Vacuole Disruption as the Primary Fungicidal Mechanism of Action of Polygodial, a Sesquiterpene Dialdehyde

Takuya Kondo[1], **Yusuke Takaochi**[1], **Yoshihiro Yamaguchi**[1,2], **Akira Ogita**[1,3], **Ken-ichi Fujita**[1], **Toshio Tanaka**[1]

[1] Graduate School of Science, Osaka City University, Osaka, Japan

[2] OCU Advanced Research Institute for Natural Science and Technology, Osaka City University, Osaka, Japan

[3] Research Center for Urban Health and Sports, Osaka City University, Osaka, Japan

Abstract

▼

The fungicidal mechanism of action of polygodial was examined in malt extract medium in which lethality against *Saccharomyces cerevisiae* was achieved at a much lower dose than in glucose-containing nutrient medium. Vacuolar membrane disruption, rather than cellular oxidative stress induction and damage to the plasma membrane, was considered the most likely lethal event underlying these observations. Vacuolar membrane disruption and polygodial-mediated lethality were similarly observed against the pathogenic fungi *Candida albicans* and *Aspergillus niger*.

Key words

polygodial · fungicidal activity · vacuole · *Saccharomyces cerevisiae*

The fungicidal activity of amphotericin B (AmB; ◐ **Fig. 1 A**), a polyene macrolide, is attributed mainly to its ability to form a molecular complex with plasma membrane-embedded ergosterol [1]. This complex functions as an ion channel, enhancing the leakage of intracellular K⁺ and other ionic substances. AmB-mediated lethality is alternatively attributed to the induction of oxidative stress via the enhanced generation of superoxide anions by this macrolide, as well as to its ability to extract ergosterol from the plasma membrane phospholipid bilayer [2,3]. In previous studies, for the first time, we found that AmB is incorporated across the plasma membrane of fungal cells, resulting in vacuolar membrane disruption with lethal effects [4–6]. Such vacuole-targeting fungicidal activity is also exerted by niphimycin from *Streptomyces* sp., as well as by polymyxin B from *Bacillus polymyxa*, with both selective and effective lethal effects against fungal cells [4]. To date, however, vacuole-disrupting activity has been reported only for microbial metabolites, not for plant-derived compounds.

Polygodial (◐ **Fig. 1 B**), a bicyclic sesquiterpene dialdehyde, was first isolated as a pungent principle from the sprout of *Polygonum hydropiper* L. (Polygonaceae), known as "tade", which is used as a food spice in Japan [7,8]. The biological activity of polygodial has been reported to include antifungal effects [9–12]. The biological mechanism of action of polygodial involves structural and functional damage to the plasma membrane, which is accompanied by the leakage of intracellular components. Polygodial-induced plasma membrane damage is attributed to the enhanced induction of cellular oxidative stress by this compound, as reflected by

a marked increase in the level of mitochondrial reactive oxygen species (ROS) production [13]. It is still not known whether polygodial-induced lethality is dependent on plasma membrane damage and ROS production.

In this study, we examined the fungicidal activity of polygodial under conditions in which cells were protected from oxidative stress and plasma membrane damage. Vacuole disruption was identified as the most likely mechanism by which polygodial exerts lethal effects against *Saccharomyces cerevisiae* and other pathogenic fungal strains.

We first examined the relationship between lethality and ROS production by polygodial treatment of *S. cerevisiae* cells in 2.5 % malt extract medium. As shown in ◐ **Fig. 2 A**, polygodial was not lethal to *S. cerevisiae* cells at a concentration of 2 µM; however, as is the case with AmB, this sesquiterpene dialdehyde was found to markedly reduce the viable cell number to around 0.1 % of the original level at 10 µM over the course of a 2-h incubation. The lethality of polygodial was far lower in nutrient-rich medium consisting of 2.0 % (w/v) glucose, 2.0 % (w/v) peptone, and 1.0 % (w/v) yeast extract (YPD); in the latter, the viable cell number was reduced to 15 % of the original level, even at a polygodial concentration of 100 µM, during 2 h of incubation [13].

