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Naturally Occurring Antifungal Agents and Their Modes of Action

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1. Introduction

Yeast fermentations are involved in the manufacturing of foods such as bread, beer, wines, vinegar, and surface ripened cheese. Most yeasts of industrial importance are of the genus *Saccharomyces* and mostly of the species *S. cerevisiae*. These ascospore-forming yeasts are readily bred for desired characteristics. However, yeasts are undesirable when they cause spoilage to sauerkraut, fruit juices, syrups, molasses, honey, jellies, meats, wine, beer, and other foods (Frazier and Westhoff, 1988). Finishing process of the fermentation is usually either through filtration or pasteurization. However, the use of the latter is limited to certain foods since it is a heat treatment and hence denaturalizes proteins, and the former is also limited to clear liquids. Neither process can be applicable to some foods such as sauerkraut and “miso” (soy bean pastes). *Zygosaccharomyces bailii*, is a food spoilage yeast species. It is known for its capacity to survive in stress environments and, in particular, in acid media with ethanol, such as in wine. In addition, spoilage of mayonnaise and salad dressing by this osmophilic yeast is well described. Therefore, safe and effective antifungal agents are still needed.

In our continuing search for naturally occurring antimicrobial agents, a bicyclic sesquiterpene dialdehyde, polygodial (**1**) (see Figure 1 for structures), was isolated from various plants (Kubo, 1995). This sesquiterpene dialdehyde exhibited potent antifungal activity particularly against yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* (Taniguchi et al., 1988), although it possessed little activity against bacteria (Kubo et al., 2005). Because of the potent antifungal activity, polygodial can be used as a leading compound to search for new antifungal drugs. This involves the study of their structure and antifungal activity relationships (SAR). However, the study of SAR required the synthesis of a series of analogues differing in the hydrophobic bicyclic portion, and because of this, polygodial may not be practical to use as a leading compound.

Subsequently, 2*E*-alkenals and alkanals were characterized from various edible plants such as the coriander *Coriander sativum* L. (Umbelliferae) (Kubo et al., 2004), the olive *Olea europaea* L. (Oleaceae) (Kubo et al., 1995a; Bisignano et al., 2001) and the cashew *Anacardium occidentale* (Anacardiaceae) (Muroi et al., 1993), and these aldehyde compounds exhibited broad antimicrobial activity (Table 1) (Kubo et al., 1995b). The maximum antimicrobial activity of 2*E*-alkenals is dependent on the balance of the hydrophobic alkyl (tail) chain length from the hydrophilic aldehyde group (head) (Kubo et al., 1995b and 2003a). The hydrophobicity of

molecules is often associated with biological action (Hansch and Dunn, 1972). However, the rationale for this observation, especially the role of the hydrophobic portion, is still poorly understood and widely debated. Although the antifungal action of polygodial may differ from those of the aliphatic aldehydes to some extent, 2*E*-alkenals with different chain lengths are a superior model for SAR study because these molecules possess the same hydrophilic portion, the enal group, which explains the role of the hydrophobic alkyl portion. In addition, a series of 2*E*-alkenals and their related analogues are common in many plants (Kim et al., 1995; Kubo and Kubo, 1995; Kubo et al., 1996 and 1999; Kubo and Fujita, 2001; Trombetta et al., 2002) and readily available. Therefore, a homologous series of aliphatic 2*E*-alkenals and the corresponding alkanals, from C₅ to C₁₃ were studied to gain new insights into their antifungal action on a molecular basis using *S. cerevisiae* ATCC 7754 as a model organism (Kubo et al., 2001a).

Fig. 1. α,β -Unsaturated aldehydes and related compounds

2. 2*E*-alkenals

The antimicrobial activity of a homologous series of 2*E*-alkenals characterized from plants has previously been reported (Kubo and Kubo, 1995; Kubo et al., 1995a; Bisignano et al., 2001) and is generally similar to being described for the corresponding alkanols (Kubo et al., 1995b). Their MIC and MFC values against *S. cerevisiae* are listed in Table 2. In general, the differences between the MIC and MFC values are not more than 2-fold, suggesting no residual fungistatic activity. As the carbon chain length increases the activity is increased, and the activity disappears after the chain length reaches the maximum activity. This so-called cutoff is a known phenomenon. For example, 2*E*-dodecenal (C₁₂) was very effective against *S. cerevisiae* with a MIC of 12.5 $\mu\text{g/mL}$, while 2*E*-tridecenal (C₁₃) no longer showed any activity up to 800 $\mu\text{g/mL}$. Interestingly, 2*E*-dodecenal exhibited the most potent MIC against *S. cerevisiae* but did not exhibit the most potent MFC. More precisely, 2*E*-dodecenal is fungistatic against *S. cerevisiae* but not fungicidal. The most potent fungicide in the 2*E*-alkenal series was 2*E*-undecenal (C₁₁) with a MFC of 6.25 $\mu\text{g/mL}$, followed by 2*E*-decenal (C₁₀) with a MFC of 12.5 $\mu\text{g/mL}$.

Microorganisms Tested	MIC (MBC or MFC)		
	2E-hexenal	2E-nonenal	2E-undecenal
<i>Bacillus subtilis</i>	400 (>800)	50 (>800)	25 (>800)
<i>Brevibacterium ammoniagenes</i>	400 (400)	100 (100)	25 (25)
<i>Micrococcus luteus</i>	400 (400)	50 (50)	12.5 (25)
<i>Streptococcus mutans</i>	800 (800)	100 (100)	50 (50)
<i>Staphylococcus aureus</i>	400 (400)	50 (50)	12.5 (12.5)
<i>S. aureus</i> (MRSA)	400 (400)	50 (50)	12.5 (12.5)
<i>Propionibacterium acnes</i>	200 (200)	25 (25)	6.25 (6.25)
<i>Escherichia coli</i>	400 (400)	200 (200)	>800 (–)
<i>Pseudomonas aeruginosa</i>	400 (400)	>800 (–)	>800 (–)
<i>Enterobacter aerogenes</i>	400 (400)	200 (200)	>800 (–)
<i>Proteus vulgaris</i>	200 (200)	25 (25)	12.5 (12.5)
<i>Salmonella choleraesuis</i>	200 (200)	25 (25)	12.5 (12.5)
<i>Saccharomyces cerevisiae</i>	200 (200)	25 (25)	25 (25)
<i>Zygosaccharomyces bailii</i>	400 (400)	50 (50)	25 (25)
<i>Candida albicans</i>	100 (100)	25 (50)	25 (25)
<i>Pityrosporum ovale</i>	50 (50)	25 (25)	12.5 (12.5)
<i>Penicillium chrysogenum</i>	50 (–)	50 (–)	6.25 (6.25)
<i>Trichophyton mentagrophytes</i>	50 (–)	12.5 (–)	1.56 (3.13)
<i>Aspergillus niger</i>	200 (–)	200 (–)	100 (200)

Numbers in *Italic* type in parenthesis are MBC or MFC. –, Not tested.

