SYNERAZOL, A NEW ANTIFUNGAL ANTIBIOTIC

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Synerazol, a new antifungal antibiotic, was isolated from cultured broth of *Aspergillus fumigatus* SANK 10588. The structure was determined based on NMR and mass spectral evidences. Synerazol was found to be a related substance to pseurotin A. Synerazol was active against *Candida albicans* and other fungi, and showed marked synergistic activity with azole-type antifungal agents.

In the course of our screening programs for new antifungal antibiotics, a new fungal metabolite, synerazol[†] was isolated. The producing organism of synerazol was identified as *Aspergillus fumigatus*, which was isolated from a soil sample collected in Thailand.

Synerazol was active against fungi including *Candida albicans*. The activity was highly potentiated by the presence of a low concentration of azole antifungal agents.

In this paper, the taxonomy of a producing strain, isolation, physico-chemical properties, structure elucidation, and biological activity of synerazol are described.

Identification of the Producing Fungus

Strain SANK 10588 was freshly isolated from a soil sample collected at Prachuap khiri khan, Thailand. The fungus was identified as *A. fumigatus*, from the characteristics described below.

Colonies on CYA medium[1] grow rapidly, attaining a diameter of 50 mm in 7 days at 25°C with velutinous appearance, deep green (27D8)[2] in color. Reverse of the colonies are grayish green (27D5). Colonies on MEA medium grow 70 mm in 7 days at 25°C, and are with velutinous appearance, dark green (27F8) in color. Reverse of the colonies are grayish yellow (2B5). At 37°C, colonies attain 70 mm in 7 days on CYA and MEA medium.

Conidia are produced abundantly on CYA and MEA media at 25 and 37°C. Conidial heads are columnar and are green to dark green in color. Conidia producing structures arise directly from mycelium. Conidiophores are smooth walled, $50 \sim 200\,\mu m \times 3.0 \sim 4.0\,\mu m$ in size, pale green in color. Vesicles are flask-shaped, $4.0 \sim 12.0\,\mu m$ i.d., and pale green in color. Metulae are not produced. Phialides are produced directly in upper half of the vesicle, $4.5 \sim 6.4\,\mu m \times 2.4 \sim 3.6\,\mu m$, pale green. Conidia are spherical to subspherical, $2.4 \sim 3.2\,\mu m$ i.d., with spinose wall, deep green in mass. No teleomorphs are observed.

† Jpn. Kokai 277,492 ('89), Nov. 7, 1989.

This strain was deposited with the Fermentation Research Institute of Agency of Industrial Science and Technology as FERM P-9956.

Fermentation

The culture of *A. fumigatus* SANK 10588 from an agar slant was inoculated into baffled 2-liter Erlenmeyer flasks containing 500 ml of a medium consisting of glycerol 5%, potato 5%, malt extract 0.5%, and yeast extract 0.5% (pH 6.0 before sterilization). The culture was incubated on a rotary shaker at 200 rpm for 6 days at 26°C and 1.5 liters of the seed culture were inoculated into each of two 600-liter fermentation tanks containing 300 liters of the same medium. The fermentation was continued for 6 days at 26°C with agitation at a rate of 100 rpm and aeration of 300 liters/minute. The production of synerazol was monitored by HPLC assay (column; Senshu Pak ODS-H-2151 (6 × 150 mm, Senshu Scientific Co., Ltd.), solvent; 44% acetonitrile-water, flow rate; 1.7 ml/minute, detection; absorbance at 254 nm) of an ethyl acetate extract of culture filtrate. Under these conditions, synerazol was eluted at 9.5 minutes. Production of synerazol reached a maximum level of 7.1 μg/ml at 144 hours (Table 1) of the cultivation.

Isolation

The purification scheme of synerazol is shown in Fig. 1. The cultured broth was filtered with an aid of diatomaceous earth, and the filtrate (630 liters) was extracted three times with ethyl acetate (each 300 liters). Then the organic layer was combined, washed with water saturated with NaCl, and concentrated *in vacuo*.

