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Multifunctional action of antifungal polygodial against *Saccharomyces cerevisiae*: Involvement of pyrrole formation on cell surface in antifungal action

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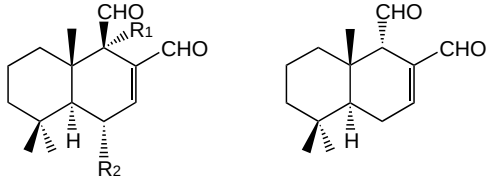
In honour of Professor Koji Nakanishi's 80th birthday

Abstract—The antifungal activity of polygodial against *Saccharomyces cerevisiae* involves multifunctions. Polygodial first acts as a surface-active agent (surfactant) and then becomes involved in biochemical processes. The ability to form a pyrrole derivative with a primary amine group of phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the outer monolayer of the plasma membrane is likely, in part, an initial step in the antifungal action of polygodial. In the lipid fraction derived from cells treated with polygodial, no PE and PS were detected, indicating a disturbance in the balance of the plasma membrane. The primary antifungal action of polygodial comes from its ability to act as a surfactant that nonspecifically disrupts the lipid–protein interface of integral proteins, denaturing their functioned conformation. Once polygodial enters the cytoplasm by destroying the membrane barrier, it reacts with L-cystein-containing cytoplasmic materials, such as a small molecule, glutathione, and a protein, alcohol dehydrogenase, to potentiate the antifungal action.

1. Introduction

A bicyclic sesquiterpene dialdehyde, polygodial (**1**) (see Fig. 1 for its structure), was first isolated as a pungent compound from the sprout of *Polygonum hydropiper* L (Polygonaceae), also known as 'tade,' which is used as spice in Japan. In addition, isotadeonal (**2**) was simultaneously isolated from the same source in minute amounts.[1] The difference between these dialdehydes is only in terms of the orientation (configuration) of the aldehyde group at C-9, though they show markedly different biological activities. For example, polygodial shows a potent fungicidal activity, particularly against yeasts, such as *Candida albicans* and *Saccharomyces cerevisiae*,[2] whereas isotadeonal (also known as epipolygodial) does not exhibit any antifungal activity.[3] In addition, polygodial induces membrane damage in human neuroblastoma cells, while isotadeonal does not.[4] The discrepancy in these observations remains to be clarified.



R1=R2=H, Polygodial (**1**)
R1=OH, R2=H, Warburganal (**3**)
R1=R2=OH, Mukaadial (**4**)

Isotadeonal (**2**)
(Epipolygodial)

Figure 1. Chemical structures of polygodial and related compounds.

Opportunistic fungal infections in humans have become serious and increasingly common problems due to the advent of broad-spectrum antibiotics. Only a relatively few drugs are available for the treatment of systemic fungal diseases, necessitating a greater need for effective antifungal agents. On account of the increase in drug resistance and prevalence of opportunistic infections, there is a need for using effective antifungal agents with

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new modes of action.[5] Antifungal agents that primarily act as surfactants have the potential to answer this requirement, since they target extracytoplasmic regions without entering fungal cells, thereby avoiding most cellular-pump-based resistance mechanisms. Primary discussion in this paper is centered on *S. cerevisiae*, but the same surfactant concept can be applied to design antifungal agents against *C. albicans*. In fact, polygodial is now available as a commercial anti-*Candida* agent. The potent antifungal activity of polygodial has recently been described to result from its multiple functions,[6] but the primary antifungal action comes from the ability of polygodial to act as a nonionic surface-active agent (surfactant). This surfactant nonspecifically disrupts the lipid–protein interface of integral proteins thereby denaturing their functional conformation. However, the primary binding site of polygodial on the plasma membrane has not yet been established.

