which no significant interference with the biosyntheses of other macromolecules and respiration was observed. P450 lanosterol C-14 demethylase ($P450_{14DM}$) activity was inhibited and furthermore, the binding of Ro 09-1470 to the heme of the enzyme was demonstrated by a difference spectrum. We conclude that Ro 09-1470 is the first natural antifungal that inhibits the $P450_{14DM}$ of fungi.

Treatment of systemic fungal diseases is limited to relatively few agents. Structurally, there are only three groups; polyene, fluorinated pyrimidine and azole which are respectively represented by amphotericin B, 5-fluorocytosine and fluconazole. All these compounds, however, have drawbacks in, for example, potency, toxicity and resistance development. Although further structure optimization of these compounds is intensively being pursued, new chemical classes of antifungals, which are safe and selectively toxic to fungi, must be identified and their target sites studied. One such site involves ergosterol, a fungal-specific sterol, and if an agent specifically inhibits ergosterol biosynthesis it will have antifungal activity with little toxicity to mammalian cells. All of the azoles inhibit cytochrome P450 lanosterol C-14 demethylase[1,2] ($P450_{14DM}$) of fungi and amphotericin B is known to interact with ergosterol in the fungal cell membrane[3], both leading to the inhibition of fungal cell growth but $P450_{14DM}$ inhibitors has not yet been isolated from the screening of natural products.

In the preceding paper[4], we reported on the isolation and structural elucidation of a series of antifungals of microbial origin. Those novel antifungals have a tetrahydropyran skeleton substituted with a nonatrienyl side chain at C-2 position. Among these, Ro 09-1470 had the highest activity against a broad spectrum of fungi. In this paper we describe the mode of action by which Ro 09-1470 inhibits the growth of fungal cells. We found that Ro 09-1470 inhibited the $P450_{14DM}$ of fungi. Treatment of *Candida albicans* with the compound caused an accumulation of C-14 methyl intermediates of ergosterol at concentrations of which no significant interference with the biosyntheses of other macromolecules and respiration was observed. Direct inhibition of $P450_{14DM}$ enzyme activity and binding of Ro 09-1470 to the heme of the enzyme were also demonstrated. Thus Ro 09-1470 is concluded to be the first $P450_{14DM}$ inhibitor isolated from a natural source.

Materials and Methods

Materials

Strains used were purchased from ATCC or IFO, or obtained as indicated in Table 1. The media

used were as follows: YNBAP medium contained Yeast Nitrogen Base (Difco) 0.67%, glucose 1%, K_2HPO_4 0.25% and low melting point agarose (LMP agarose, BRL) 0.2%. PYG contained glucose 3%, Polypeptone 0.5%, yeast extract 0.5% and KH_2PO_4 0.5%. YNB contained Yeast Nitrogen Base 0.67% and glucose 1%. [^{3}H]Adenine (20 μCi/mmol), [^{14}C]acetate (55.2 mCi/mmol) and [^{3}H]leucine (312 mCi/mmol) were purchased from Amersham. Lanosterol was purchased from Tokyo Kasei and squalene and ergosterol from Sigma. The reference drugs ketoconazole (KCZ, Sigma), fluconazole (FCZ, Pfizer), amphotericin B (AMPH B, Sigma), 5-fluorocytosine (5-FC), terbinafine and naftifine (obtained from F. Hoffmann-La Roche AG., Basle) were used in a solution of dimethyl sulfoxide (DMSO).

In Vitro Antifungal Activity

The *in vitro* antifungal activity was measured by the semi-solid agar dilution method with 96-well microtest plates. Inocula of test organisms were prepared by suspending in BSG80 (buffered saline with 0.01% gelatin and 0.1% Tween 80) spores, conidia or yeasts obtained as follows. Spores of *Aspergillus* were collected from a culture grown at 27°C for 1~4 weeks on Malt agar (Difco). *Exophiala* cells were grown on brain-heart infusion agar (Nissui) at 27°C for 1~2 weeks for the formation of yeast form cells. For *Trichophyton*, macro and micro conidia formed after cultivation at 27°C for about 1 month on the Malt agar slant were used. Other yeasts were grown at 27°C for 1~2 days on Malt agar slants and then used as an inoculum. Each suspension was diluted with YNBAP to 10^4 cfu/ml or spores/ml. 200 μl portion of inocula was mixed with 2 μl of 20 mg/ml sample and then serially diluted 2-fold with cell suspension to give compound concentrations ranging from 0.006 to 200 μg/ml. After keeping the microtest plates at 4°C for 60 minutes in order to solidify LMP agarose, they were incubated at 27°C. The MICs were determined by observing the turbidity of each well without the use of instruments. For the calculation of IC_{50}, the turbidity of each well was optically measured with an EIA reader at 630 nm. The IC_{50}s of Ro 09-1470 and KCZ for *Candida albicans* 652 and *Saccharomyces cerevisiae* ATCC 9763, which were used for biochemical experiments described below, were 0.40 and 0.05 μg/ml and 1.5 and 0.12 μg/ml, respectively.

