UK-2A, B, C, and D, Novel Antifungal Antibiotics from *Streptomyces* sp. 517-02

VII. Membrane Injury Induced by C9-UK-2A, a Derivative of UK-2A, in *Rhodotorula mucilaginosa* IFO 0001

KAZUNORI TANI, YOSHINOSUKE USUKI, KAZUHIKO MOTOBA, KEN-ICHI FUJITA and MAKOTO TANIGUCHI*

Graduate School of Science, Osaka City University,
3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

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UK-2A is a potent antifungal antibiotic and its structure is highly similar to that of antimycin A_3 (AA). UK-2A and AA inhibit mitochondrial electron transport at complex III. However, the antifungal activities of UK-2A and AA disappear after 48-hour treatment. In an attempt to improve the duration of the antifungal activity of UK-2A, several UK-2A derivatives were prepared by substituting its nine-membered dilactone ring with an *n*-alkyl or an isoprenyl moiety. Among all the derivatives tested, C9- and C10-UK-2A showed the most potent and durable antifungal activities against a strict aerobic yeast, *Rhodotorula mucilaginosa* IFO 0001. C9-UK-2A, in particular, continued to demonstrate its broad-spectrum antifungal activity after 120-hour treatment. Therefore, we focused on C9-UK-2A to further examine its mode of action against the yeast. Interestingly, C9-UK-2A did not inhibit cellular respiration of the cells even at concentrations greater than 100 μg/ml. C9-UK-2A gradually induced the efflux of potassium ions from the cells. Moreover, C9-UK-2A gradually induced the release of glucose from glucose-encapsulating liposomes. The patterns of efflux and release induced by C9-UK-2A were not as rapid as those seen with amphotericin B. These results suggest a membrane injury caused by C9-UK-2A in *R. mucilaginosa* IFO 0001.

As previously reported, *Streptomyces* sp. 517-02 produced several novel antifungal antibiotics, UK-2A, B, C and D[1], which are similar to antimycin A_3 (AA) in both chemical structure and inhibitory activity towards the electron transport chain at complex III in mitochondria[1~3]. These UK-2 compounds inhibited the growth of *Rhizopus formosaensis* IFO 4372 at 0.0125 μg/ml, while AA showed no effect at concentrations up to 100 μg/ml[1]. The UK-2 compounds were less active than AA against mouse leukemia P388, mouse melanoma B16, human oral epidermoid carcinoma KB and human colon adenocarcinoma COLO201 cells[1]. On the other hand, the UK-2 compounds, in addition to AA, have poor durability of their antifungal activities[3]. Therefore, we have prepared UK-2A derivatives to improve the durability of the antifungal activity of UK-2A. The nine-membered dilactone moiety in UK-2A was replaced by an *n*-alkyl or an isoprenyl moiety (See Fig. 1). As previously reported, among the UK-2A derivatives prepared, C8-UK-2A showed the most potent antifungal activity against a strict aerobic yeast, *Rhodotorula mucilaginosa* IFO 0001 after 24-hour treatment[6]. In this study, we report several newly-prepared derivatives. C9- and C10-UK-2A were found to exhibit the most potent antifungal activities even after 120-hour treatment among the derivatives tested. Because, C9-UK-2A had a broader antifungal spectrum than C10-UK-2A, we further examined the mode of action of C9-UK-2A against *R. mucilaginosa* IFO 0001.

* Corresponding author: makoto@sci.osaka-cu.ac.jp

Material and Methods

Strains

Schizosaccharomyces pombe IFO 0342, *Candida albicans* IFO 1061, *Rhodotorula mucilaginosa* IFO 0001, *Rhizopus oryzae* IFO 4766, *Penicillium chrysogenum* IFO 4626, and *Mucor mucedo* IFO 7684 were purchased from Institute for Fermentation, Osaka (Osaka, Japan). *Aspergillus niger* ATCC 6275 was purchased from American Type Culture Collection (Rockville, USA). All strains of yeasts and filamentous fungi were grown in a Sabouraud dextrose (SD) medium unless stated otherwise.

