Antifungal Mechanism of Polygodial

Isao Kubo,* Ken'ichi Fujita, and Sang Hwa Lee

Department of Environmental Science, Policy and Management, University of California, Berkeley, California 94720-3112

The primary antifungal action of polygodial comes in part from its ability to function as a nonionic surfactant, disrupting the lipid−protein interface of integral proteins and denaturing their conformation. As a result, the antifungal mechanism of this sesquiterpene dialdehyde is associated with the membrane functions or derangement of the membrane. For example, the glucose-induced medium acidification process of *Saccharomyces cerevisiae* was inhibited by polygodial, presumably caused by inhibition of the plasma membrane H^+-ATPase. However, the potent antifungal activity of polygodial results from its multiple functions.

Keywords: *Antifungal activity; Saccharomyces cerevisiae; polygodial; surfactant property; acidification; H*$^+$*-ATPase; lipid−protein interface*

INTRODUCTION

A bicyclic sesquiterpene dialdehyde, polygodial (**1**) (see Figure 1 for structures), was first isolated as a pungent principle from the sprout of *Polygonum hydropiper* (Polygonaceae) (*1*, *2*), known as "tade" and used as a food spice in Japan. Its congener, warburganal (**2**), was isolated from two East African *Warburgia* trees together with polygodial in minute amounts (*3*). Their potent fungicidal activity, especially against yeasts such as *Candida albicans* and *Saccharomyces cerevisiae*, was subsequently reported, although they possessed little or no activity against bacteria (*4*, *5*). The α,β-unsaturated aldehyde moiety in these sesquiterpene dialdehyde molecules was found to be responsible for their fungicidal action (*5*). In addition, this fungicidal activity was described to result from the structural disruption of the cell membrane (*6*). Similarly, both sesquiterpene dialdehydes (**1**, **2**) were reported to induce membrane leakage in the human neuroblastoma cells (*7*). Interestingly, the fungicidal action of the sesquiterpene dialdehydes against *C. albicans* and *S. cerevisiae* was dramatically enhanced through combination with phenylpropanoids such as anethole (**3**) and methyleugenol (**4**) (*8*−*10*). For example, the minimum inhibitory concentration (MIC) of polygodial against *C. albicans* was reduced from 6.25 to 0.20 μg/mL when it was combined with $^1/_2$MIC of anethole (*8*). This combination mechanism has not yet been explained on a molecular level. In addition, the binding site on the cell membrane of the sesquiterpene dialdehydes still remains to be established. With the increase in drug resistance and prevalence of opportunistic infections, there is a great need for effective antifungal agents with new modes of action (*11*). Polygodial was further studied using *S. cerevisiae* as a model in order to gain new insights into the molecular basis of its fungicidal action. Accumulation of this knowledge may provide a more rational and scientific approach to designing safe and effective antifungal agents.

* To whom correspondence should be addressed. Phone: (510) 643-6303. Fax: (510) 643-0215. E-mail: ikubo@uclink4.berkeley.edu.

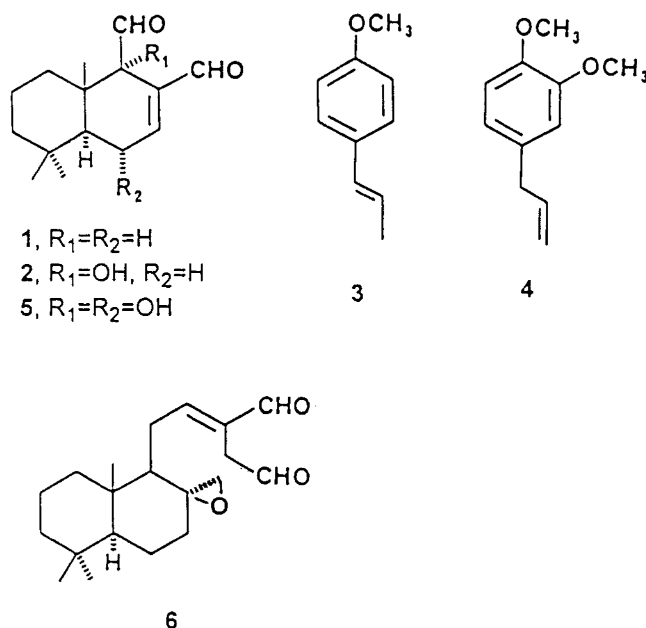

Figure 1. Chemical structures of polygodial and its related dialdehyde terpenoids, and phenylpropanoids.

