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Target Substances of Some Antifungal Agents in the Cell Membrane

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Copiamycin, an antifungal antibiotic, exhibits antimicrobial activity against a few bacteria in addition to a wide variety of fungi. The methanol extract of *Sarcina lutea*, one of the most susceptible bacteria, was found to reverse the antimicrobial activity of copiamycin. The reversing activity was associated with the phospholipid fraction of the bacteria. The *S. lutea* phospholipids also reversed the activities of azalomycin F and miconazole, but not that of clotrimazole. The effects of authentic phospholipids and fatty acids were also investigated. As the antimicrobial activities of copiamycin and azalomycin F were most strongly reversed in the same manner by phospholipids with unsaturated fatty acids and basic hydrophilic groups, the sites on the cell membrane sensitive to both antibiotics are assumed to be identical. On the other hand, the activity of miconazole was affected by different phospholipids from those which affected these two antifungal antibiotics, and the activity of clotrimazole was not affected by any of the phospholipids and fatty acids. It was postulated that the sites on the cell membrane sensitive to miconazole and clotrimazole are different from those sensitive to copiamycin and azalomycin F.

Most antibacterial chemotherapeutic agents act primarily on cell wall synthesis or protein synthesis on a ribosomal level. Polyene antifungal antibiotics, on the other hand, are well known to cause cell membrane damage. Copiamycin, a nonpolyenic antibiotic derived only from mycelia of *Streptomyces hygroscopicus* subsp. *crystallogenes* (4), possesses a broad spectrum of antifungal activity (3, 11, 15). Azalomycin F, also an antifungal antibiotic, was isolated from the culture broth of the *S. hygroscopicus* (1, 2). Both antibiotics proved to be useful for treatment of superficial mycoses and trichomoniasis. Physicochemical and biological properties of copiamycin suggested a certain relationship with azalomycin F. Further, our studies on copiamycin revealed that it inhibited some microorganisms other than fungi, and the sites on the microorganisms that were susceptible to the antibiotic were found to be in the cell membrane (12, 13).

Clotrimazole and miconazole are chlorinated imidazole derivatives which are used for the treatment of superficial and deep-seated mycoses. They inhibit the growth not only of many fungi but also of a variety of bacteria. The action mechanisms of clotrimazole and miconazole were studied by Iwata et al. (8–10), Yamaguchi et al. (21), Van Den Bossche et al. (18), and Sreedhara Swamy et al. (16, 17). There are also review papers on these compounds by Yamaguchi (20) and Holt (6). These authors postulated that these synthetic compounds also affect the cell membranes of susceptible microorganisms and alter their permeability.

Antifungal antibiotics, like polyenes, are known to interact with sterols in the membrane, whereas clotrimazole and miconazole interact with phospholipids. Our preliminary studies on copiamycin revealed that the methanol extract of *Sarcina lutea*, one of a few copiamycin-susceptible bacteria, inhibits the antimicrobial activity of copiamycin. The extract was assumed to contain the compounds related to the target substances of copiamycin in the cell membrane.

The present work was undertaken to elucidate, with special reference to clotrimazole and miconazole, both the biological characteristics of these compounds and the properties of the target substances of microorganisms susceptible to antibiotics of the copiamycin-azalomycin F group.

MATERIALS AND METHODS

Test organisms. The following organisms were selected from our culture collection for the extraction and assay of the inhibitor: *S. lutea* IFM 2066 (the primary organism used), IFM 2114, IFM 2115, IFM

2244, IFM 2245, and IFM 2246; *S. flava* IFM 2242 and IFM 2243; *S. hansenii* IFM 2247, IFM 2248, and IFM 2249; *S. subflava* IFM 2116; *S. ureae* IFM 2250, IFM 2251, IFM 2252, and IFM 2253; *Candida stellatoidea* IFM 4024; and *C. albicans* IFM 2080. The medium used for growing bacteria was heart infusion agar, and Sabouraud dextrose agar was used for *Candida*.

