

Molecular Design of Antifungal Agents

ISAO KUBO,^{*,†} PING XIAO,[†] KEN-ICHI NIHEI,[†] KEN-ICHI FUJITA,[†]
YOSHIRO YAMAGIWA,[‡] AND TADAO KAMIKAWA[‡]Department of Environmental Science, Policy and Management, University of California,
Berkeley, California 94720-3112, and Department of Chemistry, Faculty of Science and Technology,
Kinki University, Kowakae, Higashi-Osaka-shi, Osaka 577-8502, Japan

In a rational approach to the design of antifungal agents against *Saccharomyces cerevisiae*, a series of alkyl gallates (3,4,5-trihydroxybenzoates) were synthesized and assayed. Nonyl gallate (**1**) was found to be the most effective with a minimum fungicidal concentration (MFC) of 12.5 $\mu\text{g/mL}$ (42 μM), followed by octyl gallate (**2**) with an MFC of 25 $\mu\text{g/mL}$ (89 μM). These MFCs are little influenced by pH values. A time–kill curve study indicates that nonyl gallate exhibits fungicidal activity against *S. cerevisiae* at any growing stage. The antifungal activity of nonyl gallate is due primarily to its ability to act as a nonionic surface-active agent (surfactant). The length of the alkyl group is not a major contributor but plays a role in eliciting the activity to a large extent. As far as alkyl gallates are concerned, their antimicrobial spectra and potency depend largely on the hydrophobic portion of the molecules.

KEYWORDS: Antifungal activity; *Saccharomyces cerevisiae*; nonyl gallate; surfactant property; antioxidant activity

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 (1). The finishing process of the fermentation is usually through either filtration or pasteurization. However, the use of the latter is limited to certain foods because it is a heat treatment and hence denaturalizes proteins, and the former is also limited to clear liquids (2). Neither process is applicable to some foods such as sauerkraut and “miso” (soybean pastes). Therefore, safe and effective fungicides are still needed to control yeasts with new modes of action.

Safety is a primary consideration for antifungal agents, especially for those in food products, which may be utilized in unregulated quantities on a regular basis. In our continuing search for antimicrobial agents, a number of phytochemicals have been characterized as active principles against *S. cerevisiae* from edible plants, spices, and beverages. However, the individual activity of antifungal agents characterized in plants is usually not potent enough to be considered for practical use.

A possible solution to cross this hurdle is combining two or more inhibitors to increase the total biological activity or their synthetic modification. Several examples of the former have already been reported (3, 4), so the emphasis for this paper was placed on an example for the latter case, because the ideal antimicrobial agent was suggested to be produced by a rational design (5). To facilitate it, our previous structure–antimicrobial activity relationship (SAR) study with a series of alkanols was selected for this study as a model. Briefly, the maximum antimicrobial activity depends on the hydrophobic alkyl (tail) chain length from the hydrophilic hydroxyl group (head) and also on the microorganisms being tested (6). A series of alkyl benzoate derivatives were synthesized and tested for their antifungal activity against *S. cerevisiae* for comparison.

MATERIALS AND METHODS

General Methods. The procedures used for antimicrobial assay were the same as previously described (3, 4, 6). For the synthesis, the melting points were measured on a micro hot-stage Yanaco MP-S2 and are uncorrected. IR spectra were recorded on a Shimadzu FTIR-8100 or Hitachi 100-80 spectrometer, and ¹H NMR spectra were recorded on a Varian Mercury-300BB, JEOL/JNM-GX500, or JEOL-GSX270 spectrometer in CDCl₃ using TMS as the internal standard unless otherwise noted. Mass spectra were measured on a JEOL LMS-HX 100 or JEOL-JMS700 spectrometer. Silica gel TLC and column chromatography were done on a Merck precoated TLC 5715 and Merck kieselgel 60 7734, respectively. Air- and/or moisture-sensitive reactions were carried out under an argon or nitrogen atmosphere. The organic solvents were purified and dried using appropriate procedures.

Chemicals. Alkanols were available from our previous work (3, 6, 7). Gallic acid and its methyl and propyl esters used for the assay were

* Author to whom correspondence should be addressed [telephone (510) 643-6303; fax (510) 643-0215; e-mail ikubo@uclink4.berkeley.edu].

[†] University of California.

[‡] Kinki University.

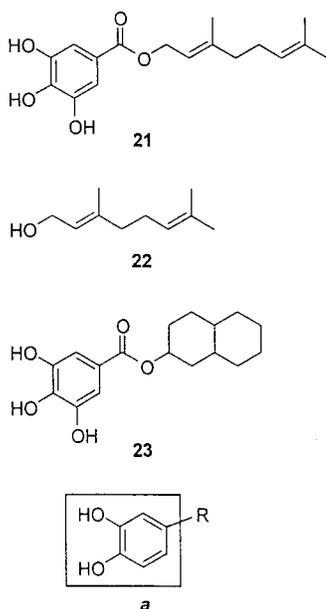
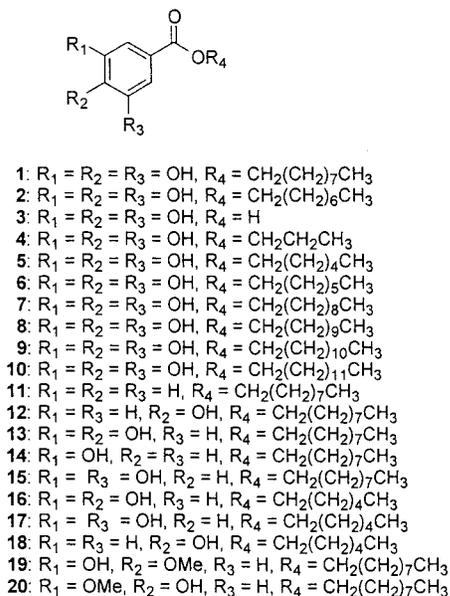


Figure 1. Chemical structures of gallates and related compounds.

purchased from Aldrich Chemical Co. (Milwaukee, WI). Benzoic acid, geraniol, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), 3,5-dihydroxybenzoic acid, 3-hydroxy-4-methoxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, and 1,1-diphenyl-2-*p*-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, MO). *N,N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, NJ).