MATERIALS AND METHODS

Chemicals. Polygodial and anethole were available from our previous work (*9*). Cycloheximide and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). $MgSO_4$ ($7H_2O$) was obtained from Aldrich Chemical Co. (Milwaukee, WI). $CaSO_4$ ($2H_2O$) and $CuSO_4$ ($5H_2O$) were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). *N,N*-Dimethylformamide (DMF) was obtained from EM Science (Gibbstown, NJ).

Test Strain. The test strain *Saccharomyces cerevisiae* ATCC 7754 used for this study was purchased from American Type Culture Collection (Rockville, MD).

Medium. *Saccharomyces cerevisiae* was maintained at −80 °C in yeast nitrogen broth (YNB; Difco Lab, Detroit, MI) containing 25% glycerol and subcultured at 30 °C in Sabouraud's dextrose agar (SDA) medium (bactopeptone 1%, dextrose 4%, bacto-agar 1.8%). A fresh culture was preincubated with shaking for 5 h at 30 °C in 2.5% malt extract (ME) broth (BBL) medium.

10.1021/jf000136g CCC: $20.00 © 2001 American Chemical Society
Published on Web 03/01/2001

Acidification Measurement. The glucose-induced medium acidification of *S. cerevisiae* was measured with a modified procedure (*12*). The test strain was cultured with shaking in YPD (glucose 2%, bactopeptone 2%, yeast extract 1%) broth overnight at 30 °C and washed twice with cold distilled water. The cells were diluted to 5×10^7 CFU/mL with cold distilled water and kept on ice. The reaction mixture contained 2.7 mL of cells and 30 μL of the DMSO sample solution, and was preincubated at 30 °C for 5 min. A 20% glucose solution of 0.3 mL was added (final 2%) to induce acidification. After 10 min of incubation, the pH of external medium was checked (Orion 8175 Ross semimicro electrode).

Antifungal Assay. The maximum extent and rate of activity is known to vary with seed culture mediums, the physiological age of the culture, and the type of culture medium. For example, the minimum inhibitory concentration (MIC) of anethole significantly varied with the inoculum size. All antifungal susceptibility tests in this study were performed under a standard condition using fresh inoculum from a 5 h shaking culture in malt extract medium, final inoculum size of 10^5 CFU/mL, and 48 h stationary incubation in malt extract medium, unless otherwise specified.

Broth macrodilution minimum inhibitory concentrations (MICs) were determined as previously described (*9*). Briefly, serial 2-fold dilutions of the test compounds were made in DMF, and 30 μL of 100× concentrated solution was added to 3 mL of ME media. These were inoculated with 30 μL of seed culture to give the final inoculum of 10^5 colony forming units (CFU)/mL. The assay tubes were incubated without shaking at 30 °C for 48 h. The MIC is the lowest concentration of test compound that demonstrated no visible growth. The minimum fungicidal concentrations (MFCs) were examined as follows. After determining the MIC, a 30-μL aliquot was taken from each clear tube and added into 3 mL of drug-free fresh medium. After 48 h of incubation, the MFC was determined as the lowest concentration of the test compounds in which no recovery of microorganism was observed. Combination studies were performed by a broth checkerboard method (*13*). A series of 2-fold dilutions of one compound was tested in combination with 2-fold dilutions of the other compounds. The assays were performed in triplicate on separate occasions.

The fraction inhibitory concentration (FIC) indices were calculated from checkerboard data. The FICs for these combinations were calculated as (MIC_a combination/MIC_a alone) + (MIC_b combination/MIC_b alone), where a and b were two compounds tested. The FIC or fractional fungicidal concentration (FFC) presented are significant values obtained from the checkerboard matrix. FIC and FFC indices were used to define the interaction of combined compounds: synergistic ($X = 0.5$), additive ($1X > 0.5$), indifferent ($4X > 1$), or antagonistic ($X > 4$).