Measurement of Syntheses of Biomolecules

About 2×10^8 cells of *C. albicans* 652 stored at −80°C was inoculated in 100 ml of PYG medium and aerobically cultivated for 5~8 hours at 37°C. Mid-logarithmic phase cells were harvested, washed twice with YNB and then resuspended in the same medium at 10^7 cells/ml. This cell suspension was used for the following experiments.

(i) DNA, RNA and Protein: To 3 ml of the cell suspension, drug and [^{3}H]adenine (4 μCi/ml) were added and incubated with gentle shaking at 30°C. At various incubation times 100 μl of sample was taken and treated with an equal volume of 10% TCA at 0°C for 30 minutes. After washing twice with cold 10% TCA, the radioactivity on the filter was counted (Aloka, LSC 1100) for the determination of total DNA and RNA amounts. For the separate determination of the amount of DNA, a 400 μl portion of cell suspension was added to 1 ml of 0.15 N NaOH and treated at 90°C for 80 minutes. The radioactivity of the second TCA-precipitate was then counted. The amounts of RNA were estimated from the values of the total and DNA amounts. For the estimation of protein synthesis, incorporation of [^{3}H]leucine (4 μCi/ml) into TCA insoluble fraction was measured.

(ii) Lipid: [^{3}H]Acetate (1 μCi/ml) was incorporated for the measurement of lipid biosynthesis. The incubation was stopped by the addition of an equal volumes of 15% KOH in 95% EtOH. After saponification at 70°C for 60 minutes, the lipids were extracted twice with an equal volume of hexane, which was then removed with a pipette. The free sterols were separated by TLC using plates having a zone for concentrating samples (silica gel 60 F_{254}, Merck) and dichloromethane as a developing solvent. The radioactivity on the plates was determined with a TLC scanner (Aloka, Radiochromanizer JTC-501).

Measurement of Oxygen Consumption

Mid-logarithmic phase cells of *C. albicans* 652 were prepared as described above and suspended in 0.4 mM KH_2PO_4 containing 10 mM glucose. Upon adding a sample, oxygen consumption by the cells (10^7 cells/ml) was measured at 30°C for 30 minutes with an oxygen electrode.

GC-MS Analysis of Sterols

The fractions of the free sterols, prepared from the cell suspension incubated with or without drugs, were isolated by preparative-TLC according to the method described above. Fractions of 4,4-dimethyl-sterols, 4α-methyl sterols or 4,4-desmethyl sterols were analyzed by using GC-MS (Jeol, JMS-DX303/JMA-DA5000) with an open column (2 m × 2 mm in diameter) packed with 1% OV-1, ran at a continuously rising temperature (20°C/minute) from 150°C ~ 300°C with helium as the carrier gas at a flow rate of 20 ml/minute.

Preparation of Microsome Fraction from *S. cerevisiae*

S. cerevisiae ATCC 9763 was cultured at 30°C for 24 hours in PYG medium and a portion was inoculated into fresh PYG medium and cultivated semi-anaerobically at 30°C. Cells were harvested at the end of exponential growth[5], washed with cold distilled water, and suspended in a minimum volume of 0.65 M sorbitol. A thick cell suspension was passed twice through a French pressure cell (Ohtake, French Press 5502) at an output pressure of 1,300 ~ 1,500 kg/cm^2. The cell-free suspension thus obtained was centrifuged at 8,000 × g for 20 minutes and the supernatant was further centrifuged at 125,000 × g for 90 minutes. The precipitate from the second centrifugation was suspended in 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, 0.25 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol and 20% glycerol. The microsomal fraction (30 ~ 50 mg protein/ml) was stored at −20°C until use.

Assay of P450 Lanosterol 14α-Demethylase ($P450_{14DM}$)

The activity of $P450_{14DM}$ was measured by the method of AOYAMA *et al.*[6]. The reaction mixture (2 ml) containing 0.1 M potassium phosphate buffer (pH 7.2), 0.15 mM NADPH, 10 mM glucose-6-phosphate, 0.19 units glucose-6-phosphate dehydrogenase, 0.2 mM EDTA, 1 mM KCN, and 5 mg protein of the microsome fraction, containing the endogenous substrate, lanosterol (about 1 nmol/mg protein). The reaction was run at 30°C with continual shaking for 60 minutes, and stopped by the addition of 2 ml of 15% KOH/EtOH. 4,4-Dimethylsterols isolated by preparative TLC as written above were analyzed by GC-MS. The procedure for GC-MS was the same as that used for other sterols, except for having a flow rate of 30 ml/minute. Lanosterol and its C-14 demethylated product 4,4-dimethyl-cholesta-8,14(24)-trien-3β-ol were determined by mass chromatography by scanning m/z 411 (the base peak of lanosterol) and m/z 412 (the molecular ion peak of the product). The conversion ratio was calculated from the peak area of the two sterols. Under these conditions the product formation proceeded linearly for at least 60 minutes, and 35 ~ 40% of the lanosterol was converted to the products during that period.