The concentrate was separated in two parts and each of them was applied on a column of silica gel (Mallinckrodt, 1 liter) equilibrated with *n*-hexane-dichloromethane (1 : 1). The column was washed with 1 liter of the same solvent mixture. The fractions containing synerazol, eluted with *n*-hexane-dichloromethane (1 : 2), were combined and concentrated *in vacuo* to yield 227 g of crude oil. The crude oil was further purified by successive HPLC treatments on System 500A (Millipore Corporation) equipped with ODS column (μBondasphere C18, particle size 15~30 μm, pore size 100 Å, 47 × 300 mm, Millipore). Initially, each 10 g of the oily material was chromatographed with 60% methanol-water at a flow rate of 100 ml/minute. Effluent containing synerazol eluted at about 25 minutes was pooled, concentrated *in vacuo*, extracted with ethyl acetate, and dried to afford 36 g of crude synerazol. Secondly, the crude synerazol thus obtained was treated with a solvent mixture of 45% acetonitrile-water at a flow rate of 120 ml/minute.

Table 1. Time course of synerazol production.

Time (hours)	pH	Mycelium (PCV[a])	Concentration[b]
0	6.1	0.7	N.D.
24	5.3	1.8	N.D.
48	3.1	2.6	N.D.
72	3.0	2.3	N.D.
96	3.2	2.8	2.8
120	3.4	2.6	5.0
144	3.8	3.0	7.1

[a] Packed cell volume (ml) per 10 ml broth at 3,000 rpm for 15 minutes.
[b] Concentration of synerazol in culture supernatant (μg/ml).
N.D.: Not determined.

Fig. 1. Isolation procedure of synerazol.

Culture filtrate (630 liters)
↓ extracted with EtOAc
EtOAc layer
↓ concd
Silica gel
↓ eluted with CH_2Cl_2-*n*-hexane (2 : 1)
Preparative LC/System 500A
↓ eluted with 60% MeOH-H_2O
Preparative LC/System 500A
↓ eluted with 45% CH_3CN-H_2O
Synerazol (720 mg)

Synerazol was eluted at about 20 minutes. The active fraction was concentrated *in vacuo*, and the antibiotic was extracted with ethyl acetate. The extract was concentrated to dryness to yield colorless powder of purified synerazol (720 mg).

Physico-chemical Properties

The physico-chemical properties of synerazol are shown in Table 2. Synerazol is soluble in methanol, acetone, ethyl acetate, dichloromethane, acetonitrile, dimethyl sulfoxide, but insoluble in water. Synerazol is unstable in aqueous solution, especially at pHs above 7 or below 4. It is also rapidly degraded in aqueous solution with SH-reagents such as 2-mercaptoethanol, dithiothreitol, and cysteine.

Structure Elucidation of Synerazol

The MW of synerazol (413) was determined by FAB-MS ($(M+H)^+$: *m/z* 414). The molecular formula of $C_{22}H_{23}NO_7$ was established by HRFAB-MS (obsd *m/z* of $(M+H)^+$, 414.1541; calcd for $C_{22}H_{24}NO_7$, 414.1553).

The ^1H NMR spectrum of synerazol is shown in Fig. 2. Two exchangeable proton signals were observed at 4.05 and 7.49 ppm. Six distinct proton spin systems comprising 15 carbons were deduced to correspond to partial structures **1**~**6**, on the basis of ^1H, ^{13}C NMR parameters summarized in Table 3

Table 2. Physical and chemical properties of synerazol.

Nature	Neutral, colorless powder
$[\alpha]_D^{25}$	+22.9° (*c* 0.55, $CHCl_3$)
MW	413 (FAB-MS $(M+H)^+$ 414)
Molecular formula	$C_{22}H_{23}NO_7$
UV λ_{max}^{EtOH} nm ($E_{1cm}^{1\%}$)	202 (567), 251 (355), 284 (249)
IR ν_{max} liq (cm^{-1})	1737, 1705, 1632, 1106

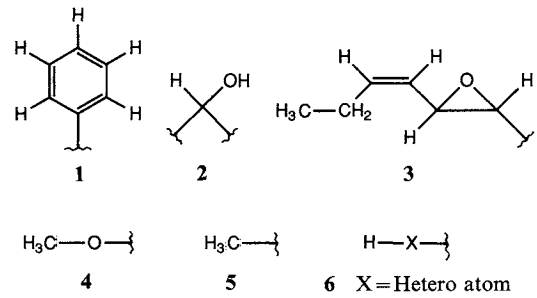


Fig. 2. ^1H NMR spectrum of synerazol in $CDCl_3$.