Synthesis. To a solution of the corresponding phenolic acid (2.00 mM) and alcohol (2.00 mM) in THF (6 mL) cooled at 0 °C was added a solution of *N,N*-dicyclohexylcarbodiimide (DCC) (4.2 mM) in THF (6 mL). After the solution had been allowed to stir for 20 h, the solvent was removed under reduced pressure. The residue was extracted with ethyl acetate several times and filtered. The filtrate was washed successively with diluted aqueous citric acid solution, saturated aqueous sodium hydrogen carbonate solution, and water, dried over MgSO₄, and evaporated. The crude products were purified by chromatography (SiO₂; elution with CHCl₃/MeOH, 98:2). Structures of the synthesized esters were established by spectroscopic methods (UV, IR, MS, and NMR; Figure 1).

Tridecyl gallate (*tridecyl 3,4,5-trihydroxybenzoate*) (**10**) was obtained in 77% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.88

(t, *J* = 6.5 Hz, 3H, -CH₃), 4.24 (t, *J* = 6.5 Hz, 2H, -OCH₂), 7.16 (s, 2H, ArH); IR (KBr) 3520, 3495, 3010, 2960, 1686, 1615, 1480, 1395, 1270, 1140 cm⁻¹.

Dodecyl gallate (*dodecyl 3,4,5-trihydroxybenzoate*) (**9**) was obtained in 65% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.88 (t, *J* = 6.5 Hz, 3H, -CH₃), 4.24 (t, *J* = 6.5 Hz, 2H, -OCH₂), 7.16 (s, 2H, ArH); IR (KBr) 3515, 3490, 3000, 2960, 1680, 1610, 1480, 1395, 1270, 1145 cm⁻¹.

Undecyl gallate (*undecyl 3,4,5-trihydroxybenzoate*) (**8**) was obtained in 63% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.88 (t, *J* = 6.5 Hz, 3H, -CH₃), 4.24 (t, *J* = 6.5 Hz, 2H, -OCH₂), 7.16 (s, 2H, ArH); IR (KBr) 3500, 3450, 2960, 2890, 1685, 1620, 1480, 1395, 1280, 1160 cm⁻¹.

Decyl gallate (*decyl 3,4,5-trihydroxybenzoate*) (**7**) was obtained in 74% yield as a colorless solid: ¹H NMR (300 MHz, CDCl₃) δ 0.88 (t, *J* = 6.5 Hz, 3H, -CH₃), 4.24 (t, *J* = 6.5 Hz, 2H, -OCH₂), 7.16 (s, 2H, ArH); IR (KBr) 3391, 2924, 1686, 1615, 1448, 1312, 1258, 1028 cm⁻¹; EI-MS, *m/z* 310 (M⁺), 282, 226, 170 (base peak), 153, 125, 83, 55; HRMS-EI, *m/z* [M]⁺ calcd for C₁₇H₂₆O₅ 310.1778, found 310.1788.

Nonyl gallate (*nonyl 3,4,5-trihydroxybenzoate*) (**1**) was obtained in 86% yield as colorless needles from benzene/*n*-pentane: mp 96.7–97.3 °C; ¹H NMR (300 MHz, CDCl₃/CD₃OD) δ 0.88 (t, *J* = 6.6 Hz, 3H, -CH₃), 4.25 (t, *J* = 6.6 Hz, 2H, -OCH₂), 7.21 (s, 2H, ArH); IR (KBr) 3489, 3439, 3333, 1670, 1625, 1604, 1531, 1300, 1256, 1024 cm⁻¹; HRMS-EI, *m/z* [M]⁺ calcd for C₁₆H₂₄O₅ 296.1630, found 296.1624.

Nonyl protocatechuate (*nonyl 3,4-dihydroxybenzoate*) (**13**) was obtained in 64% yield as a colorless solid: ¹H NMR (300 MHz, CDCl₃) δ 0.89 (t, *J* = 6.6 Hz, 3H, -CH₃), 4.24 (t, *J* = 6.6 Hz, 2H, -OCH₂), 6.66 (d, *J* = 7.8 Hz, 1H, ArH), 7.40 (dd, *J* = 2.3, 7.8 Hz, 1H, ArH), 7.53 (d, *J* = 2.3 Hz, 1H, ArH); IR (KBr) 3530, 3360, 2960, 2890, 1700, 1625, 1550, 1480, 1395, 1290, 1180 cm⁻¹.

Nonyl 3,5-dihydroxybenzoate (**15**) was obtained in 68% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.88 (t, *J* = 6.6 Hz, 3H, -CH₃), 4.21 (t, *J* = 6.6 Hz, 2H, -OCH₂), 6.34 (t, *J* = 2.3 Hz, 1H, ArH), 6.45 (d, *J* = 2.3 Hz, 2H, ArH); IR (KBr) 3340, 2960, 2890, 1690, 1610, 1480, 1395, 1255, 1165 cm⁻¹; HRMS-EI, *m/z* [M]⁺ calcd for C₁₆H₂₄O₄ 280.1673, found 280.1675.

Nonyl 3-hydroxy-4-methoxybenzoate (**19**) was obtained in 76% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.88 (t, *J* = 6.6 Hz, 3H, -CH₃), 4.20 (s, 3H, -OCH₃), 4.26 (t, *J* = 6.6 Hz, 2H, -OCH₂), 6.73 (d, *J* = 8.7 Hz, 1H, ArH), 7.43 (dd, *J* = 2.3, 8.7 Hz, 1H, ArH), 7.65 (d, *J* = 2.3 Hz, 1H, ArH); IR (KBr) 3290, 2960, 2890, 1690, 1630, 1600, 1480, 1395, 1250, 1180 cm⁻¹.

Nonyl 4-hydroxy-3-methoxybenzoate (**20**) was obtained in 61% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.88 (t, *J* = 6.6 Hz, 3H, -CH₃), 3.89 (s, 3H, -OCH₃), 4.20 (t, *J* = 6.6 Hz, 2H, -OCH₂), 6.78 (d, *J* = 8.7 Hz, 1H, ArH), 7.47 (dd, *J* = 2.3, 8.7 Hz, 1H, ArH), 7.53 (d, *J* = 2.3 Hz, 1H, ArH); IR (KBr) 3360, 2960, 2890, 1640, 1595, 1525, 1480, 1395, 1250, 1165 cm⁻¹.

Nonyl 4-hydroxybenzoate (**12**) was obtained in 64% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.88 (t, *J* = 6.6 Hz, 3H, -CH₃), 3.66 (m, 2H, -CH₂), 4.26 (t, *J* = 6.6 Hz, 1H, -OCH₂), 6.85 (d, *J* = 8.7 Hz, 2H, ArH), 7.92 (d, *J* = 8.7 Hz, 2H, ArH); IR (KBr) 3360, 2960, 2890, 1640, 1605, 1550, 1480, 1395, 1252, 1175 cm⁻¹.