Chemicals

All UK-2A derivatives tested including C9-, C10-, and C11-UK-2A as represented in Fig. 1 were prepared by a previously described method[4~6]. AA and amphotericin B (AmB) were purchased from Sigma (St. Louis, USA). Other reagents were of commercial grade. In this study, all antibiotics including UK-2A derivatives were dissolved in *N,N'*-dimethylformamide prior to biochemical experiments.

Antifungal Assay

The antifungal activities of UK-2A derivatives were determined by a two-fold agar dilution method[1,7] after 24-, 48-, and 120-hour incubation at 25°C. The MIC was defined as the lowest concentration of test compound that showed no visible growth. Effects of inoculum size on antifungal activity of C9-UK-2A and AmB in *R. mucilaginosa* IFO 0001 were also determined by a serial broth dilution method[8]. After 24-hour incubation with shaking at 30°C, the turbidity of the culture suspension was checked at 610 nm. The MIC was defined as the lowest concentration of test compound that showed no elevation of the turbidity.

Growth Studies

An overnight culture of *R. mucilaginosa* IFO 0001 was diluted into a fresh SD broth to give approximately 10^5 colony forming unit (CFU)/ml. Aliquots of this cell suspension were dispensed into each L-tube. After 15-minute incubation with shaking at 30°C, C9-UK-2A, UK-2A, and AA were added to the culture and the cultivation was continued. Portions of the culture were withdrawn at intervals, diluted with 0.9% NaCl, and then spread on YPD (1% Difco Bacto Yeast Extract, 2% Difco Bacto Peptone, and 2% glucose) agar plates to estimate CFU. CFU was determined by counting number of colonies on the plates after 48-hour incubation at 25°C.

Cellular Respiration Studies

Exponentially growing cells of *R. mucilaginosa* IFO 0001 were harvested, washed with 0.9% NaCl and then suspended in 50 mM phosphate buffer (pH 7.0) containing 1% glucose. After 1-hour incubation with shaking at 30°C, the cells were washed and resuspended in the same buffer to give 10^7 CFU/ml. At appropriate intervals, portions of the suspension were withdrawn. Cellular respiration of the yeast cells in the suspension with or without C9-UK-2A was measured polarographically at 30°C using Hansatech oxygen electrode units[1].

Cell Permeability Studies

Exponentially growing cells of *R. mucilaginosa* IFO 0001 were harvested, washed and then suspended in 50 mM succinate buffer (pH 6.0) to give 10^8 CFU/ml. After 10-minute incubation with shaking at 30°C, C9-UK-2A, UK-2A, and AA were added and then incubation was continued. At appropriate intervals, portions of the culture were withdrawn and centrifuged. The supernatant obtained by centrifugation was assayed for its content of potassium ions by using a potassium ion assay kit (Hach, Co.; Loveland, USA) based on tetraphenylborate method[9].

Liposome Studies

The effect of C9-UK-2A on artificially prepared liposomes was examined[10,11]. Liposomes were prepared by using 10 μmol phosphatidylcholine, 5 μmol phosphatidic acid, and 1 μmol ergosterol dissolved in 1 ml of chloroform. The preparation of glucose-encapsulating liposomes and the assay of trapped glucose leakage from the liposomes were carried out by the method of KINSKY *et al.*[10].

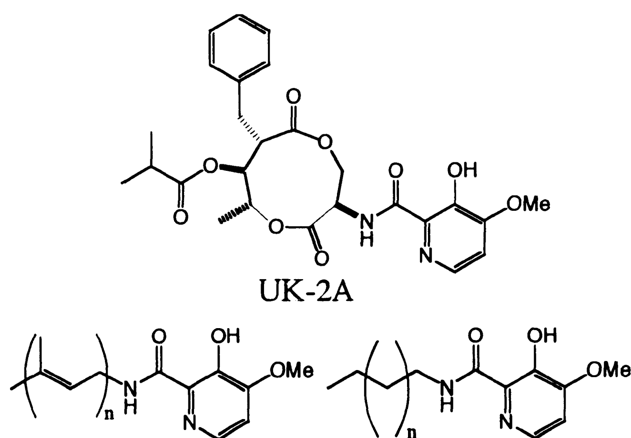
Results

Growth Inhibitory Activities of UK-2A Derivatives, UK-2A, and AA against *R. mucilaginosa* IFO 0001

