J. Microbiol. Biotechnol. (1998), **8**(3), 197-202

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY
© Korean Society for Applied Microbiology

Antifungal Activity of Medium-Chain (C_6-C_{13}) Alkenals against, and Their Inhibitory Effect on the Plasma Membrane H^+-ATPase of *Saccharomyces cerevisiae*

LEE, JAE-RAN[2], SANG-HWA LEE[1], ISAO KUBO[1]*, AND SOON-DUCK HONG[2]

[1]*Department of Environmental Science, Policy and Management, University of California, Berkeley, California 94720-3112, U.S.A.*
[2]*Department of Microbiology, Kyung-pook National University, Taegu 702-701, Korea*

Received: December 15, 1997

Abstract Aliphatic alkenals having 6 to 13 carbons were evaluated for antifungal activity against *Saccharomyces cerevisiae*. The activity was gradually increased with chain length, e.g., (*E*)-2-decenal and (*E*)-2-undecenal exhibited maximum potency, while (*E*)-2-dodecenal and (*E*)-2-tridecenal were completely inactive. Alkenals showed increasing inhibitory activity with chain length, as in the case of antifungal activity, towards glucose-induced medium acidification by the plasma membrane H^+-ATPase of *S. cerevisiae*. The group including (*E*)-2-nonenal, (*E*)-2-decenal, and (*E*)-2-undecenal exhibited maximum potency, but the potency of (*E*)-2-dodecenal and (*E*)-2-tridecenal demonstrated a sudden drop with respect to the former group. (*E*)-2-Nonenal revealed dose-reponsive inhibition to the medium acidification and inhibited over 90% at a concentration of 1.25 mM (175.3 μg/ml). In contrast to (*E*)-2-undecenal whose inhibitory efficiency increased with incubation time, inhibition by (*E*)-2-dodecenal was reversed with time. Of the tested alkenals, (*E*)-2-heptenal and (*E*)-2-octenal most highly inhibited ATP hydrolytic activity by the plasma membrane H^+-ATPase, while (*E*)-2-heptenal at 10 mM (1121.8 μg/ml) showed an inhibitory efficacy of 93.2%.

Key words: Aliphatic alkenals, plasma membrane H^+-ATPase, medium acidification, antifungal action

Polygodial isolated from the sprouts of *Polygonum hydropiper* [2], which has been used in folk medicines and food spices in some Asian countries [10, 22], has been found to have strong fungicidal activity against several systemic fungal pathogens [11, 17, 26]. When combined with other compounds including anethole [6, 7, 10], actinomycin D [9, 13], miconazole, and amphotericin B, polygodial reveals strong synergistic effect on antifungal action, except with amphotericin B in which strong antagonism is demonstrated. Regarding the antifungal mode of action, it has been reported that polygodial causes fragmentation of the cellular membrane and leakage of intracellular metabolites via destructive action on the cellular membrane [13, 27]. Polygodial has also been identified as having the inhibitory action on the plasma membrane H^+-ATPase of *S. cerevisiae*, which is assumed to cause its antifungal and membrane-destructive action.

The plasma membrane H^+-ATPase is an essential enzyme in membranes of fungi and plants [18], where it expels the expulsion of intracelluar protons into the surrounding medium and thereby creates the proton gradient used as a driving force for active nutrient uptake [4]. Another function of this P-type enzyme is to control the intracellular pH [16], and acidification of extracellular media [24] is an easily measured consequence of this activity. Recently, some reports have indicated that inhibitors of this H^+-ATPase can be effective fungicidal agents [20, 21].

Further research on the biochemical action of polygodial on the plasma membrane H^+-ATPase and its structure-activity relationship is required for the development of effective antifungal agents. The chemical structure of polygodial shows characteristic of a surfactant having the hydrophilic moiety of two aldehydes and the hydrophobicity of a drimane skeleton [2]. Similarly, aliphatic alkenals are also aldehyde surfactants and, as reported in our previous paper [8], some alkenals show antifungal activity. In addition, alkenals are widely distributed in nature, exhibit structural diversity with a variety of chain lengths, and are commercially available [8]. In order to ultimately understand the biochemical