Nonyl 3-hydroxybenzoate (**14**) was obtained in 72% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.82 (t, *J* = 6.6 Hz, 3H, -CH₃), 3.23 (m, 2H, -CH₂), 4.24 (t, *J* = 6.6 Hz, 2H, -OCH₂), 6.87 (d, *J* = 7.5 Hz, 1H, ArH), 6.95 (s, 1H, ArH), 6.98 (d, *J* = 7.5 Hz, 1H, ArH), 7.18 (t, *J* = 7.5 Hz, 1H, ArH); IR (KBr) 3360, 2960, 2890, 1640, 1590, 1551, 1480, 1395, 1252, 1170 cm⁻¹; HRMS-EI, *m/z* [M]⁺ calcd for C₁₆H₂₄O₃ 264.1721, found 264.1725.

Nonyl benzoate (**11**) was obtained in 57% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.88 (t, *J* = 6.6 Hz, 3H, -CH₃), 4.20 (t, *J* = 6.6 Hz, 2H, -OCH₂), 7.32 (t, *J* = 7.8 Hz, 2H, ArH), 7.45 (t, *J* = 7.8 Hz, 1H, ArH), 8.06 (d, *J* = 7.8 Hz, 2H, ArH); IR (KBr) 2960, 2890, 1720, 1650, 1550, 1480, 1395, 1250, 1175 cm⁻¹.

Octyl gallate (*octyl 3,4,5-trihydroxybenzoate*) (**2**) was obtained in 89% yield as a colorless solid: ¹H NMR (270 MHz, CDCl₃) δ 0.88 (t, *J* = 6.5 Hz, 3H, -CH₃), 4.24 (t, *J* = 6.5 Hz, 2H, -OCH₂), 7.15 (s,

2H, ArH); IR (KBr) 3325, 2926, 1686, 1614, 1468, 1314, 1246, 1028 cm^{-1} ; EI-MS, m/z 282 (M^+), 224, 170, 143, 99, 56 (base peak) 41; HRMS-EI, m/z [M^+] calcd for $\text{C}_{15}\text{H}_{22}\text{O}_5$ 282.1469, found 282.1467.

Heptyl gallate (heptyl 3,4,5-trihydroxybenzoate) (6) was obtained in 71% yield as a colorless solid: ^1H NMR (270 MHz, CDCl_3) δ 0.88 (t, $J = 6.5$ Hz, 3H, $-\text{CH}_3$), 4.24 (t, $J = 6.5$ Hz, 2H, $-\text{OCH}_2$), 7.16 (s, 2H, ArH); IR (KBr) 3389, 2920, 1685, 1620, 1448, 1320, 1258, 1140 cm^{-1} .

Hexyl gallate (hexyl 3,4,5-trihydroxybenzoate) (5) was obtained in 58% yield as a colorless solid: ^1H NMR (270 MHz, CDCl_3) δ 0.88 (t, $J = 6.5$ Hz, 3H, $-\text{CH}_3$), 4.18–4.28 (br, 2H, $-\text{OCH}_2$), 6.4–5.8 (br, 3H, exchangeable with D_2O , $-\text{ArOH}$), 7.20 (s, 2H, ArH); IR (KBr) 3389, 2932, 1688, 1615, 1449, 1314, 1258, 1026 cm^{-1} .

Hexyl protocatechuate (hexyl 3,4-dihydroxybenzoate) (16) was obtained in 63% yield as a colorless solid: ^1H NMR (270 MHz, CDCl_3) δ 0.89 (t, $J = 6.6$ Hz, 3H, $-\text{CH}_3$), 4.24 (t, $J = 6.6$ Hz, 2H, $-\text{OCH}_2$), 6.66 (d, $J = 7.8$ Hz, 1H, ArH), 7.40 (dd, $J = 2.3, 7.8$ Hz, 1H, ArH), 7.53 (d, $J = 2.3$ Hz, 1H, ArH); IR (KBr) 3400, 3310, 2960, 2890, 1720, 1625, 1550, 1480, 1395, 1290, 1185 cm^{-1} .

Hexyl 3,5-dihydroxybenzoate (17) was obtained in 74% yield as a colorless solid: ^1H NMR (270 MHz, CDCl_3) δ 0.89 (t, $J = 6.6$ Hz, 3H, $-\text{CH}_3$), 4.21 (t, $J = 6.6$ Hz, 2H, $-\text{OCH}_2$), 6.35 (d, $J = 2.3$ Hz, 1H, ArH), 6.45 (d, $J = 2.3$ Hz, 2H, ArH); IR (KBr) 3341, 2960, 2890, 1685, 1610, 1480, 1395, 1250, 1160 cm^{-1} .

Hexyl 4-hydroxybenzoate (18) was obtained in 66% yield as a colorless solid: ^1H NMR (270 MHz, CDCl_3) δ 0.88 (t, $J = 6.6$ Hz, 3H, $-\text{CH}_3$), 3.66 (m, 2H, $-\text{CH}_2$), 4.26 (t, $J = 6.6$ Hz, 2H, $-\text{OCH}_2$), 6.85 (d, $J = 8.7$ Hz, 2H, ArH), 7.92 (d, $J = 8.7$ Hz, 2H, ArH); IR (KBr) 3585, 3020, 2930, 2859, 1705, 1600, 1450, 1275, 1270, 1225 cm^{-1} .

Decahydro-2-naphthyl gallate (23) was obtained in 66% yield as a colorless solid: ^1H NMR (500 MHz, CDCl_3) δ 0.80–2.20 (m, 16H, $-\text{CH}_2$ and $-\text{CH}$), 4.90–5.20 (m, 1H, $-\text{OCH}$), 6.10 (br s, 3H, $-\text{OH}$), 7.30–7.35 (m, 2H, ArH); IR (Nujol) 3440, 1660, 1600, 1540, 1440, 1330, 1250, 1240, 1030 cm^{-1} ; EI-MS, m/z 306 (M^+), 224, 170 (base peak), 153, 136, 121, 95, 41; HRMS-EI, m/z [M^+] calcd for $\text{C}_{17}\text{H}_{22}\text{O}_5$ 306.1467, found 306.1444.