CO Binding Assay and Spectrophotometric Analysis

Inhibition of CO binding to $P450_{14DM}$ by Ro 09-1470 and azole compounds, and difference spectra were measured by the method of VANDEN BOSSCHE *et al.*[7].

Results

In Vitro Antifungal Activity

To mycologically characterize Ro 09-1470, which was the most active of the analogues we isolated[4], activity against 09 strains from 18 species were tested in comparison with KCZ, 5-FC, terbinafine and AMPH B (Table 1). Ro 09-1470 showed high activity against *Candida glabrata*, *Cryptococcus neoformans*, *Rhodotorula rubra*, *Trichosporon cutaneum*, *Aureobasidium pullulans*, *Exophiala dermatitidis* and *Exophiala jeanselmei*, and relatively less activity against *Candida albicans*, *Candida tropicalis*, *Saccharomyces cerevisiae*, *Kluyveromyces fragilis*, *Cryptococcus albidus* and mycelial fungi. Ro 09-1470 did not show cross-resistance with 5-FC, exhibiting the same activity against both 5-FC-resistant and -sensitive *C. albicans* strains. Mycelial fungi which were highly susceptible to terbinafine were refractory to Ro 09-1470 showing MICs as high as 200 μg/ml. The antifungal spectrum of Ro 09-1470 was overall similar to that of KCZ.

Table 1. Antifungal spectrum of Ro 09-1470 and reference drugs.

Strains	MIC (μg/ml)				
	Ro 09-1470	KCZ	5-FC	Terbinafine	AMPH B
Candida albicans ATCC 10231	200	25	1.56	>200	0.2
C. albicans 652[a]	200	12.5	>200	>200	0.2
C. tropicalis ATCC 13803	>200	>200	0.78	>200	0.2
C. glabrata IFO 0005	6.25	6.25	0.1	>200	0.1
Saccharomyces cerevisiae ATCC 9763	200	200	0.2	>200	0.2
Kluyveromyces fragilis ATCC 8635	200	50	3.13	25	0.2
Cryptococcus neoformans 159[a]	3.13	1.56	>200	3.13	0.05
C. albidus IFO 0378	200	100	>200	>200	0.39
Rhodotorula rubra IFO 0870	3.13	1.56	0.39	>200	0.78
Trichosporon cutaneum IFO 1198	0.39	<0.006	12.5	0.39	0.025
Aureobasidium pullurans IFO 5060	3.13	0.78	25	1.56	0.05
Exophiala dermatitidis IFO 8195	3.13	0.78	12.5	0.012	0.1
E. jeanselmei MTU 23016[b]	1.56	0.012	>200	<0.006	0.012
Trichophyton mentagrophytes T3[b]	100	12.5	>200	<0.006	0.39
Microsporum canis Mi-104[b]	200	12.5	>200	<0.006	0.39
M. gypseum Mi-56[b]	200	25	>200	0.1	0.78
Absidia corymbifera IFO 8084	200	50	>200	0.78	0.2
Aspergillus fumigatus MTU 06001[b]	200	12.5	200	3.13	0.2
A. niger NHL 5088[c]	100	6.25	3.13	0.05	0.2

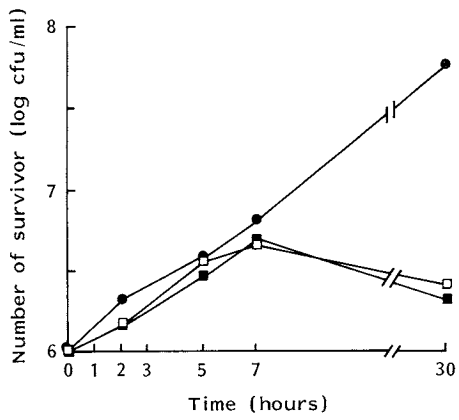
Obtained from [a] F. Hoffmann-La Roche, AG., Basle, Switzerland, [b] Faculty of Medicine, University of Tokyo, Japan, [c] National Institute of Hygiemic Science, Tokyo, Japan.

Effects on the Growth and Biosyntheses of Biomolecules

In the first 7 hours, 12 μg/ml ($\times 30\ IC_{50}$) of Ro 09-1470 did not have any effect on the growth of *C. albicans* 652 but antifungal activity was demonstrated after cultivation for 30 hours (Fig. 1). KCZ also showed a similar slow inhibition kinetic at 1.5 μg/ml ($\times 30\ IC_{50}$). Effect on the biosyntheses of DNA, RNA, protein or 4,4-demethylsterols was investigated under the condition. As seen from Fig. 2, 4,4-demethylsterol was the only biomolecule whose synthesis was markedly inhibited by Ro 09-1470. RNA and protein syntheses were not inhibited at all, while DNA synthesis was somewhat affected. This inhibition profile was essentially the same as that of KCZ.