*Corresponding author
Phone: 82-510-643-6303; Fax: 82-510-643-0215;
E-mail: ikubo@uclink.Berkeley.edu

action of polygodial and, in particular, the role of its complex hydrophobic moiety on the plasma membrane H^+-ATPase, alkenals were selected as model compounds. In this study, alkenals in the range of C_6 to C_{13} were investigated for their antifungal activity against *S. cerevisiae* and also tested for their inhibitory effect on medium acidification and on ATP hydrolysis by the plasma membrane H^+-ATPase of *S. cerevisiae.*

MATERIALS AND METHODS

Culture

Saccharomyces cerevisiae ATCC 7754 was purchased from the American Type Culture Collection (Rockville, U.S.A.). It was maintained at -80°C in yeast nitrogen broth (YNB; Difco Laboratories, Detroit, U.S.A.) containing 25% glycerol and subcultured at 30°C in Sabouraud's dextrose agar (SDA) medium (Bactopeptone 1%, Dextrose 4%, Bacto-agar 1.8%, pH 7.0).

Chemicals

Malachite green hydrochloride, EDTA, dithiothreitol, sodium ATP, and lyticase were purchased from Sigma Co. (St. Louis, U.S.A.). Alkenals including (*E*)-2-hexenal (C_6), (*E*)-2-heptenal (C_7), (*E*)-2-octenal (C_8), and (*E*)-2-nonenal (C_9) were purchased from Aldrich Chemical Co. (Milwaukee, U.S.A.), while (*E*)-2-decenal (C_{10}), (*E*)-2-undecenal (C_{11}), (*E*)-2-dodecenal (C_{12}), and (*E*)-2-tridecenal (C_{13}) were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Determination of MICs and MFCs

The susceptibility of *S. cerevisiae* to alkenals was determined by the macrobroth dilution method [10]. Briefly, 30 µl of two-fold serial dilutions of the test compounds in DMSO were mixed with 3 ml of malt extract (ME) (BBL, Cockeysville, U.S.A.) broth containing fresh inoculum (10^5 CFU/ml). The assay tubes were incubated without shaking at 30°C for 48 h. The minimum inhibitory concentration (MIC) was the lowest concentration of each compound at which no visible growth demonstrated. Minimum fungicidal concentrations (MFCs) were examined as follows. After determining the MIC, a 30 µl aliquot was taken from each clear tube and added to 3 ml of compound-free fresh medium. After 48 h of incubation, the minimum fungicidal concentration (MFC) was determined as the lowest concentration of test compound in which no recovery of microorganisms was observed. All assays were performed in triplicate on separate occasions.

Measurement of Medium Acidification

The inhibitory effect of alkenals on glucose-induced medium acidification by the plasma membrane H^+-ATPase of *S. cerevisiae* was measured with a modified procedure based on the method of Haworth [5]. The strain was cultured with shaking overnight at 30°C in YPD medium (Yeast extract 1%, Bactopeptone 2%, Glucose 2%, pH 7.0) broth and washed two times with chilled distilled water. The cells were diluted to 5×10^7 cells/ml with cold distilled water and kept on ice. The reaction mixture contained 2.7 ml of cell suspension and 30 µl of test compound in DMSO, and was preincubated for 5 min at 30°C. 0.3 ml of 20% glucose solution was added (final concentration 2%) to induce the medium acidification. After 10 min of incubation, the pH of the external medium was checked (Orion 8175 Ross semimicro electrode). All tests were performed in triplicate on separate occasions.

Isolation of Plasma Membrane H^+-ATPase

The *S. cerevisiae* strain was cultured as mentioned above and washed two times with 20 mM Tris-HCl buffer (pH 7.0) containing 10% sorbitol. The washed cells were incubated in sorbitol buffer containing 0.5 mM dithiothreitol and lyticase for 4 h at 30°C, and washed two times with sorbitol buffer. The cells were subjected to 15 min of homogenization (Branson 450 Sonifier with 1/2 horn) in a 2°C circulating water bath. After removing the debris ($3{,}000\times g$ for 5 min), the supernatant was submitted to ultracentrifugation ($53{,}000\times g$ for 1 h). The pellet was suspended in a 20% glycerol solution containing 10 mM Tris-HCl (pH 7.2) and 0.1 mM EDTA, and applied to a discontinuous sucrose gradient (53.5%:43.5% = 1:2) at $53{,}000\times g$ for 3 h (Beckman SW25.1 swinging bucket rotor) based on the method of Serrano [15]. The purified plasma membranes were recovered at the 43.5/53.5 interface and suspended in MET buffer (5 mM $MgCl_2$, 1 mM EDTA in 50 mM Tris-HCl buffer). These membrane vesicles, which showed the converted orientation predominantly (85%–90%), were used for the measurement of ATPase activity [19]. The protein concentration was determined by the Coomassie Plus protein assay (Pierce Co., Rockford, U.S.A.), and bovine serum albumin was used as a standard. The addition of sodium azide in a concentration of 5 mM did not decrease ATP hydrolytic activity of the isolated plasma membrane H^+-ATPase, indicating that there was no significant contamination by mitochondrial ATPase [23].