Geranyl gallate (3,7-dimethylocta-2,6-dienyl 3,4,5-trihydroxybenzoate) (21) was obtained in 57% yield as colorless powder: mp 67.0–68.5 $^{\circ}\text{C}$; ^1H NMR (300 MHz, CDCl_3) δ 1.60 (s, 3H, $-\text{CH}_3$), 1.67 (d, $J = 0.6$ Hz, 3H, $-\text{CH}_3$), 1.75 (d, $J = 1.2$ Hz, 3H, $-\text{CH}_3$), 2.08 (m, 4H, $-\text{CH}_2$), 4.79 (d, $J = 7.2$ Hz, 2H, $-\text{OCH}_2$), 5.09 (m, 1H, $-\text{CH}$), 5.43 (qt, $J = 7.2, 1.2$ Hz, 1H, $-\text{CH}$), 6.12 (br s, 3H, $-\text{OH}$), 7.31 (s, 2H, ArH); IR (KBr) 3549, 3406, 3288, 1686, 1614, 1537, 1312, 1244, 1229, 1198, 1022 cm^{-1} ; EI-MS, m/z 306 (M^+), 237, 170, 153 (base peak), 136, 121, 93, 69, 41; HRMS-FAB, m/z [$\text{M} + \text{H}$] $^+$ calcd for $\text{C}_{17}\text{H}_{23}\text{O}_5$ 307.11546, found 307.1561.

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

Medium. *S. cerevisiae* was maintained at -80 $^{\circ}\text{C}$ in yeast nutrient broth (YNB; Difco Laboratories, Detroit, MI) containing 25% glycerol and subcultured at 30 $^{\circ}\text{C}$ in Sabouraud's dextrose agar (SDA) medium (Bactopeptone 1%, dextrose 4%, Bacto-agar 1.8%). A fresh culture was preincubated with shaking for 5 h at 30 $^{\circ}\text{C}$ in 2.5% malt extract (ME) broth (BBL) medium.

Antifungal Assay. The maximum extent and rate of activity are known to vary with the seed culture mediums, the physiological age of the culture, and the type of culture medium. 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 10^5 colony forming units (CFU)/mL, and a 48-h stationary incubation in ME medium, unless otherwise specified.

Broth macrodilution minimum inhibitory concentrations (MICs) were determined as previously described (7). Briefly, serial 2-fold dilutions of the test compounds were made in DMF, and 30 μL of $100\times$ concentrated solution was added to 3 mL of ME medium. These were inoculated with 30 μL of seed culture to give the final inoculum of 10^5 CFU/mL. The assay tubes were incubated without shaking at 30 $^{\circ}\text{C}$ for 48 h. The MIC is the lowest concentration of test compound that

Table 1. Antifungal Activity of Alkanols against *S. cerevisiae* ATCC 7754

| alkanol tested | $\mu\text{g/mL}$ | |
|-----------------|-------------------|----------------|
| | MIC | MFC |
| C_6 | >1600 | >1600 |
| C_7 | 800 | >1600 |
| C_8 | 200 | 800 |
| C_9 | 100 | 200 |
| C_{10} | 50 | 50 |
| C_{11} | 25 | 25 |
| C_{12} | 12.5 ^a | >1600 |
| C_{13} | >1600 | — ^b |

^a This value is variable. ^b —, not tested.

demonstrated no visible growth. The MFCs were examined as follows. After the MIC had been determined, a 30- μL aliquot was taken from each clear tube and added into 3 mL of drug-free fresh medium. After 48 h of incubation, the MFC was determined as the lowest concentration of the test compound 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 $^{\circ}\text{C}$ for 5 h. A 30- μL 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 $^{\circ}\text{C}$ for 24 h before the number of CFU was determined.

Adsorption Test. The test strain was cultured with shaking in YPD broth overnight at 30 $^{\circ}\text{C}$ and washed twice with 50 mM MOPS buffer (pH 6.0). After each gallate ester was mixed with or without yeast cells (10^8 cells/mL) in the above buffer at 30 $^{\circ}\text{C}$, the suspension was vortexed for 5 s. Absorbance of the supernatants obtained by centrifugation for 2 min was measured at 272 nm.

Radical Scavenging Activity on DPPH. The reaction mixture consisted of 1 mL of 100 mM acetate buffer (pH 5.5), 1 mL of ethanol, 0.5 mL of ethanolic solution of DPPH, and 0.5 mL of the sample solution. After the mixture was allowed to stand at room temperature for 20 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity was measured as the decrease in absorbance of the DPPH expressed as a percentage of the absorbance of a control DPPH solution (8). Inhibitory activity was expressed as the mean 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration–inhibition curves.

RESULTS

Primary alkanols from C_6 to C_{13} were first tested for their antifungal activity against *S. cerevisiae*, and the results are listed in **Table 1**. Among them, undecanol (C_{11}) was found to be the most potent with the MFC of 25 $\mu\text{g/mL}$ (145 μM), whereas dodecanol (C_{12}) was completely inactive. Interestingly, fungicidal activity against *S. cerevisiae* completely disappears after dodecanol (C_{12}), and this is known as the so-called cutoff phenomenon. In the case of undecanol, no differences in MIC and MFC were noted, suggesting that no residual fungistatic activity is involved. In contrast, dodecanol still showed fungistatic activity with an MIC of 12.5 $\mu\text{g/mL}$ but did not exhibit any fungicidal activity up to 1600 $\mu\text{g/mL}$. Yeast cells appeared to adapt to dodecanol stress, eventually recovering and growing normally. It appears that the length of the alkyl group is associated with the activity to a large extent. On the other hand, the similar series of antimicrobial (2*E*)-alkenals characterized from olive oil (6) and anacardic acids identified in the cashew

Table 2. Antifungal Activity of Phenolic Acids against *S. cerevisiae* ATCC 7754

| acid tested | $\mu\text{g/mL}$ | |
|------------------------------|------------------|----------------|
| | MIC | MFC |
| benzoic acid | 800 | 1600 |
| 4-hydroxybenzoic acid | 1600 | >3200 |
| 3-hydroxybenzoic acid | 3200 | >3200 |
| 3,5-dihydroxybenzoic acid | 3200 | >3200 |
| 3,4-dihydroxybenzoic acid | 3200 | >3200 |
| 3,4,5-trihydroxybenzoic acid | >3200 | — ^a |

^a —, not tested.**Table 3.** Antifungal Activity of Alkyl Gallates against *S. cerevisiae* ATCC 7754