2. Results and discussion

During studies on the mode of the antifungal action of polygodial, we found that this sesquiterpene dialdehyde loses its antifungal activity in certain media. The result seems to provide a clue as to the location of the binding site of polygodial. So a further study was conducted to gain new insights into its antifungal action. Polygodial and two aliphatic aldehydes, (2*E*)-undecenal and undecanal, were tested for their antifungal activities against *S. cerevisiae* in three different media for comparison. As shown in Table 1, polygodial lost its antifungal activity in YPD medium, while both aliphatic aldehydes did not. The result indicates that any constituents in YPD medium probably react with the highly reactive dialdehyde moieties of polygodial and inactivate it, but not (2*E*)-alkenals. Amino acids including L-lysine are abundant in YPD medium. So various amine compounds were tested to determine if they also similarly inactivate the antifungal activity of polygodial. This postulate was confirmed by the observation that *p*-aminobenzoic acid in a minimum synthetic medium[7] also suppresses the activity of polygodial, as does ethanolamine. On the basis of the results obtained, it may be reasonable to presume that the binding sites of polygodial are, at least in part, primary amine groups in living systems.

Various lipids are the major components of plasma membranes that form continuous barriers between the outside environment and the cytoplasm. Among them, both phosphatidylethanolamine (PE) and phosphatidylserine (PS) contain a primary amine group. Polygodial may form a pyrrole with the amine groups of PE and PS in the outer leaflet of the plasma membrane, thereby disturbing the balance of the plasma membrane. This effect may be related, in part, to the antifungal action of polygodial. Although phosphatidylcholine (PC) at 70 μM did not affect the MIC of polygodial, both PE and PS at 9 μM (close to the MIC of polygodial) increased the MIC by 2-fold (Table 2), showing the inactivation of the antifungal activity. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) is used as a specific reagent for the detection of primary amines including mainly PE and PS on the cell surface since this acid cannot penetrate inside the cell. [8] TNBS itself showed a weak fungicidal activity under acidic conditions (data not shown), indicating the reaction between the sulfonic acid and a primary amine on the cell surface under such conditions. The fungicidal effect of polygodial was also measured using yeast cells pretreated with TNBS. In the pretreated cells, to impart the same fungicidal effect, a 2-fold concentration of polygodial was needed. In the cells surface-coated by TNBS, polygodial was found to hardly attack the cell surface that was partly protected by TNBS. The results obtained indicate that polygodial forms the corresponding pyrroles with primary amines mainly related to PE and PS in the outer monolayer, but not with PC. This is consistent with a previous report that polygodial forms a pyrrole derivative with compounds possessing a primary amine group.[9] It appears that the antifungal actions of polygodial differ to some extent from those of (2*E*)-alkenals since these alkenals cannot form a pyrrole but still exhibit antifungal activity. It should be noted that polygodial forms a pyrrole with a primary amine in the outer monolayer, although PE and PS are mainly located in the inner monolayer of eukaryotes. Once a pyrrole is formed, the hydrophobic decalin portion may enter the lipid

Table 1. Antifungal activity (MIC, μg/mL) of aldehydes against *Saccharomyces cerevisiae* in different media

Aldehyde tested	ME	RPMI1640	YPD
Polygodial	1.56	1.56	>100
(2*E*)-Undecenal	6.25	6.25	12.5
Undecanal	25	25	50

The yeast cells were incubated in ME, RPMI1640, and YPD broth with each drug at 30 °C for 48 h. After incubation, MFC was estimated as described in Section 4.

Table 2. Inactivation of antifungal activity of polygodial by amine-related compounds

Compound	IC[a] (μM)
L-Glycine	31
L-Arginine	16
L-Lysine	8
L-Asparagine	62
Thiamine	>500
p-Aminobeozoic acid	45
Ethanolamine[b]	25
PC[c]	>70
PE[c]	9
PS[c]	9

[a] Each chemical was mixed with polygodial in DMF:50 mM MOPS buffer (pH 7.0) (1:1, v/v), except for PC, PE, and PS (see footnote 'c' of this table), and then incubated for 24 h at 30 °C. After preincubation, the MIC was determined using the mixed solution. Each inactivation concentration (IC) was calculated to double MIC of polygodial against *S. cerevisiae*.