In YPD medium, polygodial, at a concentration of 100 µM, reduced the viable cell number via an increase in mitochondrial ROS production in cells of the parent strain, but not in the respiration-deficient mutant with polygodial-resistant phenotype [13]. We therefore examined the level of ROS production in polygodial-treated cells in malt extract medium. In agreement with a previous finding, polygodial, at a concentration of 100 µM, was found to markedly increase the level of ROS production in the medium. The observed increase was comparable to that induced by an isoprenoid farnesol due to hyperpolarization of the mitochondrial transmembrane potential [14], as shown in ◐ **Fig. 2 B**. At a concentration of 10 µM, polygodial did not elicit an elevation in cellular ROS production levels; however, lethal effects were observed at this concentration, suggesting that polygodial exhibits lethal activity against fungal cells via modes of action other than the induction of oxidative stress.

We therefore examined the possibility that polygodial damages the vacuole, a recently identified fungicidal target, resulting in
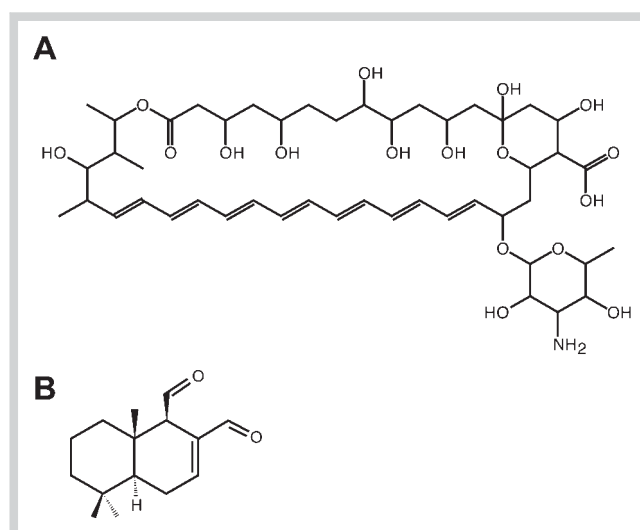

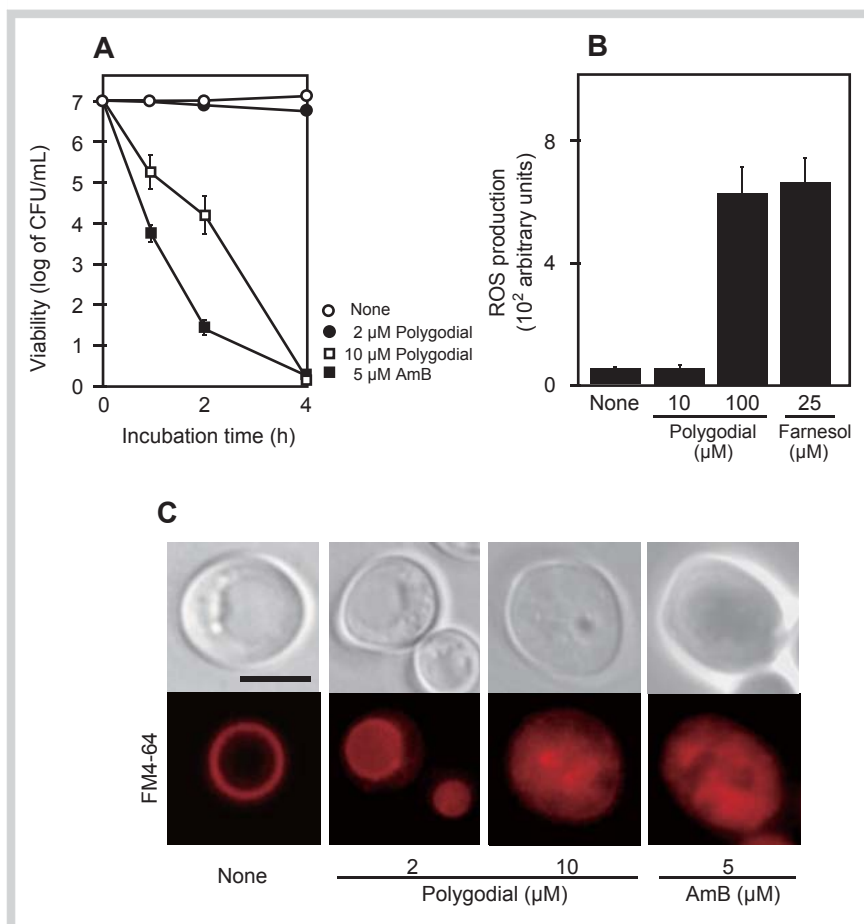**Fig. 1** Chemical structures of AmB (**A**) and polygodial (**B**).