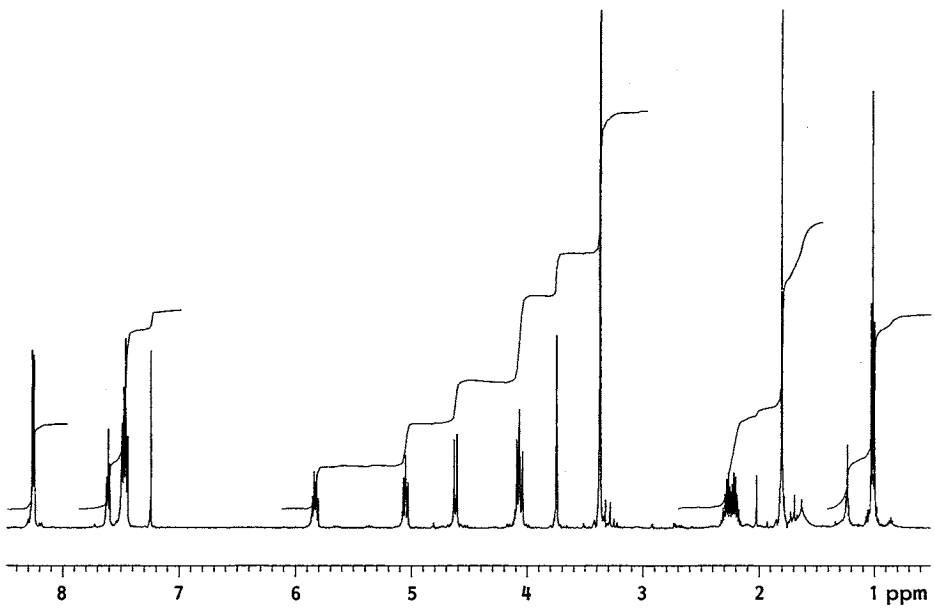
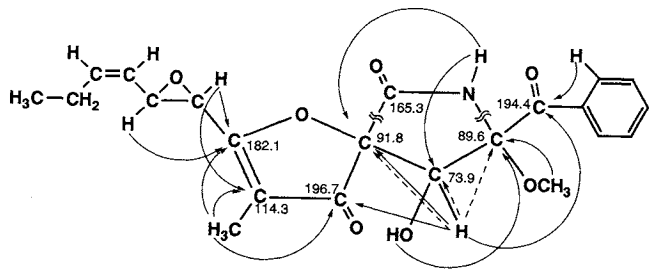


Table 3. ^{1}H and ^{13}C NMR parameters of synerazol in $CDCl_3$.

δ ^{13}C	$^{1}J_{CH}$[a] (MOB[b])	$^{n}J_{CH}$[c] (MLR[d])	δ ^{1}H	J_{HH}[e] (M[f])	Assignment
196.7	— (s)	4 (quintet)			4
194.4	— (s)	8, 3 (dt)			17
182.1	— (s)	(m)			2
165.3	— (s)	— (s)			6
141.4	154 (d)	6 (m)	5.83	11.0, 7.6, 0.9 (dtd)	13
134.6	164 (d)	7 (t)	7.61	7.4, 1.2 (tt)	21
132.4	— (s)	7 (t)			18
130.5	163 (d)	7 (t)	8.26	8.6, 1.2 (dd)	19, 23
128.7	165 (d)	7 (d)	7.46	8.6, 7.4 (dd)	20, 22
123.5	160 (d)	5, 2 (qd)	5.05	11.0, 9.0, 1.5 (ddt)	12
114.3	— (s)	7 (q)			3
91.8	— (s)	6, 5 (dd)			5
89.6	— (s)	12[g] (br)			8
73.9	154 (d)	5 (t)	4.62	12.2 (d)	9
55.1	179 (d)	13, 2 (dd)	4.08	9.0, 1.9, 0.9 (ddd)	11
52.6	181 (d)	7[g] (br)	3.74	1.9 (d)	10
51.7	144 (q)	— (s)	3.37	— (s)	O–CH_3
21.3	127 (t)	(m)	2.2~2.3	(m)	14
14.0	127 (q)	5 (q)	1.01	7.5 (t)	15
5.1	130 (q)	— (s)	1.80	— (s)	16
			7.49	— (br)	N–H
			4.05	12.2 (d)	O–H