[b] Ethanolamine was also incubated with polygodial using 50 mM MOPS buffer (pHs 3, 5, 7, and 9).

[c] PC (dipalmitoyl-DL-α-PC) was dissolved in tetrahydrofuran/ethanol (1:1, v/v). PE (dipalmitoyl-DL-α-PE) and PS (dipalmitoyl-DL-α-PS) were dissolved in the above-mentioned solvent with 1/400 vol of 10 N NaOH.

bilayer and alter the fluidity of the plasma membrane or disrupt the balance, thereby facilitating pyrrole formation of the primary amine in the inner monolayer and then causing fungal lysis and the subsequent fatal loss of intercellular materials. This has been corroborated by previous reports of the decrease in the fluidity of plasma membrane,[10] the leakage of cellular components,[11] and the disruption of membrane-bound enzymes, such as H^+-ATPase (P-type).[12] The difference in the ability to form a pyrrole may reveal why polygodial induces the leakage of 260 nm-absorbing materials from *S. cerevisiae* cells, while (2*E*)-alkenals do not (data not shown).

In contrast to the potent antifungal activity of polygodial, its congeners, isotadeonal (**3**) and mukaadial (**4**), did not exhibit any activity up to 400 μg/mL. Isotadeonal was previously reported not to form a pyrrole [9] but react with L-cystein.[3,13,14] In the current experiment, mukaadial showed a weak fungistatic activity (>200 μg/mL) up to 24-h incubation, but this activity disappeared after 48-h incubation. It appears that mukaadial did not form the corresponding pyrrole. We also tested the potency of the binding of polygodial or mukaadial to the cell surface of *S. cerevisiae*. After the cells were pretreated with each drug and then washed with a buffer, they were treated with TNBS. Polygodial apparently prevented the cells from being surface-coated with TNBS, as shown in Figure 2. On the other hand, mukaadial did not affect the coating. This result suggests that polygodial binds to the cell surface, whereas mukaadial does not. The above findings are consistent with the observations that mukaadial neither induced the leakage of cellular components nor inhibited glucose-induced acidification (data not shown).

To investigate further the effect of the reactivity of polygodial toward primary amines on its antifungal activity, we prepared the fluorescamine-coated spheroplasts of *S. cerevisiae*. This reagent with no fluorescence reacts directly with primary amines to form the same fluorophors. As it is difficult to penetrate a plasma membrane on account of its hydrophilicity, it is useful to estimate the abundance of primary amines in the outer leaflet of plasma membrane. As shown in Figure 3, the polygodial-treated cells reduced the arbitrary units of fluorescamine-derived fluorescence dose-dependently and the reduction was 86% of the DMF control when treated at 3.13 μg/mL. This result indicates the high possibility of the reactivity of polygodial to primary amines. In addition, the viabilities of the yeast cells treated with 1.56 and 3.13 μg/mL polygodial were 81% and 0.8% of control after 60-min treatment, respectively, as shown in Figure 4. The treatment with at least 3.13 μg/mL polygodial was needed for an antifungal activity. Although the 1.56 μg/mL-treatment did not significantly show any antifungal activity, a reduction in the arbitrary units of fluorescamine-derived fluorescence was observed. Therefore, the antifungal activity of polygodial was thought to be associated with other factors, except, the reactivity of this aldehyde to primary amines.