Fig. 2 Effects of polygodial on cell viability, ROS production, and vacuole disruption. Cells were incubated in 2.5 % malt extract medium containing polygodial at the indicated concentration, at 30 °C, with vigorous shaking for measurement of the viable cell number (**A**) and cellular ROS production (**B**). Data are shown as means ± SD of triplicate experiments (**A, B**). Cells were additionally used for microscopic observation of vacuole morphology (**C**); bar, 2 µm. AmB (**A, C**) and farnesol (**B**) were used as positive controls. (Color figure available online only.)

the disruption of vacuolar membrane architecture. As shown in ○ **Fig. 2 C**, the yeast vacuole was observed to possess a swollen, rounded architecture in untreated cells. In addition, no structural alteration was observed in cells treated with polygodial at the nonlethal concentration of 2 µM. However, even under nonlethal conditions, the entire luminal space exhibited abnormal staining with the fluorescent dye FM4–64, suggesting the impairment of normal transport function across the vacuolar membrane (○ **Fig. 2 C**). In cells treated with polygodial at the lethal concentration of 10 µM, the dye was found to be scattered throughout the cytoplasm, in which normal vacuolar membrane architecture could not be observed clearly, reflecting the disruption of the vacuolar membrane into smaller fragments. The observed mode of vacuolar membrane disruption by polygodial appeared to be similar to that of AmB-treated cells (○ **Fig. 2 C**).

The simultaneous addition of K^+ and Mg^{2+} is known to protect *S. cerevisiae* cells against AmB lethality, suggesting their maintenance effects on vacuole membrane integrity in addition to the compensatory effect against intracellular K^+ leakage (see ○ **Fig. 3 - A, B**) [15,16]. The putative contribution of the vacuole membrane-disrupting effect to the fungicidal activity of polygodial was thus investigated by examining the protective effects of these ions against the loss of viability of polygodial-treated cells. As shown in ○ **Fig. 3 C, D**, polygodial was found to exert a marked lethal effect with serious vacuolar membrane disruptive damage similarly in the absence as well as in the presence of both ions. The result suggests that the vacuole-targeting action of polygodial contributed to cell death more significantly than that of AmB in the malt extract medium.

We finally examined whether polygodial elicits disruption of the vacuole membrane in the pathogenic fungal strains *Candida albicans* and *Aspergillus niger*. The vacuole was found to exhibit a swollen, spherical architecture in untreated *C. albicans* cells; however, the organelles were observed as small discrete dots in the cytoplasm of cells treated with polygodial at a lethal concentration (○ **Fig. 4 A, B**). Polygodial was also found to cause vacuole disruption in the filamentous cells of *A. niger* at the higher concentration of 150 µM (○ **Fig. 4 C**). This concentration is equivalent to the value at which fungal spore germination is inhibited (data not shown).