Table 1. Antimicrobial activity (µg/mL) of 2E-hexenal, 2E-hexenal and 2E-undecenal.

Aldehydes Tested	2E-Alkenal		Alkanal	
	MIC	MFC	MIC	MFC
C ₅	100	200	–	–
C ₆	100	200	1600	1600
C ₇	100	200	400	400
C ₈	100	100	200	200
C ₉	25	25	100	100
C ₁₀	12.5	12.5	25	50
C ₁₁	6.25	6.25	25	50
C ₁₂	12.5*	100	200*	>800
C ₁₃	>800	>800	>800	>800
C ₁₄	>400	–	–	–

The cells of *S. cerevisiae* were grown in ME broth at 30 °C without shaking.

*, The values are variable. –, Not tested.

Table 2. Antifungal activity (µg/mL) of aldehydes against *S. cerevisiae*.

The fungicidal activity of 2*E*-undecenal against *S. cerevisiae* was confirmed by the time kill curve experiment. Cultures of 2*E*-undecenal, with a cell density of 5.8×10^5 CFU/mL, were exposed to two different concentrations of 2*E*-undecenal. The number of viable cells was determined following different periods of incubation with 2*E*-undecenal. The result verifies that the MIC and MFC of 2*E*-undecenal are the same. It shows that $\frac{1}{2}$ MIC slowed growth, but that the final cell count was not significantly different from the control. Notably, lethality occurred remarkably quickly, within the first 1 h after adding 2*E*-undecenal. This rapid lethality very likely indicates that antifungal activity of 2*E*-undecenal against *S. cerevisiae* is associated with the disruption of the membrane (Fujita and Kubo, 2002).

Fig. 2. Time kill curve of 2*E*-undecenal against *S. cerevisiae*. A 16-h culture was inoculated into ME broth containing 0 µg/mL (●), 6.25 µg/mL (■), and 12.5 µg/mL (▲) of 2*E*-undecenal.

Further support for the membrane action was also obtained in experiments that showed the rapid decline in the number of viable cells after the addition of 2*E*-undecenal both at the stationary growth-phase and in the presence of cell growth inhibitors, as shown in Figure 3. Namely, 2*E*-undecenal rapidly killed *S. cerevisiae* cells in which cell division was inhibited by cycloheximide. This antibiotic is known to inhibit protein synthesis in eukaryotes, thereby restricting cell division. The fungicidal effect of 2*E*-undecenal appears independent of the necessary functions accompanying the reproduction of yeast cells, which are macromolecule biosyntheses of DNA, RNA, protein and cell wall components. Hence, the antifungal mechanism of 2*E*-undecenal is associated in part with membrane functions or derangement of the membrane.

In our preliminary test, octanal showed the similar antifungal activity against *S. cerevisiae*, so that the above-mentioned antifungal activity should not be specific to 2*E*-alkenals because the conjugated double bond is unlikely essential to elicit the activity. This

prompted us to test antifungal activity of the same series of alkanals against *S. cerevisiae* for comparison. The results are listed in Table 2. The activity of alkanals is slightly less than those of the corresponding 2*E*-alkenals. Similar to 2*E*-alkenal series, dodecanal (C₁₂) was effective with a MIC of 200 µg/mL, but did not exhibit any fungicidal activity up to 800 µg/mL. Thus, *S. cerevisiae* cells appeared to adapt to dodecanal stress, eventually recovering and growing normally. In connection with this, undecanal (C₁₁) and decanal (C₁₀) were the most potent with MFCs of 50 µg/mL. Although the current study was emphasized 2*E*-alkenals because of their more structural similarity with polygodial, the data obtained with alkanals are basically the same as those obtained with 2*E*-alkenals. In the case of short (<C₉) chain 2*E*-alkenals, the activity did not increase with each additional CH₂ group in the alkyl chain, indicating their mode of antifungal action may somewhat differ from that of alkanals.

After 5.8×10^5 cells were incubated in ME broth for 16 h, compounds were added as follows; 50 µg/mL cycloheximide (○), 12.5 µg/mL 2*E*-undecenal (■), no compound (●). After further 2-h incubation, 2*E*-undecenal was added in cycloheximide treated cells (□). Viability was estimated by the number of colonies formed on YPD plate after incubation at 30 °C for 48 h.

Fig. 3. Fungicidal effect of 2*E*-undecenal in cycloheximide treated cells.

The fungicidal activity of undecanal against *S. cerevisiae* was confirmed by the time kill curve experiment as shown in Figure 4. Cultures of *S. cerevisiae*, with a cell density of 5.8×10^5 CFU/mL, were exposed to two different concentrations of undecanal. The number of viable cells was determined following different periods of incubation with undecanal. Figure 4 verifies that the MIC and MFC of undecanal are the same. It shows that $\frac{1}{2}$ MIC slowed growth, but that the final cell count was not significantly different from the control. Notably, lethality occurred remarkably quickly, within the first 1 h after adding undecanal, indicating that undecanal possesses a membrane disruptive effect, in a similar manner described for 2*E*-undecenal.

A 16-h culture was inoculated into ME broth containing 0 µg/mL (●), 25 µg/mL (■), and 50 µg/mL (▲) of undecanal. Viability was estimated by the number of colonies formed on YPD plate after incubation at 30 °C for 48 h.

Fig. 4. Time kill curve of undecanal against *S. cerevisiae*.

It is known that *S. cerevisiae* produces the acidification of the external medium during growth on glucose. This external acidification is closely associated with the metabolism of the sugar and its magnitude depends on the buffering capacity of the growth medium (Busa and Nuccitelli, 1984). The H⁺-ATPase (P-type) is important not only in the regulation of internal pH but also the energy-dependent uptake of various metabolites (Coote et al., 1994). 2E-Alkenals inhibit the external acidification by inhibiting the H⁺-ATPase as shown in Figure 5. Their antifungal activity is also partly due to the inhibition of this H⁺-ATPase. Interestingly, the potency of H⁺-ATPase inhibition in each 2E-alkenal differs and the cutoff phenomenon does not occur. It is an interesting question how these 2E-alkenals inhibit H⁺-ATPase. The 2E-alkenals with the chain length less than C₈ and longer than C₁₂ exhibited weaker fungicidal activity. This inhibition pattern is not specific to only 2E-alkenals but also that of alkanals. It seems that medium-chain (C₉-C₁₁) 2E-alkenals have a better balance between the hydrophilic and hydrophobic portions of the molecules to act as surfactants. It should be remembered here that 2E-dodecenal exhibited fungistatic activity with a MIC of 12.5 µg/mL against *S. cerevisiae* but did not show any fungicidal activity up to 100 µg/mL.