On the other hand, warburganal (**3**), which can react with L-cystein,[13] has α- and β-unsaturated dialdehydes, as well as polygodial. However, its reactivity with primary amines seems to be weaker than that of polygodial on account of steric hindrance due to it having an extra hydroxyl group at the C-4 position, similar to mukaadial. Interestingly, the rate of its reduction was lower than that of polygodial and not dose-dependent but constant as shown in Figure 3. Its antifungal activity was also slightly weaker than that of polygodial (Fig. 4). Therefore, these results suggest that a slight difference in antifungal potency between polygodial and warburganal depends on the presence of a hydroxyl group and

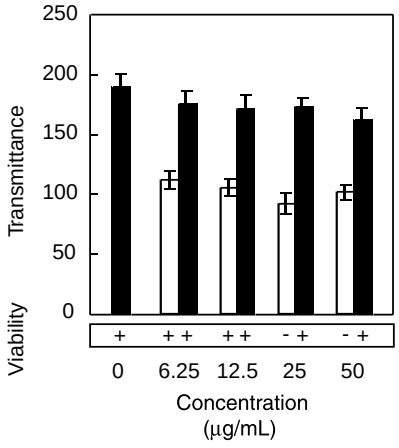

Figure 2. Protection from cell surface coating of TNBS by polygodial and mukaadial. Yeast cells (10^8 cells/mL) were preincubated with polygodial (open) or mukaadial (closed) in 50 mM MOPS buffer (pH 6.0). After preincubation, the washed cells were subjected to TNBS treatment and viability was estimated; 30 μL of the cell suspension collected after incubation was transferred to a fresh ME medium. The culture was incubated at 30 °C for 48 h prior to checking the viability. +, Growth; −, no growth. Intensity of transmittance was directly proportional to the increase in potency of the cell surface coating of TNBS. Data are presented as means ± SD ($n = 3$).

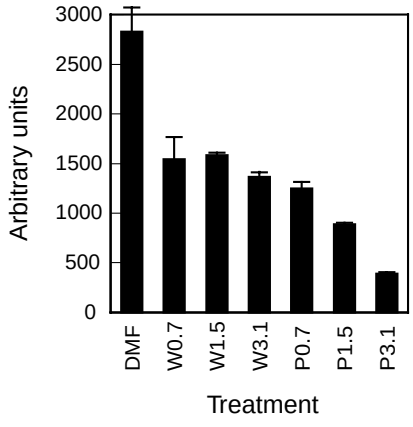

Figure 3. Fluorescamine labeling of outer leaflet of plasma membrane in spheroplasts of *Saccharomyces cerevisiae*. Yeast cells were incubated at 30 °C for 60 min in SD broth with or without the drug indicated. After incubation, spheroplasts were prepared and fluorescamine labeling was then performed. The arbitrary units were based directly on the fluorescence intensity of the fluorophors formed from the reaction of fluoroscamine with primary amines on the cell surface. DMF (control); W0.7, W1.5, and W3.1, (warburganal: 0.7, 1.5, and 3.1 μg/ml); P0.7, P1.5, and P3.1 (polygodial: 0.7, 1.5, and 3.1 μg/ml). Data are presented as means ± SD ($n = 3$).

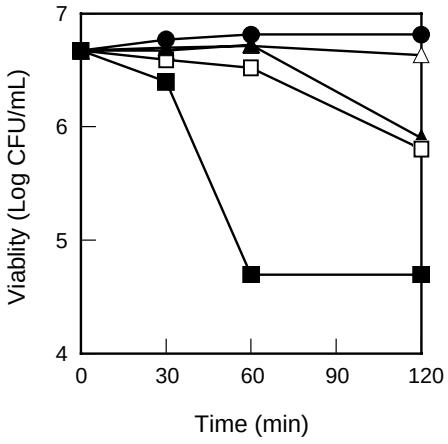

Figure 4. Effects of polygodial and warburganal on cell viability of *S. cerevisiae*. Exponentially growing *S. cerevisiae* cells were incubated at 30 °C without shaking in SD broth with or without the drug indicated. No drug added (•); polygodial: 1.56 (▲) and 3.13 (B) μg/mL; warburganal: 1.56 (△) and 3.13 (□) μg/mL.

the difference in their reactivity toward primary amines. On the other hand, the activity of mukaadial seemed to decrease on account of two additional hydroxyl groups, with these groups in mukaadial probably impeding pyrrole formation (Fig. 2).