Vacuoles are organelles that are involved in osmoregulation, ion and pH homeostasis, metabolite accumulation, and macromolecular degradation in fungal cells [17]. Vacuoles are additionally involved in autophagic processes required for the degradation of cytoplasmic components as well as the cellular stress response [18, 19]. Therefore, structural and functional damage to this organelle is considered to trigger cell death [4–6]. The antifungal property of polygodial has been attributed to its disruptive effects against the phospholipid bilayers of the plasma membrane, which is thought to occur via the induction of cellular oxidative stress. Our findings suggest that the vacuole-targeting fungicidal activity of polygodial may represent a novel mechanism of action of the compound, which is distinct from those previously reported. Our findings are additionally considered to be of potential therapeutic significance: the selective enhancement of the vacuole-targeting fungicidal activity of polygodial, via the structural modification of this compound, should enable its application in clinical settings.
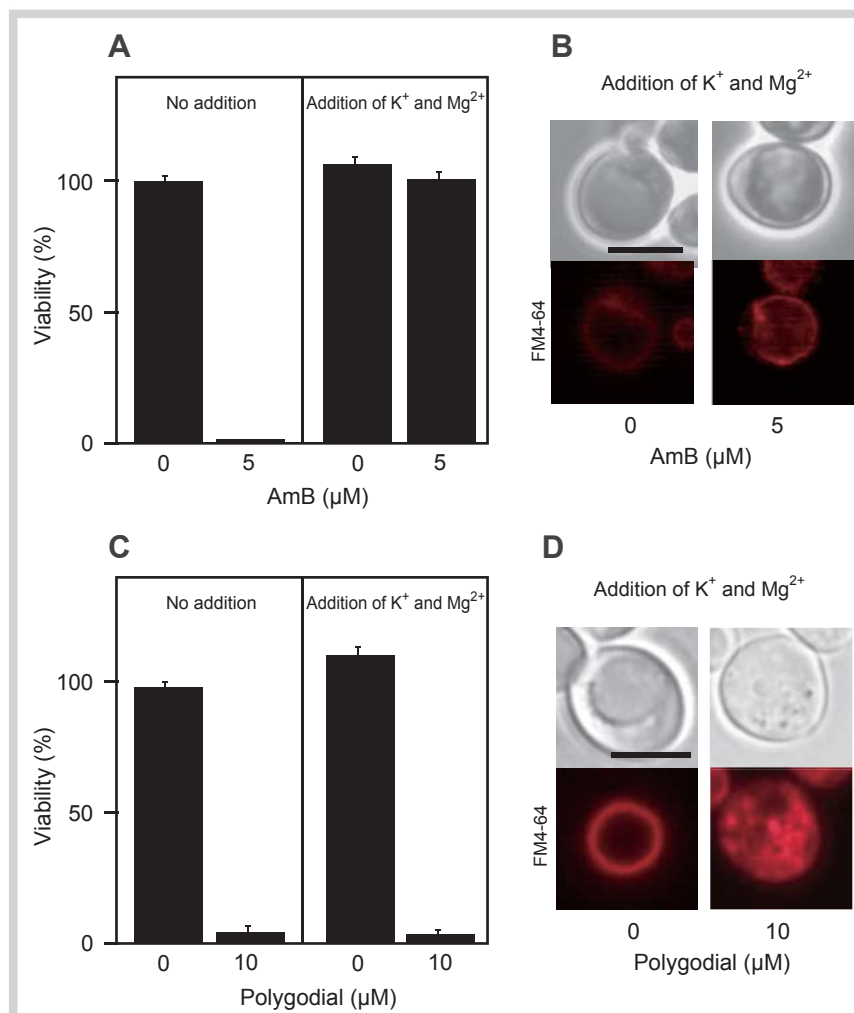
Fig. 3 Cell viability and vacuole morphology of *S. cerevisiae* cells in the absence and presence of 85 mM KCl and 45 mM MgCl₂. Cells were incubated in 2.5% malt extract medium containing AmB (**A, B**) or polygodial (**C, D**) at the indicated concentrations, at 30°C for 2 h, with vigorous shaking, for measurement of viable cell numbers (**A, C**) and microscopic observation of the vacuoles (**B, D**). Data are shown as means ± SD of triplicate experiments (**A, C**); bar, 2 µm (**B, D**). (Color figure available online only.)

Materials and Methods

▼

Measurement of cell growth and viability

S. cerevisiae W303-1A and *C. albicans* IFO 1061 cells were grown overnight in malt extract medium (2.5% malt extract, pH 5.0) at 30°C with vigorous shaking. Then, cells were inoculated to 1×10^7 cells/mL in freshly prepared malt extract medium (*S. cerevisiae*) or RPMI 1640 medium (*C. albicans*) containing polygodial or AmB at various concentrations. Cell suspensions were then incubated with vigorous shaking at 30°C, and plated on YPD medium containing 1.8% (w/v) agar. Cell viability was determined as the number of colonies formed after a 48-h incubation at 30°C.