The antifungal activities of UK-2A derivatives, UK-2A, and AA (See Fig. 1) against *R. mucilaginosa* IFO 0001 are described in Table 1. In this study, the MIC value of UK-2A against *R. mucilaginosa* IFO 0001 determined by a two-fold agar dilution method[1,7], after 24-hour treatment, was 1.56 μg/ml. This was lower than the MICs of AA and any other UK-2A derivatives tested. Although the antifungal activities of UK-2A and AA disappeared after 48-hour treatment, those of C8-, C9- and C10-UK-2A persisted. C9- and C10-UK-2A, in particular, showed the most potent

activity after 120-hour treatment among the compounds tested. On the other hand, UK-2A, AA, and any other UK-2A derivatives, except for C8-, C9- and C10-UK-2A, did not show antifungal activities up to 100 μg/ml. The hydrophobicity of Gera-UK-2A resembles that of C10-UK-2A, but an *n*-alkyl residue is thought to be more flexible than an isoprenyl one. Therefore, the flexibility of the molecule seems to be needed for expression of its antifungal activity. We also examined the antifungal spectra of C8-, C9-, and C10-UK-2A against several yeasts and fungi after 120-hour treatment. As described in Table 2, the antifungal spectrum of C9-UK-2A was the broadest among the three UK-2A derivatives. Therefore, we investigated the mode of action of C9-UK-2A against *R. mucilaginosa* IFO 0001.

Fig. 1. Structure of UK-2A and UK-2A derivatives.



Farn-UK-2A (n=3)
Gera-UK-2A (n=2)
Pren-UK-2A (n=1)

C16-UK-2A (n=13)
C12-UK-2A (n=9)
C11-UK-2A (n=8)
C10-UK-2A (n=7)
C9-UK-2A (n=6)
C8-UK-2A (n=5)
C4-UK-2A (n=1)

Effects on Growth of *R. mucilaginosa* IFO 0001

The effects of C9-UK-2A, UK-2A, and AA on the growth of *R. mucilaginosa* IFO 0001 are shown in Fig. 2. UK-2A and AA only inhibited cell growth at a high concentration, such as 100 μg/ml. On the other hand, C9-UK-2A exhibited fungicidal effect over 25 μg/ml. At a high concentration, such as 100 μg/ml, C9-UK-2A caused 99.9% loss of cell viability after 48-hour treatment. These results indicate that the action of C9-UK-2A on *R. mucilaginosa* IFO 0001 is different from that of UK-2A and AA.

Effect on Cellular Respiration of *R. mucilaginosa* IFO 0001

UK-2A and AA inhibit mitochondrial electron transport at complex III resulting in the inhibition of cellular

Table 1. Antifungal activities of UK-2A, AA, and UK-2A derivatives against *R. mucilaginosa* IFO 0001.

	MIC (μg/ml) after incubation for		
Compound*	24 hr	48 hr	120 hr
UK-2A	1.56	>100	>100
AA	3.13	>100	>100
C4-UK-2A	>100	>100	>100
C8-UK-2A	25	50	50
C9-UK-2A	12.5	25	25
C10-UK-2A	12.5	12.5	25
C11-UK-2A	25	>100	>100
C12-UK-2A	>100	>100	>100
C16-UK-2A	>100	>100	>100
Pren-UK-2A	>100	>100	>100
Gera-UK-2A	>100	>100	>100
Farn-UK-2A	>100	>100	>100

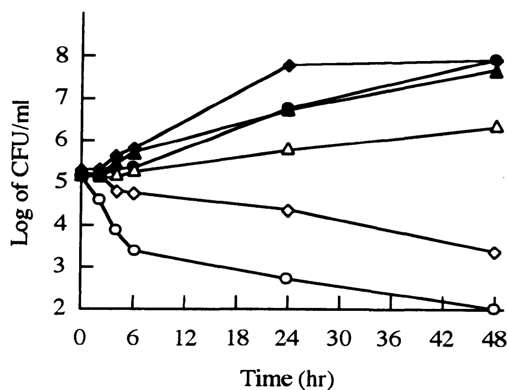
*See Fig. 1. The cells were grown on a SD agar at 25°C.