Time-kill studies were performed to examine the effects of combinations of compounds in more detail. The culture tubes were prepared as described above and incubated at 30 °C for 5 h. A 30-μL aliquot of the culture was inoculated into 30 mL of ME broth containing appropriate concentrations of the test compounds. The initial population size for *S. cerevisiae* was 5.8×10^5 CFU/mL. Samples were taken at selected times during 48 h of exposure, and serial dilutions were made in sterile saline before the samples were plated onto YPD agar plates. The plates were incubated at 30 °C for 24 h before the number of CFU was determined.

RESULTS AND DISCUSSION

In our previous study, the primary active site of antifungal polygodial was found to damage the cell membrane in *S. cerevisiae* (*6*). Subsequently, addition of excess Ca^{2+} was reported to protect from the polygodial-induced cell membrane damage in *S. cerevisiae*, but it was protected only weakly by Mg^{2+} (*14*). Therefore, polygodial was combined with EDTA, which is known as a chelating agent, to understand the role of Ca^{2+} and eventually polygodial itself. EDTA significantly synergized the antifungal activity of polygodial. The MFC of polygodial was reduced from 3.13 to 0.2 μg/mL when it was combined with 3200 μg/mL ($^1/_2$MFC) of EDTA (Table 1). Because Ca^{2+} was found to protect the cell membrane from polygodial-induced damage, and Mg^{2+} is known as a cofactor of many essential enzymes in *S. cerevisiae* (*15*, *16*), it is possible that EDTA removed these divalent cations from the membrane surface and that the antifungal activity of polygodial was enhanced as a result. The natural lipid phosphatidic acid is known to be a calcium-specific ionophore, and is the only phospholipid with this property (*17*). Interestingly, polygodial also significantly enhanced the fungicidal activity of EDTA. The MFC of the latter was lowered from 6400 to 100 μg/mL, when it was combined with 1.56 μg/mL ($^1/_2$MFC) of the former.

Table 1. Antifungal Activity of Polygodial, Anethole, and EDTA against *S. cerevisiae*

compounds tested	μg/mL	
	MIC	MFC
polygodial	3.13[a]	6.25[a]
	1.56	3.13
warburganal	3.13	6.25
mukaadial	>100	n.t.[b]
aframodial	0.78	1.56
anethole	100	200
EDTA	400	6400

[a] Inoculum size, 10^6. [b] n.t., Not tested.

It is known that the addition of glucose to an unbuffered suspension of *S. cerevisiae* cells results in the extrusion of acid. This change in external pH upon the addition of glucose is characteristic of yeast cells, and is known to be due to the action of the plasma membrane H^+-ATPase (*18*). The activation of H^+-ATPase by glucose at the molecular level is not yet fully understood, but the maintenance of internal pH homeostasis is essential for the cell to survive because intracellular pH is important for the activity of a number of enzymes with pH optima (*19*, *20*). This glucose-induced medium acidification process was inhibited by polygodial as illustrated in Figure 2. The inhibition was presumably caused by inhibition of H^+-ATPase. Therefore, it is possible that the potent antifungal activity of polygodial is, at least in part, due to its inhibition of the plasma membrane H^+-ATPase. Interestingly, the inhibitory action of polygodial to the glucose-induced acidification of the medium was suppressed strongly by Ca^{2+}, but only weakly by Mg^{2+}, as shown in Figure 3.

The above findings seem to explain several remaining problems of fungicidal action of polygodial at the molecular basis. It should be noted that the activity of polygodial is enhanced at acidic conditions (*6*). It is known that yeast cells are able to maintain a normal internal pH when suspended in an acidic medium with relatively little change in the intracellular pH. The acidic conditions appear to stimulate the plasma membrane H^+-ATPase activity, and excess protons are pumped out to the external medium, maintaining constant internal pH during growth (*18*). As a result of the inhibition of the plasma membrane H^+-ATPase by polygodial, the intracellular pH may drop into the range where phosphofructokinase is sensitive (*21*). The subsequent inhibition of glycolysis caused by this inactivation of phosphofructokinase results in a drop in ATP levels, and thus restricts growth (*19*). This rationale may explain why polygodial is more potent in the acidic