Measurement of ATP Hydrolysis

After the 250 µl of enzyme solution in MET buffer (13 µg protein/ml) and 3 µl of test compound in DMSO were mixed, the mixture was preincubated for 5 min. 50 µl of 12 mM ATP in 50 mM Tris-HCl buffer (pH 7.0) was then added (final 2 mM) and incubated for 10 min at 30°C. Inorganic phosphates generated were developed with malachite green reagent and measured by optical

absorbance at 660 nm [14]. Dehydrated disodium hydrogen phosphate was used for phosphate calibration. All assays were performed in triplicate on separate occasions.

RESULTS

Aliphatic alkenals in the range of C_6 to C_{13} were tested for antifungal activity against *S. cerevisiae* (Table 1). Activity gradually increased with alkyl chain length. Of the tested alkenals, (*E*)-2-decenal and (*E*)-2-undecenal showed maximum activity with MICs and MFCs of 25 μg/ml. However, (*E*)-2-dodecenal and (*E*)-2-tridecenal showed no antifungal activity, even at a concentration of 1600 μg/ml.

Table 1. Antifungal activity of alkenals against *Saccharomyces cerevisiae.*

Alkenals	MIC[a]	MFC[b]
(*E*)-2-Hexenal (C_6)	1600	>1600
(*E*)-2-Heptenal (C_7)	400	800
(*E*)-2-Oxenal (C_8)	200	400
(*E*)-2-Nonenal (C_9)	50	100
(*E*)-2-Decenal (C_{10})	25	25
(*E*)-2-Undecenal (C_{11})	25	25
(*E*)-2-Dodecenal (C_{12})	>1600	>1600
(*E*)-2-Tridecenal (C_{13})	>1600	>1600

[a]μg/ml. Determined by macrobroth dilution method, characterized with inoculum size of 10^5 CFU/ml, ME broth, and incubation time of 48 h.
[b]μg/ml. Determined by observation of fungal growth in 100-fold dilution after MIC determinations.

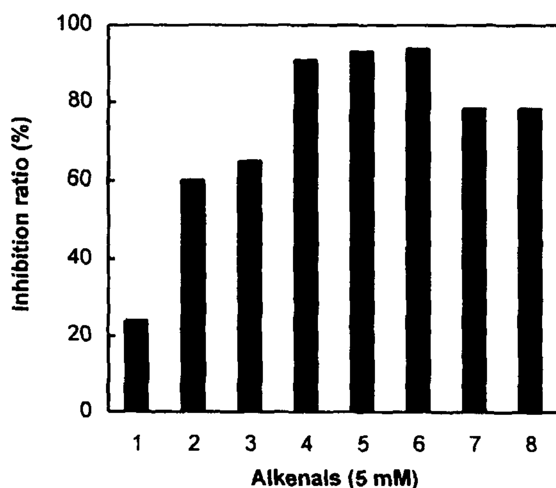

Fig. 1. The inhibitory effect of alkenals (5 mM) to the medium acidification by the plasma membrane H^+-ATPase of *S. cerevisiae.*

The medium acidification was induced by glucose (final concentration 2%) and evaluated by the mole concentration of protons calculated from external pH. The inhibition ratio (%) was calculated as follows: $(1-[H^+]_{inhibitor}/[H^+]_{inhibitor\ free})\times 100$. Alkenals: 1, (*E*)-2-hexenal; 2, (*E*)-2-heptenal; 3, (*E*)-2-octenal; 4, (*E*)-2-nonenal; 5, (*E*)-2-decenal; 6, (*E*)-2-undecenal; 7, (*E*)-dodecenal; 8, (*E*)-2-tridecenal.