Vacuole staining

Vacuoles were stained with the fluorescent probe FM4–64 [N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexa-trienyl)pyridinium dibromide] according to previously described methods [20,21], with some modifications, depending on the fungal strain used, as follows. In the case of *S. cerevisiae* and *C. albicans*, cells were grown overnight in malt extract medium and harvested by centrifugation. Cells were suspended in fresh malt extract medium to a density of 1×10^7 cells/mL and incubated with 8 µM FM4–64. After collection by centrifugation, FM4–64-stained cells were resuspended in fresh malt extract medium (*S. cerevisiae*) or RPMI 1640 medium (*C. albicans*) to obtain a density of 1×10^7 cells/mL. These cell suspensions were further incu-

bated in the absence or presence of polygodial, at the indicated concentrations, with vigorous shaking at 30°C for 2 h, and then washed and suspended in phosphate buffered saline (PBS). Cells were then observed under a phase-contrast microscope and fluorescence microscope with the excitation at 520–550 nm and emission at 580 nm. In the case of *A. niger* ATCC 6275, the mycelial suspension was directly prepared by inoculation from the agar slant into 2.5% malt extract medium for FM4–64-treatment of the fungal cells. The effect of polygodial on fungal vacuole morphology was examined by resuspending the mycelial cells with an equal volume of freshly prepared malt extract medium, followed by incubation at 30°C for 2 h. Polygodial-treated mycelial cells were washed and suspended with PBS. Cells were then observed by phase-contrast microscopy and fluorescence microscopy, as described above.

Assay of cellular ROS production

The effect of polygodial on cellular ROS production was assayed using a previously described method [22,23]. Cells from an overnight culture were inoculated into freshly prepared YPD medium to a density of approximately 1×10^7 cells/mL. Cells were then incubated with 40 µM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) at 30°C for 60 min, collected by centrifugation, and suspended in an equal volume of 2.5% malt extract medium. Cell suspensions were further treated with or without polygodial at various concentrations for 20 min, and then washed and resus-
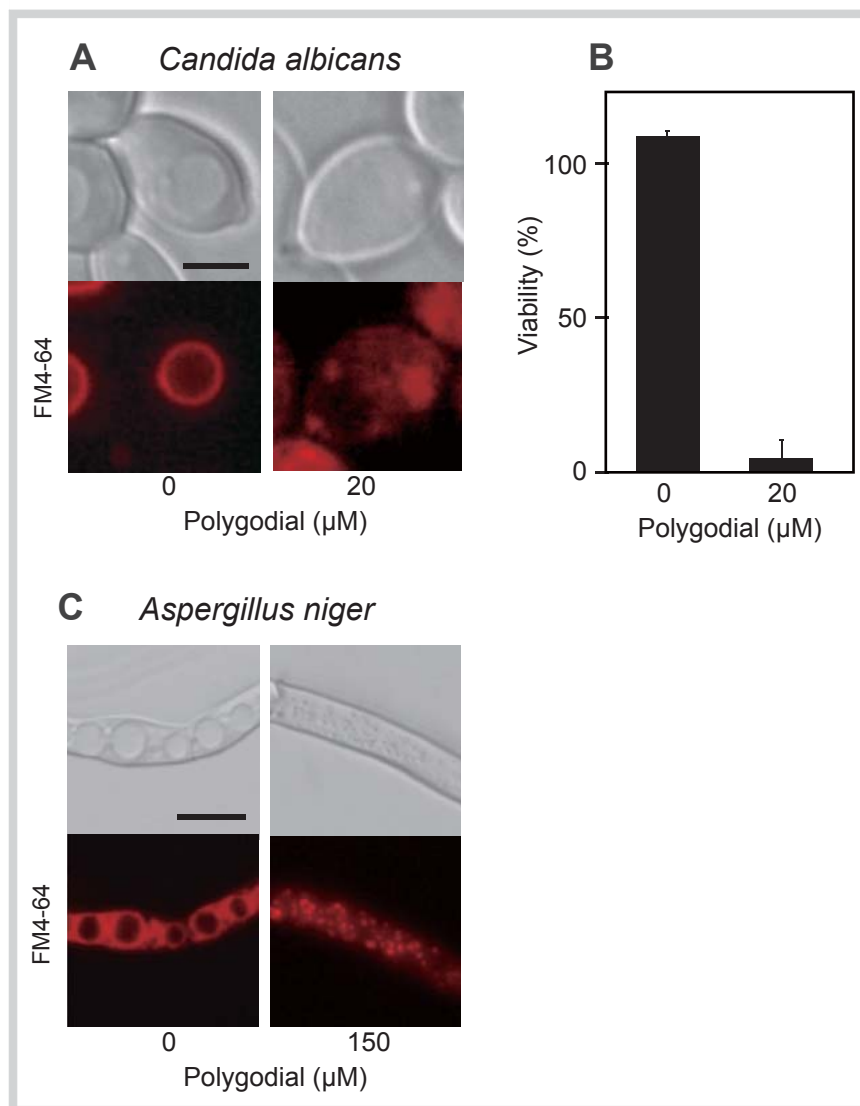
Fig. 4 Vacuole morphology (**A, C**) and viability (**B**) of *C. albicans* cells, and the mycelial cells of the filamentous fungus *A. niger*. In **A** and **B**, cells were incubated in RPMI 1640 medium containing polygodial at the indicated concentrations, at 30 °C for 2 h. In **C**, cells were incubated in 2.5 % malt extract medium containing polygodial at the indicated concentration, at 30 °C for 2 h. Data are represented as means ± SD of triplicate experiments (**B**); bars, 2 µm (**A, C**). (Color figure available online only.)