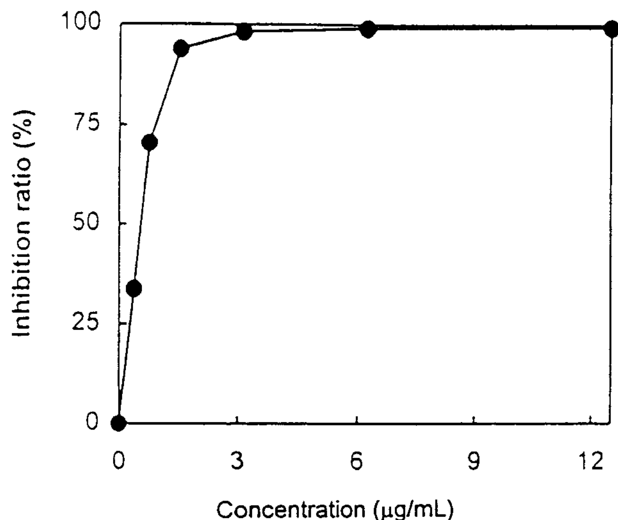

Figure 2. Inhibitory effect of polygodial to the medium acidification of the plasma membrane H^+-ATPase of *S. cerevisiae*. The medium acidification was induced by adding the glucose solution (final concentration 2%) and was evaluated with the mole concentration of protons calculated with external medium pH. The ratio of inhibition (%) was calculated as $(1 - [H^+]_{inhibitor}/[H^+]_{inhibitor\ free}) \times 100$.

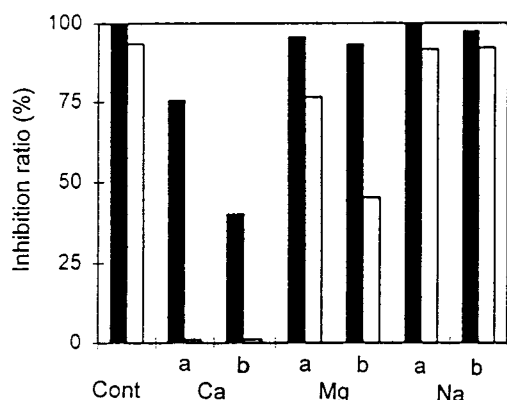

Figure 3. Metal effect on the polygodial action against the glucose-induced medium acidification of *S. cerevisiae*. Cont., without metal; a, 10 mM of each metal ion; b, 100 mM of each metal ion; and (■), 25 μg/mL of polygodial; and (□), 6.25 μg/mL of polygodial.

conditions. It is an interesting question how the plasma membrane H^+-ATPase is inhibited by polygodial.

In contrast to the potent antifungal activity of polygodial, its congener mukaadial (**5**) did not exhibit any activity up to 100 μg/mL, although warburganal (**2**) still exhibited some activity, though to a lesser extent than polygodial (*10, 22*). Thus, the activity decreased for each additional hydroxyl group "added" to the molecular framework of polygodial. The fungicidal activity of polygodial was explained as the result of the structural disruption of the cell membrane (*6*). Moreover, in a previous experiment using the human neuroblastoma cells (*7*), the increase in membrane permeability was demonstrated to depend on the accumulation of the unsaturated dialdehydes in the membranes with the reactive aldehyde groups oriented toward the membrane surface. Considering the data so far reported, the antifungal mechanism of polygodial may result, at least in part, from its ability to function as a nonionic surface-active agent (surfactant), similar to long chain alcohols (*23*). The greater activity of the dialdehydes (**1**, **2**) could be due primarily to a balance between the hydrophilicity of the unsaturated aldehyde subunit and the hydrophobicity of the Decalin portions of the molecule. It seems that mukaadial (**5**) does not possess this balance due to its increased hydrophilicity and, hence, is inactive.