In previous reports, polygodial and warbarganal were described to promote the leakage of both K^+ ions and 260-nm absorbing materials from *S. cerevisiae* cells. In contrast, both isotadeonal and mukaadial did not promote the leakage at all.[15] In contrast to polygodial, (2*E*)-alkenals did not promote the leakage (data not shown), but still exhibited an antifungal activity. The potency of antifungal activity increased with each additional CH_2 group up to (2*E*)-undecenal, suggesting that the activity can be enhanced by increasing the volume of the hydrophobic portion. Namely, a more bulky hydrophobic group may form large pores and increase the activity. From the data obtained, the hydrophilic aldehyde group can be replaced by any hydrophilic group as long as the 'head and tail' structure similar to that of a surfactant is balanced. This concept is also applied to the case of a polygodial structure. In mukaadial, two additional hydroxyl groups position on opposite sites within the molecule indicating a nonsurfactant-like structure, indicating the importance of a surfactant-like structure in addition to pyrrole formation for the antifungal activity. This prompted us to search for novel antifungal surfactants by synthetic optimization. For example, nonyl gallate promotes the leakage of K^+ ions from *S. cerevisiae* cells, but not that of 260-nm absorbing materials.[16] The leakage of K^+ ions induced by nonyl gallate was observed within 30 min of treatment accompanied by significant loss of cell viability, suggesting that nonyl gallate quickly affects the plasma membrane of *S. cerevisiae* cells, forming rather smaller size pores than the case of polygodial. Introduction of branching or unsaturation into the hydrophobic group increases the solubility of the surfactant in water. Branched-chain surfactants are generally more soluble in water and show a lower viscosity in aqueous media

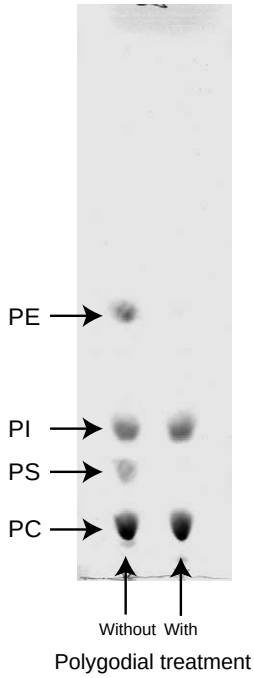

Figure 5. Effects of polygodial on phospholipid composition extracted from *Saccharomyces cerevisiae* cells. The TLC plate was developed in a solution of chloroform, methylacatate, 2-propanol, methanol, and 0.25% KCl (28:25:25:10:7, v/v).

than straight-chain materials.[17] The antifungal action of alkyl gallates is due to their surfactant properties and the binding of alkyl gallates can only involve relatively weak head group interactions, such as hydrogen bonding. It seems that the intermolecular hydrogen bonding alone is not sufficiently strong to form large pores. The ability to form a pyrrole derivative is probably specific to α- and β-unsaturated dialdehydes, polygodial, forming large pores in the plasma membrane.

If PE and PS in the membranes react with polygodial, the ratio of PE to PS to the total phospholipids should be affected. Therefore, we compared the phospholipid content between the cells treated with or without polygodial. Interestingly, in the lipid fraction derived from polygodial-treated cells for 2 h, no PE and PS were detected (Fig. 5). This result indicates the possibility that extraordinary phospholipids produced are deprived by phospholipases via unknown mechanisms. In addition, this result indirectly proves that polygodial reacts with PE and PS of the inner leaflet of the plasma membrane. The lack of PE and PS strongly supports the disruption of numerous membrane functions induced by polygodial.