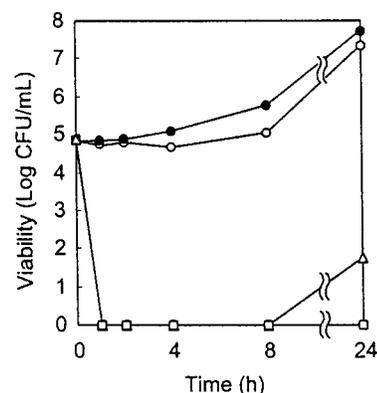
| gallate tested | $\mu\text{g/mL}$ | |
|----------------------|------------------|----------------|
| | MIC | MFC |
| C ₁ | >3200 | — ^a |
| C ₃ | 3200 | >3200 |
| C ₆ | 400 | 400 |
| C ₇ | 100 | 100 |
| C ₈ | 12.5 | 25 |
| C ₉ | 6.25 | 12.5 |
| C ₁₀ | 12.5 | 25 |
| C ₁₁ | >1600 | >1600 |
| C ₁₂ | >1600 | >1600 |
| geranyl | 50 | 50 |
| decahydro-2-naphthyl | 50 | 50 |

^a —, not tested.

apple (9) were observed to show similar SAR. These results indicate that the hydrophilic hydroxyl group can be replaced by any hydrophilic groups as long as the head and tail structure is balanced.

To create effective antifungal agents, phenolic acids were selected as the alternative hydrophilic (head) portion. The antifungal activities of the selected phenolic acids themselves against *S. cerevisiae* are listed in **Table 2**. They possessed little or no activity against *S. cerevisiae*. For example, gallic acid (3,4,5-trihydroxybenzoic acid; **3**) did not exhibit any fungicidal activity up to 3200 $\mu\text{g/mL}$. However, this phenolic acid was first selected as the hydrophilic (head) part because gallic acid is a common natural product isolated from many edible plants and, more importantly, its three esters—propyl, octyl, and dodecyl (lauryl)—are currently permitted for use as antioxidant additives in food (10). Furthermore, synthesis of a series of the esters and their related analogues is essential for SAR study, and this can be easily carried out. All of the esters used for the assay were synthesized as described under Materials and Methods. It should be noted that synthesis was achieved up to eicosanyl gallate (C₂₀), but the assay data were obtained only up to tridecyl gallate (C₁₃) (**10**), because an alkyl chain length of >11 carbon atoms did not exhibit any antifungal activity against *S. cerevisiae* in our preliminary assay. In addition, several nonyl benzoate analogues such as 4-hydroxybenzoate, 3-hydroxybenzoate, 3,4-dihydroxybenzoate (protocatechuate), and 3,5-dihydroxybenzoate were synthesized according to the same method for comparison.

The antifungal activities of the alkyl gallates synthesized against *S. cerevisiae* are listed in **Table 3**. The maximum fungicidal activity against this yeast was found in nonyl gallate (C₉) with an MFC of 12.5 $\mu\text{g/mL}$ (42 μM), whereas decyl gallate (C₁₀) (**7**) no longer showed any activity up to 1600 $\mu\text{g/mL}$. It is worth noting that undecyl (C₁₁) (**8**) and dodecyl (C₁₂) (**9**) gallates

**Figure 2.** Effect of nonyl gallate (**1**) on the growth of *S. cerevisiae* ATCC 7754: 0 (●) (control), 3.13 (○), 6.25 (△), and 12.5 (□) $\mu\text{g/mL}$ nonyl gallate. Exponentially growing cells were inoculated into ME broth and then cultured at 30 °C without shaking.

did not exhibit any antifungal activity against *S. cerevisiae*, because they may no longer possess the head and tail structure balance due to their increased hydrophobicity, but still showed antibacterial activity against Gram-positive bacteria (data not listed). The maximum activity of the carbon chain lengths in the gallates differed between the microorganisms tested, similar to those found for alkanols (6). The maximum activity seems to depend on the carbon chain lengths, reflecting differences in their cell-envelope structures. On the basis of our previous SAR study with alcohols, geranyl gallate (**21**) can be expected to show activity similar to that of nonyl gallate. The rationale for this idea is that the hydrophobic alkyl chain length from the hydrophilic hydroxy group as well as its volume is known to be a key factor in eliciting the activity (6). It seems that geraniol (**22**) nicely fits this assumption as a superior example. The introduction of branching or unsaturation into the hydrophobic group is known to increase the solubility of the surfactant in water (11). Hence, geranyl gallate was synthesized and assayed. As expected, this gallate exhibited antifungal activity against *S. cerevisiae* with an MFC of 50 $\mu\text{g/mL}$, as potent as nonyl gallate. No differences in MIC and MFC were noted, suggesting that no residual fungistatic activity is involved. In addition to its structural appropriateness, an additional reason of selection of geraniol as an alcohol moiety of this ester should be mentioned. This monoterpene alcohol is known to increase glutathione *S*-transferase activity, which is believed to be a major mechanism for chemical carcinogen detoxification (12).

The fungicidal effect of both nonyl and geranyl gallates was confirmed by the time—kill curve experiment. Cultures of *S. cerevisiae*, with a cell density of 6×10^5 CFU/mL, were exposed to two different concentrations of nonyl gallate. The number of viable cells was determined following different periods of incubation with nonyl gallate. **Figure 2** verifies that the MIC and MFC of nonyl gallate are the same. It shows that half-MIC slows growth but that the final cell count is not significantly different from the control. The result shows that lethality occurs remarkably quickly, within the first 1 h. A similar result was also obtained with geranyl gallate as shown in **Figure 3**. Noticeably, its lethality also occurs fairly quickly, similar to that of nonyl gallate. The results obtained likely indicate that both gallates are common in possessing a membrane disruptive effect.

Further evidence for this surfactant postulate was obtained in experiments that showed nonyl gallate to be fungicidal at any growing culture. Nonyl gallate at $1 \times$ MFC rapidly reduced the number of viable cells (10^6 CFU/mL within the first 2 h)

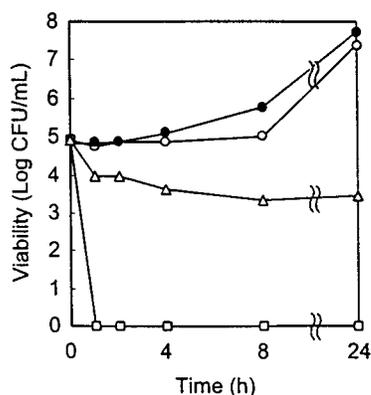


Figure 3. Effect of geranyl gallate (21) on the growth of *S. cerevisiae* ATCC 7754: 0 (●) (control), 12.5 (○), 25 (△), and 50 (□) $\mu\text{g/mL}$ geranyl gallate. Exponentially growing cells were inoculated into ME broth and then cultured at 30 °C without shaking.