As a nonionic surfactant, polygodial would likely approach the binding site with the electronegativity of the aldehyde oxygen atom. The aldehyde oxygens are potent hydrogen bond acceptors which will disrupt existing hydrogen bonds. For example, in the lipid bilayer the hydroxyl group of ergosterol, a major component of the plasma membrane, resides near the membrane−water interface and is likely hydrogen-bonded with the carbonyl group of phospholipids (*24, 25*). As ergosterol owes its membrane-fixing properties to its rigid, longitudinal orientation in the membrane, and has profound influence on membrane structure and function, if these hydrogen bonds are disrupted, the cell will die. In contrast to that of amphotericin B or miconazole, the fungicidal activity of polygodial was not suppressed by adding excess ergosterol.

Interestingly, the polygodial-induced membrane damage was protected by Ca^{2+}, but this protection is eliminated by adding EDTA (*14*). A similar Ca^{2+} induced protection was also recently reported (*26*). In addition, Ca^{2+} is known to act as a second messenger within eukaryotic cells, and it transduces cell-surface primary stimuli into intracellular events (*27*). The role of Ca^{2+} is still unclear and many mechanisms to explain it seem possible. For example, the possibility of Ca^{2+} binding to the negatively charged phosphate oxygen atoms on the membrane (*15, 17*), similar to bacterial membranes (*28*), cannot be entirely ruled out. If this is so, it would result in the formation of a cross-linked membrane structure which may impede the approach of polygodial to the binding site on the cell membrane. This can be supported by the fact that Ca^{2+} suppressed miconazole-induced leakage and slightly suppressed the amphotericin B-induced leakage (*14*). It seems that Ca^{2+} has the right size to form this cross-linked membrane structure because other divalent cations such as Mg^{2+}, Fe^{2+}, and Cu^{2+} do not have this protection activity. However, the leakage alone cannot be polygodial's potent fungicidal mechanism because it kills the cells even after protection of the leakage by Ca^{2+}.

Given the surfactant-like properties of polygodial, it is possible to suggest that polygodial also acts at the lipid−protein interface of H^+-ATPase, denaturing its functioning conformation. In a system containing both lipids and proteins, it is difficult to determine whether a conformational change of a protein is the result of a direct H^+-ATPase interaction, or of motional or conformational modification of the lipids themselves which exist at the lipid−protein interface. Nevertheless, the binding of polygodial as a nonionic surfactant can involve only relatively weak headgroup interactions, such as hydrogen bonding. It is suggested that the intrinsic proteins of immediately surrounding membranes are held in position by hydrogen bonding, as well as by hydrophobic and electrostatic forces, and that hydrogen bonding also mediates the penetration of membranes by proteins. As proposed above, hydrogen bonds may be disrupted by polygodial and redirected. Thereby, the conformation of the protein may be changed, and consequently the H^+-ATPase, in particular, may lose its functioning conformations. Although H^+-ATPase is the most abundant plasma membrane protein, constituting over 20% of the total membrane protein in *S.*

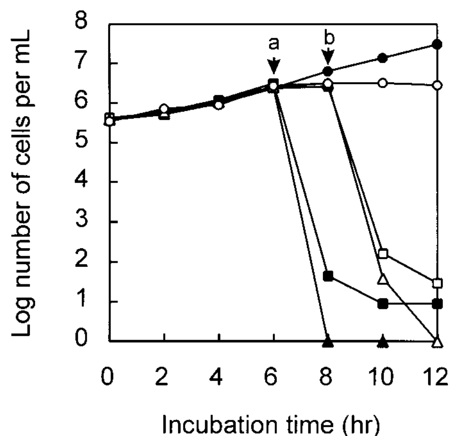

Figure 4. Decrease in viability of *S. cerevisiae* induced by polygodial. Exponentially growing cells of *S. cerevisiae* were treated with (open circle) or without (closed circle) cycloheximide (50 μg/mL) at the time point (a) in 2.5% malt extract medium. Polygodial was added to final concentration of 0 (●, ○), 3.13 (■, □), and 6.25 (▲, △) μg/mL at the time point (a) (closed circle), or (b) (open circle). Viability was estimated by the number of colonies formed on YPD plate after incubation at 30 °C for 24 h.