Table 2. Antifungal spectra of UK-2A derivatives.

Tested organisms	MIC (μg/ml)			
	C8-UK-2A	C9-UK-2A	C10-UK-2A	C11-UK-2A
Schizosaccharomyces pombe IFO 0342	>100	100	100	>100
Candida albicans IFO 1061	100	100	>100	>100
Rhodotorula mucilaginosa IFO 0001	50	25	25	>100
Aspergillus niger ATCC 6275	100	50	>100	>100
Rhizopus oryzae IFO 4766	100	12.5	100	>100
Penicillium chrysogenum IFO 4626	100	12.5	25	>100
Mucor mucedo IFO 7684	>100	>100	>100	>100

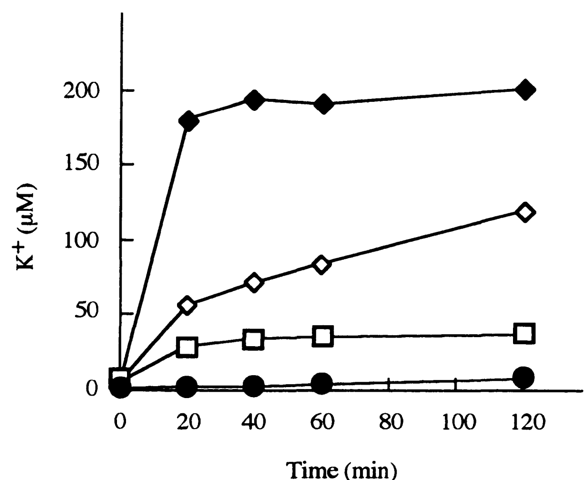
The cells were grown on a SD agar at 25°C. MICs were determined after 24-hour incubation.

Fig. 2. Effects of C9-UK-2A, UK-2A, and AA on the viability of *R. mucilaginosa* IFO 0001.



The cells were cultivated with 100 μg/ml UK-2A (●), 100 μg/ml AA (▲), 12.5 μg/ml C9-UK-2A (△), 25 μg/ml C9-UK-2A (◇), 100 μg/ml C9-UK-2A (○) or without drugs (◆) in a SD broth with shaking at 30°C. After incubation in SD broth in the presence of the agents indicated, CFU was determined by plating diluted sample on YPD agar plates followed by 48-hour incubation at 25°C before enumerating CFU.

Fig. 3. Effect of C9-UK-2A on the efflux of potassium ion from *R. mucilaginosa* IFO 0001 cells.



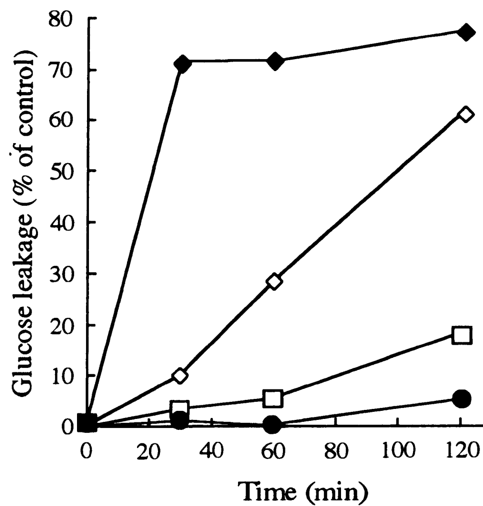
The cells (10^8 cells/ml) were incubated with 12.5 μg/ml AmB (◆), 25 μg/ml C9-UK-2A (□), 50 μg/ml C9-UK-2A (◇), or without drugs (●) in 50 mM succinate buffer (pH 6.0) at 30°C. Potassium ion concentration in cell-free supernatants was determined as indicated in Materials and Methods.

respiration. Therefore, the effect of C9-UK-2A on cellular respiration of *R. mucilaginosa* IFO 0001 was examined. Even at 100 μg/ml, C9-UK-2A did not inhibit the cellular respiration when glucose was used as a substrate.