In the aforementioned acidification inhibitory activity, the effect of the fungicidal 2E-undecenal was gradually enhanced, whereas cells treated with fungistatic 2E-dodecenal gradually recovered with time, as shown in Figure 6. Yeast cells appeared to adapt to 2E-dodecenal stress, eventually recovering and growing normally, similar to that of weak-acid stress (Holyoak et al., 1996). Among the alkanals tested, dodecanal was the most effective against *S. cerevisiae* with a MIC of 200 µg/mL but not fungicidal. This can be explained by the same manner described for 2E-dodecenal.

Fig. 5. Inhibition of medium acidification by 2E-alkenals (400 μg/mL) for short time incubation. The acidification was assayed for 10 min. The inhibition ratio (%) was calculated as follows; $(1 - [\text{H}^+]_{\text{inhibitor}} / [\text{H}^+]_{\text{inhibitor free}}) \times 100$.

Fig. 6. Effects of incubation time on the inhibition of 2E-undecenal (O) and 2E-dodecenal (●) to the medium acidification by the plasma membrane H⁺-ATPase of *S. cerevisiae*. Alkenals were tested at the concentration of 5 mM.

The data obtained so far indicates that the medium-chain 2E-alkenals act as nonionic surfactants at the lipid-protein interface, in a similar manner reported for alkanols (Kubo et al., 1995b). For example, the absence of a functioning state of the H⁺-ATPase could be due to its relative sensitivity to functional disruption by 2E-alkenals. It is suggested that the intrinsic proteins of the 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. The binding of nonionic surfactants such as the aliphatic aldehydes can only involve relatively weak headgroup interactions, such as hydrogen bonding, so that the predominant interactions will be hydrophobic, involving the alkyl tails. As proposed above, hydrogen bonds are formed or broken by the aliphatic aldehydes, and redirected. Thereby the conformation of membrane protein may be changed. For example, the H⁺-ATPase in particular could lose its proper conformation, which would lead to cell death. The H⁺-ATPase is the most abundant plasma membrane protein, constituting over 20% of the total membrane protein in *S. cerevisiae*, but the above mentioned fungicidal mechanism of the aliphatic aldehydes seems nonspecific. This can be explained as the amphipathic medium-chain aldehydes are nonionic surfactants and disrupt the hydrogen bonding in the lipid-protein interface in *S. cerevisiae*. As surfactants, the binding site of the aliphatic aldehydes should not be specific and their broad antimicrobial spectrum supports this postulate.

Further supporting generalized surfactant action at the plasma membrane is that the 2E-alkenals do not appear to inhibit the major energy production pathway. *S. cerevisiae* is a facultative anaerobic organism that is able to survive without a functional respiratory chain by relying on the fermentation of sugars to supply its energy demand, which is the state yeast prefer when sugars are present in significant amounts. 2E-Alkenals are inhibitory to the yeast while in this fermentative state. 2E-Alkenals also inhibit the growth of *S. cerevisiae* growing on non-fermentable carbon sources such as ethanol-, lactate-, acetate- and glycerol-containing media. Since no suppression of fungicidal activity seen as would be expected by removal of the potential target, it is unlikely that 2E-alkenals' lethal action in yeast is caused by inhibiting components of the respiration or fermentation pathway.

In addition, further support for the surfactant concept was obtained in an additional experiment that indicates antifungal 2E-undecenal rapidly adsorbed onto the surface of *S. cerevisiae* cells but 2E-hexenal did slightly, as shown in Figure 7. It appears that *S. cerevisiae* showed different affinities to 2E-alkenal having different alkyl chain length. The hydrophilic enal moiety was adsorbed by an intermolecular hydrogen bond by attaching itself to hydrophilic portion of the membrane surface. The adsorbing sites may not be specific but need to be clarified.

Given the surfactant-like properties of medium-chain 2E-alkenals, it is possible to suggest that 2E-alkenals act at the lipid-protein interface of integral proteins, such as ion channels and/or transport proteins, denaturing their functional conformation in a similar manner found for alkanols (Kubo et al., 1995b and 2003a). The common nature among these 2E-alkenals should be considered in that the electron negativity on the aldehyde oxygen atom forms an intermolecular hydrogen bond with a nucleophilic group in the membrane, thereby creating disorder in the fluid bilayer of the membrane. The fluidity of the cell membrane can be disturbed maximally by hydrophobic compounds of particular hydrophilic enal group. Thus, the amphipathic medium-chain 2E-alkenals disrupt the

hydrogen bonding in the lipid-protein interface in *S. cerevisiae*. The data obtained are consistent with an effect on the bulk membrane rather than a direct interaction of the specific target proteins, and 2*E*-alkenals' non-specificity of antimicrobial activity supports this assumption. The possibility of the antimicrobial activity of the medium-chain 2*E*-alkenals is due to their nonionic surfactant property, but this may not be the case for short-chain (<C₉) 2*E*-alkenals. The short chain 2*E*-alkenals enter the cell by passive diffusion across the plasma membrane and/or through porin channels (Schulz, 1996). Once inside the cell, their α,β -unsaturated aldehyde (enal) moiety is chemically highly reactive and hence, they may readily react with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl (Schauenstein et al., 1977). Sulfhydryl groups in proteins and lower molecular weight compounds such as glutathione are known to play an important role in the living cell. Microorganisms protect themselves against hydrogen peroxide in various ways (Brul and Coote, 1999), and some of the most ubiquitous systems include glutathione. 2*E*-Alkenals causes depletion of cytoplasmic and mitochondrial glutathione, which functions in eliminating reactive oxygen species, similar to found for polygodial (Machida et al., 1999). This 2*E*-alkenal mediated depletion of intercellular glutathione can be explained by a direct interaction between the enal moiety and the sulfhydryl group of glutathione by a Michael-type addition. This may reveal the reason why 2*E*-alkenals exhibit in general more potent and broader antimicrobial activity than those of the corresponding alkanals and alkanols. In the case against *S. cerevisiae*, 2*E*-hexenal exhibited the fungicidal activity against this yeast with an MFC of 200 $\mu\text{g/mL}$, whereas hexanol did not show any activity up to 1600 $\mu\text{g/mL}$.

Fig. 7. Binding of 2*E*-hexenal (a) and 2*E*-undecenal (b) to *S. cerevisiae* cells. After each 2*E*-alkenal was mixed with (●) or (○) without yeast cells (10^8 cells/mL), the suspension was vortexed for 5 sec. Absorbance of the supernatant obtained by centrifugation for 5 min was measured.