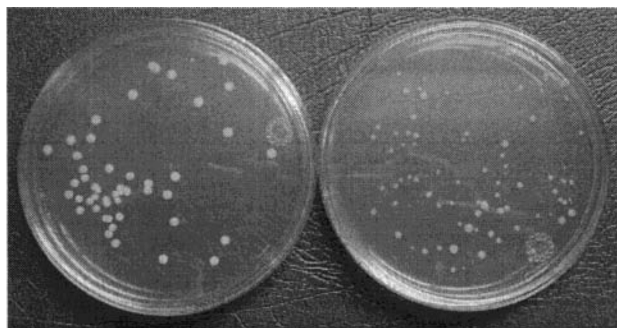

Figure 5. Effect of polygodial on colony formation of *S. cerevisiae* cells. The cells (5×10^5 cells/mL) were treated with (right) or without (left) 3.13 μg/mL of polygodial in 2.5% malt extract medium for 2 h. After treatment, 100 μL of the cell suspension was diluted adequately and then spread on the YPD plate (∅ 80 mm). Colonies were formed after 24 h of incubation.

cerevisiae, other plasma membrane proteins may also be disrupted by polygodial. The data obtained are consistent with an effect on the lipid−protein interface rather than a direct interaction of H^+-ATPase. All of this is agreeable with the previous report that the primary active site of polygodial is at the membrane (*6*).

Further support for this postulate was also obtained in experiments that showed the rapid decline in the number of viable cells after the addition of polygodial at the exponential-growth phase as shown in Figure 4. Furthermore, polygodial rapidly killed *S. cerevisiae* cells in which cell division was inhibited by cycloheximide. This observation excludes several modes of action for polygodial, such as inhibition of DNA, RNA, protein, or cell wall component synthesis in vivo. The antifungal mechanism of polygodial is likely associated with the membrane functions or derangement of the membrane. It should be noted that the size of colonies formed in the presence of polygodial was variable and smaller than that in the absence of polygodial as shown in Figure 5.

All the data so far obtained can be explained by the surfactant concept. It should be remembered that polygodial showed potent fungicidal activity, particularly against yeasts, but the above-mentioned surfactant mechanism should not be specific. The specificity of polygodial against yeasts is likely on the basis of its hydrophobic Decalin moiety. To prove this hypothesis, modification of the bicyclic moiety would be necessary, though it may not be practical. However, indirect evidence in support of this hypothesis already exists in the fact that a more bulky labdane diterpene dialdehyde, aframodial (**6**), exhibited more potent activity. The activity of polygodial can be increased by modifying its hydrophobic bicyclic moiety, but experimentally this may not be practical. More specifically, a series of (2*E*)-alkenals were reported as antifungal agents against *Saccharomyces cerevisiae,* and the result obtained indicates that the hydrophobic alkyl group obviously plays an important role in the activity (*29*).

In addition, α,β-unsaturated aldehydes are highly reactive substances, and they readily react with biologically important nucleophiles, such as sulfhydryl, amino, and hydroxyl groups. The main reaction appears to be 1,4-addition under physiological condition, although the formation of Schiff bases is also possible. An earlier report demonstrated a good correlation between the antifungal activity and the papain inhibitory activity of polygodial. Both activities appear to result from their highly specific reactivity with sulfhydryl groups at the enal group (*22*). Yeast plasma-membrane H^+-ATPase is reported to contain nine cysteines. Polygodial may bind directly to the plasma membrane H^+-ATPase, possibly with sulfhydryl groups of the three cysteines in the presumed transmembrane segments (C148, C312, C867). However, Petrov and Slayman (*30*) reported that no single cysteine is required for the enzyme activity on the basis of their site-directed mutagenesis study. This does not exclude, however, the possibility that polygodial breaks the hydrogen bond as a surfactant and then reacts with the freed sulfhydryl group of the H^+-ATPase. This is supported by the previous report by Monk and his colleagues (*31*) that covalent modification of the conserved C148 in the transmembrane segment 2 may be important for inhibition of H^+-ATPase activity and cell growth. The involvement of this kind of biochemical reactions still remains unclear. In addition, the possibility that polygodial enters the cell by passive diffusion across the plasma membrane is unlikely but cannot be entirely ruled out. The question of how polygodial interacts with the membrane functions needs to be clarified.