Efflux of Potassium Ions from *R. mucilaginosa* IFO 0001 Cells

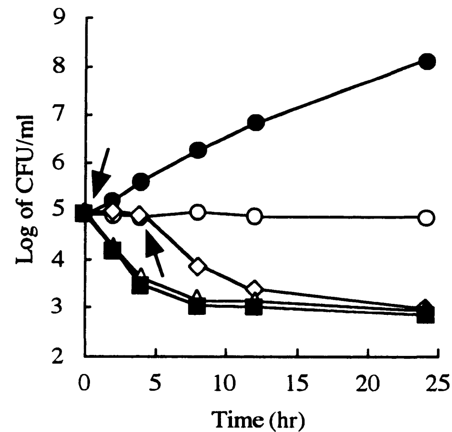
AmB selectively binds to the plasma membrane ergosterol of yeast and filamentous fungi[12,13] and then forms pores that allow potassium ions to leak out of the cytoplasm. The MIC values of C9-UK-2A and AmB

Fig. 4. Effect of C9-UK-2A on glucose release from liposomes.



The liposome suspensions were incubated with 12.5 μg/ml AmB (◆), 25 μg/ml C9-UK-2A (□), 50 μg/ml C9-UK-2A (◇) without drugs (●) at 30°C. Glucose released was determined as described in Materials and Methods.

Fig. 5. Effect of C9-UK-2A on the cycloheximide-treated cells of *R. mucilaginosa* IFO 0001.



Cells were cultivated with or without cycloheximide at a concentration of 100 μg/ml in a SD broth with shaking at 30°C. C9-UK-2A was added at 100 μg/ml to cycloheximide-containing cultures at 0 hour (△) and at 4 hours (◇). Controls included cultures with no drugs (●), cycloheximide only (○) or C9-UK-2A only added at 0 hours (■). CFU was determined by taking samples at the times indicated, and plating diluted samples on YPD agar plates followed by 48-hour incubation at 25°C before enumerating CFU. The arrows indicate the time of addition of C9-UK-2A.

against *R. mucilaginosa* IFO 0001 was 25 and 6.25 μg/ml, respectively, in SD broth with shaking at 30°C after 24-hour cultivation when inoculum size was 10^8 cells/ml. As shown in Fig. 3, the 2×MIC of AmB greatly induced the efflux of potassium ions from the yeast cells and the efflux level reached a maximum after 20-minute treatment with AmB. On the other hand, the 2×MIC of C9-UK-2A gradually induced the efflux of potassium ions. After 120-minute treatment, the efflux induced by C9-UK-2A was 63% of that by AmB.

Effect on Release of Glucose from Liposomes

The efflux of potassium ions from the cytoplasm of *R. mucilaginosa* IFO 0001 cells in an energy-dependent manner might be induced in response to stimulation such as external environmental changes[14]. In order to investigate whether C9-UK-2A directly affects stability or permeability of the yeast plasma membrane, the effect of C9-UK-2A on glucose-encapsulating liposomes was examined. AmB at 2×MIC greatly induced the release of trapped glucose from the liposomes after a 30-minute treatment with the drug as shown in Fig. 4. C9-UK-2A at 2×MIC gradually induced the release. After 120-minute treatment, the release of glucose in AmB and C9-UK-2A at 2×MICs was 76% and 61% of the control level released by Triton X-100, respectively. The pattern of the release of trapped glucose from liposomes induced by C9-UK-2A was significantly different from that by AmB.

Effect on Non-growing Cells of *R. mucilaginosa* IFO 0001

An antifungal antibiotic benanomicin A is fungicidal and disrupted the cell permeability barrier, including leakage of intracellular K^+ and ATP in growing cells, while the antibiotic has none of these effects in non-growing cells[15]. We also investigated the effect of C9-UK-2A on the non-growing cells in *R. mucilaginosa* IFO 0001. Cycloheximide restricts cell division thereby blocking cytoplasmic protein synthesis[16]. As shown in Fig. 5, C9-UK-2A reduced viability of the cycloheximide-pretreated cells as well as the non-treated cells after addition of C9-UK-2A. This result indicates that non-growing yeast cells are also susceptible to fungicidal action of C9-UK-2A. This type of a reduction in viability has been reported in other membrane-injury

chemicals, such as poligodial[16] and gallate esters[17].