Moreover, the leakage of carboxyfluorescein (CF) in liposomes of phosphatidylcholine (PC) following exposure to 2*E*-alkenals was previously reported (Trombetta et al., 2002). Interestingly, 2*E*-alkenals caused rapid CF leakage from PC liposomes and the effectiveness order correlated well with the alkyl chain length. Thus, 2*E*-nonenal was more effective in inducing CF leakage from PC liposomes than that of 2*E*-hexenal. This previous report also supports the surfactant concept. Short chain 2*E*-alkenals are involved more in biochemical processes.

The process by which antimicrobial agents reach the action sites in living microorganisms is usually neglected in the cell-free experiment, but this must be taken into account in the current study. The inner and outer surfaces of the membrane are hydrophilic while the interior is hydrophobic, so the increased lipophilicity of 2*E*-alkenals should affect their movement further into the membrane lipid bilayer portions. It should be logical to assume that most of the lipophilic 2*E*-alkenal molecules being dissolved in the medium are partially incorporated into the lipid bilayers (Franks and Lieb, 1986) in which they may react with biologically important substances. The amount of 2*E*-alkenals entering into the cytosol or lipid bilayer is dependent on the length of the alkyl chain. Hence, the length of the alkyl chain is associated with eliciting activity to a large extent (Kubo and Kubo, 1995; Kubo et al., 1995a).

The current SAR study of 2*E*-alkenals was initiated largely as a model to understand the modes of the potent antifungal action of polygodial. The data described so far demonstrates the similarity between polygodial and 2*E*-alkenals in many aspects, but there are also significant differences. For example, polygodial loses its potent antifungal activity in YPD medium but not aliphatic aldehydes as shown in Table 3. This observation is consistent with the previous report that a primary amino group reacts with the dialdehyde moiety of polygodial and inactivates it (Cimino et al., 1987; Fujita and Kubo, 2005), but not 2*E*-alkenals. YPD contains very high levels of components with amine groups. The result indicates that polygodial forms a pyrrole derivative with the compounds possessing a primary amino group. Therefore, the binding site of polygodial may be, at least in part, a primary amino group in living systems.

Aldehydes Tested	ME	RPMI1640	YPD
Polygodial	1.56	1.56	>100
2 <i>E</i> -Undecenal	6.25	6.25	12.5
Undecanal	25	25	50

Table 3. Antifungal (MIC) activity (μg/mL) of aldehydes against *S. cerevisiae* in different media.

On the other hand, neither 2*E*-alkenals nor alkanals can form a pyrrole derivative. Notably, isolated mitochondrial ATPase (F-type) is strongly inhibited by polygodial (Lunde and Kubo, 2000), while it is weakly inhibited by the 2*E*-alkenals. In connection with this, 2*E*-alkenals and alkanals were found to inhibit the succinate-supported respiration of intact mitochondria isolated from rat liver, similar to those found for alkanols (Hammond and Kubo, 2000). However, results already discussed above show that these slight mitochondrial inhibitory activities are not primary responsible for cellular inhibition. The antifungal mechanism of polygodial seems to be associated in part with its specific dialdehyde structural features and differs from aliphatic 2*E*-alkenals.

The volume of the hydrophobic portions also seems to be related to the activity since antifungal activity of aliphatic α,β -unsaturated aldehydes are weaker than that of bicyclic sesquiterpene, polygodial. For example, the best fungicidal activity of the 2*E*-alkenal series against *S. cerevisiae* is 2*E*-undecenal with a MFC of 6.25 $\mu\text{g/mL}$, which is 2-fold less potent than that of polygodial. In the case of alkanals, the most potent undecanal is 16-fold less effective. There are two ways to increase the activity. First, the activity can be enhanced by combining with synergists. For example, the MFC of 2*E*-undecenal against *S. cerevisiae* was enhanced 16-fold when it was combined with $\frac{1}{2}$ MFC of anethole (2) (Kubo and Kubo, 1995). This combination strategy may be superior to enhance and broaden the total biological activity and, more importantly, it may hinder the development of resistant mechanisms in microorganisms. It should be noted that fungistatic compounds did not provide the stable enhancing activity in combination with other antifungal compounds. In fact, the combination data of the above mentioned fungistatic 2*E*-dodecenal varied. Second, the activity may be enhanced by increasing the volume of the hydrophobic portion through synthetic modification. For example, the volume of polygodial is unlikely the maximum since a more bulky labdane diterpene dialdehyde, aframodial (3), exhibited even more potent activity as listed in Table 4. It seems that the activity increased with increasing the volume of the hydrophobic portions. On the other hand, mukaadial (4) did not exhibit any activity up to 200 $\mu\text{g/mL}$ but warburganal (5) still exhibited the activity but lesser extent than polygodial (Kubo and Himejima, 1992). It is therefore apparent that the activity decreased for each additional hydroxyl group to polygodial. However, the rationale for these still remains unclear.

Aldehydes Tested	MIC	MFC
Polygodial (1)	1.56	3.13
Aframodial (3)	0.78	1.56
Mukaadial (4)	>200	–
Warburganal (5)	3.13	6.25

The cells of *S. cerevisiae* were grown in ME broth at 30 °C without shaking. –, Not tested.

Table 4. Antifungal activity ($\mu\text{g/mL}$) of polygodial and its related compounds against *S. cerevisiae*

Safety is a primary consideration for antifungal agents, and hence, the aldehydes characterized as antifungal agents from edible plants should be superior compared to non-natural antifungal agents. In addition, aldehydes have another superior property as antifungal agents compared to sorbic acid, a common commercial antifungal agent. As a weak acid antifungal agent, the activity of sorbic acid is pH dependent and increases as the pH of the substrate decreases (Sofos et al., 1983), as shown in Table 5. At higher pH values (>5), sorbic acid did not show any antifungal activity up to 1600 µg/mL due to a higher degree of dissociated molecules. In contrast, the aldehydes are not affected by pH. This would appear to be of greater overall value than other pH-sensitive antimicrobials, since many foods have near neutral pH values.

pH	2E-Undecenal	Sorbic acid	Undecanal
3	3.13	400	25
5	6.25	1600	50
7	6.25	>1600	50
9	6.25	>1600	50

Table 5. pH Effect of fungicidal (MFC) activity (µg/mL) of 2E-undecenal, sorbic acid and undecanal against *S. cerevisiae*.