Alkenals were investigated for their inhibitory effect on medium acidification by the plasma membrane H^+-ATPase of *S. cerevisiae*. Medium acidification was induced by adding glucose to cell suspension in distilled water and evaluated by determining the mole concentration of protons based on the external medium pH. Inhibitor-free cell suspension was acidified from pH 6.0 to 3.61, which is equivalent to the production of 23.4×10^{-5} M of protons. The inhibitory effect of alkenals (final concentration 5 mM) on the medium acidification are presented in Fig. 1. Overall, inhibitory activity gradually increased with chain length. The group including (*E*)-2-nonenal, (*E*)-2-decenal, and (*E*)-2-undecenal exhibited the maximum potency (>90%). However, (*E*)-2-dodecenal and (*E*)-2-tridecenal demonstrated a suddenly dropped activity of 78.4%. The inhibitory activity of (*E*)-2-nonenal on medium acidification was investigated at various concentrations (Fig. 2). (*E*)-2-Nonenal showed a dose-responsive inhibitory curve, exhibiting 57.1% and 95.2% inhibition at concentrations of 0.31 mM (43.47 μg/ml) and 1.25 mM (175.29 μg/ml), respectively. The inhibitory effects of (*E*)-2-undecenal and (*E*)-2-dodecenal during 240 min of incubation were compared. Medium acidification was increasingly inhibited over time by (*E*)-2-undecenal, whereas (*E*)-2-dodecenal showed gradually decreasing activity with time (Fig. 3).

The effect of alkenals on ATP hydrolysis by the plasma membrane H^+-ATPase was investigated (Fig. 4). The specific activity of the H^+-ATPase isolated from *S. cerevisiae* was 8.9 μmole/min/mg protein. The inhibitory activity of the alkenals (final concentration 10 mM) on the ATP hydrolysis is presented in Fig. 4. The maximum

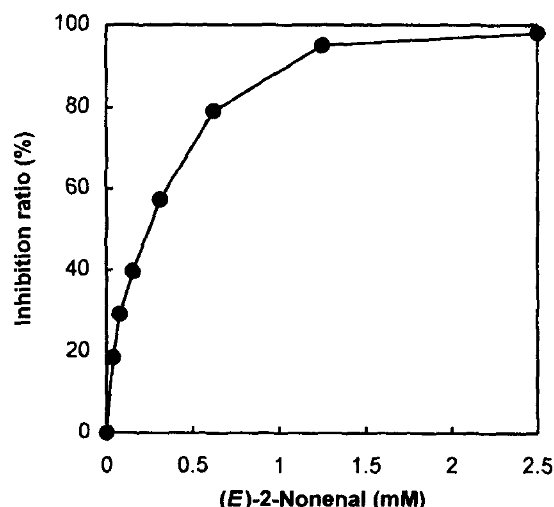

Fig. 2. The inhibitory activity of (*E*)-2-nonenal to the medium acidification by the plasma membrane H^+-ATPase of *S. cerevisiae.*

The inhibition ratio (%) was calculated as follows: $(1-[H^+]_{inhibitor}/[H^+]_{inhibitor\ free})\times 100$.

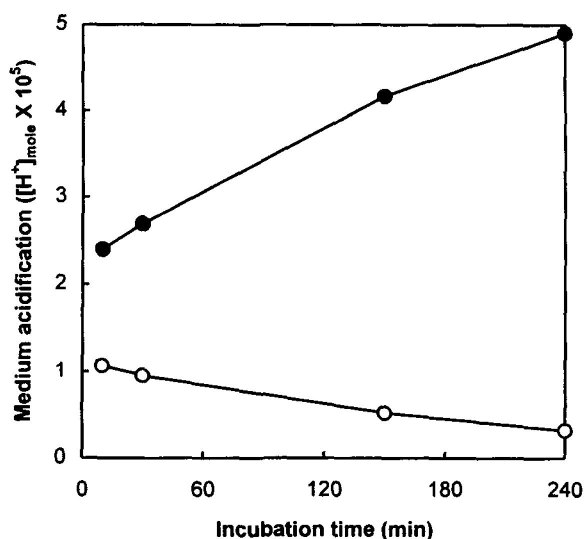

Fig. 3. The effects of incubation time on the inhibition of (*E*)-2-undecenal (○) and (*E*)-2-dodecenal (●) to the medium acidification by the plasma membrane H^+-ATPase of *S. cerevisiae.*

Alkenals were tested at the concentration of 5 mM.