Fig. 1. Effects of Ro 09-1470 and KCZ on the growth of *Candida albicans* 652.

□ KCZ (1.5 μg/ml), ■ Ro 09-1470 (12 μg/ml), ● none.



These results suggest that Ro 09-1470, like KCZ, may inhibit an enzyme or enzymes involved in the biosynthesis of ergosterol.

Effect of Ro 09-1470 on the Respiration of *C. albicans*

No inhibition of oxygen consumption was detected with Ro 09-1470 up to 100 μg/ml, whereas KCZ, antimycin A and gramicidin S showed IC_{50}s of 37, <0.34 and 3.65 μg/ml, respectively.

Fig. 2. Effect of Ro 09-1470 and KCZ on the syntheses of biomolecules.

Biosyntheses of DNA (○), RNA (●), protein (□) and 4,4-demethylsterols (■) were measured. (A) Ro 09-1470 (12 μg/ml), (B) KCZ (1.5 μg/ml).

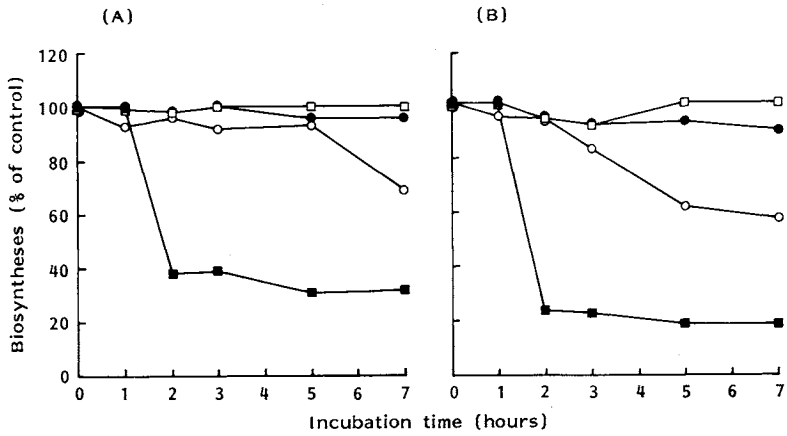
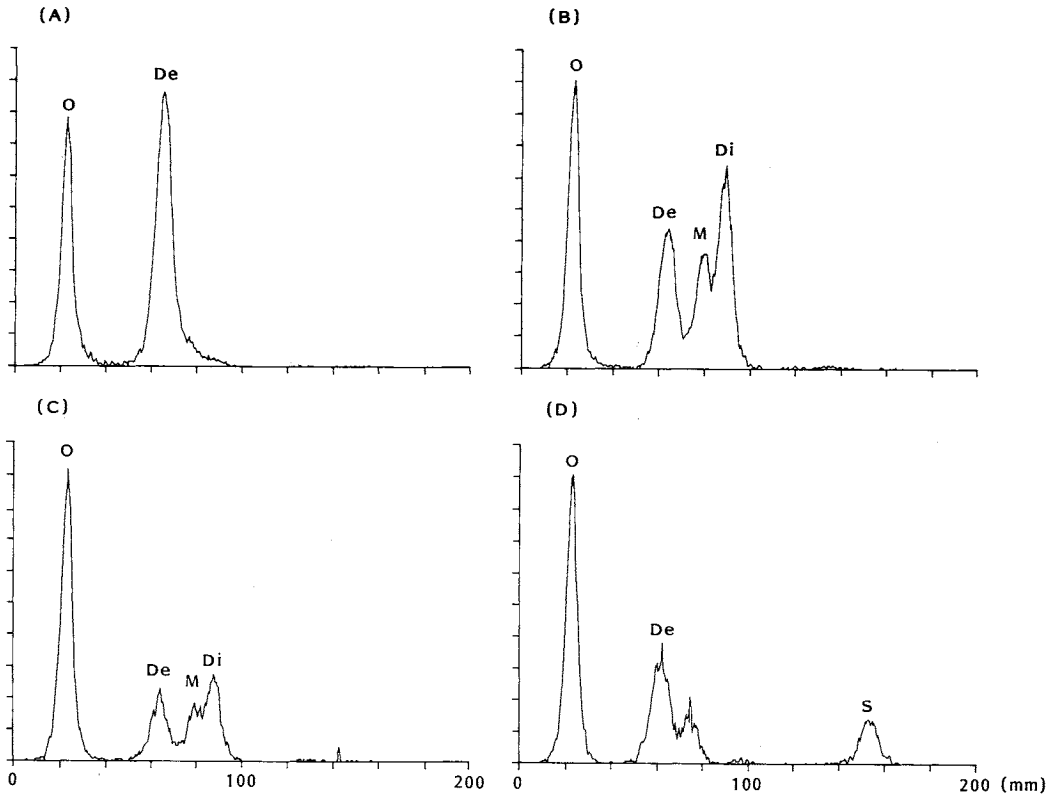


Fig. 3. Effects of Ro 09-1470, KCZ and naftifine on the [^{14}C]acetate incorporation into squalene and sterols by *Candida albicans* 652.