Antifungal agents and reagents. Copiamycin and azalomycin F were kindly supplied by Kyowa Fermentation Co. Ltd. and Sankyo Pharmaceuticals Co. Ltd., respectively. Clotrimazole, 2,4-bis-phenyl-(2-chlorophenyl)-1-imidazoyl methane, of Bayer AG, West Germany, and miconazole, 1-(2,4-dichloro-β-[2,4-dichloro-benzyloxy]-phenyl) imidazole nitrate, of Janssen Pharmaceutica, Belgium, were donations procured through Eisai Co. Ltd., Japan. Phosphatidic acid-dipalmitoyl, phosphatidic acid-dioleoyl, phosphatidylcholine, egg lecithin, phosphatidylcholine-dilinoleoyl, phosphatidylcholine-dipalmitoyl, phosphatidylcholine-distearoyl, phosphatidylcholine-dioleoyl, phosphatidylglycerol, phosphatidylinositol, cardiolipin, oleic acid, and linoleic acid were purchased from Serdary Research Laboratories, Canada.

Extraction of copiamycin-inactivating principles. *S. lutea* IFM 2066 was shake cultured in heart infusion broth at 27°C for 48 h. The cells were then harvested by centrifugation, washed several times with saline, and extracted with an equal volume of methanol for 30 min by stirring. The total complex lipid was extracted from wet cells of *S. lutea* IFM 2066 by the modified Bligh-Dyer method (5), and 100 mg of the complex lipid was further purified by diethylaminoethyl-cellulose column chromatography (7, 14). The column was successively developed with the solvents and solvent mixtures as indicated in Fig. 1.

Assay of the reversal of the antimicrobial activity. Paper disks were immersed in the methanol solution of antifungal agents and the inhibitor solution. They were then placed on agar plates seeded with *S. lutea* IFM 2066 or *C. stellatoidea* IFM 4024 as indicator organisms. Finally, the plates were incubated at 37°C for 18 h, and the diameters of the inhibition zones were measured with a slide caliper. The reversal of antimicrobial activity of the antifungal agents was expressed as percent decrease in antimicrobial activity.

RESULTS

The effects of *Sarcina* extracts on the activities of antifungal agents are shown in Tables 1

TABLE 1. *Effect of Sarcina extract on the activity of copiamycin and azalomycin F*[a]

Methanol extract from	*S. lutea* IFM 2066	
	Copiamycin	Azalomycin F
None	13.8	14.7
S. flava		
IFM 2242	0	0
IFM 2243	0	0
S. hansenii		
IFM 2247	0	0
IFM 2248	0	0
IFM 2249	0	0
S. lutea		
IFM 2066	0	0
IFM 2114	0	0
IFM 2115	0	0
IFM 2244	(9.1)	0
IFM 2245	(10.2)	0
IFM 2246	(10.0)	0
S. subflava		
IFM 2116	(9.1)	0
S. ureae		
IFM 2250	0	0
IFM 2251	0	0
IFM 2252	(9.0)	0
IFM 2253	(9.3)	0

[a] Figures indicate the diameter of inhibition zones in millimeters. Parentheses denote incomplete inhibition. Copiamyicn: 200 μg/ml. Azalomycin F: 500 μg/ml.