pended in 50 mM succinate buffer (pH 6.0). Fluorescence from the cells was then measured with the excitation at 485 nm and emission at 530 nm.

Chemicals

Polygodial (97 % purity) and farnesol (89 % purity) were purchased from Wako Pure Chemical Industries, Ltd. AmB (80 % purity) and FM4–64 was obtained from Sigma-Aldrich. DCFH-DA was purchased from Molecular Probes. All other reagents were of analytical grade. Malt extract medium was purchased from Oriental Yeasts Co.

Statistical analysis

Statistical evaluation was performed using Student's *t*-test; $p < 0.05$ was considered to represent statistical significance.

Acknowledgements

Conflict of Interest

▼
The authors declare no conflicts of interest.

References

1 *Carrillo-Muñoz AJ, Giusiano G, Ezkurra PA, Quindós G.* Antifungal agents: mode of action in yeast cells. Rev Esp Quimioter 2006; 19: 130–139
2 *Mesa-Arango AC, Trevijano-Conador N, Román E, Sánchez-Fresneda R, Casas C, Herrero E, Argüelles JC, Pla J, Cuenca-Estrella M, Zaragoza O.* The production of reactive oxygen species is a universal action mechanism of amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug. Antimicrob Agents Chemother 2014; 58: 6627–6638
3 *Anderson TM, Clay MC, Cioffi AG, Diaz KA, Hisao GS, Tuttle MD, Nieuwkoop AJ, Comellas G, Maryum N, Wang S, Uno BU, Wildeman EL, Gonen T, Rienstra CM, Burke MD.* Amphotericin B forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol 2014; 10: 400–406
4 *Ogita A, Fujita K, Tanaka T.* Enhancing effects on vacuole-targeting fungicidal activity of amphotericin B. Front Microbiol 2012; 3: 100
5 *Kang CK, Yamada K, Usuki Y, Ogita A, Fujita K, Tanaka T.* Visualization analysis of the vacuole-targeting fungicidal activity of amphotericin B against the parent strain and an ergosterol-less mutant of *Saccharomyces cerevisiae.* Microbiology 2013; 159: 939–947
6 *Yoshioka M, Yamada K, Yamaguchi Y, Ogita A, Fujita K, Tanaka T.* The fungicidal activity of amphotericin B requires autophagy-dependent

targeting to the vacuole under a nutrient-starved condition in *Saccharomyces cerevisiae.* Microbiology 2015; 162: 848–854