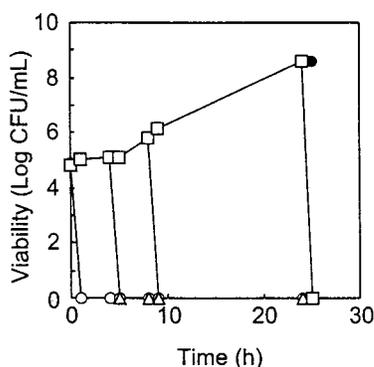


Figure 4. Fungicidal effect of nonyl gallate (1) on the growth stage of *S. cerevisiae* ATCC 7754. Yeast cells (10^5 cells/mL) were inoculated to ME broth medium. Nonyl gallate (concentrations: 0, ●; MFC, ○; 2 \times MFC, △; and 4 \times MFC, □) was added after 0 (●), 4 (△), or 24 h (□) of incubation.

when added to the culture at any stage of growth. Remarkably, nonyl gallate rapidly reduced the number of viable cells even when it was added to the cultures in the late-stationary growth phase (10^8 CFU/mL) as shown in **Figure 4**.

Among the food preservatives currently used, sorbic acid and its salts are some of the most commonly used as yeast inhibitors (13). Hence, the activity of nonyl gallate was compared with that of sorbic acid. Despite their wide application in various foods, especially as yeast and mold inhibitors, sorbates are generally static (14). In our preliminary screening, sorbic acid needs 3200 $\mu\text{g/mL}$ to exhibit lethal activity (MFC) against *S. cerevisiae*. This lack of potency limits their use, although sorbic acid is considered to be one of the least harmful preservatives in use. The fungicidal activity of nonyl gallate (C_9), 50 $\mu\text{g/mL}$, is 64-fold more potent than that of sorbic acid. More importantly, nonyl gallate has another superior property. As a weak acid antifungal agent, the activity of sorbic acid is pH dependent and increases as the pH of the substrate decreases (13), as shown in **Table 4**. At higher pH values (>5), sorbic acid did not show any antifungal activity up to 1600 $\mu\text{g/mL}$ due to a higher degree of dissociated molecules. In contrast, nonyl gallate was little influenced by pH values.

Propyl, octyl, and dodecyl gallates are currently permitted for use as antioxidant additives in food, so that the gallates synthesized were tested for their antioxidant activity (15). All of the gallates tested, regardless of their alkyl chain length, showed potent scavenging activity on the DPPH radical, indicating that the alkyl chain length was not directly related

Table 4. pH Effect of Antifungal Activity of Nonyl Gallate and Sorbic Acid against *S. cerevisiae* ATCC 7754

| pH | nonyl gallate | | sorbic acid | | benzoic acid | |
|----|---------------|------|-------------|------|--------------|------|
| | MIC | MFC | MIC | MFC | MIC | MFC |
| 3 | 6.25 | 12.5 | 400 | 1600 | 800 | 1600 |
| 5 | 6.25 | 12.5 | 800 | 3200 | 800 | 1600 |
| 7 | 12.5 | 25 | >1600 | — | >1600 | — |
| 9 | 25 | 50 | >1600 | — | >1600 | — |

Table 5. Antifungal Activity of Nonyl and Hexyl Esters of Various Phenolic Acids against *S. cerevisiae* ATCC 7754

| ester tested | $\mu\text{g/mL}$ | |
|-----------------------------|------------------|----------------|
| | MIC | MFC |
| nonyl | | |
| benzoate | >400 | — ^a |
| 4-hydroxybenzoate | >400 | — |
| 3-hydroxybenzoate | >400 | >400 |
| 3,5-dihydroxybenzoate | >400 | >400 |
| 3-hydroxy-4-methoxybenzoate | >400 | >400 |
| 4-hydroxy-3-methoxybenzoate | >400 | >400 |
| 3,4-dihydroxybenzoate | 6.25 | 12.5 |
| 3,4,5-trihydroxybenzoate | 6.25 | 12.5 |
| hexyl | | |
| 3,4-dihydroxybenzoate | 400 | 400 |
| 4-hydroxybenzoate | >800 | — |
| 3,5-dihydroxybenzoate | >800 | — |

^a—, not tested.

to this activity. Therefore, all of the gallate esters can be used as antioxidants in food. It appears that the potency of the antifungal activity of the gallates against microorganisms depends on their alkyl chain length, as described above, but not their scavenging activity. The amount required for antioxidant activity (IC_{50}) is slightly less compared to the amounts needed to control microorganisms.

The gallates were designed on the basis of the consideration of the head and tail concept similar to alkanols (6). Therefore, their mode of antifungal action can be expected to act as a surfactant. However, additional functions may need to be considered for newly synthesized phenolic esters. For example, the ester group did not exist in the original alkanol structure and may be related to eliciting the additional activity. As can be seen in **Table 1**, nonanol itself exhibits antifungal activity, so the possibility of *S. cerevisiae* exuding an esterase that hydrolyzes nonyl gallate (C_9) to the original gallic acid and antifungal nonanol was first taken into account. This possibility can be readily ruled out because neither nonyl benzoate (11) nor nonyl 4-hydroxybenzoate (12) exhibited any antifungal activity against *S. cerevisiae* up to 800 $\mu\text{g/mL}$ as shown in **Table 5**. If these benzoates were hydrolyzed by esterase, the freed nonanol should show the activity. In connection with the ester group, this group is essentially not related to the activity.