In conclusion, the data so far obtained indicate that polygodial initially acts as a nonionic surfactant. For example, polygodial induces leakage by disrupting the membrane surface. More specifically, it inhibits the plasma membrane H^+-ATPase by disrupting and disorganizing the hydrogen bonds at the lipid bilayer−protein interface. It should be kept in mind, however, that polygodial does not act by a single defined process, but, rather, has multiple functions by which it exerts its potent fungicidal action. Last, it is worthwhile to note that polygodial very likely targets the extracytoplasmic region and thus does not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms.

ACKNOWLEDGMENT

The authors are indebted to Dr. J. Snyder and Dr. M. Lewin for their critical reading of the manuscript.

LITERATURE CITED

(1) Barnes, C.; Loder, J. The structure of polygodial, a new sesquiterpene dialdehyde from *Polygonum hydropiper* L. *Aust. J. Chem.* **1962**, *15*, 322−327.
(2) Ohsuka, A. The structure of tadeonal and isotadeonal components of *Polygonum hydropiper* L. *Nippon Kagaku Zasshi* **1963**, *84*, 748−752.
(3) Kubo, I.; Lee, Y. W.; Pettei, M.; Pilkiewicz, F.; Nakanishi, K. Potent army worm antifeedants from the East African *Warburgia* plants. *J. Chem. Soc., Chem. Commun.* **1976**, 1013−1014.
(4) McCallion, R. F.; Cole, A. L.; Walker, J. R. L.; Blunt, J. W.; Munro, M. H. G. Antibiotic compounds from New Zealand plants II. Polygodial, an anti-*Candida* agent from *Pseudowintera colorata*. *Planta Med.* **1982**, *44*, 134−138.
(5) Taniguchi, M.; Adachi, T.; Oi, S.; Kimura, A.; Katsumura, S.; Isoe, S.; Kubo, I. Structure−activity relationship of the *Warburgia* sesquiterpene dialdehydes. *Agric. Biol. Chem.* **1984**, *48*, 73−78.
(6) Taniguchi, M.; Yano, Y.; Tada, E.; Ikenishi, K.; Oi, S.; Haraguchi, H.; Hashimoto, K.; Kubo, I. Mode of action of polygodial, an antifungal sesquiterpene dialdehyde. *Agric. Biol. Chem.* **1988**, *52*, 1409−1414.
(7) Forsby, A.; Walum, E.; Sterner, O. The effect of six sesquiterpenoids unsaturated dialdehydes on cell membrane permeability in human neuroblastoma SH-SY5Y cells. *Chem.-Biol. Interact.* **1992**, *84*, 85−95.
(8) Himejima, M.; Kubo, I. Antimicrobial agents from *Licaria puchuri-major* and their synergistic effect to polygodial. *J. Nat. Prod.* **1992**, *55*, 620−625.
(9) Kubo, I.; Himejima, M. Anethole, a synergist of polygodial against filamentous microorganisms, *J. Agric. Food Chem.* **1991**, *39*, 2290−2292.
(10) Kubo, I.; Himejima, M. Potentiation of antifungal activity of sesquiterpene dialdehydes against *Candida albicans* and two other fungi. *Experientia* **1992**, *48*, 1162−1164.
(11) Sternberg, S. The emerging fungal threat. *Science* **1994**, *266*, 1632−1634.
(12) Haworth, R. S.; Cragoe, E. J., Jr.; Fliegel, L. Amiloide and 5-(*N*-ethyl-*N*-isopropyl) amiloride inhibit medium acidification and glucose metabolism by the fission yeast *Schizosaccharomycess pombe*. *Biochim. Biophys. Acta* **1993**, *1145*, 266−272.
(13) Norden, C. W.; Wenzel, H.; Keleti, E. Comparison of techniques for measurement of in vitro antibiotic synergism. *J. Infec. Dis.* **1979**, *140*, 441−443.