7 *Barnes C, Loder J.* The structure of polygodial, a new sesquiterpene dialdehyde from *Polygonum hydropiper* L. Aust J Chem 1962; 15: 322–327

8 *Ohsuka A.* [The structure of tadeonal and isotadeonal components of *Polygonum hydropiper* L]. Nippon kagaku zasshi 1963; 84: 748–752

9 *Anke H, Sterner O.* Comparison of the antimicrobial and cytotoxic activities of twenty unsaturated sesquiterpene dialdehydes from plants and mushrooms. Planta Med 1991; 57: 344–346

10 *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

11 *Lee SH, Lee JR, Lunde CS, Kubo I. In vitro* antifungal susceptibilities of *Candida albicans* and other fungal pathogens to polygodial, a sesquiterpene dialdehyde. Planta Med 1999; 165: 205–208

12 *Kubo I, Fujita K, Lee SH.* Antifungal mechanism of polygodial. J Agric Food Chem 2001; 49: 1607–1611

13 *Machida K, Tanaka T, Taniguchi M.* Depletion of glutathione as a cause of the promotive effects of polygodial, a sesquiterpene on the production of reactive oxygen species in *Saccharomyces cerevisiae.* J Biosci Bioeng 1999; 88: 526–530

14 *Machida K, Tanaka T.* Farnesol-induced generation of reactive oxygen species dependent on mitochondrial transmembrane potential hyperpolarization mediated by F_0F_1-ATPase in yeast. FEBS Lett 1999; 26: 108–112

15 *Brajtburg J, Medoff G, Kobayashi GS, Elberg S.* Influence of extracellular K^+ or Mg^{2+} on the stages of the antifungal effects of amphotericin B and filipin. Antimicrob Agents Chemother 1980; 18: 593–597

16 *Ogita A, Yutani M, Fujita K, Tanaka T.* Dependence of vacuole disruption and independence of potassium ion efflux in fungicidal activity induced by combination of amphotericin B and allicin against *Saccharomyces cerevisiae.* J Antibiot (Tokyo) 2010; 63: 689–692

17 *Kilonsky DJ, Herman PK, Emr SD.* Fungal vacuole: composition, function, and biogenesis. Microbiol Rev 1990; 54: 266–292

18 *Reggiori F, Klionsky DJ.* Autophagic processes in yeast: mechanism, machinery and regulation. Genetics 2013; 19: 341–361

19 *Stauffer B, Powers T.* Targets of rapamycin signaling mediates vacuolar fission caused by endoplasmic reticulum stress in *Saccharomyces cerevisiae.* Mol Biol Cell 2015; 26: 4618–4630

20 *Vida TA, Emr SD.* A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J Cell Biol 1995; 128: 779–792

21 *Ogita A, Matsumoto K, Fujita K, Usuki Y, Tanaka T.* Synergistic fungicidal activities of amphotericin B and *N*-methyl-*N*''-dodecylguanidine: a constituent of polyol macrolide antibiotic niphimycin. J Antibiot (Tokyo) 2007; 60: 27–35

22 *Machida K, Tanaka T, Fujita K, Taniguchi M.* Farnesol-induced generation of reactive oxygen species via indirect inhibition of mitochondrial electron transport chain in the yeast *Saccharomyces cerevisiae.* J Bacteriol 1998; 180: 4460–4465

23 *Ogita A, Hirooka K, Yamamoto Y, Tsutsui N, Fujita K, Taniguchi M, Tanaka T.* Synergistic fungicidal activity of Cu^{2+} and allicin, an allyl sulfur compound from garlic, and its relation to the role of alkyl hydroperoxide reductase 1 as a cell surface defense in *Saccharomyces cerevisiae.* Toxicology 2005; 215: 205–213

Correspondence
Prof. Dr. Toshio Tanaka
Graduate School of Science
Osaka City University
3–3–138 Sugimoto, Sumiyoshi-ku
Osaka 558–8585
Japan
Phone: + 81 6 66 05 31 63
Fax: + 81 6 66 05 31 64
tanakato@sci.osaka-cu.ac.jp

License terms