Both nonyl 3,4,5-trihydroxybenzoate and nonyl 3,4-dihydroxybenzoate (13) showed both antifungal and scavenging activity on the DPPH radical. Their potencies of both activities are almost comparable. On the other hand, nonyl benzoate, nonyl 3-hydroxybenzoate (14), nonyl 4-hydroxybenzoate, and nonyl 3,5-dihydroxybenzoate (15) exhibited neither antifungal nor scavenging activity on the DPPH radical, indicating that catechol or pyrogallol moieties are associated with the activities tested. Both hexyl 3,5-dihydroxybenzoate (17) and hexyl 4-hydroxybenzoate (18) were synthesized and tested for their antifungal activity against *S. cerevisiae*. Neither benzoate showed any activity up to 800 $\mu\text{g/mL}$. In addition, nonyl 3-hydroxy-4-

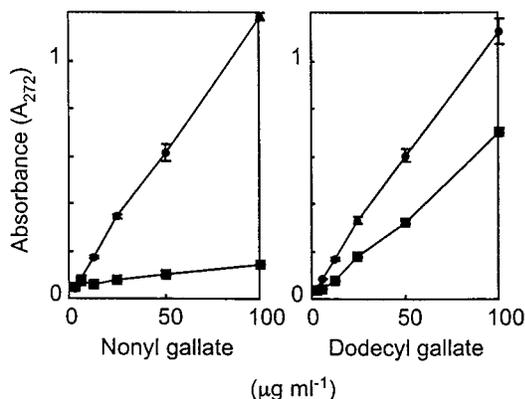


Figure 5. Adsorption of nonyl (**1**) and dodecyl (**9**) gallates to the cells of *S. cerevisiae* ATCC 7754. After each gallate was mixed with (■) or (●) without yeast cells (10^8 cells/mL), the suspension was vortexed for 5 s. Absorbance of the supernatant obtained by centrifugation for 2 min was measured.

methoxybenzoate (**19**) and nonyl 4-hydroxy-3-methoxybenzoate (**20**) did not show any antifungal activity, although both exhibited scavenging activity on the DPPH radical. On the basis of the data obtained, it appears that at least a catechol moiety seems to be essential to elicit the antifungal activity against *S. cerevisiae* and that the alkyl chain length also plays an important role. The results obtained demonstrate that the structural criteria of antifungal hydroxybenzoates differ from those of aliphatic alkanols. For instance, alkanols do not have any scavenging activity, but both 3,4,5-trihydroxybenzoates and 3,4-dihydroxybenzoates possess potent antioxidant activity. The antifungal mechanism of these benzoates seems to be associated with their specific structural features. The length of the hydrophobic alkyl group is associated with their antifungal activity against *S. cerevisiae* to a large extent but not antioxidant activity.

Further support for the surfactant concept was obtained in an additional experiment that indicates antifungal nonyl gallate rapidly adsorbed onto the surface of *S. cerevisiae* cells but dodecyl gallate did much less, as shown in **Figure 5**. The pyrogallol moiety adsorbed by an intermolecular hydrogen bond in attaching itself to hydrophilic portion of the membrane surface. This creates, as a surfactant, disorder in the fluid bilayer of the membrane. It seems that amphiphilic alkyl gallate unlikely disrupts specific target proteins such as cell-surface receptors or signal transduction proteins. Their adsorbing sites are unlikely specific but need to be clarified. On the other hand, most of the dodecyl gallate molecules did not adsorb onto the cell surface and remained in the water-based medium, probably in the form of insoluble monolayer or spread film (*16*). This may reveal why dodecyl gallate did not show any effects on eukaryotic microorganisms such as *S. cerevisiae*. The length of the alkyl chain is not a major contributor but plays an important role in eliciting the activity.

DISCUSSION

In view of the present investigation it appears that nonyl gallate's antifungal activity is due primarily to its surfactant property, similar to alkanols (*6*). Evidence for this postulate can be provided by the time-kill curve experiment, which showed that nonyl gallate is fungicidal against *S. cerevisiae*. It killed *S. cerevisiae* cells notably quickly, and no viable cells were detected after exposure to $50 \mu\text{g/mL}$ ($177 \mu\text{M}$) for 2 h. In addition, this fungicidal activity against *S. cerevisiae* was observed at any stage of growth. In addition, the length and

volume of the hydrophobic alkyl group are also associated with its antifungal activity to a large extent. As long as the catechol moiety of "a" exists, the hydrophobic portion seems to be flexible. For example, the alcohol portion of alkyl gallate can be replaced by a bicyclic alcohol, decahydro-2-naphthol (**23**), as well as a monoterpene alcohol, geraniol (**Table 3**). The result may provide a hint to design more appropriate antimicrobial agents because branched-chain or ring-containing surfactants are generally more soluble in aqueous media than straight-chain materials with the same number of carbon atoms (*11*). In addition, the straight-chain surfactants are much more biodegradable than the branched-chain or ring-containing materials. The hydrophobic portion is not a major contributor but obviously related to the activity.

Although the current study was focused against *S. cerevisiae*, the gallates synthesized showed a broad antimicrobial spectrum. Their antimicrobial activity is nonspecific (*17*), and hence binding sites seem to be nonspecific as surfactants. The results obtained indicate that microorganisms having different membrane structures showed different susceptibilities to gallates having different chain lengths. For example, dodecyl gallate did not inhibit the growth of *S. cerevisiae* but still shows antibacterial activity against Gram-positive bacteria and scavenging activity on the DPPH radical, indicating possibly different inhibition mechanisms between yeasts and bacteria.

The aforementioned primary alkanols were recently found to inhibit the succinate-supported respiration of intact mitochondria isolated from rat liver (*18*). 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. The data obtained with freeze-thaw (broken) mitochondria distinguish effects on the mitochondrial substrate carrier from that on the electron transport chain, suggesting that inhibition originates from interference with the dicarboxylate carrier, which must transport succinate across the mitochondrial membranes. It seems that alkanols inhibit this plasma membrane transporter and other membrane-bound proteins as nonionic surfactants (*18*). Antifungal hydroxybenzoates such as nonyl 3,4,5-trihydroxybenzoate and nonyl 3,4-dihydroxybenzoate can be expected in part to inhibit the same respiration as nonionic surfactants. However, it cannot be inferred that membrane damage is the only cause of the lethal effect of these hydroxybenzoates. It appears that the possibility of additional mechanisms may need to be taken into consideration. Further studies are currently underway and will be reported separately.

It should be noted that the hydrolyzable ester group can be an ideal food additive to prevent undesired side effects, particularly endocrine-disrupting activity such as alkylphenolic compounds (*19*), which are environmentally persistent estrogen mimics (*20*). In general, the gallates—such as octyl and nonyl—act as multifunctional agents in foods—at least as antifungal and antioxidant agents. After the gallates are consumed together with the food to which they are added as additives, these esters are hydrolyzed to the original gallic acid and the corresponding alcohols. The former still acts as a potent antioxidant, and the latter is a common plant component. More specifically, the freed gallic acid acts as an antioxidant; for instance, it scavenges superoxide anions generated enzymatically and nonenzymatically—even more potent than its ester forms. In addition, nonyl alcohol likely prevents the generation of superoxide anion and hydrogen peroxide by mitochondria in the resting state, as an uncoupler similar to fatty acids (*2*). The rationale of this