3. Conclusion

The binding of polygodial to primary amines on a cell surface by itself cannot explain its antifungal mechanism but should be considered to be a large part of it. Polygodial may also enter the cells through pores derived from membrane damage by forming the aforementioned pyrrole. Similarly, polygodial acts, in part, as a nonionic surfactant, disrupting membrane functions.[6,12] It is also

conceivable that polygodial permeates across the plasma membrane by passive diffusion. Once inside the cells, polygodial may react with various intercellular components forming pyrroles with primary amines, for example, in PE and PS mainly located in the inner monolayer. More importantly, sulfhydryl groups in proteins and lower-molecular-weight compounds, such as glutathione, play an important role in living cells.[18] An α,β-unsaturated aldehyde group reacts with sulfhydryl groups mainly by 1,4-addition under physiological conditions.[18] Polygodial also inhibits alcoholic fermentation by inhibiting alcohol dehydrogenase competitively[14] and produces reactive oxygen species by depleting glutathione (GSH).[19] Many studies have defined the role of GSH as a cellular protectant against oxidative-stress-induced xenobiotics.[20] In addition, polygodial has recently been found to inhibit mitochondrial ATPase (F-type), but not the respiratory process.[21,22] *S. cerevisiae* is a facultative anaerobic organism that is able to survive without a functional respiratory chain. Maltose is the main carbon source in the malt extract broth used in this study, hence, *S. cerevisiae* cells acquire energy using glycolysis and grow anaerobically.[23] If *S. cerevisiae* were grown under fermentative conditions, the polygodial fungicidal activity cannot then be explained by the inhibition of mitochondrial ATPase as this inhibition does not affect the growth of *S. cerevisiae* cells. In summary, polygodial first acts as a surfactant and only then inhibits various cellular functions. As polygodial is thought to affect the various essential functions described above in humans, it is important to reduce its toxicity for therapeutic use by combining it with other chemicals, such as *trans*-cinnamic acid,[24] *trans*-anethole,[25,26] and EDTA,[27] which have been reported to show a synergistic effect.

4. Experimental

4.1. Chemicals

Mukaadial, polygodial, and warburganal[28] were available from our previous work. (2*E*)-Undecenal, undecanal, 2,4,6-trinitrobenzenesulfonic acid (TNBS), *p*-aminobenzoic acid, fluorescamine, dimethylsulfoxide (DMSO), dipalmitoyl-DL-α-phosphatidylcholine (PC), dipalmitoyl-DL-α-phosphatidylethanolamine (PE), dipalmitoyl-DL-α-phosphatidylserine (PS), and RPMI1640 were purchased from Sigma Chemical (St. Louis, MO). *N*,*N*-Dimethylformamide (DMF) was obtained from EM Science (Gibbstown, NJ). 3-(*N*-Morpholino) propanesulfonic acid (MOPS) was purchased from Fisher Biotech (FairLain, NJ).

4.2. Test strain

The test strains, *Saccharomyces cerevisiae* ATCC 7754, were purchased from the American Type Culture Collection (Manassas, VA).

4.3. Media

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

4.4. Antifungal assay

The maximum extent and rate of activity are known to vary with the seed culture media, physiological age of culture, and type of culture medium. All antifungal susceptibility tests in this study were performed under standard conditions 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 concentration (MIC) was determined, as previously described.[6] Briefly, serial 2-fold dilutions of the test compounds were prepared in DMF and 30 μL of 100 × concentrated solution was added to 3 mL ME media. They were then inoculated with 30 μL seed culture to give a 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 concentration (MFC) was examined as follows: After determining the MIC, a 30 μL of aliquot was taken from each clear tube and added into 3 mL of drug-free fresh medium. After 48-h incubation, the MFC was determined as the lowest concentration of the test compounds in which no recovery of microorganism was observed.