[a] One-bond ^{13}C-^{1}H coupling constants in Hz.
[b] ^{13}C signal multiplicity attributable to one-bond ^{13}C-^{1}H coupling.
[c] Long-range ^{13}C-^{1}H coupling constants in Hz.
[d] ^{13}C signal multiplicity attributable to long-range ^{13}C-^{1}H coupling.
[e] ^{1}H-^{1}H coupling constants in Hz.
[f] ^{1}H signal multiplicity.
[g] Peak width in Hz at half-height.

Fig. 3. Summary of observed long-range ^{13}C-^{1}H couplings (⟶) and ^{13}C-{^{1}H} NOE (⇢) of synerazol.



and ^{1}H-^{1}H, ^{13}C-^{1}H COSY. Since all the carbons having one-bond ^{13}C-^{1}H coupling are involved in the structures **1**~**5**, proton at 7.49 ppm must be bonded to a hetero atom.

Besides the structures **1**~**6**, seven carbon, four oxygen, and one nitrogen atoms are in a molecule of the compound. Connection between these atoms and structures **1**~**6** were deduced mainly from long-range ^{13}C-^{1}H couplings and ^{13}C-{^{1}H} NOE correlations summarized in Fig. 3. The carbons at 196.7 and 194.4 ppm were considered to be conjugated carbonyl carbons whereas the carbon at 182.1 ppm was assigned to the oxygen-attached olefinic carbon atom on the basis of ^{13}C chemical shifts and ^{13}C-^{1}H long-range couplings. The carbon at 91.8 ppm was assigned to be an oxygen-attached sp^3 carbon atom.

Fig. 4. FAB-CAD MS/MS spectrum of $(M+H)^+$ of synerazol.

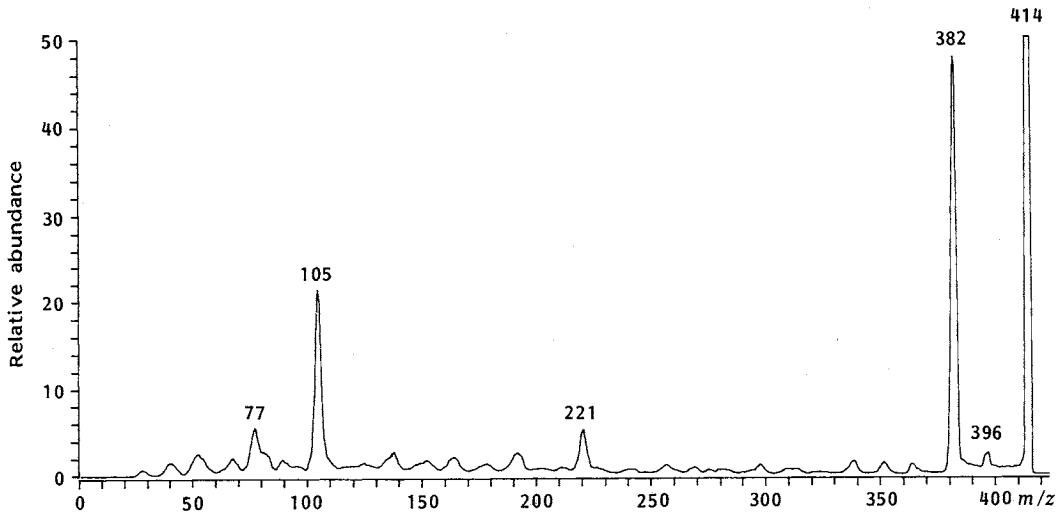
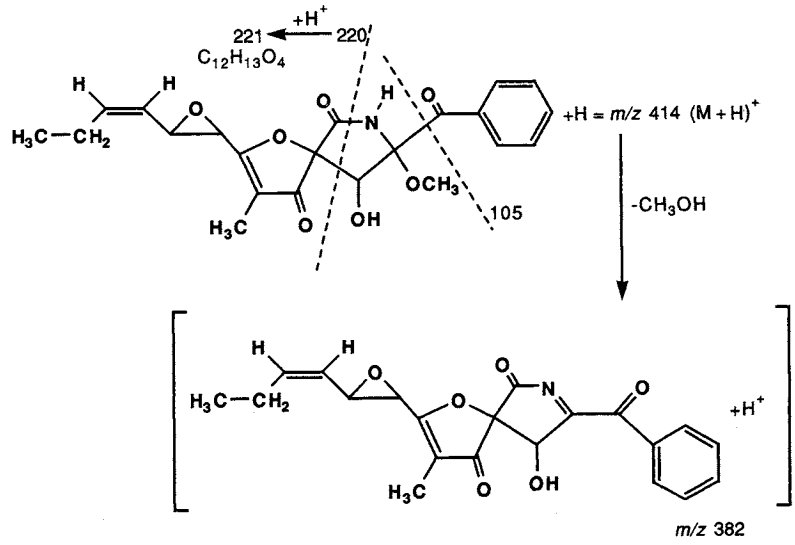