The lipid fractions from the cells treated with solvent (A), 12 μg/ml of Ro 09-1470 (B), 1.5 μg/ml of KCZ (C) or 25 μg/ml of naftifine (D) were separated by TLC, and the radioactivity on the TLC plates were analyzed by a TLC scanner. Peaks O, De, M, Di and S represent the origin, 4,4-demethylsterols 4α-methylsterols, 4,4-dimethylsterols and squalene, respectively.

Effect on Sterol Biosynthesis

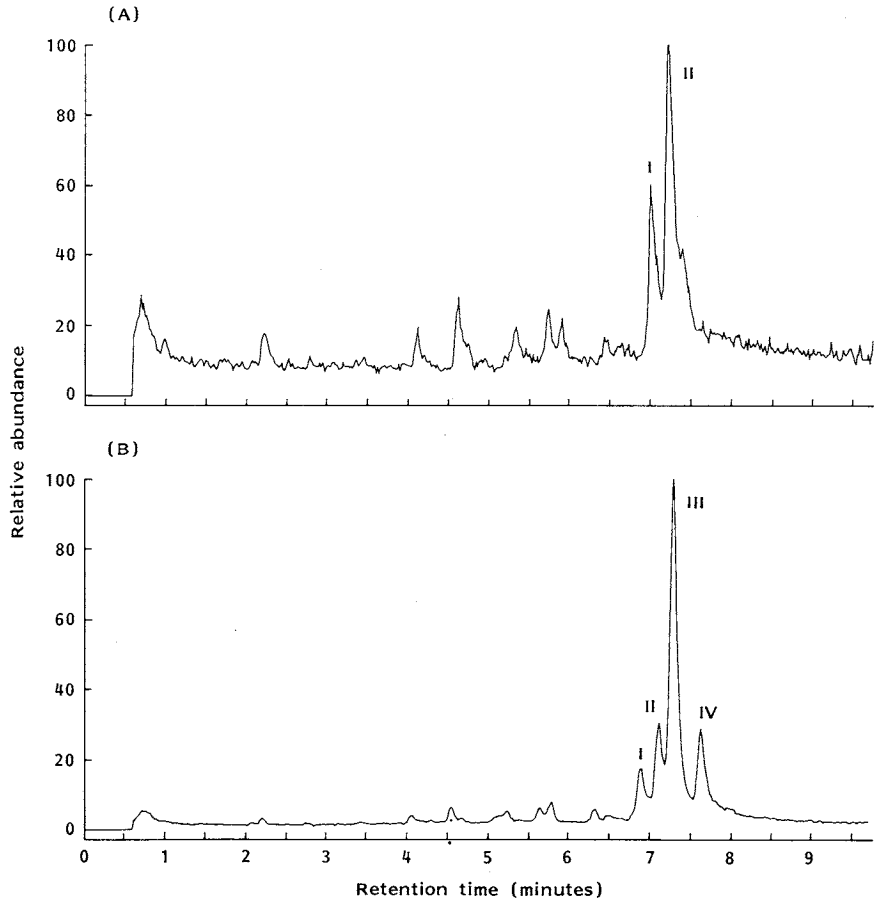
(1) Change in Sterol Components

After a 3-hour pulse-treatment of cells with [^{14}C]acetate, sterols were extracted and the distribution of radioactivity was analyzed by TLC (Fig. 3). In contrast with the control, in which only 4,4-demethylsterols were detected, 4-mono- and 4,4-dimethylsterols were dominant in the cells treated with Ro 09-1470. Naftifine and KCZ, known to inhibit squalene epoxidase[8] and $P450_{14DM}$[2,3], respectively, also diminished the incorporation of [^{14}C]acetate into 4,4-demethylsterols but accumulated squalene, and mono- and dimethylsterols, respectively. To determine which intermediates were accumulated by the treatment with Ro 09-1470, we analyzed the TLC-fractionated sterols by GC-MS.