preventing mechanism at molecular levels is not yet clear, but the similar concept of the fatty acids cycle (22, 23) is also very likely applicable to this alcohol. Gallic acid is found in many plants such as blackberry bark, henna, tea, and uva ursi. Geraniol is reported in a large number (>160) of essential oils—such as lemon grass, coriander, lavender, carrot, and geranium—and used as food flavor for baked goods, soft and hard candies, gelatin, pudding, and chewing gum. As mentioned, the three gallates—propyl, octyl, and dodecyl—are currently permitted for use as antioxidant additives in foods; therefore, the above gallates may also be safe to use as food preservatives. In addition, it is evident that the volume of the hydrophobic portion also contributes to the activity. This suggests that even more optimization is possible through the synthetic approach. However, the most pertinent volume to elicit the maximum activity needs to be established. Antioxidant, gallic acid, and a glutathione *S*-transferase inducer, geraniol, may contribute to reduce cancer risk as well as oxidative damage related diseases. The complete scavenging activity of gallic acid was observed in the range of 10–30 μ M, which is much less than the amounts needed to control *S. cerevisiae*.

Compared to sorbic acid, nonyl gallate is odorless, as well as colorless and tasteless, with superior antifungal activity, and this antifungal activity is not influenced by the pH values in contrast to sorbic acid. In addition to this fungicidal activity, the gallates have potent antioxidant activity, which is also an important property to protect food, but sorbic acid does not possess this activity. Therefore, nonyl gallate should have a wider application potential as a food additive, particularly to control *S. cerevisiae* (1). It should be noted that this antifungal agent—as a nonionic surfactant—targets the extracytoplasmic region and thus does not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms.

ACKNOWLEDGMENT

We are grateful to Dr. N. Masuoka for performing the DPPH antioxidant assay and R. J. Lee for obtaining preliminary antimicrobial activity data of some alkyl gallates at an earlier stage of the work.

LITERATURE CITED

- Fleet, G. Spoilage yeasts. *Crit. Rev. Biotechnol.* **1992**, *12*, 1–44.
- Frazier, W. C.; Westhoff, D. C. *Food Microbiology*, 4th ed.; McGraw-Hill: New York, 1988; pp 410–439.
- Kubo, I.; Himejima, M. Potentiation of antifungal activity of sesquiterpene dialdehydes against *Candida albicans* and two other fungi. *Experientia* **1992**, *48*, 1162–1164.
- Kubo, I.; Lee, S. H. Potentiation of antifungal activity of sorbic acid. *J. Agric. Food Chem.* **1998**, *46*, 4052–4055.
- Spratt, B. G. Resistance to antibiotics mediated by target alterations. *Science* **1994**, *264*, 388–393.
- Kubo, I.; Muroi, H.; Kubo, A. Structural functions of antimicrobial long-chain alcohols and phenolics. *Bioorg. Med. Chem.* **1995**, *3*, 873–880.
- Kubo, I.; Himejima, M. Anethole, a synergist of polygodial against filamentous microorganisms. *J. Agric. Food Chem.* **1991**, *39*, 2290–2292.
- Blois, M. S. Antioxidant determination by the use of a stable free radical. *Nature* **1958**, *181*, 1199–1200.
- Kubo, I.; Muroi, H.; Himejima, M.; Yamagiwa, Y.; Mera, H.; Tokushima, K.; Ohta, S.; Kamikawa, T. Structure-antibacterial activity relationships of anacardic acids. *J. Agric. Food Chem.* **1993**, *41*, 1016–1019.
- Aruoma, O. I.; Murcia, A.; Butler, J.; Halliwell, B. Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *J. Agric. Food Chem.* **1993**, *41*, 1880–1885.
- Rosen, M. J. *Surfactants and Interfacial Phenomena*, 2nd ed.; Wiley-Interscience: New York, 1989; pp 1–32.
- Zheng, G. Q.; Kenney, P. M.; Lam, L. K. Potential anticarcinogenic natural products isolated from lemongrass oil and galanga root oil. *J. Agric. Food Chem.* **1993**, *41*, 153–156.
- Sofos, J. N.; Busta, F. F. Sorbates. In *Antimicrobials in Food*; Brannen, A. L., Davidson, P. M., Eds.; Dekker: New York, 1983; pp 141–175.
- Robach, M. C.; Sofos, J. N. Use of sorbates in meat products, fresh poultry and poultry products: A review. *J. Food Prot.* **1982**, *45*, 374–383.
- Kubo, I. Molecular design of antioxidative and antimicrobial agents. *CHEMTECH* **1999**, *29*, 37–42.
- Jones, M. N.; Chapman, D. *Micelles, Monolayers, and Biomembranes*; Wiley-Liss: New York, 1994.
- Kubo, I.; Xiao, P.; Fujita, K. Antifungal activity of octyl gallate: Structural criteria and mode of action. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 347–350.
- Hammond, D. G.; Kubo, I. Alkanols inhibit respiration of intact mitochondria and display cutoff similar to that measured *in vivo*. *J. Pharm. Exp. Ther.* **2000**, *293*, 822–828.
- Soto, A. M.; Juticia, H.; Wray, J. W.; Sonnenschein, C. *p*-Nonylphenol: an estrogenic xenobiotic released from “modified” polystyrene. *Environ. Health Perspect.* **1991**, *92*, 167–173.
- White, R.; Jobling, S.; Hoare, S. A.; Sumpter, J. P.; Parker, M. G. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **1994**, *135*, 175–182.
- Korshunov, S. S.; Korkina, O. V.; Ruuge, E. K.; Skulachev, V. P.; Starkov, A. A. Fatty acids as natural uncouplers preventing generation of O₂^{•-} and H₂O₂ by mitochondria in the resting state. *FEBS Lett.* **1998**, *435*, 215–218.
- Jezeq, P.; Engstova, H.; Zackova, M.; Vercesi, A. E.; Costa, A. D. T.; Arruda, P.; Garlid, K. Fatty acid cycling mechanism and mitochondrial uncoupling proteins. *Biochim. Biophys. Acta* **1998**, *1365*, 319–327.
- Skulachev, V. P. Uncoupling: new approaches to an old problem of bioenergetics. *Biochim. Biophys. Acta* **1998**, *1363*, 100–124.

Received for review January 25, 2002. Revised manuscript received April 16, 2002. Accepted April 17, 2002. This work was presented in part at the Symposium of Chemistry of Antimicrobials in the Division of Agricultural and Food Chemistry at the 220th National Meeting of the American Chemical Society in Washington, DC. K.F. thanks Osaka City University for financial support during his study at UCB.

JF020088V