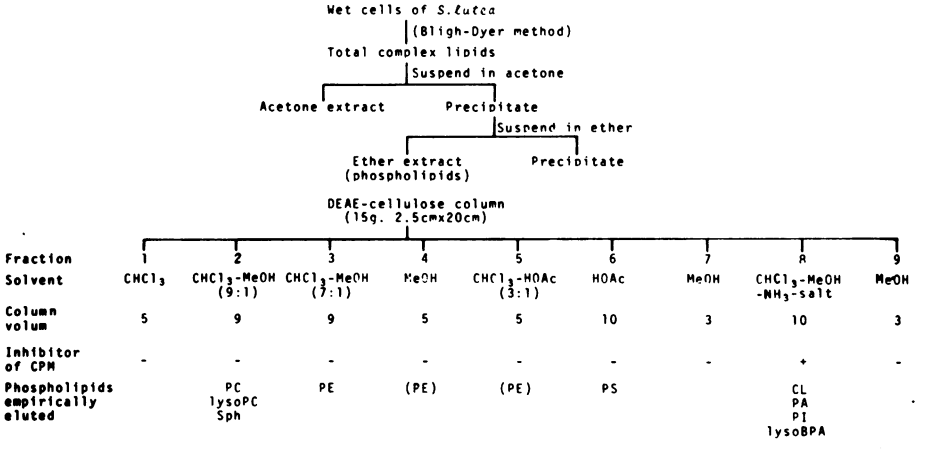

FIG. 1. *Purification of S. lutea IFM 2066 phospholipids. PC, Phosphatidylcholine; lysoPC, lyso-phosphatidylcholine; Sph, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; CL, cardiolipin; PA, phosphatidic acid; PI, phosphatidylinositol, lysoBPA, lyso-bis-phosphatidic acid; CPM, copiamycin.*

2. The methanol extracts from all of the test *Sarcina* species showed a complete or partial reversal of copiamycin and azalomycin F activities. When *C. stellatoidea* was used as an indicator organism, a complete reversal was demonstrated. A partial reversal was observed with miconazoles, and almost no effect was observed with clotrimazole when either *S. lutea* or *C. stellatoidea* was used as indicator organism. The reversing activity was associated with phospholipid components of *S. lutea* and was purified as summarized in Fig. 1. The reversing potency was detected in the fraction eluted with the solvent mixture of chloroform and methanol (4:1) containing 0.1 M CH_3COONH_4. The lipids empirically eluted in this solvent mixture were cardiolipin, phosphatidic acid, phosphatidylglycerol, phosphatidyl-inositol, and lyso-bis-phosphatidic acid. The thin-layer chromatogram of this fraction was compared with those of some authentic phospholipids. Three spots revealed by the Vaskovsky-Kostetsky (19) reaction gave R_f values of 0.71, 0.58, and 0.25, which approximately corresponded to those of cardiolipin, phosphatidylglycerol, and phosphatidylinositol, respectively. All three fractions had reversal potency for copiamycin and gave negative reactions to amino acid, imino group, choline, sugar, and sterol.

TABLE 2. *Effect of Sarcina extract on the activity of clotrimazole (CTZ) and miconazole (MCZ)*[a]

Methanol extract from	*S. lutea* IFM 2066		*C. stellatoidea* IFM 4024
	MCZ	CTZ	CTZ
None	15.5	19.6	(30.7)
S. flava			
IFM 2242	11.8	16.8	(26.1)
IFM 2243	11.2	13.7	(22.7)
S. hansenii			
IFM 2247	9.9	13.7	(23.7)
IFM 2248	(9.7)	14.7	(22.8)
IFM 2249	12.7	16.4	(23.4)
S. lutea			
IFM 2066	0	13.7	(25.2)
IFM 2114	11.9	12.0	(23.3)
IFM 2115	11.2	12.3	(24.0)
IFM 2244	11.8	14.7	(24.3)
IFM 2245	12.0	14.5	(22.3)
IFM 2246	12.6	14.6	(24.7)
S. subflava			
IFM 2116	10.6	14.6	(25.2)
S. ureae			
IFM 2250	12.7	15.2	(25.2)
IFM 2251	12.3	(11.3)	(22.6)
IFM 2252	11.6	13.6	(23.6)
IFM 2253	11.2	17.3	(28.6)

[a] Figures indicate the diameter of inhibition zones in millimeters. Parentheses denote incomplete inhibition. CTZ: 25 μg/ml. MCZ: 100 μg/ml.

Spots of R_f 0.25 and 0.58 gave, respectively, moderate and slight reactions to vicinal-OH with NaIO-Schiff. Authentic phosphatidylinositol also exhibited significant inhibition of the activity of copiamycin and azalomycin F against *S. lutea* and *C. albicans*, and gave a positive reaction to vicinal-OH. Thus, the fraction of R_f 0.25 was assumed to resemble phosphatidylinositol. The final identification of these three phospholipids, however, awaits further chemical characterization.