(2) GC-MS Analysis of the Accumulated Metabolites

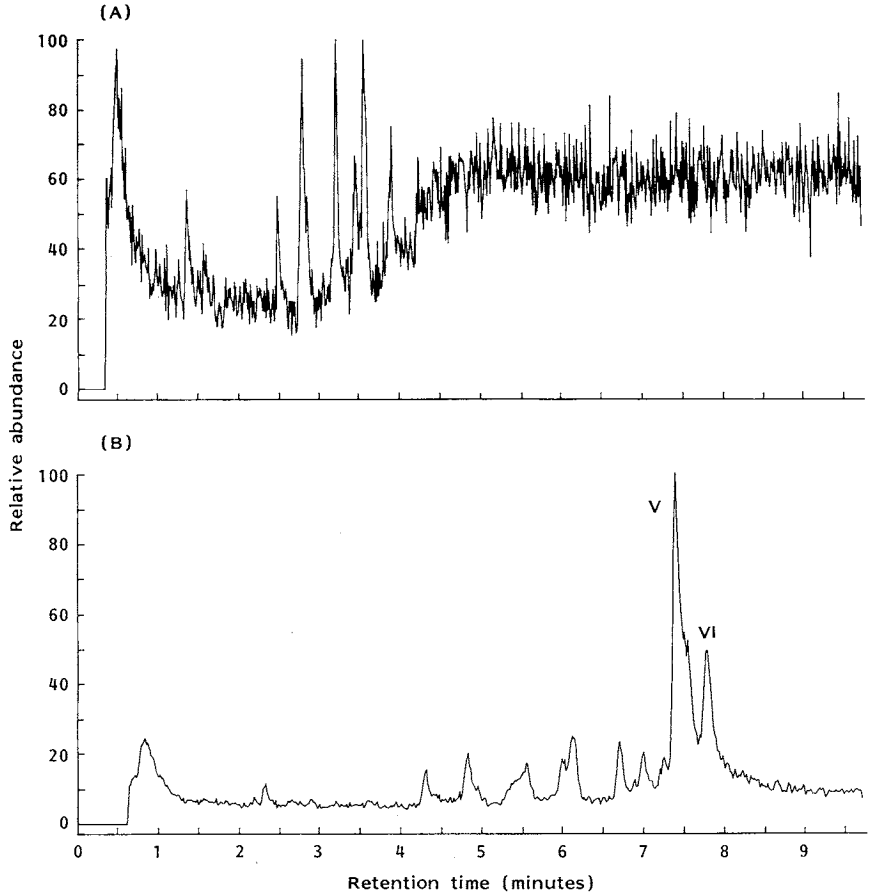
(a) 4,4-Dimethylsterols: As seen from Fig. 4, the major component (peak II) in the gas chromatogram of 4,4-dimethylsterols from the control cells was identified to be lanosterol by its retention time and by mass-spectrography in which the molecular ion peak (M^+) of m/z 426 and fragmentation pattern were identical to those of lanosterol. Peak I (M^+, m/z 400) in the control was not identified. Treatment with

Fig. 4. GC-MS scans showing relative proportions of 4,4-dimethylsterols.



4,4-Dimethylsterols were isolated by TLC from the lipid fractions of *Candida albicans* 652 treated with solvent (A) or 12 μg/ml of Ro 09-1470 (B). The description of each peak is given in Table 2.

Fig. 5. GC-MS scans showing relative proportions of 4α-methylsterols.



Further details are given in Fig. 4.

Ro 09-1470 resulted in the appearance of two additional peaks; peak III (M^+, *m/z* 440) and peak IV (M^+, *m/z* 442), the former having the higher intensity. The compound corresponding to peak III was identified as a eburicol (4,4,14α-trimethylergosta-8,24(28)-dien-3β-ol) by the fragmentation analysis of the mass-spectrum. 1) The molecular ion peak (*m/z* 440) corresponded to the MW of eburicol and 2) the fragmentation pattern coincided with those reported by RAGSDALE[9]. Peak IV was identified to be 24(28)-dihydroeburicol, since the molecular ion peak (*m/z* 442), base peak (*m/z* 427) and the fragmentation pattern were the same as those of eburicol.

(b) 4α-Methylsterols: In the GC-MS scan of 4α-methylsterols (Fig. 5), peak V (M^+, *m/z* 426) and VI (M^+, *m/z* 428) appeared in the samples from the cells treated with Ro 09-1470. The mass spectrum of peak V was identical with that of obtusifoliol (4α,14α-dimethylergosta-8,24(28)-dien-3β-ol)[9]. In contrast with cells treated with Ro 09-1470, 4α-methylsterols were not detected in the control.

(c) 4,4-Demethylsterols: Ergosterol (peak VII) was the main peak in the control sample, whereas no measurable amount of 4,4-desmethylsterols were detected in the treated cells.

Relative Quantities of Each Intermediates

To compare the quantity of each metabolite between the control and the samples treated with Ro

Table 2. Relative amounts of sterols in control and antifungal-treated cells.

Sterols	MW	Peak[a]	Relative amounts (lanosterol = 100)		
			Control	Ro 09-1470 (12.0 μg/ml)	KCZ (1.5 μg/ml)
4,4-Dimethylsterols					
Unknown	400	I	55	55	78
Lanosterol	426	II	100	100	100
Eburicol[b]	440	III	nd	366	279
Dihydroeburicol	442	IV	nd	95	71
4α-Methylsterols					
Obtusifoliol[c]	426	V	nd	134	69
Dihydroobtusifoliol	428	VI	nd	59	52
4,4-Demethylsterols					
Ergosterol	396	VII	909	nd	nd

[a] Peak numbers correspond to those in Figs. 4 and 5.
[b] 4,4,14α-Trimethylergosta-8,24(28)-dien-3β-ol.
[c] 4α,14α-Dimethylergosta-8,24(28)-dien-3β-ol.
nd: Not detected.