Fig. 5. Fragmentation pathways from $(M+H)^+$ of synerazol.



Since the compound shows no basic nature, the nitrogen atom seems to be involved in an amide group rather than in an imino group. The carbon at 165.3 ppm is assignable to the amide carbonyl from its chemical shift, though no spin-spin coupling was observed between the carbon and any protons. The exchangeable proton at 7.49 ppm is not an alcoholic proton but an amide proton, because no free valence remains on any of oxygen atoms.

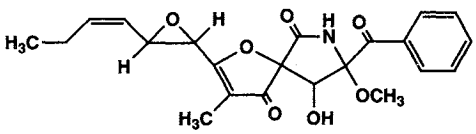
The following mass spectral evidences support the presence of amide group and show its direction.

Distinct peaks at *m/z* 382 and at *m/z* 221 were observed both in the FAB mass spectrum and in the FAB collisionally activated dissociation (CAD) tandem mass (MS/MS) spectrum of $(M+H)^+$ (Fig. 4). The compositions of the ions were determined to be $C_{21}H_{20}NO_6$ (($(M+H)^+ - CH_3OH$): obsd *m/z* 382.1308; calcd *m/z* 382.1291) and $C_{12}H_{13}O_4$ (obsd *m/z* 221.0759; calcd *m/z* 221.0804), by HRFAB-MS, respecively.

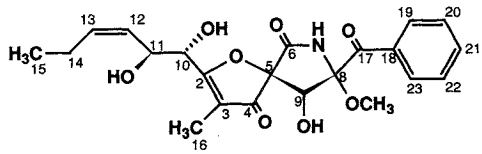
Table 4. ^{13}C chemical shifts of synerazol and pseurotin A[3] in $(CD_3)_2SO$.

Assignment	Synerazol	Pseurotin A	Assignment	Synerazol	Pseurotin A
C-2	179.6	186.8[a]	C-13	140.3	134.0
C-3	113.6	111.6	C-14	20.5	20.6
C-4	196.2[b]	196.7	C-15	14.0	14.1
C-5	92.5[c]	92.4	C-16	4.8	5.6
C-6	165.9	166.6[a]	C-17	195.7[b]	196.4
C-8	91.9[c]	91.1	C-18	133.4	133.6
C-9	74.6	75.0	C-19, 23	130.3	130.2
C-10	51.6	72.0	C-20, 22	128.4	128.4
C-11	54.0	68.3	C-21	133.8	133.6
C-12	124.3	128.4	O–CH_3	51.6	51.7

[a] Original assignments[3] of C-2 and C-6 were interchanged on the basis of present result.
[b] Assignments of C-4 and C-17 may be interchanged.
[c] Assignments of C-5 and C-8 may be interchanged.