The effects of several phospholipids and fatty acids on the activities of copiamycin, azalomycin F, clotrimazole, and miconazole against *C. albicans* are compared in Fig. 2. These phospholipids and fatty acids exercised almost the same effect on copiamycin and azalomycin F. Phosphatidic acid-dipalmitoyl and phosphatidylcholine-dipalmitoyl had no effect on the activity of the two antibiotics, whereas phosphatidic acid-dioleoyl, phosphatidylcholine-dioleoyl, egg lecithin, oleic acid, and linoleic acid showed a moderate effect. Phosphatidylglycerol, phosphatidylinositol, and cardiolipin were the most effective. The antimicrobial activity of these antibiotics was completely reversed by these phospholipids against copiamycin at a molar ratio of 1:4. On the other hand, all the test phospholipids and fatty acids had almost no effect on the activity of clotrimazole. Antimicrobial activity of miconazole was also affected by these phospholipids and fatty acids; however, the mode of inactivation by these phospholipids was quite different from that of copiamycin and azalomycin F. In this case, phosphatidylglycerol, phos-

TABLE 3. *Summary of the effects of fatty acids and phospholipids on the activities of antifungal agents against C. albicans*[a]

Lipid	CPM	AZM	CTZ	MCZ
Oleic acid	+	+	−	++
Linoleic acid	+	+	−	++
PA-dipalmitoyl	−	−	−	−
PA-dioleoyl	+	+	−	+
PC-dipalmitoyl	−	−	−	−
PC-distearoyl	−	−	−	−
PC-dioleoyl	+	+	−	+
PC-dilinoleoyl	+	+	−	+
Egg lecithin	+	+	−	+
Phosphatidylglycerol	++	++	−	−
Phosphatidylinositol	++	++	−	−
Cardiolipin	++	++	−	−
Phospholipid (R_f 0.25) of *S. lutea*	++	++	−	−

[a] ++, High inactivation; +, low inactivation, −, no inactivation. PA, Phosphatidic acid; PC, phosphatidyl choline; CPM, copiamycin; AZM, azalomycin F; CTZ, clotrimazole; MCZ, miconazole.