Fig. 6. Inhibitory effects of Ro 09-1470, KCZ and FCZ on $P450_{14DM}$.

○ Ro 09-1470, ● KCZ, □ FCZ.

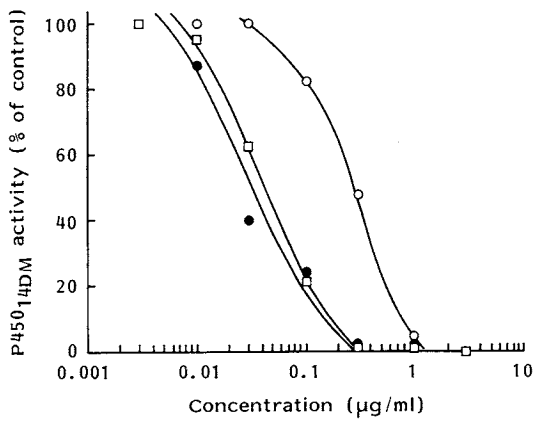
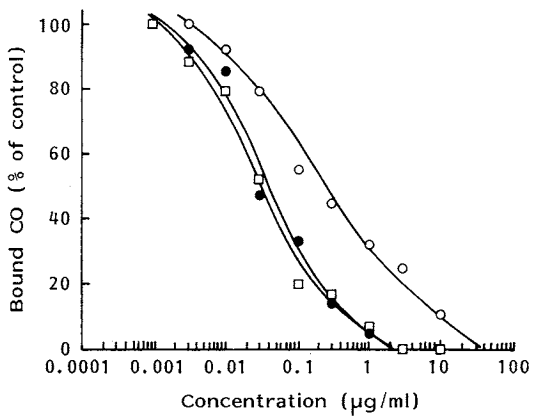


Fig. 7. Inhibitory effects of Ro 09-1470, KCZ and FCZ on CO binding to $P450_{14DM}$.

○ Ro 09-1470, ● KCZ, □ FCZ.



09-1470, a relative amount of sterols detected in each sample was calculated on the basis of the amount of total ion in GC-MS and is summarized in Table 2. The table shows that Ro 09-1470 inhibited the biosynthesis of ergosterol by interfering with the reaction of demethylation at C-14 of sterol. The same accumulation pattern of sterols was observed by the treatment with KCZ.

Interaction with Cytochrome $P450_{14DM}$ of *S. cerevisiae*

(a) Inhibition of lanosterol 14α-demethylation catalyzed by $P450_{14DM}$: The catalytic activity of $P450_{14DM}$ in microsome fraction was assayed by measuring the conversion of lanosterol to 4,4-dimethylcholesta-8,14(24)-trien-3β-ol. Fig. 6 shows the tritration curve of Ro 09-1470 and two $P450_{14DM}$ inhibitors against $P450_{14DM}$ activity. Ro 09-1470 inhibited the enzyme in a dose dependent manner giving an IC_{50} of 0.3 μg/ml. This inhibitory activity was 5 to 7 times lower than that of KCZ and FCZ, which inhibited the enzyme with IC_{50}'s of 0.05 and 0.06 μg/ml, respectively.

Fig. 8. Difference spectra of ferric $P450_{14DM}$ induced by 10 μg/ml of Ro 09-1470 (solid line) and KCZ (dashed line).

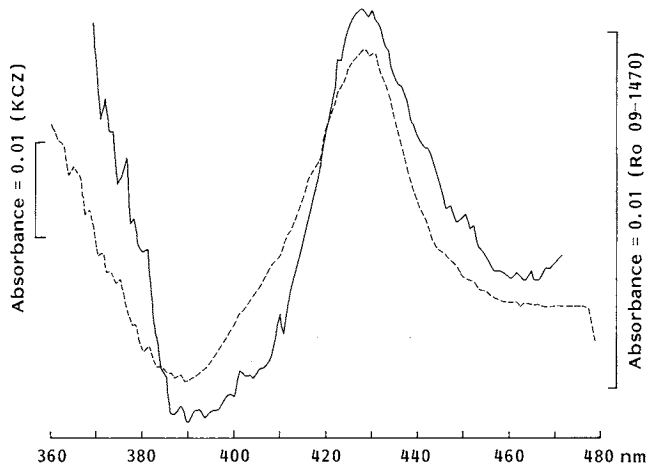
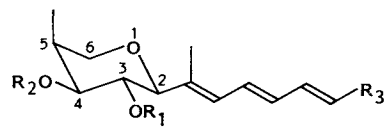


Table 3. Activities of Ro 09-1470 analogues against *S. cerevisiae* ATCC 9763 and its $P450_{14DM}$.