Synerazol (**7**)



Pseurotin A (**8**)

Table 5. Activity of synerazol against fungi.

Test organism	MIC (μg/ml)
Candida albicans YU 1200	12.5
C. albicans IFO 0583	25
C. glabrata IFO 0622	25
C. tropicalis SANK 59263	25
Schizosaccharomyces pombe SANK 57362	25
Saccharomyces cerevisiae SANK 50265	25
Cryptococcus neoformans SANK 58063	12.5
Aspergillus oryzae SANK 11147	>100
A. fumigatus SANK 10662	>100
Fusarium oxysporum SANK 17076	>100
Penicillium chrysogenum SANK 12768	>100
Pyricularia oryzae SANK 11468	25
Trichophyton rubrum SANK 11768	25
T. mentagrophytes SANK 11868	>100

Incubation time: 48 hours (yeasts), 120 hours (fungi).
Incubation temperature: 30°C (yeasts), 26°C (fungi).
Medium: Sabouraud agar + 0.3% yeast extract.
Inoculum size: 1×10^6 cells/ml.

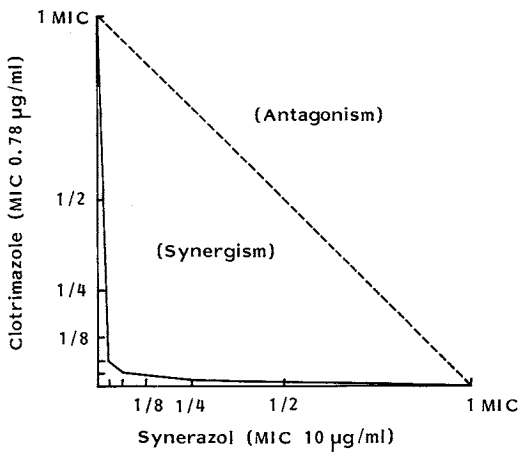
Another distinct peak at *m/z* 105 shows the presence of the benzoyl group in the molecule. Since *m/z* 105 was not found in the FAB-CAD MS/MS spectrum of *m/z* 221, the benzoyl group is not involved in the ion at *m/z* 221 and the ion should be assigned to the left-half moiety of the molecule including the amide carbonyl group (Fig. 5). The abundance of $((M+H)^+ - CH_3OH)$ ion may be attributable to the adjacent position of the amide nitrogen to the methoxylated carbon. Consequently, structure of synerazol was determined as **7**.

The structure of synerazol is closely related to that of pseurotin A (**8**), a metabolite of *Pseudeurotium ovalis* STOLK[3,4]. The structure of pseurotin A including absolute stereochemistry has been determined by means of single crystal X-ray analysis of its 12,13-dibromo derivative[4]. Pseurotin A corresponds to the hydrolysis product of the epoxide ring in synerazol. The ^{13}C NMR chemical shifts of **7** in $(CD_3)_2SO$ agree well with those of **8**[3] except for the values around the epoxide moiety (Table 4). Hence, synerazol seems to have a stereochemical resemblance to pseurotin A.

Biological Properties

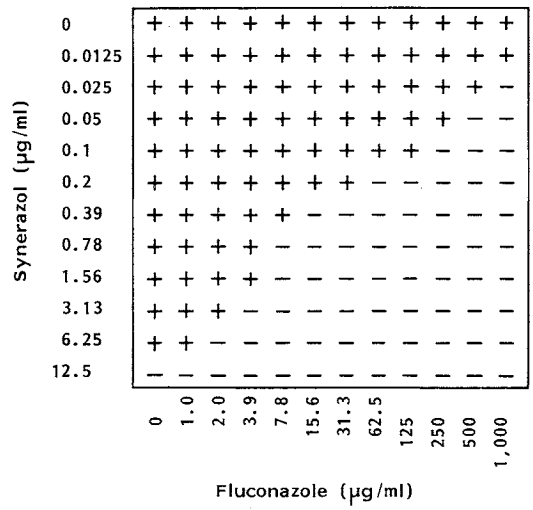
The MICs of synerazol for yeasts and fungi are listed in Table 5. These were determined by the agar dilution method in Sabouraud agar medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 0.3%

Fig. 6. Isobologram between synerazol and clotrimazole.