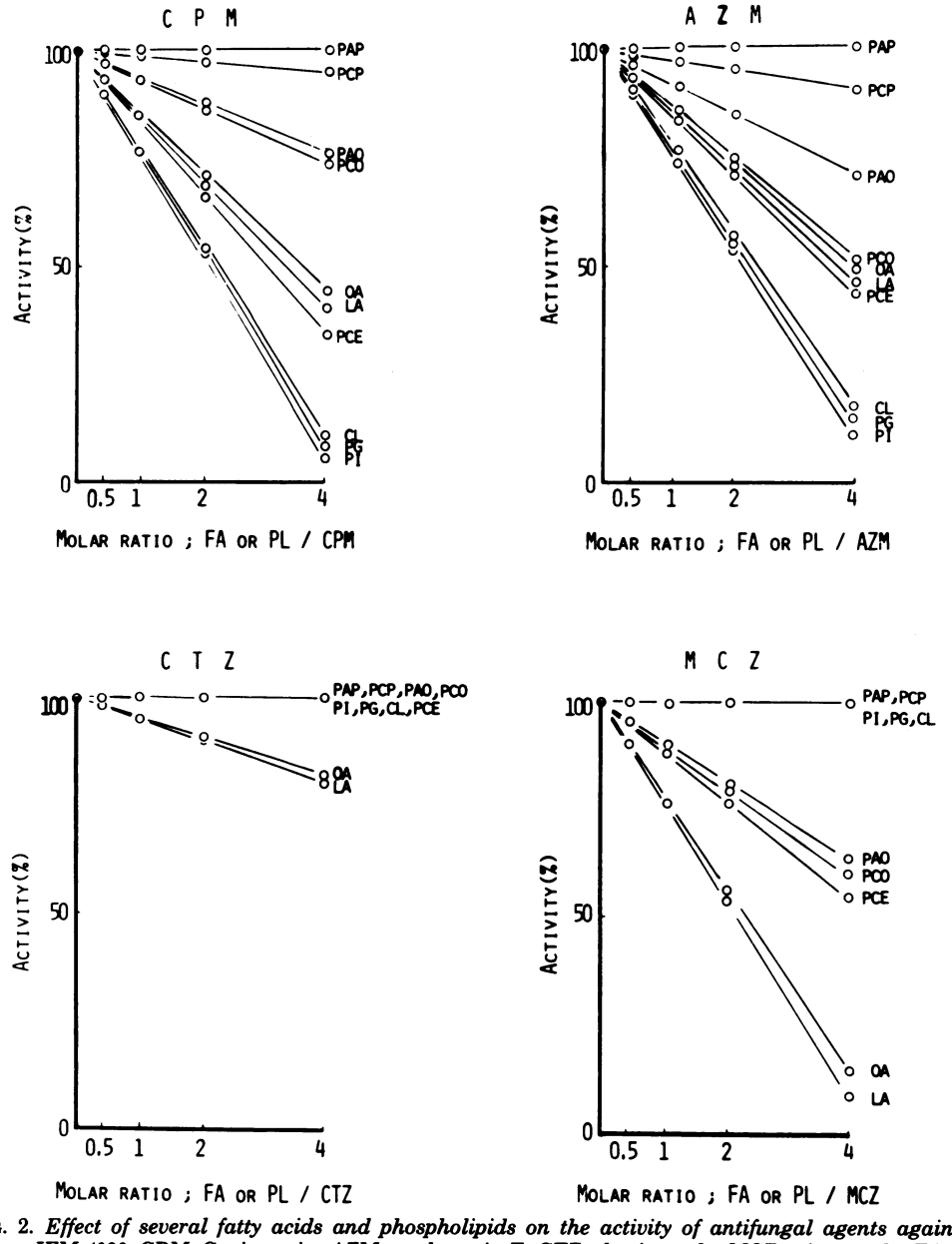

FIG. 2. *Effect of several fatty acids and phospholipids on the activity of antifungal agents against C. albicans IFM 4080. CPM, Copiamycin; AZM, azalomycin F; CTZ, clotrimazole; MCZ, miconazole; FA, fatty acid; PL, phospholipid; PAP, phosphatidic acid-dipalmitoyl; PCP, phosphatidylcholine-dipalmitoyl; PAO, phosphatidic acid-dioleoyl; PCO, phosphatidylcholine-dioleoyl; OA, oleic acid; LA, linoleic acid; PCE, egg lecithin; CL, cardiolipin; PG, phosphatidyl glycerol; PI, phosphatidyl inositol.*

phatidylinositol, and cardiolipin had no effect, whereas oleic acid and linoleic acid showed the strongest effect.

A similar antagonizing effect of phospholipids and fatty acids on antifungal agents was observed when *S. lutea* was used as an indicator organism (Fig. 3). Phosphatidylcholine-dipalmitoyl showed moderate antagonistic activity with *S. lutea*, and the effect of egg lecithin was more marked than with *C. albicans*. No difference was noticed between the effects of other phospholipids and fatty acids with *S. lutea* and *C. albicans*. These experimental results are summarized in Table 3.