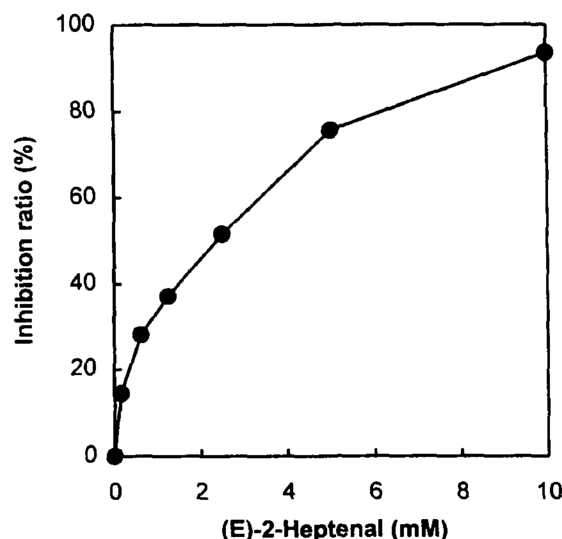

Fig. 5. The inhibitory activity of (*E*)-2-heptenal to the ATP hydrolysis by the plasma membrane H^+-ATPase of *S. cerevisiae.*

The amount of enzyme used (specific activity 8.9 μmole/min/mg protein) was 0.029 U (μM P_i/min), and the inhibition ratio (%) was calculated as follows: $[1 - (OD_{660\ nm})_{inhibitor}/(OD_{660\ nm})_{inhibitor\ free}] \times 100$.

activity (>90%) was shown by (*E*)-2-heptenal and (*E*)-2-octenal, and alkenals in the range of C_9-C_{13} exhibited decreasing activity with chain length. (*E*)-2-heptenal exhibited dose-reponsive inhibition of ATP hydrolysis by the isolated H^+-ATPase (2.9×10^{-2} U) showing 51.5% and 93.2% inhibition at concentrations of 2.5 mM (80.45 μg/ml) and 10 mM (1121.8 μg/ml), respectively (Fig. 5).

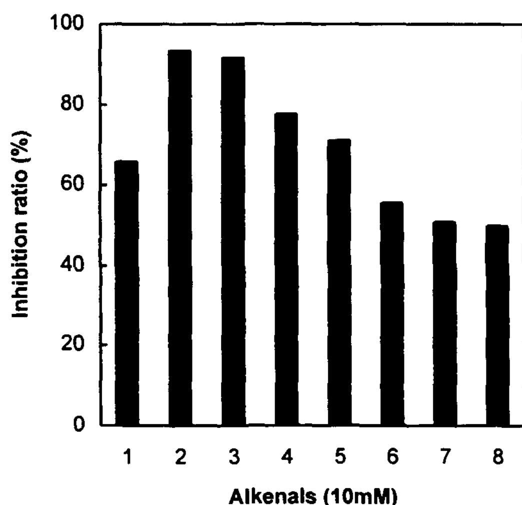

Fig. 4. The inhibitory effect of alkenals (10 mM) to the ATP hydrolysis by the plasma membrane H^+-ATPase of *S. cerevisiae.*

The amount of enzyme used (specific activity 8.9 μmole/min/mg protein) was 0.029 U (μM P_i/min), and the inhibition ratio (%) was calculated as follows: $[1 - (OD_{660\ nm})_{inhibitor}/(OD_{660\ nm})_{inhibitor\ free}] \times 100$. Alkenals: 1, (*E*)-2-hexenal; 2, (*E*)-2-heptenal; 3, (*E*)-2-octenal; 4, (*E*)-2-nonenal; 5, (*E*)-2-decenal; 6, (*E*)-2-undecenal; 7, (*E*)-2-dodecenal; 8, (*E*)-2-tridecenal.

DISCUSSION

This paper describes the effect of hydrophobic chains of alkenals on antifungal action against, and inhibitory action on, the plasma membrane H^+-ATPase of *S. cerevisiae.* With reagards to antifungal action, alkenals in the range of C_6 to C_{11} exhibited gradually increasing activity with chain length, but (*E*)-2-dodecenal, which has one more methyl group than (*E*)-2-undecenal, was completely inactive (cut off). Thus it can be noted that alkenals show the chain-length dependence on antifungal action including the "cut off" phenomenon, as seen with several biological actions of other aliphatic compounds [1, 12, 25].