FIC index 0.078. Test organism: *Candida albicans* YU 1200 (1×10^4 cells/ml). Incubation time: 18 hours. Incubation temperature: 30°C. Medium: Sabouraud medium + 0.3% yeast extract.

Fig. 7. Checkerboard between synerazol and fluconazole.



FIC index $\leqq 0.039$. Test organism: *Candida albicans* YU 1200 (1×10^4 cells/ml). Incubation time: 18 hours. Incubation temperature: 30°C. Medium: Sabouraud medium + 0.3% yeast extract.

yeast extract and the incubation was carried out at 30°C for 48 hours for yeasts and 26°C for 120 hours for fungi.

The activity of synerazol was potentiated strongly by co-presence of azole compounds such as clotrimazole. The synergistic activity was evaluated by the checkerboard method using broth dilution in Sabouraud liquid medium with 0.3% yeast extract after incubation at 30°C for 18 hours. The isobologram between synerazol and clotrimazole is shown in Fig. 6. The synergistic effect was very strong and the FIC index was calculated to be 0.078. Further studies using other imidazoles and triazoles such as ketoconazole and fluconazole have shown the synergistic effect with synerazol. Fig. 7 shows the checkerboard study between synerazol and fluconazole. However other ergosterol synthesis inhibitors such as naftifine and ML-236B did not show any synergistic effect with synerazol (data not shown).

Synerazol showed no toxicity for male ICR mice (5 weeks old) by intraperitoneal administration at a dose of 200 mg/kg. However, at the same dose it showed no therapeutic effect with experimental intravenous infections with *C. albicans* in mice.

Discussion

The strucure of synerazol is found to be very similar to that of pseurotin A, a fungal metabolite reported by BLOCH *et al.*[3,5]. Since the cultured broth of *A. fumigatus* SANK 10588 contained pseurotin A as well, these compounds would share the same biosynthetic pathway. Despite the close similarity of the structures, pseurotin A was reported to have neither antibacterial nor antifungal activity[5], and thus the antifungal activity of synerazol might be ascribed to the epoxide moiety. This was supported by the fact that the antibiotic exhibited a high reactivity to SH-reagents.

Synerazol exhibited marked synergism with azoles such as clotrimazole and fluconazole. A possible mechanism of the synergism can be explained by the fact that the two agents act on the common biosynthesis pathway as reported by SUD and FEINGOLD[6] with synergism between ergosterol biosynthesis inhibitors.

It might not be the case for synerazol, because it did not show any distinct synergistic effect with naftifine or ML-236B. Recently azole compounds are reported to increase sensitivity of *C. albicans* to various types of antifungal agents under sub-MIC level[7,8]. The mechanism is explained as follows. Fungal cells treated with azole compounds accumulate abnormal sterols in the membrane owing to inhibition of cytochrome P-450, whereby the affected membrane becomes more permeable leading to hypersensitivity to various agents. It is also comfirmed that mutant strains of *C. albicans* accumulating 14-methylated sterols showed increased susceptibility to other antifungal compounds[8,9]. The synergism between synerazol and azole compounds could rather be explained under this mechanism.

Experimental

FAB mass spectra and FAB-CAD MS/MS spectra were obtaned with a Jeol JMS-HX100 tandem mass spectrometer as described elsewhere[10] using a 3-nitrobenzyl alcohol matrix. HRFAB mass spectra were measured by means of a dual target method using the reference compound reported previously[11].

All NMR spectra were recorded at 27°C on a Jeol JNM-GX500. $CDCl_3$ was used as the solvent unless otherwise stated. The chemical shifts are given downfield from internal TMS. The 45° sequence[12] was used for ^1H-^1H COSY experiment. The standard sequence[13] was used for ^{13}C-^1H COSY. Both the proton detection sequence[14] and the COLOC sequence[15] were used for observation of long-range ^{13}C-^1H couplings. The coupling correlations were verified by means of long-range selective proton decoupling experiments. ^{13}C-$\{^1H\}$ NOE difference spectra were obtained with a complete decoupling mode.

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