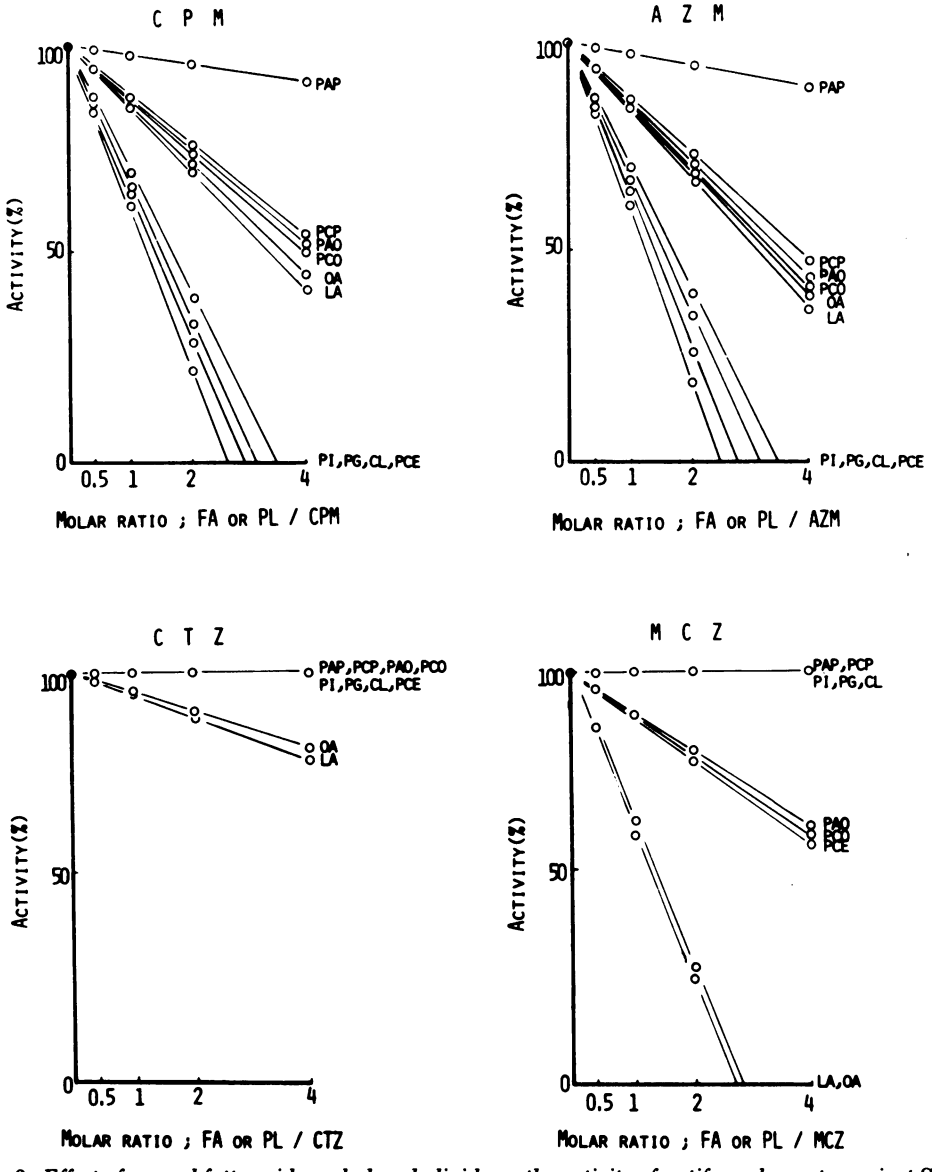

FIG. 3. *Effect of several fatty acids and phospholipids on the activity of antifungal agents against S. lutea IFM 2066. Abbreviations as in Fig. 2.*

DISCUSSION

Copiamycin and azalomycin F are apparently related but not identical antibiotics from the viewpoint of their ultraviolet absorption spectra, elemental analysis (including their molecular weights), and antibacterial spectra. They were affected by phospholipids and fatty acids in a similar manner, thus suggesting similar mechanisms of action. The most significant reversal of their antimicrobial activity was attained with phospholipids such as phosphatidylglycerol, phosphatidylinositol, and cardiolipin. It should be noted that all are acidic phospholipids with unsaturated fatty acids on one hand and hydrophilic groups on the other within their molecular structure. As Yamaguchi pointed out (20), the presence of unsaturated fatty acids seems to be a prerequisite for these phospholipids and fatty acids to antagonize these antifungal agents. The reversal of clotrimazole activity by these phospholipids and fatty acids, however, was not observed in the present experiments.

Moderate reversal of the activity was observed

with unsaturated fatty acids and phospholipids without basic hydrophilic groups. The activity of miconazole was likewise affected by the phospholipids and fatty acids. The phospholipids with basic hydrophilic groups, however, had no effect, and the unsaturated fatty acids showed the most significant effects.

These experimental results suggested that the susceptibility of microorganisms to copiamycin, azalomycin F, and miconazole is determined by the permeation of these antifungal agents to the membrane and binding with such phospholipids in the membrane. The target phospholipids in the membrane, however, appear to be different with the copiamycin and azalomycin F group of antibiotics than with miconazole. Studies on the characterization of these phospholipids are now in progress and will be reported elsewhere. Despite the fact that clotrimazole and miconazole are chlorinated imidazole compounds with a similar broad antimicrobial spectrum, the effects of phospholipids were found to be surprisingly different. This might suggest a difference in their respective target substances, provided both agents act on the cell membrane.