As previously indicated, polygodial is structually characteristic of an aldehyde surfactant [2] and shows inhibitory activity to medium acidification. Alkenals were also identified as having such inhibitory activity, which may be expected based on common structural characteristics [8]. As in the case of antifungal activity, inhibition of medium acidification gradually increased with chain length and maximum inhibitory activity was shown in the same alkenal group except for (*E*)-2-nonenal. However, the activities of (*E*)-2-dodecenal and (*E*)-2-tridecenal were not completely cut off; they were just reduced relative to (*E*)-2-undecenal. It is interesting to note that the inhibitory effects of (*E*)-2-undecenal and (*E*)-2-dodecenal over time were significantly different. Namely, the inhibitory effect of (*E*)-2-undecenal was gradually increased, whereas (*E*)-2-dodecenal's activity

was gradually reversed with time. In a previous work, polygodial with complete inhibitory activity (>90%) exhibited strong antifungal activity, but diethylstilbestrol with maximum inhibition below 60% did not show any antifungal activity. This indicates that the maximum inhibitory activity of plasma membrane H^+-ATPase inhibitors is very important for antifungal activity. Thus, although (*E*)-2-dodecenal shows inhibitory activity on medium acidification, it may be inactive on antifungal action. This is because (*E*)-2-dodecenal did not show the complete and continuous inhibition of the medium acidification.

Alkenals were investigated for their inhibitory action on ATP hydrolysis, as a possible explanation for the inhibition of medium acidification by plasma membrane H^+-ATPase. Inhibitory activity of ATP hydrolysis was found to be chain dependent, but was considerably different from the inhibition of medium acidification. Namely, maximum activity was not consistent and alkenals in the range of C_6-C_{13} gradually exhibited decreasing activity with chain length. Therefore, it can not be concluded that the inhibitory activity of alkenals on medium acidification is directly caused by the inhibition of ATP hydrolysis.

In summary, alkenals in the range C_6 to C_{11} exhibited increasing antifungal activity with chain length, but the activity was cut off at (*E*)-2-dodecenal and (*E*)-2-tridecenal. Alkenals also exhibited inhibitory activity to medium acidification, and the chain dependence was similar to that of antifungal activity. Thus, it can be proposed that antifungal activity of alkenals is due to the action which inhibits the medium acidification of plasma membrane H^+-ATPase. However, it is likely that the alkenal's inhibition of medium acidification is not due to the inhibition of ATP hydrolysis. Thus, the inhibitory mechanism of alkenals on medium acidification (*in vivo* activity) by the plasma membrane H^+-ATPase [3] remains to be solved.