Compound	R_1	R_2	R_3	Antifungal activity	$P450_{14DM}$ inhibition
				IC_{50} (μg/ml)	IC_{50} (μg/ml) (% inhibition at 10 μg/ml)
Ro 09-1469	H	CH_3	C_3H_7	>100	>10 (0)
Ro 09-1470	$COCH_2NH_2$	CH_3	C_3H_7	1.5	0.34
Ro 09-1543	$COCH_2NHCOCH_3$	CH_3	C_3H_7	46	>10 (12.9)
Ro 09-1544	H	CH_3	$CH=CHCH_3$	>100	>10 (0)
Ro 09-1545	$COCH_2NHCH_2CH_2COCH_3$	CH_3	C_3H_7	1.2	0.35
Ro 09-1547	H	H	C_3H_7	>100	>10 (0)
Ro 09-1549	C-5 demethyl derivative of Ro 09-1469			>100	>10 (0)

(b) Binding of Ro 09-1470 to $P450_{14DM}$: To learn whether Ro 09-1470 directly interacts rats with $P450_{14DM}$, the compound was tested for its activity to bind to the heme of the enzyme. As shown in Fig. 7, an increase in the concentration of Ro 09-1470 prevented the binding of CO to the enzyme, indicating that the compound bound to the heme. In this binding assay, too, KCZ and FCZ had a higher affinity for the P450 heme.

More direct evidence to indicate the interaction between Ro 09-1470 and heme was obtained by the difference spectrum. Ro 09-1470 induced a difference spectrum with an absorption maximum and minimum at 428 and 390 nm, respectively (Fig. 8), as KCZ did.

Structure-activity Relationship

Table 3 shows the activities of analogues of Ro 09-1470 against *S. cerevisiae* ATCC 9763 and its $P450_{14DM}$. Beside Ro 09-1470, Ro 09-1543 and Ro 09-1545 were the only compounds that showed activity.

These three active compounds had an aminoacetoxy moiety in the side chain at C-3.

Discussion

From a *Penicillium* sp.[4] we have isolated a new series of antifungal agents, among which Ro 09-1470 showed the highest activity. In the long history of antifungal screening of microbial products, there have been no compounds reported that inhibit $P450_{14DM}$. In this report we have proved that Ro 09-1470 blocks the biosynthesis of ergosterol in fungi by inhibiting $P450_{14DM}$, which catalyzes demethylation of C-14 methyl group of lanosterol, an intermediate of ergosterol. Thus, Ro 09-1470 is the first ergosterol biosynthesis inhibitor discovered from a natural source.

The mode of action of Ro 09-1470 inhibiting $P450_{14DM}$ was elucidated by several lines of evidence; i) ergosterol biosynthesis was predominantly inhibited by the compound under the condition where neither DNA, RNA, protein, nor respiration were inhibited, ii) C-14 methyl intermediates of ergosterol biosynthesis accumulated, iii) $P450_{14DM}$ enzyme activity was inhibited, and iv) Ro 09-1470 bound to heme of $P450_{14DM}$. This evidence indicates that Ro 09-1470 directly interfered with $P450_{14DM}$ and showed inhibition of ergosterol biosynthesis in cells.

The complex of Ro 09-1470 with ferric $P450_{14DM}$ gave rise to a typical Type II difference spectrum with an absorption maximum at 428 and minimum at 390 nm. The Type II spectral change has been postulated to result from the binding of a basic amino nitrogen to the sixth coordination position of a heme iron[5]. YOSHIDA *et al.*[10~12] and HITCHCOCK *et al.*[13] observed that azoles, which inhibit $P450_{14DM}$, induced a Type II difference spectra when bound to ferric $P450_{14DM}$. These results strongly suggest that the nitrogen atom of the amino group in the side chain at C-3 of Ro 09-1470 should coordinate to the heme iron of $P450_{14DM}$.

Using several minor components isolated from the same producer strain, a limited structure-activity relationship was studied. Of the seven natural analogues, the only compounds that have a glycyl ester moiety in the C-3 side chain showed activity. However, a difference was seen in the activity; Ro 09-1543 having an acetylglycyl ester was much weaker than the other two. The results suggest that existence of an glycyl ester moiety is not sufficient for the activity of the compound, but the basicity in the nitrogen of glycyl moiety is crucial for the activity against fungi and $P450_{14DM}$. When antifungal activities of the seven analogues were compared with the IC_{50} for $P450_{14DM}$, good correlation was observed. Ro 09-1470 and Ro 09-1545 were the two most active compounds against *S. cerevisiae*, and were also the most potent inhibitors of $P450_{14DM}$ activity. Ro 09-1543 was less potent in both antifungal and enzyme inhibition activities. This parallelism together with the above evidence which showed direct interaction between Ro 09-1470 and $P450_{14DM}$ suggests that $P450_{14DM}$ is a primary target of Ro 09-1470.

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