3. Alkanols

As long as the antifungal activity is concerned, medium chain length 2E-alkenals may be potent enough to use as antifungal agents. However, it still needs to be considered, especially from practical points of view. For example, since α,β-unsaturated aldehyde is a chemically highly reactive group and readily reacts with biologically important nucleophilic groups, such as sulfhydryl, amino, or hydroxyl groups (Schauenstein, 1977), some practical application may limit the use of 2E-alkenals. As aforementioned the surfactant concept (biophysical processes) is a major contributor to their antifungal activity, so it is worth testing alkanols because they are usually considered as surfactants. In addition, alkanols are chemically stable compounds and unlikely react with any biologically important substances in the cytosol or lipid bilayer. Their maximum antimicrobial activity is dependent upon the hydrophobic alkyl (tail) chain length from the hydrophilic hydroxyl group (head). It should be noted that antimicrobial agents, which primarily act as surfactants, may have the potential, since they may target the extracytoplasmic region, and thus do not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms. Alkanols are considered to be stable, colorless, inexpensive, biodegradable (Swisher, 1970), and essentially nontoxic to humans (Opdyke, 1973). More importantly, alcohols are among the most versatile of all organic compounds, and free and esterified alcohols are known to occur widely in nature.

A series of aliphatic primary alkanols from C₆ to C₁₃ against *S. cerevisiae* were tested for their antifungal activity against *S. cerevisiae* using a 2-fold serial broth dilution method. The results are listed in Table 6, indicating basically similar to those found for 2*E*-alkenals. In agreement with many other studies of the homologous series of alkanols, the antifungal activity of the alkanols increased with number of carbons in the chain until dodecanol and undecanol, which had the best MIC and MFC, in this experiment. Noticeably, the activity disappeared after the chain length reached the best MIC and MFC, known as the so-called cutoff phenomenon. For example, dodecanol (C₁₂) was the most effective with an MIC of 12.5 µg/mL, while tridecanol (C₁₃) showed no activity up to 1600 µg/mL. Dodecanol is the most effective fungistatic but did not show any fungicidal activity up to 1600 µg/mL. The MIC of dodecanol slowed growth for the first 24 h, but the growth recovered shortly after and became no longer different from the control. The cutoff point may migrate by the slight difference in growth conditions such as inoculum size of yeast cells or medium composition. Namely it seems to be important that the cutoff point exists but not crucial where is the cutoff point. Alkanols can form hydrogen bonds with water and as a result, simple alkanols are fairly soluble in water. However, as the hydrocarbon content increases, especially to more than six carbons, there is a general decline in solubility. As the hydrocarbon chain becomes longer, its hydrophobic properties come to dominate the properties of the molecule so that the medium-chain (C₉-C₁₁) alkanols are amphipathic molecules.

Alkanols Tested	µg/mL		log <i>P</i>	Remarks
	MIC	MFC		
C ₆	1600	>1600	1.938	<div>↓</div> Surfactant
C ₇	800	>1600	2.469	
C ₈	400	>1600	3.001	
C ₉	50	100	3.532	
C ₁₀	25	50	4.063	<div>↓</div>
C ₁₁	12.5	25	4.595	
C ₁₂	12.5 ^{**}	>1600	5.126	
C ₁₃	>1600	-	5.657	Partially soluble in phospholipid

-, Not tested. **, The value is variable.

Table 6. Antifungal activity and log *P* of alkanols against *S. cerevisiae*.

Among the alkanols tested, undecanol (C_{11}) was the most potent against *S. cerevisiae* with a MFC of 25 $\mu\text{g/mL}$ (0.15 mM). No differences in MIC and MFC were noted, suggesting that undecanol's activity was fungicidal. This fungicidal effect was confirmed by a time kill curve method as shown in Figure 8. Cultures of *S. cerevisiae*, with a cell density of 6×10^5 CFU/mL, were exposed to two different concentrations of undecanol. The number of viable cells was determined following different periods of incubation with undecanol. The results show that $\frac{1}{2}$ MIC slowed growth but the final cell count was not significantly different from the control. At the MFC lethality occurred quickly, within the first 8 hours, indicating a membrane disruptive effect. Similar results were obtained with hexanol but fungicidal activity was not seen until 24 h, indicating that short-chain alkanols act in somewhat different ways.

Fig. 8. Time kill curve of undecanol against *S. cerevisiae*. A 16-h culture was inoculated into ME broth containing 0 $\mu\text{g/mL}$ (▲), 12.5 $\mu\text{g/mL}$ (■), and 25 $\mu\text{g/mL}$ (●) of undecanol.

It is known that *S. cerevisiae* produces the acidification of the external medium during growth on glucose. This external acidification is closely associated with the metabolism of the sugar and its magnitude depends on the buffering capacity of the growth medium. The H^+ -ATPase is important not only in the regulation of internal pH but also the energy-dependent uptake of various metabolites. Interestingly, alkanols were found to inhibit this acidification process by inhibiting the H^+ -ATPase (Kubo et al., 2003b). As a result, the antifungal activity of alkanols is, at least in part, due to their inhibition of the H^+ -ATPase shown in Figure 9. Interestingly, the inhibitory value of each alkanol differs and the cutoff phenomenon occurred between C_{12} and C_{13} . The alkanols of the chain length less than C_8 and longer than C_{12} exhibited much weaker inhibition activity. This inhibition pattern is not specific to only alkanols but also that of alkanals and fatty acids. The longer chain ($>C_{12}$) alkanols are soluble in the membrane phospholipid, and is thought to be incorporated into the hydrophobic domain of the membrane. In contrast, the shorter chain ($<C_9$) alkanols enter the cell by passive diffusion across the plasma membrane. It seems that only amphipathic medium-chain (C_9 - C_{11}) alkanols act as surface-active compounds (surfactants). It should be remembered that dodecanol exhibited fungistatic activity with MIC of 25 $\mu\text{g/mL}$ but did not show any fungicidal activity up to 1600 $\mu\text{g/mL}$. This alkanol inhibited the external acidification when tested after 5 min but not after 4 h. More specifically, the acidification inhibitory activity of fungicidal undecanol was

gradually enhanced, whereas cells treated with fungistatic dodecanol gradually recovered with time, as shown in Figure 10. Yeast cells appeared to adapt to dodecanol stress, eventually recovering and growing normally, similar to that of other stress.

Fig. 9. Inhibition of medium acidification by alkanols (400 µg/mL) for short time incubation. The acidification was assayed for 10 min. The inhibition ratio (%) was calculated as follows; $(1 - [H^+]_{inhibitor} / [H^+]_{inhibitor\ free}) \times 100$.

Fig. 10. Inhibition of medium acidification by undecanol (●) and dodecanol (□) (400 µg/mL) during long time incubation. The inhibition ratio (%) was calculated as follows; $(1 - [H^+]_{inhibitor} / [H^+]_{inhibitor\ free}) \times 100$.