Discussion

UK-2A and AA restrict the growth of eukaryotic cells by inhibiting mitochondrial electron transport at complex III. However, this effect is temporary (Table 1 and Fig. 1). The direct binding sites of UK-2A and AA to the complex III have been reported to be associated with a 3-hydroxy-4-methoxy-pyridine-2-carboxylyl and a 3-formylamino-salicylyl group, respectively[18,19]. The nine-membered dilactone rings of UK-2A and AA are thought to be needed for their hydrophobicity in order to pass through the barrier of the biomembrane and for the tight binding of the drugs to the complex III[18,19]. However, these dilactone residues are subject to enzymatic degradation. Poor duration of the antifungal activity of UK-2A and AA could be due to this susceptibility. We replaced the dilactone with stable *n*-alkyl and isoprenyl in derivatives of UK-2A. Although all the UK-2A derivatives prepared showed weaker antifungal activity against *R. mucilaginosa* IFO 0001 than UK-2A and AA after 24-hour treatment, C8-, C9-, and C10-UK-2A continued to show the activity after 120 hours (Table 1). In our previous report, inhibitory activity against the uncoupler-stimulated respiration of bovine heart submitochondrial particles was examined[6]. The activity of C8-UK-2A was about 1% of that of UK-2A and AA[6]. Moreover, C9-UK-2A did not affect cellular respiration of *R. mucilaginosa* IFO 0001. C9-UK-2A caused the reduction of viable cell number, but UK-2A and AA did not. These results suggest that the fungicidal action of C9-UK-2A would be significantly different from the growth inhibitory effect of UK-2A.

C9-UK-2A acts on the cycloheximide-treated cells of *R. mucilaginosa* IFO 0001 indicating its action against non-growing cells. C9-UK-2A induced the efflux of potassium ions from the yeast cells and also the release of glucose from glucose-encapsulating liposomes. These results suggest the possibility of membrane injury caused by C9-UK-2A. However, the rate of the efflux and the release caused by C9-UK-2A was slower than that by AmB. The rate of decrease in viability induced by C9-UK-2A was also gradual. Therefore, the phenomena of the efflux, the release of glucose and the decrease in viability seem to be synchronized, strongly supporting the above possibility.

All of UK-2A derivatives prepared in our studies did not act on Gram-negative and Gram-positive bacteria at concentrations up to 100 μg/ml[6]. The lipid compositions of the membrane between prokaryotic and eukaryotic cells are different[20~22]. The selectivity of the UK-2A derivatives for eukaryotic cells can be hypothesized to be due to unique features of eukaryotic membranes. Alternatively, it might affect both prokaryotic and eukaryotic membranes similarly, but the main target is at the eukaryotic electron transport chain. It is possible that neither of the above are targets for the UK-2A derivatives but rather their effects are due to some other as yet unidentified targets. Further studies are needed to reveal the basis for the selective toxicity of this compound on yeast.

We succeeded in improving the duration of the antifungal activity of UK-2A. In order to achieve this goal, the nine-membered ring of UK-2A was replaced by an *n*-alkyl residue to give C9-UK-2A as a more structure. Further synthesis of newly designed UK-2A derivatives is now in progress.

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Reference

1) Ueki, M.; K. Abe, M. Hanafi, K. Shibata, T. Tanaka & M. Taniguchi: UK-2A, B, C and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02. I. Fermentation, isolation and biological properties. J. Antibiotics 49: 639~643, 1996
2) Hanafi, M.; K. Shibata, M. Ueki & M. Taniguchi: UK-2A, B, C and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02. II. Structural elucidation. J. Antibiotics 49: 1226~1231, 1996
3) Ueki, M. & M. Taniguchi: The mode of action of UK-2A and UK-3A, novel antifungal antibiotics from *Streptomyces* sp. 517-02. J. Antibiotics 50: 1052~1057, 1997
4) Shimano, M.; T. Shibata & N. Kamei: Enantioselective total synthesis of the antifungal dilactone, UK-2A: The determination of the relative and absolute configurations. Tetrahedron Lett. 39: 4363~4366, 1998
5) Spencer, T. A.; T. J. Onofrey, R. O. Cann, J. S. Russel, L. E. Lee, D. E. Blanchard, A. Castro, P. Gu, G. Jiang & I. Shechter: Zwitterionic sulfobetaine inhibitors of squalene synthase. J. Org. Chem. 64: 807~818, 1999
6) Usuki, Y.; K. Tani, K. Fujita & M. Taniguchi: UK-2A, B, C and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02. VI (1). Structure-activity relationship of UK-2A. J. Antibiotics 54: 600~602, 2001
7) Shafi, M. S.: Determination of antimicrobial MIC by paper diffusion method. J. Clin. Pathol. 258: 989~992, 1975