4.5. Inhibition of TNBS cell coating by polygodial

Precultured cells were washed twice with 50 mM MOPS buffer (pH 6.0). One milliliter of the washed cell suspension (10^8 cells/mL) was incubated in 50 mM MOPS buffer (pH 6.0) with or without polygodial and mukaadial at 30 °C for 24 h. After incubation, a 30 μL of aliquot was withdrawn from each culture and added into 3 mL of a drug-free ME broth to check for cell viability. The rest of the polygodial-treated cells were washed twice with 50 mM MOPS buffer (pH 6.0). The washed cells were incubated in 1 mL of 50 mM MOPS buffer (pH 6.0) with 0.5% TNBS at 30 °C for 24 h in the dark. The TNBS-coated cells were washed twice with 50 mM MOPS buffer (pH 6.0) and then once with 50 mM MOPS buffer (pH 9.0). The washed cells were collected in a 1.5 mL Eppendorf tube by centrifugation. To estimate the strength of yellow color derived from TNBS coated on the cell surface, digital images were taken as RGB image files by FinePix 1500 (Fuji film, Tokyo, Japan) under fluorescence light (2000 lx) and then analyzed as follows: A digital image was transferred to a CNYK image from the RGB one and the image containing only a yellow (Y) component was extracted from the CNYK image using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA). Transmissivity in the image was estimated using the public domain NIH image (developed at the U.S. National Institutes of Health and available

from the Internet by an anonymous FTP at zippy.nimh.nih.gov, Springfield, Virginia, Part Number PB95-500195GEI).

4.6. Fluorescamine labeling

For the labeling of total aminophospholipids of the outer leaflet of the plasma membrane in yeast cells, the fluorescamine labeling of spheroplasts was performed, as described below. Fluorescamine reacts directly with primary amines to form the same fluorophors.[29] As it is difficult to penetrate the plasma membrane on account of its hydrophilicity, it is useful to estimate the abundance of primary amines in the outer leaflet of the plasma membrane. Exponentially growing yeast cells were harvested by centrifuging at 2000*g* for 3 min, washed once with SD broth (1% glucose and Difco yeast nitrogen base without amino acids), and resuspended in the same broth with or without the drug indicated at 4.7×10^6 CFU/mL. The cell suspension was incubated at 30 °C without shaking for 60 min. After incubation, the cells were washed once with 20 mM potassium phosphate buffer (pH 7.4) containing 1.2 M sorbitol and then resuspended in the same buffer with 25 μg/mL zymolyase (KIRIN Brewery Co., Ltd.). After gentle swirling for 45 min at 30 °C, the spheroplasts were collected by centrifugation and washed twice with 50 mM potassium phosphate buffer (pH 9.0) containing 1.2 M sorbitol. The washed cells were collected in a 1.5 mL Eppendorf tube by centrifugation. The cell suspension was cooled to 10 °C by gentle swirling for 10 min in a water bath maintained at 10 °C. A solution of 0.8 M fluorescamine in dry DMSO was added dropwise with constant swirling over 30 s. The reaction was stopped by the addition of equal volumes of 1 M ammonia solution in 1.2 M sorbitol. The spheroplasts were washed with 100 μL of 20 mM potassium phosphate buffer (pH 9.0) containing 1.2 M sorbitol. To estimate the abundance of primary amines in the outer leaflet of the plasma membrane, the fluorescence intensity of the cell suspension was read with a Cytofluor 2300 fluorescence spectrophotometer (Millipore Co.) with excitation at 390 nm and emission at 475 nm. The arbitrary units were based directly on fluorescence intensity.

4.7. Effect of polygodial on cellular phospholipids

Exponentially growing cells were harvested by centrifugation, washed once with 50 mM succinate buffer (pH 6.0), and then resuspended in the same buffer to give a cell density of 10^7 cells/mL. The cell suspensions (200 mL) were incubated at 30 °C for 2 h with or without 25 μg/mL polygodial and then washed once with the same buffer. The cells were harvested and cellular lipids were then extracted according to the method of Bligh and Dyer.[30] An aliquot from each extract was spotted onto a silica gel plate (Silica Gel 60; Merck) and lipids were then separated in a solution of chloroform, methylacetate, 2-propanol, methanol, and 0.25% KCl (28:25:25:10:7, v/v). Phospholipids were detected using the Dittmer–Lester reagent.[31]

Acknowledgments

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