Based on the data obtained, it seems logical to assume that alkanols act at the lipid-protein interface of H⁺-ATPase as nonionic surfactants. The absence of a functioning state of the H⁺-ATPase could be due to its relative sensitivity to denaturation by alkanols. The binding of

alkanols as nonionic surfactants can only involve relatively weak head group interactions such as hydrogen bonding. It is suggested that the intrinsic proteins of the membranes are held in position by hydrogen bonding, as well as by hydrophobic and electrostatic forces. As proposed above, hydrogen bonds are formed or broken by alkanols and then redirected. As a result, the conformation of the membrane protein may change. In particular, the H⁺-ATPase could lose its proper conformation. In addition to H⁺-ATPase, alkanols may destroy the native membrane-associated functions of integral proteins, such as ion channels and transport proteins. This can be supported, for instance, by alkanols inhibit the uptake of glucose and other nutrients by *S. cerevisiae* in a noncompetitive way. It appears that alkanols, as well as the corresponding alkanals and 2E-alkenals, are nonionic surfactants. Because of the lack of specificity, alkanols act in an unspecific manner on the lipid-protein interaction. This can be explained as the amphipathic medium-chain alkanols are considered as more appropriately balanced nonionic surfactants and more strongly disrupt the lipid-protein interface in *S. cerevisiae*. The shorter chain (<C₉) alkanols enter the cell by passive diffusion across the plasma membrane. In contrast, the longer chain (>C₁₂) alkanols are soluble in the membrane phospholipid and is thought to be incorporated into the hydrophobic domain of the membrane without perturbing the lipid. The partitioning of radiolabelled long-chain alcohols into biological membranes and lipid bilayers can support this. The cutoff in antifungal activity observed could be due to a corresponding cutoff in the absorption of long chain alcohols into lipid-bilayer portions of membranes. It should be noted that the carbon number for cutoff slightly varies by experimental conditions, tridecanol shows no antifungal activity against *S. cerevisiae* under any conditions. The precise explanation for the role of alkyl chain length - that must be related to antifungal activity - still remains obscure.

The nonspecific antimicrobial mechanism of alkanols is apparently due to their nonionic surface-active properties. The common nature among these alkanols should be considered in that the electron negativity on the oxygen atom forms an intermolecular hydrogen bond with a nucleophilic group in the membrane, thereby creating disorder in the fluid bilayer of the membrane. The fluidity of the cell membrane can be disturbed maximally by hydrophobic compounds of particular hydrophilic hydroxyl group. They could enter the molecular structure of the membrane with the polar hydroxyl group oriented into the aqueous phase by hydrogen bonding and nonpolar carbon chain aligned into the lipid phase by dispersion forces. Eventually, when the dispersion force becomes greater than the hydrogen bonding force, the balance is destroyed and the activity disappears. Concerning this, the hydrophobic bonding energy between an average fatty acid ester and a completely hydrophobic peptide is approximately 12 kcal/mol. Addition of a hydrogen bond between a peptide and a fatty ester's carbonyl adds another 3-6 kcal/mol. Furthermore, alkanols first approach the binding site with the electron negativity of the hydroxyl oxygen atom. This hydrogen bond acceptor will affect the hydrogen bonds that regulate the permeability of the lipid bilayer. For example, in the lipid bilayer, the hydroxyl group of ergosterol resides near the membrane-water interface and is likely to bind to the carbonyl group of phospholipids. Alkanols may function by disrupting and disorganizing these hydrogen bonds. Ergosterol is a major component of the plasma membrane of *S. cerevisiae* and owes its membrane-closing properties to its rigid longitudinal orientation in the membrane. Since ergosterol has a profound effect on membrane structure and function, cell function will be impaired if the hydrogen bond is broken. The similar hydrogen bond-breaking concept was proposed to explain the anesthesia cutoff phenomenon.

Further support for the surfactant postulate was also obtained in experiments that showed a rapid decline in the number of viable cells after the addition of undecanol at the exponential growth-phase as shown in Figure 11. In addition, the effect of undecanol was tested during holding viable cell number in the presence of cycloheximide. This drug is known to restrict cell division by inhibiting protein synthesis; its effect against *S. cerevisiae* cells is fungistatic. Undecanol rapidly killed *S. cerevisiae* cells in which cell division was inhibited by cycloheximide. This observation excludes several modes of action for undecanol such as inhibition of DNA, RNA, protein, or cell wall component synthesis *in vivo*. The result observed indicates that the antifungal mechanism of undecanol is due primarily to its surfactant property, although it is not possible to confirm that membrane damage is the only cause of the lethal effect. It can be concluded that the medium-chain alkanols target, in part, the extracytoplasmic region as surfactants. This conclusion is highly desirable since they do not need to enter the cell, thus avoiding most resistance mechanisms based on cellular pump.

After 10^4 cells were incubated in ME broth for 14 h, compounds were added as follows; 50 $\mu\text{g/mL}$ cycloheximide (■), 25 $\mu\text{g/mL}$ undecanol (▲), no compound (●). After further 2-h incubation, undecanol was added in cycloheximide treated cells (□).

Fig. 11. Fungicidal effect of undecanol in cycloheximide treated cells.

The time-kill curve study showed that undecanol was fungicidal against *S. cerevisiae* at any growth stage. Figure 12 shows the effect of undecanol at various growth stages. Undecanol at MFC rapidly reduced the number of viable cells when added to the culture at the exponentially growing culture (10^6 CFU/mL) within the first 4 h. Thus, no viable cells were detected within 2 h after adding undecanol. However, undecanol was not fungicidal when added to the stationary growing culture (10^8 CFU/mL). This alcohol rapidly reduced the number of viable cells, but slowed thereafter and complete lethality did not occur. As the number of viable cells increases, the amount of drug's molecules needs to be increased to retain the fungicidal activity. The results indicate that undecanol disrupts the lipid-protein interface nonspecifically as a surfactant rather than in a direct interaction with specific target proteins such as cell-surface receptors or signal transduction proteins.

Fig. 12. Effect of undecanol (25 µg/mL) at the various growth stages of *S. cerevisiae*. A 16-h culture was inoculated into ME broth. Undecanol was added at 0 h (□), 4 h (▲), 24 h (◇), and control (●).