LITERATURE CITED

1. **Arai, M.** 1960. Azalomycin B and F, two new antibiotics. I. Production and isolation. J. Antibiot. **13A**:46–50.
2. **Arai, M.** 1960. Azalomycin B and F, two new antibiotics. II. Properties of azalomycin B and F. J. Antibiot. **13A**:51–56.
3. **Arai, T., Y. Katoh, and M. Miyaji.** 1965. Biological activities of antifungal antibiotic "Copiamycin." Jpn. J. Med. Mycol. **6**:134–136 (in Japanese).
4. **Arai, T., S. Kuroda, H. Ohara, Y. Katoh, and H. Kaji.** 1965. Copiamycin, a new antifungal antibiotic derived from *S. hygroscopicus* var *crystallogenes*. J. Antibiot. **18A**:63–67.
5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipide extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
6. **Holt, R. J.** 1976. Topical pharmacology of imidazole antifungals. J. Cutaneous Pathol. **3**:45–59.
7. **Huston, C. K., P. W. Albro, and G. B. Grindey.** 1965. Lipids of *Sarcina lutea*. III. Composition of the complex lipids. J. Bacteriol. **89**:768–755.
8. **Iwata, K., Y. Kanda, H. Yamaguchi, and M. Osumi.** 1973. Electron microscopic studies on the mechanism of action of clotrimazole on *Candida albicans*. Sabouraudia **11**:205–209.
9. **Iwata, K., H. Yamaguchi, and T. Hiratani.** 1973. Mode of action of clotrimazole. Sabouraudia **11**:158–166.
10. **Iwata, K., H. Yamaguchi, Y. Kanda, and M. Osumi.** 1972. Action mechanism of clotrimazole, p. 315–322. *In* K. Iwata (ed.), Yeasts and yeast-like microorganisms in medical science. Proceedings of Specialized Symposium on Yeasts, Tokyo.
11. **Kubota, S.** 1968. In vivo and in vitro activities of "Copiamycin" and its derivatives. J. Chiba Med. Soc. **44**:50–67 (in Japanese).
12. **Kuroda, S., and T. Arai.** 1970. Sensitive sites of microorganisms to copiamycin, p. 211–216. *In* H. Umezawa (ed.), Progress in antimicrobial and anticancer chemotherapy. Unversity of Tokyo Press, Tokyo.
13. **Kuroda, S., and S. Yamanaka.** 1969. Sensitivity of actinomycetes to copiamycin. Ann. Rep. Inst. Food Microbiol. Chiba Univ. **22**:51–58.
14. **Rouser, G., G. Kritchevsky, and A. Yamamoto.** 1967. Column chromatographic and associated procedures for separation and determination of phosphatides and glycolipids, p. 99–162. *In* G. V. Marinetti (ed.), Lipid chromatographic analysis, vol. 1. Marcel Dekker, New York.
15. **Seiga, K., and K. Yamazi.** 1970. Studies on copiamycin. Jpn. J. Med. Mycol. **11**:127–131 (in Japanese).
16. **Sreedhara Swamy, K. H., M. Sirsi, and G. Ramananda Rao.** 1974. Studies on the mechanism of action of miconazole: effect of miconazole on respiration and cell permeability of *Candida albicans*. Antimicrob. Agents Chemother. **5**:420–425.
17. **Sreedhara Swamy, K. H., M. Sirsi, and G. Ramananda Rao.** 1976. Studies on the mechanism of action of miconazole. II. Interaction of miconazole with mammalian erythrocytes. Biochem. Pharmacol. **25**:1145–1150.
18. **Van Den Bossche, H., G. Willemsens, and J. M. Van Cutsem.** 1975. The action of miconazole on the growth of *Candida albicans*. Sabouraudia **13**:63–73.
19. **Vaskovsky, V. E., and E. Y. Kostetsky.** 1968. Modified spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. **9**:396.
20. **Yamaguchi, H.** 1976. Action mechanism of empecid. Med. von Heute **60**:85–94 (in Japanese).
21. **Yamaguchi, H., Y. Kanda, and K. Iwata.** 1974. Antifungal agents which interact with the cell membrane with special reference to clotrimazole. Jpn. J. Med. Mycol. **15**:18–25 (in Japanese).