(14) Yano, Y.; Taniguchi, M.; Tanaka, T.; Oi, S.; Kubo, I. Protective effects of Ca^{2+} on cell membrane damage by polygodial in *Saccharomyces cerevisiae*. *Agric. Biol. Chem.* **1991**, *55*, 603−604.
(15) Brockerhoff, H. Hypothesis: Control of intracellular calcium level. *Chem. Phys. Lipids* **1986**, *39*, 83−92.
(16) Kocková-Kratochvílová, A. *Yeasts and Yeast-like Organisms*. VCH: Weinheim, Germany, 1990.
(17) Serhan, C.; Fridovich, J.; Doetzl, E. J.; Dunham, P. B.; Weissmann, G. Leukotriene B_4 and phosphatidic acid are calcium ionophores. Studies employing arsenazo III in liposomes. *J. Biol. Chem.* **1982**, *257*, 4746−4752.
(18) Eraso, P.; Gancedo, C. Activation of yeast plasma membrane ATPase by acid pH during growth. *FEBS Lett.* **1987**, *224*, 187−192.
(19) Busa, W. B.; Nuccitelli, R. Metabolic regulation via intracellular pH. *Am. J. Physiol.* **1984**, *246*, 409−438.
(20) Ramos, S.; Balbín, M.; Raposo, M.; Valle, E.; Pardo, L. A. The mechanisms of intracellular acidification induced by glucose in *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **1989**, *135*, 2413−2422.
(21) Madshus, I. H. Regulation of intracellular pH in eukaryotic cells. *Biochem. J.* **1988**, *250*, 1−8.
(22) Taniguchi, M.; Adachi, T.; Haraguchi, H.; Oi, S.; Kubo, I. Physiological activity of warburganal and its reactivity with sulfhydryl groups. *J. Biochem.* **1983**, *94*, 149−154.
(23) Kubo, I.; Muroi, H.; Kubo, A. Structural functions of antimicrobial long-chain alcohols and phenolics. *Bioorg. Med. Chem.* **1995**, *3*, 873−880.
(24) Brockerhoff, H. Model of interaction of polar lipids, cholesterol, and proteins in biological membranes. *Lipids* **1974**, *9*, 645−650.
(25) Chauhan, V. P. S.; Ramsammy, L. S.; Brockerhoff, H. Molecular interactions in the hydrogen belts of membranes glucose-6-phosphate, lysophosphatidylcholine, and cholesterol. *Biochim. Biophys. Acta* **1984**, *772*, 239−243.
(26) Ibrahim, H. R.; Higashiguchi, S.; Sugimoto, Y.; Aoki, T. Role of divalent cations in the novel bactericidal activity of the partially unfolded lysozymes. *J. Agric. Food Chem.* **1997**, *45*, 89−94.
(27) Pitt, D.; Kaile, A. Transduction of the calcium signal with special reference to Ca^{2+}-induced condition in *Penicillium notatum*. In *Biochemistry of Cell Walls and Membranes in Fungi;* Kuhn, P. J., Trinci, A. P. J., Jung, M. J., Goosey, M. W., Copping, L. G., Eds.; Springer-Verlag: Berlin, 1990; pp 238−298.
(28) Asbell, M. A.; Eagon, R. G. Role of multivalent cations in the organization, structure, and assembly of the cell wall of *Pseudomonas aeruginosa*. *J. Bacteriol.* **1966**, *92*, 380−387.
(29) Kubo, A.; Kubo, I. Antimicrobial agents from *Tanacetum balsamita*. *J. Nat. Prod.* **1995**, *58*, 1565−1569.
(30) Petrov, V. V.; Slayman, C. W. Site-directed mutagenesis of the yeast pma1 H^+-ATPase. *J. Biol. Chem.* **1995**, *270*, 28535−28540.
(31) Monk, B. C.; Mason, A. B.; Abramochkin, G.; Haber, J. E.; Seto-Young, D.; Perlin, D. S. The yeast plasma membrane proton pumping ATP-ase is a viable antifungal target. I. Effects of the cysteine-modifying reagent omeprazole. *Biochem. Biophys. Acta* **1995**, *1238*, 81−90.

Received for review January 31, 2000. Revised manuscript received January 12, 2001. Accepted January 16, 2001. K.F. thanks Osaka City University for financial support during his study at UCB.

JF000136G