The same series of alkanols were recently found to inhibit the succinate-supported respiration of intact mitochondria isolated from rat liver. The potency increased with increasing chain length up to undecanol. Given each alkanol's nearly identical effect on State 3 and uncoupled respiration, action is not directly on ATP synthetase, but earlier in the respiratory process. Hexanol and decanol were also assayed against freeze-thawed (broken) mitochondria to distinguish effects on the mitochondrial substrate carrier from that on the electron transport chain. Both alcohols were only weak inhibitors of respiration in broken mitochondria, suggesting that inhibition originates from interference with the dicarboxylate carrier, which must transport succinate across the mitochondrial membranes. Alkanols may inhibit this transporter in the inner membrane as nonionic surfactants. The concentrations found to inhibit mitochondrial respiration are slightly lower than those causing fungicidal activity against *S. cerevisiae*, prompting the question whether decreased mitochondrial functions might in some way contribute to *S. cerevisiae* death. It is uncertain, however, if the respiratory inhibition mechanism is the primary mode of antifungal action of alkanols against *S. cerevisiae* because we do not know if alkanols can reach the mitochondria in vivo. The results with mitochondria also support the alkanols' nonionic surfactant concept because enzyme systems related to transport of solutes and electron transfer are located in the inner membrane of the cell envelope.

In addition, *S. cerevisiae* is a facultative anaerobic organism that is able to survive without a functional respiratory chain, by falling back on the fermentation of sugars to supply its energy demand. This latter mechanism is used preferentially, since when a combination of fermentable and non-fermentable carbon sources is available, respiration is greatly reduced and fermentation accounts for the major fraction of sugar catabolism. *S. cerevisiae* is thus able to rapidly adjust its metabolism to its environment and in particular to the availability of carbon sources. Alkanols also inhibit the growth of *S. cerevisiae* growing on non-fermentable carbon sources such as ethanol-, lactate-, acetate- and glycerol-containing media. As surfactants, alkanols exhibit fungicidal activity when *S. cerevisiae* is growing on both fermentable and non-fermentable condition.

Safety is a primary consideration for *S. cerevisiae* control agents, especially concerning their use in food products that may be utilized in unregulated quantities regularly. The phytochemicals characterized as antifungal agents against *S. cerevisiae* from edible plants should be superior to non-natural preservatives. Incidentally, alcohols are among the most versatile of all organic compounds, free and esterified alcohols occur widely in nature. In addition, alcohols have another superior property as antifungal agents compared to commonly use antifungal agents such as sorbic acid and benzoic acid. As a weak acid antifungal agent, the activity of sorbic acid is pH dependent and increases as the pH of the substrate decreases as shown in Table 7. At higher pH values (>5), sorbic acid did not show any antifungal activity up to 1600 µg/mL due to a higher degree of dissociated molecules. In contrast, undecanol and geraniol are not influenced by pH values.

pH	Undecanol	Sorbic acid	Geraniol
3	12.5	800	800
5	25	1600	800
7	12.5	>1600	800
9	12.5	>1600	800

Table 7. pH Effect of fungicidal (MFC) activity (µg/mL) of undecanol, sorbic acid, geraniol against *S. cerevisiae*.

4. Antifungal mechanisms

The fluidity of the lipid bilayer is partly regulated by hydrogen bonding. For example, the hydroxyl group of ergosterol resides near the membrane-water interface in the lipid bilayer and is likely to be bonded with the carbonyl group of phospholipids (Brockerhoff, 1974; Chauhan et al., 1984). As nonionic surfactants, the aliphatic aldehydes first approach the binding site with the electron negativity of the aldehyde oxygen atom and may function by disrupting and disorganizing the hydrogen bonds such as the above mentioned. Ergosterol is a major component of the plasma membrane of *S. cerevisiae* and owes its modulation of membrane fluidity to its rigid longitudinal orientation in the membrane. Since ergosterol has profound influences on membrane structure and function, if the hydrogen bond is broken, cell function will be impaired. If the aldehydes target the extracytoplasmic region, it is highly desirable since they do not need to enter the cell, thus avoiding most cellular pump-based resistance mechanisms. The similar hydrogen bond-breaking concept was proposed to explain the anesthesia cutoff phenomenon (Chiou et al., 1990).

In previous reports, the antifungal activity of the same series of alkanols against *S. cerevisiae* was described (Kubo et al., 1995b). Similar to 2*E*-alkenals, the short chain alkanols enter the cell by passive diffusion across the plasma membrane and/or through porin channels

(Schulz, 1996), and the long chain alkanols enter in part into the lipid bilayers (Franks and Lieb, 1986). The amount of alkanols entering into the cytosol or lipid bilayer is dependent on the length of the alkyl chain. Nonetheless, alkanols are chemically stable compounds and may not react with any biologically important substances in the cytosol or lipid bilayer. Hence, the primary antifungal action of alkanols comes largely from their ability to function as nonionic surfactants (physical disruption of the membrane). In the case of 2*E*-alkenals, their α,β -unsaturated aldehyde group should not be overlooked because this group is chemically highly reactive and readily reacts with biologically important nucleophilic groups, such as sulfhydryl, amino, or hydroxyl (Schauenstein et al., 1977). For example, the yeast plasma-membrane H⁺-ATPase was reported to contain nine cysteines. 2*E*-Alkenals may bind directly to the plasma membrane H⁺-ATPase probably with sulfhydryl groups of the three cysteines in the presumed transmembrane segments (C148, C312, C867). However, Petrov and Slayman (1995) reported that no single cysteine is required for activity based on their site-directed mutagenesis study. This previous result does not exclude the possibility to assume that 2*E*-alkenals first break the hydrogen bond as nonionic surfactants and then react with the freed sulfhydryl group of the H⁺-ATPase as well as other plasma membrane proteins. This can be supported by the previous report that covalent modification of the conserved C148 in the transmembrane segment 2 may be important for inhibition of H⁺-ATPase activity and cell growth (Monk et al., 1995). However, the observation that alkanals and 2*E*-alkenals exhibit similar antifungal activity against *S. cerevisiae* as shown in Table 2 and also inhibit glucose-induced acidification, may not support the above assumption because the conjugated double bond is not essential to elicit the activity. The possibility of this concerted function of 2*E*-alkenals is unlikely but cannot be excluded.

The leakage of carboxyfluorescein (CF) in liposomes of phosphatidylcholine (PC) following exposure to 2*E*-alkenals was previously reported (Trombetta et al., 2002), similar to those described for alkyl gallates (Fujita and Kubo, 2002). Interestingly, 2*E*-alkenals tested caused rapid CF leakage from PC liposomes and the effectiveness order correlated well with the alkyl chain length. Thus, 2*E*-nonenal was more effective in inducing CF leakage from PC liposomes than that of 2*E*-hexenal (Trombetta et al., 2002). This also supports the surfactant concept.