REFERENCES

1. Alifimoff, J. K., L. L. Firestone, and K. W. Miller. 1989. Anesthetic potencies of primary alkanols: Implications for the molecular dimensions of the anesthetic site. *Br. J. Pharmacol.* **96:** 9–16.
2. Barnes, C. S. and J. W. Loder. 1962. The structure of polygodial: A new sesquiterpene dialdehyde from *Polygonum hydropiper* L. *Aust. J. Chem.* **15:** 322–327.
3. Coote, P. J., M. V. Jones, I. J. Seymour, D. L. Rowe, D. P. Ferdinando, A. J. McArthur, and M. B. Cole. 1994. Activity of the plasma membrane H^+-ATPase is a key physiological determinant of thermotolerance in *Saccharomyces cerevisiae. Microbiol.* **140:** 1881–1890.
4. Eddy, A. A. 1982. Mechanisms of solute transport in selected eukaryotic microorganisms. *Adv. Microb. Physiol.* **23:** 1–78.
5. Haworth, R. S., E. J. Cragoe, Jr., and L. Fliegel. 1993. Amiloride and 5-(*N*-ethyl-*N*-isopropyl) amiloride inhibit medium acidification and glucose metabolism by the fission yeast *Schizosaccharomyces pombe. Biochim. Biophys. Acta.* **1145:** 266–272.
6. Himejima, M. and I. Kubo. 1992. Antimicrobial agents from *Licaria puchuri-major* and their synergistic effect with polygodial. *J. Nat. Prod.* **55:** 620–625.
7. Himejima, M. and I. Kubo. 1993. Fungicidal activity of polygodial in combination with anethole and indole against *Candida albicans. J. Agric. Food Chem.* **41:** 1776–1779.
8. Kubo, A. and I. Kubo. 1995. Antimicrobial agents from *Tanacetum balsamita. J. Nat. Prod.* **58:** 1565–1569.
9. Kubo, I. 1995. Antifungal sesquiterpene dialdehydes from the *Warburgia* plants and their synergists, pp. 233–249. *In* Atta-ur-Rahman (ed.), *Studies in Natural Products Chemistry: Structure and Chemistry*, vol. 17. Elsevier Science, Amsterdam, New York.
10. Kubo, I. and M. Himejima. 1991. Anethole, a synergist of polygodial against filamentous microorganisms. *J. Agric. Food Chem.* **39:** 2290–2292.
11. Kubo, I. and M. Himejima. 1992. Potentiation of antifungal activity of sesquiterpene dialdehydes against *Candida albicans* and two other microorganisms. *Experientia.* **48:** 1162–1164.
12. Kubo, I., H. Muroi, and A. Kubo. 1995. Structural functions of antimicrobial long-chain alcohols and phenols. *Bioorg. Med. Chem.* **3(7):** 873–880.
13. Kubo, I. and M. Taniguchi. 1988. Polygodial, an antifungal potentiator. *J. Nat. Prod.* **51:** 22–29.
14. Lanzetta, P. A., L. J. Alvarez, P. S. Reinach, and O. A. Candia. 1979. An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* **100:** 95–97.
15. Malpartida, F. and R. Serrano. 1981. Phosphorylated intermediate of the ATPase from the plasma membrane of yeast. *Eur. J. Biochem.* **116(2):** 413–417.
16. Malpartida, F. and R. Serrano. 1981. Proton translocation catalysed by the purified yeast plasma membrane ATPase reconstituted in liposomes. *FEBS Lett.* **131:** 351–354.
17. McCallion, R. F., A. L. J. Cole, J. R. L. Walker, J. W. Blunt, and M. H. G. Munro. 1982. Antibiotic substances from New Zealand plants. II. Polygodial, an anti-*Candida* agent from *Pseudowintera colorata. Planta Med.* **44:** 134–138.
18. McCusker, J. H., D. S. Perlin, and J. E. Haber. 1987. Pleiotrophic plasma membrane ATPase mutations of *Saccharomyces cerevisiae. Mol. Cell. Biol.* **7:** 4082–4088.
19. Menendez, A., C. Larsson, and U. Ugalde. 1995. Purification of functionally sealed cytoplasmic side-out plasma membrane vesicles from *Saccharomyces cerevisiae. Anal. Biochem.* **230(2):** 308–314.
20. Monk, B. C., A. B. Mason, T. B. Kardos, and D. S. Perlin. 1995. Targeting the fungal plasma membrane proton pump. *Acta Biochim. Pol.* **42(4):** 481–496.

21. Monk, B. C., A. B. Mason, G.Abramochkin, J. E. Haber, D. Seto-Young, and D. S. Perlin. 1995. The yeast plasma membrane proton pumping ATPase is a viable antifungal target. I. Effects of the cysteine-modifying reagent omeprazole. *Biochim. Biophys. Acta.* **1239:** 81–90.
22. Ohsuka, A. 1963. The structure of tadeonal and isotadeonal components *of Polygonum hydropiper* L. *Nippon Kagaku Zasshi.* **86:** 748–752.
23. Serrano, R. 1978. Characterization of the plasma membrane ATPase of *Saccharomyces cerevisiae. Mol. Cell. Biochem.* **22:** 51–63.
24. Serrano, R. 1984. Plasma membrane ATPase of fungi and plants as a novel type of proton pump. *Curr. Top. Cell. Regul.* **23:** 87–126.
25. Schultz, T. W., L. M. Arnold, T. S. Wilke, and M. P. Moulton. 1990. Relationships of quantitative structure-activity for normal aliphatic alcohols. *Ecotoxicol. Environ. Saf.* **19(3):** 247–253.
26. Taniguchi, M. and I. Kubo. 1993. Ethnobotanical drug discovery based on medicine men's trials in the African savanna: Screening of East African plants for antimicrobial activity II. *J. Nat. Prod.* **56:** 1539–1546.
27. Yano, Y., T. Makoto, T. Toshio, O. Susumu, and I. Kubo. 1991. Protective effects of Ca^{2+} on membrane damage by polygodial in *Saccharomyces cerevisiae. Agric. Biol. Chem.* **55(2):** 603–604.