8) TANIGUCHI, M. & Y. SATOMURA: Structure and physiological activity of carbostyril compounds. Agri. Biol. Chem. 36: 2169~2175, 1972
9) PAFF, P. & W. BROTZ: Uber die bestimmung des kaliums als tetraphenyl-bor-kalium. Z. Anal. Chem. 133: 241~248, 1951
10) KINSKY, S. C.; J. A. HAXBY, D. A. ZOPF, C. R. ALVING & C. B. KINSKY: Complement-dependent damage to liposomes prepared from pure lipids and Forssman hapten. Biochemistry 8: 4149~4158, 1969
11) RUFINI, S.; P. CESARONI, A. DESIDERI, R. FARIAS, F. GUBENSEK, J. M. GUTIERREZ, P. LULY, R. MASSOUD, R. MORERO & J. Z. PEDERSEN: Calcium ion independent membrane leakage induced by phospholipase-like myotoxins. Biochemistry 31: 12424~12430, 1992
12) KRUIJFF, B. D. & R. A. DEMEL: Polyene antibiotic-sterol interaction in membranes of *Acholeplasma laidlawwii* cells and lecithin liposomes. Biochim. Biophys. Acta 339: 57~70, 1974
13) KRUIJFF, B. D.; W. J. GERRISTEN, A., OERLEWANS, R. A. DEMEL & L. L. M. VANDEENEN: Polyene antibiotic sterol interaction in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by the polyene antibiotics. Biochim. Biophys. Acta 339: 30~43, 1974
14) ENRÌQUEZ-FREIRE, E.; R. LÛPEZ & A. PEÒA: Potassium ion efflux induced by cationic compounds in yeast. Biochim. Biophys. Acta 1418: 147~157, 1999
15) WATANABE, M.; H. TOHYAMA, T. HIRATANI, H. WATANABE, S. INOUE, S. KONDO, T. TAKEUCHI & H. YAMAGUCHI: Mode of antifungal action of benanomicin A in *Saccharomyces cerevisiae*. J. Antibiotics 50: 1042~1051, 1997
16) KUBO, I.; K. FUJITA & S. H. LEE: Antifungal mechanism of polygodial. J. Agric. Food Chem. 49: 1607~1611, 2001
17) KUBO, I.; P. XIAO & K. FUJITA: Antifungal activity of octyl gallate: structural criteria and mode of action. Bioorg. Med. Chem. Lett. 11: 347~350, 2001
18) MIYOSHI, H.; N. TOKUTAKE, Y. IMAEDA, T. AKAGI & H. IWAMURA: A model of antimycin A binding based on structure-activity studies of synthetic antimycin A analogues. Biochim. Biophys. Acta 1229: 149~154, 1995
19) UEKI, M.; K. MACHIDA & M. TANIGUCHI: Antifungal inhibitors of mitochondrial respiration: Discovery and proepectives for development. Current Opinion in Anti-infective Investigational Drugs 2: 387~398, 2000
20) RATTRAY, J. B.; A. SCHIBECI & D. K. KIDBY: Lipids of yeasts. Bacteriol. Rev. 39: 197~231, 1975
21) GOLDFINE, H.: Bacterial membranes and lipid packing theory. J. Lipid Res. 25: 1501~1507, 1984
22) SHAW, N.: Lipid composition as a guide to the classification of bacteria. Adv. Appl. Microbiol. 17: 63~108, 1974