In general terms, aldehydes may enter the cell by passive diffusion across the membrane. Once inside the cells, the following reactions are known for the 2*E*-alkenals. Reactions with the sulfhydryl group, for which primary addition to the α,β -olefinic group occurs exclusively. Reactions with amino groups, where the formations of Schiff bases and 1,4-addition products is possible. It is generally true, however, that the reaction with the sulfhydryl groups takes place much faster, in fact by several orders of magnitude. These reactions may lead to the deactivation of enzymes – in particular of sulfhydryl enzymes. The main problem of general significance remains the certain and unequivocal experimental proof that 2*E*-alkenals recognized as highly reactive compounds is in fact essential bioregulators of metabolism. For example, sulfhydryl groups in proteins and lower molecular weight compounds such as glutathione are known to play an important role in the living cell. Bacteria protect themselves against hydrogen peroxide in various ways (Brul and Coote, 1999), and some of the most ubiquitous systems include glutathione. 2*E*-Alkenals causes depletion of cytoplasmic and mitochondrial glutathione, which functions in eliminating reactive oxygen species, similar to found for polygodial (Machida et al., 1999).

This 2*E*-alkenal mediated depletion of intercellular glutathione can be explained by a direct interaction between the enal moiety and the sulfhydryl group of glutathione by a Michael-type addition. This may reveal the reason why 2*E*-alkenals exhibit in general more potent and broader antimicrobial activity than those of the corresponding alkanals and alkanols. Thus, 2*E*-hexenal exhibited the fungicidal activity against *S. cerevisiae* with an MFC of 200 µg/mL, whereas hexanal showed the same activity at only 1600 µg/mL.

5. Antifungal assay

The maximum extent and rate of activity is known to vary with the seed culture mediums, the physiological age of the culture, and the type of culture medium. The initial assay was achieved using two-fold serial dilution method against *Saccharomyces cerevisiae* ATCC 7754. This yeast was purchased from American Type Culture Collection (Manassas, VA).

5.1 Medium

Saccharomyces cerevisiae was maintained at -80 °C in yeast nutrient broth (YNB; Difco Laboratories, 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 16 h at 30 °C in 2.5% malt extract (ME) broth (BBL) medium.

5.2 Acidification measurement

The glucose-induced medium acidification of *S. cerevisiae* was measured with a modified procedure (Haworth et al., 1993). 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 X 10⁷ colony forming units (CFU) per mL with cold distilled water and kept on ice. The reaction mixture contained 2.7 mL of cells and 30 µL of the inhibitor in DMSO, 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 incubation, the pH of external medium was checked (Orion 8175 Ross semimicro electrode).

5.3 Antifungal assay

The test compounds were first dissolved in DMF and the concentration of DMF in each medium was always 1%. The highest concentration tested was 1600 µg/mL, unless otherwise specified. The maximum extent and rate of activity is known to vary with the 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 ME medium, final inoculum size of 10⁵ CFU/mL, and 48 h stationary incubation in ME medium, unless otherwise specified.

Broth macrodilution minimum inhibitory concentrations (MICs) were determined as previously described (Kubo and Himejima, 1992). Briefly, serial 2-fold dilutions of the test compounds were made in DMF and 30 µL of 100 X conc. solution was added to 3 mL of ME

media. These were inoculated with 30 μ L of seed culture to give the final inoculum of 10^5 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 the MIC had been determined, a 30 μ L 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.

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 16 h. A 30 μ L aliquot of the culture was inoculated into 3 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 48 h before the number of CFU was determined.

5.4 Adsorption test

The test strain was cultured with shaking in YPD broth overnight at 30 °C and washed twice with 50 mM MOPS buffer (pH 6.0). After each 2*E*-alkenal was mixed with or without *S. cerevisiae* cells (10^8 cells/mL) in the above buffer at 30 °C, the suspension was vortexed for 5 seconds. Absorbance of the supernatants obtained by centrifugation for 5 min was measured at 255 nm.

6. Summary

The antifungal activity of alkanols comes from their ability to act as surfactants and the maximum activity can be designed by selecting a alkyl chain length to give the appropriate partition coefficient (log P) as a standard. Similarly, 2*E*-alkenals also act as surfactants, but their α,β -unsaturated aldehyde moiety needs to be taken into account. 2*E*-Alkenals may not act by a single defined process but have multiple functions (Figure 13). The surfactant concept, disrupting and disorganizing the lipid bilayer-protein interface nonspecifically, can be extended to answer many other problems related to membrane-bound enzymes and receptors, and the fluidity of the membrane lipids. For example, the anesthesia cutoff phenomenon among alkanols is well known and a long standing problem. Anesthesia involves many membrane-bound proteins such as synaptosomal ATPases and acetylcholine receptor (Edelfors and Ravn-Jensen, 1990; Elliott and Haydon, 1989). The same surfactant concept, disrupting and disorganizing the lipid bilayer-protein interface, seems applicable to explain the anesthesia cutoff phenomena of alkanols. The knowledge obtained may provide insights into fungicidal action of aldehydes and alkanols on a molecular basis, and a more rational and scientific approach to use or design efficient and safe antifungal agents. Based on the data obtained, the hydrophilic head portion can be replaced by any hydrophilic groups as long as the “head and tail” structure is balanced. Hence, various additional biological activities can be introduced mainly by selecting appropriate head portions. For example, each series of alkyl gallates (Kubo et al., 2001b; Fujita and Kubo, 2001 and 2002) and alkyl protocatechuates (Nihei et al., 2003) were synthesized as antioxidation antifungal agents.

Fig. 13. Antifungal action involves multifunction. The amphipathic medium-chain aldehydes and alcohols are nonionic surfactants and disrupt the hydrogen bonding in the lipid-protein interface of integral proteins, such as ion channels and/or transport proteins, denaturing their functional conformation. 2E-Alkenals react with biologically important nucleophilic groups such as sulfhydryl, amino, or hydroxyl. For example, sulfhydryl groups in proteins and lower molecular weight compounds such as glutathione are known to play an important role in the living cell and 2E-alkenal mediated depletion of intercellular glutathione can be explained by a direct interaction between the enal moiety and the sulfhydryl group of glutathione by a Michael-type addition. Aldehydes are known to inhibit alcohol dehydrogenase competitively but not phosphofructokinase or pyruvate decarboxylase.

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A fungicide is a chemical pesticide compound that kills or inhibits the growth of fungi. In agriculture, fungicide is used to control fungi that threaten to destroy or compromise crops. Fungicides for Plant and Animal Diseases is a book that has been written to present the most significant advances in disciplines related to fungicides. This book comprises of 14 chapters considering the application of fungicides in the control and management of fungal diseases, which will be very helpful to the undergraduate and postgraduate students, researchers, teachers of microbiology, biotechnology, agriculture and horticulture.

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